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Fungicides

Edited by Odile Carisse

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Meet the editor

Dr Carisse obtained a baccalaureate in agronomy in 1987, a master in plant pathology in 1989 and a doctorate in epidemiology in 1992 from McGill University. Dr Carisse works as plant pathologist with Agriculture and AgriFood Canada since 1992. She is section editor for the Canadian Journal of Plant Pathology. She is chair of the international committee in plant disease epidemiology. Dr Carisse is an active member of the Canadian, American and International societies of phytopathology. Dr Carisse published over hundred scientific papers, presented numerous conferences to scientists, agronomists and growers. She wrote several field guides, factsheets and book chapters. She is involved in the implementation of a scouting networks for fruit and vegetable diseases management based on molecular detection of airborne inoculum and various risk indicators. Her research topics are: development of forecasting systems and improved management schemes for fruit, grapes and vegetable diseases; molecular detection of fungal plant pathogens and fungicide resistance genes; epidemiology and aerobiology of airborne fungal diseases; and decision making theory for better plant disease management.

Contents

Preface

During the pre-synthetic pesticides era, crop yields depended almost entirely on in-farm resources. Pests were present but production was secured from pest outbreaks by growing a diversity of crops. As agricultural modernization progressed, monocultures became the norm and pest control relies more and more on pesticides. These production systems although extremely productive and competitive generated a panoply of economic, environmental and social problems. On the other end, by increasing agricultural yields, pesticides have helped several countries become self-sufficient in food production. There is disagreements in the scientific community as to fungicides should remain part of the arsenal to fight against fungal diseases or banned to safeguard human and environmental health. Nevertheless, fungicides are still a key component of several disease management programs worldwide. This book was put together to provide science base knowledge on major issues and challenges related to fungicides.

Crop plants are attacked by a vast number of plant pathogens that caused various types of yield losses ranging from blemishes to complete crop losses. In some systems, plant pathogens are difficult to manage because their populations vary in time, space, and genotype. Chapters 1 to 9 addressed issues related to fungicide efficacy on various crops and in various environments. Plant pathogens evolve, often overcoming the action of fungicides. The first families of fungicides introduced prior to 1970 were mostly multi-sites fungal inhibitors most often used to protected plant from pathogen's attack. Despite their widespread use, resistance to these compounds was rarely reported. However, following the introduction of single-site fungicides, resistance has become a major problem. Problems related to fungicide resistance will be addressed in chapters 10 to 13 and to some extend in chapter 9.

Depending on the fungicide chemistry, persistence and quantities used, residues may persist in the soil or contaminate waterways affecting health of terrestrial and aquatic ecosystems. As more food is increasingly needed to feed the populations and agricultural lands diminished, maintaining agro-ecosystem health is becoming an issue. Chapters 14 to 16 addressed some of the problems related to the impact of fungicides in the environment. Pesticides are developed to kill or inhibit fungi, hence they are toxics that can enter in food chain and cause injury to human and animal health. Chapters 17 to 22 describe the toxicity of fungicides to humans and animals. Because of the high value of fungicide as tools to manage crop diseases and of the risk they pose to human, animal and ecosystems health, new chemistries are needed. The last three chapters of this book present new development in fungicide science.

The book is a result of efforts by many experts. I would like to acknowledge the authors, who are from different countries, for their contribution to the book. We hope that this book will contribute to a more informed and safe use of fungicides.

Odile Carisse

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Part 1

Fungicide Efficacy

1

Curative and Eradicative Effects of Fungicides

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1. Introduction

Fungi and oomycetes are the causal agents of numerous fungal plant diseases, causing losses in agricultural production worldwide. Plant pathogenic fungi and oomycetes have co-evolved with their plant hosts, and have developed extremely efficient mechanisms to cause an infection, to grow, multiply and spread on living plants. Most of these plant pathogens have an exceptional potential to reproduce within the plant host. For example, *Pytophthora infestans* can produce 50 000 to 117 000 sporangia per linear centimeter of infected potato stem length (Johnson et al., 2000). Larger apothecia of *Sclerotinia trifoliorum* can release about 4.7 million ascospores during their active period (Raynal, 1990). In late vegetation stages, the number of *Fulvia fulva* conidia per square centimeter of infected tomato leaf can exceed 17 000 (Veloukas et al., 2007), while *Pseudoperonospora cubensis* can produce even 195 000 sporangia per square centimeter of cucumber leaf seven days after infection (Wang et al., 2009). Considering the prevalence, ubiquitous nature and the ability of fungi and oomycetes to cause epidemics in relatively short period of time, disease management strategies are needed to secure the productivity in today's agriculture. Chemical control measures are particularly common in management of fungal plant diseases, and most of these measures rely on the use of fungicides.

No matter of many differences in biology of different fungal and oomycetous plant pathogens, they share certain similarities. Most of the fungi produce various kinds of spores, which come in contact with plant tissue, germinate, and penetrate into the plant during the infection process. Some plant pathogenic fungi (e.g. *Rhizoctonia solani*, *Sclerotium rolfsii*, or *Macrophomina phaseolina*) rarely produce spores and infect plants through penetration of mycelia into plant tissue. After infection, fungi and oomycetes continue to grow as mycelium in or on plant organs, where they produce their new vegetative or generative, propagation or dormant structures. Nearly all fungicides used in agriculture today show their best effect if applied before the infection occurs. When present on the surface of plant organs, fungicides destroy fungal spores or suppress germination tubes, hyphae, and other fungal structures. As a rule, control of fungal diseases with fungicides is aimed to prevent an infection and subsequent disease development, and in such way the use of fungicides in plant protection differ from the use of antibiotics or antimycotics in medicine and animal health.

However, in agricultural practice, there are many cases in which disease control measures are needed after the infection has occurred, after the pathogen is already sporulating or after

the symptoms have appeared. Until the late 60s and 70s of the 20th century, possibilities for therapeutic treatment of infected plants with fungicides were limited, as most of the fungicides developed and available for use in agriculture were protectant fungicides. Such fungicides remain on the surface of the plant, do not penetrate in it or translocate within it, and have little or no effect on the pathogen established in plant tissue. Systemic fungicides, introduced into the market in the 60s and 70s of the 20th century, are absorbed by the plant and translocated in it over the shorter or longer distance. In such way, fungitoxic active substance can come into the contact with the pathogen already established in the plant, which can result in pathogen's elimination.

Movement and translocation of fungicides within the plant differs between various fungicide active compounds, and delineation between systemic and non-systemic fungicides is often not clear. Most of the true systemic fungicides translocate through the xylem, and relatively small number of them has an ability to translocate basipetally in the phloem (Jacob & Neumann, 1987). Some of the fungicides found on the market today are only locally mobile, translocating only within the organ of application (Jacob & Neumann, 1987). Many modern fungicides classified as non-systemic show translaminar activity, i.e. they penetrate through the leaf and move from the upper to the lower surface of the leaf.

Discovery and development of systemic fungicides offered some new possibilities in disease management, important from practical point of view - to achieve a relatively efficient control after the infection has already occurred. Such therapeutic effect of fungicides is usually referred as "curative effect" or "curative activity". Theoretically, fungicidal activity on a fungal parasite within the plant can result in pathogen's eradication, i.e. the elimination of the pathogen from the host. Although complete eradication of plant pathogens within the host following fungicide application is relatively rarely achieved in practice, such effect of fungicides is referred as “eradicative effect” or “eradicative activity”. In plant protection terminology, application of a fungicide after the infection has occurred is often called “curative treatment”, while application after the symptoms have appeared is sometimes called “eradicative treatment”.

Curative and eradicative effects or performance of fungicides in chemotherapy of plants is dependent on a number of factors, out of which the most important are uptake, translocation and distribution of the fungicide in the plant, sensitivity of the pathogen to the fungicide, dose or concentration used, the position of the pathogen in plant tissue, and the stage of the pathogen development in the plant. Significant differences were recorded for different fungicides, different plant diseases, and different plant species investigated. Generally, results obtained in practice and recorded so far in experimental work showed that therapeutic treatments of plants with fungicides are usually less efficient than chemical control on preventive, protective basis. No matter of that, systemic fungicides with curative and eradicative effects contributed to some significant advantages in plant protection, and in efficient and economical control of certain fungal plant diseases.

2. Effect of systemic fungicides on pathogens established in plants

Curative activity of fungicides implies the effect of a fungicide on fungal mycelium located in the plant. To affect mycelium of fungi and oomycetes in plant tissue, fungicide must come into contact with the pathogen inside the plant. This might not always be the case, but if fungitoxic compound reaches vegetative or generative structures of a sensitive pathogen in a plant, certain effects on pathogen's physiology and morphology are inevitable to occur.

The amount of damage to the pathogen in the plant following fungicide application is usually measured by monitoring further symptom development or decrement in spore production. Nevertheless, several studies revealed morphological, physiological or biochemical alterations in structures of fungi and oomycetes inside the plant after postinfection fungicide treatment. Triazole fungicides tebuconazole and myclobutanil caused breakdown of subcuticular and intercellular mycelia of *Diplocarpon rosae* in infected rose leaves (Gachomo et al., 2009). In the same experiment, less systemic strobilurin fungicides pyraclostrobin and trifloxistrobin also caused collapse of *Diplocarpon rosae* inside rose leaves, but their effects were evident only on subcuticular mycelia of the fungus (Gachomo et al., 2009). Abnormal growth and changes in hyphal morphology was observed in mycelia of *Venturia inaequalis*, causal agent of apple scab, on plants treated with bitertanol, fenarimol, triforine and benomyl (O'Leary & Sutton, 1986; Kelley & Jones, 1981). Similar effects on *Venturia inaequalis* subcuticular hyphae in infected apple leaves following postinfection treatments with different triazole fungicides were recorded in another study (Thakur et al., 1992). Triadimefon lead to swelling and distortion of hyphae of *Hemileia vastatrix*, causal agent of coffee rust, when applied 24 or 48 hours after inoculation of coffee leaves with the fungus (Coutihno & Rijkenberg, 1995). Incomplete and multiperforate septa, as well as extensive wall thickening were observed in mycelia of *Uromyces fabae* and *Puccinia recondita* inside the infected leaves of broad bean and wheat after treatment with triadimefon (Pring, 1984). Similar changes were recorded in another wheat rust fungus, *Puccinia striiformis*, after application of tebuconazole three days after infection (Han et al., 2006). Vacuoles inside mycelial cytoplasm increased and cell walls thickened, following by collapse of cytoplasm in fungal cells (Han et al., 2006). Four days after the treatment, destruction of *Puccinia striiformis* intercellular mycelia and haustoria was evident (Han et al., 2006). Application of kresoxim-methyl and penconazole on grapevine leaves infected with powdery mildew, *Uncinula necator*, resulted in reduced mycelial density of the fungus and in partial collapse of hyphae (Leinhos et al., 1997). Accumulation of electron dense deposits adjacent to mitochondria, followed by mitochondrial degeneration were observed in hyphae of *Phytophthora infestans* in infected tomato leaves after treatment with oxadixyl (Jing & Grossmann, 1991). Cell walls of *Phytophthora infestans* hyphae became thickened, and degeneration of haustoria was recorded (Jing & Grossmann, 1991). Abnormal shrinking of cells, separation of membrane from the hyphal wall, breakdown of cell membrane and indistinct structure of cell organelles were noted in *Phytophthora capsici* mycelium inside the pepper seedlings after treatment with metalaxyl (Hwang et al., 1990). Five days after metalaxyl application on pea plants infected with downy mildew, *Peronospora pisi*, haustoria of the parasite were deformed, and vacuoles were visible in the cytoplasm of haustorial cells (Hickey & Coffey, 1980).

As it can be seen, most of the studies in which the effects of fungicides on fungal mycelium inside the infected plant were studied were conducted on biotrophic and hemibiotrophic plant pathogens. Many of biotrophic plant pathogenic fungi an oomycetes can not be cultivated *in vitro*, and the effects of fungicides on their development often can not be investigated on usual artificial nutrient media. Beside this, such pathogens are and interesting object of research for biologists and plant pathologist because of their very close contact with the host plant. Changes in their morphology and physiology can also reveal some new aspects of the host-pathogen interaction.

Suppression of pathogen's spore production on infected plant tissue can be viewed as an indirect consequence of fungicide activity on fungi and oomycetes established in the plant.

Numerous studies showed such effect of various systemic fungicides, and these studies can be regarded as especially valuable in disease management practice. For example, tebuconazole, cyproconazole, flusilazole and prochloraz reduced the number of *Septoria tritici* pycnidia on wheat leaves when applied after inoculation of plants with the fungus (Schöfl & Zinkernagel, 1997). Three sprays of propiconazole destroyed all teliosori of *Puccinia horiana* when this fungicide was applied after the symptoms of white rust occurred on chrysanthemum leaves (Dickens, 1990). When tebuconazole was applied three days after inoculation of wheat leaves with *Puccinia striiformis*, no uredia and urediniospores were formed (Han et al., 2006). Postinfection treatment with propiconazole and difenconazole inhibited sporulation of *Cercospora arachidicola* on peanut plants for 100 % and 90 %, respectively (Dahmen & Staub, 1992). Production of *Venturia inaequalis* conidia on apple leaves was almost completely inhibited when fenarimol, bitertanol and triforine were applied up to 120 hours after infection (Schwabe et al., 1984). Similar results for the same three fungicides and benzimidazole benomyl were recorded in another study (O'Leary & Sutton, 1986). Lesions of *Venturia inaequalis* sprayed twice in a week with bitertanol two days after their emergence contained much more immature and non-viable conidia (Kelley & Jones, 1981). Low germination of *Venturia inaequalis* conidia was also recorded after postsymptom application of bitertanol, fenarimol, triforine and benomyl (O'Leary & Sutton, 1986). Propiconazole, tebuconazole, myclobutanil, flusilazole, triforine and fenarimol reduced *Monilinia fructicola* conidial production from 58 % to 95 % when applied 72 hours after inoculation of sour cherry blossoms with the fungus (Wilcox, 1990). Application of kresoxim-methyl and penconazole on grapevine leaves infected with powdery mildew, *Uncinula necator*, resulted in inhibition of conidial formation on fungal mycelia (Leinhos et al., 1997). Difenconazole and azoxystrobin suppressed *Cercospora beticola* sporulation after treatment of sugar beet plants on which symptoms of Cercospora leaf spot emerged (Anesiadis et al., 2003). When propiconazole and azoxystrobin were applied to ryegrass plants infected with *Puccinia graminis*, they reduced urediniospore production by 73 % and 95 %, respectively (Pfender, 2006). After treatments of grapevine seedlings inoculated with *Guignardia bidwellii*, myclobutanil and azoxystrobin suppressed formation of pycnidia in lesions caused by the fungus (Hoffman & Wilcox, 2003). Application of myclobutanil resulted in 100 %, 91 % and 75 % reduction of *Guignardia bidwellii* pycnidium formation when applied six, eight or ten days after inoculation of plants with the fungus (Hoffman & Wilcox, 2003). Trifloxystrobin significantly reduced sporulation of *Fulvia fulva* on tomato leaves when applied on symptomatic plants (Veloukas et al., 2007). Kresoxim-methyl inhibited sporulation of *Venturia inaequalis* and *Uncinula necator* when applied two to four days after inoculation of apple and grapevine leaves (Ypema & Gold, 1999). Azoxystrobin, pyraclostrobin and kresoxim-methyl significantly reduced sporulation of *Sclerospora graminicola* on infected pearl millet plants (Sudisha et al., 2005), while trifloxystrobin and pyraclostrobin significantly reduced sporulation of *Cercospora beticola* on infected sugar beet plants (Karadimos et al., 2005). When applied up to five days after inoculation of grapevine seedlings with *Plasmopara viticola*, azoxystrobin reduced downy mildew sporulation by 96 %, and metalaxyl by 94 % (Wong & Wilcox, 2001). When treatment with these two fungicides was performed after the incubation period, on already sporulating downy mildew lesions, azoxystrobin and metalaxyl inhibited resporulation from the lesions by 85 % and 84 %, respectively (Wong & Wilcox, 2001). One day after fungicide treatment, metalaxyl suppressed sporulation of *Peronospora pisi* on pea plants infected with downy mildew (Hickey & Coffey,

1980). Dimetomorph reduced sporangial production when applied one day after inoculation of potato and cucumber plants with *Phytophthora infestans* and *Pseudoperonospora cubensis*, respectively (Cohen et al., 1995). The same fungicide inhibited *Pseudoperonospora cubensis* sporangial production on cucumber leaf disks treated three days after the inoculation (Wang et al., 2009). Postinfection treatment with dimetomorph, propamocarb and cymoxanil significantly reduced sporulation of *Phytophthora infestans* on potato plants (Johnson et al., 2000), while dimetomorph, mandipropamid, benthivalicarb and iprovalicarb suppressed sporulation of *Bremia lactucae* on infected lettuce leaf discs (Cohen et al., 2008).

Beside effects on development of reproductive structures of different fungi and oomycetes inside the plants, it is interesting to mention that postsymptom application of certain fungicides can sometimes lead to long-term effect on further development of the pathogen inside the plant tissue. Such long-term effect was recorded in *Venturia inaequalis* after treatment with ergosterol biosynthesis inhibitors bitertanol, fenarimol and triforine (O'Leary & Sutton, 1986). *Venturia inaequalis* produce conidia on apple leaves during the vegetation, while during the winter period it continue to develop in fallen dead leaves and produce pseudothecia, fruiting bodies with asci and ascospores. It was noted that the fungus exposed to the above mentioned fungicides during vegetation produced smaller pseudothecia in fallen apple leaves, with many undeveloped asci containing fewer ascospores (O'Leary & Sutton, 1986).

Antisporulant activity on fungi and oomycetes established in plants is not a property attributed only to systemic fungicides. Fungicides regarded as protectant can also show similar effects. Reproductive structures of the majority of parasitic fungi and oomycetes develop on the surface of plant organs, providing the pathogen to spread its spores in environment. In such way, protectant fungicides on the surface of plant tissue can come into contact with condiophores, sporangiophores, ascocarps, acervuli, pycnidia, sori or other fungal structures, which can result in reduction of sporulation. How much fungicidal compound will reach reproductive structures of fungi and oomycetes, depends on the position of a certain fungal species in infected plant. For example, it can be expected that protectant fungicides will have an effect on mycelia of many powdery mildews (Erysiphales), plant parasites which develop their hyphae and conidia on the surface of plant organs.

3.1 Curative and eradicative effects of fungicides in seed treatment and treatment of planting material

The first systemic fungicides introduced to the market were carboxin fungicides (Kulka & von Schmeling, 1987). After their discovery, carboxin fungicides were proven to be extremely effective in seed treatments against loose smuts of barley and wheat, caused by *Ustilago nuda* and *Ustilago tritici* (Maude, 1996; Kulka & von Schmeling, 1987). These *Ustilago* species infect wheat and barley flowers, and become established in embryo of developing kernels, without causing visible symptoms. As *Ustilago nuda* and *Ustilago tritici* mycelia are located deeply in infected seed, fungicides that do not penetrate in seed embryo could not be effective in controlling these diseases. Carboxin fungicides enabled an eradication of loose smuts pathogens inside the seed, and this was the first example of fungicidal therapeutic activity used in fungal disease management practice worldwide. Soon after the introduction of carboxin, other systemic fungicides were developed for seed treatment, like guazatine, ethirimol, benzimidazoles thiabendazole, benomyl or carbendazim, triazole

fungicides, fenpropimorph, metalaxyl, fosetyl-Al or fludioxonil. Eradication of seed-borne pathogens with systemic fungicides has become much more effective compared to the majority of non-systemic seed fungicides. Although protectant fungicides are still used successfully, most of the fungicides used for seed treatment on the market today are systemic fungicides. The advantages of systemic fungicides in seed treatment are not demonstrated only in better eradication of seed-borne parasites. Systemic fungicides are uptaken and equable redistributed inside the seedlings, which leads to better distribution of fungitoxic compound in all young plant tissues. Their movement to cotyledons and first leaves offers protection of the aboveground parts of the plant for a certain period. Such period of protection can be relatively long, and it can contribute to more efficient management of various foliar diseases. Nevertheless, eradication of different fungal pathogens established in the seed can be also accomplished with protectant fungicides. For example, systemic benomyl and protectant iprodione and vinclozolin equally effectively eradicated seed-borne *Sclerotinia sclerotiorum* from the sunflower seed (Herd & Phillips, 1988). The main reason for this is the position of parasitic fungi and oomycetes inside the seed. Generally, there are not many economically important fungal pathogens which are usually established deeply in the seed. Many seed-borne fungi and oomycetes are present as spores or mycelium on the surface of the seed or inside the superficial tissues of seeds, and often the infection is located in pericarp (Maude, 1996). Relatively smaller number of seed-borne fungal parasites is usually located in deeper seed tissues, like endosperm or embrionic axes (Maude, 1996). Therefore, protectant fungicides can come into contact with fungal structures of fungi and oomycetes which develop close to the seed surface, like relatively many *Alternaria*, *Fusarium*, *Botrytis*, *Sclerotinia*, *Phoma* or *Pythium* species (Maude, 1996). Eradicative effect of non-systemic fungicides is dependent how effectively and to which extent will fungicidal compound reach the pathogen inside the seed.

Comparing it to eradication in chemical control of foliar, fruit, root or stem diseases, eradication of fungal pathogens is much easier and more successfully achieved in seed treatment. Chemotherapeutic effect in seed treatment is often total, with the pathogen completely destroyed inside the seed tissues. There are several reasons for this. First of all, seed has a small volume compared to adult plants. When fungicides are applied, they can effectively reach each part of the seed and come into contact with the target pathogens. Seed treatment machinery is technically very advanced today, and all surfaces of the seed can be uniformly covered with a fungicide. Due to small volume of the seedling, concentration of a fungicide inside the seedling remains high for a relatively long period, which decreases the possibility of the pathogen to survive. At the same time, the biomass of the pathogen inside the seed is small and in such way easier to exterminate. Not less important, it must be pointed out that chemical companies which produce certain fungicide for a seed treatment are testing, calculating and accurately establishing dose rate needed for a treatment to be effective.

Treatment of planting material is another common measure where eradicative effects of fungicides can be used in disease management practice. Some important and potentially destructive plant pathogens can be present in tubers, bulbs, cuttings, seedlings or other types of planting material, very often as symptomless infection. Disease control in nurseries and phytosanitary control of mother plants' health status are the main preventive measure intended to secure that economically important fungi and oomycetes are not present in planting material. However, in many cases it is relatively hard to prevent and control the presence of certain fungal diseases in planting material, especially considering the fact that they may not cause any visible symptoms for some time. The efficacy of fungicides in

eradication of pathogens from planting material basically depends on the same factors as it is with seed treatment. Due to some important differences, the efficacy of such treatments is usually not as high as the treatment of seed. First, all types of planting material have much larger volume than the seed. Fungicide must redistribute in all parts of plant tissue in sufficient fungitoxic amounts. Fungal biomass in different types of planting material is much larger, and more fungicide is needed for complete eradication of the pathogen. The technology of fungicide application on planting material is not as advanced, precise and practical as it is with the seed treatment. Finally, there are much less fungicides on the market registered for the treatment of specific types of planting material. This can lead to problems in assessment of appropriate dose, concentration, or exposure time, as well as to the higher risk of possible phytotoxicity.

Several studies showed the lack of complete pathogen's eradication following the treatment of planting material with fungicides. Application of thiophanate-methyl to potato seed pieces infected with *Phytophthora infestans* significantly reduced the amount of tuber surface area colonized by the oomycete, but such reduction was not very high (Inglis et al., 1999). An incidence of *Phaeomoniella chlamydospora* and *Phaeoacremonium* species in grafted grapevine cuttings and uprooted nursery vines was reduced following drench in benomyl, although such reduction was not significant compared to plants drenched only in water (Fourie & Halleen, 2004). Prochloraz, propiconazole and difenconazole dip resulted in 11.5 %, 53.5 % and 57.2 % mortality of strawberry transplants caused by *Colletotrichum acutatum*, compared to 80 % mortality in untreated transplants (Freeman et al., 1997). The percentage of *Colletotrichum acutatum* recovery from strawberry runners dipped in prochloraz was less than 5 % after three days, but it increased to 60 % after 12 days (Freeman et al., 1997). Nevertheless, fungicide treatment can also be exceptionally effective in eradication of the pathogens from planting material. For example, 10 and 20 days after dip of chrysanthemum cuttings in myclobutanil, no pustules of *Puccinia horiana* appeared on treated plants, while more than 90 % of control plants showed symptoms (Bonde et al., 1995). Thirty days after the dip, *Puccinia horiana* was visible on only two out of the 720 treated plants, while 94 % of untreated plants were infected (Bonde et al., 1995).

3.2 Curative effects of fungicides in management of root and crown roots

Generally, root and crown rots are plant diseases which are difficult to control with chemical measures. Caused by different soil-borne pathogens, symptoms of such diseases can develop on the aboveground parts of the plant relatively long after the infection has occurred, often after considerable damage has already been done to plant's root or crown tissue. Symptoms of many root and crown rots are unspecific and it can be relatively hard to make an accurate diagnosis without laboratory analysis. Root and crown rots can develop unevenly over the production area - in some parts of the field a disease can be especially severe, while in other parts it may be absent. Soil-borne pathogens which are causal agents of root and crown diseases can sometimes attack plants during the whole year, and it is relatively complicated to develop a reliable forecast model which could predict infection risk periods. On the other hand, a small number of systemic fungicides have an ability to translocate downward through the plant in the phloem sap. In such way, foliar treatments with the most of systemic fungicides against root or crown diseases are not effective, as fungicidal compound does not reach the pathogen in roots or basal parts of a stem or a trunk. If fungicides are applied as soil treatment, plants can uptake a relatively small amount of a fungitoxic compound. This can cause difficulties in calculation of an appropriate dose and concentration of a product. While

lower doses can be ineffective, larger doses of a fungicide can cause phytotoxicity problems. High amount of the fungicide applied to a soil can be rapidly degraded, and repeated applications of the fungicide may be required, which can significantly influence the cost-benefit ratio and economic justifiability of soil treatments. A relatively small number of granular-formulated fungicides are labelled for soil treatment on the market today, and they may not be available in all countries. Comparing it to foliar treatments, agricultural mechanization for soil treatment or trunk application has not been intensively developed. Trunk paints, trunk injections, or root drench of separate plants is labour-intensive and not practical in many cases. These are some of the reasons why chemical control of root and crown diseases could be problematic, and why management of root and crown diseases is relied primarily on prophylactic non-chemical methods, like host resistance, crop rotation, or regulation of water regime in soils. During the last two decades, much work has been done on biological control of such diseases, and it is obvious that biological control of soil-borne pathogens will become increasingly important in practice.

Theoretically, preventive chemical control of root and crown rots could be efficient if fungicides are present in all susceptible root and stem plant tissue in effective fungitoxic amounts during the period when infection is possible. The other possibility for chemical control of soil-borne diseases would be eradication of pathogens in soil prior to cultivation. Fungicides are used in management of certain root and crown diseases, like those caused by *Phytophthora* or *Rhizoctonia*, but nearly all of these fungicide treatments are intended to be used on preventive basis. In case of such diseases, options for curative treatments of already infected plants are limited. When symptoms begin to develop on the aboveground parts of the plant, parasitic fungus or oomycete has often developed a relatively high amount of biomass, and high amounts of a fungicide are needed to be effective. Fungicide must be applied and redistributed in a plant to reach the zones where pathogen's mycelium is active. If a part of mycelium survives fungicide treatment, or revive after fungistatic activity of a fungicide has ceased, the disease can continue to advance.

Despite many difficulties and limitations in therapeutic treatments of plants affected with root or crown rots, producers are often looking for each solution which could stop the disease progress if such disease emerge in a field and start to threaten the production. After the discovery and development of systemic fungicides, some studies have been done in order to explore the possibilities for curative treatments and the possible eradication of root and crown rot pathogens after the disease has started to develop. The majority of such research has been done on root and crown rots caused by various *Phytophthora* species, especially after introduction of potent systemic compounds metalaxyl and fosetyl-Al to the market (Erwin & Ribeiro, 2005). Fosetyl-Al was shown to be efficient in control of cankers caused by *Phytophthora citricola* on avocado (El-Hamalawi et al., 1995). One week after fosetyl-Al application, expansion of cankers on infected plants was stopped, and six months after the treatment it was not possible to recover the oomycete from canker lesions (El-Hamalawi et al., 1995). Mean disease ratings of avocado trees, infected with *Phytophthora cinnamomi* and trunk-injected with fosetyl-Al, decreased dramatically, from 5.3 in the first year to zero in the fourth year of the experiment (Darvas et al., 1984). Apple trees in the initial stage of root and crown rot caused by *Phytophthora cactorum* and drenched with metalaxyl remained alive and productive, while control untreated trees died within a three-year period (Utkhede, 1987). Survival of azaleas, infected with *Phytophthora cinnamomi* and treated with three foliar sprays of fosetyl-Al and two drenches of metalaxyl, was monitored for three years after transplanting the plants in disease-free landscape beds (Benson, 1990).

A small number of treated plants developed symptoms of root rot after three years in beds, while mortality of control plants reached 39 % (Benson, 1990).
Curative effects of fungicides on root and crown rots other than those caused by *Phytophthora* species have not been studied intensively. Certain effectiveness of soil drench with benomyl and tridemorph on plants affected with *Rosellinia necatrix* root rot has been recorded (ten Hoopen & Krauss, 2006). An interesting study has been conducted on propiconazole treatment of almond plants affected with *Armillaria mellea* root rot, using medical intravenous bags hanged around the trunk (Adaskaveg et al., 1999). Five months after the treatment, all five treated trees were alive, while four out of five control trees died (Adaskaveg et al., 1999). In the second year, four out of five treated trees were alive, while four out of five control trees died, and disease severity was significantly lower on treated trees (Adaskaveg et al., 1999).

3.3 Curative effects of fungicides in management of vascular wilts, cankers and wood rots

Chemical control of fungal vascular wilts, cankers, and wood rots more or less share the same problems related with the use of fungicides in management of root and crown rots. In fact, certain fungi and oomycetes which can cause canker diseases can also cause root and crown rots, and delineation among such diseases is sometimes not sharply defined. This is especially evident among diseases caused by different *Phytophthora* species, some of which are able to attack roots, stems, branches, or fruits of various cultivated plants. *Sclerotinia sclerotiorum* on sunflower or soybean, or *Colletotrichum acutatum* on strawberry are the other examples of such diseases.
As it is with root rots, symptoms of vascular wilts and wood rots can become evident when development of the pathogen inside the plant is already in advanced stage. In cases of vascular wilts, fungus is growing through the xylem tissue, and can be present in different parts of the plant. Wood rots are caused by fungi which degrade lignin and cellulose inside the wood, and can also develop large amounts of mycelia before a tree starts to wither. It is often hard to determine the periods when infection is possible, as vascular wilt pathogens or wood-rotting fungi penetrate the plant from the soil through the roots, through the wounds on the aboveground parts of the plant, or can be carried by insect vectors. Cankers are more easily to detect on plants, but one of the significant problems in chemical management of such diseases can be the fact that these diseases frequently develop throughout the whole year, often even more intensive out of the vegetation period. Relatively large amount of pathogen's mycelia inside the diseased plants, invisible early stages of infection, location of the pathogen deep inside the plant tissues, lack of adequate machinery needed for special types of fungicide application, and a small number of fungicides labelled for use are the major constraints associated with chemotherapy of vascular wilts, wood rots and canker diseases. Beside this, it must be pointed out that protective or curative treatments in cases of such diseases are usually labour-intensive, and can often be economically unjustifiable. As it is with many root rots, it can be cheaper and more practical to eradicate the diseased plant than to eradicate the disease from the plant.
After the introduction of systemic fungicides to the market, their curative and eradicative potential in treatments of certain vascular wilts, wood rots and cankers has started to be studied. For the above mentioned reasons, during the next twenty years such experiments were remitted, and the research on management of such diseases focused on other possibilities. Nevertheless, some results have been achieved in therapeutic treatments of

various cankers caused by *Phytophthora* species and vascular wilt diseases of landscape ornamental trees like oaks and elms. For example, cankers caused by *Phytophthora palmivora* on cocoa trees were significantly reduced following potassium phosphonate trunk injections (Guest et al., 1994). Metalaxyl and fosetyl-Al, applied in granular formulations, or fosetyl-Al applied as foliar spray, significantly reduced cankers on peach caused by *Phytophtora cactorum* when applied as curative treatments (Taylor & Washington, 1984). Both fungicides were also effective in reducing cankers following postinfection treatments as trunk paints (Taylor & Washington, 1984). Almost no active lesions were found on trees treated with granular formulations of metalaxyl and fosetyl-Al (Taylor & Washington, 1984), which indicates that these fungicides were capable of complete eradication of the oomycete from infected trees. Fosetyl-Al and mefenoxam, active isomer of metalaxyl, applied as topical treatments with paint brush or as a spray, reduced canker expansion on almond inoculated with *Phytophthora cactorum* or *Phytophthora citricola* by 36 - 88 % (Browne & Viveros, 2005). Active cankers treated with fosetyl-Al sprays expanded 71 - 77 % less, while those sprayed with mefenoxam expanded 54 - 79 % less than on control trees (Browne & Viveros, 2005). In other variant, fosetyl-Al reduced canker expansion by 86 - 88 %, while mefenoxam reduced canker development by 52 - 80 % (Browne & Viveros, 2005). Curative bark drench applications of phosphonate on beech saplings inoculated with *Phytophthora citricola* significantly limited canker expansion on infected trees (Weiland et al., 2009).

Several experiments have been done on chemical control of oak wilt, caused by *Ceratocystis fagacearum*, and Dutch elm disease, caused by *Ophiostoma ulmi*. Trunk injections with thiabendazole were somewhat effective as therapeutic treatment of elms affected with Dutch elm disease (Lanier, 1987). Trunk injections with benomyl to elm trees with various stages of decline led to remission of disease in many trees, but disease development was not affected if application was performed on trees which showed more than 5 % of crown damage prior to the treatment (Smalley et al., 1973). Generally, it is proven that thiabendazole and propiconazole can be effective in therapeutic treatments of trees affected with Dutch elm disease (Scheffer et al., 2008), but these fungicides need to be applied in relatively early stages of infection. These two fungicides are labelled for chemical control of Dutch elm disease in the USA, but are not recommended to be used on severely affected trees. On thiabendazole label, it is written that treatments may not be effective when tree shows more than 5 % of crown symptoms, while on propiconazole label it is stated that treatment may not be effective when applied to trees in advanced stages of disease development (Scheffer et al., 2008). The same triazole fungicide, propiconazole, has shown to be effective in curative treatments of oak wilt (Appel & Kurdyla, 1992). Following intravascular injection of infected oaks with propiconazole, level of crown loss 9 to 36 months after the injection ranged from none to 41 %, while it reached 61 - 100 % in untreated trees (Appel & Kurdyla, 1992). Injections in presymptomatic stage of the disease resulted in better control (Appel & Kurdyla, 1992).

It is recorded that curative treatments can be effective in control of Fusarium wilt on certain vegetables, caused by different forms of *Fusarium oxysporum*. Prochloraz and carbendazim were tested in control of Fusarium wilt of tomato in hydroponic system by adding fungicides in nutrient solution one week after plants were artificially inoculated with the fungus (Song et al., 2004). Two weeks after the treatment, prochloraz showed 50 % control, while carbendazim gave 34.4 % disease control (Song et al., 2004). Benomyl applied as soil drench reduced the quantity of mycelium in muskmelon plants infected with *Fusarium*

oxysporum, and it is reported that plants were generally cured when fungicide was applied before symptoms occurred (Maraite & Meyer, 1971).
Grapevine cultivation is somewhat different from fruit cultivation. In case of grapevine grown for vine production, breeding for resistance and development of disease-resistant cultivars is virtually not conducted. Some vineyard areas and locations are particularly valuable for winegrowing and appreciated in the vine market. In such perspective, labour-intensive curative treatments of certain grapevine diseases could be a measure regarded as acceptable for some producers. Eutypa canker, caused by *Eutypa lata*, and esca, complex disease caused by wood-rotting *Fomitiporia* species, *Phaeomoniella chlamydospora* and *Phaeoacremonium* species, cause considerable damage to grapevine production in many areas of the world, and it is not surprising that the possibility of their management with fungicides has been explored. Trunk injections of cyproconazole resulted in better productivity and lower mortality in vines affected with esca which were subjected to trunk removal and injection of fungicides for three consequent years (Calzarano et al., 2004). The number of vines showing symptoms of esca decreased after trunk injections with propiconazole, difenconazole and thiabendazole (Dula et al., 2007). Syringe treatments with cyproconazole, flusilazole, penconazole and tetraconazole of vines with early symptoms of esca significantly reduced foliar symptoms appearance of the disease (Di Marco et al., 2000). However, treatments of old infected vines were not effective, and neither were trunk paints or foliar applications of fungicides (Di Marco et al., 2000). Similarly, almost no effects were recorded following trunk injections of propiconazole and difenconazole in vines affected with *Eutypa lata* (Darrieutort & Lecomte, 2007).

3.4 Curative and eradicative effects of fungicides in management of foliar and fruit diseases

Most of the fungicides are developed and registered for management of diseases which develop on aerial plant parts. Chemical control of various diseases affecting leaves, stems, branches, flowers or fruits on nearly all cultivated plants has become regular measure in agricultural production, and it is obvious that it will remain so in the future. Machinery for pesticide treatments is technically advanced, while the assortment of fungicides available on the market offers numerous possibilities for effective and economical disease control. These are the main reasons why curative and eradicative effects of fungicides have been most intensively studied in cases of foliar and fruit diseases.
As mentioned before, most of the fungicides are more efficient when applied prior to infection than after the infection has occurred, or after the symptoms have appeared. No matter of this, many fungicides are able to stop pathogen development inside the infected plant tissue. If applied shortly after infection, during the early stages of pathogenesis, fungicide treatment can sometimes completely eradicate the parasite from the plant, although such cases are actually rare. Generally, it can be stated that all the fungicides which are able to penetrate the plant can have curative or eradicative activity on certain plant diseases. True systemic fungicides, locally systemic fungicides, fungicides with translaminar activity, and in some cases even the fungicides regarded as protectants can reveal curative or eradicative effects in postinfection treatments.
One of the most important factors which implicate the curative effects of different fungicides is the period passed from the infection to the fungicide application. During the early stages of pathogenesis, fungal biomass inside the plant is relatively small, but mycelium of parasitic fungi and oomycetes is growing, proliferating and spreading with the time within

the plant tissue. While fungitoxic compound can be effective on small amounts of pathogen's mycelia located near the plant surface, larger amounts of mycelia deeper inside the plant tissue can be much less affected. In nearly all experiments and field trials on postinfection treatments, the efficacy of fungicides decreased as the period between the infection and fungicide application became longer. When trifloxystrobin was applied 24, 48 or 96 hours after inoculation of tomato with *Fulvia fulva* at concentration of 10 µg a.i./ml, its effectiveness was 89 %, 76 % and 60 %, respectively (Veloukas et al., 2007). Similar decrease of disease control level was also observed for other concentrations of trifloxystrobin applied (Veloukas et al., 2007). Fluazinam and boscalid applied two days after inoculation of peanut plants with *Scleortinia minor* reduced disease incidence, but no reduction was recorded when these fungicides were applied four days after the inoculation (Smith et al., 2008). Applied one and three days after inoculation of cucumber plants with *Pseudoperonospora cubensis*, dimetomorph gave 67 and 32 % disease control, respectively, while the efficacy of azoxystrobin decreased from 96 % when applied one day after inoculation to 39 % when applied three days after inoculation (Wang et al., 2009). Mandipropamid and dimetomorph applied 24 hours after inoculation of potato and tomato leaflets with *Phytophthora infestans* significantly reduced sporulation of the oomycete, but no curative effects were observed when fungicides were applied 48 hours after the infection (Cohen & Gisi, 2007). When azoxystrobin, pyraclostrobin, mefenoxam and phosphonate were applied 13, 24, 36 or 48 hours after strawberry plants were inoculated with *Phytophthora cactorum*, disease incidence on azoxystrobin- and pyraclostrobin-treated plants was about 40 % when applied 13 hours after inoculation, comparing it to 70 % on control plants and zero on mefenoxam- or phosphonate-treated plants (Rebollar-Alviter et al., 2007). When azoxystrobin and pyraclostrobin were applied 24 hours after the inoculation, disease incidence on treated plants was the same as on control (Rebollar-Alviter et al., 2007). Mefenoxam and phosphonate provided significant disease control when applied even 48 h after the inoculation of plants (Rebollar-Alviter et al., 2007). Pyraclostrobin applied 3 or 8 hours after inoculation of strawberry plants with *Colletotrichum acutatum* provided excellent disease control, but was less effective when applied 24 or 48 hours after inoculation (Turechek et al., 2006). Trifloxystrobin, pyraclostrobin and difenconazole were efficient in control of *Cercospora beticola* when applied 24 hours after inoculation of plants, but their efficiency decreased if 96 hours has passed from the inoculation to the application time (Karadimos et al., 2005). When propiconazole, tebuconazole, myclobutanil, flusilazole, triforine, fenarimol, vinclozolin and iprodione were applied 24 hours after inoculation of sour cherry with *Monilinia fructicola*, they reduced or completely prevented blossom blight. When application was prolonged to 72 hours after inoculation, none of the fungicides provided significant disease control (Wilcox, 1990). One day after inoculation of tomato with *Alternaria solani*, difenconazole provided more than 90 % disease control, but the level of control fell to 60 % when the fungicide was applied two days after inoculation (Dahmen & Staub, 1992). Reduction of necrotic leaf area caused by *Septoria tritici* on wheat plants rapidly decreased the longer was the period between infection and application of tebuconazole, cyproconazole, flusilazole and prochloraz (Schöfl & Zinkernagel, 1997). Similar decrement in efficacy was recorded when pyrimethanil, azoxystrobin and fludioxonil were applied to lemon fruit 9, 12, 15, 18 and 21 hours after inoculation with *Penicillium digitatum* (Kanetis et al., 2007). These are only some of the examples which show the relation of curative effects of different fungicides and postinfection application time.

The effect of fungicides on pathogen established in the plant will typically be more pronounced when higher dose or concentration is used. Such effects can easily be measured *in vitro*, by monitoring mycelium growth in the presence of different fungicide concentrations on agar media, in broths, or on various detached leaf assays. Numerous *in vitro* studies of this kind have been conducted, often as preliminary screening to further glasshouse or field experiments on living plants. The impact of dose or concentration applied on efficacy of postinfection fungicide treatment can be clearly seen from several studies. Bitertanol applied at 150 µg a.i./ml was less effective in suppressing apple scab lesion development than the same fungicide applied at concentration of 300 µg a.i./ml (Kelley & Jones, 1981). When applied one day after inoculation of cucumber plants with *Pseudoperonospora cubensis*, dimetomorph in concentrations of 250, 500 and 1000 µg a.i./ml provided 33, 43 and 62 % of disease control, respectively (Cohen et al., 1995). An increase in suppression of sporulation and lesion development was recorded when higher concentrations of difenconazole, penconazole and propiconazole were used in postinfection treatment of *Venturia inaequalis* on apple and *Cercospora arachidicola* on peanut (Dahmen & Staub, 1992). When trifloxystrobin was applied one day after inoculation of tomato with *Fulvia fulva* at different concentrations, level of disease control was 70 % for 5 µg a.i./ml, 89 % for 10 µg a.i./ml, and 93 % for 20 µg a.i./ml (Veloukas et al., 2007). Level of postinfection *Puccinia horiana* control on chrysanthemum was different when myclobutanil was applied at different concentrations (Bonde et al., 1995).

Similarly like curative effects of fungicides are more pronounced with higher fungicide dose or concentration, higher inoculum level can lead to decrement in efficacy of postinfection fungicide treatment. In cases of higher diseases pressure, multiple infections occur, and disease development is faster. In such conditions, there are less possibilities for a fungicide to efficiently affect all the infection sites. Moreover, faster development of a pathogen within the plant leads to faster accumulation of more mycelia, incubation period is shorter, and sporulation is more rapid. Studies conducted in controlled conditions show such effects. Propiconazole, tebuconazole, myclobutanil, flusilazole, triforine, fenarimol, vinclozoline and iprodione were very effective in reducing blossom blight when applied 48 hours after inoculation of sour cherry with 5000 *Monilinia fructicola* conidia, but they were less effective when plants were inoculated with 50 000 conidia (Wilcox, 1990). Postinfection disease control with azoxystrobin was low when grapevine seedlings were inoculated with million *Guignardia bidwellii* conidia/ml, while it was relatively high when plants were inoculated with 20 000 conidia/ml (Hoffman & Wilcox, 2003). Effectiveness of curative treatments of tebuconazole, cyproconazole, epoxiconazole, flusilazole and prochloraz in control of *Septoria tritici* on wheat was different depending on the environmental factors which have conditioned disease development in different experiments (Schöfl & Zinkernagel, 1997).

It is not easy to evaluate the differences in curative effects among the fungicides from the same chemical groups, or to compare fungicides from different chemical groups. General conclusions regarding the therapeutic potential of a certain fungicide can be made based on its uptake and movement within the plant, mode of action, toxicity to a certain plant pathogen, results of laboratory experiments or field trials, and experience in practice, but the performance of each fungicide can vary depending on specific conditions in which it is applied. Many differences in performance of fungicides are recorded among different active compounds and in cases of different fungal plant diseases. For example, among strobilurin fungicides, azoxystrobin showed better effectiveness than trifloxystrobin or kresoxim-methyl in treatments of pearl millet plants infected with *Sclerospora graminicla* (Sudisha et al.,

2005). In foliar treatments, azoxystrobin gave 97 % of disease control, while trifloxystrobin and kresoxim-methyl gave 92 % and 69 %, respectively. When applied to roots of infected pearl millet plants, azoxystrobin gave 36 % of control, trifloxystrobin about 10 %, while kresoxim-methyl gave only about 4 % of disease control (Sudisha et al., 2005). On the other hand, trifloxystrobin was more efficient than azoxystrobin in reducing fruit rot of apple caused by *Alternaria alternata* when applied 24 or 48 hours after inoculation of fruits with the fungus (Reuveni & Sheglov, 2001). In experiments with strawberry plants inoculated with *Phytophthora cactorum*, azoxystrobin and pyraclostrobin showed mostly the same level of postinfection disease control (Rebollar-Alviter et al., 2007). In several trials, triazole fungicides showed higher effectiveness in postinfection treatments than strobilurin fungicides. Higher levels of disease control was achieved with difenconazole comparing it to azoxystrobin in curative treatments of sugar beet inoculated with *Cercospora beticola* (Anesiadis et al., 2003), and the same was recorded comparing curative effects of tebuconazole, myclobutanil, pyraclostrobin and trifloxystrobin on *Diplocarpon rosae* (Gachomo et al., 2009). When applied to grapevine seedlings inoculated with *Guignardia bidwellii*, myclobutanil provided complete control of lesion development up to six days after inoculation, while azoxystrobin provided only 61 % control of lesion formation (Hoffman & Wilcox, 2003). On the other hand, there are examples of other diseases where better results in postinfection treatments were obtained with strobilurin fungicides than with triazoles. Azoxystrobin significantly reduced severity of *Puccinia graminis* on ryegrass when applied up to 14 days after infection, while propiconazole was effective only up to seven days after infection (Pfender, 2006). The highest reduction of *Rhizoctonia solani* foliar blight on inoculated soybean plants was achieved with pyraclostrobin, followed by azoxystrobin, trifloxystrobin, difenconazole, bromuconazole, tebuconazole, benomyl, thiabendazole, fluazinam and carbendazim (Meyer et al., 2006). Propiconazole and triadimefon significantly reduced *Puccinia hemerocallidis* severity on daylilies when applied up to five days after inoculation, myclobutanil only up to three days after inoculation, while azoxystrobin significantly reduced disease severity when applied even up to seven days after inoculation of plants with the fungus (Mueller et al., 2004). Superior performance of azoxistrobin compared to myclobutanil, propiconazole and triadimefon was also recorded in postinfection treatments of sunflower plants inoculated with *Puccinia helianthi* (Mueller et al., 2004).

An influence of different pathogens on curative effects of fungicides can be showed in examples with the same fungicides applied as curative treatments on plants affected with different diseases. Benomyl was effective when applied up to 72 hours, while fenbuconazole and azoxystrobin were effective when applied up to 48 hours after inoculation of citrus seedlings with *Elsinoe fawcettii*, causal agent of citrus scab. None of the fungicides was effective in postinoculation treatments on melanose, another disease of citrus, caused by the fungus *Diaporthe citri* (Bushong & Timmer, 2000). Triadimefon significantly reduced lesions caused by *Puccinia pelagonii-zonalis* on geranium plants when applied up to seven days after inoculation of plants with the fungus (Mueller et al., 2004). The same fungicide significantly reduced lesions of another rust, *Puccinia hemerocallidis* on daylily, when applied up to five days after inoculation, while significant effect of this triazole fungicide on sunflower rust, *Puccinia helianthi*, was observed only up to one day after inoculation of plants with the fungus (Mueller et al., 2004). Applied one day after inoculation of tomato with *Alternaria solani*, difenconazole showed more than 90 % of disease control, while control level decreased to 60 % when the same fungicide was applied two days after inoculation of plants (Dahmen & Staub, 1992). In the same study, difenconazole completely prevented the

appearance of disease symptoms when applied up to three days after inoculation of apple with *Venturia inaequalis* or inoculation of peanut with *Cercospora arachidicola* (Dahmen & Staub, 1992).
As all the above mentioned studies show, an effect of a fungicide applied as curative treatment is dependent on many factors. In addition, it must be considered that the results of studies performed in controlled conditions can somewhat differ form the results which will be obtained in practical, field conditions. No matter of this, all of the scientific research on therapeutic treatments show what can be expected form the certain fungicide compound in cases of different foliar and fruit diseases.

4. Conclusions

Development of systemic fungicides led to some significant advantages in disease management in modern agriculture. Systemic fungicides, uptaken by the plant, translocated and redistributed within the plant, are able to protect new growth of the plant for a certain period. The performance of such fungicides is generally less influenced by application method, which make their use more practical. Further advantage of fungicides which penetrate into the plant and translocate within it is their potential to affect the parasite already established in the plant, their theoretical potential to "cure the disease". Curative and eradicative effects of fungicides are commonly used in today's agriculture, with a range of clear benefits. The most important one is the flexibility in plant disease control. For a producer, it is usually hard to determine when the infection occurs. Even with the most accurate disease-warning or forecasting system, practical limitations may prevent the treatment to be conducted in optimal period. Being effective after the infection, curative treatments offered much more flexible control of numerous economically important plant diseases. Curative or eradicative activity of systemic fungicides can lead to reduced number of treatments, and can contribute to more economical and ecologically acceptable use of plant protection products. Second, if the symptoms are already present, most of the fungicides which are used for curative treatments show significant antisporulant activity. Reducing the spore production, fungicides reduce an inoculum needed for further disease spread. In such way, curative treatments can contribute to the delay of epidemic phase of the disease, which usually leads to considerable yield losses. Finally, systemic fungicides are often effective in eradication of certain seed-borne pathogens, or pathogens present in bulbs, tubers, stolons, or other types of planting material. Eradicative effects of fungicides contribute to the production and use of seed or planting material of higher quality. In such way, development of systemic fungicides offered some new possibilities in control of seed-borne diseases, in disease management in nurseries, in establishment of field crops, vegetables, ornamentals, orchards or vineyards, as well as in disease control strategies in various areas of cultivation.
Although all systemic fungicides can have a potential therapeutic effect, curative treatments are generally less effective than protective treatments. Considering the biology of plant pathogenic fungi and oomycetes, and some common facts in epidemiology of plant diseases, it is clear that complete eradication of the pathogen in practical conditions is rarely achieved, with some exceptions. Economic constraints regarding the costs are further limiting factors in plant chemotherapy. Therefore, it must be pointed out that no matter of their potential to affect disease development after an infection has occurred or when symptoms have appeared, systemic fungicides show their best effect if applied to prevent an

infection. In such way, strategies for the most efficient disease control with protectant and systemic fungicides mostly remain the same.

Research and development of new fungicides is constant, as well as is exploring for the new possibilities which different fungicidal compounds can offer. Numerous new active ingredients or combinations are introduced to the market, while other are being withdrawn for the ecotoxicological reasons. Different fungicides are being labelled for new uses, and other lose their permission. Various disease-warning systems are being continuously improved. Plant pathogenic fungi and oomycetes continue to change, evolve, and adapt to new agricultural practices, with populations changing, new pathotypes emerging, resistant forms developing and spreading to new areas. Many plant diseases are emerging or becoming more important, while other decline and become less significant. Agricultural practice is advancing, with development of new strategies in disease management. Production is becoming more and more market-oriented, and the number of people producing food and other agricultural products for their own needs is rapidly decreasing. These are the reasons why the possibilities of therapeutic treatments of plants with different fungicides are intensively studies for more than fifty years, and it will continue to be investigated in the future. Numerous studies are conducted on active compounds in chemical companies. Fungicides are subjects of studies in scientific research on universities, institutes, and other scientific institutions. A number of field trials is performed each year by plant pathologists, plant protection experts or agronomists. Such dynamics makes a more complete summary of current knowledge regarding curative and eradicative effects of fungicides almost impossible. Beside this, it must be said that almost all agricultural producers have their own experiences and considerable knowledge about the use of various fungicides in practice, and such experiences are seldom published. Based on their experience, they often know which fungicides are effective if a disease emerged or if there is a risk that an infection has already taken place. In such way, producers often indirectly witness the curative or eradicative effects in the field, without knowing much about fungicide biochemistry, mode of action or interaction with the plant and the pathogen. Research, experiments and experience will contribute to the further advance and benefits in use of curative and eradicative activity of modern fungicides.

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2

Factors Affecting Fungicide Efficacy in the Tropics

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1. Introduction

Fungicide efficacy at tropical conditions is influenced by pathogen population dynamics, abundant vegetative growth of the crops, several pathogen species attacking the whole cycle of the plant. Factors affecting fungicide efficacy can be summarized as the amount of fungicide that reach the plant tissue into a dense canopy, cultural management of the crop, dynamics of pathogen and plant population, and alternative or intermediate hosts management. The result of the fungicide spray is measured by absolute or relative residual. Most agriculturally important crops are sprayed with fungicides from 2 to 10 times every season. Such volume suggests negative impacts on the environment, difficult sustainability of the farmer, costly production system and need for continuous development of the fungicide technology. The most prominent characteristic of any fungicide is the efficacy that is closely related with the residual period along with the range of pathogens controlled. The residual of a fungicide depends on the chemical characteristic of each chemical group. The period of fullest fungicide activity is the absolute residual, while the effective residual will vary according to the interaction among fungicide intrinsic characteristics, stage of the pathogenesis and general physiological conditions of the plant at the moment of the spray.
The tropical environment is optimum for fully fungal development providing stunting pathogenic activity during the whole cycle of a single crop. The synergistic effect showed by some fungicide mix shows longer relative residual than the observed when the fungicides are sprayed alone. In the absence of synergisms the residual observed will depends on the target mostly controlled. The mix of different fungicides is an alternative to avoid resistance risk along with pushing the control do higher efficacy levels.

2. Timing of spray: preventative x curative

The chemical control success depends on the disease pathogenesis stageat any moment when the fungicide is applied. The fungicide application prior any contact between pathogen and host is considered to be preventative. After inoculation and just before initial symptoms, the application is curative. All applications made after the onset of symptoms is eradicative (Figure 1).The preventative spray protects host leaf area from pathogen invasion, providing greater control period and maximizing the residual period. As the infection takes

place and the fungi develop into the plant tissue, the fungicide shows lower efficiency and the residual period decrease (Figure 2).

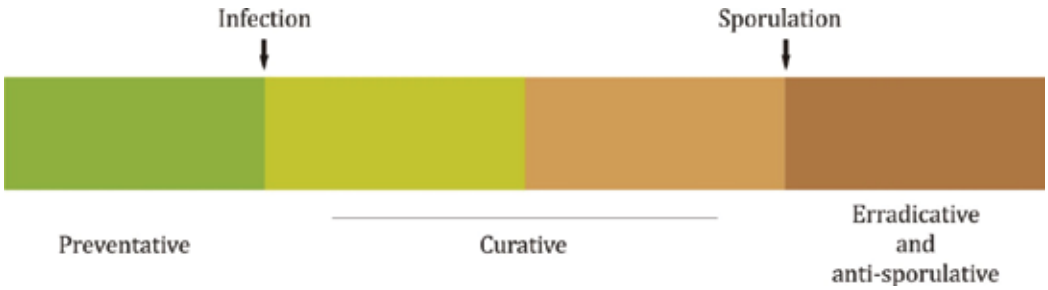


Fig. 1. Timing of fungicide application according to the host-pathogen interaction stages.

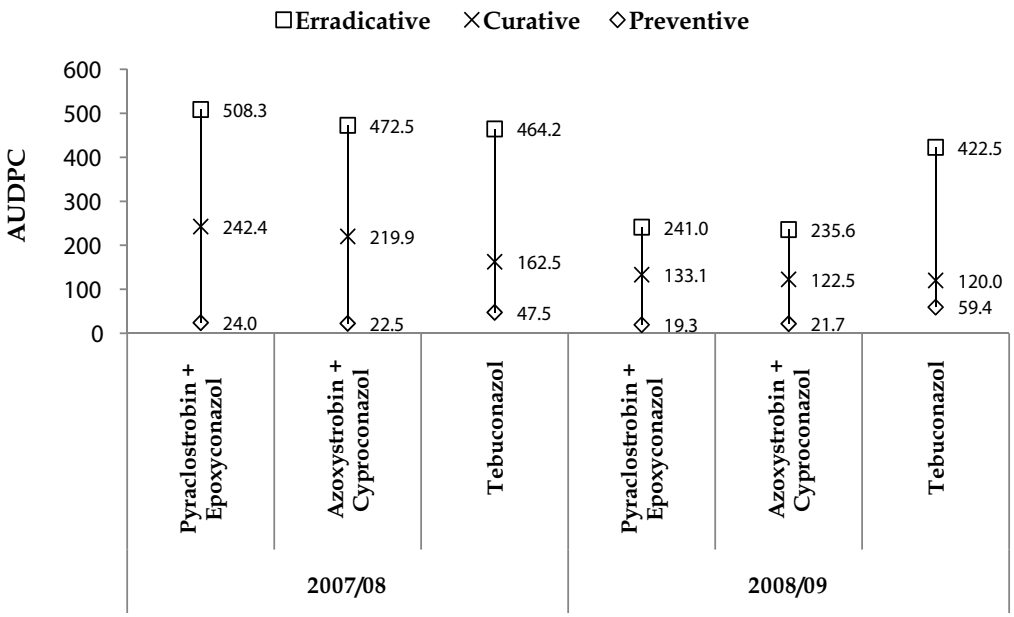


Fig. 2. Area Under Disease Progress Curve (AUDPC) determined for soybean rust after preventative application (14 days before inoculation), curative (3 days after inoculation) and eradicative (9 days after inoculation) of triazole + strobilurin and triazole fungicides.

After disease detection any delay on fungicide application will generate damage and efficacy reduction. Fungicide sprayed to control Soybean Asian Rust(SAR) seven days after first symptoms detection increased the defoliation up to 82%. When the application was 14 days later the defoliation increased by 155% (Andrade & Andrade, 2002).
Fungicide application made at the third day after inoculation(curative timing) reduced SAR severity and the disease progress of the rust, but with compromised efficiency. Higher curative / eradicative effect is observed at 48-72 hours after inoculation (Azevedo, 2001).

Larger differences among active ingredients are also observed at the early stages of the infection. Such difference might be related to the early inhibition efficiency of leaf tissues, or even due to the fungistatic effect.
According to Ugalde (2005) the preventative control gives greater period of effective residual when *Phakopsora pachyrhizi* was inoculated two hours after fungicide spray. Similar results were observed even for inoculations performed at 10 and 14 days after fungicide spray. The period of effective residual of strobys + triazoles varies from 36 to 42 days after application at preventative sprays, reducing to 15 days when the application was done curatively.
The triazole + strobilurin mixture showed greater efficacy and longer residual in preventative applications comparing to the efficacy observed when the single active ingredients were sprayed separately(Andrade & Andrade, 2002;Soares et al.,2004;Ugalde, 2005,Balardin, 2005).

3. Fungicide application: spray technology

The optimum residual period of a fungicide will varies according to host physiological characteristics, pathogen life cycle, and environmental characteristics. However, the correct placement of the fungicide droplet on the leaf tissue will also be decisive.
The establishment of pathogenesis requires a film of water for spore germination, conditions usually observed at the lower portion of the canopy. However, at this part of the plant coverage and penetration by the fungicide tend to be more difficult to achieve. Directly, when the product is placed at the target, and indirectly when the redistribution process takes place systemically.
Studies on droplet size for a particular operation are helpful for better control. This choice depends on weather conditions, leaf area index and plant architecture. A better choice of a spectrum of spraying is related to characteristics of the drops, which are important for canopy penetration and coverage. The spectrum of the spray is characterized by parameters such as volume median diameter (VMD), number median diameter (NMD) and relative amplitude (RA) that allow expressing the numerical size and uniformity of drops.
The VMD is the value expressed in micrometers (mm), which represents the droplet diameter that divides the total into two equal halves, i.e. half the volume consists of sprayed droplets smaller than the VMD and the other half in drops larger than this value. Note that the value VMD is located closer to the upper range of diameter classes, because the volume of a few big drops is equivalent to that of many small droplets.
The NMD is the value also expressed in micrometers (mm) and corresponds to the drop diameter, which divides the total number of droplets in two equal portions, where half the number of drops in the series is larger than the NMD and the other half of the droplets are smaller. Thus, the value of the NMD will necessarily be less than or equal to the NMD because the measure is independent of volume and the small droplets are considered in the same way that large.
Factors influencing the production of a particular droplet spectrum are: nominal flow rate, spray angle, pressure of the liquid properties of the syrup and type of spray tip. A particular point does not produce all the drops of the same size but a size range of droplets. In a spray with droplets of similar size spectrum is considered homogeneous, since with different sizes of droplets produced it is called heterogeneous (Christofoletti, 1999).
Under favorable environmental conditions (temperature <30^{o}C and relative humidity>60%) drops of thin spectrum maximize the penetration and coverage in all parts of the plant,

especially to the bottom part of the canopy (Table 1). However, it should be noted that due to the small size of these droplets, there is a risk of drift due to wind speed above recommended. In the case of large drops, they usually deposit the drops on the upper surfaces and drip of the solution to the soil.
The application with coarse droplet spectrum can be an alternative for applications that target size is small and therefore does not require fine droplets to reach them. Likewise, in regions where the conditions of application are not appropriate due to the high potential risk of drift, the heterogeneous spectrum of drops has good performance, however, must take into account that this type of application provides less coverage of the target.

Spray class	Symbol	Color Code	VMD ASAE (μm)
Very Fine	MF	Red	<100
Fine	F	Orange	100 - 175
Medium	M	Yellow	175 - 250
Large	C	Blue	250 - 375
Very Large	VC	Green	375 - 450
Extremely Large	XC	White	>450

Table 1. Droplet size according to the classification from ASAE S-572.

Coverage is considered the number of drops per unit area (or percentage of covered area), obtained from the spray and the final result of spraying.
The ideal coverage should consider:

- The target to be controlled: diseases are mostly undercover requiring larger area of contact between product and plant. Also, initial infections are mostly established at lower parts of the plant canopy where the number of droplets is reduced. So, after disease established the chance of survival in oculum is as higher as fungicide is sprayed later.
- Fungicide mobility: it varies from none to full mobility into the plant tissue (contact or systemic).Contact fungicides require a direct deposition of the droplet on the target, so there is a need for a large number of droplets/cm^2.Systemic fungicides are absorbed by the leaves and redistributed through the xylem. However, because of the negative balance between mobility and residual period is necessary large number of droplets/cm^2.

The Coverage index (C) is given by the formula $C = 15.V.R.K^2/A.D$, so C = coverage (% of area), V = volume of spray ($L.ha^{-1}$), R = recuperation tax, K = droplets spread, A = surface plant exists in hectare, and D = droplet diameter (Courshee, 1967 quoted by MATUO et al., 2005).
According to the parameters adopted by the C index, to increase coverage just raises the volume of application (V).The recovery rate (R), corresponding to the percentage of applied volume captured by the target is dependent on several factors inherent to the technology of application and timing of application, droplet size and arrangement of plants appropriate. The droplets spread (K) is a quadratic function that can be maximized by adding surfactants to the spray reducing the surface tension of the drop by reducing the angle of contact with the leaf surface.
Besides providing a larger area covered by drop, the reduction in surface tension allows the product to penetrate local and otherwise unable epidermis with dense trichomes. Regarding the denominator, the ratio is mathematically reverse, i.e., coverage will be difficult with the increase of leaf area per hectare, if maintaining the other conditions.

Penetration is the ability to transpose dense layers of foliage, reaching the points of infection or healthy tissue inside the canopy. In general, small droplets penetrate easier the dense canopy of leaves.

During the crop cycle the leaf area index (LAI) increases. Adjustments must be made in the other parameters of the C index formula; otherwise the effectiveness of the application is greatly affected. Balardin (2005) observed better disease control at the end of the cycle with an increase in application volume as it increases the index leaf. Along with adjusting the volume of application (V), the change in drop size (D) and the addition of adjuvants to the spray solution benefiting the droplets spread (K) are possible strategies to be adopted.

Another way that expresses the target coverage is the droplets.cm^{-2}. This parameter is more easily determined either in the form of manual counting or through specific software. It establishes the minimum number of droplets to allow minimum efficacy level of the fungicide. However, this measure varies widely depending on product characteristics and the addition of adjuvants, finding many discrepancies between researchers.

In the specific case of fungicides, Ozeki & Kunz (1998) suggest a minimum of 30-50 droplets.cm^{-2} (systemic) and over 70 (contact). Christofoletti (1999) indicates the need of 30-40 gotas.cm^{-2} (systemic) and between 50-70 (contact). Ugalde (2005) worked with application rates of 120 and 160 L.ha^{-1}and showed a minimum coverage for systemic fungicide of 45 and 60 droplets.cm^{-2}, respectively. Madalosso (2007) observed proportionality between increased row spacing and the coverage and penetration of droplets with greater fungicide efficacy (Table 2).

Treatments		Nº/cm²[2]		AUDPC[3]	
Rowspacing (cm)	Nozzle[1]	Average		Average	
30		13[4]	a	101.33	C
45	XR 110 01	17	b	88.89	B
60		56	c	71.56	A
CV (%)		8.48		11.53	
30		21	a	85.33	C
45	TJ-60 110 02	24	a	62.22	B
60		64	b	53.56	A
CV (%)		5.26		7.35	
30		25	a	89.78	B
45	TXA 8002	26	a	65.56	A
60		53	b	61.89	A
CV (%)		7.62		9.64	
30		5	a	119.56	C
45	Duo (XR+TT)	13	b	80.00	B
60		19	c	65.56	A
CV (%)		8.74		13.63	

[1]Nozzles: XR 11001 (172 kPa), TJ-60 11002 (206 kPa), TXA 8002 (241 kPa)e o Turbo TeeJet® Duo - TT 11002 + XR 11002 (206 kPa) to 200 L.ha^{-1}. [2]Fungicide foliar coverage (number of drops.cm^{-2}. [3]Area Under Disease Progress Curve. [4]Means followed by the same letter, within the variable number of drops.cm^{-2} and AUDPC for each spray tip, do not differ by Tukey test (P≤0,05).

Table 2. Coverage of droplets generated by spray nozzles at spaced 30, 45 and 60 cm between rows and Area Under Disease Progress Curve (*Phakopsorapachyrhizi*).Santa Maria, 2007.

The biological efficacy of the fungicide depends on the quality of coverage and penetration as well as reducing losses through evaporation and drift, induced by droplet diameter. The increase in droplet diameter reduces drag drift by presenting fewer problems with evaporation on the path from the tip to the target, but promotes a lower coverage of the surface being treated.

4. Fungicide application: cultural practices and plant physiology

Soybean cultivars present different physiological characteristics. Cultural management equally extended to all varieties has reduced the crop yield potential, sometimes by a deficiency of the capture of solar radiation by poor distribution of plants in the area, sometimes by residual decline in disease control because of inadequate disposal of product or even both. Uneven distribution of yield components in the canopy generates a concentration of energy drag in the middle and upper part of the canopy. The bottom part of the canopy presents lower physiological influence on the final yield serving as substrate for infection and spread of pathogens. The environmental conditions observed in the bottom part of the canopy of the plants where the temperature undergoes minor changes and the dew has slowed its evaporation, determining a time of leaf wetness exceeding 8 hours per day are ideal for spore germination and infection (Balardin, 2005).These epidemiological parameters are achieved quickly with the density of plants in the area, either through higher densities, close spacing, or both. In this sense, this region of the plant has huge importance on chemical disease control. There are important points that can maximize the residual period of any fungicide application that go beyond the active ingredient, such as understanding the pathogenesis, correct application technology and physiological activity of the plant.

Several authors mention the fact that fast close space between lines result in increased incidence or severity of disease due to microclimate conditions formed within the canopy. Diseases like white mold (*Sclerotiniasclerotiorum*) (Elmore, 2004; Lee et al., 2003), anthracnose (*Colletotrichumtruncatum*) (EMBRAPA, 2006) and Asian Soybean Rust (*Phakopsorapachyrhizi*) (Madalosso et al., 2006) are examples of pathogens favored by higher plants density.

The most appropriate plant arrangement provide adverse epidemiological conditions slowing the rate of disease progress, due to greater distance between the lines, lower relative humidity, higher incidence of radiation, high temperature and consequently low leaf wetness.

The intensification of farming might facilitate the pathogen infection resulting in lower residual of the fungicide. The physical barrier imposed by the high rate of leaf area makes difficult the penetration and coverage of the fungicide on the leaves at the bottom part of the canopy. As a result, the amount of active ingredient can not reach the adequate quantity and quality to maintain the lethal effect on the pathogen (Table 3).

The number and size of droplets.cm^{-2}that reach the biological target determine the effectiveness of the fungicide application. Parameters such as penetration and coverage of the entire plant especially where the onset of the disease, are fundamental. Thus, the reduction in the spacing between lines increasing the number of leaves per m^2,will reduce both the deposition of the fungicide and the residual control.

Several techniques are unsuccessful employed to maximize the deposition of the fungicide within the plant canopy. The alternative is to expose all the foliage of the plant to the fungicide through a better distribution of plants in the area, either by the greater spacing or by the lower plant density.

Row spacing (cm)	Canopy portion	N°/cm²	Rate*
30	Upper	221	3.167
	Medium	15	0.214
	Bottom	11	0.157
45	Upper	214	3.043
	Medium	20	0.286
	Bottom	13	0.186
60	Upper	216	3.093
	Medium	57	0.814
	Bottom	43	0.614

* 70 drops/cm²

Table 3. Penetration and coverage of fungicide in thirds of the plant subjected to three different row spacing. Santa Maria / RS, 2007.

Larger distance between soybean rows maximizes the residual product, regardless of nozzles used, reducing the quantity and final establishment of disease (Figure 3).In this experiment, two fungicide applications were not enough to reduce the amount of disease at 30 cm space row, compared to 45 and 60 cm. Fungicide sprayed on plants at 60 cm space row showed higher residual period along with longer green foliage and higher yield.

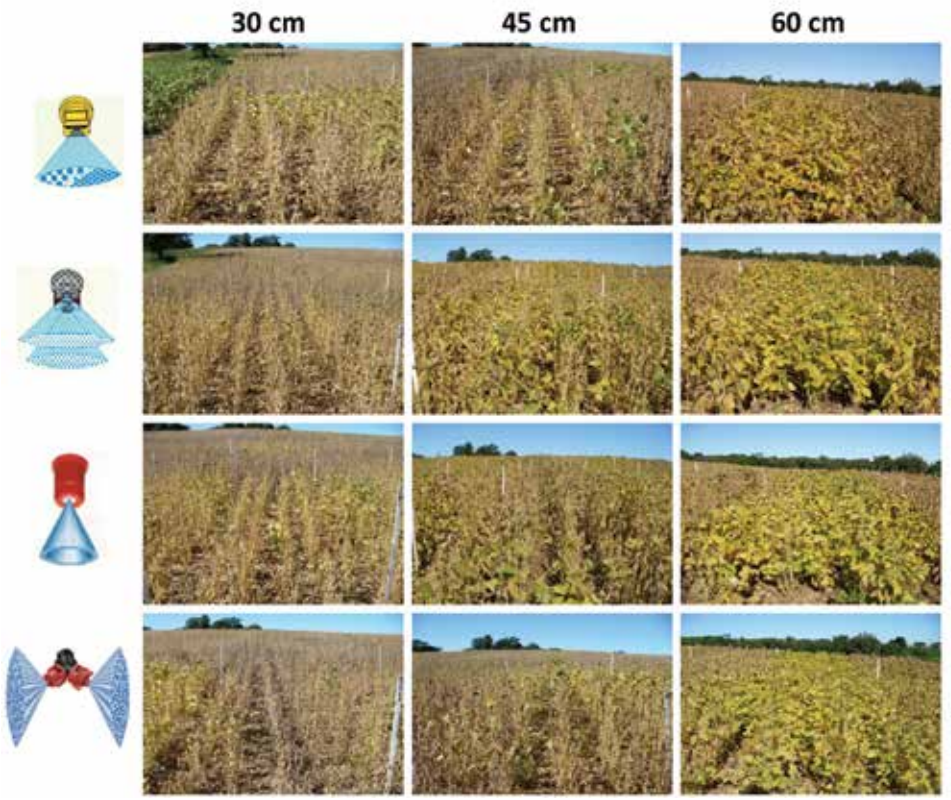


Fig. 3. Control of *Phakopsorapachyrhizi* (A 8000 RG) after two applications of fungicide in three rows using four nozzles (Madalosso & Balardin, 2007).

Adjusting the density of plants according to the plant architecture and leaf area index maximizes the residual of the fungicide, and allows greater physiological expression of the material. An experiment carried out with seven cultivars studied the effect of density of plants on the residual period after two fungicide applications showed the relationship of plant density, architecture and disease development (Figure 4).Besides the lower density of plants corresponding to a larger residual period, this condition allows the photosynthetic activity of the middle and lower will increase the yield. It was observed cultivars with high responsiveness to changes in densities opposite the positioning and pressure chemical disease and others where the variation between 16 and 30 pl/m² did not showed similar result. So, residual control must be built not only focusing on the effectiveness of the fungicide, but also on the cultural management as a whole discouraging the pathogen and the emphasis on active photosynthetic area.

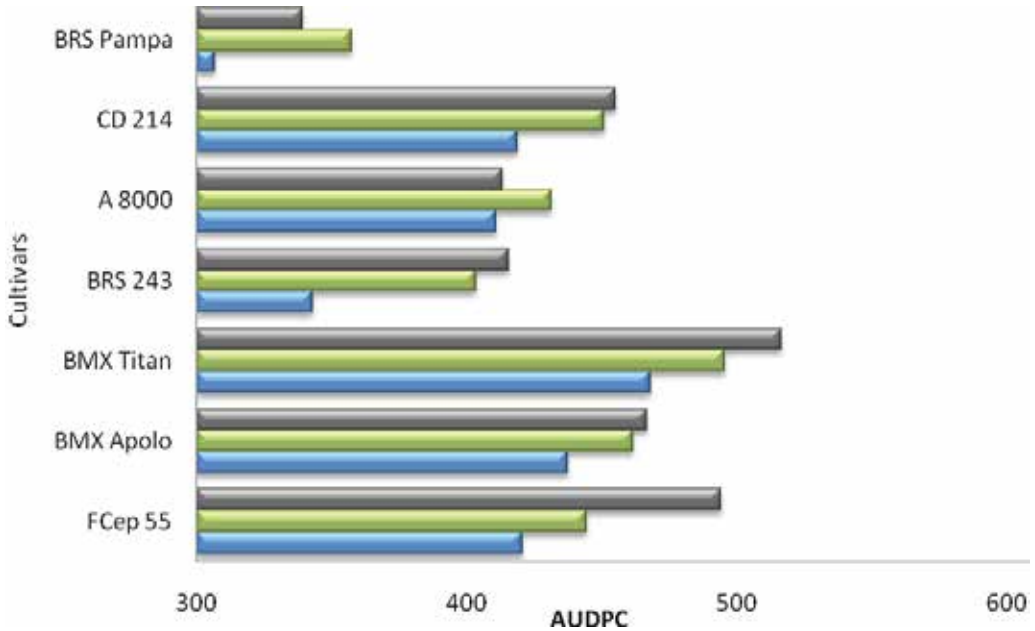


Fig. 4. Area Under Disease Progress Curve (*Phakopsorapachyrhizi*) evaluated on seven cultivars and three plant densities submitted two applications of fungicide. *Gray - 44pl/ha; Green - 30pl/ha; Blue - 16pl/ha.*

The production of soybeans is a function of canopy photosynthetic rate. This, in turn, depends on the amount of solar radiation intercepted and reaches the maximum, around 95% interception (Rodrigues et al., 2002).

The variation of plant density changes the configuration of plant architecture through the formation of branches. In more dense cultures, the proximity of the plants reduces the capacity to issue lateral branches, while a larger distance encourages them to branch (Table 4).

Beyond the capacity of the plant emit branches, leaf area index gained importance in the analysis of product penetration (Table 3) and light inside the canopy. According to Figure 5, seven soybean cultivars responded differently as light penetration, taking into account the

branching and leaf area. These cultivars can be grouped by their physiological plasticity as the presence or absence of response to plant density. The highest penetration of radiation are in a group of cultivars in lower densities in response to better distribution of plants, and another at higher densities, but without the issue of lateral branches (Table 4).There is also a group of cultivars that do not respond to variation in density, pointing to high leaf area as a factor that may be interfering with the deposition of fungicide compromising the residual. In this context, the reduced spacing affect the duration of residual cultivars of this group (Figure 3).

Cultivars	Density		
	160000 plants	300000 plants	440000 plants
FCep 55	3,75	2,30	2,34
BMX Apolo	1,81	0,51	0,02
BMX Titan	3,92	3,09	1,71
BRS 243	3,16	1,64	0,94
A 8000	3,77	1,95	1,16
CD 214	4,56	2,80	1,94
BRS Pampa	2,13	1,01	1,20
C.V. %		17,23	

Table 4. Number of branches per plant of soybean subjected to three plant densities.Santa Maria / RS, 2010.

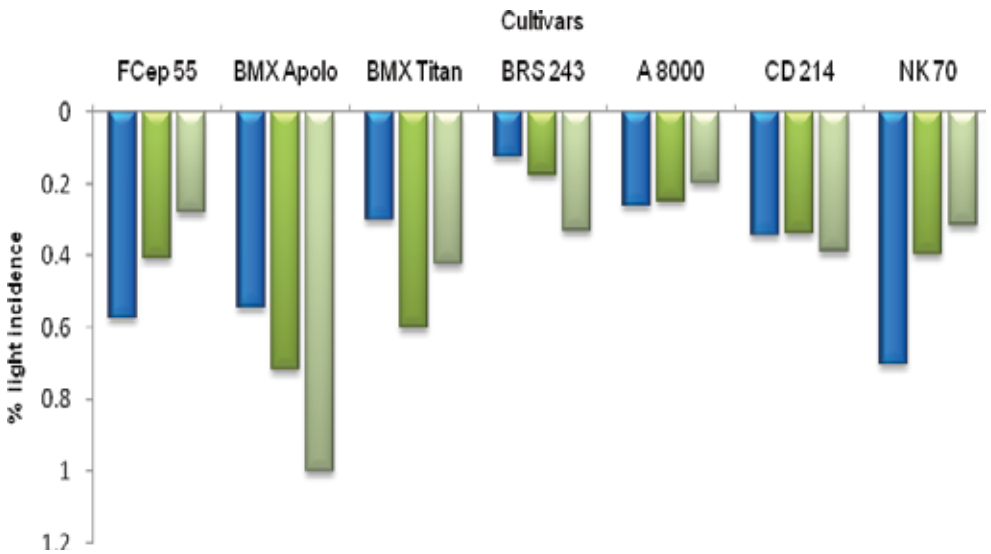


Fig. 5. Percentage of light penetration in the canopy of soybean subjected to three plant densities. *Gray - 44pl/ha; Green - 30pl/ha; Blue - 16pl/ha.*

The maintenance of leaf photosynthetic active in middle and bottom part of the plant canopy promotes higher number of pods in any profile of the plant (Figure 6). For Kantolic and Carmona (2006) the difference between the amount of pods is due to a deficient

distribution of carbohydrates by reducing incident solar radiation. Besides the direct consequence on the pathogenesis, the decrease in total production of pods with the narrowing between the lines is directly related to early defoliation and shading of the lower third, resulting in a decrease in photosynthetic activity, sudden withdrawalthe setting of flowers, formation and grain filling (Taiz; Zeiger 2004; Parcianello et al., 2004).

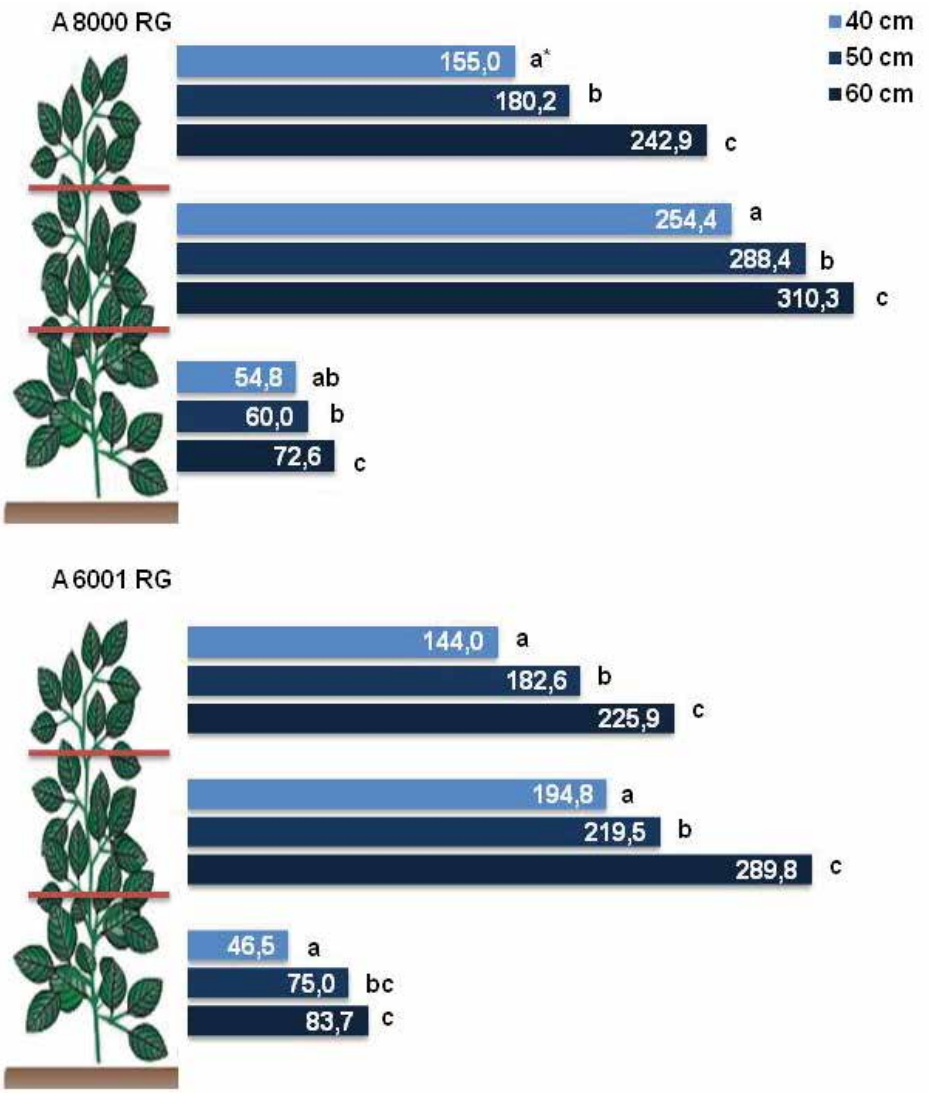


Fig. 6. Number of pods inthe cultivars A 8000 and A 6001 and three row spacing.

Taiz and Zeiger (2004) mentioned that the leaves shaded at the bottom decrease the photosynthetic activity not producing energy for absorption of nutrients. Moreover, the deficit of light allows a predisposition to attack by diseases. The greater vulnerability provided by the deficiency of radiation passes through the change in mechanical barriers

such as plant protective waxy cuticle and stomata index (Martinset al. 1999; Vida et al., 2004).Under lower light, the cuticle tends to be thinner and also a lower deposition of waxes and may facilitate the penetration of pathogens.

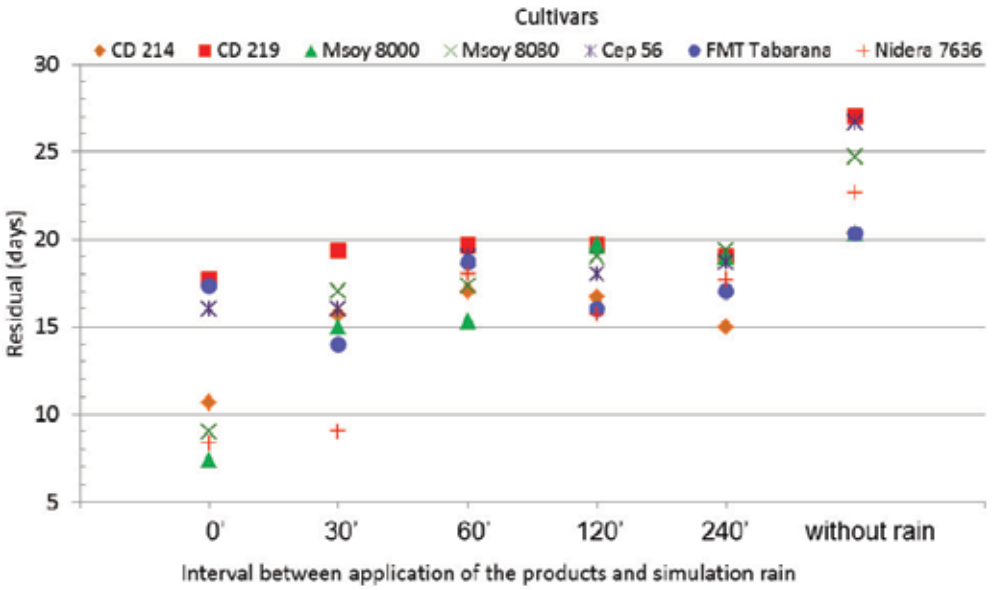
Moreover, the leaves shaded at the bottom decrease photosynthetic activity, reducing the production of photo assimilates (sucrose) and cellular respiration, not producing energy for absorption of nutrients (Taiz & Zeiger, 2004).The velocity of aging of cells is influenced by the reduction in the incidence of light triggering a cascade of physiological processes that will culminate in leaf abscission. One of these components is the phytochrome polypeptide responsible for the perception of light. Basically has two forms, a more stable and inactive form and the other more volatile and active, so the phytochrome may act as "biological switches", activating and deactivating reactions. With the shading of the leaf tissue there is a reduction of the perception of effective radiation, causing the inactivation of phytochrome. The high concentration of inactive phytochrome promotes the expression of genes that lead to the redistribution of hormones such as cytokinin, auxin and abscisic acid synthesis; there was an increase in ethylene concentration sensitizing the abscission layer.

From this new arrangement hormone, the cells of this layer become morphologically and biochemically differentiated by synthesizing and secreting enzymes into vesicles by the Golgi apparatus that will degrade these cells (Taiz & Zeiger, 2004).Thus, there is a disruption of the cells of leaf insertion, been dropped by the wind or by its own weight (Floss, 2004).From the standpoint of production, the earlier the defoliation occurs the smaller the grain size and, consequently, the greater the damage on yields and grain quality (Yorinori, 2004).Thus, understanding the system (pathogenesis, technology application and plant physiology) in addition to the fungicide itself, determines a sum of strategies that maximize the residual control and culminate in increased revenue, strengthening the concept of integrated management of diseases.

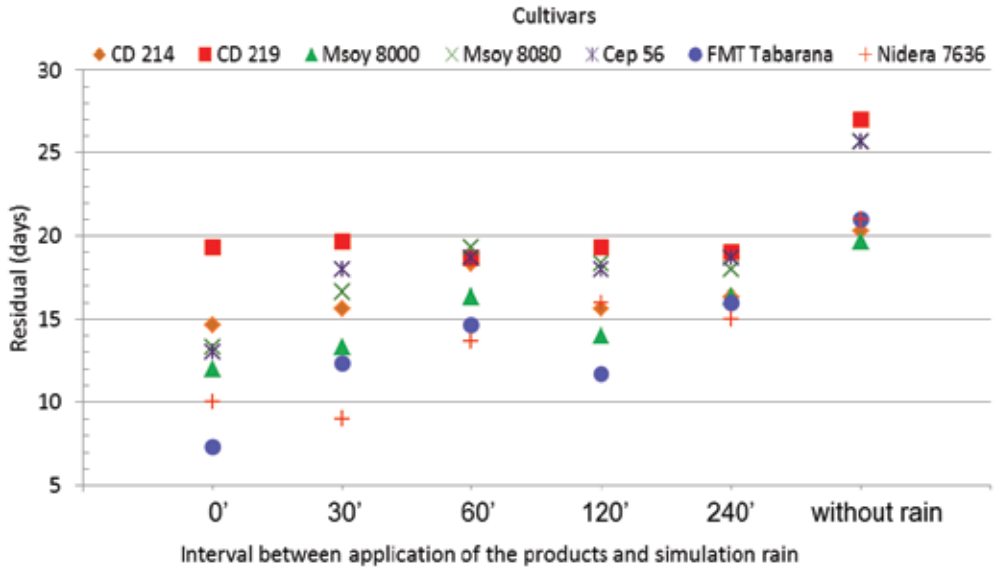
5. Rate of absorption

In contrast to fungicide application, the residual period may be negatively influenced by several chemical factors, physical and biological removal or degradation of the active ingredient in the tissues of plants. The absorption rate of fungicides is dependent on several factors among which may be cited the active ingredient, formulation, chemical group, number of droplets cm^{-2} obtained by spraying, biological target, cultivars, age of tissues and interval between application and rainfall. Among all cases the rain has the greatest effect on residual activity and efficacy of products for foliar application. The occurrence of rain may affect the structure and activity of the product by dilution, redistribution, or physical removal by the removal of tissues.

Rain is considered the biggest detractor of fungicide deposits on the leaf surface and in this context, several factors affect the relationship between rain / residual control of fungicides, the most important being the intensity (mm / hour), quantity, time interval between treatment and rain, the formulation of commercial products, the solubility product in water and the culture in question. Knowledge of the persistence of the products in the tissues after the occurrence of rainfall is essential to optimize use of them. This fact allows us to estimate more precisely their residual activity and helps to establish parameters to define the need for reapplication after rain events.



(A)



(B)

Fig. 7. Residual Control *Phakopsorapachyrhizi* in seven soybean cultivars.
(A) Azoxystrobin + Cyproconazole+ Nimbus; (B)Pyraclostrobin + Epoxiconazole + Assist.

The control of *Phakopsorapachyrhizi* in seven soybean cultivars was studied through the application of Azoxystrobin + Cyproconazole + Nimbus (A) and Pyraclostrobin + Epoxiconazole + Assist (B), both submitted at intervals of 0', 30', 60', 120 'and 240' between the application of the fungicides and a simulated rain (Figure 7). One can see that the residual varies so much depending on the time between application and rainfall simulation as a function of cultivars and fungicides. A reduction of residual period between 7 to 10 days was observed if the simulated rain occurs 2 hours after the spray. Fungicide spray on soybean varieties has showed a difference up to 5 days.

Formulation and adjuvant also influence the rate of absorption of systemic fungicides. In general, fungicides formulated in emulsifiable concentrates more easily transposed to the cuticle layer of tissues, just as adjuvants to more easily dissolve wax layer of the epidermis. The epidermis is the main barrier to penetration of fungicides. The epidermis is composed of a cuticle that coats the leaves with a layer of 0.5 to 14 microns thick, consisting of cutin, intracuticle lipids, polysaccharides, polypeptides and phenols. However, the main barrier to penetration of fungicides is lipids intracuticle.

The epidermis of leaves is not adapted to the absorption of water-soluble substances. The entry of substances in the leaves is processed more efficiently in the lower epidermis in contrast to the upper, facilitated by lower content of lipids intracuticule and greater number of stomata. Likewise, new plant tissue offers less resistance to the absorption of fungicides, since they have thinner cuticles, which will determine a barrier more easily translatable to the fungicide. In addition to this, one should also consider that new plant tissue have a more intense physiological activity.

Control of *Phakopsorapachyrhizi* measured on soybean trifoliate leaves of different ages were submitted to the application of mixture of triazole + strobilurin considering large, medium and fine droplet spectra (Figure 8).It is clearly shown by the analysis of each set of data, comparing the age of first trifoliate leaves, that the number of days of residual increases as you considers the newest plant tissues. This relationship is seen for all droplets sizes and that even with the use of VMD drops high and consequently, a smaller number of droplets cm^{-2}, new plant tissue has higher fungicide absorption. Biological efficiency of mplementation depends on the quality of coverage and penetration and reduces losses by drift and evaporation provided by the droplet diameter.

The droplet size affects the coverage, penetration and deposition thus small droplets provide better coverage, however, they may suffer more easily drift or evaporation and large drops can present problems due to the tendency of flowing and few droplets cm^{-2} with consequent reduced contact between product and plant. In determining the target to be reached, the product must exercise its action on the pathogen that wants to control. The goal of fungicide application is to produce a droplet size that allows good balance of coverage, penetration and droplet deposition. An implementation is one that, when performed correctly, it provides sufficient coverage of the target and it deposits the amount of defense needed to eliminate or slow down safely, a particular problem, so that economic damage is avoided.

Whenever a droplet spectrum is used and provides a greater number of droplets.cm^{-2}a greater coverage of the leaf area is observed and higher absorption and higher rate, which results in greatly increased number of days remaining. Although the younger tissues show higher rates of absorption even with the use of large drops, the greater the difference in increase of residual as they use less drops of NMD.

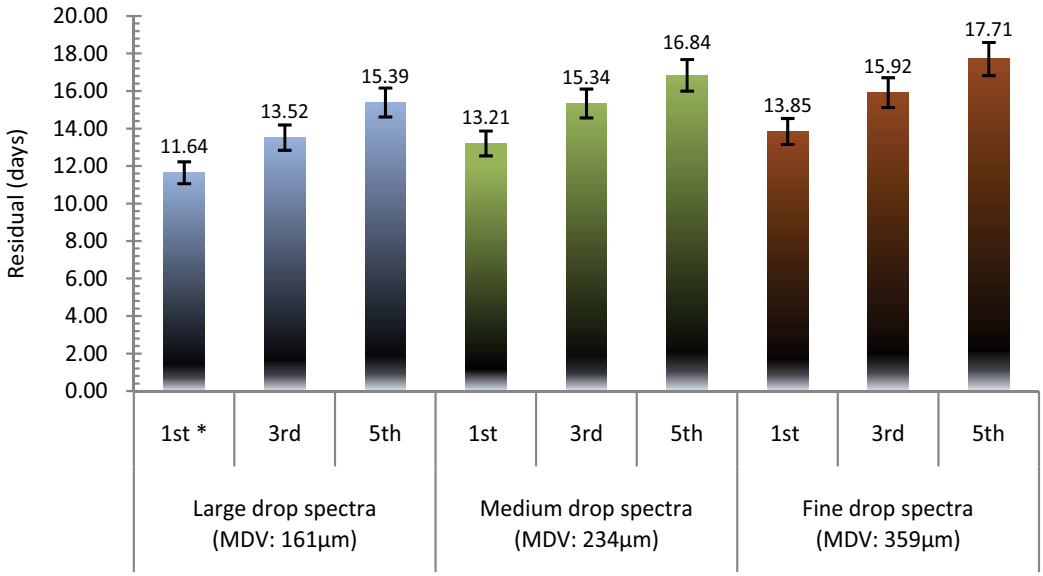


Fig. 8. Residual period to control *Phakopsorapachyrhizi* in trifoliate leaves of different ages undergoing spectra drops large, medium and fine. Santa Maria, 2010.
* 1st: first leaflet; 3rd: third leaflet; 5th: fifth leaflet. The first leaflet represent the oldest.

6. Concluding remarks

The effectiveness of fungicidal spray must be measured considering the gap between absolute and residual period. The combination between intrinsic characteristics of the fungicide and the established strategies of control as the moment of fungicide spray, host and pathogen population density, general characteristics of the plant as age, nutrition cultural crop practices, and the genetics expression of the resistance in the plant are among the main factors regulating the result of the fungicide spray on crops in tropical environment.

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3

Fungicides and Biological Products Activities towards Fungi Causing Diseases on Banana and Vegetable in Côte d'Ivoire

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1. Introduction

Banana and plantain are among important crop playing a major role as respectively exportation crop and foodstuff. Plantain are produced for local consumption and represent the fourth important crop of developing world (Arias *et al*. 2003). African bananas are either grown as a subsistence crop, often in backyard gardens or in intercropped agricultural systems. Besides providing food and nutritional security, bananas, because of their ability to be harvested all year round, produce a surplus and thus constitute an important financial resource for small-scale farmers. On a continental scale, the value of bananas exported from African countries was, in 2005, estimated at US$ 78 millions (FAO, 2006). However, the majority of the crop (up to 85 %) is normally used for local consumption and marketing (Ploetz and Mourichon, 1999). Banana is an important food for the populations in Côte d'Ivoire. Exports of bananas are established in around 360 000 tons (FAO, 2007). The production areas are almost 6 000 ha (Kobenan *et al*., 2006). As for plantain, its production is estimated at 1 510 778 tons and it is classified 3rd foodstuff behind yam and cassava (FAO, 2007). This production makes Côte d'Ivoire the 3rd producer country of plantain in West Africa after Nigeria and Ghana (FAO, 2007), and the principal supplier of the countries in West Africa.

Tomato represents the second vegetable behind the traditional cultivar of eggplant (N'drowa) in Côte d'Ivoire (Ndabalishye, 1995). Tomato is the first condiment in the world according to its alimentary importance and its therapeutics virtues (FAO, 2005; Rao and Agarwal, 2000). The world production of tomato raises 120 millions tons / year according to FAO (2005).

Fungi belonging to *Mycosphaerella* genus cause the most important leaf diseases of banana and plantain (Stover and Simmonds, 1987; Carlier *et al*., 1996; Koné, 2008) worldwide *Mycosphaerella fijiensis* [anamorph: *Pseudocercospora fijiensis* (Stewart *et al*., 1999)] causes black

leaf streak disease (black Sigatoka) and *M. musicola* (anamorph : *Pseudocercospora musae*) is the causal agent of yellow Sigatoka (Stover and Simmonds, 1987). *Mycosphaerella musae* causes a leaf speckle disease which is considered of minor importance except in Australia (Stover, 1972). A more recently identified Mycosphaerella species, *M. eumusae* (anamorph: *Pseudocercospora eumusae*) is the causal agent of septoria leaf spot of banana (Carlier *et al*., 2000). Black leaf streak disease caused by *Mycosphaerella fijiensis* is present in all areas where bananas or plantains are grown. The infection process of these *Mycosphaerella* species is similar, except that symptoms develop faster and are more severe on banana infected with *M. fijiensis* and *M. eumusae* than with *M musicola* (Balint-Kurti *et al*., 2001). Since it was observed in Fiji in the early 1960s (Rhodes, 1964; Mourichon and Fullerton, 1990), *M. fijiensis* has spread rapidly to new banana- and plantain-growing areas, being the most pathogenic and of greatest concern to both commercial banana growers and in countries where banana and plantain are staple crops. Although yellow Sigatoka has been reported to be a significant problem at higher altitudes and cooler temperatures (Mouliom-Pefoura *et al*., 1996), most of the banana and plantain production areas in West Africa are located in lower altitude zones characterized by high temperature regimes. Struggle methods for those diseases are pesticides. In Côte d'Ivoire, Banana leaves are also colonized by *Cladosporium musae* causing Cladosporium leaf speckle and disease development is more frequent on Pisang mas or Figue Sucrée (*Musa* AA), (Koné *et al*., 2006; Koné *et al*., 2007a). *Deightoniella torulosa* considered as a minor disease is more frequently described on plantain (Koné *et al*., 2007b). The fungus can alone induce symptoms particularly on plantain (Koné *et al*., 2007b) and was also described in Savannah (South Georgia) in USA (Kone *et al*., 2008a, b).

Crop protection by using pesticides is one of the most important ways to increase yield in reducing pathogenic fungi impact.

Foliar disease management strategies are focused on agronomic, use of resistant cultivar and chemicals in simple or integrated approaches. The protectant fungicide Mancozeb is primarily used, while systemic fungicides such as propiconazole (Tilt, Bumper and Aurora), tebuconazole (Folicur) and benomyl (Benlate) may be applied during the wet season.

In banana fileds fungicides are used alone or in mixture for foliar disease control. In Côte d'Ivoire several companies are involved in fungicides commercialization (Table 1). Nowadays, *Mycosphaerella* pathogens are controlled by developing an integrated approach to control the pathogens. Disease control strategies are focused on *Mycosphaerella fijiensis*, the most foliar pathogenic fungi in Côte d'Ivoire banana field (Koné *et al*., 2008c). Several applications of fungicides occur according to the geographic regions base on monitoring program consisting of a field survey to detect resistance. Fungicides application is related to disease evolution.

Fungicides belonging to triazoles (propiconazole, Bumper), benzimidazoles (Peltis), Strobilurin (Trifloxystrobine), Spiroketalamins (Spiroxamine) are the essential products used for the control of banana foliar diseases. There is a concern to control Cladosporium leaf speackle and leaf black spot caused by *Deightoniella torulosa* because any fungicide was recommended and evaluated against *Cladosporium musae* and *Deightoniella torulosa* exept the works of Kone *et al*. (2008c) and Camara *et al*. (2007 and 2010). To recommend fungicides, investigations were performed *in vitro* using synthesis and biological fungicides (Camara *et al*., 2007, Koné *et al*., 2008c; Camara *et al*., 2010).

Tomato production is strongly influenced by some viruses, bacteria and soil born fungi in Côte d'Ivoire. The severity of soil born fungi parasites become more important in view of

Groupe	Commercial name	Active compound	quantity	Recommended dose
Systemic, IBS				
Triazoles	Tilt 250 EC/ Bumper 25 EC Référence 250 EC	Propiconazole	250 g/l	0.4 l/ha
	Folicur 250 EW-Junior 250 EW	Tébuconazole	250 g/l	0.4 l/ha
	Opal 7.5 EC	Epoxiconazole	75 g/l	1 l/ha
	Bayfidan 250 OL	Triadimenol	250 g/l	0.4 l/ha
	Sico 250 EC Difecor 250 EC	Difénoconazole	250 g/l	0.3 – 0.4 l/ha
	Trical 250 EC	Triadimefon	250 g/l	0.4 l/ha
	Eminent	Tétraconazole	250 g/l	0.4 l/ha
	Punch 40 EC	Flusilazole	400 g/l	0.25 l/ha
Benzimidazoles	Peltis 40/ Callis 400 OL			
	Fungis 400 SC	Méthyl-thiophanate	400g/l	0.7 - 1 l/ha
	Benlate OD / Flash OD	Bénomyl	500 g/kg	0.3 - 0.5 kg/ha
Strobilurines	Bankit	Azoxystrobine	250 g/l and 0.4 l/ha	1 l /ha
	Téga 075 EC	Trifloxystrobine	75 g/l	1 l /ha
Spiroketalamines	Impulse 800 EC	Spiroxamine	800 g/l	0.4 l/ha
Penetrants, IBS				
Morpholines	Calixine 75 EC	Tridémorphe	750 g/l	0.6 l/ha
	Volley 88 0L	Fenpropimorphe		0.5 l/ha
Protectants				
Anilino-pyrimidines	Siganex 600 SC	Pyriméthanil	600 g/l	0.5 l/ha
	Banko 720 SC	Chlorothalonil*	720 g/l	1 - 2 l/ha
Dithiocarbamates	Antracol 70 WG	Probineb	70 g/l	2 - 3 Kg/ha
	Ivory 80 WP / Dithane F 448 SC	Mancozèbe*		2 kg/ha

EC : Emulsionnable EW : Emulsion OL : liquid dissolve in water SC : Concentrated suspension
OD : Powder disperse in water WG: Autodispersible compounds
*: Fungicides use for both vegetables and banana diseases.

Table 1. Fungicides recommended to control black leaf streak disease of banana in Côte d'Ivoire

damages in field (Soro *et al.*, 2008). Nevertheless it is difficult to practice gardening market in the tropical countries because there are a lot of pathogens including *Pythium*, *Fusarium* and *Macrophomina* (Soro *et al.*, 2008).

In San José, there was much discussion about the potential of new fungicides, rational ways of using them, the advantages of forecasting systems, and the management of resistance to

fungicides (Jacome *et al*., 2003). It was emphasized that effective and rational control required a greater knowledge of different aspects of the pathogen epidemiology. The use of fungicides remains the strategy by which other strategies are compared. In the past, the selection pressure by different active ingredients has given rise to the disastrous situation where fungicide-resistant pathotypes are continuously selected. This strategy is no longer acceptable in a society increasingly concerned about the environment (Romero, 2007). The pesticides become more important to control the diseases and pests. There is increasing public concern over the level of pesticide residues in food. The bad utilization of pesticides causes the apparition of some resistant viruses, bacteria and fungi, why it is necessary today to found alternative products to control pests (Rose *et al*., 2003; Punja, 2003). This concern has encouraged us to look for other solutions instead of synthetic pesticides in the second hand. Recently there has been considerable interest in GRAS (generally regarded as safe) compounds. Naturally occurring biologically active compounds from plants are examples of GRAS compounds. These plant extracts are generally assumed to be more acceptable and less hazardous than synthetic compounds. This means that essential oils could be used as alternative anti-fungal and anti-bacterial treatments for fresh produce. The potential for these types of plant extracts is considerable. It is a resource that has not been fully explored.
In the majority of the tropical countries, forest destruction and agriculture cause a strong reduction of forest cover. The majority of the market gardening is practiced on fallow; what makes the pressure parasitic very strong in tomato culture in Côte d'Ivoire. The use of the natural extracts in the control of the parasites of the cultures will make it possible on the one hand to reduce the degradation of these ecosystems and on the other hand, to improve the vegetable productions (Bakayoko, 2005; Ouattara, 2006; Makumbelo *et al*., 2008). In order to contribute to the reduction of the degradation of the wooded areas, some woody species were compared to the fungicides assessed *in vitro* and *in vivo* for their antifungal activities against three telluric fungi strains (*Pythium*, *Fusarium* and *Macrophomina*) in tomato culture. Some authors showed that the powder extracts and essential oils are capable to repress the parasites of the cultures (Smolinska and Horbowicz, 1999; Smolinska, 2000; Soro *et al*., 2008; Soro *et al*., 2010).
This book chapter is first a contribution about synthetic fungicides and biological fungicides evaluated against foliar fungi of banana and recommended for the control of vegetables diseases by using natural products.

2. Material and methods

2.1 Material

2.1.1 Pathogenic fungi

Banana

Pathogenic fungi of banana including *Mycosphaerella fijiensis*, *Deightoniella torulosa* and *Cladosporium musae* isolated from plantain have been used to assess funigicide and biological products activities.
Vegetables
Four fungi including *Pythium aphanidermatum*, *Macrophomina phaseoli*, *Fusarium oxysporum* f. sp. *radicis lycopersici* and *Sclerotium rolfsii* were used. *Pythium aphanidermatum* was characterized by a whitish thalle constituted of non partitioned mycelial filaments (Fig. 1 A and E). *Fusarium oxysporum* f. sp *radicis lycopersici* (Forl) has a purplish red thalle on the PDA

culture medium (Fig. 1 B). To the microscopic level, it presents the macro or microconidies (Fig. 1 F) and of the chlamydospores. *Macrophomina phaseoli* is a fungus that presents a blackish gray thalle on the PDA culture medium (Fig. 1 C). To the microscope, the fungus doesn't present any spores but of the microsclerotes (Fig. 1 G). *Sclerotium rolfsii* present the filaments of flaky aspect of white coloration on the PDA culture medium (Fig. 1 D). These filaments condense to give white mycelia with brown or black coloration sclerotes (Fig. 1 H).

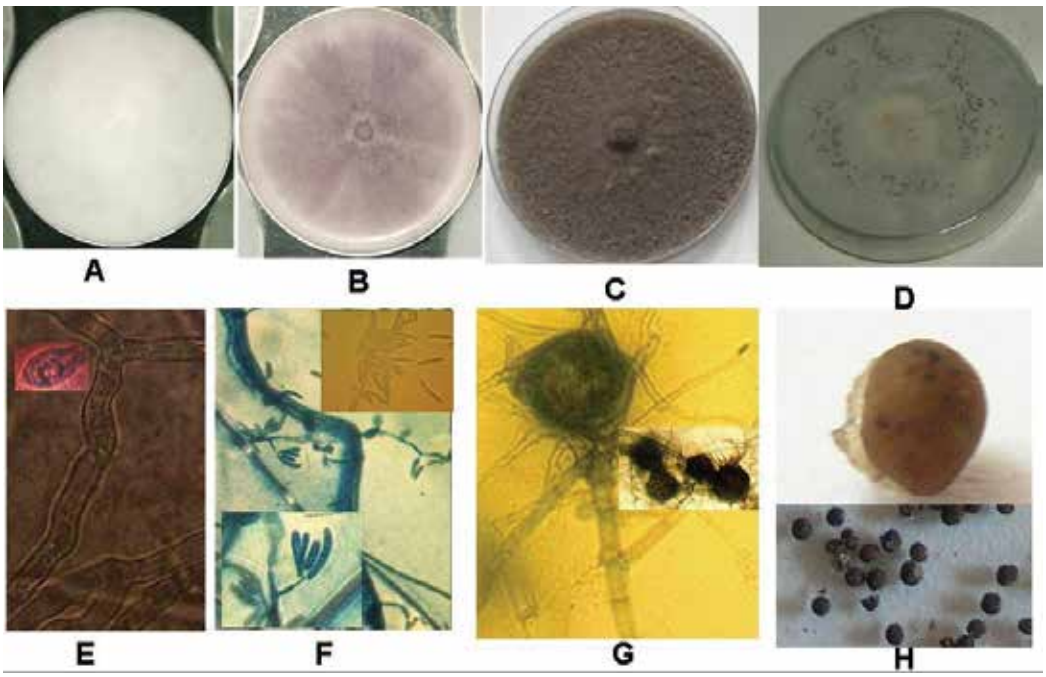

Fig. 1: Fungi isolated from tomato roots and stem at Songon in South Côte d'Ivoire
A: Thalle of *Pythium aphanidermatum*; B: *Fusarium oxysporum radicis lycopersici*; C: *Macrophomina phaseoli*; D: *Sclerotium rolfsii*; E: Mycelial filament of *Pythium* sp.; F: Conidies of *Fusarium oxysporum* f. sp. *radicis lycopersici*; G: Sclerotes of *Macrophomina phaseoli*; H: Sclerotes of *Sclerotium rolfsii* brown or black.

2.1.2 Fungicides products

2.1.2.1 Banana

Fungicides used *in vitro* and in field traitements in Côte d'Ivoire used to control Mycosphaerella disease were listed in table 1. The essential oils of *Monodora myristica, Eucalyptus torelliana, Eucalyptus platyphylla, Melaleuca quinquenervia, Eucalyptus citriodora* and *Ocimum gratissimum* were assessed *in vitro* against *Deightoniella torulosa* and *Mycosphaerella fijiensis*.

2.1.2.2 Vegetables

Antifungal chemicals
Two fungicides of synthesis have been used during all these experiments; "Banko-Plus" of which the formulation of basis is the chlorothalonil (550 g/L) more of the carbendazime (100

g/L) in the dose of 1.5 L/ha and Ivory 80WP whose chemical formula is the Mancozeb (80%) with the "Callicuivre" composed of 56% of cuprous oxide used to the dose of 2 kg/ha.
Antifungal natural products
Ten plants were tested (Table 2). These ten (10) natural extracts were ranged in 3 basic groups: spices plants including *Peper guinense* L., *Xylopia aethiopica* L., *Zingiber officinalis* L.; aromatic plants such as *Ocimum basilicum* L., *Ocimum gratissimum*, *Melaleuca quinquenervia* L. and non spicy and non aromatic plants are *Cola nitida* L., *Cola acuminata* L., *Combretum racemosum* L., *Ricinus communis* L. These plants were used in oil, powder and acqueous extracts formulation.

Scientific name	Family	Gone of the used plant	Place of the harvest	Excerpt produces
Xylopia aethiopica (Dunal) A. Rich.	Annonaceae	fruits	Bingerville	powder / essential oil
Peper guinense Schum et Tonn.	Piperaceae	fruits	Bingerville	essential oil
Ocimum basilicum L.	Labiaceae	leaves	Agnibilékro	essential oil
Ocimum gratissimum L.	Labiaceae	leaves	Dabou	essential oil
Melaleuca quinquenervia L.	Myrtaceae	leaves	Abidjan	essential oil
Zingiber officinalis L. (Roscoe)	Zingiberaceae	rhizomes	Divo	powder / essential oil
Combretum racemosum P. Beauv	Combretaceae	leaves	Dabou	powder
Cola nitida (Vent.) Schott & Endl	Sterculiaceae	walnut	Anyama	powder
Cola acuminata (Vent.) Schott & Endl	Sterculiaceae	walnut	Anyama	powder
Ricinus communis L.	Euphorbiaceae	seeds	Abidjan	siccative oil

Table 2. Plant species used for the antifungal tests

2.2 Methods

2.2.1 Banana

2.2.1.1 Field traitement and observation

Fungicides applications were performed by using air plan. Observations consisted of disease evolution based on symptoms presence on the leaves.

2.2.1.2 *In vitro* activitiy of fungicides against *Mycosphaerella fijiensis, Cladosporium musae* and *Deightoniella torulosa*

Tests were performed first against *Mycosphaerella fijiensis.* Propiconazole was used at recommended concentration (0.4 L/ha) followed by testing concentrations 1.5 or 2 times

inferior to the recommended one. According to the different concentration, tests were performed with 4 fungicides. Because of total inhibition due to the synthetic fungicides in relation with active ingredient of each product, stock solutions were prepared at 100 ppm., 10 ppm., 1 ppm. After autoclaving media, stock solutions were used to amend media to the following final concentrations: 0.01 ppm., 0.05 ppm., 0.1 ppm., 0.5 ppm. and 1 ppm. Seventeen millilitres of amended media were poured in 9 cm diameter plates. Susceptibility of each fungus was evaluated by measuring mycelium growth of each colony. A mycelium disk of 6-mm-diameter was removed at the edge of the colony and placed at the center of plate containing amended medium. Plates with non amended medium were used as control. Five plates were used for each fungicide. Inhibition rate was estimated. Experimentations were repeated 3 times.

2.2.1.2.1 In vitro activitiy of essential oils against Mycosphaerella fijiensis and Deightoniella torulosa

In the case of foliar diseas of banana, PDA culture medium was autoclaved and cooled in a water bath to 40 °C. The essential oils were mixed with sterile molten PDA to obtain final concentrations of 1000 ppm, 3000 ppm, 5000 ppm, 7000 ppm and 10000 ppm. The PDA was poured into 9 cm plates (15 ml/plate).

2.2.1.2.2 Measurement of mycelia growth and germination of the spores

Susceptibility of each fungus was evaluated by measuring mycelium growth of each colony. A mycelium disk of 6 mm diameter was removed at the edge of the colony and placed at the center of plate containing amended medium. Plates with non amended medium were used as control. Five replicate plates were used for each fungicide, natural extracts or essential oils. The plates were incubated at 28 °C and photoperiod 12/12. The colony radius was measured every 24 h for ***Deightoniella torulosa*** and 72 h for ***Mycosphaerella fijiensis***, excluding the plug. An average was taken of two measurements made on each plate. The assessment of the inhibition rate was estimated. Experiments were repeated 3 times. The rate of inhibition was calculated using the following formula (Hmouni *et al*., 1996):

$$\text{Inhibition rate} = (To - E / To) \times 100 \quad (1)$$

To = mean value of control treatments radial growth
E = mean value of assay treatments radial growth
The minimal inhibition concentration (MIC) is calculated from the smallest concentration that inhibits the mycelial growth of every fungus. The sufficient concentrations to kill the resumption of the mycelial growth have been used to calculate the lethal doses of CI_{50} and CI_{90} with the help of the linear equations of inhibition of the mycelial growth according to the reviewed formula of (Paranagama *et al*., 2003). Experiments were performed three times.
Activity of fungicide has been evaluated on spore's germination using spores suspension calibrated to 10^5 ou 10^6 conidia/ml. Different dilutions were realized until the final dilution estimated to 10^2 conidia/ml. Eight hundred µL (800 µL) of this final dilution were spread out on plates containing amended or non-amended medium and incubated at 28 ± 2 °C. Germination was evaluated in counting 20 spores among those germinating conidia were counted. The rate of germination has been evaluated by the percentage of germinating conidia. For mycelia or conidia germination, the concentration that inhibits 50% was calculated by lenear regression.
For *Deightoniella torulosa,* after 21 days of culture growing on various media, each plates according to the concentration of the fungicides is scraped using a curved Pasteur pipette, in

the presence of 10 ml of distilled water. A drop is then taken and assembled between blade and plate evaluated qualitatively to under the optical microscope the presence of spore.

2.2.2 Vegetables

2.2.2.1 *In vitro* evaluation on mycelial radial growth

Concerning pathogenic fungi of vegetables, five concentrations (1, 2, 4, 6, 8 g/L or 250, 500, 1000, 2000, 4000 ppm) have been kept either for the excerpts of powder or for the essential oils according to the behavior of the different fungi strains (Neri *et al*., 2006). A mycelial disk of 5 mm was removed at the edge of the colony and placed in a plate of 9-cm-diameter containing 20 ml of PDA. The set of these plates kept at 27±2 °C, under photoperiod 12 h during 7 days (Paster *et al*., 1993). At the end of the 7th day, the plug was transfert on a new medium and the product is fungicide if there is a not repulse mycelial. In the contrary case, product is said fongistatic. This experiment has been repeated 3 times (Neri *et al*., 2006; Hmouni *et al*., 1996). Measurement of the mycelia growth is done as describe in the case of banana.

2.2.2.2 *In vivo* evaluation of natural products

The seeds of the three varieties of tomato have been treated to the ethanol 70% during 3 minutes then they have been rinsed with the sterile distilled water 3 times during 3 min for every rinsing. Under the hood in presence of a flame, 25 seeds have been deposited on the paper blotter in plates of 11 cm of diameter. The seedling is kept at the steamroom in 27±2 °C to the obscurity and watered once per day until the apparition of the leaves (Hibar *et al*., 2005). To this stage, the seedlings are planted out in containing ferries of the sterile soil of Songon-Dabou and conserved under shelter at the ambient temperature until the transplantation.
The fungal inoculum has been deposited directly all around of the hypogeal part of the stem of the tomato plant. Five plants out of the ten that account every variety have been inoculated with 10 ml of mycelial solution of each of the three fungi. The seedlings of the same age and the same plant species are planted out in ferries control containing the same soil sterilized two times at 121 °C during 30 min to the pressure of 1.5 bars.
Control with the pesticides were performed using 4 weeks old seedlings.
The synthesis fungicides, Mancozeb and Banko-Plus (the control positive) are used to the concentrations of formulation for the set of the three fungi strains.
In using powder of plant, the non spicy plant extracts (*Cola nitida* and *Combretum racemosum*) was brought to 25 g/dm^3 whereas those of the plant to spices (*X. aethiopica* and *Z. officinalis*) were brought of 10 g/dm^3.
The essential oils (*Xylopia aethiopica, Zingiber officinalis and Ocimum gratissimum*) are brought all to the concentration of 20 µl/dm^3. The seedlings are treated by 20 µl/dm^3 of the essence homogenized in 100 ml of "Tween 20" and the whole is mixed in 900 ml of distilled water. The treatment consists in watering the root system of the plant with 100 ml of this solution. The death rate has been valued 30 days after the inoculation. The rate of the dead plants served to value the infectious potential of the different fungi. This experience was repeated 3 times.

2.2.2.2.1 Index of withering (IW) and index of mortality (IM) of the tomato plants in greenhouse were recorded to assess product efficiency.

The assessment of the symptoms is achieved 30 days after the transplantation of the seedlings (Woo *et al*., 1996) while being based on a scale of notation of the symptoms

proposed by Vakalounakis & Fragkiadakis (1999) and that understands four active securities of zero to three:
0 : healthy plant,
1 : light yellowing, light rot of the pivot and the secondary roots and rot of the collar,
2: yellowing important of the leaves with or without withering, stunted of the plants, stern rot of the pivot and the secondary roots, rot important of the collar and brown discoloration of the vessels of the stem,
3: dead of the plant.
These notations acted as basis to calculate the index of mortality that corresponds to the average of the securities assigned to the ten plants (number of repetition by elementary treatment). Besides, the percentage of the plants that has a notation of the symptoms superior or equal to 2 is taken like criteria to value the severity of the attacks of *Fusarium*, *Pythium* and *Macrophomina*. The severity of the mycopathogen is valued like follows:

- fungus strain is said very virulent when the index of mortality is between 50 and 100%, the cultivar is said very sensitive;
- fungus strain is said virulent when the index of mortality is consisted between 20 and 50%, the cultivar is said sensitive;
- fungus strain is said fairly virulent when the index of mortality is between 10 and 20%, the cultivar is said moderately resistant;
- fungus strain is said little virulent when the index of mortality is between 5 and 10%, the cultivar is said resistant;
- fungus strain is said very little virulent when the index of mortality is between 1 and 5%, the cultivar is said very resistant;
- fungus strain is said non virulent when the index of mortality is equal to 0 and the cultivar is said immune according to the formula modified of Bambang (1987). The experiment has been repeated three times.

2.2.2.2.2 Dry biomass

The dry biomass foliar and root is valued 60 days after the seedling to the level of the biologic struggle test. The plant matter is put to dry at the steamroom in 105 °C until obtaining of a constant weight on 3 days (Woo *et al*., 1996).

2.3 Stastitical analysis

Data were first tested for normality and then subjected to analysis of variance (ANOVA). Significant differences between values were determined using Newman-keuls test ($p<0.05$), following ANOVA. Statistical analysis was performed using SAS V7 and graphs were produced using Microsoft Office Excel.

3. Results

3.1 Control of Banana foliar diseases

3.1.1 Field disease control strategies against banana leaf spot diseases in Côte d'Ivoire

Disease control by fungicide application varies according to geographic location because of the monitoring program. The number of application of fungicides is also different according the wet or dry season. The number of fungicide application is higher in the wet season. During black sigatoka disease evolution, a number of measures are implemented to ensure

the pathogen. Agronomic practices include plant destruction by burning the leaves in order to reduce the sources of inoculum.
In commercial plantation, foliar disease management is undertaken through combine methods including cultural and chemical practices. Due to the higher cost of hemical and the lack of sufficient knowledge, small holder farmers do not use them. An important diversity of systemic fungicides is available according to their efficacy and the distribution. In Côte d'Ivoire, fungicides used against Sigatoka disease, particularly black leaf streak disease are more utilised. All the fungicides available were only evaluated and homologation is focused on *Mycosphaerella fijiensis*. Resistance to protectant fungicide is lower due to the multisite action of these products. Protectant fungicides are well efficient on pathogen present on the leaves at the moment of their application.The lack of systemic activity make them more efficient during a time exceeding a week. Mancozeb and chlorothalonil belonging to the protectant fungicides groups are particularly used alone or in mixture in the strategy to ovoid resistance strains appearance.
Sytemic fungicides contain diverse actives coumpounds that can remain 14 days in humid period and 28 days during dry period after application. Fungicides belonging to benzimidazoles are different.
Benzimidazoles containing benomyl (Benlate) and methyl-thyophanate (Peltis, Callis) are the first systemic fungicides used in 70's for the control of black leaf streak disease caused by *Mycosphaerella fijiensis*. The fungicides are unisite inhibitors and resistant strains have been detected in Côte d'Ivoire.
Morpholines represent the second group of systemic fungicides commercialized in 80's and used in Côte d'Ivoire is represented by Tridemorph (Calixine) as active coupounds.
Fungicides used are ergosterol biosynthesis inibitors. Several products possessing different compounds have the same inhibition site that consiste to inhibit cytochrom P-450. The inhibition leads to eliminate methyl groupment in position 14.
Products containing propiconazole (triazole fungicide) are systemic and have a good activity. Propiconazole shows more efficacy in june where inoculum development begining in may could be a factor of severity because of humidity and night temperature elevation.
Fungicides containing Tebuconazole are systemic and can easily spread out on the leaf surface.
Fungicides belonging to strobulirines have two active compounds including azoxystrobine (Bankit) and Trifloxystrobine (Tega). Trifloxystrobine is conidia germination inhibitor. Disease evolution is stopped and the product reduces inoculum level.
Fungicides of Spiroketalamine family are considered as a new group recommended to the control of sigatoka disease. Spiroxamine is one of the fungicides having preventive and curative action.

3.1.2 *In vitro* essay

3.1.2.1 Fungicide effect

3.1.2.1.1 Inhibition of mycelial growth

Mycelium growth is inhibited at the recommended concentration that is 0.4 L/ha) and also at fungicide amount reaching 1.5 to 2 times inferior to recommended concentration and 1.5 times superior to recommended amount (Fig. 2 A, B and C). The inhibition rate is 100 % for all the fungi whatever the concentration. After 3 weeks, all the plugs that have been inhibited on

media amended with differents concentrations of propiconazole did not grown after transplantation on a new medium without fungicide. The inhibition rate of mycelium becomes higher when the product concentration is higher. Media amended with tebuconazole, trifloxystrobine and propiconazole gave higher inhibition rate compare to media amended with spiroxamine (Fig. 2 A and B). A total inhibition of *Mycosphaerella fijiensis* mycelium reaching 100% at 0.5 and 1 ppm that do not cause inhibition in *Cladosporium musae* were observed (Fig. 2 A and B). The inhibition of mycelium growth reaching 50 % is inferior to 0.6 ppm and is 0.14, 0.4 and 0.52 ppm respectively for tebuconazole, trifloxystrobine and propiconazole (Table 3). Spiroxamine gave less inhibition on *Cladosporium musae* and *Mycosphaerella fijiensis* compare to other molecules. Reduction of *Cladosporium musae* mycelium growth can reach 70 to 80% on media amended with trifloxystrobine and propiconazole (Fig. 2 B). Inhibition rate of *Deightoniella torulosa* mycelium can reach 15 to 70% and 0.5, 0.63 and 0.72 are the inhibiting concentration that can affect 50% of the mycelial growth.

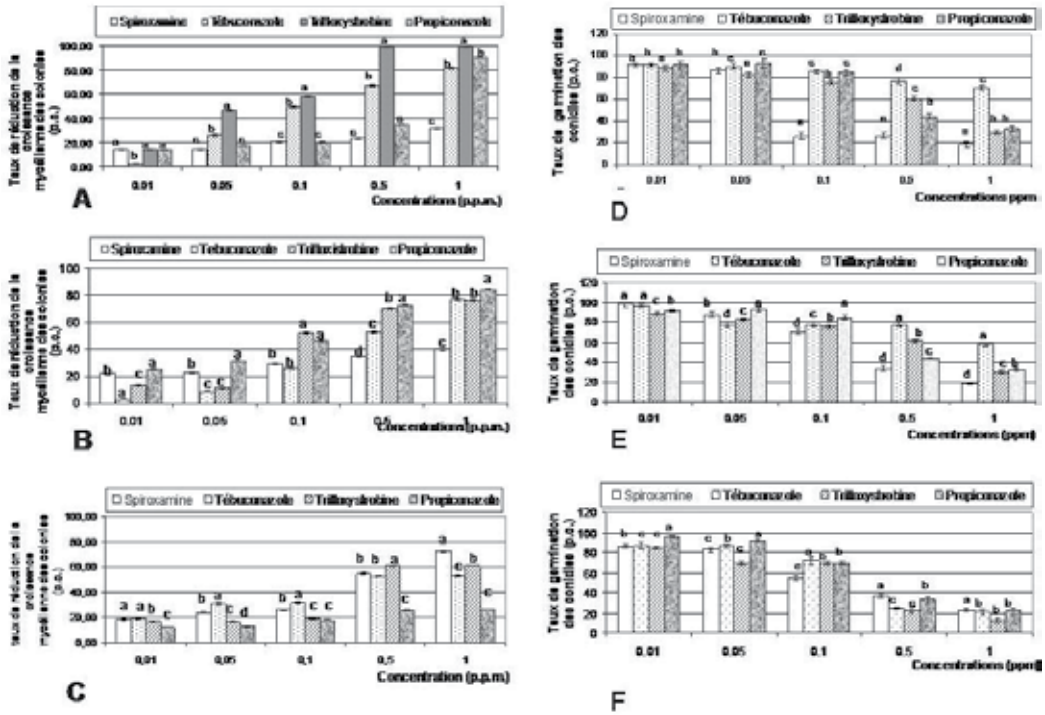

Fig. 2. *In vitro* effect of different molecule on foliair fungi growth and conidia germination
A and D : *Mycospherella fijiensis* ; B and E : *Cladosporium musae* ; C and F : *Deightoniella torulosa*

3.1.2.1.2 Activity on conidia gemination

In *Mycosphaerella fijiensis* (Fig. 2 D), conidia germination is different according to product concentration. Germination rate can reach 80% at 0.01 and 0.05 ppm. Tebuconazole gave less activity compare to other fungicides. Germination of *Cladosporium musae* conidia depend on the product (Fig. 2 E). Germination is lesser in tebuconazole. Germination rate of *Deightoniella torulosa* (Fig. 2 F) conidia can reach 80% at 0.01 and 0.05 ppm. Concentrations of

product required to inhibit 50% of conidia germination are inferior to those inhibiting 50% of mycelium growth.

actives compound	Ci_{50} of mycelium growth (ppm)					
	Mycosphaerella fijiensis		*Cladosporium musae*		*Deightoniella torulosa*	
	Ci50	R^2	Ci50	R^2	Ci50	R^2
Spiroxamine	2.06	0.91	1.50	0.83	0.54	0.97
Tébuconazole	0.40	0.75	2.32	0.93	0.72	0.77
Trifloxystrobine	0.14	0.71	0.39	0.71	0.63	0.82
Propiconazole	0.52	0.94	0.29	0.89	2.44	0.79

Table 3**.** Ci50 Values according to the fungicide

3.1.2.1.3 In vitro activity of essential oils on Mycosphaerella fijiensis and Deightoniella torulosa

3.1.2.1.3.1 *Mycosphaerella fijiensis*

Essential oils of *Monodora myristica*, *Eucalyptus torelliana*, *Melaleuca quinquenervia*, *Eucalyptus citriodora* and *Ocimum gratissimum*, were compared for their *in vitro* antifungal activity. The inhibitory effects on the mycelial growth of *Mycosphaerella fijiensis* are respectively shown in the fig. 3. *Monodora myristica* essential oil showed a good aptitude to reduce the mycelial growth of *Mycosphaerella fijiensis*. The growths of mycelium were reduced of 50% and 70% after 6 days, respectively for 1000 and 3000 ppm. The amounts of 7000 ppm inhibited strongly the growth of the fungus (Fig. 4 A) with a rate of 100% during the 18 first days and 97% three days later. At 10000 ppm, there was a total inhibition (100%) during all the experiment. The CI_{50} parameter which corresponds to the amount inhibiting for half the mycelial growth, is 744.046 ppm (Table 4).The essential oil of *Eucalyptus torelliana* showed high fongitoxic activity on the mycelial growth with the concentrations of 5000, 7000 and 10000 ppm. These amounts allowed a reduction ratio of growth superior to 70%. A moderate toxicity was observed with 3000 ppm which reduced the growth of half 17 days after. At 1000 ppm this oil is slightly fongitoxic. With this last concentration, the growth was reduced of 30% six days after (Fig. 4 B). The CI_{50} was 3158.900 ppm (Table 4).The essential oil of *Melaleuca quinquenervia* showed a good aptitude for the reduction of the mycelial growth of *M. fijiensis* for the whole of the concentrations used (Fig. 4 C). The rates of inhibition vary between 60 and 100% according to time and of the concentrations. The strongest concentrations (5000, 7000 and 10000 ppm) completely inhibited the mycelial growth during the first 15 days after the start of the experiment. From this date, the mycelial growth slightly restarts. This one was reduced by 60% the 6^{th} day for the concentration of 1000 ppm. CI_{50} is equal to 738.58 ppm (Table 4). Essential oils of *Eucalyptus citriodora* and *Ocimum gratissimum* inhibited all the mycelial growth of *M. fijiensis* at all the concentrations (Fig. 4 D and E).

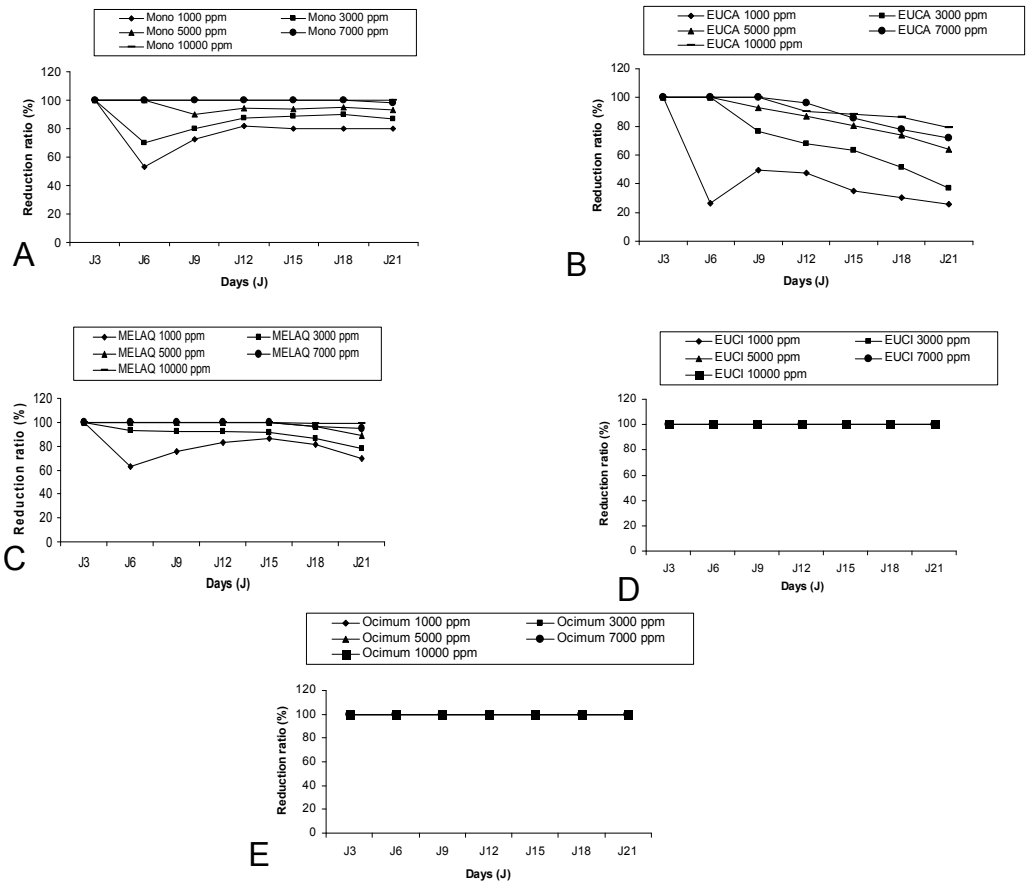

Fig. 3. Reduction of the mycelial growth of *Mycosphaerella fijiensis* at differents concentrations of *Monodora myristica* (A), *Eucalyptus toreilliana* (B), *Melaleuca quinquenervia* (C), *Eucalyptus citriodora* (D) and *Ocimum gratissimum* (E) essential oils

3.1.2.1.3.2 *Deightoniella torulosa*

Antifungal activity of essential oils is different from one and another. The essential oil of *Monodora myristica* was shown fairly fongitoxic with the concentrations of 5000, 7000 and 10000 ppm (Fig. 4 A). For each one of the last amounts, 50% of reductions of the mycelial growth are respectively obtained the 11th, 13th and 15th day. This essential oil is slightly fongitoxic with the concentrations of 1000 and 3000 ppm. With these concentrations, 50% of reductions of the mycelial growth are reached respectively to the 2nd and 8th day. The CI_{50} at the 14th day is of 23 860 ppm and CI_{90} at the same time is of 40450 ppm (Table 5). With the essential oil of *Ocimum gratissimum*, the mycelial growth was inhibited to 100% with the concentrations higher or equal to 3000 ppm (Fig. 4 B). The fungus grows on the culture medium with essential oil at the concentration of 1000 ppm, and filled all surface of the plate at the 17th day of the experiment. A reduction of 50% of the mycelial growth was reached to the 12th day. CI_{50} at the 14th day is of 2020 ppm and CI_{90} at the 14th day is of 14940 ppm. No mycelial growth is observed with the essential oil amounts higher or equal to 3000 ppm. The mycelial fragments taken in limp of plates to this last concentration and transferred on a

new culture medium PDA start again their growth. The rate of resumption of mycelial growth was 66.7% after 24 h, it reached 100% 48 hours after their transfer. The essential oil amounts higher than 3000 ppm do not allow a resumption of growth of the mycelium once this one transferred on new culture medium PDA.

The essential oil of *Melaleuca quinquenervia* showed a good aptitude for the reduction of the mycelial growth of *Deightoniella torulosa* for the whole of the concentrations used (Fig. 4 C). The rates of inhibition vary from 0 to 100% according to time and from the concentrations. The mycelial growths were reduced by 50% at the 11th, 15th and 20th days respectively for the concentrations of 1000, 3000 and 5000 ppm. The amounts of 7000 ppm and 10000 ppm have a marked effect on the reduction of the fungus growth with a rate of inhibition higher than 50%. The 14th day of the culture in the presence of essential oil of *Melaleuca quinquenervia* have CI_{50} of 7997 ppm and CI_{90} is equal to 19383 ppm (Table 5). The essential oil of *Eucalyptus platyphylla* was shown highly fongitoxic on the mycelial growth at the concentrations of 7000 and 10000 ppm. These amounts made it possible to obtain a reduction ratio of growth superior to 50%. An average toxicity, observed with 5000 ppm, reduced the growth of half, at the 16th day. At 1 000 and 3000 ppm this oil is slightly fongitoxic. With these last concentrations, the growth was reduced 50% respectively at the 3rd day and the 10th day (Fig. 4 D). The 14th day, the CI_{50} and CI_{90} are respectively 12256 and 20 080 ppm. *Deightoniella torulosa* is thus more sensitive to the essential oils of *Melaleuca quinquenervia* than with that of *Eucalyptus platyphylla*.

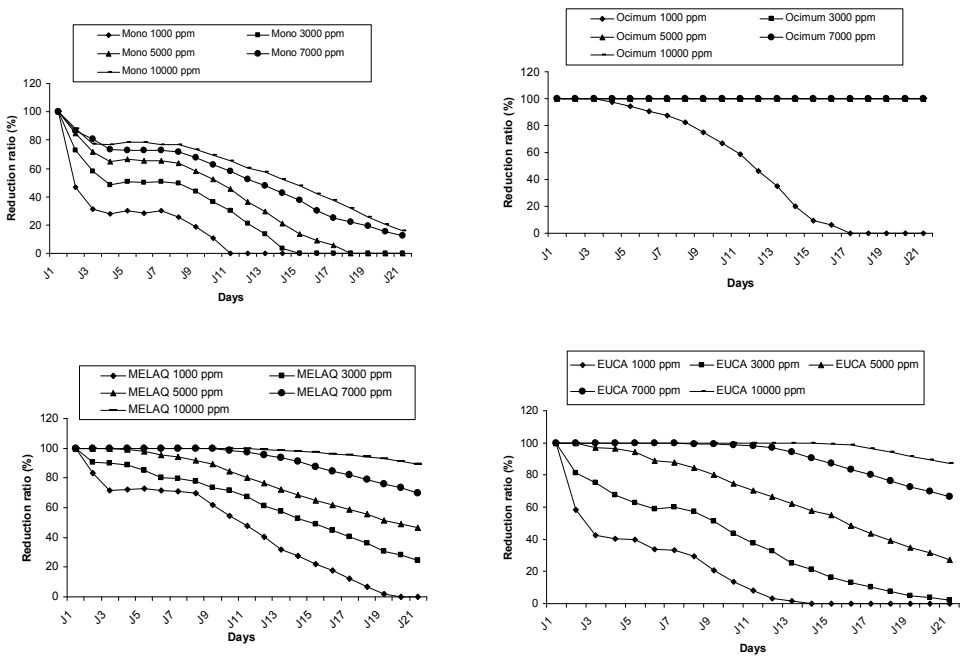

Fig. 4. Reduction of mycelial growth of Deightoniella torulosa according to time and the concentrations of *Monodora myristica* (A), *Ocimum gratissimum* (B) *Melaleuca quinquenervia* (C) and *Eucalyptus platyphylla* (D) essential oils

Antifongic parameters	Essential oils			
	Melaleuca	*Eucalyptus*	*Ocimum*	*Monodora*
CI_{50} (ppm)	7 997 a	12 256 b	2 020 a	23 860 b
CI_{90} (ppm)	19 383 a	20 080 b	14 940 a	40 450 b

NB: On the same line, the concentrations followed by the same letters has, a and b are not significantly different with $p<0.05$ according to the test of Newman-Keuls.

Table 5**.** Concentrations of essential oils (in ppm) reducing 50% (CI_{50}) and 90% (CI_{90}) the mycelial growth at the 14th day of incubation of *Deightoniella torulosa* on the cultures medium.

3.1.2.1.3 Conidia production

The essential oil of *Ocimum gratissimum* at all the concentrations prevents the production of the spores of *Deightoniella torulosa*. The essential oil of *Monodora myristica* is ineffective on the production of spores at all the concentrations. As for those resulting from *Melaleuca quinquenervia* and *Eucalyptus platyphylla* essential oils, they prevented the sporulation of the fungus at the concentrations of 7000 and 10000 ppm (Table 6).

Essential oils	**Concentrations (ppm)**				
	1000	3000	5000	7000	10000
Ocimum gratissimum	-	-	-	-	-
Monodora myristica	+	+	+	+	+
Melaleuca quiquenervia	+	+	+	-	-
Eucalyptus platyphylla	+	+	+	-	-

+ means that there is presence of spores
- means that there is absence of spores

Table 6**.** Production of spores after 21 days on medium culture PDA containing essential oils at various concentrations

3.2 Control of vegetable diseases

3.2.1 *In vitro* effects of fungicides, natural extracts and essential oils on soil born pathogens

3.2.1.1 Mancozeb

The rate of inhibition of mancozeb illustrated by table 6 shows a low sensitivity of *Fusarium* compared to *P. aphanidermatum* and *M. phaseoli* to the concentrations lower than 3 g/L. The threshold of significance is largely exceeded to 1 g/L for *Pythium* and *Macrophomina* whereas it is reached only with 1.2 g/L at *Fusarium*. All the 3 fungi strains are completely

inhibited for the concentrations higher than 2 g/L and no other resumption of mycelial pastille was observed. The rate of germination of the spores of *Fusarium* is null for all the inhibiting concentrations at 100%. The mancozeb is thus well a fungicide of synthesis for these three mycopathogens.

3.2.1.2 Banko-Plus

The rate of inhibition of Banko-Plus illustrated by table 6 shows a strong sensitivity of *Fusarium* compared to *P. aphanidermatum* and *M. phaseoli* to the concentrations lower than 1000 ppm. The threshold of significance is largely exceeded with 2000 ppm for the three fungi. The rate of inhibition to 100% is reached with 4000 ppm for the whole of the fungi. All the concentrations higher than 4000 ppm completely inhibited the resumption and the growth of the mycelial pastilles. The rate of germination of the spores of *Fusarium* is null for all the inhibiting concentrations at 100%. Banko-Plus is thus well a fungicide of synthesis for these three mycopathogens.

3.2.1.3 Aqueous extract of *Cola nitida* (Vent.) Schott & Endl (Sterculiaceae), *Cola acuminata* (Vent.) Schott & Endl (Sterculiaceae) and *Combretum racemosum* P. Beauv (Combretaceae)

The aqueous extract of *C. nitida* inhibits in various manners the mycelial growth of the three fungi. The minimum threshold of significance (25%) is reached for *Pyhtium* and *Fusarium* with 10 g/L. The inhibiting minimal concentration (MIC) with 50% is reached to 20 g/L and no fungus was inhibited at 90% by this aqueous extract. The aqueous extract of *C. nitida* is more effective on *Fusarium* than the two others fungi and this difference is significant at 15 and 25 g/L. *Cola nitida* spring like fongistatique and not a fungicide for the whole of these three fungi (Table 6).

The aqueous extract of *Cola acuminata* is significantly effective on *Macrophomina* with the concentration of 15 g/L. As for *Fusarium* and *P. aphanidermatum* they did not raise any inhibiting concentration with the threshold of significance (25%). No concentration could inhibit the mycelial growth of only one fungus with 50%. For all the concentrations lower or equal to 15 g/L, the aqueous extract of *C. acuminata* is rather activator of the mycelial growth of *Pythium* and *Fusarium*.

The simultaneous repiquate of the mycelial pastilles of the three mycopathogenes in the Petri dishes with various concentrations of *Combretum racemosum* shows a stronger inhibition at *Fusarium* compared to *P. aphanidermatum* and *M. phaseoli* (Table 6). The aqueous extract of *C. racemosum* is significantly effective on *Fusarium* with the concentration of 0.7 g/L. As for *M. phaseoli* and *P. aphanidermatum* they have minimal concentrations of 0.9 g/L and 1.9 g/L respectively. The rate of inhibition at 100% is reached to 6 g/L for the strains of *Fusarium* and *P. aphanidermatum*; on the other hand *M. phaseoli* reaches his rate of maximum inhibition to 8 g/L. Beyond 6 g/L all the 3 fungi strains are completely inhibited and no other resumption of mycopathogen is noted (Table 6).

The minimal inhibiting concentrations (MIC) and the CI_{50} and CI_{90} vary between 0.03 g/L to 0.52 g/L for the MIC and between 0.25 to 1.26 g/L for the CI_{50} and 2.25 to 3.54 for the CI_{90}. The fungi strains of *Pythium*, *Fusarium* and *Macrophomina* have a very significant sensitivity with respect to the aqueous extract of *Combretum racemosum*.

The aqueous extract of *Combretum racemosum* can thus be used as well like a fungicide just like antifongistatique for these three mycopathogens.

3.2.1.4 Powders fruits of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) and the rhizomes of *Zingiber officinalis* L. (Roscoe) (Zingiberaceae)

The inhibiting capacity of the powder of the fruits of *Xylopia aethiopica* varies from 30 to 60% for the smallest concentration of 1 g/L (Table 6). The inhibition of the mycelial growth increases with the concentration of the extract for all fungi. *Pythium aphanidermatum* has a very strong sensitivity to the powder of the fruits of *X aethiopica*. Indeed, it is the only strain which was inhibited with more than 90% (25 g/L).

No lethal inhibiting concentration was given with the powder of the fruits of *Xylopia* against the whole of these mycopathogens. The inhibiting minimal concentrations (MIC) and the inhibiting concentrations with 50% and 90% were given to 1 g/L; 4.6 g/L and 12.4 g/L respectively. The fungi strains of *Pythium*, *Fusarium* and *Macrophomina* thus have a significant sensitivity with respect to the powder of the fruits of *X aethiopica*.

The minimal inhibiting concentrations (MIC) of *Z. officinalis* with the threshold of significance (25%) vary from 5 to 10 g/L for *Pythium*, *Fusarium* and *Macrophomina*

Products		**Mycelia growth**		
	Mycopathogens	MIC (g/L)	CI_{50} (g/L)	CI_{90} (g/L)
Mancozeb	*P. aphanidermatum*	< 0.5 mg/L	1 mg/L	0.70
	Forl	0.69	1.53	3.34
	M. phaseoli	< 0,5 mg/L	< 0.5 mg/L	< 1 mg/L
C. racemosum	*P. aphanidermatum*	0.52	1.26	3.04
	Forl	0.03	0.25	2.25
	M. phaseoli	0.17	0.78	3.54
Z. officinalis	*P. aphanidermatum*	1.24	3.63	10.61
	Forl	1.49	4.61	14.28
	M. phaseoli	1.42	4.55	14.49
C. nitida rouge	*P. aphanidermatum*	0.57	5.08	> 25
	Forl	0.59	3.04	15.80
	M. phaseoli	1.04	5.84	> 25
X. aethiopica	*P. aphanidermatum*	0.94	2.09	4.66
	Forl	1.25	3.82	11.67
	M. phaseoli	1.20	3.10	7.98

Table 6. Minimal inhibiting concentrations (MIC), CI_{50}, CI_{90} (g/L) of the natural extracts and mancozeb on *Pythium aphanidermatum*, *Fusarium. oxysporum* f. sp. *radicis-lycopersici* and *Macrophomina phaseoli*

respectively. The rough extract of *Z. officinalis* could not inhibit only one fungus at 90%. On the other hand all the fungi are inhibited with more than 50% with the concentration of 20 g/L. The fungi strains *Pythium*, *Fusarium* and *Macrophomina* thus have a significant sensitivity with respect to the rough extract of *Z. officinalis* at the strong concentrations (Table 6).

3.2.1.5 Siccative oil of the fruits of *Ricinus communis* L. (Euphorbiaceae)

The siccative oil of *R. communis* is not active to 5 ml/L on the stocks of *P. aphanidermatum*, *Fusarium* and *M. phaseoli*. The significant minimal inhibiting concentration for the fungi is at 15 ml/L. All the fungi were resistant to the siccative oil of *R. communis* for all the concentrations. Minimal inhibiting concentration (MIC) raised is obtained to 10 ml/L.
The inhibiting concentrations with 50% are obtained with the strongest concentration (25 ml/L) and no concentration is raised to 90%. The sensitivity of these fungi to the siccative oil of *R. communis* is very low. It is nonfungicidal oil for these fungi (Table 6).

3.2.2 Essential oils

3.2.2.1 *Xylopia aethiopica*

The table 6 illustrates the rate of inhibition of the mycelial growth of *Pythium*, *Fusarium* and *Macrophomina* by the essential oil of *X. aethiopica*. The inhibition of the mycelial growth increases with the concentration. The essential oil presents a total inhibition of *Fusarium* and *Pythium*. On the other hand *Macrophomina* could not be inhibited completely by the essential oil of *X. aethiopica*. Indeed, the mycelial pastilles of *Fusarium* and *Pythium* could not take again their growth in the box of Petri to the concentration of 4000 ppm and even after its transfer on a neutral culture medium. The lethal inhibiting concentration thus determines the fungicidal capacity of the essential oil of *Xylopia* against the mycopathogens *Fusarium* and *Pythium*.
The weakest inhibiting concentrations with 50% and 90% were given with 94 ppm and 2761 ppm. The fungi strains *Fusarium*, *M. phaseoli* and *Pythium* thus have a very significant sensitivity with respect to essential oil of *X. aethiopica* (Table 7).

3.2.2.2 *Peper guinense*

The inhibition of the mycelial growth only increases with the concentration of the oil for the fungus *Pythium aphanidermatum*. The essential oil presents a total inhibition of *Pythium* at 2000 ppm. On the other hand *Fusarium* and *Macrophomina* could not be inhibited completely by the essential oil of *P. guinense*. Indeed, no mycelial resumption of *Pythium* was raised in the boxes of Petri to the concentrations of 2000 and 4000 ppm and even after its transfer on a neutral culture medium. The essential oil of *P. guinense* is thus fungicidal for the mycopathogen *P. aphanidermatum*.
The minimal inhibiting concentrations (MIC) vary from 100 ppm to 200 ppm. The inhibiting concentrations with 50% and the weakest 90% are obtained with *Pythium*. Those (CI_{50} and CI_{90}) obtained with *Fusarium* and *Macrophomina* are given at 250 ppm. All the fungi strains were inhibited with more than 75% to 2000 ppm. The sensitivity of these fungi is thus very significant with respect to the essential oil of *Peper guinense* (Table 7).

3.2.2.3 *Zingiber officinalis*

No fungus was completely inhibited. However, *Pythium* was very significantly inhibited (75%) by the essential oil of *Z. officinalis*. The essential oil of *Z. officinalis* is thus not

fungicidal for the mycopathogens *P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*.

The table 7 presents the minimal inhibiting concentrations (MIC), the CI_{50} and CI_{90} which were given with 270 ppm, 350 and with 1050 ppm respectively for *Pythium*. *Pythium aphanidermatum* is the only strain which raised a CI_{50} with 500 ppm. All the fungi strains were inhibited to 50% only that with 4000 ppm. The sensitivity of *Pythium* is very significant with respect to the essential oil of *Z. officinalis*; on the other hand *Fusarium* and *Macrophomina* are significantly less sensitive to this oil. Indeed, no inhibiting concentration with 75% was raised for two fungi with essential oil and no fungus strain presented a CI_{90}.

3.2.2.4 *Melaleuca quinquenervia*

All the three fungi were inhibited completely by the essential oil of *M. quinquenervia*. On the other hand, it is only at *Pythium* that no resumption of the mycelial growth was observed.

The minimal inhibiting concentrations (MIC) vary from 100 ppm with 200 ppm. The inhibiting concentrations with 50% and the weakest 90% are obtained with *Pythium*. Those (CI_{50} and CI_{90}) obtained with *Fusarium* and *Macrophomina* are given with 600 ppm and 4000 ppm respectively. All the fungi strains were inhibited with more than 75% to 2000 ppm. The sensitivity of these fungi is very significant with respect to the essential oil of *Melaleuca quinquenervia* (Table 7).

3.2.2.5 *Ocimum basilicum*

All the strains were inhibited completely by the essential oil of *Ocimum basilicum*. However, all the mycelial pastilles took again their growth. The essential oil of *O. basilicum* is thus fongistatic for these three fungi. The essential oil of *Ocimum basilicum* is thus not fungicidal for the mycopathogens *P. aphanidermatum*, *F oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*.

The minimal inhibiting concentrations (MIC) are all lower than 200 ppm. The inhibiting concentrations with 50% and the weakest 90% are obtained with *Pythium*. The CI_{50} and CI_{90} obtained with *Fusarium* and *Macrophomina* are given below 500 ppm and only *Pythium* could reach the CI_{90} (<4000 ppm). All the fungi strains were inhibited with more than 75% to 2000 ppm. The sensitivity of these fungi is thus very significant with respect to the essential oil of *Ocimum basilicum* (Table 7).

3.2.2.6 *Ocimum gratissimum*

No significant difference was raised between the five concentrations for all the three fungi strains. The very significant threshold of inhibition (75%) is reached starting from the smallest concentration 250 ppm.

In addition, no resumption of the mycelial growth was observed at only one stock. The minimal inhibiting concentrations (MIC) are all lower than 100 ppm. The inhibiting concentrations with 50% and 90% are largely exceeded with the weakest concentrations (250 ppm). The threshold of inhibition very highly significant (75%) is reached by all the fungi strains with 250 ppm. The sensitivity of these fungi is thus very significant with respect to the essential oil of *Ocimum gratissimum*.

In addition, No resumption of the mycelial growth was observed at only one stock. The essential oil of *O. gratissimum* is fungicidal for *Pyhtium aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli* (Table 7).

		Mycelia growth		
	Mycopathogens	MIC (ml/L)	CI_{50} (ml/L)	CI_{90} (ml/L)
	P. aphanidermatum	2 µl	0.06	1.58
X. aethiopica	Forl	0.05	0.46	3.77
	M. phaseoli	7 µl	0.05	3.93
	P. aphanidermatum	0.86	1.84	3.91
P. guinense	Forl	0.87	2.29	5.99
	M. phaseoli	090	2.33	6.01
	P. aphanidermatum	0.56	1.58	4.43
Z. officinalis	Forl	3.04	7.07	16.44
	M. phaseoli	1.55	9.04	52.92
	P. aphanidermatum	5 µl	0.13	3.08
M. quinquenervia	Forl	0.06	0.60	6.37
	M. phaseoli	0.26	1.04	4.12
	P. aphanidermatum	0.02	0.29	5.39
O. basilicum	Forl	0.11	1.03	9.63
	M. phaseoli	8 µl	0.27	9.77
	P. aphanidermatum	5 µl	0.11	2.25
O. gratissimum	Forl	< 1 µl	< 1 µl	0.01
	M. phaseoli	< 1 µl	< 1 µl	< 1 µl
	P. aphanidermatum	0.01	0.15	1.98
Banko Plus	Forl	0.24	0.87	3.19
	M. phaseoli	0.03 µl	6 µl	1.20

Table 7. Minimal inhibiting concentrations (MIC), CI_{50}, CI_{90} (g/L) of the essential oils and Banko-Plus on *Pythium aphanidermatum, Fusarium. oxysporum* f. sp. *radicis-lycopersici* and *Macrophomina phaseoli*

3.2.3 *In vivo* effects of fungicides, natural extracts and essential oils on soil born pathogens

3.2.3.1 Effect of Mancozeb on diseases

The transplantation of the tomato seedlings in the substrate treated by the mancozeb and inoculated by each of the three mycopathogens (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis lycopersici* and *M. phaseoli*) generated a weak attack of the latter (Table 8). Indeed the index of disease is practically null for the three isolates at the three cultivars except *Pythium* on the level of the cultivars Caraïbo (0.1) and Tropimech (0.1); nevertheless the percentage of the seedlings having a higher notation of the symptoms or equalizes to two is null. The fungicide of synthesis significantly reduced the index of the diseases (rot pythienne, fusariose and black rot) on the tomato seedlings.

The effect of mancozeb on the dry biomass of the seedlings of tomato and/or inoculated and/or treated is consigned in table 4. The biomass produced during the treatment of the fusariose by the mancozeb is a function of the variety of tomato. Mongal presents the strongest foliar biomass for all the treatments. All the inoculated seedlings and treated have a biomass higher than that of inoculated and the seedlings inoculated with Forl (6.53±0.31 g) and treated produced the strongest biomass. The seedlings inoculated for all the three varieties produced a biomass significantly reduced compared to the healthy control or positive control.
The mancozeb reduced the impact of fungi on the dry biomass root on the level of the treated inoculated seedlings. Indeed, all the treated inoculated seedlings gave a biomass root higher than that of the seedlings inoculated at all the cultivars. The dry biomass root produced at Tropimech is significantly lower than that of Mongal and Caraïbo.

3.2.3.2 Effect of Banko-Plus on diseases

The transplantation of the tomato seedlings in the substrate treated by Banko-Plus and inoculated by each of the three mycopathogenes (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis lycopersici* and *M. phaseoli*) generated a weak attack of the latter (Table 8). The index of the diseases varies according to the fungi and the cultivars. It is null at Mongal and is higher at Tropimech (0.2) with Pythium and Forl (0.6). The percentage of the seedlings having a higher notation of the symptoms or equalizes to two is null for all the cultivars. The fungicide of synthesis significantly reduced the incidence of the fusariose and entailed a null mortality of the tomato seedlings at all the cultivars.
The biomass produced during the treatment of the fusariose by Banko-Plus is a function of the variety of tomato. Mongal presents the strongest foliar biomass for all the treatments. All the inoculated seedlings and treated have a biomass higher than that of inoculated and the seedlings inoculated with Forl (5.59±0.18 g) and treated produced the strongest biomass. The seedlings inoculated for all the three varieties produced a biomass significantly reduced compared to the healthy or positive control.
Banko-Plus reduced the impact of fungi on the root dry biomass on the level of the treated inoculated seedlings. Indeed, all the treated inoculated seedlings gave a root biomass higher than that of the seedlings inoculated at all the cultivars. The root dry biomass produced at Tropimech is significantly lower than that of Mongal and Caraïbo.

3.2.3.3 Effect of the aqueous extract of the leaves of *Combretum racemosum* on diseases

The table 8 shows the tomato seedlings mended in the substrate treated by the aqueous extract of *Combretum racemosum* and inoculated by each of the three mycopathogenes (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*). No mortality was raised at the positive control. The inoculated seedlings treated with the aqueous extract of *C. racemosum* strongly reduced the attacks of the fungi. The percentage of the seedlings which had a notation of symptoms higher or equal to two is null at Mongal inoculated with Forl and *Macrophomina*. The aqueous extract of *C. racemosum* significantly reduced the index of the diseases (rot pythienne, fusariose and black rot) on the tomato seedlings.
The tests carried out *in vivo* with the extract of the powder of *Combretum racemosum* made it possible to establish a positive relation between the product and the vegetative growth of the tomato seedlings. The foliar and root dry biomass are evaluated over 30 days after inoculation of the seedlings. The aqueous extract of *C. racemosum* significantly ($p<0.05$) increased the foliar and root biomass of the tomato seedlings inoculated by *P. aphanidermatum*, *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) and *M. phaseoli* compared

to the healthy control. Indeed, the seedlings inoculated by the three fungi produced a biomass varying from 2.08 g to 3.54 g of foliar matter and 1.02 g to 1.67 g of root matter respectively. No significant difference on the level of the foliar biomass is raised between the treated and inoculated seedlings and the positive control at all the cultivars. On the other hand, the root biomass of the seedlings treated and inoculated has a varietal sensitivity according to fungi. The weakest biomass are obtained with Tropimech treated and inoculated with Forl (1.65±0.10 g) and *M. phaseoli* (1.95±0.14 g).
The aqueous extract of *C. racemosum* thus reduced the impact of fungi on the tomato seedlings; what supported an increase in the foliar and root biomass.

3.2.3.4 Effect of the powder of *Xylopia aethiopica* on diseases

The table 8 shows the tomato seedlings mended in the substrate treated by the powder of the fruits of *Xylopia aethiopica* and inoculated by each of the three mycopathogens (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*). No mortality was raised at the positive control. The inoculated seedlings treated with the rough extract of *Xylopia aethiopica* strongly reduced the attacks of fungi on the tomato seedlings. The percentage of the seedlings which had a notation of symptoms higher or equal to two is null with Forl and *Macrophomina. Pythium aphanidermatum* showed the highest death rate at Caraïbo (5.6%) and Tropimech (4.2%). The rough extract of *X. aethiopica* significantly reduced the index of the diseases (rot pythienne, fusariose and black rot) on the tomato seedlings. An effect of phytotoxicity was raised with the powder of *X. aethiopica* without that not causing mortality at the tomato seedlings.
The biomass produced with the application of the powder of *X. aethiopica* on the tomato seedlings varies according to varieties'. The powder significantly increased the foliar and root biomass inoculated seedlings and treated or seedlings treated (pilot positive) compared to the healthy control and with inoculated. The three varieties react positively with the powder of *Xylopia*. Indeed, the three tomato cultivars carried out profits of 33.6%; 30.6%; 43.1% and 47.1%; 51.8%; 35.5% in foliar and root biomass at Mongal, Caraïbo and Tropimech respectively. The repressive effect of the powder of *Xylopia aethiopica* on the fungi is the result of this tendency in rise of the biomass of the tomato seedlings.

3.2.3.5 Effect of the powder of *Ziniber officinalis* on diseases

No mortality was raised at the positive control; on the other hand an effect of toxicity was observed at all the cultivars Mongal (0.2) Caraïbo (0.3) and Tropimech (0.3). All seedlings inoculated by each of the three fungi (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis lycopersici*) and treated with the rough extract of *Z. officinalis*, concerned mortalities varying from 30% to 60%. The rough extract of *Z. officinalis* did not have any effect on the incidence of the diseases of the tomato seedlings. The phytotoxicity of the extracts of *Z. officinalis* caused the chlorosis of the sheets of the treated tomato seedlings.
The biomass is reduced with the application of the powder of *Zingiber officinalis* on the tomato seedlings at all the cultivars. This fall of the foliar biomass is significant with the inoculum of *P. aphanidermatum* at Mongal (1.77 ± 0.21 g), Caraïbo (1.81 ± 0.41 g) and Tropimech (1.51 ± 0.14 g) compared to the respective healthy witnesses (7.25 ± 0.45 g; 6.88±0.23 g; 6.16±0.28 g). The powder of *Z. officinalis* had a negative effect on the foliar biomass of the tomato seedlings.
The dry root biomass also significantly lowered at all the cultivars treated with the powder of *Z. officinalis* compared to the healthy control. No significant difference is raised between the inoculated controls and inoculated treated at all the cultivars. The healthy control

presents also a dry root biomass significantly higher than that of the positive control except at Tropimech. Indeed, the same dry root biomass produced at the healthy control (3.33±0.22 g) does not present any significant difference with that of the positive witnesses (2.97±0.04 g). The rough extract of the rhizomes of *Z officinalis* significantly reduced the biomass of the tomato seedlings.

3.2.3.6 Effect of the aqueous extract of the nuts of *Cola nitida* on diseases

The index of the disease is null at Mongal and it did not exceed 0.01 at Caraïbo and Tropimech for the three fungi. The percentage of the seedlings having a notation of the symptoms higher or equal to two is null also at Mongal and varies from 2.08% to 3.13% at the two other varieties. The biofongicide extracts aqueous from *C. nitida* significantly reduced the index of the diseases on the tomato seedlings. Nevertheless, there was an effect of toxicity raised with the aqueous extract of *Cola*.

The aqueous extract of *Cola nitida* significantly increased the foliar and root biomass of tomato seedlings inoculated with *P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli* compared to those inoculated and untreated. Indeed, the seedlings inoculated with the fungi produced a foliar biomass varying from 2.08 to 3.37 g. of foliar dry matter and of 1.02 to 1.67 g of matter dry racinaire respectively. On the other hand, those inoculated with mushrooms and treaties with the aqueous extract of *C. nitida* produced a biomass varying between 3.04 and 4.28 g from foliar dry matter and 2.24 and 2.97 g of matter dry racinaire respectively. The aqueous extract of *C. nitida* thus reduced the impact of the fungi on the tomato seedlings; what supported an increase in the foliar biomass and racinaire.

	No product			E_1= *Combretum racemosum*			E_2= *Xylopia aethiopica*			E_3= *Zingiber officinalis*			E_4= *Cola nitida*			E_5= Mancozeb		
Treatments	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**
Inoculated by *Pythium aphanidermatum*	1.6	1.5	2.5															
	60	60	90															
Inoculated by *Pythium aphanidermatum* treated by E_n				0.7	1.1	0.9	0.4	1.9	1.7	1.0	1.3	2.4	0	0.6	0.1	0	0.1	0.2
				30	40	30	10	30	40	40	50	60	0	20	10	0	0	0
Inoculated by *Fusarium oxysporum* f. sp. *radicis-lycopersici*	1.0	1.3	2.4															
	40	50	90															
Inoculated by *Fusarium oxysporum* f. sp. *radicis-lycopersici* treated by E_n				0.2	0.4	0.9	0.3	0.6	0.6	0.5	1.4	1.3	0	0.5	0.3	0	0.1	0.6
				0	20	30	0	0	0	30	50	50	0	20	20	0	0	0
Inoculated by *Macrophomina phaseoli*	0.8	1.0	1.9															
	30	40	70															
Inoculated by *Macrophomina phaseoli* treated by E_n				0.4	0.6	0.3	0.3	0.5	0.4	0.9	1.3	1.0	0	0.1	0.1	0	0.3	0
				10	20	10	0	0	0	30	40	30	0	0	0	0	0	0
Treated by E_n				0	0.1	0.1	0.1	0.2	0.3	0.2	0.3	0.3	0	0.1	0.1	0	0	0
				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Healthy control	0	0	0															
	0	0	0															

Index of wiltering

Index of mortality (%)

M=Mongal; **C**=Caraïbo; **T**=Tropimech

Table 8. Effects of natural extracts and mancozeb on the incidence of the diseases of tomato varieties at 30 days after transplantation

3.2.3.7 Effect of the essential oil of the fruits of *Xylopia aethiopica* diseases

The table 8 shows the tomato seedlings mended in the substrate treated by the essential oil of the fruits of *Xylopia aethiopica* and inoculated by each of the three mycopathogenes (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*). No mortality was raised at the positive control just like at the healthy control. The essential oil of *Xylopia aethiopica* strongly reduced the attacks of fungi on the inoculated tomato seedlings and treated. With share *P. aphanidermatum* which caused the death of 30% (Mongal) 40% (Caraïbo) and 50% (Tropimech) of the seedlings, the percentage of the seedlings which had a notation of symptoms higher or equal to two is null at *Fusarium* and *Macrophomina*. The essential oil of *X. aethiopica* significantly reduced the incidence of the diseases (rot pythienne, fusariose and black rot) of the tomato seedlings however it presented an effect of toxicity.
The biomass produced during the treatment of the fusariose by the essential oil of *X. aethiopica* is a function of the variety of tomato. Mongal presents the strongest foliar biomass for all the treatments, 28.80 g (Pilot healthy); 9.70 g (Inoculated); 17.10 g (Pilot positive); 15.90 g (Inoculated and treated with essential oil). The seedlings inoculated for all the three varieties produced a biomass significantly reduced compared to the healthy or positive control and the treated inoculated seedlings. The Tropimech variety inoculated treated with essential oil produced the double of the root biomass (2.10 g) of the untreated inoculated seedlings (1.10 g).

3.2.3.8 Effect of the essential oil of the rhizomes of *Zingiber officinalis* on diseases

The table 8 shows the tomato seedlings mended in the substrate treated by the essential oil of the rhizomes of *Zingiber officinalis* and inoculated by each of the three mycopathogenes (*P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* and *M. phaseoli*). No mortality was raised at the positive control just like at the healthy control. The essential oil of *Z. officinalis* did not have any effect on the attacks of fungi on the level of the inoculated tomato seedlings and treated. *P. aphanidermatum* raised the index of mortality highest with 70% at Mongal; 80% at Caraïbo and Tropimech. No fungus was repressed by the essential oil of *Z. officinalis*.
The foliar and root dry biomass are evaluated over 30 days after inoculation of the seedlings.
The essential oil of *Z. officinalis* did not have any significant effect on the foliar and root biomass of the tomato seedlings treated and inoculated with *P. aphanidermatum*, *F. oxysporum* f. sp. *radicis-lycopersici* or *M. phaseoli* compared to the control. Indeed, the seedlings inoculated with mycopathogens produced a foliar biomass varying from 2.08 to 3.37 g of foliar dry matter and of 1.02 to 1.67 g of root dry matter respectively. On the other hand, those treated and inoculated with fungi produced a biomass varying between 3.04 and 3.82 g from foliar dry matter and 0.68 and 1.45 g of root dry matter respectively. The essential oil of the rhizomes of *Z. officinalis* did not have any effect on the attacks of fungi on the tomato seedlings; what supported a reduction of the foliar and root biomass.

3.2.3.9 Effect of the essential oil of the sheets of *Ocimum gratissimum* on diseases

The effect of the oil essential of *Ocimum gratissimum* on the incidence of the diseases at 30 days after transplantation is consigned in table 8. The tomato seedlings mended in the substrate treated by the essential oil of *O. gratissimum* just like do not present any mortality at the positive control at the healthy control. The essential oil of *O. gratissimum* strongly repressed the attacks of fungi on the inoculated tomato seedlings and treated. With share *P. aphanidermatum* which caused the death of 10% of the seedlings at Mongal, the percentage of the seedlings which had a notation of symptoms higher or equal to two is null with *Macrophomina* and

Fusarium. The essential oil of *O. gratissimum* significantly repressed the incidence of the diseases (rot pythienne, fusariose and black rot) on the tomato different cultivars.

The biomass produced with the application of the essential oil of the sheets of *Ocimum gratissimum* on the tomato seedlings varies according to cultivars. Essential oil significantly increased the foliar and root biomass inoculated seedlings and treated or seedlings treated (pilot positive) compared to the healthy control and with inoculated. The three varieties react positively to the essential oil of the sheets of *O. gratissimum*.

The three tomato cultivars produced foliar biomass at the positive control, significantly no different from those of the healthy control except at Mongal (7.25±0.49 g and 6.37±0.35 g respectively). All the inoculated seedlings treated by the essential oil of *O. gratissimum* presented a better vegetative growth compared to the inoculated control. The difference between the treated inoculated seedlings and the healthy control is significant at all the cultivars.

The essential oil of *O. gratissimum* significantly increased the root dry biomass of the inoculated tomato seedlings treated of all the cultivars. No significant difference is noted between the healthy control Mongal (4.30±0.29 g) and same the cultivar inoculated by Forl (3.83±0.38 g). The essential oil of *O. gratissimum* significantly increased the biomass of the tomato seedlings at all the treated inoculated cultivars.

	No product			O_1= *Xylopia aethiopica*			O_2= *Zingiber officinalis*			O_3= *Ocimum gratissimum*			O_4= Banko-Plus		
Treatments	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**	**M**	**C**	**T**
Inoculated by *Pythium aphanidermatum*	1.6	1.5	2.5												
	60	60	90												
Inoculated by *Pythium aphanidermatum* treated by O_n				1.1	1.6	1.5	1.8	1.6	1.3	0.4	0	0	0	0.1	0.2
				30	40	50	70	80	80	10	0	0	0	0	0
Inoculated by *Fusarium oxysporum* f. sp. *radicis-lycopersici*	1.0	1.3	2.4												
	40	50	90												
Inoculated by *Fusarium oxysporum* f. sp. *radicis-lycopersici* treated by O_n				0.5	0.9	1.1	1.0	1.3	1.9	0	0	0	0	0.1	0.6
				0	0	0	40	40	80	0	0	0	0	0	0
Inoculated by *Macrophomina phaseoli*	0.8	1.0	1.9												
	30	40	70												
Inoculated by *Macrophomina phaseoli* treated by O_n				0.7	0.9	0.8	1.2	1.8	1.2	0	0.3	0.2	0	0.3	0
				0	0	0	30	60	50	0	0	0	0	0	0
Treated by O_n				0.2	0.2	0.3	0.6	0.6	0.6	0.1	0.1	0.1	0	0	0
				0	0	0	0	0	0	0	0	0	0	0	0
Healthy control	0	0	0												
	0	0	0												

Index of wiltering

Index of mortality (%)

M=Mongal; **C**=Caraïbo; **T**=Tropimech

Table 9. Effects of essential oils and Banko-Plus on the incidence of the diseases of tomato varieties at 30 days after transplantation

3.2.4 Activity of essential oil compared to fungicides on *Sclerotium rolfsii*

Inhibition of mycelium growth is different according to the product and their concentration (Fig. 5). Between essential oil, all concentrations (250 to 6000 ppm) of *Chenopodium ambrosioides* and *Zingiber officinalis* inhibited mycelial growth. The most effective

concentration of *Melaleuca quinquenervia* and *Monodora myristica* are respectively 4000 and 6000 ppm. Mancozeb is the most effective fungicide inhibiting mycelium growth followed by Banko plus and Callicuivre. Callicuivre effect is different to others fungicides. There is no inhibition but the mycelium turned in grren color from 1000 ppm more marked with the concentration. *Zingiber officinalis* and *Chenopodium ambrosioides* gave similar effect than

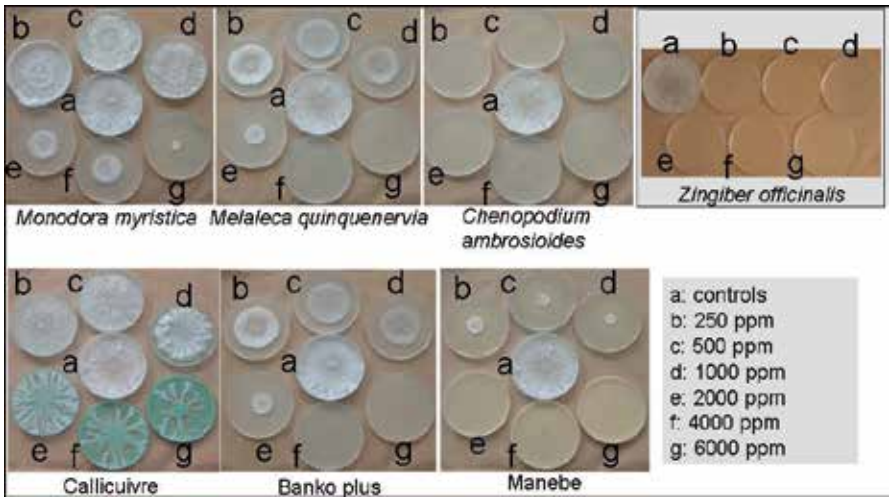

Fig. 4. *In vitro* activity of fungicides on mycelium growth of *Sclerotium rolfsii*

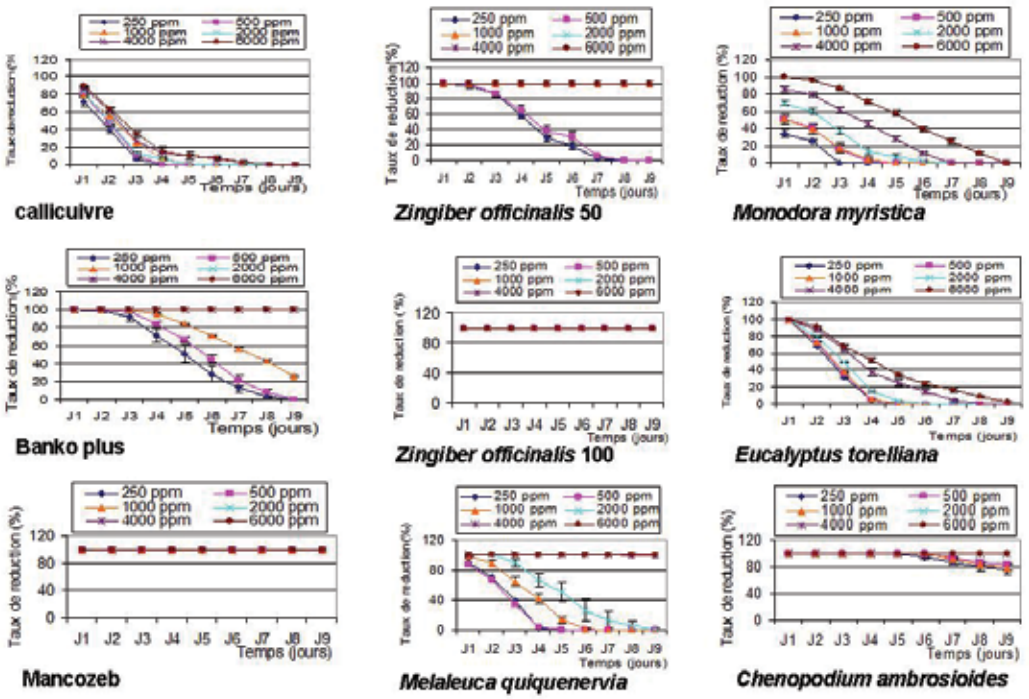

Fig. 5. Inhibition rate of essential oil and fungicides on mycelium growth of *Sclerotium rolfsii*

mancozeb. Essential oil of *Zingiber officinalis* and *Chenopodium ambrosioides* gave similar effect than Mancozeb (Fig. 6). Essential oils of *Melaleuca quinquenervia* gave similar effect than banko plus while essential oil of *Monodora myristica* reacted as Callicuivre (Fig. 5 and 6).

4. Conclusion

Control of *Mycosphaerella* sp. pathogens and others pathogenic fungi of banana and vegetable need to use an integrated approach that will incorporate both cultural practices and the application of fungicides. In *Côte d'Ivoire* fungicide application in commercially growing banana varies according to geographic location. Around 20 applications of fungicides were performed during the year. Systemic fungicides including Tilt, Bumper (propiconazole), folicur (tebuconazole) were applied during wet periods while protectant fungicides such mancozeb is used during dried periods. Because of appearance of resistant strain, development of strategy base on the use of fungicide in mixture (systemic and biological) is an approach to encourage. It is required to use less fungicide to get banana free fungicide and to control *Mycosphaerella* leaf spots in decreasing pesticide input.

The various extracts tested *in vitro* gave results significantly different according to the fungi Forl, *P. aphanidermatum* and *M. phaseoli*. On the other hand no significant difference was raised between fungicides of synthesis and the aqueous extracts of *Combretum racemosum*. The tests of inhibition carried out with the aqueous extract of *C. racemosum* revealed antifongistatic and fungicidal activities significantly higher than the extracts of *Xylopia aethiopica* on these three mycopathogenes. The strongest positive correlation observed between the amounts of the powder of *C. racemosum* and the inhibition of the three fungi was raised with *Pythium* and *Fusarium*. Compared to fungicides of synthesis, the aqueous extract of *C. racemosum* is fungicidal for three fungi.

The study carried out in greenhouse made it possible to highlight the infectious potential of the soils and to come out the need from it for adopting a method of control in order to reduce mortalities. Three of the identified telluric fungi parasites belong to the kinds *Pythium*, *Fusarium*, *Sclerotium*. A new fungus ever announced in Côte d'Ivoire on market gardenings was also identified; it is about *Macrophomina phaseoli*. The study of the infectious potential showed that the presence of the telluric fungi parasites tiny room the growth of the seedlings and induces a mortality whose rate varies according to varieties'. This reduction of growth is stronger at Tropimech with respect to 4 mycopathogenes on these soils. On the other hand the test of inoculation with *Pythium* alone showed that Caraïbo is the variety most sensitive to this fungus. The seedbeds of 20 and 25 days arise like the best ages for the transplantation of the seedlings of these three varieties of tomato. Indeed, the seedlings of 20 and 25 days of seedbed resist better the attacks of the mycopathogene *Pythium* sp.

This study showed the inhibiting effect of *Combretum racemosum* on Forl, *P. aphanidermatum* and *M. phaseoli*, all telluric mycopathogenes of the tomato cultures. They are agents responsible for the rot of the roots, tomato collet and stem. The tests of inhibition carried out with the natural extract gross of the powder of *C. racemosum* revealed an antifongistatic and fungicidal activity significant on these three mycopathogenes. The strong correlation observed between the concentration of the powder of *C. racemosum* and the inhibition of the three fungi will be used as a basis to determine the lethal amounts usable *in vivo* in order to extend its use to the plantations of tomato cultures. The natural extract of the powder of *C. racemosum* can be used as a biopesticide against these fungi for the alternative control in tomato plantation once that its antifongic activity will have been proven in cultures *in vivo* on tomato. The interest of this study locates at two levels; on the one hand,

the advantage that *Combretum racemosum* meets easily in the forest belts of the Côte d'Ivoire and on the other hand it is a nontoxic plant for human consumption. A method of use on a large scale of the powder of this plant would be thus a good prospect to be considered for the market gardenings in Côte d'Ivoire.

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4

A Multi-aspect Comparative Investigation on the Use of Strobilurin and Triazole - based Fungicides for Winter Wheat Disease Control

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1. Introduction

Fungicides continue to be essential for the effective control of plant diseases. The azoles class of fungicides has been the leading agents for the control of fungal pathogens of plants since their introduction in the mid 1970s. This class of fungicides is also called sterol demethylation inhibitors (DMIs) because they inhibit the formation of cytochrome P450 sterol 14 alpha-demethylase (450 14DM), an enzyme required for the biosynthesis of ergosterol. Ergosterol is an important compound required for fungal membrane integrity and cell cycle progression (Dahl et al., 1987). DMIs represent one of the largest groups of systemic fungicides that have been used to control agriculturally important fungal pathogens (Zhan et al., 2006). In the mid 1980s, the first DMIs fungicide propiconazole has been investigated and introduced in the agronomic practice of Lithuania for winter wheat disease control (Šurkus et al., 1988) and currently 10 DMIs active substances alone or in mixtures are registered for cereal disease control. In spite of their long-term use, a widespread resistance to azoles in plant pathogenic fungi has not occurred. The shift to decreasing sensitivity propiconazole to *Phaeosphaeria nodorum* the causal agent of Stagonospora blotch of wheat has been reported since 1994 (Peever et al, 1994) although until 1994 only minor changes in the sensitivity of *Mycosphaerella graminicola* populations – c.a. Septoria leaf blotch, to DMIs fungicides in European countries was confirmed (Turner et al., 1996). The slightly resistant field isolates were not cross-resistant to the DMI fungicides, which act at a different stage of sterol biosynthesis (Hollomon, 1994). Sometimes the cross-resistance is only partial. Whilst cross-resistance extends to all DMIs (Gisi et al., 2004) a recent rapid decline in the efficacy of some DMIs fungicides in controlling powdery mildew and Septoria leaf blotch of wheat has been noted. Some fungicides, notably epoxyconazole and prothioconazole, are still very effective in controlling *M. graminicola* (Cools & Fraaije, 2008).

In the beginning of 90s a new class of fungicides, generated on the basis of the fungus *Strobilurus tenacellus* secondary metabolites was developed (Ammermann et al., 1992; De Vleeschauwer et al., 1996). Strobilurins, with a chemistry based on a natural product from a mushroom, are fungicide of new generation and proved to be quite effective, protective, eradicant and potential broad-spectrum substances against foliar diseases of winter wheat.

They have low mammalian toxicity and are environmentally safe. In addition to disease control, strobilurins have useful non-fungicidal physiological effects: they improve nitrogen metabolism and also inhibit ethylene biosynthesis. This latter effect is responsible for the greening effect which results in delayed senescence with higher amount of chlorophylls (Habermeyer et al., 1998) and index of photosynthesis (Häuser-Hahn et al., 2004; Oerke et al., 2005). According to Inagaki et al. (2009), strobilurins can act on delaying root water uptake, resulting in postponement of soil dehydration, which contributes to a slight increase of grain yield in some wheat genotypes in the field under water deficit conditions.

These broad-spectrum strobilurins or QoI (quinone outside inhibitors) fungicides based on affecting the mitochondrial respiration of the fungus cell by inhibiting the electron transport at the cytochrome-bc_1 complex have been registered in Lithuania since the year 2000 and nowadays 7 active substances mostly in mixture with azoles are used in agricultural practice. It is known that the foliar strobilurin treatment in wheat had resulted in both the suppressing foliar diseases and maintenance of green leaf area as a result - period of grain filling being extended, and thus likely contributing to a yield increase (Gooding et al., 2000; Dimmock & Gooding 2002). QoI fungicides gave adequate control of the main cereal foliar diseases despite the disease pressure. Strobilurins have a single-site mode of action and have therefore been classified as at high risk of resistance (Brent & Hollomon, 2007). Consequently, for a short time the strains of powdery mildew (*Blumeria graminis*) and Septoria (*M. graminicola)* with reduced sensitivity to QoIs were identified (Sierotzki et al., 2000; Gisi et al., 2002). In recent years, the efficacy of strobilurin fungicides against Septoria leaf blotch and powdery mildew has declined markedly because of widespread cross-resistance (Sierotzki & Gisi, 2006). Fungicides composed of several active ingredients are characterised by a wider mode of action, provide a versatile and lasting protection against fungal diseases, moreover, they prevent development of pathogen resistance (Kendall & Hollomon, 1994).

Septoria leaf blotch (*Mycosphaerella graminicola* (Fuckel) J. Schröt., syn. *Septoria tritici* Berk. & M.A. Curtis*)*, Stagonospora blotch (*Phaeosphaeria nodorum* (E. Müll.) Hedjar. syn. *Stagonospora nodorum* (Berk.) E. Castell. & Germano; *Septoria nodorum* (Berk.) Berk.*)* and tan spot *(Pyrenophora tritici repentis* (Died.) Drechsler, syn. *Drechslera tritici-repentis* (Died.) Shoemaker*)* are the main foliar diseases and can be a constraint to wheat yields in Lithuania and in many countries with a temperate climate. Despite the differences in biology, these diseases frequently occur together. Both Septoria and Stagonospora blotch are economically important worldwide (Cunfer & Ueng, 1999). *P. nodorum* is known to be more pathogenic at late growth stages and can cause high levels of leaf and glume blotch at higher temperatures. The cooler temperatures of spring favour development of leaf blotch caused by *M. graminicola.* The greater prevalence of *P. nodorum* later in the season may be a consequence of increasing susceptibility of wheat plants to pathogen (Shaner & Beuchley, 1995). The prevalence of *P. nodorum* later in the season also can be predicted by interaction between pathogens. As Nolan et al. (1999) reported *P. nodorum* inhibits *M. graminicola* and it was concluded that the latter has a stimulatory effect on spore production by *P. nodorum*. Tan spot has recently become recognised as one of the important and widespread diseases of wheat. Losses due to tan spot have been chronically 3–15% and as high as 50% of grain yield (Hosford et al., 1987). The pattern of diseases development varied in each year under the influence of different rainfall and temperature patterns.

In Central Europe severe epidemics of Fusarium Head Blight (FHB) occur once or twice a decade only, but it can sharply reduce yield and quality traits of cereals (Miedaner at al.,

2001). Surveys carried out between 1951 and 1985 recorded 19 FHB outbreaks with a wheat grain yield reduction of 5-15% in the years when moderate epidemics of FHB were recorded and up to 40% in years when disease epidemics were severe (Pirgozliev et al., 2003).
Under suitable conditions the foliar diseases as well as FHB cause dramatic symptoms and can result in substantial losses in both yield and quality through the production of undersized grain either by affecting grain processing qualities or by producing a range of toxic metabolites. This paper provides the results of a multi-aspect comparative investigation on the use of strobilurin and triazole - based fungicides for winter wheat disease control. The aim was to study yield losses resulting from foliar and ear diseases epidemics and define the relationships between disease severity, green canopy retention, grain quality and yield.

2. Winter wheat leaf disease control with strobilurin-based and triazole

On the basis of field trials conducted during the period 2002–2004 at the Lithuanian Institute of Agriculture in Dotnuva the efficacy of fungicides against fungal leaf diseases of winter wheat cv. Zentos was explored. The impact of strobilurin based Allegro Plus, Rombus, Amistar, Opera, Acanto and triazoles Opus and Tilt (Table 1) on the epidemic progress of Septoria leaf blotch (*M. graminicola)* and Stagonospora blotch (*P. nodorum)* – both diseases were not separated, and tan spot (*P. tritici-repentis)* was surveyed. The trials were arranged in randomized blocks in the plots 10 m in length and 2.5 m in width with four replicates. The fungicides, except Folicur, were applied at the end of booting (BBCH 47), Folicur was applied at anthesis for FHB control.

Commercial name	Applied at BBCH	Active ingredients ($g\ l^{-1}$)	Rate	
			fungicide ($l\ ha^{-1}$)	active ingredients ($g\ ha^{-1}$)
Allegro Plus	47	Krezoxim–methyl; epoxyconazole; fenpropimorf (125+125+150)	1.0	125+125+150
Rombus	47	Trifloxystrobin; propiconazole (125+125)	1.0	125+125
Amistar	47	Azoxystrobin (250)	1.0	250
Opera	47	Pyraclostrobin + epoxyconazole (133+50)	1.5	200+75
Acanto	47	Picoxystrobin (250)	1.0	250
Opus	47	Epoxyconazole (125)	1.0	125
Tilt	47	Propiconazole (250)	0.5	125
Folicur	65	Tebuconazoze (250)	1.0	250

Table 1. Characteristics of fungicides used for the winter wheat disease control

Disease assessments on leaves were conducted periodically with 7-10 days interval from the end of booting (BBCH 47) to late milk stage (BBCH 79). Plant growth stages were identified according to the BBCH scale (Phenological growth stages..., 1997). Percent of leaf area showing symptoms of leaf diseases was used to quantify disease severity. Disease severity

was assessed on each plot in five randomly selected places on three adjacent tillers on three upper leaves using a percentage scale 0, 1, 5, 10, 25, 50, 75. The leaf positions on tillers were numbered relative to the uppermost leaf - the flag leaf. Thus the leaf immediately below the flag leaf (F) was designated F–1, the second leaf below the flag leaf – F–2. AUDPC (Area Under the Disease Progress Curve) was calculated by trapezoidal integration in accordance with 7-10 days interval disease severity data over the season.

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i-1}}{2} \right) (t_{i+1} - t_i) \quad (1)$$

where: y_i – disease severity %, t_i – interval of data records (days), n – number of assessments (Campbell & Madden, 1990). The plots were harvested and yields in t ha^{-1} were adjusted to 15% moisture content, thousand grain weight (TGW) was calculated. The significance of data was determined by the Fisher's criterion with a significance level of $P \leq 0.01$ and ≤ 0.05. Significant differences from untreated in Tables are marked as **($P \leq 0.01$) and *($P \leq 0.05$). Linear correlation analysis was used to examine the relationships between grain yield and AUDPC.

In 2002, fungal leaf diseases incidence and severity in winter wheat were slight due to extremely dry and unusually hot weather until harvest. In both 2003 and 2004, the pressure of fungal leaf diseases was severe. The unusually hot period during the wheat ripening stage in 2003 provoked the intense outbreak of tan spot *(P. tritici-repentis)*. At milk ripe abundant and severe tan spot infection dwarfed Septoria leaf and Stagonospora blotch on wheat leaves. However, the warm and rainy prolonged ripening season in 2004 promoted the severe infection of Septoria disease (Stagonospora blotch and Septoria leaf blotch). Both diseases were not separated, though in years 2002-2004 Stagonospora blotch *(P. nodorum)* considerably prevailed over Septoria leaf blotch *(M. graminicola)*. AUDPC allows expression of entire season's leaf diseases epidemic on different fungicide treatments. AUDPCs were plotted for each of the topmost three leaves from the data of the fungicide use.

Our experimental findings suggest that both strobilurin and triazole fungicides significantly suppressed the epidemic progress of Septoria disease (*P. nodorum* and *M. graminicola)* and tan spot *(P. tritici-repentis)* on the upper three leaves of winter wheat irrespective of dissimilar diseases pressure in particular years. In 2002 the development of both Septoria disease and tan spot was slight due to droughty weather and the AUDPC values in fungicide treated plots were low and comparable (Table 2). In both 2003 and 2004, infection of leaf diseases was vigorous. The lowest AUDPC value was recorded in the treatment with Opera (pyraclostrobin + epoxyconazole), while the highest – in the treatment applied with Tilt (propiconazole). AUDPCs of the other strobilurin fungicides and triazole Opus (epoxyconazole) were comparable. Our results suggest that the weakest control of Septoria disease ant tan spot was in Tilt treatment. As was reported by Milus and Chalkley (1997) propiconazole was the least effective treatment against *P. nodorum*. In order of precedence between DMI fungicides in 1994 epoxyconazole showed the best efficacy against Septoria both *M. graminicola* and *P. nodorum* (Kendall & Hollomon, 1994). Still recent epoxyconazole and prothioconazole are referred as very effective in controlling *M. graminicola* (Cools & Fraaije, 2008). According to sensitivity test of 42 isolates from Sweden, collected in 2003-

2005, the *P. nodorum* population is still sensitive to propiconazole, prothioconazole and cyprodinil, even though some isolates varied in sensitivity to triazoles (Blixt et al., 2009). The results of semi-field trial carried out in Denmark showed that propiconazole + fenpropimorf had only a residual preventive effect for 10 days in comparison to azoxystrobin, which showed a very effective, long preventive effect against *P. nodorum*, lasting for three weeks or more, however against *M. graminicola* both propiconazole and azoxystrobin had a similar curative effect (Jorgensen et al., 1999). After the strobilurin-based fungicides were introduced in 1994, this new class of fungicides provided a superior disease control and additional favourable effects on the physiology of the plant (Fraaije et al., 2003). In the years 2003 and 2004 the high efficacy of Qol fungicides was confirmed by our results (Table 2).
The first QoI-resistant isolates of *M. graminicola* were detected in the UK in 2001 at low frequency in QoI-treated plots (Fraaije et al., 2005), and subsequently in 2002 in five European countries (Gisi et al., 2004). The G143A mutation causing QoI resistance was first detected during 2002 in all tested populations (Torriani et al., 2009). During 2003 and 2004, the frequency of resistant isolates increased rapidly in northern Europe. The resistance is now widespread across the entire UK, whereas in France and Germany resistance levels are higher in the north than in the south (Fraaije et al., 2005). Resistance to QoI fungicides in *P. tritici-repentis* was detected in 2003 in Sweden and Denmark. The rapid increase of the frequency of the mutations F129L and G143A in pathogen population was observed and in 2005 both mutations were found in a significant proportion of the isolates from Sweden, Denmark and Germany (Sierotzki et al., 2007). A first report of variability in sensitivity of *P. nodorum* to azoxystrobin was made by Blixt et al. (2009). In the majority of the *P. nodorum* isolates, collected in 2003-2005 in Sweden, the mutation G143A, associated with loss of sensitivity to strobilurins was found. Recently Lithuanian population of *M. graminicola* has been showing medium resistance levels and *P. tritici-repentis* resistance levels are highly variable (www.frac.info). As for the increment of pathogen resistance to fungicides, the results of efficacy of the tested fungicide nowadays presumptively may be different than our presented. In commercial practice in Lithuania Qol-based fungicides are widely used and till now have given a good response to grain yield due to adequate disease control and maintenance of green leaf that act as grain filling prolongation.
Our results suggest that the highest yield and thousand grain weight (TWG) increase in experimental years 2002-2004 were recorded in Opera (pyraclostrobin + epoxyconazole) treatment, as top effective against Septoria disease and tan spot, and the lowest in Tilt (propiconazole) treatment, where it gave the lowest efficacy against these diseases (Table 8). In the case of high disease pressure in 2003 and 2004, the grain yield (X) and AUDPC (Y) of both Septoria disease and tan spot on F, F–1 and F–2 leaves showed strong negative linear correlations at a probability level of $P \leq 0.01$. Regression coefficient R2y/x for Septoria *(P. nodorum* and *M. graminicola)* ranged between 73.3-91.9% and for tan spot – 83.6-92.7%. However, in the droughty year 2002, regarding slight disease severity only Septoria disease AUDPC on F-1 leaves against yield showed a significant ($P \leq 0.01$) negative correlation (R2 71.4%). Our experimental findings on the correlation between the disease severity and yield agree with those obtained in Australia. As Bhathal et al. (2003) reported the different rates of progress of both tan spot and Stagonospora blotch caused similar losses in grain yield, ranging from 18% to 31%. The infection by either disease on the flag or penultimate leaf provided a good indication of yield losses.

Treatment[#]	AUDPC					
	Septoria disease (*P. nodorum* and *M. graminicola)*			Tan spot (*P. tritici-repentis*)		
	F §	F–1 §§	F–2 §§§	F §	F–1 §§	F–2 §§§
2002						
Untreated	8.6	9.6	4.3	18.4	14.7	1.1
Allegro Plus	0	0.2	0.3	9.0	5.8	1.3
Rombus	0.1	0.7	0.6	7.1	3.8	0.7
Amistar	0.4	2.2	0.6	3.1	3.9	0.6
Opera	0.1	0.2	0.3	2.7	2.2	1.2
Acanto	1.0	5.2	0.3	6.7	4.7	1.0
Opus	0.1	0	0.6	8.5	6.4	0.4
Tilt	0.2	1.6	0	7.0	4.7	1.4
2003						
Untreated	43.3	10.9	151.8	287.6	411.3	446.3
Allegro Plus	0.2	2.1	30.5	110.9	60.8	136.0
Rombus	0.9	1.5	42.8	109.2	62.5	128.8
Amistar	0.8	0.9	39.6	79.4	47.0	106.5
Opera	0	0.2	35.8	41.7	12.4	33.9
Acanto	4.2	1.6	36.9	107.8	62.0	103.8
Opus	0.1	0.4	38.6	89.3	30.9	96.6
Tilt	8.7	1.4	37.6	208.6	180.2	291.9
2004						
Untreated	135.2	490.1	649.7	108.6	155.5	153.8
Allegro Plus	5.5	45.0	98.8	33.4	48.9	52.2
Rombus	12.3	60.5	133.5	31.1	63.7	76.3
Amistar	8.9	53.3	100.1	28.0	40.8	49.0
Opera	1.2	19.2	40.8	9.9	14.0	28.6
Acanto	29.2	84.7	127.3	39.9	40.7	43.0
Opus	7.8	35.5	103.2	22.6	35.4	57.8
Tilt	39.1	156.2	306.7	51.7	63.6	75.1

In all treatments AUDPC value difference significant at P ≤0.01; #See Table 1 for fungicide rates; §F – flag leaf; §§F-1 – first below flag leaf; §§§F-2 – second below flag leaf.

Table 2. The effect of strobilurin and triazole fungicides on the epidemic progress of Septoria disease (*P. nodorum* and *M. graminicola)* and tan spot (*P. tritici-repentis*) in winter wheat.

3. The incidence of Stagonospora blotch *(P. nodorum)* on winter wheat ears and grain

The epidemic incidence of Stagonospora blotch in winter wheat canopy is determined by the amount of rainfall, number of rainy days, previous crop and straw residue on soil surface, infected seeds and other factors (Hansen et al., 1994). Gradual epidemics of Stagonospora blotch were characterized by disease arising on successive leaf layers as they appeared during sustain periods of weather suitable for inoculum's transport and infection. Ordinary spores from infected plants are transported by rain splash to 0.5 m and to 40 cm

above ground (Griffits & Ao, 1976). Wheat seed infection by *P. nodorum* is common, and infected seeds can be an important source of primary inoculum for foliar epidemics. According to Griffiths and Ao (1976) under the conditions prevailing to *P. nodorum* the significant distance for spreading from infected plant is about 2 m until heading. With this degree of spread less than 1000 infected plants per hectare are needed at the pre-heading stage to generate a widespread and severe attack and this situation arises if only 0.016% of seeds sown gave rise to infected plants. Transmission of the pathogen from infected seeds to coleoptiles can approach 100% over a wide soil temperature range; transmission to the first leaves is less than 50% and is most efficient at soil temperature below 17 °C. At least 44% of infected first leaves can be symptomless. (Shah & Bergstrom, 2000).

On the basis of field trials the efficacy of strobilurin-based and triazole fungicides on Stagonospora blotch on ears and laboratory analyses of grain infestation with *P. nodorum* is discussed. Percent of affected ears was used to quantify the disease incidence. Disease severity was assessed on each plot in five randomly selected places on three adjacent ears using a percentage scale 0, 1, 5, 10, 25, 50, 75. Grain infestation with *P. nodorum* in 2003 was tested using blotter-freezing method (Kietreiber, 1981) and in 2004 - both blotter-freezing and SNAW agar method (Manandhar & Sunfer, 1991).

As our experiments indicated, both strobilurin and triazole fungicides significantly suppressed the incidence and severity of Stagonospora blotch on leaves (Table 2). However, in the case of sudden outbreak of infection on ears, the incidence of glum blotch was irresistible. In 2003, abundant and severe tan spot infection prevailed and blanketed out Stagonospora blotch on wheat leaves until milk ripe. After some heavy rains at milk ripe Stagonospora blotch developed rapidly and fully occupied ears not only in untreated, but also in fungicide treated plots. In contrast, during 2004 disease developed rapidly before milk ripe and slowly later in the season. Rainy period during stem elongation and from heading to fruit development stage in that year caused a severe Stagonospora blotch infection on leaves (Table 2). Later in the season lack of precipitation and hot weather limited disease severity on glumes. Irrespective of Stagonospora blotch dissimilar incidence and severity on ears in 2003 and 2004 (Table 3), *P. nodorum* infection on wheat seeds was comparable. Similar results were obtained in Poland: correlation of level of seed infestation and subsequent disease severity was in many cases statistically insignificant (Arseniuk et al., 1998). In 2003, in spite of similar fungicide efficacy on leaves and ears only in Opera treatment significant decrease of *P. nodorum* seed infection was observed. In 2004, seed infection in parallel was tested by Blotter-freezing test and on SNAW (Septoria nodorum agar for wheat). Both Blotter-freezing and SNAW showed comparable results. In both experimental years strobilurin fungicides gave better protection of grain against *P. nodorum* infection, in comparison with triazoles.

Wheat seed infestation by *P. nodorum* is common, the extent and range depending mainly on rainfall during the production season (Shah & Bergstrom, 2000). According to Shaw et al., (2008) long term (1844-2003) archived samples analysis fluctuations in amount of *P. nodorum* in grain were related to changes in spring rainfall, summer temperature and national SO_2 emission. *P. nodorum* seed infection is influential primary infection source in wheat crop. *P. nodorum* transmission to seedling coleoptile and leaves occurred over a broad temperature range. Under the high densities at which wheat is sown, a significant number of infected seedlings per unit area may originate from relatively low initial seed infection levels and transmission efficiencies (Shah & Bergstrom, 2000). As Arseniuk et al. (1998) reported planting of highly infected seed resulted in more severe Stagonospora blotch than did the

planting of healthy or only slightly infected seed; however, environmental conditions contributed considerably to disease severity. Planting in clean fields, using seeds with a low level of seed-borne inoculum in addition treated with fungicides and applying foliar fungicide application contributed toward reducing leaf infestation by *P. nodorum*, severity of leaf and glume blotch and incidence *P. nodorum* in the harvested seed (Milus & Chalkley, 1997).

Treatments[#]	Infection on ears		Infected grain (%)	
	Incidence (%)	Severity (%)	Blotter test	SNAW
2003				
Untreated	100	28.8	22.3	-
Allegro Plus	100	19.4**	17.5	-
Rombus	100	17.6**	21.5	-
Amistar	100	12.8**	19.8	-
Opera	100	12.9**	9.7*	-
Acanto	100	12.1**	18.0	-
Opus	100	18.8**	23.2	-
Tilt	100	21.65	26.2	-
2004				
Untreated	100	3.65	31.5	27.4
Allegro Plus	85.0**	1.12*	22.0	25.6
Rombus	81.6**	1.08*	15.6**	23.4
Amistar	73.3**	0.80**	18.0*	13.3**
Opera	58.4**	0.58**	19.8*	11.9**
Acanto	76.7**	1.03*	18.0*	11.9**
Opus	80.0**	1.22*	21.7	21.2
Tilt	90.0*	2.73	21.0	26.1

* Difference significant at P ≤0.05. ** Difference significant at P ≤0.01;
#See Table 1 for fungicide rates;

Table 3. The incidence and severity of Stagonospora blotch on winter wheat ears and grain infestation with *P. nodorum* in response to strobilurin-based and triazole fungicides

During the last several years in Lithuania a clear predomination of *M. graminicola* over *P. nodorum* has been observed (unpublished data). It may be determined by mild winters and agronomic factors. As Shaw et al. (2008) reported, annual variability among of *M. graminicola* or *P. nodorum* was dominated by weather factors occurring over a period longer than the growing season.

4. The impact of triazole and strobilurin-based fungicides on the incidence of Fusarium head blight toxic fungi and mycotoxins on winter wheat grain.

The *Fusarium* species predominantly found associated with FHB in wheat and other small-grain cereals all over Europe are *F. graminearum, F. avenaceum, F. culmorum, F. poae.* Among the less encountered species are *F. cerealis, F. equiseti, F. sporotrichioides, F. tricinctum* and several others witch are less pathogenic, but also toxigenic (Bottalico & Perrone, 2002; Birzele et al., 2002). In addition to true *Fusarium* species, *Monographella nivalis* may also cause

head blight and can be particularly prevalent where cooler and wetter conditions prevail (Nicholson et al., 2003). The predominant *Fusarium* species on cereal grain during 1999–2002 under Lithuanian conditions were *F. culmorum, F. equiseti, F. avenaceum, M. nivalis* and *F. oxysporum.* The frequency of detection of *Fusarium* spp. micromycetes on cereal grain was 93.5 % (Lugauskas et al., 2004). Most species producing inocula, grow best, and are more pathogenic to cereal heads at warm temperatures and under humid conditions (Doohan et al., 2003). FHB is of particular concern because of the ability of the *Fusarium* species to produce mycotoxins in the grains that are harmful to human and animal consumers. The most frequently encountered mycotoxins in FHB in Europe have proved to be deoxynivalenol (DON) and zearalenone (ZEA) produced by *F. graminearum* and *F. culmorum*. Nivalenol was usually found associated with deoxynivalenol and its derivatives, formed by *F. graminearum, F. cerealis, F. culmorum* and, in northern areas, by *F. poae*. Moreover, from central to northern European countries moniliformin has been consistently reported, as a consequence of the widespread distribution of *F. avenaceum*, whereas the occurrence of T-2 has been recorded in conjunction with sporadic epidemics of *F. sporotrichioides* and *F. poae*. The beauvericin has recently been found in Finnish wheat colonized by *F. avenaceum* and *F. poae* (Bottalico & Perrone, 2002; Nicholson et al., 2003).

The control of *Fusarium* spp. and *M. nivalis* and the production of DON, including both naturally and artificially inoculated trials, were reported. Application of fungicides to reduce FHB gave a different control of these fungi. Tebuconazole selectively controlled *F. culmorum* and *F. avenaceum* and reduced levels of DON, but showed little control *M. nivalis.* Application of azoxystrobin, however, selectively controlled *M. nivalis* and allowed greater colonisation by toxigenic *Fusarium* species. This treatment also led to increased levels of DON detected. Azoxystrobin application two days post-inoculation increased the production of DON per unit of pathogen in artificially inoculated field trials. This result indicates the potential risk of increasing DON contamination of grain following treatment with azoxystrobin to control head blight in susceptible cultivars (Simpson et al., 2001). The effect of different fungicide treatments on FHB, grain yields and DON was evaluated after artificial inoculation under field conditions with a mixture of *F. graminearum* and *F. culmorum* on five different cultivars of soft and durum wheat. Treatments with cyproconazole + prochloraz and mixture tebuconazole + azoxystrobin significantly reduced the FHB disease severity (by 25 and 77 %) and DON content (by between 32 and 89 %) in the grain as compared with the inoculated control. Yields and thousand grain weight were higher in the plots subjected to fungicide treatments (Haidukowski et al., 2005).

The aim of our experiments was to investigate the efficacy of strobilurin-based and triazole fungicides on FHB in winter wheat and laboratory analyses of grain contamination with micromycetes which can occur on ripening ears of wheat and mycotoxins in relation to the application timing and environmental factors. Furthermore, control of these fungi, and in particular the prevention of increasing concentration of mycotoxins are discussed.

For investigation of mycoflora of grain at different maturity stages (BBCH 75 and BBCH 85–87) non–disinfected grain, taken directly from ears randomly from each plot (10 grains per ear, 10 ears per plot) were planted on PDA (potato dextrose agar). The plates were incubated in the dark at 25 ˚C for 7 days. The infection level of grain was evaluated in percent (0 – all grains healthy, 100% – all grains infected). Microscopic studies of *Fusarium* fungi were carried out after 7–8 days and were identified on the basis of their cultural and morphological characteristics according to Leslie and Summeral (2006).

The mycotoxins DON, ZEA, and T-2 were analysed by the CD-ELISA (competitive direct enzyme-linked immunosorbent assay) method. The Veratox® quantitative test kits (Neogen corporation, Food Safety Diagnostics), approved by the AOAC Research Institute (Certificate N 950702) were used for the analysis. The optical densities of samples and controls from standard curve were estimated by a photometer Neogen Stat Fax®303 Plus, using filter of 650 nm. Measured absorbances were automatically converted to the mycotoxin concentration units - μg kg^{-1}. The results were estimated taking into account the lowest calibration curve's mycotoxin concentration value (LOD-limit of detection), which is for: DON – 100.0 μg kg^{-1} (ppb); ZEA – 10.0 μg kg^{-1} (ppb); T-2 – 7.5 μg kg^{-1} (ppb).
The occurrence of *Fusarium* spp. in wheat ears depended mostly on the climatic conditions during flowering. Continuous rainfall during and after flowering with lower temperatures and longer period of open inflorescence increased grain infection level (Birzele et al., 2002). In 2003, wheat flowered from 20 to 26 of June. During the three ten–day periods before flowering warm weather conditions prevailed, except for several days with weak rain during the heading stage. Only the showery rainfall before and during the flowering occurred. Folicur was applied on 23 June. In 2004, continuous rainfall predominated before, during and after flowering, with lower temperatures. That year wheat flowered from 24 June to 2 July. Folicur was applied on 28 June.
In 2003, the appearance of FHB was late and slight. At dough stage only 12 % of ears showed slight symptoms of FHB (Table 4).

Treatment [#]	BBCH 75		BBCH 85	
	FHB Incidence (%)	Grain surface infection (%)	FHB Incidence (%)	Grain surface infection (%)
2003				
Untreated	0	50.5	12.0	33.3
Opera	0	45.3	1.0**	40.8
Tilt	0	45.3	4.0	12.5**
Opera and Folicur	0	35.8*	4.0	34.0
Tilt and Folicur	0	35.0*	4.0	14.3**
2004				
Untreated	55.0	5.8	38.3	23.5
Opera	31.7*	8.5	25.0*	56.0*
Tilt	53.3	10.0	41.6	50.0
Opera and Folicur	41.7	6.0	23.3*	24.0
Tilt and Folicur	61.7	9.3	31.6	15.8

* Difference significant at $P \leq 0.05$; ** Difference significant at $P \leq 0.01$;
[#]See Table 1 for fungicide rates and application time

Table 4. Incidence of FHB and grain infection in wheat at milk (BBCH 75) and dough (BBCH 85) ripe stages in 2003 and 2004

A significant reduction in diseased ears in Opera treated plots at booting was obtained. Slow disease incidence might have resulted from dry plant debris before flowering, because when soil moisture content is below 30 %, ascospore production is not possible. When it is greater

than 80 %, ascospore production is at its maximum. Peak ascospore release occurred 2–4 days after rainfall and its dispersal is associated with rainfall too (Xu, 2003). In 2004, due to the rainy weather, FHB appearance was early and abundant. The first symptoms of infected glumes were spotted at milk ripe. At that time half of the ears were diseased. Later in the season, at dough stage the incidence of FHB was similar to that at milk ripe. Only in Opera treated plots the incidence of FHB was significantly lower than in untreated. The consistent control of FHB, achieved through fungicides under field conditions, depends on the application timing. Studies where fungicides were applied between BBCH 32 and 50 failed to reveal any significant reduction of FHB (Hutcheon & Jordan, 1992). However, when fungicides were applied between BBCH 59 and 70 significant reductions were achieved in both the severity of FHB and concentration of mycotoxins in harvested grain. According to Mathies and Buchenauer (2000), applications of either tebuconazole or prochloraz, 2 days post inoculation (BBCH 65) reduced disease severity by 56 and 41 %, whilst application 8 days pre–inoculation and 9 days post–inoculation were less effective.

In 2003, in spite of unfavourable conditions for FHB at milk ripe in fungicide untreated plots *Fusarium* spp. was detected on 50.5 % non–disinfected grain, taken directly from ears (Table 4). Application with fungicides at the end of booting did not influence grain infection level, however, additional application with tebuconazole significantly decreased grain infection. Later, at hard dough stage grain contamination with *Fusarium* spp. was lower than at milk ripe. It might be related to glum residual surface infestation, which at milk ripe together with grain got into PDA. Significant decrease of grain contamination at that stage was recorded in the treatment with Tilt applied at booting and additionally with Folicur at flowering. In 2004, at milk ripe there were found only 5.8–10 % *Fusarium* spp. contaminated grain, nevertheless at dough ripe the infection level increased markedly, especially in the plots treated with fungicides at booting. In the plots applied with Folicur at flowering *Fusarium* spp. contaminated grain content was twice as low as in the plots treated only at booting.

In spite of different levels of FHB infection, *Fusarium* infected grain content (internal infection) after harvesting in untreated plots was found similar to that in 2004 and 2003 (Table 5). It might have been determined by severe epidemics of foliar diseases, early dried leaves and early matured plants in theses plots. In 2003, *Fusarium* spp. infected grains in Folicur treated plots decreased significantly. That year in all grain samples there was detected DON and a slight grain contamination with ZEA and T-2 was found. In all fungicide treated plots ZEA concentration in grain was significantly lower, and especially low in Folicur treatment. T-2 was detected in all grain samples, but at negligible quantities and the influence of fungicide treatment was inconsistent. In 2004, in fungicide treated plots in post–harvested samples *Fusarium* spp. infected grain made up 12.3–20.8 %, while in untreated 7.8 %. In Opera treatment the increase (20.8 %) was significant. DON concentration in grain samples taken from fungicide treated plots at booting was significantly higher than in untreated and treated with Folicur at flowering. That year relatively high concentration (65.4 μg kg^{-1}) of ZEA was found in untreated grain samples. ZEA in fungicide treated harvested grain samples was not detected.

Survey of results of two years' investigation showed that chemical control of FHB and grain contamination with *Fusarium* spp. under field conditions might be inconsistent. Field trials conducted to assay the efficacy of fungicides against FHB gave conflicting results. Effective chemical control of FHB is confounded by the fact that disease is caused by a complex of pathogens which interact with one another and with saprophytic species such as *Alternaria*

Treatment #	*Fusarium* infected grain (%)	Content of mycotoxins µg kg^{-1}		
		DON	ZEN	T-2
2003				
Untreated	7.5	160.3	27.7	<LOD
Opera	7.8	129.8*	13.8*	7.5
Tilt	4.0	125.0*	12.5*	<LOD
Opera and Folicur	2.3	122.0*	n.d.	<LOD
Tilt and Folicur	3.3	144.8	<LOD	8.1
2004				
Untreated	7.8	126.1	65.4	n.d.
Opera	20.8	135.0	n.d.	7.9
Tilt	15.0	142.0	n.d.	<LOD
Opera and Folicur	12.3	125.0	n.d.	8.0
Tilt and Folicur	15.8	n.d.	n.d.	n.d.

* Difference significant at $P \leq 0.05$; ** Difference significant at $P \leq 0.01$;
#See Table 1 for fungicide rates and application time

Table 5. Effect of different mode and action fungicides on internal *Fusarium* spp. infection and contamination with mycotoxins DON, ZEN and T-2 in harvested grain

spp. and *Cladosporium* spp. (Pirgozliev et al., 2003). According to Milus and Parsons (1994) fungicides benomyl, chlorothalonil, fenbuconazole, flusilasole, propiconazole, tebuconazole had no effect on FHB, mycotoxins levels or yield of harvested grain. Conversely, Siradinou and Buchenauer (2001) showed that applications of tebuconazole 2 days before and 2 days post-inoculation of wheat plots with *F. culmorum* reduced severity of FHB and DON content in wheat grain by 61–89 % and 50–70 %, respectively and metconazole by 69 % and 71 %, respectively. Application of fungicides to reduce FHB gave a differential control of *Fusarium* spp. and *Monographella nivalis*, which is known as non–toxigenic causal agent of FHB. Tebuconazole selectively controlled *F. culmorum* and *F. avenaceum* and reduced levels of DON, but showed little control of *M. nivalis*. Application of azoxystrobin, however, selectively controlled *M. nivalis* and allowed greater colonization by toxigenic *Fusarium* spp. This treatment also led to increased levels of DON detected (Simpson, 2001).

Due to the lack of consistently effective control measures, FHB continues to pose a significant threat to the yield and quality of wheat. Based on our experimental evidence from both field experiments and laboratory investigations, in the future we have to focus on some of the complex interactions between *Fusarium* infections, differential effects of fungicides, environmental factors and associated mycoflora which can occur on ripening ears of wheat. Experimental years were not conducive to the spread of FHB, as a result, efficacy of fungicide use against this disease did not stand out sufficiently, especially in mycotoxin tests. Discussions have been raised in scientific literature recently about the "masked" or conjugated mycotoxins (Berthiller et al., 2007; Galaverna et al., 2009). It is believed that in growing plants, especially under the effects of natural and anthropogenic factors (including fungicides) the already formed toxic metabolites can biotransform into other very similar chemical compounds that are simply not detected by the methods used by us. As a result, our future research will be focused on masked mycotoxins in naturally contaminated cereals.

5. Efficacy of strobilurin based and triazole treatments on the winter wheat senescence, grain yield and quality.

Yield losses due to diseases vary between crops and regions and are often 10–20 % (Hewitt, 2000). Research evidence on the effects of disease incidence and fungicide use on grain quality and safety are diverse and sometimes controversial (Mesterhazy et al., 2003; Covarelli et al., 2004; Wang et al., 2005; Polišenská & Tvaružek 2007; Gärtner et al., 2008; Clarck, 2003; Everts et al., 2001; Ruske et al., 2003). This part of our study was aimed to estimate the effects of fungicides containing strobilurins and triazoles on the senescence of winter wheat cv. Zentos leaves, grain yield and quality.

Measurements of the concentrations of chlorophylls, total nitrogen in flag leaves of fungicides - treated plants were started on the third day after treatment and were repeated weekly until the end of vegetation (5-6 weeks). Photosynthetic pigments and nitrogen analyses were not done on the vegetative mass of the treatments that had lost greenness. Chlorophylls a, b and carotenoids concentration was determined according to Ermakov (Ermakov et al., 1987). Samples for the total nitrogen concentration determination in flag leaves were dried, ground by a mill with 1 mm sieve and analysed by the Kjeldahl method, using FOSS Tecator Kjeltec system with 1002 Distilling Unit. Protein concentration in grain was calculated by multiplying the total nitrogen content, measured after Kjeldahl, by the coefficient 5.7. Wet gluten content was determined in flour (milled with Perten 3100 mill) by hand washing with excess water according to AACC method 38-10. Gluten deformation index was measured by IDK-1M instrument (standard LST:1522). The falling number and the sedimentation value according to Zeleny (Zeleny index) were determined following the ICC standard methods 107/1, 118 and 116/1.

The experimental data were compared by the analysis of variance (ANOVA), where the F-ratio was significant, the least significant difference (LSD) was calculated for $P \leq 0.05$ (symbol *) and $P \leq 0.01$(symbol **). The weather conditions after the resumption of vegetative growth of winter wheat were diverse and were discussed in the previous section of the chapter.

In the absence of fungicide, green leaf area decline was associated with drought or infection with a number of foliar pathogens (Gooding et al., 2000). Loss of chlorophyll is the classical indicator of senescence in plants. Our observation showed very low and inconsistent differences in the concentrations of chlorophylls a+b as well as the differences in nitrogen concentrations between treatments during the first weeks after fungicide spraying. As a result, the current paper will discuss the data of only the last three assessments. Lower concentrations of chlorophylls were identified in the leaves from untreated plots compared with those in the samples taken from nearly all fungicide treated ones starting with the fourth week from the spray application in 2003 and 2004 and the fifth week in 2002 (Table 6). No advantage of strobilurins over triazoles to prolong green leaf area retention was noted under the conditions of the year 2002. Inappreciably lower concentrations of chlorophylls in 2003 were identified in the 5th week after the fungicide spraying in wheat leaves of the treatments sprayed with triazole fungicide Opus (epoxyconazole), and especially with Tilt (propiconazole). In the 6th week in 2002 in all treatments, in 2003 in the untreated and Tilt treated plots, in 2004 in the control treatment only, the leaves had lost their greenness, whereas the vegetation of the rest of the treatments in 2003 and 2004 still continued. Alterations in nitrogen concentrations subject to fungicide application or harvest year have the similar trend like those of chlorophylls. Crop infection with foliar fungal diseases cause leaf drying, colour alterations and reduction of greenness, i.e. leaf pigment loss.

Year	2002		2003			2004		
Week after F treatment	4	5	4	5	6	4	5	6
Fungicide[#]	Chlorophylls a+b (mg /100 g fresh matter)							
Control	238	157	263	204	NA	261	357	NA
Allegro Plus	241	201	310	280	171	310	360	168
Rombus	271	167	340	306	239	284	375	175
Amistar	226	169	346	305	193	301	385	164
Opera	241	173	331	301	219	283	365	187
Acanto	209	199	317	273	207	302	365	169
Opus	205	205	311	266	190	297	361	172
Tilt	223	179	325	256	NA	291	370	151
	Nitrogen (g kg^{-1} dry matter)							
Control	25.2	25.6	35.6	29.8	NA	33.1	27.8	NA
Allegro Plus	26.4	28.2	37.6	32.4	24.7	30.6	30.1	24.8
Rombus	27.4	27.4	37.9	35.7	28.0	31.7	30.1	29.8
Amistar	25.3	27.1	38.7	33.4	24.7	35.4	30.1	27.6
Opera	27.9	27.0	39.4	34.5	25.0	34.6	31.9	27.8
Acanto	25.2	26.5	35.8	33.4	25.5	31.3	35.1	29.1
Opus	25.4	26.5	37.9	32.0	24.5	32.4	32.2	27.3
Tilt	27.3	26.7	38.5	29.5	NA	33.4	31.1	26.9

[#]See Table 1 for fungicide rates; ¶NA – not analysed

Table 6. Alteration of the concentrations of chlorophylls a+b and nitrogen in the flag leaves of winter wheat cv. Zentos as affected by the fungicide applied

Strobilurins inhibit respiration of fungi, while triazoles and morpholines arrest biosynthesis of fungi sterols. These fungicides exert protecting and eradicant effects. Strobilurins and triazoles fungicides application significantly suppressed the epidemic progress of Stagonospora leaf blotch and tan spot on the upper three leaves, including flag leaf both in 2003 and 2004 (Gaurilčikienė & Ronis, 2006). Besides, the weakest control of leaf diseases was in propiconazole treatment.

In sum we can conclude, that all fungicides tested prolonged retention of green canopy of wheat plants in 2003-2004. Chlorophylls concentrations in flag leaves at the end of plant vegetation, i.e. in the fifth week after fungicide treatment, were higher in the plots treated with strobilurins compared with those treated with triazole Tilt and untreated plots. There is abundant information in literature that strobilurins have considerably stronger positive effect on plant metabolic activity than any other compounds (e.g. azoles) (Beck, 2004), with the extension of flag leaf life, related to higher chlorophyll content (Habermeyer et al., 1998; McCartney et al., 2007) and higher CO_2 consumption, i.e. photosynthetic index (Häuser-Hahn et al., 2004; Oerke et al., 2005). According to Zhang et al. (2010) fungicide, including strobilurins, treatments appeared to delay the senescence of wheat and increase the grain yield of wheat, owing to retarding the enhancement of active oxygen species and the decrease of antioxidative enzyme activity during aging of wheat. However, slow up of leaf senescence depends on a variety (Beck, 2004; Habermeyer et al., 1998; Oerke et al., 2005; Ruske et al., 2003). Two-way analysis of variance (ANOVA) was carried out according to the following scheme: A factor fungicide applied, B factor harvest year were used to reveal the significance of

factors for winter wheat grain yield and quality components tested, as well as for protein and gluten yields (Table 7). The tested factors were significant at $P \leq 0.01$ probability for wheat grain, protein and gluten yields. The interactions of these factors significantly at $P \leq 0.01$ affected grain yield and at $P \leq 0.05$ protein yield.

Factor	Fisher's test							
	GY	Pr	Glu	GluDI	ZI	FN°	PrY	GluY
Fungicides (F)	16.24**	0.1	1.29	0.59	0.69	0.63	11.67**	7.55**
Harvest year (Yr)	524.5**	90.76**	149.4**	93.9**	266.0**	57.42**	544.1**	202.1**
Interaction FxYr	2.48**	0.93	0.47	0.54	1.02	0.75	2.14*	1.22

Table 7. Significance of the effect of the fungicide applied and harvest year (2002-2004) on grain yield (GY), protein (Pr), gluten (Glu) concentrations, gluten deformation index (GluDI), Zeleny index (ZI), falling number (FN°), grain protein and gluten yield (PrY, GluY) according to Fisher's test

However, plant protection with fungicides composed of different active ingredients did not have any impact on grain quality parameters. The interaction between the use (treatments) of plant protection against fungal diseases and harvest year was also statistically insignificant. This observation does not contradict the inferences about the significance of the air temperature and amount of precipitation at different plant development periods for the wheat grain yield and quality, including protein concentration, made by other researchers (Guttieri et al., 2001; Cesevičienė & Mašauskienė, 2008; Triboi et al., 2003; Souza et al., 2004). So, fungicide efficacy on grain quality depended firstly on the weather conditions being conducive to the occurrence of fungal diseases and then on fungicide applied.

Fungicide application significantly increased grain yield in all fungicide-treated plots in 2003 and 2004, and in most plots - in 2002, and especially in the conditions of 2004 (Table 8). The last year was conducive to the occurrence of fungal diseases (Gaurilčikienė & Ronis, 2006). The extra yield amounted to 0.70 and 2.16 t ha^{-1}. In 2002, the effect of not all the fungicides used was considerable on the yield increase: the extra yield was statistically insignificant in the plots treated with fungicide, containing only one active ingredient - strobilurin Amistar and Acanto, and triazole Tilt. Both in 2003 and 2004, in all fungicide-treated plots the yield significantly increased at 99% probability level, except for propiconazole treatment where yield increase was of 95% probability level. The data from 2003-2004 as well as the data averaged over all experimental years suggest that the highest winter wheat yield increase was obtained from the plots treated with the fungicide Opera, while the least increase was recorded for the plots applied with triazole Tilt. The plots sprayed with the other fungicide containing strobilurins and triazole Opus gave a similar average grain yield increase. The yield increased due to flag leaf's senescence delay, which was related to chlorophylls concentration (Table 6 and 8). Because the flag leaf makes up nearly 75% of the effective leaf area that contributes to grain fill (Kelley, 2001), keeping it free of diseases with foliar fungicide application can be beneficial for kernel development.

Fungicides help to retain vegetation of the flag leaves, allowing conditions for larger grains growth. Since in 2002 drought and heat occurred, upper leaves withered up early in all plots, including fungicide-treated ones and TGW increase in most of the treatments was minor (Table 8). However, in 2003 and 2004 the TGW increase was significant in fungicide treated plots, with the two exceptions in 2003 – plots sprayed with strobilurin based fungicide

Treatments[#]	2002		2003		2004	
	Yield increase	TGW increase	Yield increase	TGW increase	Yield increase	TGW increase
Untreated	7.52[§]	47.1[§]	6.11[§]	45.2[§]	8.50[§]	49.7[§]
Allegro Plus	0.94	0.4	0.78	1.2	1.85	3.8
Rombus	0.62	0.2	0.73	2.2	1.39	4.3
Amistar	0.28	0.6	1.02	2.4	1.84	5.5
Opera	0.88	1.0	1.19	3.0	2.16	5.9
Acanto	0.22	0.2	0.93	2.5	1.54	5.5
Opus	0.67	1.0	0.86	2.5	1.56	4.8
Tilt	0.34	0.5	0.58	1.4	0.70	2.5
$LSD_{0.05}$	0.461	0.88	0.475	1.42	0.549	1.12
$LSD_{0.01}$	0.616	1.17	0.635	1.90	0.734	1.50

[#]See Table 1 for fungicide rates; [¶]TGW – thousand grain weight g; [§]Grain yield and TGW in untreated plots

Table 8. Effects of fungicide treatment on grain yield (t ha^{-1}) and thousand grain weight (g) increase

Allegro Plus and triazole Tilt. The largest grains grew and the highest TGW increase due to the use of fungicides was achieved in 2004, thanks to prolonged period of grain filling and maturation through cool and rainy weather.

Our findings suggest that compared with untreated plots, all fungicides applied in the experiment had only an insignificant effect on all the tested wheat grain quality parameters with rare exceptions (Table 9). Since the protein and gluten are the most important and obligatory indicators describing grain quality, we paid more attention to their variation peculiarities. The tables do not present the data of protein as well as gluten concentration and other quality parameters values for separate treatments of each year since quality variation among treatments was insignificant. In relation to the fungicides use, protein concentration in grain fluctuated within the similar range: 126-148 g kg^{-1} DM in the plots treated with strobilurins, 130-146 g kg^{-1} DM – with triazoles and 128-145 g kg^{-1} DM in untreated plots, even though fungicide prolonged green leaf area retention compared with untreated plots. kg^{-1} DM in untreated plots, even though fungicide prolonged green leaf area retention compared with untreated plots. Similar regularities and conclusions are found and discussed in literature: the effects of fungicides on grain protein concentration and its relationship with green area retention of the flag leaf were inconsistent over years (Kelley, 2001; Everts et al., 2001). Protein concentration depended on the year. Protein concentration in the grain of 2003 harvest, averaged across all treatments, was 138 g kg^{-1} and ranged from

Quality parameter	2002			2003			2004		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Protein, g kg^{-1}	130	126	132	138	136	141	146	144	148
Gluten, g kg^{-1}	245	240	254	299	287	311*	274	268	278
Gluten DI, units	85.1	84.0	89.2	78.4	76.0	81.6	67.3	63.2	70.6
Zeleny Index, ml	43.5	39.8	47.2*	63.6	62.1	65.2*	57.3	54.8	58.8
Falling N°, s	417	398	426	468	461	475	373	366	383

Table 9. Variation of wheat grain quality as affected by the fungicide applied and harvest year

136 g kg^{-1} in the plots treated with Rombus (trifloxystrobin+propiconazole), Opera (pyraclostrobin+epoxyconazole) and Opus (epoxyconazole) to 141 g kg^{-1} in the plots treated with Acanto (picoxystrobin) and the fungicide, containing three active ingredients of different chemical groups, i.e. Allegro Plus (kresoxim-methyl + epoxyconazole + fenpropimorph).

In the yield of 2004 protein concentration varied from 144 g kg^{-1} (strobilurin Amistar and triazole Tilt treatments) to 148 g kg-1 in Rombus treated plots, with 146 g kg^{-1} on average. The grain from the 2002 harvest contained less protein, but the differences between treatments did not exceed 95% confidence limits. Some literature sources suggest that if wheat does not receive nitrogen compounds during grain ripening period, less nitrogen will accumulate in grain (Triboi et al., 2003). In 2002, during grain ripening period the weather was hot and sunny, however, shortage of rainfall and the drought interrupted plant vegetation, markedly accelerated grain ripening and at the same time nitrogen flow into grain. It is known that factors and measures aimed to maximize the yield exert an opposite effect on grain quality, especially protein concentration. For example, environmental factors that lead to high yields can also lead to a reduction in protein concentration, partly because it appears that nitrogen quantity per grain is relatively conserved when grain weight is modified by increased temperatures and restricted water availability (Gooding et al., 2003; Triboi & Triboi-Blondel, 2002). The differences in mean annual values of grain quality parameters show that harvest year conditions affected wheat grain quality formation more significantly, than fungicides use. The fact that fungicide-treated wheat plots produced higher grain yield with undiminished protein concentration, indicates that plant protection against fungal diseases exerts a positive effect not only on dry matter but also on nitrogen accumulation in grain. This agrees with the data in literature: there were several instances where grain protein concentration was unaffected despite large (1.5 t ha^{-1}) increases in grain yield following fungicide use (Ruske et al., 2003). While comparing the data of grain yield and protein concentration of the year 2002 and 2003, the consequences of "dilution" effect, i.e. higher yield has a lower protein concentration, could be discerned. However, this phenomenon cannot explain such relationship between the yield and protein concentration of the grain grown in 2004. Dilution of grain protein concentration following fungicide use, when it did occur, was small compared with what would be predicted by adoption of other yield increasing techniques such as the selection of high yielding cultivars (based on currently available cultivars) or by growing wheat in favourable climates (Ruske et al., 2003). The year 2004 was favourable not only for dry matter accumulation but also for effective utilisation of mineral nitrogen for protein biosynthesis. Compared with the control treatment, in the fungicide-sprayed crops photosynthesis was more intensive, during which carbohydrate accumulation and protein synthesis occurred uniformly, which prevented the consequences of "dilution" effect in significantly higher grain yield produced in the plots protected from fungal diseases by fungicide treatments.

Since gluten is some portion of proteins – it is based on a water-insoluble fraction of ones - glutenins and gliadins, there are the presumable consistent patterns of variation the same as for protein concentration. Actually, fungicides tested against fungal diseases mostly insignificantly affected wet gluten concentration in grain (Table 9). Only in 2003 in plots treated fungicide Allegro plus, composed of three active ingredients, grain contained more gluten on average than grain in other plots and significantly (at $P \leq 0.05$) more than in plots treated with only one strobilurin azoxystrobin containing fungicide Amistar and fungicide-

untreated plots. Grains matured in fungicide Allegro plus treated plots exhibited the highest gluten content in 2002 also, and in Amistar – sprayed plots grain had less gluten than the rest treatments all three years of investigation. This observation agrees with the inferences made by other researchers: none of the fungicides caused any significant changes in the wet gluten content or had only minor effect (Tanács et al., 2005; Wang et al., 2004).

Sedimentation is a protein property to swell in weak acid solutions. This is the important bread baking characteristics describing indicator. By averaged data of replications Zeleny index varied in a range 39.8 - 65.2 ml (Table 9) and met the local requirements for first class of grain quality. Due to the use of different fungicides statistically significant differences between the treatments were found in only two cases: in 2002 in the strobilurin-containing fungicide Allegro plus treated plots Zeleny index was higher than that in the other treatments and significantly (at $P \leq 0.05$) higher than in plots treated with Opus, Opera, Amistar and untreated plot. In triazole Tilt fungicide treated plots of 2003 yield matured grains exhibited the highest sedimentation (65.2 ml), which was significantly (at $P \leq 0.05$) higher than in the untreated (63.0 ml), treated with strobilurins Amistar (63.2 ml), Opera (62.1 ml) and triazole Opus (63.0 ml) plots. In Amistar - sprayed treatment matured grain Zeleny index was the lowest or one of the least every year.

For buying wheat in Lithuania the falling number must be not less than 200 seconds. This indicator of grain quality in all treatments satisfied requirements of local standard and was significantly greater than 200s: from 366-382 s in 2004 to 461-475 s in the yield of 2003 harvest (Table 9). In 2002-2003, sunny and dry weather during crop maturation and harvest time could be a factor underlying the very low alpha-amylase, i.e. a high falling number. Plant protection products use did not reveal any reliable influence in any year. Grain yield of 2004 in all treatments exhibited high quality, elastic gluten. Grains matured in 2002 had weaker gluten, i.e. gluten deformation index was higher, but satisfied the local requirements of food grains; the differences among fungicide-treated and untreated plots were statistically unreliable.

Although fungicide use had only a minor effect on the grain quality, the response of protein and wet gluten yield to the plant protection was marked each experimental year (Fig. 1). In most cases protein and gluten yields were higher for the strobilurin-treated plots (with a small exception mostly in 2002) than for those treated with triazole Tilt and untreated. Protein and gluten yields in the plots treated with Opus (3481 and 6896 kg ha^{-1} during 3 years) were similar to those produced in the plots treated with fungicides containing some strobilurins like Rombus, Amistar and Acanto (3440-3500 and 6735-6804 kg ha^{-1} respectively). The highest average protein yield, like that of gluten, was obtained when the fungicide used contained both strobilurin and epoxyconazole (Fig. 1, treatments 2 and 5). When estimating protein and gluten yield according to years, it is obvious that in 2004 protein yield was by approximately 1.43-1.57 times higher than that in 2003 and gluten yield - by 1.26-1.48 times higher than that in 2002.

Having estimated the relationship of FHB incidence and grain *Fusarium* spp. (%) contamination with grain quality, statistically significant correlations were found with many grain quality indicators (Table 10). However, the data of these correlations should be viewed critically. Having calculated respective correlations for separate years, a weak but also negative correlation was established only between FHB incidence and Gluten DI and Zeleny index both in 2003 and 2004.

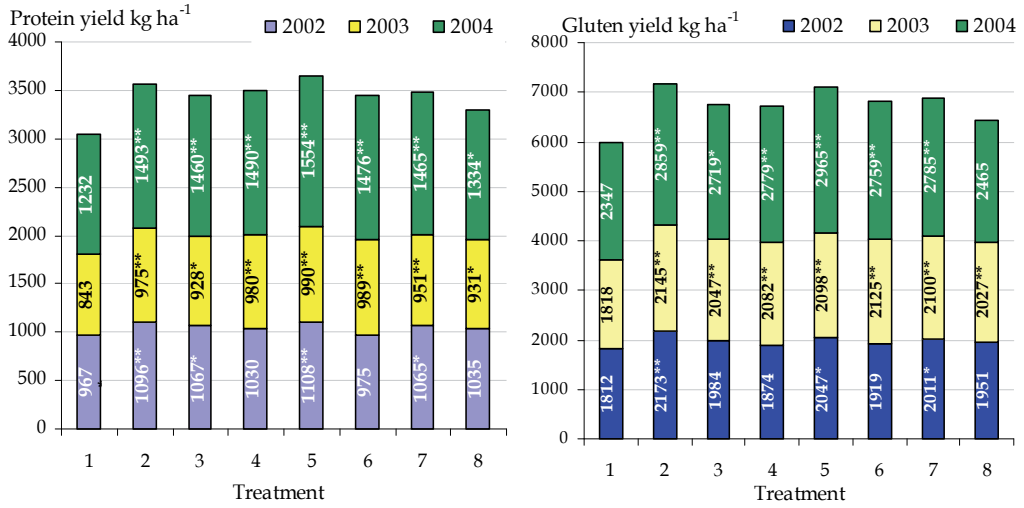

Fig. 1. Effect of fungicide treatment on grain protein and gluten yields. 1- no fungicides, 2- Allegro Plus, 3- Rombus, 4- Amistar, 5- Opera, 6- Acanto, 7- Opus, 8- Tilt; *, **difference from fungicide untreated plot significant at $P \leq 0.05$ and at $P \leq 0.01$ respectively; fungicide a.i. see in Table 1

FHB incidence (%) with:	Coefficient of linear correlation r	*Fusarium* spp. % with:	Coefficients of linear correlation r
Fusarium spp	0.76**		
Protein	0.842**	Protein	0.604**
Gluten	-0.772**	Gluten	-0.004
Gluten DI	-0.888**	Gluten DI	-0.697**
Zeleny Index	-0.867**	Zeleny Index	-0.147

Table 10. FHB incidence (2003-2004) and grain contamination with *Fusarium* spp. (2002-2004) correlation with grain quality indicators. Calculated by the data averaged over replications.

In our experiments, as well as in those reported in the scientific literature, fungal diseases, like FHB, rusts (*Puccinia* spp.) and powdery mildew (*Blumeria graminis*) incidence and fungicide use had only a minor, and harvest year considerable effect on the grain quality (Dimmock & Gooding, 2002**;** Gärtner et al., 2008). Gärtner et al. (2008) found that the falling number is more dependent on the prevailing climatic conditions than on FHB infection. Due to the FHB, the protein content declined, but only slightly, and the Zeleny index decreased only in some varieties. The Fusarium infection did not noticeably influence either the protein content or the water absorption ability of wheat flour (Wang et al., 2005). The fact that fungicide application significantly increased protein and gluten yields is related to the grain yield increase in fungicide treated plots, especially in the year 2004 which was conducive to the occurrence of fungal diseases. To make summarising conclusions on this subject, more detailed further research is needed, since too narrow range of quality data variation among treatments appeared each year.

6. Conclusions

The results of the multi-aspect comparative investigation on the use of strobilurin and triazole - based fungicides for winter wheat disease control suggest that the foliar application of both strobilurin-based and triazole fungicides gave an adequate control of Septoria *(P. nodorum, M. graminicola)* and tan spot *(P. tritici-repentis)*. The foliar fungicide application contributed towards reducing incidence of *P. nodorum* in the harvested seed; however, both the control of FHB and assay of mycotoxin contaminations yielded contradictory results. Under the fungicide use a substantial grain yield increase was obtained. The grain yield showed the significant negative linear correlation with AUDPC for both Septoria and tan spot.

Fungicides prolonged green leaf area retention. This effect depended on the year and fungicide origin. The application of fungicides (both strobilurins and triazoles) at the end of booting – heading stages (BBCH 47-55) significantly increased grain protein and gluten yields, but did not diminish grain quality, however, meteorological conditions of the year played a decisive role for grain quality, compared with fungicide treatments. The highest average protein yield, like that of gluten, was obtained when the fungicide used contained both strobilurin and epoxyconazole.

7. References

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5

Fungicides for Wood Protection – World Viewpoint and Evaluation/Testing in Slovakia

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1. Introduction

Hardwoods (e.g. ash, beech, oak, poplar) and softwoods (e.g. fir, pine, spruce) consist of various types of cells: vessel elements, tracheids, libriform fibres, axial parenchyma cells, ray parenchyma cells, epithelial cells, etc. These cells exhibit various orientations in relation to the standing tree and provide various functions, such as, imparting mechanical strength, acting as water-conducting elements, or functioning as storage cells. Wood cells, their cell walls and lumens have typical shapes and dimensions. Cell walls usually consist of primary and secondary wall layers which are made up from basic natural polymers: cellulose, hemicelluloses and lignin. Extractives of durable wood species (such as terpenoids, tropolones, stilbenes, flavonoids or chinones) are usually present in the cell lumens, or sometimes even in the cell walls (Fengel & Wegener 1984).
Wood is a natural organic material and as such it can be degraded by biological agents: bacteria, fungi and insects. The use of wood is thereby limited by its susceptibility to organisms that may damage its structure and deteriorate its properties.
Wood-destroying fungi, such as brown-rot (*Serpula lacrymans, Coniophora puteana, Antrodia vaillantii, Gloeophyllum trabeum, Lentinus lepideus*, etc.) and white-rot (*Trametes versicolor, Trametes hirsuta, Schizophyllum commune,* etc.) basidiomycetes, or soft-rot (*Chaetomium globosum, Monodictys putredinis*, etc.) ascomycetes, destroy polysaccharides (cellulose and hemicelluloses) present in the cell walls. They may also, in different degrees, degrade the lignin (Arantes *et al*. 2010, Schmidt 2006). Fungi attain the depolymerization of polysaccharides by producing various types of hydrolytic enzymes, e.g. degradation of crystalline cellulose is achieved using etracellular endo-1,4-ß-glucanases, exo-1,4-ß-glucanases, and 1,4-ß-glucosidase. Brown rot-fungi - which do not have exo-1,4-ß-glucanases - use the low molecular non-enzymatic Fenton chelator-mediated system consisting of oxalic acid, iron cations and hydrogen peroxide (Eriksson *et al*. 1990, Goodell *et al*. 2007, Hastrup *et al*. 2010, Messner *et al*. 2003). Rotten wood has lower density and lower strength (Reinprecht 1992, Wilcox 1978). In parallel to the changes above, rot-fungi also change the moisture, colour, acoustic and other properties of wood, with an expressive influence on wood quality. However, these fungi can only degrade the structural components of wood in situations when its moisture content (MC) is 20 % or more (Carll & Highley 1999).

Other wood-degrading fungi, such as blue stain fungi (commonly called staining fungi – growing inside of wood) and microscopic fungi (commonly called moulds – growing on wood surfaces), are not able to depolymerise the cellulose and other building polymers in the cell walls of wood. These fungi metabolize simple sugars and starch present in ray cells and axial cell lumens. Staining fungi (*Ceratocystis pilifera*, *Aureobasidium pullulans*, *Alternaria alternata*, etc.) release pigments and cause colour changes in inner parts of the wood, while simultaneously increasing its permeability as a result of fine perforation of cell walls by penetration hyphae and also by disruption of pits in tracheids or in other cell elements. Some of these strains are also known to be able to cause soft rot in hardwoods under optimal conditions (e.g. *Phialophora* spp.). Colonization of wood by staining fungi occurs at moisture contents (MC) above 30 %, e.g. sapwood of softwoods is optimally attacked at MC of 100 – 130 %, while sapwood of tropical and other hardwoods at MC of 40 – 80 % (Fougerousse 1985, Zabel & Morrell 1992). Moulds (*Aspergillus niger*, *Penicillium brevi-compactum*, *Trichoderma viride*, etc.) produce masses of coloured spores on wood surfaces, and some of them also yield pigments (Reinprecht 2008). Moulds have only a minor influence on wood quality. Growth of moulds on wood surfaces is not so dominantly dependant on the wood's MC, as they are influenced mainly by the relative humidity (RH) of the surrounding air, which has to be minimally about 80 % (Viitanen & Ritschkoff 1991).

Protection of solid wood and wooden materials (plywood, laminated veneer lumbers, particleboards, fibreboards, etc.) prior to fungal attacks can be accomplished by the use of one of the following procedures:

- exposure of wood and wooden products to convenient environmental conditions, e.g. without contact with ground and without influence of rain using various design tools (Foliente *et al.* 2002), when MC of wood is stable under 20 – 26 % and RH of air is stable under 80 – 90 % (Wang & Morris 2010),
- usage of more durable wood species (EN 350-2, Taylor *et al.* 2002, Van Acker *et al.* 2003),
- thermal, chemical or enzymatic modification of wood (Hill 2006, Reinprecht & Vidholdová 2008),
- bio-control of wood with antagonist organisms (Phillips-Laing *et al.* 2003, Singh & Chittenden 2008),
- application of fungicides.

In the present, the service life of wooden products exposed to wet conditions is usually increased by using more durable wood species or by treatment of wood with effective and ecologically convenient fungicides (Brischke & Rapp 2008). However, in the future a bigger emphasis will be given to the use of modified woods, without any negative influence on the environment (Suttie 2008).

Sleepers, poles, fences, shingles, decking, cladding, windows, log cabins, and many other wooden products exposed to exterior commonly have an MC above 20 %. The requirement of their fungicide protection in the 2nd till 5th classes of exposure by the EN 335-1 depends on the natural durability of used wood species by the EN 350-2. For wooden structural elements it is complexly given in the EN 460. Wood species with a lower amount of decay-inhibiting heartwood extractives (terpenoids, stilbenes, etc.) have a minimal natural durability, e.g. beech, birch, hornbeam, poplar, fir, spruce, and more others (EN 350-2). In this fact, wooden products from less durable wood species intended for higher risk in-service expositions have to be made either from modified wood substances (e.g. plywood from thermally modified or acetylated veneers), or from wood substance, glue or paint treated with a suitable fungicide (see Chapter 2).

2. Fungicides used for wood protection – world viewpoint

Fungicides are inorganic or organic substances acting against fungi (Tab. 1). Their toxicant or retardant mode of action depends on their chemical structure from which all important biocidal properties are derived. Some fungicides have simultaneously bactericidal, insecticidal or other biocidal effects, e.g. boric acid (Lloyd 1998).

2.1 Modes of action of fungicides and their efficacy

Activity of the wood-degrading fungi can be suppressed by more biochemical modes of action of fungicides (Eaton & Hale 1993, Reinprecht 2008):

- *Inhibitors of respiration,* by which either the formation of acetyl coenzyme A (CoA) is inhibited or the respiratory chain phosphorylation is interrupted, and simultaneously production of the high-energy intermediate "adenosine triphosphate" (ATP) is suppressed. Cupric ion Cu^{2+} from copper sulphate, copper oxide, copper naphthenate, copper-8-hydroxyquinolinate, Cu-HDO, etc. is a typical inhibitor of respiration processes in fungal cells. It has affinity to different chemical groups in the cells of fungi, particularly to thiol groups, resulting in the non-specific denaturation of proteins and enzymes. Similar inhibitory effects were found at the arsenic compounds, 2-phenylphenol, pentachlorophenol and other phenolic compounds, carboxamides, tributyltin compounds, or isothiazolones.
- *Inhibitors of polysaccharide biosynthesis*, and/or inhibitors of protein, lipid and nucleic acid biosynthesis. Well known inhibitors of the chitin (polysaccharide from N-acetylglucosamine units) synthesis in the cell walls of fungi are polyoxins, antibiotics derived from a streptomycete. Lipid synthesis is suppressed by imidazoles, pyrimidines, or triazoles.
- *Inhibitors of cell division* act by inhibiting the synthesis of microtubules. During cell division (mitosis) the genetic information stored in the nucleus in DNA (deoxyribonucleic acid) must be copied and the products transformed to the two daughter cells. The benzimidazole derivatives (e.g. carbendazim, benomyl) interfere with microtubule subunit polymerization and such preventing mitosis, at which depress the DNA synthesis, as well.
- *Disruptors of cell membranes* of fungi, in which structure and function the sterols (especially ergosterol) play an important role. Tiazoles (e.g. azaconazole, propiconazole, tebuconazole) act as inhibitors of sterol biosynthesis, and this process is connected with disruption of cell membranes. Tar oils also disrupt the cell membranes when the lipids in membranes are soluble in the non-polar oil liquids. Semi-permeable membranes of fungi can be dehydrated and damaged by quarternary ammonium compounds (QAC), e.g. by didecyl-dimetyl-ammonium chloride (DDAC), in connection with leaking of cell constituents.
- *In-activators of enzymes* usually inhibit several enzymatic processes at the same time. They often react with thiol groups in proteins (e.g. mercury-based fungicides used in the past, dicarboximides), inhibit glycolysis (e.g. mercury Hg^{2+} and copper Cu^{2+} cations), or inhibit other enzymatic reactions (e.g. boric acid and various boron compounds which form stable complexes with vitamins, coenzymes or other biological molecules having e.g. poly-ols groups (Lloyd *et al.* 1990), and simultaneously inhibit metabolic activity, enzymatic function and growth of fungi – so they act more as fungistatics rather than fungicides).

- *Retardants of Fenton depolymerisation* of polysaccharides in cells of wood are substances which chemically bond Fe^{3+} ions (e.g. tropolon, β-tujaplicin), and in this fact decrease the activity of brown-rot fungi (Gérardin *et al*. 2002).
- *Retardants of fungal spread* in wood and other materials in buildings, e.g. non-toxic amino acid analogue "AIB" α-aminoisobutyric acid targets adaptive nitrogen redistribution mechanisms that are unique to rot-fungi, and which are essential to their ability to spread in buildings and colonise fresh wood sources poor in nitrogen (Watkinson & Tlalka 2008).

Efficacy of fungicides depends on their ability to damage fungal cells, suppress growth, enzymatic or other activities of rotting-fungi in wooden or in model testing substrates, etc. In laboratory and field tests, and also in practice it is determined as a critical minimal concentration (%) or as a minimal critical retention in wood (g/m^2, kg/m^3). However, efficacy is not a constant value, because it is influenced by more biological and environmental factors presented inside and outside of the fungal cells (Reinprecht 2008):

- *velocity of adsorption of fungicide on the surface of fungal cells*, which depends e.g. on the pH value of wood,
- *velocity of accumulation of fungicide into fungal cells*, which can be increased in presence of conditioners,
- *species of fungus,* when individual fungi non-seldom have a selective resistance to the molecule of fungicide (e.g. borates are effective against rotting-fungi but not against moulds; copper compounds are effective against soft-rot fungi, staining fungi and moulds but they have a lower efficacy against white-rot and brown-rot fungi, especially against fungi from the families of *Antrodia* and *Serpula* which create non-toxic crystals of copper oxalate (Hastrup *et al.* 2005),
- *amount of fungal cells*, because the fungicide in interaction with cells of a living fungus can be sometimes inactivated and its concentration is brought down (e.g. fungicide for curative usage have to be applied in a higher concentration than for preventive usage),
- *amount of the applied fungicide*, because its under-critical concentrations are not usually effective for a long time, when the fungus can be adapted on molecules of the fungicide, *presence of other substances*, which catalyse or retard efficacy of the fungicide (e.g. efficacy of QAC is synergistically increased in presence of inorganic copper compounds (Härtner & Barth 1996)),
- *temperature, moisture, UV light and other environmental factors*, which influence the activity of fungal cells (e.g. cells are disposed to damage at higher temperatures – so the necessary of fungicide molecules is lower), the transport of fungicide to fungal cells (e.g. diffusion transport of water soluble fungicides is more intensive in wetter and warmer wood substance – so the necessary of fungicide molecules is lower), etc.

2.2 The most used fungicides for wood protection

Natural decay resistance of wood, plywood and other wooden materials against fungi can be increased by various synthetic and natural chemicals having fungicide or fungistatic effects.

In the 20th century, protection of wood against fungi included the use of the following chemicals: traditional coal tar oils, various mercury-based compounds ($HgCl_2$, ...), fluorine compounds (NaF, KF, $CuSiF_6.6H_2O$, $MgSiF_6.6H_2O$, $ZnSiF_6.6H_2O$, $Na_2SiF_6.6H_2O$, ...), arsenic-based compounds (As_2O_3, As_2O_5, FeAsS, Na_2HAsO_4, ...), copper-based compounds ($CuSO_4.5H_2O$, CuO, $CuCO_3.Cu(OH)_2$, Cu-HDO = bis/N-

cyclohexyldiazeniumdioxy/copper, CuN = copper naphthenates, copper-8-quinolinolate, ...), zinc-based compounds ($ZnCl_2$, ZnO, ...), chromium-compounds ($Na_2Cr_2O_7$, $K_2Cr_2O_7$, CrO_3, ...), boron compounds (H_3BO_3, $Na_2B_4O_7.10H_2O$, ...), quarternary alkyl-ammonium compounds (QAC, e.g. DDAC = didecyl-dimetyl-ammonium chloride), pentachlorophenol (PCP) and its sodium salt sodium pentachlorophenolate, organotin compounds (TBTO = bis/tributyltin/oxide, ...), etc. In the 70-90th years of the 20th century new organic substances have been tested and subsequently used for protection of wooden structures against fungi, e.g. sulfamides (dichlofluanid, tolylfluanid, …), carbamates (IPBC = 3-iodo-2-propynyl-butyl-carbamate), dithiocarbamates, but first of all various hetero-cycles – e.g. benzothiazoles (TCMTB = 2-thiocynomethylthio-benzothiazole, …), benzoimidazoles (carbendazim, thiabendazole = 2-thiazol-4-yl-1H-benzoimidazole, …), triazoles (propiconazole, tebuconazole, …), isothiazolones (DCOIT = 4,5-dichloro-2-n-octyl-4-isothiazol-3-one), or furan derivatives with the biologically active group CH_3-C=C-C=O (furmecyclox, …) (Eaton & Hale 1993, Reinprecht 1996, 2008, Richardson 1993, Unger *et al.* 2001).

Fungicides containing the fluorine, arsenic, copper or zinc molecules with predestination for exterior expositions were usually combined with chromium, which fixed them into stable in water non-soluble complexes, e.g. F/Cr = Wolmanit U, Cu/Cr = CC salts, Cu/Cr/B = CCB salts, Cu/Zn/Cr/B = CBZ salts, Cu/Cr/As = CCA salts, etc. (Dahlgren & Hartford 1972, Peek & Willeitner 1984). In present years the chromium can be, by the Biocidal Product Directive 98/8/EC, used only as a fixative agent in the form of chromic acid CrO_3, not as a fungicide (Jüngel & Melcher 2006, Jüngel & Hellkamp 2008). Applications of the mercury, fluorine, pentachlorophenol, organotin and also of some other compounds including traditional compositions of tar oils with the carcinogen benzo-α-pyrene, have been stopped already during the 20th century due to environmental, health or other aspects.

Today in the 21st century, the industrial protection of wood against fungi has responded to environmental, legislative and economic pressures to utilize more environmentally sensitive formulations (Wallace & Dickinson 2006). Usage of arsenic in the copper-chromated-arsenate (CCA) and in other preservatives was restricted (Preston 2000, Evans 2003). Timber preservation is carried out either with modified coal tar oils "creosotes" without benzo-α-pyrene and having only a minimal amount of easily evaporated naphtalene derivatives (tar oils only for pressure impregnation of sleepers and poles), and with selected types of inorganic and organic fungicides having oral LD_{50} above 500 ppm for the animal organisms, at which these fungicides may not have mutagenic and cancer effects on animals (Tab. 1).

Coming out from the health and environmental demands on biocidal products determined in the Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998, and also in its other Commission Regulation (EC) reports No. 1896/2000, 2032/2003, 1048/2005, 1849/2006 1451/2007, only a limited number of active substances will be permitted in the European market after 2014 (Krajewski & Strzelczyk-Urbańska 2009). Already today in the Annex I to the Directive 98/8/EC there is only a small number of active substances from PT 8 (wood preservatives), e.g. fungicides IPBC, K-HDO, propiconazole, tebuconazole, dichlofluanid, or thiabendazole.

As a result of these regulations, in the future will probably be a reduced interest for investigation of new biocidal – fungicide active substances, mainly those on the basis of heavy metals (not only chromium, tin or arsenic, but also copper, etc.), which usage is already now limited in a practice. According to Leithoff & Blancquaert (2006) the price for notification of existing active substances varies from 3.3 to 6.0 million Euro, at which the

BIOCIDE	EFFICACY	
	Fungicide	Insecticide
❑ Boron compounds (boric acid, borax, ...)	+	+
❑ Carbamates (IPBC, ...)	+	
❑ Copper inorganic compounds (copper oxide, ...)	+	
❑ Copper naphtenates and citrates	+	
❑ Creosotes	+	+
❑ Isotiazolones (DCOIT, ...)	+	
❑ N-organodiazeniumdioxy-metals (Cu-HDO, ...)	+	(+)
❑ Quarternary ammonium compounds – QAC (DDAC, ...)	+	(+)
❑ Sulfamides (dichlofluanid, tolylfluanid, ...)	+	
❑ Triazoles (propiconazole, tebuconazole, ...)	+	
❑ Natural substances (chitosan, essential oils, ...)	+	(+)

Note: + it is a basic biocidal activity; (+) it is an additional biocidal activity

Table 1. The most important fungicides for wood protection used today in the Europe and also in the world (Reinprecht & Tiralová 2007a)

highest 85-90 % expenses are connected with the toxicology and eco-toxicology studies. Lower prices are needed for registration of existing (0.2-0.5 million Euro) or new preservative products (0.3-1.4 million Euro). On the other hand, the price for evaluation, notification and registration of a new active substance is usually from 30 to 40 million Euro (Reinprecht 2008).

Commercial wood preservatives used in the present contain mainly boron compounds (boric acid, disodium tetraborate, disodium octaborate, glycol borates, etc. often modified with fixatives, water repellents or other protective additives against leaching), quarternary alkyl-ammonium compounds, triazoles (azaconazole, propiconazole, tebuconazole), benzoimidazoles, isothiazolones, sulfamides, carboxamides, or 3-iodo-2-propynyl-butyl-carbamate, and to this time also various copper-based compounds (CuO, $CuCO_3.Cu/OH/_2$, Cu-HDO = bis/N-cyclohexyldiazeniumdioxy/copper, etc.). Interesting for the future could be also other compounds with biological efficiency, e.g. aqueous based silver compositions or nano-silver particles – especially those between 100-200 nm (Ellis *et al.* 2007), triazines (Milata *et al.* in press), aminosilicone macro emulsions – ASMaE (Ghosh *et al*. 2008), 1,3-dimethylol-4,5-dihydroxy-ethyl-urea – DMDHEU (Xie *et al.* 2005), or from natural substances especially the chitosan (Schmidt *et al.* 1995, Eikenes *et al*. 2005).

Nanotechnologies are surely promising for wood preservation, as well. Nanometal characteristics may be totally different from the characteristics of the elemental metals and potentially may perform in an unusual manner. Nanometal preparations have several characteristics (e.g. size and charge), that may improve their performance in wood protection applications. If their particle size is smaller than the diameter of pores in the bordered pits or in the wood cells, complete penetration and uniform distribution in wood can be expected (Akhtari & Arefkhani 2010). However, in solid wood with a small amount of trace elements (Mn, Fe, Co, Ni, Cu, Zn, Mo, Pb, etc), which have a positive or negative physiological influence on the mycelium growth of fungi, the additional treatment of wood tissue with nano-particles of silver, copper, zinc, aluminium or other metals (nanobiocides) can be effective only if their concentration will be under-threshold toxicity range (Važny & Kundzewicz 2008).

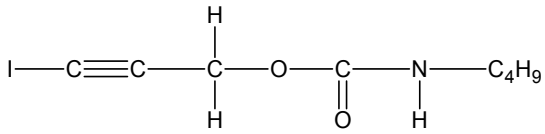
From the point of view of efficacy and ecology of fungicides it is also very important their stability in treated wood. The main problem associated with the development of environmentally friendly wood preservatives for high hazard end uses, based on organic compounds, is the observed biotransformation of these chemicals. Some strains of proteobacteria (*Alcaligenes*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, etc.) are able to degrade IPBC, propiconazole, chlorothalonil and other organic fungicides (Wallace & Dickinson 2006). Propiconazole and tebuconazole are partly degradable also by the black-stain fungus *Epicoccum purpurascens* (Stirling & Morris 2010). According to these authors, if it proves to be possible to disrupt the mechanism of detoxification processes of organic fungicides, this could herald a new generation of environmentally friendly wood preservatives. On the other hand, intentional detoxification of organic fungicides can be important at reconstruction of old heritage buildings, and at remediation of carbon-based preservative-treated wood at the end of its service life.

Boron compounds: Boric acid H_3BO_3, disodium-tetraborate-decahydrate $Na_2B_4O_7.10\ H_2O$ (borax), disodium-octaborate-tetrahydrate $Na_2B_8O_{13}.4H_2O$ (timbor), zinc borates $Zn(BO_2)_2$ (Kirkpatrick & Barnes 2006, Lin & Furuno 2001), and also some other boron compounds belong to traditional preservatives for wood protection against wood-destroying fungi and insects in interior exposures (Dickinson & Murphy 1989, Lloyd 1998, Luo *et al.* 2005). Boron compounds have also certain fire retardant effect. They are usually applied in water solutions, or by diffusion methods at treatment of wet wood. Advantage of these compounds is their low toxicity to humans and the environment. In order to reach a better efficacy, they have to be applied in higher amounts, approximately from 3 to 20 kg/m^3 of wood (Lyon *et al.* 2009, Pallaske 2004, Reinprecht 2007). Their disadvantage is a lower efficacy against moulds (Reinprecht *et al.* 1986).

Inorganic boron compounds used in outdoor exposures have to be modified with fixatives or with water repellents, polymerizable monomers or other protective additives to reduce their leachability (Babuder *et al.* 2004, Lloyd *et al.* 2001). Polyvinyl alcohols belong to the most known fixatives which form stable complex with the borate ions (Mohareb *et al.* 2010a). Complexes with boron create also tannins (Pizzi & Baecker 1996). Fixation of boron in wood can be secured also by silicone gels (Furuno & Imamura 1998) and animal proteins (Mazela *et al.* 2007, Thevenon & Pizzi 2003).

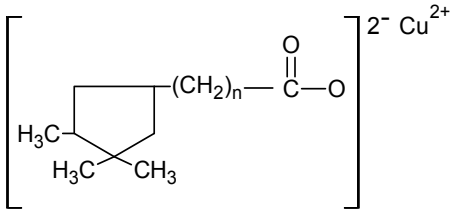
Organic compounds of boron, e.g. in esters (tetramethyl-ammonium bis/salicyl/borates – Humphrey *et al.* 2002; ammonium borate oleate "ABO" – Lyon *et al.* 2009), are usually more stable against leaching with water. The newly developed didecyl-dimethyl-ammonium tetrafluoroborate (DBF) is acceptably stable as well as effective against white-rot and brown-rot basidiomycetes (Kartal *et al.* 2005) and in soil-bed tests against soft-rot fungi (Kartal *et al.* 2006). The DBF with or without incorporation with acryl-silicon type resin emulsion showed a good decay resistance against basidiomycetes after severe weathering (Kartal *et al.* 2004, 2006).

Carbamates: 3-iodo-2-propynyl-butyl-carbamate (IPBC) is an organic fungicide, which can be applied in organic solvents (acetone, xylene), and also in water emulsions. The IPBC is effective against various types of wood-degrading fungi (Hansen 1984), however it is mainly used against moulds and staining fungi. Activity of IPBC against fungi can be enhanced with borates (Cassens & Eslyn 1981), or against the dry rot fungus *Serpula lacrymans* approximately about 50 % in the presence of 2g/l of α-aminoisobutyric acid (Bota *et al.* 2010).

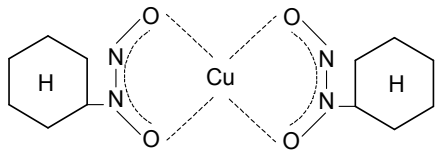


IPBC

Copper compounds: Copper is effective against fungi either in various inorganic compounds: $CuSO_4.5H_2O$, CuO, $Cu(OH)_2.CuCO_3$, Cu/Cr/B = CCB salts, Cu/Cr/As = CCA salts, Ammonia/Cu/As = ACA salts; or in various organic compounds: CuN = copper naphthenates, copper-8-quinolinolate, Cu-HDO = bis/N-cyclohexyldiazeniumdioxy/copper, ACC = ammoniacal copper carboxylates with the structure of R-COO-Cu-OOC-R, e.g. ammoniacal copper citrates, ACQ = alkaline copper quat formulations /e.g. consisted from CuO and DDAC/ (Eaton & Hale 1993, Pankras *et al.* 2009). Copper in the form of Cu^{2+} cations is especially effective against soft-rot fungi (Ray *et al.* 2010).



CuN



Cu-HDO

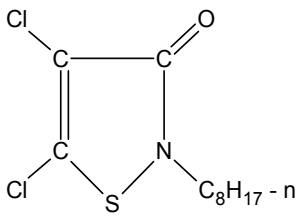
Copper compounds are mainly used in water, amine or organic solutions, but in recent years also in the form of micronized copper particles "MCQ", usually ranging from 10 to 700 nm (Matsunaga *et al.* 2007, McIntyre 2010). For example copper carbonate used in the form of MCQ is fixed to wood creating octahedral complexes with six oxygen atoms surrounding the central copper (Xue *et al.* 2010). From studies made by Chen (2010), it is evident that both oxidation of hemiacetals by Cu^{2+} in cellulose and hemicelluloses, and oxidation of guaiacyl lignin by Cu^{2+} to quinone methides took place, which led to complex formation of copper with all wood components. However, for long term efficacy of copper compounds in exterior exposures it is important not only the fixation of copper ions to polysaccharide and lignin in cell walls of wood, but also of their possible migration in the wood, e.g. from the surface of treated wood onto untreated check surfaces (Choi *et al.* 2001).

However, copper preservatives are not effective against all types of wood-inhabiting fungi. It is well known, that most brown-rot fungi (*Serpula lacrymans*, *Serpula himantioides*, *Antrodia radiculosa*, *Oligoporus placentus*, *Fomitopsis palustris*, etc.), which use Fenton reaction at depolymerization of cellulose, are tolerant towards copper-based wood preservatives as a consequence of creation the non-active copper oxalate crystals, as well as other gene predictions (Hastrup *et al.* 2005, Tang *et al.* 2010, Schilling & Inda 2010, Woo & Morris 2010). Copper oxalate is insoluble in water and copper in this form has a greatly reduced inhibitory effect on fungal growth (Humar *et al.* 2001). Thereby, broad-spectral preservatives require the addition of suitable co-fungicide (e.g. boron-compound), to protect wood against copper tolerant fungi.

Creosotes: Coal tar oils – creosotes, patented in 1836 by German chemist Franz Moll and first used for wood impregnation by John Bethell in 1838. In the 19th and 20th centuries, creosotes were the most commonly used wood preservatives throughout the world, with a worldwide

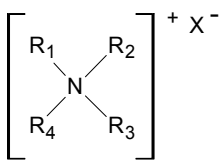
production of approximately 16 million metric tons (Eaton & Hale 1993). Creosotes consist from 200 to 800 different compounds, mainly from neutral polycyclic aromatic hydrocarbons (anthracene, phenantrene, pyrene, chrysene, ...), tar phenols (cresol, naphtol, ...) and tar bases (quinoline, acridine, ...). They have good fungicide and insecticide effects at wood retention level between 30 kg/m^3 (against insects) and 120 kg/m^3 (against soft-rot fungi). In Europe, their composition and usage is controlled by the West European Institute for Wood Impregnation (WEI). However, now and mainly in the future, their application will be significantly restricted because of their negative influences on the environment.

Isotiazolones: DCOIT (4,5-dichloro-2-n-octyl-4-isothiazol-3-one), Kathone (2-n-octyl-4-isothiazol-3-one), Kathone CG (5-chloro-2-methyl-4-isothiazol-3-one) and others isotiazolones are used for wood protection as bactericides and fungicides in retentions from 0.15 to 1.28 kg/m^3. Some of them can also be used against termites. They are applied either in organic solvents or in water emulsions (Hegarty *et al.* 1997).



DCOIT

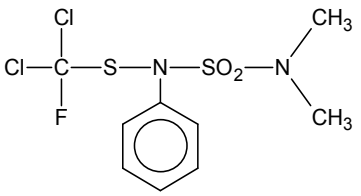
Quarternary ammonium compounds: QAC with various alkyl or aryl groups (R_1, R_2, R_3, R_4), and with chloride, tetrafluoroborate, nitrate, acetate or other anion X^- (Zabielska-Matejuk & Skrzypczak 2006), have been used approximately 30 years for wood protection (Nicholas & Preston 1980). QAC are usually more effective against staining fungi and moulds (Micales-Glaeser *et al.* 2004), and less effective against wood-destroying fungi. However, DDAC (didecyl-dimetyl-ammonium chloride) has a sufficient efficiency against rotting-fungi, as well. QAC are soluble in water and miscible with alcohol. In wood are fixed by ionic reactions with carbonyl groups of lignin and hemicelluloses, and by interactions with its –OH groups (Nicholas *et al.* 1991). Due to their lower stability in environment, their rapid fixation close to the wood surface, and their influence on higher absorption of water by wood from environment, the QAC are not convenient for treatment of wood in contact with ground. Nowadays, QAC are used for treatments of structural timbers in interior and exterior above ground expositions, usually in combination with copper-compounds (e.g. $CuCO_3.Cu/OH/_2$), boron-compounds (e.g. H_3BO_3) or triazoles (e.g. propiconazole) (Reinprecht & Tiralová 2007a, 2007b).



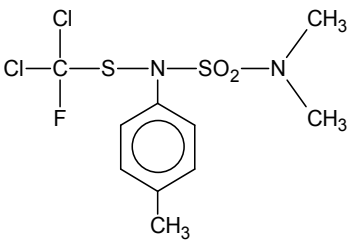
QAC

R_1, R_2 - alkyl groups with 1 to 6 carbon atoms
R_3 - alkyl group with 1 to 20 carbon atoms, or benzyl group
R_4 - alkyl group with 8 to 22 carbon atoms

Sulfamides: These compounds (dichlofluanid = N,N-dimethyl-N′-phenyl-N′-/flurodichloromethylthio/-sulfamide, tolylfluanid = N,N-dimethyl-N′-p-tolyl-N′-/fluorodichloromethylthio/-sulfamide, etc.) have typical active biological groups –S-C-(Cl_2)F or –S-C-(Cl_3), which are connected with nitrogen atom. They are more effective against staining fungi and moulds (Buschhaus 1992), but in higher concentrations they are able to suppress activity of rotting-fungi, as well. Sulfamides are used in organic solvents or water emulsions, and they are often added also to exterior coatings for the preventive treatment of structural elements without soil contact.

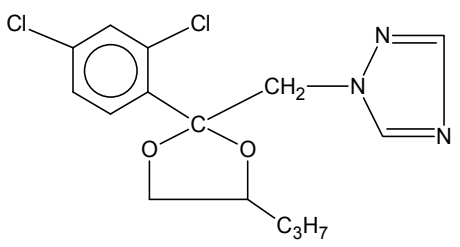


Dichlofluanid

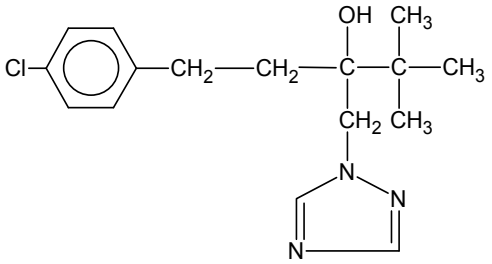


Tolylfluanid

Triazoles: Propiconazole ±cis/trans(1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]-methyl]-1H-1,2,4-triazole), tebuconazole (Alpha-[2-(4-chlorophenyl)-ethyl]-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol) and azaconazole are well known organic fungicides often used in various commercial formulations for wood protection against all types of wood-degrading fungi (Wüstenhöfer *et al.* 1993). They are applied in non-polar and semi-polar organic solvents, or in water emulsions. The 1,2,4-triazoles are stable in environment, and have only a low toxicity to animals. Their efficacy can be increased with antioxidants or with metal chelators (Bakhsous *et al.* 2006). A typical natural antioxidant is caffeine (1,3,7-trimethylxanthine) which induces a strong alteration of cell wall architecture of fungi, inhibiting their growth. Its combination with propiconazole is effective against wood-destroying basidiomycetes (Lekounougou *et al.* 2007).



Propiconazole



Tebuconazole

Metal azole complexes have been known from the 70th years of the 20th century. Now, after restriction of CCA salts, the use of copper in combination with organic co-biocide come into interest of more researches (Evans *et al.* 2008). Metal-centred azole complexes formed from propiconazole or tebuconazole with copper acetate, e.g. Cu(tebuconazole)$_2$(OAc)$_2$, or other metal substances create crystals which by more studies and patents have either lower or higher efficiency against fungi in comparison to original compounds.

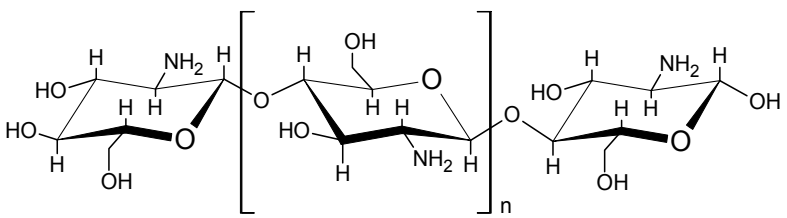
Natural substances: Interest in the exploration and use of natural products as fungicides is rapidly growing worldwide. Different natural substances are potentially suitable for wood protection before attack by fungi: plant extracts, essential oils, heartwood extractives, waxes and resins from bark, and other bioproducts, e.g. chitosan (Singh & Singh 2010). However, industrial uptake of these compounds by wood preservation has been limited until now due to the following reasons: - incompatibility in efficacy of a compound on nutrient medium at screening tests and on wood in a practice; - narrow spectrum of fungicidal activity of some of these compounds, e.g. only against selected species of moulds; - variability in chemical composition; - incompatibility with legislation and registration of new compounds for market. In spite of these limitations, the potential is enormous for developing protection technologies based on the use of natural compounds as fungicides.

Natural organic compounds established as safe to human health and environment such as chitosan, essential oils, etc. have been investigated with aim of replacing inorganic and synthetic organic fungicides. Problems with their high leachibility from treated wood can be solved either with their in situ polymerization with wood tissues, e.g. by enzymatic polymerization of essential oil phenols with wood lignin, or by their nano-formulation in a form of micronized particles (Singh & Singh 2010). Their fungicidal or commonly biological activity can also be improved using a combination of natural products (Maoz *et al.* 2007).

Essential oils are commercially used as perfumes, flavouring food additives, or pharmaceutical products. Some of them have good antifungal properties against wood-degrading fungi, e.g. cinnamon, geranium, lavender, oregano, thyme. Essential oils with phenolic or aldehyde and ketone components like thymol, carvone or carvacrol show evidently higher activity against wood-rotting basidiomycetes as those with hydrocarbon monoterpenes or ester monoterpenes (Amusant *et al.* 2009). Carvone, citronellol, geraniol, thymol and borneol are effective inhibitors of mould spore germination (Clausen *et al.* 2010).

Extracts from heartwood of more durable tropical or temperate wood species (*Cupressus lusitanica, Dalbergia sissoo, Eperua falcata, E. grandiflora, Milicia excelsa, Taiwania cryptomerioides, Tectona grandis, Thuja plicata*, etc.) contain bioactive compounds: polyphenolics (e.g. flavonoids, sterols), terpenics (e.g. cedrol, agathadiol, epimanool, bornyl acetate, cedrene), alkaloids, or stilbenes. These extracts inhibit activity of various types of wood-degrading fungi (Amusant *et al.* 2005, Chang *et al.* 2003, Kazemi *et al.* 2006, Mohareb *et al.* 2010b). However, alteration of their chemical structures during extraction processes from durable trees, during pressure or unpressured treatment of less durable wood species, or during exposure of treated wood products to environment can lead to loss of their original antifungal activity.

Chitosan is 1-4 linked heterogeneous polymers of D-glucosamine ($F_A = 0$), usually also with N-acetyl-D-glucosamine units ($F_A \neq 0$). This natural compound is derived from crustacean shells. To some extent is water soluble under acidic conditions. It can act both fungistatically and as a fungicide at higher concentrations (Eikenes *et al.* 2005).



Chitosan (type $F_A = 0$)

Wood treated with 1-5 % concentrations of chitosan proved effective against the brown-rot fungi *Coniophora puteana* and *Gloeophyllum trabeum* (Schmidt *et al.* 1995), and also against the white-rot fungus *Trametes versicolor* (Maoz & Morrell 2004). Efficacy of chitosan depends also on its molecular weight (MW). Chitosan with lower MW has been found to be the most effective against *Trametes versicolor* and *Poria placenta* basidiomycetes (Mohareb & Badawy 2008).

3. Evaluation and testing of fungicides for wood protection in Slovakia

In Slovakia, after its formation from Czechoslovakia in 1993, there has been a relatively poor interest for investigation of new active compounds with fungicide effects for wood preservation. Basic research at a limited level has been performed either in the capital Bratislava – in the Slovak Forest Products Research Institute, in the Chemical-Technological Faculty of the Slovak Technical University, in the Pharmacy faculty of the University of Comeniana, and simultaneously in Zvolen – in the Faculty of Wood Sciences and Technology of the Technical University in Zvolen.
The following compounds have been prepared (in Slovakia or also in Czech Republic) and tested by screening methods (poisoned agar-malt soil, poisoned filter papers, etc.) and European standards (EN 113, P ENV 839 – with treatment of wood samples) in the mycological laboratory of TU in Zvolen:

- more organic compounds with a potential fungicide efficiency (e.g. tributyltin-N,N-diethyl-dithiocarbamate and other organotin-dithiocarbamates, copper and zinc dithiocarbamates, N-salicylidene-L-glutamato-copper(II) and other copper chelates, or 1,3,5-triazines with three identical hetero-cyclic groups),
- traditional boron fungicides in mixtures with inorganic ammonium salts (fire retardants),
- waste glycols modified with boron compounds (B-glycol-complexes), or with boron and waste copper compounds (Cu-B-glycol-complexes), together with selected additives.

Boric acid, disodium tetraborate, tributyltinnaphtenate (TBTN), 2-thiocyanomethyltiobenzothiazole (TCMTB) and some other commercial fungicides have been used and tested also for antifungal protection of wooden composites – plywood, particleboards, etc. During the laboratory experiments interest was given to necessary changes related with technological parameters of their preparation (e.g. temperature and time of pressing), and on their effects against fungi. Tests showed that fungicides added to wooden composites in higher amounts provided successful antifungal activity. However, some fungicides had a negative influence on the moisture and strength properties of composite products with phenol-formaldehyde or melamine-urea-formaldehyde glues (Reinprecht & Štefka 1989, Reinprecht & Perlác 1995).

3.1 Evaluation and antifungal tests of new organic compounds

Between 1993 and 2008, the antifungal efficacy of newly prepared organotin compounds, metal dithiocarbamates, copper chelates and 1,3,5-triazines was tested at Zvolen's TU. These organic compounds were prepared in Bratislava in the Chemical-Technological Faculty of STU, or in the Pharmacy faculty of UC, and some of them also in Brno - Czech Republic in the Chemical faculty of VUT.
Organotin-dithiocarbamates: During 1993-1998, the antifungal efficacy of more organotin compounds, e.g. tributyltin-N,N-diethyl-dithiocarbamate (TBT-DEDTK), triphenyltin-N,N-

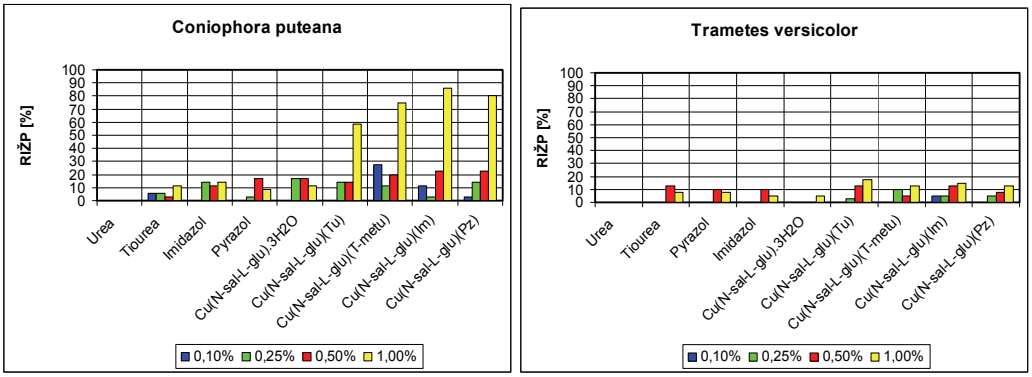
diethyl-dithiocarbamate (TPT-DEDTK), tribenzyltin-N,N-diethyl-dithiocarbamate (TBzT-DEDTK), tributyltin-N-pyrrolidinyl-dithiocarbamate (TBT-PoDTK), tributyltin-N-piperidinyl-dithiocarbamate (TBT-PiDTK), tributyltin-N-morpholinyl-dithiocarbamate (TBT-MfDTK), dibutyltin-di-N-pyrrolidinyl-dithiocarbamate (DBT-DPoDTK), tributyltin-N,N-dipropyl-dithiocarbamate-dipropionate (TBT-DPrDTK-DPr), among others, was valued in screening and standard tests (Kizlink *et al.* 1996a, 1996b, Reinprecht 1996, Reinprecht & Kizlink 1996, 1999), Their activity was compared with the commercial organotin fungicides bis/tributyltin/oxide (TBTO) and tributyltinnaphtenate (TBTN).

Anti-mould tests were carried out against a mixture of moulds (*Aspergillus amstelodami*, *Aspergillus niger*, *Paecilomyces varioti*, *Penicillium brevicompactum*, *Penicillium cyclopium* and *Trichoderma viride*), firstly by screening method on poisoned agar-malt soil and then by standard method STN 49 0604 with beech samples 50 x 20 x 5 mm treated with organotin compounds by dipping or pressure impregnation technology. Anti-decay tests were performed against the brown-rot fungi *Serpula lacrymans* and *Coniophora puteana* and the white-rot fungus *Trametes versicolor*, firstly by screening method on poisoned agar-malt soil and then by modified EN 113 standard using pressure impregnated beech samples 120 x 8.5 x 8.5 mm. Before carrying out the antifungal tests, treated beech samples were naturally aged without contact with ground under an angle 45° during the period of 0, 2 or 4 months. From the organotin-dithiocarbamates tested, only the TBT-DEDTK had sufficient antifungal efficiency comparable with the commercial organotin compounds TBTO and TBTN (Reinprecht 1996).

<u>*Copper and zinc dithiocarbamates:*</u> Metal dithiocarbamates with aromatic or hetero-cyclic groups were prepared and then tested in screening tests against wood-destroying fungi *Coniophora puteana* and *Trametes versicolor* (Reinprecht *et al.* 2003). However, their antifungal efficacy, e.g. of copper/Cu^{2+}/-N-morpholinyl-dithiocarbamate, zinc/Zn^{2+}/-N-morpholinyl-dithiocarbamate, copper/Cu^{2+}/-N-piperidinyl-dithiocarbamate, zinc/Zn^{2+}/-N-piperidinyl-dithiocarbamate, and some others, was not sufficient. Antifungal effect of these metal dithiocarbamates was evidently lower in comparison with the organotin-dithiocarbamate TBT-DEDTK.

<u>*N-salicylidene-L-glutamato-copper(II) and other copper chelates:*</u> Antifungal and antimicrobial activity of various copper chelates does not show promising results against growth inhibition of wood-rotting fungi from a practical perspective (Kizlink *et al*. 2003, Reinprecht *et al*. 2003, Švajlenová *et al*. 1997). In screening tests the activity of newly prepared copper chelates, e.g. of (Cu/salicylidene/-glycin)-chelate, (Cu/chlorobenzaldehyde/-glycin)-chelate, (Cu/anizaldehyde/-glycin)-chelate, (Cu/fural/-glycin)-chelate or (Cu/chlorbenzaldehyde/-beta-alanine)-chelate, against the fungi *C. puteana* and *T. versicolor,* was slightly lower that the well-known (Cu_2/salycilidene/-beta–alanine)-chelate. However, antifungal activity of all these chelates was evidently lower in comparison with the commercial fungicide Tebuconazole (Reinprecht *et al.* 2003).

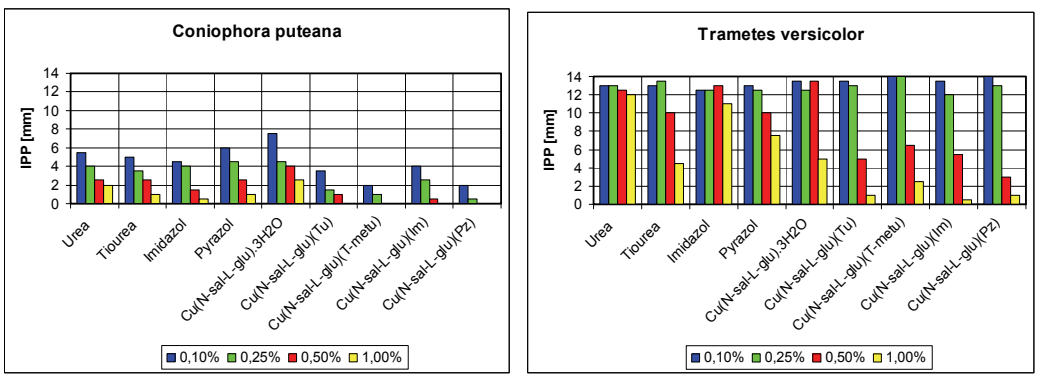
In antifungal screening tests with other copper chelates (Reinprecht *et al.* 2009), it was determined that activity of the N-salicylidene-L-glutamato-copper(II) chelate complexes (Cu/N-salicylidene-L-glutamato/X, where X = ligand) against the fungi *Coniophora puteana* and *Trametes versicolor* was better in comparison to the parent substance monohydrate diaqua/N-salicylidene-L-glutamato/copper(II) complex (Cu/N-sal-L-glu/.$3H_2O$), and also to the free uncoordinated ligands X: urea, tiourea (TU), tetramethyltiourea (T-metu), imidazole (Im) or pyrazole (Pz) (Fig. 1 and 2). Activity of these copper chelates increased at higher concentrations from 0.1 % to 1.0 %. Their efficiency was better against the brown-rot fungus *C. puteana*. However, activity of the tested copper chelate complexes was 4 to 20 times lower, when compared with the commercial fungicides IPBC and Tebuconazole.



Notes:

- Screening tests with poisoned filter papers Whatman 3 CHR ∅ 14 mm treated with solutions of copper chelates or ligands X, which were located 20 mm from fungus inoculate
- Each value in the figure corresponds to the arithmetic mean of three samples.

Fig. 1. Growth inhibition indexes of fungus mycelium on the malt-agar soil (RIŽP) of selected N-salicylidene-L-glutamato-copper(II) chelates Cu/N-sal-L-glu/X between the 0 and 7th day of screening test (Reinprecht *et al.* 2009)



Notes:

- Screening tests with poisoned papers Whatman 3 CHR ∅ 14 mm treated with solutions of copper chelates, or ligands X
- Each value in the figure corresponds to the arithmetic mean of three samples.

Fig. 2. Indexes of fungus mycelium growth on poisoned filter papers (IPP) of selected N-salicylidene-L-glutamato-copper(II) chelates Cu/N-sal-L-glu/X between the 7th and 14th day of screening test (Reinprecht *et al.* 2009)

1,3,5-triazines with three identical hetero-cyclic groups: Symmetrical triazines (s-triazines or 1,3,5-triazines) are a larger class of compounds exploited in many applications, most of them due to their biological properties (Afonso *et al.* 2006, Milata *et al.* 2001). New 1,3,5-triazines with three identical groups: benzotriazol-1/2-yl, imidazol-1-yl, pyrazolyl-1-yl, 3,5-dimethylpyrazolyl-1-yl, 4,5-diphenylimidazol-1-yl, benzimidazolyl-1-yl, 2-methylbenzimidazolyl-1-yl, or 2-phenylbenzimidazolyl-1-yl were synthesised. Their biological activity against wood-destroying fungi *Serpula lacrymans*, *Coniophora puteana* and

Trametes versicolor was tested in screening tests by the method of poisoned filter papers. *S. lacrymans* occurred as the most sensitive fungus on presence of 1,3,5-triazines.
Triazines with three imidazole or three 4,5-diphenylimidazole groups were a slightly more effective than other ones. However, their efficacy in comparison with the commercial fungicides Tebuconazole and IPBC was insufficient (Milata *et al.* – in press).

3.2 Antifungal efficiency of boron compounds in mixtures with inorganic ammonium salts

The aim of the experiment was to propose a suitable type of the preservative for treatment of wood in interiors with a complete fungicide, insecticide and fire-retardant effect (Reinprecht & Pánek 2007). Chemicals based on boron (boric acid and disodium tetraborate decahydrate /borax/) were used as biocides "fungicide + insecticide", and chemicals based on inorganic ammonium salts (ammonium sulphate and dihydrogenammonium phosphate) were used as fire-retardants. Fungicide efficacy of inorganic boron-amino preservatives with different portions of boron and ammonium salts was valued against the wood-destroying fungi *Coniophora puteana* and *Trametes versicolor* (by screening test, and then by the P ENV 839 – Tab. 2), and also against the mixture of moulds *Alternaria alternata, Aspergillus niger, Aspergillus amstelodami, Penicillium cyclopium* and *Penicillium brevi-compactum* (by screening test, and then by the Slovak standard STN 49 0604 – Tab. 3).

Fungus	Wood preservative	Concentration of boron compounds in the preservative c_B (%)	Retention of boron compounds by wood R_B (g/m²)	Weight loss of sap-pine wood samples at decay Δm (%)
Coniophora puteana	AS	-	-	20.80
	AS + B	0.25	0.45	18.92
		0.5	1.07	19.03
		1.0	1.96	12.40
		2.5	4.06	8.15
		5.0	8.76	2.30
Trametes versicolor	AS	-	-	12.22
	AS + B	0.25	0.47	14.11
		0.5	1.07	11.69
		1.0	1.75	7.74
		2.5	4.52	4.32
		5.0	8.94	3.54

Notes:

- AS = ammonium salt (50 % $NH_4H_2PO_4$ – dihydrogenammonium phosphate, and 50 % /$NH_4/_2SO_4$ – ammonium sulphate) applied in the form of 35 % water solution.
- B = mixture of boron compounds 1:1 (50 % H_3BO_3 – boric acid, and 50 % $Na_2B_4O_7.10H_2O$ – disodium tetraborate decahydrate), added to the AS preservative in various amounts.
- Each value in the table corresponds to the arithmetic mean of four samples.
- Weight losses of control untreated samples by *C. puteana* = 34.36 %, and by *T. versicolor* = 20.72 %.

Table 2. Fungicide efficacy of wood preservatives containing ammonium salts (AS) and boron compounds (B) – standard test by P ENV 839 (Reinprecht & Pánek 2007)

Results of the screening and standard mycological tests showed that sufficient efficacy of the inorganic boron-amino preservative against brown-rot (*C. puteana*) and white-rot (*T. versicolor*) fungi can be achieved only if at least 5 % amount of boron compounds (2.5 % of boric acid and 2.5 % of borax) are used. In this situation the weight losses of treated sap-pine samples were under 3 %, or under 4 % (Tab. 2). On the other hand, the 5 % concentration of boron in the boron-amino preservatives is not a guarantee of a sufficient efficacy of treated wood against the mould attack (Tab. 3). This can be explained by a lower efficacy of boron against moulds than against wood-destroying fungi. An alternative explanation might be that the presence of nitrogen in ammonium salts can often support growth of moulds.

Mixture of moulds	Wood preservative	Concentration of boron compounds in the preservative c_B (%)	Retention of boron compounds by wood R_B (g/m²)	Growth of moulds on sap-pine wood surfaces Moulds (0 – 4)
A. alternata	AS	-	-	4
A. amstelodami	AS + B	0.25	0.43	4
A. niger		0.5	1.02	3.8
P. cyclopium		1.0	2.11	3.8
P. brevi-compactum		2.5	4.18	3.6
		5.0	8.94	3.2

Notes:

- Each value in the table corresponds to the arithmetic mean of 20 samples.
- Growth of moulds on untreated samples was always maximal (Moulds = 4), it means that more as 50 % of wood surfaces was covered with microscopic fungi – moulds.

Table 3. Efficacy of wood preservatives containing ammonium salts (AS) and boron compounds (B) against moulds – test by the national standard STN 49 0604 (Reinprecht & Pánek 2007)

3.3 Antifungal efficiency of waste glycols modified either with boron compounds (B-glycol-complexes), or with boron and waste copper compounds (Cu-B-glycol-complexes)

Exploitation of metals (Cu, Cd, Co, Fe, Mn, Ni, Pb, Ti, V, W, Zn, Zr) and their salts which can be obtained by treatment of electrical and electronical (E&E) wastes is now one of the main interests of researchers and technical workers. This interest is a direct result of the European Union Direction No 2002/96/ES (about wastes from electrical and electronical devices), which gives not only demands on recycling of metals obtained from wastes, but also informs about possibilities of how to use salts of these metals and their mixtures for chemical products. Some metal salts from electrical and electronical wastes can be used as wood preservatives, plant preservatives, anti-corrosive protective agents of steels, or stabilizing agents of plastics (Edenbaum 1992).

The aim of the experiment was to prepare modified waste substances (copper and glycols) obtained from electrical and cooling waste products, and use them as wood preservatives

with antifungal effects (Reinprecht & Kizlink 2007). Waste glycols "Fridex" were mixed with boron compounds H_3BO_3 and $Na_2B_4O_7.10H_2O$ (B-glycol-complexes), or also with copper compounds (Cu-B-glycol-complexes). Both types of these compounds were modified as well as with the QAC fungicide "Althosan MBO – lauryl-benzyl-dimethyl-ammonium chloride", or also with some other additives. For complexes of the type I., II. and III., the Cu(water-waste) products, or also the cupric-salicylate, were added (Tab. 4).

1) 20 % Boron-glycol +80 % Ethanol-Water
2) 20 % Boron-phosphor-glycol + 80 % Ethanol-Water
3) 10 % Boron-glycol +10 % Boron-phosphor-glycol + 80 % Ethanol-Water
4) 3 % H_3BO_3 + 2 % $Na_2B_4O_7.10H_2O$ +5 % Fridex + 1 % Althosan MBO + 0.25 % KSD + 88.75 % Water
5) 3 % H_3BO_3 + 2 % $Na_2B_4O_7.10H_2O$ + 5 % Fridex + 2 % Althosan MBO + 0.25 % KSD + 87.75 % Water
6) 3 % H_3BO_3 + 2 % $Na_2B_4O_7.10H_2O$ + 5 % Fridex + 2 % A0lthosan MBO + 1 % KSD + 1 % UR + 86 % Water
7) 3.5 % H_3BO_3 + 2 % $Na_2B_4O_7.10H_2O$ +5 % Fridex + 2 % Althosan MBO + 1 % UR + 86.5 % Water
8) 3 % H_3BO_3 + 1.5 % $Na_2B_4O_7.10H_2O$ + 5 % Fridex + 2 % Althosan MBO + 0.5 % UR + 88% Water
9) 3 % H_3BO_3 + 1.5 % $Na_2B_4O_7.10H_2O$ + 5 % Fridex + 2 % Althosan MBO + 1 % TUR + 87.5 % Water
10) 3.5 % H_3BO_3 + 2 % $Na_2B_4O_7.10H_2O$ + 5 % Fridex + 2 % Althosan MBO + 1 % TUR + 86.5 % Water
11) 3 % $Cu(OH)_2.CuCO_3$ +2 % H_3BO_3 + 5 % Fridex + 5 % Althosan MBO + 5 % MEA + 80 % Water
12) 3 % $Cu(OH)_2.CuCO_3$ + 2 % H_3BO_3 + 8 % Fridex + 2 % Althosan MBO + 5 % MEA + 80 % Water
13) 3 % $Cu(OH)_2$ + 2 % H_3BO_3 + 8 % Fridex + 2 % Althosan MBO + 5 % MEA + 80 % Water
14) Complex I. = 1 % $CuCO_3$ + 1 % H_3BO_3 + 3 % MEA + 5 % glycol + 90 % Cu(water-waste)
15) Complex II. = 1 % $Cu(OH)_2.CuCO_3$ + 1 % H_3BO_3 + 3 % MEA + 5 % glycol + 90 % Cu(water-waste)
16) Complex III. = 95 % Complex II. + 5 % cupric-salicylate

Notes:

- Boron-glycol was prepared by the reaction from boric acid, borax and ethyleneglycol, and boron-phosphor-glycol was prepared by the reaction from boric acid, borax, phosphoric acid and propyleneglycol (Bukovský *et al.* 1998).
- Additives: Fridex (waste glycols), Cu(water-waste) waste products with approximately 0.7 % of copper, Althosan MBO (QAC = lauryl-benzyl-dimethyl-ammonium chloride), KSD (potassiumhydrogensulphate), UR (urea), TUR (thiourea), MEA (monoethanolamine), SA (sulphuric acid) → only drops for pH value regulation between 7 and 10.

Table 4. Composition of the B-glycol and Cu-B-glycol complexes (Reinprecht & Kizlink 2007)

Efficacy of all prepared complexes No. 1-16 against the brown-rot fungus *Coniophora puteana* and the white-rot fungus *Trametes versicolor* was tested by the method of poisoned filter papers Whatman 3 CHR ∅ 14 mm, using 10 %, 33 % or 100 % water solution of these complexes. Three poisoned filter papers and one control paper were situated on agar-malt soil in each Petri dishes with a diameter of 120 mm, where inoculate of used fungus was previously situated in its centre (Fig. 3). Table 5 presents results obtained with the 10 % solutions of tested compounds.

Antifungal effect of the Cu-B-glycol-complexes was nearly comparable with the standard Wolmanit CX-H 200 (mixture of 2.5 % Cu-HDO, 4.2 % $Cu(OH)_2.CuCO_3$ and 25 % H_3BO_3 in water). Copper in the glycol-complexes had an apparent positive antifungal effect, comparing a higher efficiency of the Cu-B-glycols (No. 11-13) with a lower efficiency of the B-glycols (No. 4-10). Activity of copper was slightly better against the white-rot fungus *T. versicolor* than against the brown-rot fungus *C. puteana* (No. 11-13). However, the complexes I., II. and III., based on the waste copper products: $Cu(OH)_2.CuCO_3$-H_3BO_3-ethanolamine-

glycol-Cu(water-waste) and $CuCO_3$-H_3BO_3-ethanolamine-glycol-Cu(water-waste), and without the QAC "Althosan MBO", were less effective against the wood-destroying fungi, specially against the *T. versicolor* (No. 14-16).

Tested compound	*Coniophora puteana*				*Trametes versicolor*			
	Inhibition zone (mm)		Growth on papers (mm)		Inhibition zone (mm)		Growth on papers (mm)	
c = 10 %	7th day	14th day	7th day	14th day	7th day	14th day	7th day	14th day
B-glycol complexes								
1	1-5	0	0	8-12	0-2	0	0-2	8-14
2	1-3	0	0	10-12	0	0	1-4	14
3	1-3	0	0	9-11	0	0	1-4	12-14
4	4-6	0	0	5-7	0-4	0	0	2-7
5	4-8	0	0	4-7	1-4	0	0	1-4
6	3-4	0	0	5-9	1-2	0	0	2-6
7	2-4	0	0	6-11	0-1	0	0	3-7
8	3-7	0	0	5-8	0-2	0	0	3-7
9	2-7	0	0	4-9	1-3	0	0	2-6
10	3-6	0	0	5-8	2-3	0	0	2-4
Cu-B-glycol complexes								
11	6-12	0	0	3-5	5-8	2-3	0	0
12	5-10	0	0	2-5	6-9	1-2	0	0-3
13	6-10	0	0	2-5	4-7	0-1	0	0-2
14	7-12	0	0	0-6	0-2	0	0-1	6-10
15	4-6	0	0	4-7	0-2	0	0-2	8-12
16	5-10	0	0	2-5	0-3	0	0-2	10-12
Standard								
Wolmanit CX-H 200	8-15	3-9	0	0	3-6	0-1	0	0-3
Controls	0	0	0-4	14	0	0	10-14	14

Note:

- Each value in the table corresponds to the arithmetic mean of nine poisoned papers situated in three Petri dishes.

Table 5. Screenings of the antifungal efficacy of B-glycol and Cu-B-glycol complexes against the fungi *C. puteana* and *T. versicolor*, at using their c = 10 % water solutions in poisoned filter papers (Reinprecht & Kizlink 2007)

Waste glycol compounds and waste copper compounds can be in exceptional cases used for preparation of wood preservatives: glycols as solvents and copper as fungicides. However, the chemical composition of all waste compounds used as wood preservatives with fungicide activity has to be exactly known, because it is very important from the ecological aspects and also from the point of view of the stability and durability of antifungal or other protection effects.

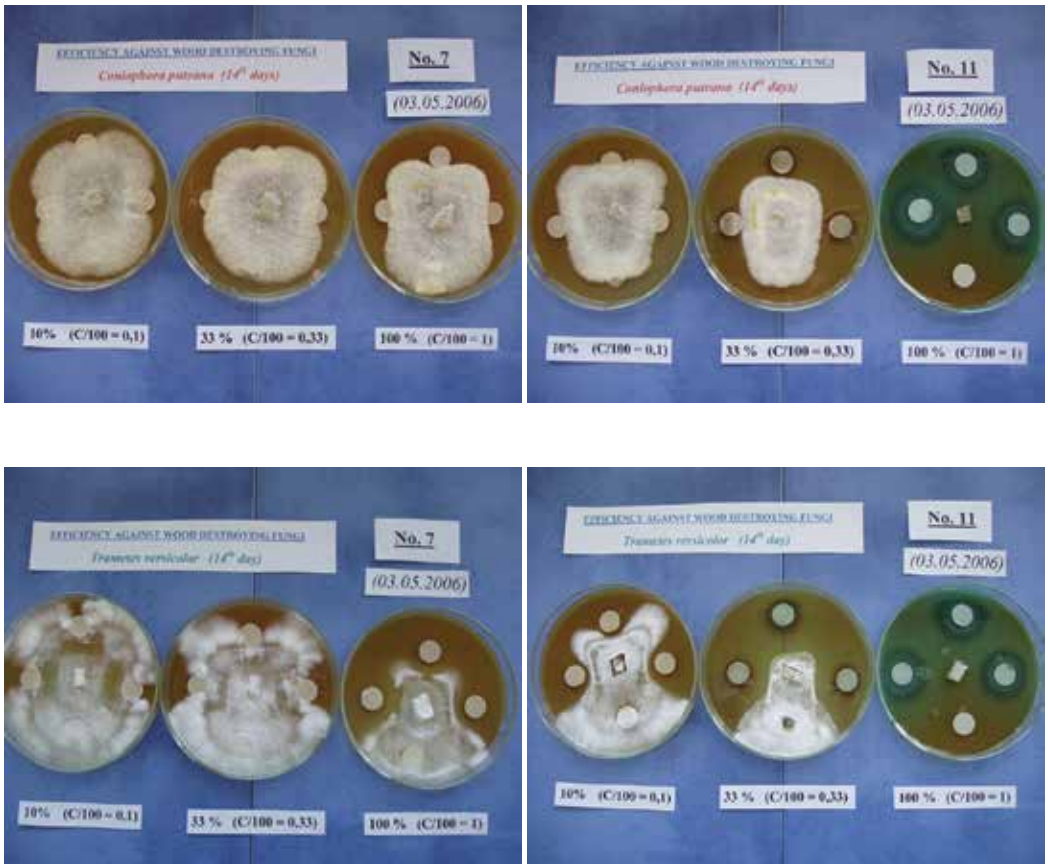


Fig. 3. Activity of the B-glycol-complex (No. 7) and the Cu-B-glycol complex (No. 11) against the fungi *C. puteana* and *T. versicolor*, determined by the screening test with poisoned filter papers in the 14th day

4. Conclusion

In present various types of inorganic and organic fungicides are commonly used for preservation of wood and wooden composites against moulds, staining fungi and rotting-fungi. However, in future the timber industry will need new environmentally more friendly preservatives. Among the most perspective fungicides probably belong the natural substances (chitosan, essential oils, ...) and synthetically prepared organic compounds (hetero-cycles, carbamates, ...). Research in these fields have to be complex with aim to give on market only healthy-safe, stable and effective products.

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6

Challenges of Fungicide Control on Wheat Rusts in Kenya

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1. Introduction

Stem rust or black rust (caused by *Puccinia graminis* Pers. f. sp. *tritici* Erikss. & E. Henn.) is a serious wheat disease causing a decrease of wheat production in many areas of the world (Roelfs, 1978). The effective barberry eradication in the early 20th century and the infrequent occurrence of favourable temperature in Europe has resulted in a decline in importance of stem rust. Until recently, the disease has been under effective control through the wide spread use of resistant varieties (Jin, 2007). The re-emergence of a new virulent race TTKSK (Ug99) in Eastern Africa region (Pretorius et al., 2000), and the subsequent detection of its many variants in Kenya has rendered important commercial varieties susceptible (Jin, 2008). Wide-scale epidemics have been frequent, being recorded in 2004, 2005, 2006, 2007, 2008, 2009 and 2010. Fungicides sprays have become part of the production practice of wheat growers in Kenya, although many are not effective. The fungicides used for the control of wheat leaf diseases have increased costs of production, because of the multiple applications required to protect the crop before it matures. Unlike in other regions e.g. United States, Canada, Australia and South Africa (Table 1), there is currently no fungicide registered for the control of wheat stem rust in Kenya, this is because the effects of fungicide on loss prevention are poorly understood and data is limited (Wanyera et al., 2009). However, fungicides are used extensively by producers in other countries to control stripe and leaf rusts, but only a few are registered for the control of stem rust.

Many fungicides have been found to be effective at specific growth stages. Until recently, studies have indicated that up to one-third of applications may be too late for optimal effectiveness. Application timing is critical in managing stem rust. Stem rust activity needs to be closely monitored both in individual fields and across regions. This is because preventive applications consistently provide better results than applications made after disease infection has occurred. Unlike stripe and leaf rust, foliar fungicide applications targeting stem rust must be applied as soon as the disease is detected as opposed to targeting key yield determining leaves. It is important to note that, the severity of any disease is related to inoculum pressure, weather conditions and variety susceptibility (Cook et al., 1999). According to Milne et al. (2007) the fungicide choice and the timing of application are often poor in commercial crops. Sometimes, crops receiving up to three

Active ingredient	Trade names	Stem Rust	Stripe Rust	Leaf Rust
Flutriafol	Flutriafol 250 EC, Force, Impact, Jubilee	*	250-500 mL/ha	250-500 mL/ha
Flusilazole/ carbendazim	Punch Xtra 125/250 EC	*	400 mL/ha	400 mL/ha
Propiconazole	Aurora 250 EC, Bumper 250 EC, Prestige, Propiconazole 250 EC, Slipstream 250 EC, Tilt 250 EC, Tyrant 250 EC	500 mL/ha	250-500 mL/ha	150-500 mL/ha
Tebuconazole	Folicur 430 SC, Orius 430 SC, Stingray, Tebuconazole 430 SC	*	145-290 mL/ha	145-290 mL/ha
Triadimefon	Accord, Bayleton 125 EC, Slingshot, Triad 125 EC, Triadimefon 125 EC, Turret	*	500-1000 mL/ha	*
Azoxystrobin	Amistar 250 SC	*	300 mL/ha	300 mL/ha
Azoxystrobin + Cyproconazole	Amistar Xtra 280 SC	400-800 mL/ha	400-800 mL/ha	400-800 mL/ha
Azoxystrobin + Difenoconazole	Amistar Top 200/125 SC	*	500 mL/ha	500 mL/ha
Trifloxystrobin	Twist 500 SC	*	100 mL/ha	100mL/ha
Cyproconazole	Alto 100 SL	*	400 mL/ha	400 mL/ha
Propiconazole + Cyproconazole	Tilt Xtra 250 EC	500 mL/ha	250-500 mL/ha	250-500 mL/ha
Epoxiconazole	Opus 125 SC	*	800 mL/ha	800 mL/ha
Hexaconazole	Anvil 50 SC	*	480 mL/ha	480 mL/ha

* Denotes not recommended

Table 1. Foliar fungicides and recommended rates used to control rust diseases of wheat in Australia and South Africa

badly-timed sprays suffer as much disease as those untreated, suggesting that either the fungicides are applied too late or do not control the disease effectively. Research has shown that fungicides applied during the period from flag leaf emergence to ear emergence (GS 37-59) offer the best prospects for cost-effective rust control in wheat (Bradley, 2004).

The management of stem rust by growing resistant varieties is undoubtedly the most desirable method of control. However, fungicides have been used against various diseases of cereals for over 100 years. With the introduction of systemic fungicides in the 1970's, fungicides became an integral part of cereal crop production. They have become established as an essential input in the growing of cereals and it is not possible to grow a profitable crop of wheat in stem rust "hot-spot" areas without the application of fungicide (Wanyera et al., 2009). Despite global concerns and restrictions on chemical development and usage in food

production, such limitations have tended to give impetus to developments in fungicide chemistry and delivery systems (McDonald, 2006). This was synergised by the development of new fungicide classes with novel modes of action in the 1990's. These include the strobilurins, phenypyrroles, anilinopyrimides, phenoxyquinolines and other compounds that induce defence mechanisms in the plant (Knight et al., 1997).

The Eastern Africa epidemics of wheat stem rust due to race Ug99 of *Puccinia graminis* f. sp. *tritici* (Pretorius et al., 2000) have stimulated interest in the search for effective and economically sustainable chemicals to manage this disease. In recent past the efficacy of various fungicides to control stem rust has been reported (Mayfield, 1985; Rowell, 1985; Loughman et al., 2005 and Wanyera et al., 2009). Fungicides can play a major role in integrated management of stem rust disease before new varieties with effective host resistance are released. Currently only limited studies have been conducted to determine the effectiveness of foliar fungicides on stem rust severity and grain yield in the Eastern Africa region (Wanyera et al., 2009), and none on the usefulness of seed and fertilizer treatments for the control of stem rust (Dill-Macky & Roelfs, 2000). The lack of fungicides specifically labelled for the control of stem rust in Kenya has hindered the immediate release of recommendations for fungicide use to manage the disease.

1.1 Nature and recurrent of wheat rusts

The history of wheat production in Kenya has been mainly an account of attempts to control stem rust, *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., using resistant varieties (Green et al., 1968). Further, little is known of the sources of variation of stem rust in East Africa. There is circumstantial evidence that some variation is due to somatic recombination of existing virulence (Harder et al., 1970). The early wheat growers were confronted by frequent stem rust epidemics; this necessitated the search for resistant varieties and chemicals to manage rust diseases. Wheat stem rust has been studied at Njoro, Kenya, since 1927 (Guthrie, 1966). Kenya's wheat growing areas are unique in that they lie within the Great Rift Valley which extends from Mediterranean region in the north to South Africa. There is a possibility of urediniospores being transported throughout this mega-region, which ensures the presence of a large reservoir of inoculum, but if there was little or no exchange of inoculum, it was thought possible to limit rust development in any one area by controlling agricultural practices. This was attempted earlier in Kenya, where alternate host of rust (*Barberry vulgaris* L.) does not function (Green et al.,1970) by discouraging double cropping (two crops a year) to decrease the movement of rust from one crop to the next. Before the current effects of climate change (global warming), wheat was planted at designated times of the year to make maximum use of rainfall while the crop was growing, yet be ready for harvest during a dry period. This has since changed with the unreliability and low amount of rainfall received in wheat belts, therefore farmers have resulted in planting wheat throughout the year (multi-cropping). It is common to find within a short distance crops of all growth stages, this ensures the availability of "green-bridges" for urediniospore production and continuous supply of inoculum for new crops. This coupled with rapid change in altitude in wheat growing areas; entails localised epidemics occurring in many areas in the country depending on the growing season forming a stem rust migration route, where the disease pathogen moves back and forth in these areas. Therefore, high host resistance to all races of the pathogen would be required to withstand sustained disease attack; otherwise to grow wheat under such conditions without an effective fungicide protection programme is a serious challenge.

Ecological conditions in Kenya favour cereal rust development. Most of the wheat-growing areas are located in highlands (1,800 to 3,000 meters above sea level) known as "hot-spot'' for the evolution and survival of new rust races (Saari & Prescott, 1985). The growing season extends over 12 months, temperature range from 18 to 30°C, days are uniformly about 12.5 hours long, dews are heavy, and precipitation occurs frequently as showers during the main growing season. Stem rust survives on volunteers or other hosts including, barley *(Hordeum spp.)*, triticale *(xTriticosecale)* and other related grasses. Normally stem rust is more active in the warmer parts of the cropping season or in lower altitude areas compared to stripe rust and leaf rust. It has been observed that race Ug99 is equally aggressive in the high cool elevations (2,700-3,000 meters above sea level) of Kenya.

1.2 Resistance/susceptibility of current varieties

According to Reynolds and Borlaug (2006), about 25% of the world's wheat area is at risk from stem rust attack, accounting for an estimated 19% of global production. In Kenya, since 2002 virtually all the growing areas; experiences devastating stem rust epidemic every year. Extensive screening of thousands of global wheat varieties for resistance to Ug99 in key sites in Kenya and Ethiopia for the last five years, only about 5% have exhibited appreciable resistance (Table 2). This indicates the vulnerability of all the countries producing wheat to the new stem rust pathogen. In Kenya, for instance, all the commercial varieties have succumbed to race Ug99, the farmers are advised to grow moderately susceptible varieties, which need fungicide protection at the appropriate growth stages in order to harvest an economic yield.

Resistance/susceptibility group	Estimated area	
	million ha	%
Unknown	30.71	41.1
Susceptible	38.54	51.6
Moderately susceptible	1.73	2.3
Resistant	1.04	1.4
Moderately resistant	2.72	3.6

* Singh et al., 2008

Table 2. Estimated areas planted to wheat varieties in 18 African and Asian countries resistance/susceptibility grouping to Ug99, based on screening data 2005 and 2006 in Njoro, Kenya.

1.3 Diagnosing stem rust disease in wheat

Stem rust fungi has been changing continuously, producing new races. These races are detected when previously resistant variety becomes infected. Therefore, when a resistant variety is sown, the crop should be monitored for disease on regular basis. This should start around the 2nd node stage (GS 32) and continue to flag leaf (GS 39) (Peterson et al., 1948). The protection of the shoots is important in contributing to the yield and quality of wheat. In "hot-spot" areas such as Kenya where the crop is under constant bombardment by urediniospores throughout the year, disease scouting starts at seedling and continues up to grain-filling growth stage. High infections around head emergence have been reported to

cause significant yield losses (Barlett et al., 2002). It is important for the grower to be able to identify the disease early; however, this is not always possible without relevant skills for the correct disease identification, and rust severity assessment that are prerequisite to informed decisions on fungicide use. While fungicide treatment is meant to control the disease, there is evidence that some have been shown to have beneficial effect on the plants by delaying senescence, thereby prolonging the duration of the green-leaf area and increasing yield (Burgeno et al., 2000; Bertelsen et al., 2001). However, distinguishing between the yield grain because of disease suppression and yield grain because of any direct physiological effects is difficult in field experiments. Quantitative evidence in representative environments is not available (Niks & Lindhout, 2006). However, the yield improvements are economically important and cannot be overlooked.

1.4 Fungicides for controlling rusts

The best way to control stem rust is to use resistance varieties, which is universally accepted. But its practical implementation remains a scientific challenge, because the fungus that causes wheat stem rust disease is difficult to control due to its nature of constantly evolving and mutating into new races. It is also important to note that the level of resistance expressed by varieties to stem rust can be significantly different to the one expressed to leaf diseases. Alternative control measures might prove economically feasible if areas of potential loss were identified in time to reduce damage by application of effective fungicides. Fungicides have become an integral part of disease-management programmes on cereal crops in many countries of the world. Most of the fungicides currently used for stripe rust and other wheat leaf disease control are not registered for the control of stem rust in Kenya (Table 1). Therefore the choice of an appropriate chemical is difficult (Viljanen-Rollinson et al., 2006).

The use of appropriate fungicide is an effective but least employed method of rust management. Foliar fungicides can achieve economic control as long as they are applied at an early disease onset (Loughman et al., 2005). Early intervention reduces damage to the leaves, stem and transport system ensuring translocation of nutrients, and therefore proper filling of grains. Chemical control is more effective when rust diseases are identified on susceptible varieties early in the growing season. Their effectiveness depends on varietal susceptibility, level of infection and stage of crop growth at application. In fields planted with moderately resistant (slow rusting) or resistant varieties, a fungicide application may not be necessary even if some disease occurs. However, fields planted with moderately susceptible or susceptible varieties should be scouted regularly, and any sign of disease may warrant a fungicide application. Although it is not advisable to plant very susceptible varieties, if sown two or more applications may be necessary to achieve a moderate level of control. Yield and quality studies with fungicides on grains have documented other benefits including bigger grain size and better milling quality (Bartlett et al., 2002).

1.5 Foliar fungicides and thresholds

In Kenya, farmers who afford fungicides for stem rust control commonly use calendar-based fungicide spray schedules. Under heavy stem rust epidemics some farmers in wheat growing areas have been reported to apply as many as 5 times per crop cycle and sometimes they alternate contact and systemic compounds. In other crops, studies indicate that fungicide spray regimes should be tailored to host resistance than depending on blanket recommendation. But for stem rust where wheat resistance has broken down, fungicide

application should begin when the first rust pustule appears. This is repeated every 7 to 10 days for contact or 14 to 21 days for systemic fungicides depending on rain and temperatures. Carisse et al. (2009) have recommended the use of action thresholds for rapid and accurate classification of the incidence of apple scab on leaves, and aid in scab management decision making. It has also been suggested that, stem rust infection thresholds be established to correctly determine the level of resistance in varieties in order to reduce the amount of fungicide treatments. But to gather sufficient data for this purpose, a massive national variety resistance evaluation process, and monitoring of the pathogen virulence is needed. This is costly and takes time. Paveley et al. (2003) demonstrated that it is possible to predict fungicide efficacy of a two spray programmes from the performance of their component single spray. In a related study, Kromann et al. (2009) concluded that timing fungicide sprays based on rainfall thresholds could be used to control Potato Late Blight. However, in the case of wheat stem rust in Eastern Africa where there is continuous cropping throughout the season it is difficult to predict an epidemic. The threshold system is used as a guide for whether or not there is adequate disease pressure to justify fungicide application. Fungicides are beneficial only if rust is present at high levels and occurs early in the season to cause yield loss. Timing of application is critical in managing stem rust, whose activity needs to be closely monitored. Unlike stripe and leaf rust, foliar fungicide applications targeting stem rust must be applied as soon as the disease is detected. The more important times for application of foliar fungicides are usually flag leaf emergence (GS 37) and full boot (GS 45), the latter is considered the ideal timing for a single spray. A follow up spray may be necessary 3 to 4 weeks later, if conditions continue to favour disease development. Preventative applications consistently provide better results than applications made after disease infection has occurred.

2. Fungicide Case Studies in Kenya

The following is an assessment of commercial and two new foliar fungicides available in Kenya for efficacy against Ug99 pathogen. This article reports field experiments conducted under natural infection to determine the effect of foliar fungicides on wheat stem rust severity, grain yield, and 1,000-kernel weight.

2.1 Materials and methods

2.2 Commercial fungicides

Field experiments were conducted in 2005 and 2006 in three locations represented by trial sites at (1) Kenya Agriculture Research Institute (KARI) - Njoro (0^{o} 20′ S, 35^{o} 56′) at 2,185 meters above sea level (MASL), with mean minimum and maximum temperatures of 9.7 and 23.5^{o}C respectively, and mean average rainfall of 900 mm; (2) Eldoret (0^{o} 31′ N, 35^{o} 15′ E) at 2,180 MASL, with mean annual rainfall 1,250 mm with minimum and maximum temperatures of 12 and 23^{o}C, respectively; and (3) Mau-Narok (0^{o} 39′ S, 35^{o} 57′ E) at 2,900 MASL an annual rainfall with 1,200 to 1,400 mm, minimum and maximum temperatures of 6 to 14 and 22 to 26^{o}C, respectively.

Nine commercial fungicides labeled for the control of stripe rust, leaf rust, and other leaf diseases of wheat were evaluated for control of stem rust in field experiment. The experimental units were 8 row field plots of 9 m^{2} with an inter-row spacing of 20 cm. The cultivar was the widely grown bread wheat Duma, which is highly susceptible to stem rust. The cultivar is moderately resistant to stripe rust (*P. striiformis* Westend. f. sp. *tritici* Erikss.). A randomized complete block design with four replicates was used in all the locations. An

experimental seed-drill planter was used at a seeding rate of 100 g/plot. Planting was done on May 19 at KARI-Njoro, June 4 at Eldoret and August 22 at Mau-Narok in 2005. In 2006, the planting dates were May 18 at Eldoret, May 30 KARI-Njoro and September 19 at Mau-Narok (Table 3).

		2005			2006	
Activity, GS[Z]	KARI-Njoro	Eldoret	Mau-Narok	KARI-Njoro	Eldoret	Mau-Narok
Planting	19-May	4-Jun	22-Aug	3-May	18-May	14-Sep
Fungicide application						
GS 55	4-Aug	10-Aug	15-Nov	9-Aug	3-Aug	...
GS 65	18-Aug	26-Aug	30-Nov	23-Aug	18-Aug	...
GS 65	...	...	...	...	...	15-Dec
GS 73	...	...	...	...	...	2-Jan, 2007
Disease assessment						
GS 55	4-Aug	10-Aug	15-Nov	9-Aug	3-Aug	...
GS 65	18-Aug	26-Aug	30-Nov	23-Aug	18-Aug	8-Nov
GS 65	...	...	...	...	...	15-Dec
GS 73	...	...	...	...	...	2-Jan, 2007
GS 77	1-Sep	13-Sep	14-Dec	8-Sep	12-Sep	16-Jan, 2007
Harvesting	12-Nov	4-Nov	26, March, 2006	6-Nov	31-Oct	6-April, 2007

[Z]GS= growth stage

Table 3. Schedule on planting, fungicide application, disease assessment, and harvesting at Kenya Agricultural Research Institute (KARI)-Njoro, Eldoret and Mau-Narok location in Kenya

The experimental fields were fertilized with Di-ammonium phosphate (18% N,46% P, 0% K) at the recommended rate of 150 kg/ha at planting. A pre-emergence herbicide, Stomp 500 E (pendimethalin), labeled for weed control in wheat was applied soon after planting at 3 liters/ha. Buctril MC (bromoxynil +MCPA) labeled for control of broad-leaved weeds was applied at 1.25 liters/ha at growth stage GS 24 (tillering). Metasystox 250 EC (oxy-demeton-s-methyl) was applied at 0.5 liters/ha to control cereal aphids at GS 24 (tillering), GS 65 (flowering), and GS 73 (milk) Zadoks et al., 1974).

The commercial fungicides tested were : (1) epoxiconazole + carbendazim (Swing 250 EC), (2) cyproconazole 80 g/L + propiconazole 250 g/L (Artea 330 EC), (3) tebuconazole + tridimenol (Silvacur 375 EC), (4) tebuconazole (Folicur 250 EC), (5) trifloxystrobin + propiconazole (Stratego 250 EC), (6) epoxiconazole 125 g/L + carbendazim 125 g/L (Soprano C 250 EC), (7) tebuconazole (Orius 25 EW), (8) hexaconazole (Cotaf 5 EC), and (9) azoxystrobin 200 g/L + cyproconazole 80 g/L (AmistarXtra 280 SC) all at the rate of 1.0 L/ha. The untreated plots served as the control. The fungicides were applied using a knapsack sprayer at recommended water volume of 200 liters/ha at GS 55 (heading) and GS 65 (flowering) (Zadoks et al., 1974).

Stem rust severities based on modified Cobb scale (Peterson et al., 1948) were scored three times on whole plots at GS 55, 65, and 73 or 77 (late milk) before fungicide application and at two 14 to 18 day intervals following the application, according to the schedule in Table 1. However, the third reading at Eldoret in 2006 was delayed due to logistical problems. Area under the disease progress curve (AUDPC) was calculated by a computer program developed at the International Centre for Maize and Wheat Improvement (CIMMYT) that calculates the area of the curve created by the disease scores taken three times during the crop cycle (Tables 4, 5, and 6).

Every plot was harvested using a Hans-Ulrich Hege plot combine harvester (Saatzuchtmaschinen Hohebuch). Grain weight measurements were taken after harvest and cleaning at 13 to 14% moisture content. A sample of grain was taken from each plot for determination of kernel weight based on the weight of 1,000 grains (1,000-kernel weight). Data on disease severity, grain yield, and 1,000-kernel weight were analyzed according to the analysis of variance procedure using SAS Statistical package (PROC-ANOVA) (SAS Institute, Inc. 1999). Differences in treatment effect were compared at ($P \leq 0.05$) using the least significant difference test (Steel and Torrie, 1980).

2.3 New fungicides (Nativo 300 SC and Prosaro 250 EC)

Field experiments were conducted at two locations (KARI-Njoro and Mau-Narok) during the 2006 and 2007 growing seasons to assess the efficacy of two new foliar fungicides; viz. trifloxystrobin 100 g/L + tebuconazole 200 g/L (Nativo 300 SC) and prothioconazole 125 g/L + tebuconazole 125 g/L (Prosaro 250 EC) each applied at three rates; 0.6 L/ha, 0.75 L/ha, and 1.0 L/ha. Two standard fungicides; azoxystrobin 200 g/L + cyproconazole 80 g/L (AmistarXtra 280 SC) and tebuconazole (Folicur 250 EC), each applied at the rate of 1.0 L/ha for comparison and untreated plots served as the control. The field operations, disease assessment and data analysis was conducted as described in materials and methods section.

3. Results

3.1 Rust severity

In 2005, the onset of stem rust disease was early in the growing season at KARI-Njoro, resulting in a severe infection of 77.5% at GS 77 in the untreated plots. In the same year severity at Eldoret and Mau-Narok was moderate, 30.0 and 35.0%, respectively (Tables 4, 5, and 6). In 2006, disease severity in untreated plots was 28.8% at KARI-Njoro, 45.0% at Eldoret, and 40.0% at Mau-Narok (Tables 4, 5, and 6). There were no other diseases observed in the experimental plots.

3.2 Effect of fungicides on stem rust severity

In both 2005 and 2006, fungicide treatments for AUDPC were significant ($P \leq 0.05$) at KARI-Njoro. Differences in AUDPC for stem rust were observed among fungicides at Eldoret and Mau-Narok in 2006. In 2005, non significant ($P \geq 0.05$) effects of fungicide treatments for AUDPC were obtained at Eldoret and Mau-Narok (Tables 4, 5, and 6). AUDPC values indicated that AmistaXtra 280 SC, Orius 25 EW, Folicur 250 EC, and Silvacur 375 EC, reduced rust severity across the three locations.

The trend of reduction on stem rust severity was maintained after first and second fungicide application. However, the reductions were more pronounced in the latter. Overall,

	2005							2006						
	MRS[v]				Grain yield[w]		TKW[x]	MRS[v]				Grain yield[w]		TKW[x]
Treatment[y]	1st	2nd	3rd	AUDCP[z]	t/ha	G (%)	Wt. (g)	G (%)	1st	2nd	3rd	AUDCP[z]	t/ha	G (%)	Wt. (g)	G (%)
Untreated	23.8a	57.5a	77.5a	709 b	0.6b	-	29.8d	-	10.3a	11.3a	28.8a	518a	0.8b	-	36.9b	-
Swing 250 EC	25a	47.5a	42.5b	792a	0.9ab	31.5	33.2cd	10.1	5a	7.8bc	6.3cd	191cd	2.0a	61.5	45.1a	22.2
Artea 330 EC	32.5a	15.0b	22.5cd	210c	1.4a	55.8	37.6a	20.6	11.2a	13.8a	15.0bc	271bc	2.2a	65.1	42.0a	13.8
Silvacur 375 EC	27.5a	12.5b	15.0de	290c	1.2ab	47.9	36.7ab	18.6	6.3a	2.0bc	7.5cd	116d	2.6a	71.3	45.2a	22.5
Folicur 250 EC	17.5a	8.8b	11.3e	154c	1.0ab	37.1	36.4abc	18	12.5a	1.0c	6.3cd	154cd	2.3a	66.9	45.2a	22.5
Stratego 250 EC	22.5a	21.3b	32.5bc	381bc	1.4a	57.3	36.6abc	18.5	6.3a	5.5 abc	8.8cd	191cd	2.5a	70.2	43.3a	17.3
Soprano 50 EC*	31.25a	40.0a	62.5a	815a	0.8ab	26.5	33.2bcd	10.2	12.5a	10.3ab	12.5bc	292bc	1.9a	60.3	42.4a	14.9
Orius 5 EW*	20a	10.0b	11.3e	189c	1.2ab	49.6	37.0a	19.2	11.3a	2.0bc	8.8cd	156cd	2.0a	62.3	44.8a	21.4
Cotaf 5 E*	20a	10.0b	16.3de	225c	1.3ab	51.6	35.9abc	16.7	7.5a	12.5a	18.8ab	276ab	0.5b	38.8	35.6b	-3.5
AmistarXtra 280 SC	17.5a	10.0b	12.5de	210c	1.4a	55.1	36.9a	19.3	7.8a	1.0c	4.0d	105d	2.2a	66.2	44.9a	21.7
Mean[h]	23.8	19.5	25.2	363	1.2	45.8	35.9	16.8	8.9	6.2	9.8	206	2	62.5	43.2	17
LSD (0.05)	-	1.6	1.1	32.3	0.6	-	3.6	-	-	1.5	1.2	4.9	0.9	-	4.2	-

[u] Treatment means within columns followed by the same letter are not significantly different at P≤ 0.05 according to least significant difference (LSD) test; – = no data.

[v] MRS = mean rust severity (modified Cobb scale); 1st, 2nd, and 3rd disease notes were recorded at growth stages 55, 65, and 77, respectively.

[w] Yield gain (G) (%) = (fungicide-treated – untreated) × 100/treated.

[x] TKW = 1,000-kernel weight; gain (G) (%) = (fungicide-treated – untreated) × 100/treated.

[y] Active components of the fungicides are as follows: Swing 250 EC, epoxiconazole + carbendazim; Artea 330 EC, cyproconazole at 80 g/liter + propiconazole at 250 g/liter; Silvacur 375 EC, tebuconazole + tridimenol; Folicur, tebuconazole; Stratego 250 EC, trifloxystrobin + propiconazole; Soprano C 250 EC, epoxiconazole at 125 g/liter + carbendazim at 125 g/liter); Orius 25 EW, tebuconazole; Cotaf 5 E, hexaconazole; and AmistarXtra 280 SC, azoxystrobin at 200 g/liter + cyproconazole at 80 g/liter. Soprano 250 EC, Orius 25 EW, and Cotaf 5 E are generic chemicals.

[z] Area under the disease progress curve (AUDPC) values are means of four replications.

Table 4. Effect of fungicide on stem rust severity, area under disease progress curve (AUDCP), grain yield, and 1,000-kernel weight on wheat cv. Duma at Kenya Agricultural Research Institute - Njoro[u]

	2005							2006						
	MRS[v]				Grain yield[w]		TKW[x]	MRS[v]				Grain yield[w]		TKW[x]
Treatment[y]	1st	2nd	3rd	AUDCP[z]	t/ha	G (%)	Wt. (g)	G (%)	1st	2nd	3rd	AUDCP[z]	t/ha	G (%)	Wt. (g)
Untreated	12.5a	30.0a	30.0a	643a	0.7c	-	25.0d	-	12.5a	14.0ab	45.0a	975a	2.3ab	-	40.7
Swing 250 EC	5a	5.0cd	5.0bc	195a	2.1b	67	37.3ab	32.8	13.8a	5.3bc	18.8cd	510ab	2.2b	8.4	43.3
Artea 330 EC	7.5a	20.0cd	5.5bc	425a	1.8b	62	35.9 bc	30.3	7.5a	4.3bc	13.8cd	335b	2.4ab	2.1	41.1
Silvacur 375 EC	7.5a	7.5bcd	5.5bc	235a	2.1b	66.4	36.1bc	30.7	6.8a	5.3bc	12.5cd	357b	2.7ab	14	43.3
Folicur 250 EC	7.5a	3.0cd	7.5bc	195a	2.3b	69.4	35.8bc	30.1	13.8a	6.5bc	11.3cd	381b	2.5ab	7.5	42.9
Stratego 250 EC	10a	5.0cd	10.0b	257a	1.5b	54.6	33.0c	24.1	13.8a	5.5bc	25.0cd	221b	2.3ab	2.6	41.9
Soprano C 250 EC*	7.5a	12.5bc	12.5b	580a	1.6b	55.1	33.7bc	25.8	6.3a	2.0c	7.5d	652ab	2.2b	6.4	40.6
Orius 5 EW*	5a	2.5d	5.0bc	172a	2.2ab	68.5	34.1bc	26.6	6.3a	6.3bc	25.0bc	618b	2.4ab	0.9	41.8
Cotaf 5 E*	7.5a	7.5cd	5.5bc	221a	2.2ab	67.4	35.1bc	28.6	7.5a	21.3a	37.5ab	1020a	2.2b	7.4	40.4
AmistarXtra 280 SC	10a	3.0cd	3.0c	156a	3.0a	76.3	40.1a	37.5	7.5a	3.3c	11.3cd	335b	2.9a	18.2	42.9
Mean[h]	8	7.3	6.6	271	2.1	65.2	35.7	29.6	9.2	6.6	18.1	492	2.4	7.5	42
LSD (0.05)	1.6	1.6	1.3	571	0.8	-	3.6	-	NS	1.5	1.8	517	0.6	-	NS

[u] Treatment means within columns followed by the same letter are not significantly different at $P \leq 0.05$ according to least significant difference (LSD) test;

– = no data.

[v] MRS = mean rust severity (modified Cobb scale); 1st, 2nd, and 3rd disease notes were recorded at growth stages 55, 65, and 77, respectively.

[w] Yield gain (G) (%) = (fungicide-treated – untreated) × 100/treated.

[x] TKW = 1,000-kernel weight; gain (G) (%) = (fungicide-treated – untreated) × 100/treated.

[y] Active components of the fungicides are as follows: Swing 250 EC, epoxiconazole + carbendazim; Artea 330 EC, cyproconazole at 80 g/liter + propiconazole at 250 g/liter; Silvacur 375 EC, tebuconazole + tridimenol; Folicur, tebuconazole; Stratego 250 EC, trifloxystrobin + propiconazole; Soprano C 250 EC, epoxiconazole at 125 g/liter + carbendazim at 125 g/liter); Orius 25 EW, tebuconazole; Cotaf 5 E, hexaconazole; and AmistarXtra 280 SC, azoxystrobin at 200 g/liter + cyproconazole at 80 g/liter. Soprano C 250 EC, Orius 25 EW, and Cotaf 5 E are generic chemicals.

[z] Area under the disease progress curve (AUDPC) values are means of four replications.

Table 5. Effect of fungicide treatment on stem rust severity, area under the disease progress curve (AUDPC), grain yield, and 1,000-kernel weight on wheat cv.Duma at Eldoret [U]

Treatment[y]	2005 MRS[v] 1st	2nd	3rd	AUDCP[z]	Grain yield[w] t/ha	G (%)	TKW[x] Wt. (g)	G (%)	2006 MRS[v] 1st	2nd	3rd	AUDCP[z]	Grain yield[w] t/ha	G (%)	TKW[x] Wt. (g)
Untreated	9.0ab	11.5a	35.0a	367.3a	1.3c	-	38.2 b	-	5a	35.0a	40.0a	833a	2.1b	-	34.5
Swing 250 EC	10.0ab	10.0a	13.8b	316.5a	3.0ab	54.8	46.6a	18	1a	12.5bcd	11.3c	262cd	3.0a	30	38.2
Artea 330 EC	10.0ab	117.5	9.0b	392.0a	3.4a	60.5	44.0a	13	4a	32.5a	32.5ab	699ab	2.4ab	13.9	36.1
Silvacur 375 EC	10.0ab	8.0a	6.5b	236.8a	3.3a	59.8	43.7a	12.6	0.8a	16.3abcd	18.8bc	350cd	3.0a	30.5	38.7
Folicur2 50 EC	13.8a	12.5a	9.0b	347.5a	3.2ab	57.9	45.1a	15.3	0.8a	5.3d	11.3c	180d	2.9a	28.6	37.2
Stratego 250 EC	13.8a	13.8a	8.0b	358.8a	3.3a	59.7	45.6a	16.1	1a	23.8ab	32.5ab	528abc	2.5ab	14.3	36.8
Soprano C 250 EC*	6.3b	12.5a	16.5b	344.0a	3.1ab	56.6	44.1a	13.4	0.5a	15.0bcd	21.3bc	392bcd	2.8ab	25.5	38.1
Orius 5 EW*	10.0ab	10.0a	3.0b	241.0a	3.1 ab	56.3	44.4 a	14	0.8a	13.8 bcd	13.8 c	309cd	3.0 a	29.8	38.6
Cotaf 5 E*	6.3b	11.3a	7.8b	264.5a	3.1 ab	56.5	44.3 a	13.7	1a	21.3 abc	30.0 ab	492bcd	2.4 ab	13.6	36.7
AmistarXtra 280 SC	6.3b	10.0a	5.5b	230.8a	3.2ab	37.9	44.2a	13.5	1a	8.8cd	13.8c	264cd	3.1a	31.2	37.6
Mean[h]	9.5	11.9	11.4	309.9	3	55.6	44.7	14.4	1.2	16.6	20.6	386	2.8	24.2	37.6
LSD (0.05)	1.0	1.7	1.9	7.2	1	-	3.2	-	NS	1.8	1.4	322	0.7	-	NS

[u] Treatment means within columns followed by the same letter are not significantly different at P ≤ 0.05 according to least significant difference (LSD) test;
– = no data.
[v] MRS = mean rust severity (modified Cobb scale); 1st, 2nd, and 3rd disease notes were recorded at growth stages 55, 65, and 77, respectively.
[w] Yield gain (G) (%) = (fungicide-treated – untreated) × 100/treated.
[x] TKW = 1,000-kernel weight; gain (G) (%) = (fungicide-treated – untreated) × 100/treated.
[y] Active components of fungicides are as follows: Swing 250 EC, epoxiconazole + carbendazim; Artea 330 EC, cyproconazole at 80 g/liter + propiconazole at 250 g/liter; Silvacur 375 EC, tebuconazole + tridimenol; Folicur, tebuconazole; Stratego 250 EC, trifloxystrobin + propiconazole; Soprano C 250 EC, epoxiconazole at 125 g/liter + carbendazim at 125 g/liter; Orius 25 EW, tebuconazole; Cotaf 5 E, hexaconazole; and AmistarXtra 280 SC, azoxystrobin at 200 g/liter + cyproconazole at 80 g/liter; Soprano C 250 EC, Orius 25 EW, and Cotaf 5 E are generic chemicals.
[z] Area under the disease progress curve (AUDPC) values are means of four replications.

Table 6. Effect of fungicide treatment on stem rust severity, area under the disease progress curve (AUDPC), grain yield, and 1,000-kernel weight on wheat cv. Duma at Mau-Narok [U]

the pattern of stem rust suppression by fungicide treatments was observed at all locations for the 2 years. The lowest mean disease severity after the second treatment were obtained in plots sprayed with AmistaXtra 280 SC followed by Folicur 250 EC, Orius 25 EW and Silvacur 375 EC.

3.3 Effect of fungicides on grain yield

In 2005, fungicide treatments with reduced stem rust severity yielded significantly ($P \leq 0.05$) more grain at Eldoret and Mau-Narok (Table 5 and 6) whereas only Artea 330 EC, Stratego 250 EC and AmistarXtra 280 SC significantly *(*$P \leq 0.05$) increased grain yield at KARI-Njoro (Table 5). Similarly, in 2006, treatments that had reduced stem rust severity significantly ($P \leq 0.05$) increased grain yield, except for Cotaf 5 E at KARI-Njoro and Eldoret; and Swing 250 EC and Soprano C 250 EC at the latter site. The mean grain yield among the treatments ranged from 1.3 to 2.6 t/ha. Highest yield, 2.6 t/ha, was obtained in plots of AmistarXtra 280 SC, which was 50% higher than the untreated control.

3.4 Effect of fungicides on kernel weight

All fungicide treatments that had significantly ($P \leq 0.05$) higher grain yield also had higher 1,000-kernel weight. In 2005, 1,000-kernel weights from treated plots at all locations were significantly ($P \leq 0.05$) higher than those from untreated control plots. The trend for both grain yield and 1,000-kernel weight in 2006 was similar, except at Eldoret and Mau-Narok (Table 4, 5, and 6).

3.5 Effect of Nativo 300 SC and Prosaro 250 EC on stem rust severity

All the foliar fungicide treatments reduced stem rust on wheat cultivar 'Duma'(data not shown). Fungicide applications significantly ($P \leq 0.05$) reduced mean rust severity (MRS) compared to the untreated control, with Prosaro 250 EC at 1.0 L/ha, Nativo at 0.75 L and 1.0 L/ha and the standards (Folicur 250 EC and AmistarXtra 280 SC), performing better than Prosaro 250 EC at 0.6 L/ha, 0.75 L/ha and Nativo 300 SC at the rate of 0.6 L/ha. The highest disease severity reduction in 2006 was generally observed in plots that were sprayed with Prosaro 250 EC at 1.0 L/ha (59.4%), Nativo 300 SC and AmistaXtra 280 SC at 1.0 L/ha (55.6% each) at KARI-Njoro and Prosaro 250 EC at 0.6 L/ha (75.7%), Prosaro 250 EC at 1.0 L/ha (75.5%) and Nativo 300 SC at 1.0 L/ha (73.5%) at Mau-Narok. In 2007, the highest disease severity reduction was in plots sprayed with Folicur 250 EC at 1.0 L/ha (85.3%), followed by Prosaro 250 EC at 0.75 L/ha (82.6%) and 1.0 L/ha (77.8%) at KARI-Njoro and Prosaro 250 EC at 0.6 L/ha (76.5%), Prosaro 250 EC at 1.0 L/ha (75.5%), and Nativo 300 SC at 1.0 L/ha (73.47%) at Mau-Narok .

The average grain yields and 1,000-kernel weights across the locations varied from one treatment to another, ranging from 1.2 - 4.0 t/ha and 28.5 - 45.4 g, respectively. Significant ($P \leq 0.05$) grain yield increases of 57.3% and 49.7% were obtained at KARI-Njoro and Mau-Narok in 2006, while 54.1% and 44.7% increases occurred in 2007, respectively. Similar increases in 1,000-kernel weight occurred at both sites; KARI-Njoro (24.5% and 25.1%) in 2006, and 10.4% and 23.3% in 2007.

4. Discussion

There is very little information published on the use of fungicide to control wheat stem rust, specifically related to the race TTKSK (Ug99) that is prevalent in the Eastern Africa region.

The occurrence of stem rust and the onset of epidemics differed from year to year and location to location. In this study, three locations, KARI-Njoro, Eldoret, and Mau-Narok, were selected because of their stem rust favourable weather conditions. Generally, these areas have hot days of 23°-30°C, mild night temperatures below 15°C and adequate moisture for night dews. Under such favourable conditions, the growth and spread of the stem rust pathogen can greatly reduce grain yield and 1,000-kernel weight in susceptible cultivars. Stem rust epidemics were high in 2005 and 2006 at KARI-Njoro. The rust development began early due to the warm and moist environmental conditions that prevailed. At Eldoret, rust severity was moderate in 2005 but high in 2006. Even though the Mau-Narok location experienced cooler weather and frequent rainfall during the growth seasons, rust severity was moderate before application of fungicides.

Stem rust caused 32 to 57% of grain yield loss and 17 and 24% of 1,000- kernel weight reduction in the experiments at the three locations in 2005 and 2006, respectively. These results were consistent with losses reported in other studies (Dill-Mackey et al., 1990; Loughman et al., 2005, and Paveley et al., 2003).

Yield losses of 50% due to stem rust infection have been reported in a recent study (Expert Panel , 2005). Previously, Pretorius (1983) had reported that yield losses caused by stem rust ranged from 7 to 35% depending on cultivar. Dill-Mackey et al. (2000) induced severe stem rust epidemics in barley and wheat and observed yield losses of 50 to 58%. Mayfield (1985) found a clear relationship between grain yield and disease severity by demonstrating that prevention of a 1% increase in rust severity saved a 2% loss in grain yield. Different fungicides vary in their efficacy to control stem rust. Loughman et al., 2005 reported that Folicur (tebuconazole) was more effective than Triad (triadimefon) or Impact (flutriafol) in disease reduction and yield increase. In the present study, all fungicide applications resulted in lower disease severity and higher yields than untreated check plots. However, AmistarXtra 280 SC, Folicur 250 EC, Silvarcur 375 EC and Orius 25 EW were more effective than other treatments in reducing disease and increasing yield. The effectiveness of Stratego 250 EC, Cotaf 5 E, Swing 250 EC, Artea 330 EC, and Soprano C 250 EC was inconsistent. Therefore, growers should choose fungicides based on their efficacy.

Stem rust severity was relatively low in 2005 at Eldoret and Mau-Narok than in KARI- Njoro but treatments significantly increased grain yield. The yield increase by fungicide applications under low disease pressure could be due, in part, to phytonic effect of fungicides. This stimulatory effect of fungicide treatments on growth may result in significant yield increases even in the absence of the disease (Wegulo et al., 1998). Fungicide treatments, if applied under high and moderate disease pressure at critical growth stages may reduce large yield losses by suppressing or eliminating stem rust pathogen (Mayfield, 1985). However, greater rust control and greater yield increases may have been possible using higher rates and more applications of fungicides as well as spraying at an earlier stage of the rust epidemic. Under field conditions, a stem rust severity level greater than 5% in 'hot- spot' regions should be controlled to reduce yield losses (Loughman et al., 2005). For this study, there was a sudden explosion of the disease within a few days due to ideal climatic conditions, which contributed to the high stem rust severities recorded at the KARI-Njoro location before the first fungicide application. This provided ideal conditions for assessing efficacies of the fungicides.

For Nativo 300 SC and Prosaro 250 EC, stem rust severity was relatively low in 2007 in the trials at KARI-Njoro and Mau-Narok compared to 2006, but there was grain yield increase in response to fungicide applications. A similar yield increase under relatively low disease

pressure was recorded during the assessment of commercial foliar fungicides. Therefore, the two new fungicides were recommended for commercial use in the control of stem rust in Kenya.

The impact of fungicide application in the management of stem rust was well illustrated at the three locations. Significant differences were found among fungicides in their ability to suppress disease development and protect crop canopy, which is vital for dry matter accumulation and yield (Viljanen-Rollinson et al., 2006). The efficacy differences among the tested fungicides were probably related to their fungicidal activity. The two studies demonstrated that fungicide treatments applied under high and moderate disease pressure at critical crop growth stages increase grain yield of susceptible varieties by suppressing or eliminating the negative effects of the rust. The adoption of effective foliar fungicides to combat stem rust pathogen as a short term control strategy until resistant varieties are developed should be encouraged. However, more research is required to identify the precise timing of fungicide sprays and doses.

5. The future

The demand for new innovative chemicals will continue to be strong, but these alone will not be the answer to reduced crop losses. On-going studies show that amongst all the management strategies, slow rusting wheat varieties are providing the most effective, long-term and cost effective control over stem rust (Singh et al., 2006). Fungicides can be a valuable tool in increasing yields and profitability of wheat production, especially if disease susceptible varieties are grown and, where the disease pressure is only moderate. But combining fungicides with host resistance offers the best monetary return when disease pressure is high (Ransom et al., 2008). This should be linked to accurate disease forecasting and timely application of chemicals (Sbragia, 1975). While the impetus on breeding high yielding varieties will continue, the emphasis on disease resistance will even be higher. Appropriate management of the few effective resistance genes remaining through deployment and pyramiding will require greater attention to prevent their rapid breakdown.

6. Acknowledgment

The authors thank the agrochemical companies and Borlaug Global Rust Initiative (BGRI) for partial funding of the studies; and our colleagues in the pathology section, specifically D. Onyango and P. Kinyanjui, for their tireless support during the implementation of the experiments.

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7

Fungicides Application against Fusarium Head Blight in Wheat and Barley for Ensuring Food Safety

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1. Introduction

Fusarium head blight (FHB), or scab, caused by several *Fusarium* species, especially *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch], is a widespread and destructive disease of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and other small-grain cereals (Liddell 2003; Parry et al. 1995; Pirgozilev et al. 2003). These pathogens infect spikes and reduce grain yield and quality. Moreover, *Fusarium* species that cause FHB produce trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV), which are toxic to humans and other animals. The Joint WHO/FAO Expert Committee on Food Additives evaluated the risk of DON and set a provisional maximum tolerable daily intake of DON as 1 μg/kg body weight in 2001. Accordingly, the Japanese government determined provisional guidelines for DON content in unpolished wheat grains as 1.1 mg kg^{-1} in 2002. Many other countries have also established maximum allowed levels for DON in cereals and cereal products.

There is currently no robust single control measure by which to manage either FHB or mycotoxin contamination in barley and wheat. Fungicide application is one measure available to reduce the risk; however, results have not been highly effective or consistent (Horsley et al. 2006; Jones 2000; McMullen et al. 1997; Mesterhazy 2003). To obtain increased chemical control of FHB, the timing of fungicide application is an important factor, as well as fungicide selection, application rate, and good coverage of the spike (Mesterhazy 2003).

This chapter introduces FHB disease and its related mycotoxins, and summarizes our research on the chemical control of mycotoxin contamination.

2. Fusarium head blight of cereals

Symptoms caused by the fungus in wheat include premature bleaching of the spikelets or entire spike (Fig. 1a). These white heads are very conspicuous in a green field. Frequently, only part of the head is affected by FHB. These partly white and partly green heads are diagnostic. Additional indications of FHB infection are pink to salmon-orange spore masses of the fungus often seen on the infected spikelet and glumes during prolonged wet weather (Fig. 1a). Bleached spikelets usually are sterile or contain shriveled and/or discolored seeds

(Fig.1c). These kernels are sometimes called "tombstones" because of their chalky, lifeless appearance. Other Fusarium-infected kernels may be more normal in size, if infection occurred late in kernel development. In barley, FHB infections are not always readily apparent in the field. Infected spikelets may show a browning or water-soaked appearance (Fig. 2a). Infected barley kernels show a brown discoloration similar to that caused by other kernel blighting organisms (Fig. 2c).

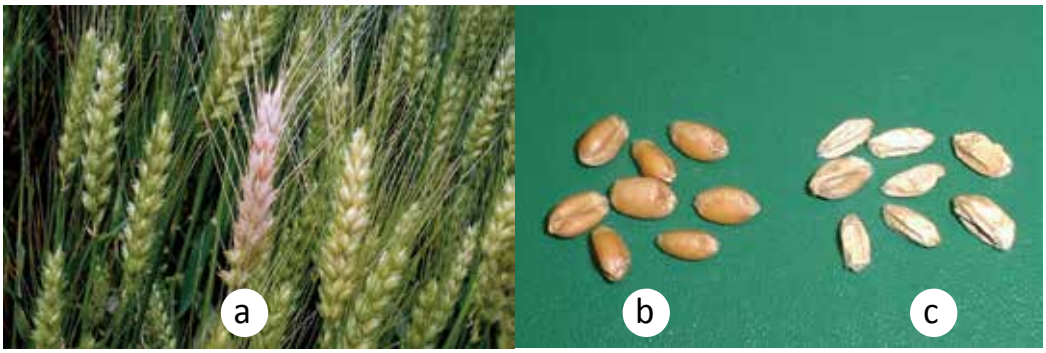
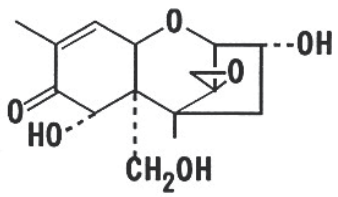


Fig. 1. Symptom of Fusarium head blight (a), healthy seeds (b) and diseased seeds (c) in wheat.

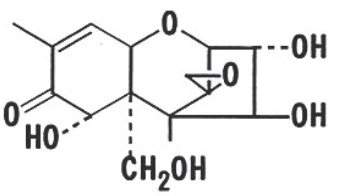
Fig. 2. Symptom of Fusarium head blight (a), healthy seeds (b) and diseased seeds (c) in barley.

3. Toxicity of mycotoxins

Trichothecene mycotoxins produced by FHB pathogens, especially DON and NIV (Fig. 3) possess common biochemical and cellular toxicities. These have a strong inhibitory effect on protein synthesis by binding to the ribosomes, an inhibitory effect on RNA and DNA synthesis, and toxic effects on cell membranes (Sugita-Konishi & Kumagai, 2005). Furthermore, their capacity to inhibit protein synthesis may induce apoptosis in thymus, lymphatic, and hematopoietic tissue via mitogen-activated protein kinase. Crops contaminated with trichothecene may result in serious food poisoning accompanied by nausea, vomiting, and diarrhea. Immunotoxicity, which is a chronic effect of trichothecene mycotoxins, decreases host resistance. Selective upregulation of serum IgA caused by dietary exposure to DON or NIV induces IgA nephropathy. Its cancer-promoting effects seem to be responsible for the immunotoxicity.



Deoxynivalenol (DON)



Nivalenol (NIV)

Fig. 3. Chemical structure of deoxynivalenol (DON) and nivalenol (NIV)

The toxicity of mycotoxins can be compared to that of agricultural fungicides for FHB control using acceptable daily intake (ADI) and provisional tolerable dietary intake (PTDI); both are estimates of the amount of a substance in a food or crop that can be ingested daily over a lifetime without appreciable health risk. The ADI of four main fungicides for FHB control are much higher than the PTDI of DON or NIV, indicating that the risk of mycotoxins are much higher than that of fungicides. Therefore, appropriate application of fungicides for FHB control is better for ensuring food safety. This is good example for explanation of benefit of fungicides.

Fungicide	**Acceptable daily intake** (μg/kg bw/day)	**Maximum residue limit in wheat** (mg/kg)
Thiophanate-methyl	120 a)	0.6 a)
Tebuconazole	29 a)	0.5 a)
Propiconazole	18 a)	1.0 a)
Metconazole	40 a)	0.2 a)
Mycotoxin	**Provisional tolerable dietary intake** (μg/kg bw/day)	**Provisional standard in wheat** (mg/kg)
Deoxynivalenol	1.0 b)	1.1 a)
Nivalenol	0.7 c)	N.D.

Table 1. Comparison of toxicity between Fusarium head blight (FHB) mycotoxins and fungicides for FHB control, as a) determined by the Japanese government, b) evaluated by Joint FAO/WHO Expert Committee on Food Additives, and c) evaluated by European Food Safety Authority.

4. Screening of fungicides for the reduction of mycotoxins

Since the provisional standard of 1.1 ppm for DON in wheat was set by the Japanese government in 2002, the endpoint of our research had to be changed from disease severity to mycotoxin contamination. Therefore, a re-evaluation of registered fungicides and screening of new candidates to control mycotoxin contamination became mandatory. We tested 24 fungicides with different modes of action. Three experiments were conducted for 2 years (Nakajima, 2004).

In a paddy field, we sprayed fungicides 2 days before flowering and 5 days after flowering. Inoculations of *F. graminearum* were conducted at flowering and 7 days after flowering. In 2002, we used DON producer, and in 2003, we used a mixture of DON and an NIV

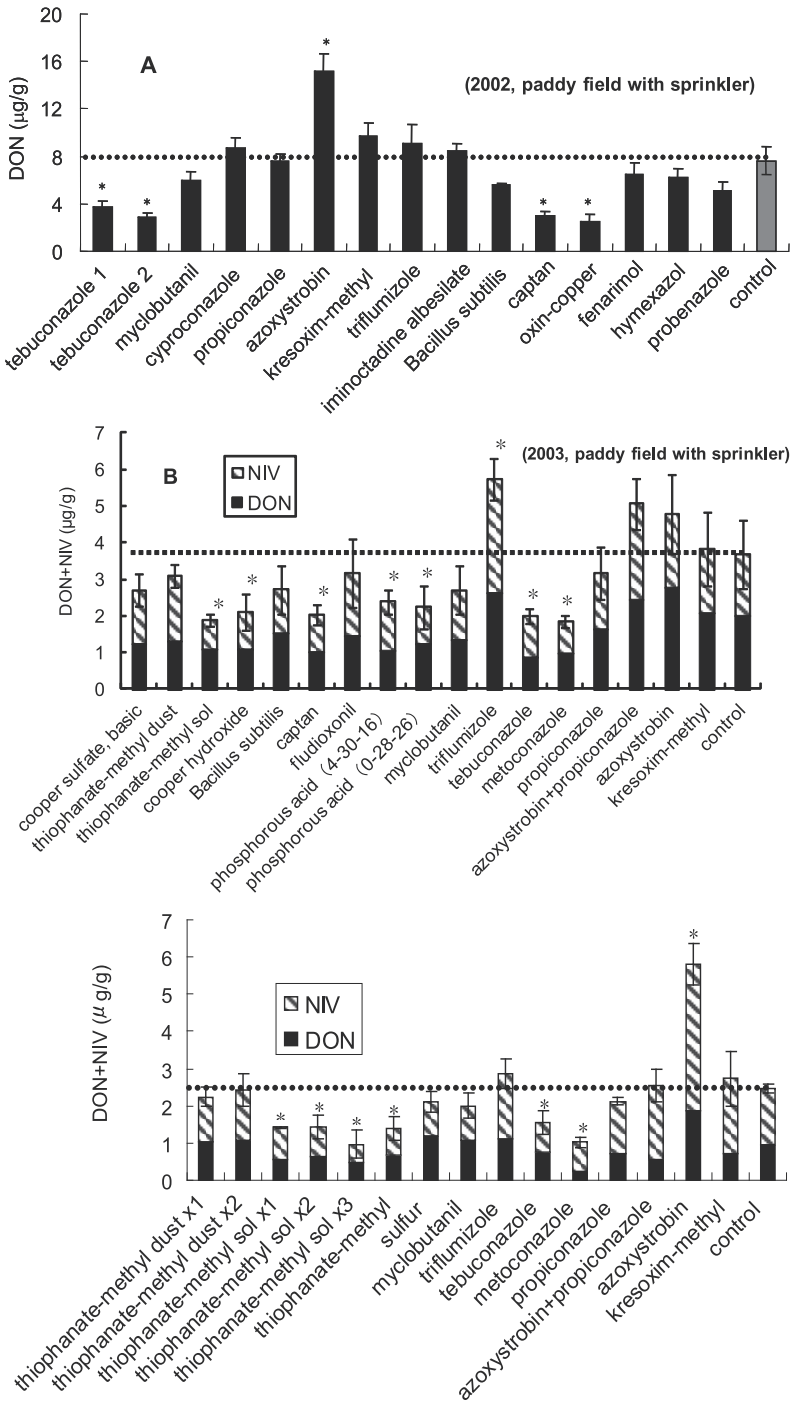


Fig. 4. Effects of fungicides on the level of deoxynivalenol (DON) and nivalenol (NIV) (Nakajima, 2004). * significantly different from control plot at $p < 0.05$. Bars indicate standard error.

producer. A sprinkler was used to promote disease development. In addition, an upland field experiment was performed in 2003. Corn grain inoculum of a mixture of DON and an NIV producer were used under natural rainfall conditions.

Most of the fungicides controlled FHB disease severity. In particular, tebuconazole, captan, and oxin-copper were highly effective. Azoxystrobin was not so effective but its efficacy was about 40%. In general, the fungicides were less efficacious against DON than against disease severity. However, tebuconazole, captan, and oxin-copper decreased the DON level significantly compared to the control plot (Fig. 4-A). In contrast, azoxystrobin increased the DON level significantly. Other fungicides did not affect the DON level.

In the paddy field, most fungicides except trifulumizole were highly effective in 2003. The reason for the failure of trifulumizole was unknown. Control of DON + NIV was more difficult than control of disease severity. In 2003, two applications were insufficient to decrease mycotoxin levels. Thiophanate-methyl sol, cooper hydroxide, captan, and two-kinds of phosphorous acid, tebuconazole and metoconazole decreased significantly DON+NIV level than control plot (Fig.4-B). Trifulumizole was not effective for disease or toxin control. Azoxystrobin and a mixture of azoxystrobin and propiconazole were effective for disease control but not as effective against mycotoxins.

We inoculated a corn grain inoculum with a DON + NIV mixture in 2003 in an upland field to simulate a natural infection. In this case, most of the fungicides were highly effective. Two applications was enough to control disease severity. However, control of DON + NIV was more difficult. The efficacy of toxin control was lower than that in the paddy field in which spore inoculation was performed (Fig. 4-C). Corn inoculum probably supplies conidiospores continually during the maturation period. Therefore, a nonvisible infection might increase mycotoxin levels. Thiophanate-methyl sol significantly decreased the DON + NIV level compared to thiophanate-methyl dust. Tebuconazole and metoconazole decreased the DON+NIV level significantly compared to the control plot. In contrast, azoxystrobin increased the DON + NIV level, especially the NIV level, significantly. In this case, a mixture of propiconazole and azoxystrobin did not increase or decrease mycotoxin levels. Interestingly, the mode of action of kresoxim-methyl was similar to that of azoxystrobin but its effect on mycotoxin levels seemed to be different.

Several studies have reported that some fungicides stimulate DON accumulation in wheat grains infected by FHB fungi (Magan et al., 2002; Simpson et al., 2001). Simpson et al. (2001) suggested that eliminating the competition between DON-producing *Fusarium* species and *M. nivale* by selectively controlling *M. nivale* increases the proportion of DON producers in the host plant. In our tests, however, *M. nivale* was not responsible for FHB. Ramirez et al. (2004) also reported a direct effect of fungicides on DON production by *F. graminearum* when cultured in vitro with viable wheat grains. Five fungicides stimulated DON production under certain water activities, temperatures, fungicide concentrations, and durations of incubation (Ramirez et al., 2004). Microarray analysis revealed that trichothecene biosynthesis genes were highly expressed in *F. culmorum* grown under optimal conditions and under mild temperature and water stress (Schmidt-Heydt et al., 2008). Thus, suboptimal growth conditions generated by a particular fungicide application and environmental stress enhanced DON production by *Fusarium* species.

Therefore, to screen fungicides, we must consider the effects they have on DON or NIV levels. These results suggest that a new fungicide evaluation system based on efficacy for mycotoxin contamination should be introduced.

5. Critical control point for the control of the mycotoxins in wheat

In our field evaluation of fungicides, the efficacy for DON and NIV control was consistently lower than the efficacy for FHB severity (Nakajima, 2004). Based on this finding, we hypothesized that the critical control point of DON and NIV might be different than that of FHB severity. The general recommendation for fungicide application timing is the beginning of flowering, because plants are most susceptible to infection at this stage. An additional application at 7–10 days after the first application is recommended in Japan. The frequency of fungicide application is also crucial for mycotoxin reduction.

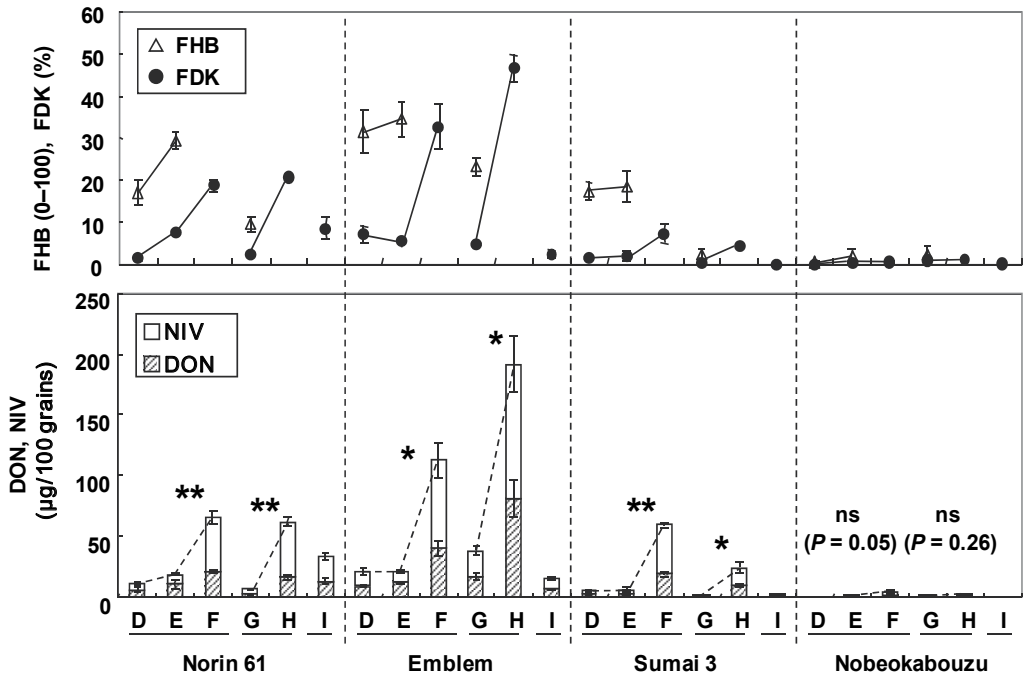


Fig. 5. Deoxynivalenol (DON) and nivalenol (NIV) concentrations in grains, Fusarium head blight (FHB) severity, and the percentage of Fusarium-damaged kernels (FDK) during host development after different timings of inoculation for the four cultivars in the greenhouse experiments in 2004 (Yoshida & Nakajima, 2010). In treatments (Trts) D, E, and F, plants were inoculated at anthesis and sampled 10 days after anthesis (DAA), 20 DAA, and maturity (38 to 40 DAA), respectively. In Trts G and H, plants were inoculated at 10 DAA and sampled at 20 DAA and at maturity, respectively. In Trt I, plants were inoculated at 20 DAA and sampled at maturity.

To develop effective timing for controlling FHB in wheat, particularly for reducing the risk of toxin contamination, it is necessary to elucidate the manner in which toxin accumulation occurs in wheat grain.

Four cultivars were tested in a greenhouse environment (Yoshida & Nakajima, 2010), where the plants were spray-inoculated at three different stages with a mixture of *F. graminearum* DON and NIV chemotypes. To determine the actual manner in which the toxin increases in the developing grain, the toxin content of grains after inoculation of each cultivar was

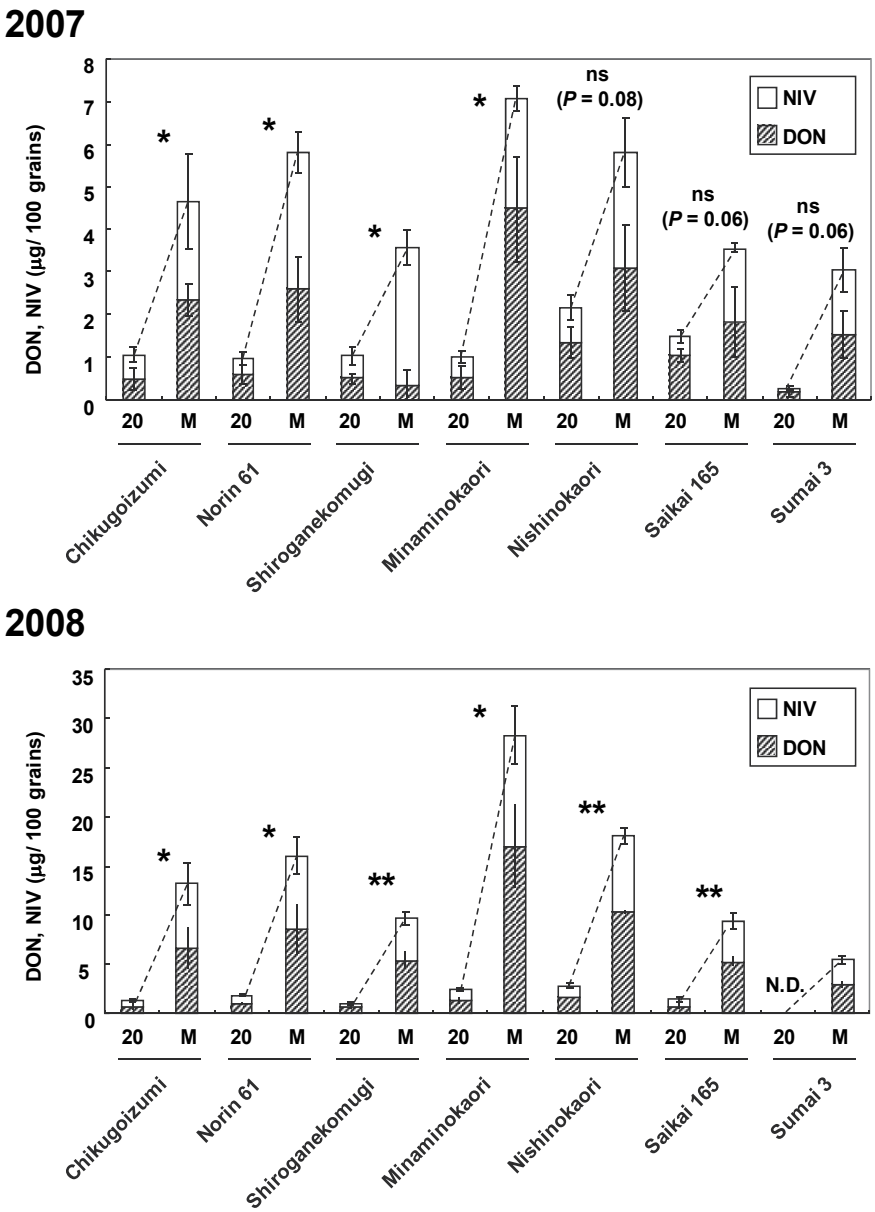


Fig. 6. Deoxynivalenol (DON) and nivalenol (NIV) concentration in grains at two developmental stages for the seven cultivars in the field experiments in 2007 and 2008 (Yoshida & Nakajima, 2010). Signs below the graph (20 and M) indicate the timing when the samples were harvested, 20 days after anthesis (DAA) and maturity (40 DAA), respectively. Each data point represents the mean of three replications. Bars represent standard errors. N.D., not detected; * and ** indicate $P < 0.05$ and 0.01, respectively, for differences in toxin (DON + NIV) concentration in grains (micrograms per 100 grains) between the two harvests (i.e., 20 DAA and at maturity) (one-tailed *t*-test); ns, difference not significant.

measured on a grain number basis (μg/100 grains) instead of a grain weight basis (e.g., μg g^{-1}, ppm, etc.), considering the influence of grain-weight increase during development. The results indicated that high levels of DON and NIV were produced beyond 20 days after anthesis (DAA), even following early infection (Fig. 5). The results of field experiments performed on seven cultivars, in which the inoculation was conducted using colonized maize kernel inoculum, were consistent with the greenhouse results (Fig. 6). Furthermore, in the greenhouse experiments, late infection, at least as late as 20 DAA, caused grain contamination with these toxins, even without clear disease symptoms on the spike (Fig. 5).

Strategies for controlling FHB and toxin contamination have been developed, mostly focusing on infection during the host flowering stage. However, our results indicate the importance of the late stages of grain development for toxin contamination in addition to the early stage, showing that the amount of DON and NIV largely increases after 20 DAA (late milk stage), even with infection at earlier stages, and that infection at late stages, at least as late as 20 DAA, can cause non-negligible levels of contamination, even without clear FHB symptoms. Thus, control strategies should be established covering the late stage as well as the time around the flowering stage to effectively reduce the risk of DON and NIV contamination.

Application of fungicides or other control agents at the late stage may be an effective measure for reducing the final level of toxin accumulation. Agents used for such 'late control' are expected to possess effects not only to prevent primary infection at late stages but also to prevent toxin production in late stages by previous fungal infection. Studies to test the effectiveness of such 'late control' for reducing toxin contamination are needed. The use of resistant cultivars with a lower risk of toxin contamination throughout the developmental period is also important. To properly assess the total risk of toxin contamination in wheat cultivars, inoculation tests that cover the late as well as the early stages are required. Among the tested cultivars in this study, 'Nobeokabouzu', which is a FHB-resistant cultivar widely used in FHB-resistance breeding, as well as 'Sumai 3' (Rudd et al., 2001; Shi et al., 2008) showed the highest resistance to mycotoxin accumulation for all timings of inoculation (Fig. 5). 'Nobeokabouzu' seems to possess consistent resistance to mycotoxin accumulation as well as to FHB during grain development. Such a cultivar may be a useful resistance source, especially for introducing resistance to mycotoxin accumulation during all grain development stages.

6. Extrusion of the spent anthers in the cleistogamous barley cultivar

Anthesis is considered the optimal growth stage for fungicide application to control FHB in wheat (Mesterhazy 2003; Paul et al. 2007). This is reasonable because wheat is most susceptible to FHB during anthesis (Atanasoff, 1920; Parry et al., 1995; Pugh et al., 1933; Sutton 1982), at which time anthers extrude from the florets. An initial *Fusarium* infection commonly occurs on extruded anthers (Pugh et al. 1933). In most studies on the chemical control of FHB in barley, fungicides were applied at or near anthesis (Havlova et al. 2006; Ioos et al. 2005; Jones 2000; Vanova et al. 2007) similar to the case of wheat, or at full head emergence (Havlova et al. 2006; Jones 2000), which may or may not be concurrent with the time of anthesis. Although barley usually undergoes anthesis after heading similar to wheat, in some barley-growing regions anthesis occurs while the spike is still enclosed within the flag leaf sheath (McCallum and Tekauz 2002; Steffenson 2003). In such cases, the time of full head emergence should be after anthesis is completed. In Japan, where anthesis in barley

usually occurs at or a few days after full heading, fungicide application for FHB has been performed around anthesis regardless of cultivar. However, the influence of fungicide application timing on reducing FHB and mycotoxin accumulation in barley has not been investigated to date.

While wheat is generally chasmogamous (open-flowering type) and extrudes anthers at anthesis, barley has two flowering types: chasmogamous and cleistogamous (closed-flowering type). In cleistogamous cultivars, the florets do not open and anthers are not extruded at anthesis (Fig.7). However, the anthers may be pushed out from the tips of florets by the developing grain several days after anthesis (Fig.7). In Japan, this phenomenon is observed commonly in cleistogamous cultivars. Thus, it can be said that the timing of anther extrusion differs with flowering type in barley.

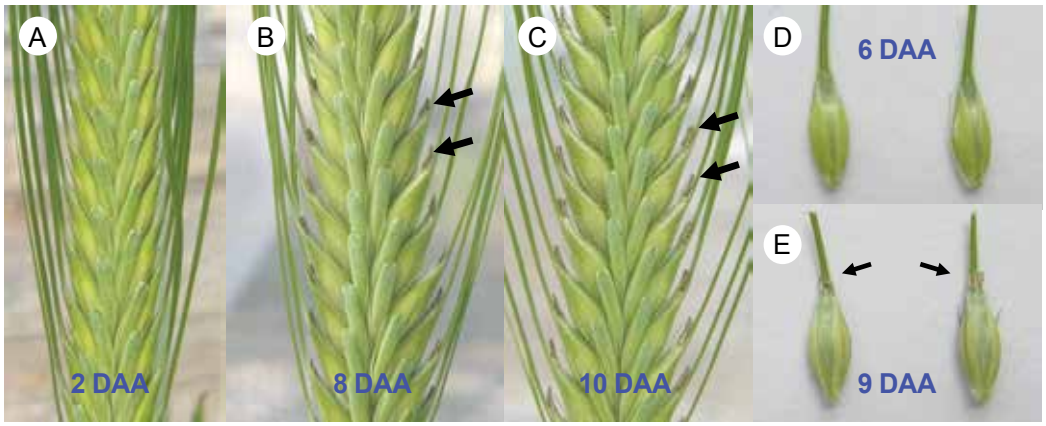


Fig. 7. Extrusion of the spent anthers in the cleistogamous barley cultivar 'Nishinochikara' (Yoshida et al., 2007). A–C, Spikes (A) 2 days after anthesis (DAA), (B) 8 DAA, and (C) 10 DAA. D and E, Appearance of the ventral side of florets (D) 6 DAA and (E) 9 DAA. Anthers were still contained within the closed florets at 6 DAA; most anthers were partially extruded at 8 DAA; most anthers were completely extruded at 10 DAA. Arrows indicate the extruded spent anthers.

In Japanese barley cultivars, there is a strong association between row type and flowering type; most two-rowed cultivars are cleistogamous, whereas most six-rowed cultivars are chasmogamous. In most cases, the former are relatively resistant and the latter are susceptible to FHB (Yoshida et al., 2005), consistent with the general observation that two-rowed types are more resistant than six-rowed types (Choo et al., 2004; Heta & Hiura, 1963; McCallum, et al., 2004; Steffenson, 2003; Takeda & Heta, 1989 & Zhou et al., 1991). Cleistogamy contributes to the resistance of barley cultivars to FHB, at least for infection that occurs at anthesis (Yoshida et al., 2005). Even in the two-rowed cleistogamous cultivars with their relative resistance, however, high levels of DON and NIV contamination can occur (Yoshida et al., 2007).

In the cleistogamous cultivars, anthers did not protrude at anthesis; however, as the grain developed after pollination, the spent anthers were extruded from the tip of the floret between the tip of the palea and lemma (Fig. 7). Spent anther extrusion began around 7 DAA and was completed by 10 DAA.

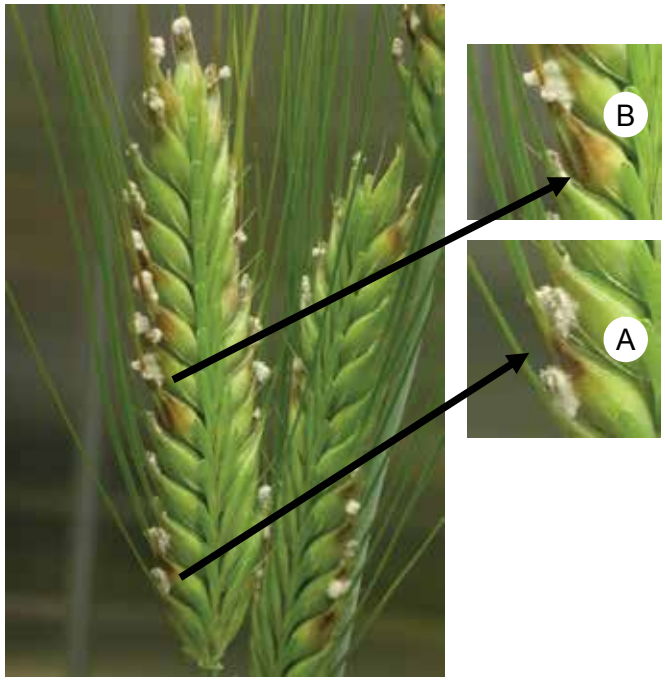


Fig. 8. Initial symptoms of Fusarium head blight (FHB) appeared in two-rowed closed-flowering cultivars inoculated at the timing of spent anther extrusion. (A) Fungal colonization of the spent anthers and initial symptoms appeared around the tips of florets. (B) The discoloration progressed toward the bases of the florets.

7. Effect of infection timing on Fusarium head blight and mycotoxin accumulation in barley

Thirteen barley cultivars (nine two-rowed and four six-rowed cultivars shown in Fig. 9) were evaluated for FHB resistance at anthesis and 10 DAA. Among the cultivars, all six-rowed cultivars are chasmogamous and the two-rowed varieties are cleistogamous, except for the chasmogamous two-rowed cultivar Satsuki Nijo. The cultivars were planted in pots (four plants per pot) and grown in a greenhouse. Japanese isolates of *F. graminearum* Schwabe (DON chemotype) was used as the inoculum. The spikes were spray inoculated at the respective stages and were nursed overnight in a greenhouse at 18–25°C and 95–100% humidity. After inoculation, the plants were placed in the greenhouse (18–25°C) equipped with a sprinkler system that intermittently produces a fine mist to keep the spikes wet during the test. FHB severity was assessed 2 weeks after inoculation on a scale of 0 to 100 (0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100), according to a visual rating of the percentage of infected florets.

The results showed that infection timing differentially influenced FHB severity among cultivars (Fig. 9). The two-rowed, cleistogamous cultivars were severely diseased by 10 DAA inoculation, whereas they showed good resistance to infection at anthesis. The drastic decline in resistance during the period seemed to be related to the extrusion of old anthers, which occurred around 10 DAA in the cleistogamous cultivars.

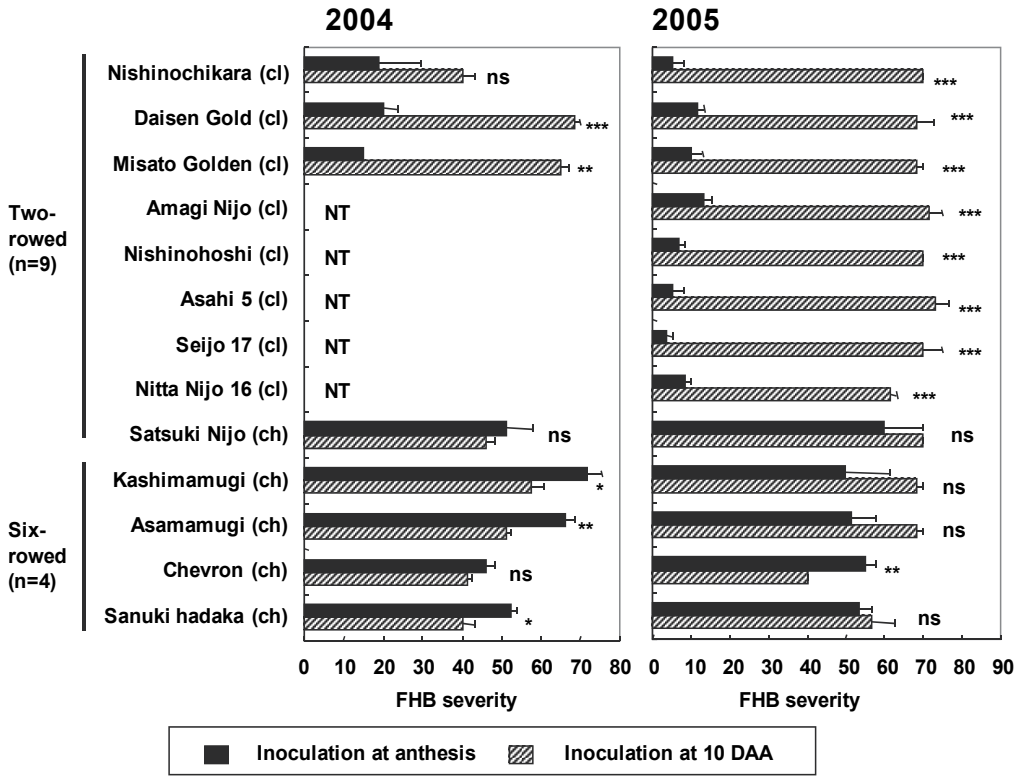


Fig. 9. Fusarium head blight (FHB) severity (0–100 scale) for 13 cultivars inoculated with *Fusarium graminearum* at anthesis and 10 days after anthesis (DAA) in 2004 and 2005 (Yoshida et. al, 2007). Bars represent standard errors. *P < 0.05, **P < 0.01, *** P < 0.001 for differences between the two inoculation times (t-test). Ns, the difference was not significant; NT, not tested; cl, cleistogamous cultivar; ch, chasmogamous cultivar.

Five barley cultivars (three two-rowed and two six-rowed cultivars shown in Fig. 10) were tested. The cultivars were planted in pots (five to six plants per pot) and at the heading stage, late tillers of each plant were removed, thereby allowing 7–12 spikes that appeared to be at the same stage to grow per pot. A mixture of two Japanese isolates of *F. graminearum* Schwabe differing in trichothecene chemotype (DON chemotype and NIV chemotype) was used as the inoculum. Spray inoculation was conducted at anthesis (= 0 DAA), 10 DAA, and 20 DAA. The inoculated plants were kept wet for 6 days after inoculation in a greenhouse equipped with a sprinkler system. Then the plants were returned to the greenhouse in which they were grown. FHB severity was visually assessed 20 DAA. Sampling was conducted at maturity (33 to 35 DAA), and the grain samples were analyzed for DON and NIV content using ELISA (Yoshizawa et al. 2004).

In this experiment, the two-rowed, cleistogamous cultivars accumulated much more mycotoxins when inoculated 10 DAA or 20 DAA than when inoculated at anthesis, whereas the six-rowed, chasmogamous cultivars accumulated mycotoxins the most when inoculated at anthesis (Fig. 10). Based on these results, we concluded that the most critical timing of infection for FHB and mycotoxin accumulation in barley differs with cultivar, probably associated with flowering type. This was probably because the extruded spent anthers

provide an initial base for the colonization of *F. graminearum* in cleistogamous cultivars (Fig. 8). These results suggest that the optimal timing of chemical control may depend on cultivar, although fungicide application to control FHB has been performed uniformly around anthesis, which is several days after anthesis in Japan.

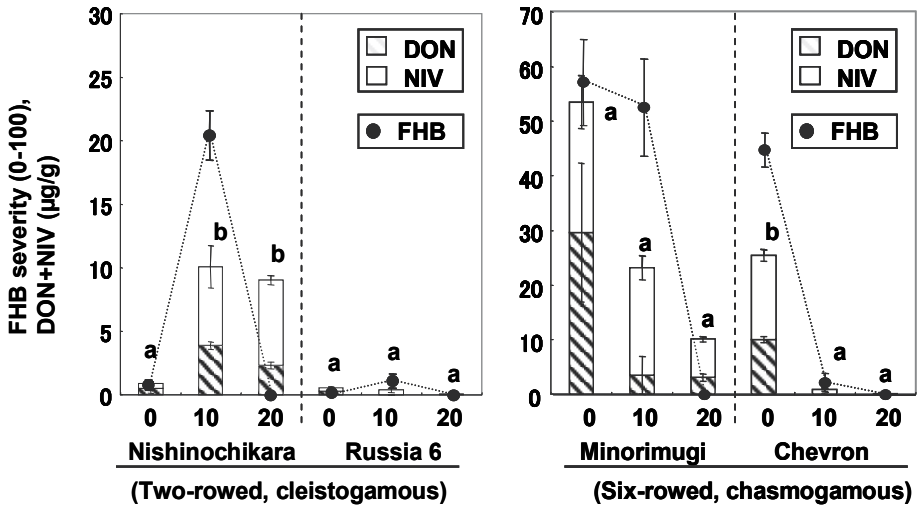


Fig. 10. Fusarium head blight (FHB) severity caused 20 days after anthesis (DAA) and mycotoxin concentration in five barley cultivars and different inoculation times in 2004 (Yoshida et. al 2007). Numbers below the graph indicate the timing of inoculation in DAA. Each point represents the mean of three replicates; bars represent standard errors. Within cultivars, mycotoxin concentrations (the total of deoxynivalenol [DON] plus nivalenol [NIV]) with the same letter were not significantly different at P = 0.05 (Tukey-Kramer multiple comparison).

8. Effect of the timing of fungicide application in barley

The two-row cleistogamous cultivar 'Nishinochikara' was used for the field trials in 2005 and 2006. The timings of fungicide application (see Table 2) were represented by codes from I to VI, which were arranged by the authors. A wettable powder of thiophanate-methyl (1050 g ai/ha) was used as the fungicide treatment. Treatments (different timings of fungicide application and no-fungicide control) were assigned to 4-m single-row plots that were arranged at 0.5-m intervals in a randomized complete block design in the field consisted of 12 50-m-long rows spaced 0.8 m apart. Adjacent rows were left untreated as border rows. Inoculation of *F. graminearum* was performed using colonized maize kernels inoculum (Champbell and Lipps 1998, Dill-Macky 2003), which generate ascospores over a period of testing season in the field. Mist irrigation for promoting ascospores production and the fungal infection was performed in the field. Disease assessments were done at 22 and 24 DAA in 2005 and 2006, respectively. After the harvest, the percentage of discolored kernels (DK), which showed tan to dark brown discoloration on more than 5 % of the grain surface, thousand kernel weight (TKW, g), DON and NIV content were also measured for each grain sample.

Code	Plant growth stage description	ZGS[e]	2005		2006	
			DAA	Date	DAA	Date
I	Before anthesis	56–57	–3	15-Apr	–2	16-Apr
II	Anthesis	(59,) 64–65	0	18-Apr	0	18-Apr
III-1	4–5 DAA[a]	71	4	22-Apr	5	23-Apr
III-2	9 DAA, before spent anther extrusion		9	27-Apr	9	27-Apr
IV-1	Beginning of spent anther extrusion[b]	73	11	29-Apr	12	30-Apr
IV-2	Spent anther extrusion half-way[c]		–	–	14	2-May
IV-3	Spent anther extrusion complete[d]	75	15	3-May	16	4-May
V	Late milk	77	21	9-May	21	9-May
VI	Soft dough	85	29	17-May	30	18-May

Table 2. Timing of fungicide application for closed-flowering barley (cv. 'Nishinochikara') grown in the test field in Koshi Japan in 2005 and 2006.

[a] Days after anthesis.

[b] Spent anther tips emerged in 50% of spikes.

[c] Spent anthers fully extruded in 50% of spikes.

[d] Spent anthers fully extruded in > 90% of spikes.

[e] Zadoks growth stage (ZGS) corresponded to each timing. ZGS 56–57: 3/4 of the inflorescence emerged in > 50% of spikes; ZGS 59: emergence of inflorescence completed; ZGS 64–65: anthesis half-way in > 50% of spikes; ZGS 71: caryopsis water ripe; ZGS 73: early milk; ZGS 75: medium milk; ZGS 77: late milk; ZGS 85: soft dough.

The disease level of the no-fungicide control plots was moderately severe in 2005 (FHB incidence: 98.0%, FHB severity: 13.7), whereas it was slight in 2006 (FHB incidence: 59.5%, FHB severity: 3.5). The mycotoxin content in grains from the control plots was also higher in 2005 (DON + NIV: 25.0 μg/g) than in 2006 (DON + NIV: 6.1 μg/g). The DK percentage, however, was higher in 2006 (11.1%) than in 2005 (8.7%). In both years, the effect of fungicide timing was significant (ANOVA; $P < 0.05$) for all disease and post-harvest parameters evaluated except TKW ($P = 0.10$ in 2005 and $P = 0.16$ in 2006). To assess the effects of fungicide application timing, we adopted the inoculation method using colonized maize kernels and sprinkler irrigation. It would be difficult to assess the effects of fungicide application timing with experiments using spray inoculation or under natural infection conditions, because with such methods, the timing of spray inoculation or natural precipitation would affect the results. The inoculation method used in this study, which provides periodic moist conditions and generates *F. graminearum* spores throughout the testing season, was most appropriate for the purposes of this study.

In both test years, fungicide application around the beginning of spent anther extrusion resulted in the best efficacy for controlling FHB and mycotoxin content in a cleistogamous barley cultivar (Fig. 10). In addition, it is noteworthy that fungicide application at later stages (21 and 29–30 DAA, ZGS 77 and 85, respectively) resulted in significantly lower DON and NIV content compared to the no-fungicide control, although it did not significantly affect disease levels. These results suggest that the optimal time for chemical control of FHB and mycotoxin contamination in cleistogamous barley is around the beginning of spent anther extrusion, rather than at anthesis. In addition, fungicide application as late as 30 DAA may also be effective for controlling mycotoxin accumulation in grain.

Although the effectiveness of late-stage fungicide application for controlling mycotoxins without controlling disease was indicated, such late application can lead to residues in the harvested grain. Thus, for commercial barley production, the use of some fungicides at late stages may be restricted. Nevertheless, our results demonstrate the potential of the late timing of treatment for controlling mycotoxin contamination in barley.

The type of fungicide may affect the results of application. We used Topsin M (thiophanate-methyl, a benzimidazole), which is a broad-spectrum systemic fungicide with both preventive and curative properties. Thiophanate-methyl is converted to methyl benzimidazole carbamate (carbendazim), and this compound interferes with nuclear division in sensitive fungi. It is likely that the systemic and/or curative properties of Topsin M are critical to effectively control mycotoxin accumulation in grain, especially when

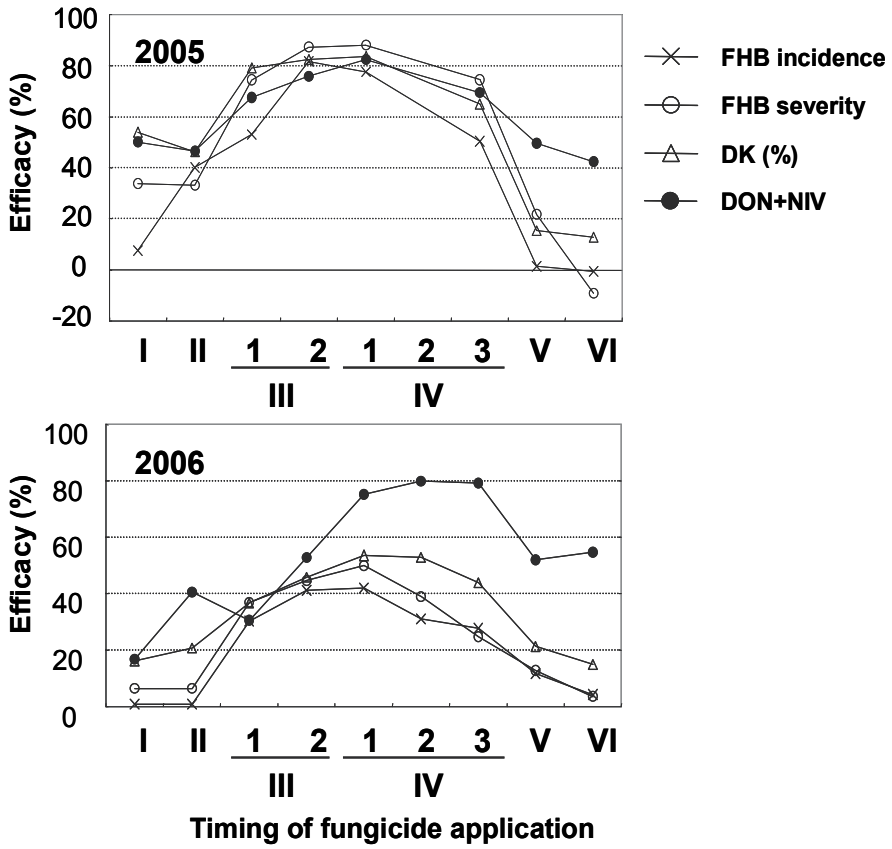


Fig. 11. Control efficacy for *Fusarium* head blight (FHB) incidence, FHB severity, percentage of discolored kernels (DK), and concentration of deoxynivalenol (DON) plus nivalenol (NIV) at various times of thiophanate-methyl fungicide application in each study year (Yoshida et al., 2008). The timing of fungicide application is indicated in the stage codes described in Table 1 (i.e., I: before anthesis; II: anthesis; III-1: 4–5 days after anthesis [DAA]; III-2: 9 DAA; before spent anther extrusion; IV-1: beginning of spent anther extrusion; IV-2: spent anther extrusion half-way; IV-3: spent anther extrusion complete; V: late milk; VI: soft dough).

applied at later stages. Further studies are needed to evaluate the efficacy of other fungicides with different properties or different modes of action in applications at the beginning of spent anther extrusion and at later stages in cleistogamous barley.
In conclusion, spent anther extrusion has not been given adequate attention thus far and is poorly documented worldwide. Not only the timing, but also the degree of extrusion may differ among cultivars or environments. It would be helpful, not only in Japan but also in other barley-growing regions, to observe spent anther extrusion in cleistogamous barley cultivars to understand the cultivar's response to FHB and to determine the timing of chemical control against FHB and mycotoxin contamination. Figure 12 shows the information transfer sheet for fungicide application timing by regional extension center. Our findings have already been confirmed by field trials by some prefectural researchers and have been distributed to farmers through the extension center.

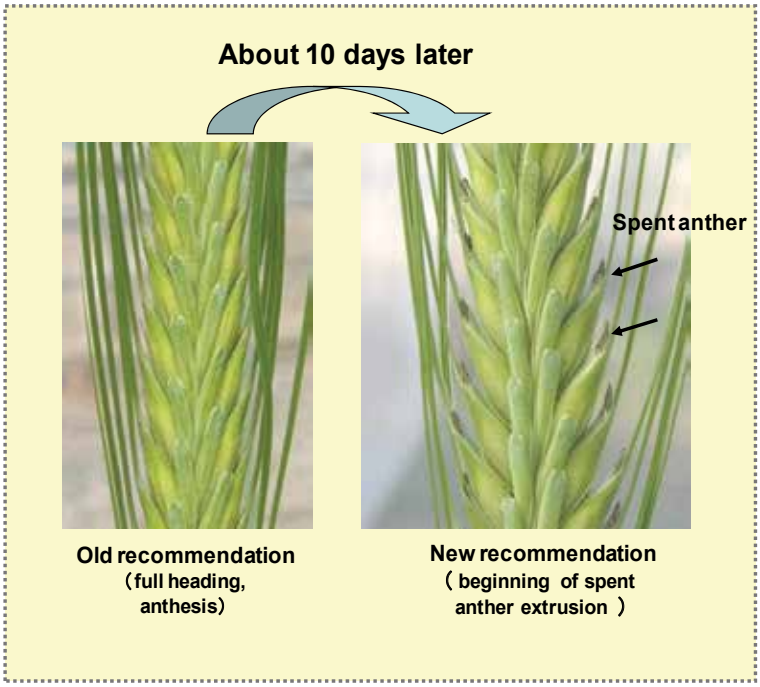


Fig. 12. Information transfer sheet prepared by the regional extension center for fungicide application timing to control FHB in closed-flowering barley.

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8

Advances of Fungicide Application for Winter Oilseed Rape

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1. Introduction

The area of oilseed rape has increased dramatically during the last twelve years in Latvia: from 400 ha in 1997 to 93 900 ha in 2009. This is the reason why development of diseases has become one of the most important risk factors for oilseed rape cultivation under intensive management in Latvia.

White stem rot (Sclerotinia stem rot) caused by *Sclerotinia sclerotiorum*, **Phoma stem canker** (blackleg, stem rot, Phoma leaf spot) caused by *Leptosphaeria* spp., and **Alternaria spot** caused by *Alternaria* spp. are the most important diseases of rape all over the world, including the Baltic region and Latvia (Brazauskiene & Petraitiene, 2004; Balodis et al., 2007). **Downy mildew** (caused by *Peronospora parasitica)* has been detected in Poland, Latvia and other regions where rape is cultivated (Sadowski et al., 2002; Bankina et al., 2008). **Powdery mildew** (caused by *Erysiphe cruciferarum)* is a significant disease in the warmer part of Europe (Mert-Türk et al., 2008), but in a temperate climate it is seldom observed, e.g., a high level of the infection was observed in Poland in the years 1998 and 1999 (Sadowski et al., 2002). **Gray mould** (caused by *Botrytis cinerea*) and **wilt** (caused by *Verticillium dahliae)* have been observed very sporadically (Sadowski et al., 2002). **Clubrot** (caused by *Plasmodiophora brassicae*) as an important disease and serious threat to oilseed rape production is recognized in many regions of Poland, especially if rape and other crucifers occupy a large part of the sowing structure (Korbas et al., 2009; Jedrycka et al., 2002). **Snow mould** (caused by *Typhula* spp.), the same as downy mildew, powdery mildew, gray mold, wilt, and clubrot, has been found also in Latvia; however, those diseases are recognized as economically unimportant in this country at present (Bankina et al., 2008).

Sclerotinia stem rot has been reported as one of the most harmful diseases in many countries (United Kingdom, France, Germany, etc.). Different opinions exist regarding economic importance of the disease, as, in some cases, yield losses of up to 50% have been reported (Koch et al., 2007). However, there are data that at least half of fungicide applications have been unnecessary (Young et al., 2007). Analysis of the dataset from official field trials of the German state extension service during 1991–2003 has shown that only 33% of fungicide treatment had been economically effective (Dunker & Tiedemann, 2004).

The development of Sclerotinia stem rot is dependent on meteorological conditions, therefore incidence of the disease differs greatly among the years. The first symptoms of disease are not visible at the time of fungicide application.

Fungicides from different chemical groups are used to control Sclerotinia stem rot: metconazole, tebuconazole, boscalid, and others. Some differences in the efficacy of

fungicide application have been observed, but the problem lies not in the choice of a fungicide but in the decision about the necessity of spraying.

Different approaches (including forecast methods and decision-support systems) have been developed to improve efficacy of fungicide treatment and to avoid unnecessary fungicide application. Different risk indicators have been compared to establish the most important and suitable ones for the growers. Part of these systems is based on biological peculiarities of *Sclerotinia sclerotiorum* (germination of apothecia, amount of ascospores in the air, etc.). Agrometeorological computer models have also been developed basing mostly on the weather data and growth stages of rape. The ScleroPro system is easy-to-handle, fully computerized and based on the weather and field-site-specific data; this program has been available for growers and advisors since 2006 (Koch et al., 2007). D. Makowski et al. have analysed systems that are based on determination of rape flowers' infection and on mathematical models. The researchers have found that percentage of infected flowers is more accurate than the calculated algorithm which is based on the development of rape and weather conditions, but the former method is costly and time consuming (Makowski et al., 2005).

The risk-point system developed in Sweden is relatively simple: factors that affect infection by *S. sclerotiorum* are expressed as risk points which, in their turn, for each specific field are summarized and compared to predetermined threshold values. According to this system, the most important factors are pre-crop of rape in a long period, development of rape, level of infection in the previous year, amount of rainfall two weeks before flowering and at the flowering time, weather forecast, and germination of sclerotia (Twengstrom et al., 1998).

Phoma stem canker caused by *Leptosphaeria* spp. (anamorph *Phoma lingam*) is an internationally important disease of oilseed rape causing serious losses of yield in Europe, Australia, and North America (Gugel & Petrie, 1992; West et al., 2001). Basal stem canker and leaf spots are the most important symptoms of the disease; also seed infection and damping of seedlings are observed in some cases. Phoma stem canker is now known to be caused by a complex that comprises at least two species: *Leptosphaeria maculans* (formerly classified as A group of *L. maculans*), and *L. biglobosa* (formerly classified as B group of *L. maculans*). The B group probably includes several subspecies of *L. biglobosa*. *L. maculans* is described as more aggressive and more harmful. While there are differences between A and B group, their life cycle is similar and they coexist on oilseed rape in Europe. Necroses on the upper and lower parts of the stem, slightly different symptoms on leaves, and especially colour and morphology of pure cultures confirm occurrence of *L. maculans* and *L. biglobosa* in Latvia (Balodis et al., 2008).

The source of primary infection is ascospores from the stem debris. Ascospores release through the vegetation season is the reason why effective chemical control is difficult (Gugel & Petrie, 1992). Under conditions of Latvia, ascospores start to differentiate during October, and in late October they are fully differentiated and ripe. Ascospores are found after overwintering and throughout the whole season of rape growing. A similar situation is observed also in other countries. Yield losses are mostly associated with the development of stem canker that restricts uptake of water and nutrients during the seed filling (West et al., 2001). In Australia, root rot has also been detected, but the research results indicate that it causes no additional reduction in the yield (Sprague et al., 2010). In general, the crucial point of possible development of Phoma stem canker epidemic is autumn, when release of ascospores and leaf infection take place.

It has been found that most popular active ingredients against Phoma stem canker are flusilazole, metconazole, prochloraz, and tebuconazole.

Though J. R. Hood et al. have observed poor correlation between severity of Phoma leaf spot and stem basal canker (Hood et al., 2007), there are also results which confirm correlation between early occurrence of leaf spot in autumn and severe basal stem canker (Steed et al., 2007). The efficacy of fungicide sprays depends on the timing of treatment in relation to the plant and disease development. It has been observed that accumulated thermal time determines maturation of ascospores, rate of pathogen growing along leaf petioles and progress of canker in stems, but rainfall mostly influences discharge of ascospores (Evans et al., 2008). The researches suggest that the possible threshold for fungicide application can be 10% incidence of leaf spot in autumn. The most harmful is infection of middle leaves, because senescence of the oldest leaves occurs before the pathogen has reached the stem, whereas the pathogen from younger leaves having reached the upper part of the stem causes less harmful damage (Steed et al., 2007). Most of the authors emphasise the importance of fungicide treatment in autumn, but there are converse data that the greatest additional yield has been obtained by spraying in spring at the beginning of flowering (Karolewski et al., 2009). There are very complicated relationships between the pathogen, the plant development, and the weather conditions which determine development of Phoma stem canker epidemic. Relationships between disease severity and yield losses are unclear, because the data are controversial. In general, fungicide application decreases severity of the disease, but not always increases the yield (Sprague et al., 2010).

There are attempts to develop a forecasting model for improvement of the efficacy of fungicide treatment against Phoma stem canker. The SIPROM-WOSR model has been developed including different factors which describe development of plants, initial source of infection, infection, and yield loss. Different knowledge about the infection process is necessary to implement this model in oilseed rape production (Lô-Pelzer et al., 2010). The Blackleg Sporacle model has been developed to predict onset of seasonal ascospores release: the number of ascospores in the air can be counted by the Burkard spore sampler, or by determining maturation of pseudotechia under a microscope. This model is based on the weather conditions (temperature and rainfall). The results show that the data of ascospores release can be predicted by a weather-based computer model with a set of parameters adapted to different conditions (Salam et al., 2007).

Alternaria leaf and pod spot (blackspot) (caused by *Alternaria* spp.) is one of the most widespread diseases in Poland, Lithuania, and other regions (Sadowski et al., 2002; Brazauskiene&Petraitiene, 2006; Balodis et al., 2008). The disease has been observed on the leaves, stems, and siliques; also seeds can be infected. Development of the disease depends on moisture periods – four hours of continuous wetness are necessary for the development of infection (Hong&Fitt, 1995). Frequent precipitation and high relative air humidity at the time of silique ripening promote development of Alternaria pod spot. Fungicides from different chemical groups are used for the disease control: prochloraz, tebuconazole, and azoxistrobin. Application of fungicides has significantly increased the rape yield in Lithuania (Petraitiene&Brazauskiene, 2007).

The most important diseases of oilseed rape and possibilities of control are widely described in the literature; however, a strong system of disease control has not been developed yet, and the data about necessity of fungicide application are inconsistent. There are very few data about disease control possibilities in the Baltic region, where climatic conditions differ from those in the West Europe: autumns are shorter and winters are longer and cooler, which determines different development of rape and most likely also different development of pathogens.

The aim of our investigations was to clarify peculiarities of the development of winter oilseed rape diseases and to work out recommendations for control of the diseases.

2. Winter oilseed rape production monitoring in farms of central Latvia

2.1 Materials and methods

Monitoring of winter oilseed rape diseases (Alternaria leaf and pod spot, caused by *Alternaria* spp., Phoma leaf spot and stem canker, caused by *Leptosphaeria* spp., and Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*), described above as important, was carried out during the years 2005–2008 in several randomly chosen farms (9 farms in 2006, and 15 farms in 2007 and 2008) located in central Latvia, which is the region of the greatest sowing area of this crop. Incidence and severity of the diseases were evaluated at different development stages of the crop:

- at 14-19 GS (growth stage according to BBCH scale) – incidence and severity of Alternaria spot and Phoma leaf spot in the autumn of the sowing season; 100 randomly chosen plants per field were evaluated, and severity was expressed in percent of leaf area;
- at 80-85 GS – incidence of Alternaria pod spot; 1000 pods per field were evaluated;
- directly after harvesting – Phoma stem canker and Sclerotinia stem rot were inspected per one meter long row at 10 places in the field. Disease incidence was expressed in percent from totally evaluated plants.

In addition, data on the growing technology (soil tillage method, pre-crops, proportion of area under rape in the total sown area in the farm, fertilization, used cultivar, sowing date and rate, and control of harmful organisms) were collected to explain the obtained data on disease incidence and severity better.

Meteorological conditions during the monitoring years were different. The years 2006 and 2008 were not favourable for *S. sclerotiorum* development; however, in 2007, precipitation two weeks before rape flowering and during flowering stimulated stem rot development. Moisture and temperature conditions in 2007 favoured development also of other above-mentioned diseases.

2.2 Results and discussion

Alternaria spot and Phoma leaf spot were found in the autumn of each sowing year. Incidence of these diseases showed tendency to increase (Alternaria spot – 77% in 2005, 85% in 2006, and 53% in 2007; Phoma leaf spot – 1.1% in 2005, and 19% in 2006 and 2007 on average), but severity remained insignificant (only few percent for both diseases). These data did not give evidence of sharp Phoma stem canker disease spreading during the succeeding year (Balodis et al., 2008). Fungicide (metconazole, 90 g L^{-1}, or tebuconazole, 250 g L^{-1}) application in autumn was based only on the necessity for rape plant growth regulation, and there was no intention of controlling any disease at that time.

The obtained data on Alternaria pod spot incidence fluctuated during the monitoring years (average incidence was 1.8% in 2006, 73% in 2007, and 7.3% in 2008; Table 1), and severity of this disease also remained insignificant. Incidence of Phoma stem canker and Sclerotinia stem rot was registered directly after harvesting. Sclerotinia stem rot initially was assumed as the most harmful rape disease in Latvia, but our observations clearly showed that incidence of the disease fully depends on meteorological conditions, and is low in unfavourable years (2006 and 2008; Table 1). Also incidence of Alternaria pod spot was at

least partly connected with meteorological conditions; besides, it should be noted that another reason for its low incidence in 2008 might have been the wide fungicide application in the inspected fields (see below).

Phoma stem canker incidence increased from 42% in 2006 to 83% in 2007, but in the year 2008 it was on average 56% per all inspected fields. Consequently, it can be concluded that Phoma stem canker incidence had decreased compared to the year 2007 (Table 1). For the first time in Latvia's conditions, more attention to this disease was paid during our investigations in 2006. Phoma stem canker caused by *Leptosphaeria* spp. is considered as an important disease of winter oilseed rape in most places where this crop is grown (West et al., 2001; Salam et al., 2003). This conclusion from the literature together with our observations of the disease spread was the reason for our deeper interest in the possible disease control ways.

Field, No	Disease incidence, %								
	Alternaria pod spot			Phoma stem cancer			Sclerotinia stem rot		
	2006	2007	2008	2006	2007	2008	2006	2007	2008
1	×	99.7	5.7	×	91	66	×	68.9	0.0
2	2.0	95.8	5.6	46	79	39	0.0	36.0	6.0
3	1.6	76.1	×	21	96	×	3.0	14.1	×
4	×	82.9	5.3	×	74	43	×	63.1	1.0
5	×	29.1	4.2	×	49	44	×	17.9	0.0
6	2.6	–	4.1	34	96	62	3.0	8.1	2.0
7	1.0	48.1	5.6	47	94	58	3.0	23.7	1.0
8	1.0	38.8	8.2	22	94	52	1.0	28.9	4.0
9	×	92.2	15.2	×	39	56	×	78.2	2.0
10	×	96.0	10.4	×	98	47	×	8.6	1.0
11	4.1	91.4	5.5	25	92	44	3.0	7.5	0.0
12	0.7	67.5	7.0	56	68	65	1.0	36.7	14.2
13	1.6	19.8	6.3	88	86	79	1.0	8.3	0.0
14	×	91.3	11.5	×	89	66	×	25.1	0.0
15	×	92.6	×	×	95	×	×	24.1	×
Average	**1.8**	**73.0**	**7.3**	**42**	**83**	**56**	**1.9**	**29.9**	**2.4**

Table 2.1. The incidence of Alternaria pod spot, Phoma stem canker, and Sclerotinia stem rot on winter rape in production fields during the disease monitoring years 2006–2008, Latvia

In 2006, farmers did not apply any fungicides against the observed diseases. Whereas during the next two years, fungicides (boscalid, 500g kg^{-1}, or protioconazole, 125 g L^{-1}, + tebuconazole, 125 g L^{-1}, or azoxystrobin, 200 g L^{-1}, + cyproconazole, 80 g L^{-1}) were applied: in seven fields during the season of 2007, and in twelve fields in 2008. The year 2007 was favourable (mild temperatures and very even distribution of precipitation throughout the whole season) for the development of all mentioned diseases, and in subsequent years farmers started to apply fungicides on more occasions, sometimes even without particular need and motivation.

As production of modern oilseed rape started only in 1997 in Latvia, we had very little information about and practically no experience of the disease development cycle in Latvia's

conditions as well as of the best ways to control the most widespread diseases. The collected data range was nearly sufficient to clarify some further research directions, and one of them was to develop specific fungicide application schemes against Phoma stem canker, Sclerotinia stem rot, and Alternaria pod spot.

3. Materials and methods

3.1 Trial site, conditions, and crop management

Two-factor (A – cultivar; B – fungicide treatment; see below subsection *Experimental design*) field trials testing several fungicide application schemes (Tables 3.2. and 3.3.) were carried out at the Research and Study Farm (RSF) "Vecauce" (latitude: N 56° 28′; longitude: E 22° 53′) of the Latvia University of Agriculture during 2005–2008.

Winter rape plots were established in the fields with no oilseed rape growing in crop rotation before.

Soil at the trial site was strongly-altered-by-cultivation loam with agro-chemical parameters summarized in Table 3.1.

Parameters	2005/2006	2006/2007	2007/2008
Humus content, g kg^{-1}	19	32	38
pH KCl	7.0	7.2	7.4
P, mg kg^{-1}	147	100	115
K, mg kg^{-1}	122	169	194

Table 3.1. Soil agrochemical parameters on the trial site at RSF "Vecauce" from 2005 to 2008

Winter barley (in 2005) and barley-oat mixture for green forage (in 2006–2007) were used as pre-crops. Traditional soil tillage with mould-board ploughing a month before rape sowing was done in all trial years. Rototilling was used directly before sowing. The crop was fertilized with mineral fertilizers before sowing depending on a year: N at the rate of 12–33 kg ha^{-1}, P – 18–30 kg ha^{-1}, and K – 57–103 kg ha^{-1}. Top-dressing with nitrogen fertilizer was done twice: at the start of vegetation (70 kg ha^{-1} N), and at GS 30–31 (another 70 kg ha^{-1} N). Sowing was done at an optimal time (close to 20 August) for Latvia's conditions. Having obtained more information and experience, the sowing rate was changed during the trial years: 5.0 kg ha^{-1} in 2005, 4.0 kg ha^{-1} in 2006, and 60 germinate able seeds per m^2 for hybrid cultivars and 80 germinate able seeds per m^2 for line cultivars in 2007. Dicotyledonous weeds were controlled using herbicide metazachlor, 333 g L^{-1}, + quinmerac, 83 g L^{-1} (2.5 L ha^{-1}), throughout all trial years; monocotyledons were controlled only in autumn 2005 applying propaquizafop, 100 g L^{-1} (1.0 L ha^{-1}). Insects (mainly *Meligethes aeneus* and *Ceutorhynchus* spp.) were controlled by alpha-cypermethrin, 100 g L^{-1} (0.15 L ha^{-1}), or lambda-cyhalothrin, 50 g L^{-1} (0.15 L ha^{-1}), or thiacloprid, 100 g L^{-1}, + deltamethrin, 10 g L^{-1} (0.75 L ha^{-1}), depending on a year.

The yield was harvested at GS 90-92 (on average from middle of July till early August depending on a year) and was recalculated to 100% of purity and 8% of moisture.

3.2 Meteorological conditions

Meteorological conditions, observed by a local meteorology station at "Vecauce", considerably differed during the research years. In 2005 and 2007, amount of precipitation

was enough for successful seed germination. Summer of 2006 was extremely dry and the air temperature was high, which negatively affected seed germination during autumn. An unusually long-lasting autumn and warm winter were observed in the season of 2007/2008. The October of all trial years was characterized by a sufficient number of rainy days with enough precipitation favouring successful oilseed rape disease development. Only in spring-summer of 2007, both the recorded precipitation and temperature were favourable for successful Sclerotinia stem rot development.

3.3 Experimental design

The trials were arranged in randomised blocks in four replications. The plot size was 7–10 m^2. Every year each treatment was compared with untreated check.

With the aim to investigate the action of fungicides as growth regulators in autumn, metconazole, 90 g L^{-1}, and tebuconazole, 250 g L^{-1}, were used during 2005–2007 and 2006–2007 respectively (Table 3.2).

Scheme No	Name	Growth stage according to BBCH scale	
		14–16 GS – autumn	65 GS
1	J_R+J	0.5 L ha^{-1} metconazole, 90 g L^{-1}	0.5 L ha^{-1} metconazole, 90 g L^{-1}
2	F	0.5 L ha^{-1} tebuconazole, 250 g L^{-1}	×

Table 3.2. Application of fungicides as growth regulators in autumn

Scheme No 1 was supplemented with the 2nd treatment against Sclerotinia stem rot (initial idea) during rape flowering (Table 3.2). Decrease in incidence of all three diseases, mentioned in the previous sections, in comparison with untreated check was evaluated. Several cultivars were used in this scheme: 'Excalibur' (F1), 'Falstaff' (line), 'Californium' (line), and 'Elixir' (F1). In the scheme No 2, cultivars 'Aviso' (line) and 'Falstaf' (line) were used in 2006/2007, but 'Elixir' and 'Californium' – in 2007/2008.

With the aim to decrease incidence of diseases Alternaria pod spot, Phoma stem canker, and Sclerotinia stem rot, another eight fungicide application schemes were used (Table 3.3). As our knowledge on the disease causal agents and disease development cycle under Latvia's conditions was incomplete, initially we applied standard schemes. This is the reason why the applied schemes and used cultivars were changed during the trial years.

Schemes No 5, using cultivars 'Aviso' and 'Falstaf' (both are lines), and No 9 (according to Dacom advice), using cultivars 'Californium' (line) and 'Elixir' (F1), in addition to the schemes mentioned in Table 3.2, were applied in 2006/2007. Schemes Nos 3–10 using cultivars 'Californium' (line) and 'Elixir' (F1) were applied in 2007/2008. Schemes Nos 3–7 are standard or routine schemes with preventive fungicide application without deeper idea on the real disease incidence and severity. Oilseed rape growers hope that such application helps protect rape against the main diseases, especially against Sclerotinia stem rot, but schemes No 3, and Nos 6–7 with fungicide application in spring – against Phoma stem canker.

Schemes Nos 8–10 were planned for Sclerotinia stem rot control. The only difference between schemes No 9 (PP) and No 10 (PP_{mod}) was in the applied fungicide dose (Table 3.3); both of them are based on the Dacom (the Netherlands) "*Sclerotinia sclerotiorum* in oilseed rape" control system (commercial program; decision-support system) that provides the

grower with advice through a computer program. The user has to input data on field conditions, used cultivar, development of crop (emergence, crop density, crop growth – according to special recording scale), and disease incidence in the closest environment into the program.

Scheme No	Name	Growth stage according to BBCH scale		
		31 – 33 GS	61 GS	65 GS
3	J_p	0.7 L ha^{-1} metconazole, 90 g L^{-1}	×	×
4	C_{61}	×	0.5 kg ha^{-1} boscalid, 500 g kg^{-1}	×
5	C_{65}	×	×	0.5 kg ha^{-1} boscalid, 500 g kg^{-1}
6	J_p+C_{65}	0.7 L ha^{-1} metconazole, 90 g L^{-1}	×	0.5 kg ha^{-1} boscalid, 500 g kg^{-1}
7	J_p+J_{65}	0.7 L ha^{-1} metconazole, 90 g L^{-1}	×	1.0 L ha^{-1} metconazole, 90 g L^{-1}
8	C_{sign}	0.5 kg ha^{-1} boscalid, 500 g kg^{-1}		
9	PP	0.7 L ha^{-1} metconazole, 90 g L^{-1}, – according to Dacom advise		
10	PP_{mod}	1.0 L ha^{-1} metconazole, 90 g L^{-1}, – according to Dacom advise		

Table 3.3. Fungicide application in the succeeding spring and summer

The program is connected with the Dacom automatic meteorological station which provides it with the current weather data and weather prognosis. The system determines whether the present conditions favour an outbreak of disease – commonly referred to as critical periods. When the program has calculated enough risk, advice is given to apply a fungicide, and also the particular fungicide type against Sclerotinia stem rot is recommended.
Scheme No 8, which is a slightly modified system worked out by Swedish researchers (Twengstrom et al., 1998), was included in the trial of 2007/2008. Also this scheme is built on actual observations in the field and on risk calculations, but without a special computer program, which is money consuming. Data on pre-crops in a long period, disease incidence in pre-crop, rape density, amount of precipitation two weeks before rape flowering, weather prognosis, and regional risk (number of apothecia from 100 sclerotia) are evaluated according to the scale in points from 0 (low risk) to 10-15 (high risk – depending on the evaluated parameter). All points are summarized and the result shows necessity to spray:
<40 points – it is not necessary to spray;
40–50 points – decision has to be made on the basis of previous experience;
>50 points – spraying is mandatory.

3.4 Analysis and observations

To evaluate the growth regulation effect of a fungicide, 10-plant (treated according to the Nos 1–2 fungicide application schemes; Table 3.2.) samples were taken randomly from each plot for biometrical analysis at the end of autumn vegetation in the years 2005–2007. Number of leaves per plant, plant, root and shoot weight (g), root length (cm), diameter of root neck (mm), and height of growth-point (mm) were measured in a laboratory.

Disease incidence and severity were evaluated in autumn and in the following summer. Disease incidence was expressed in percent from the evaluated plants. Disease severity was evaluated as damaged leaf area in percent (Alternaria and Phoma leaf spots) or in points (Phoma stem canker) according to Formula (1):

$$R = \frac{\sum a \times b}{N}, \quad (1)$$

where R – disease severity, % or points;
$\sum a \times b$ – sum of multiplication of the infected plants by the respective severity;
N – total number of inspected plants.

Fifty plants per plot were evaluated near the end of the vegetation period (late October) to detect Alternaria spot and Phoma leaf spot incidence and severity.

Ten pods in 10 places per plot at GS 85-89 (BBCH) were evaluated in the summer of 2006 and 2008 to detect incidence of Alternaria pod spot.

In all trial years, fifty randomly selected stems per plot were evaluated directly after harvest to detect incidence of Sclerotinia stem rot and Phoma stem canker.

Phoma stem canker severity (in points 0–4) also was evaluated (according to Formula (1)) after the harvest, but only in August 2008. As more knowledge and experience were obtained in the previous research years, we were able to visually identify both shapes of Phoma stem canker in 2008. A hypothesis was proposed that fungicide application can cause at least decrease in the severity of Phoma stem canker even if the incidence is not decreased. Severity scale: 0 – without any signs of disease; 1 – small lesion was observed; 2 – lesion included ½ of stem; 3 – lesion included 75% of stem; 4 – stem was broken or prematurely ripen (Chigogora & Hall, 1995).

Efficacy of fungicide application was evaluated as biological efficacy which was expressed in percent and was calculated by Formula (2):

$$\left(\frac{D_{check} - D_{treatment}}{D_{check}} \right) \times 100 \quad (2)$$

where D_{check} – severity or incidence of the disease in check plot;
$D_{treatment}$ – severity or incidence in a specific treatment.

The obtained results were statistically processed using analysis of variance.

4. Results and discussion

4.1 Effect of fungicides applied as growth regulators in the autumn of the sowing year

Over-wintering is the key factor for successful winter rape production in the conditions similar to those in Latvia. Wintering of rape depends on the plant development stage in the autumn, which could be affected by the growing manner (including used cultivar, sowing time and rate, and any treatment) and agro-climatic factors. Before the winter period, a rape plant should create a sufficient aboveground and root mass, but, on the other hand, it should not be overgrown. Important characteristics are: root-neck diameter (should reach 8 to 10 mm), height of growth-point above the soil (should be less than 30 mm), and number of leaves (at least 6 to 8 leaves). Researchers from Lithuania, under conditions very similar to those in Latvia, reported that application of a growth regulator increases the number of leaves per plant and the root-neck diameter, and decreases the height of the growth-point of

winter rape, thus favouring winter-hardiness of the crop (Gaveliene et al., 2002; Miliuviene et al., 2004). As the sowing time in Latvia's farms differs greatly (from early August, sometimes even late July, to middle of September), plant development regulation might be needed. It is suggested that azole-group fungicides applied in autumn act also as plant growth regulators.

From the results of all our different trials, it is evident that winter rape biometrical parameters were influenced by fungicide application in the autumn period and the used cultivar as well as by the particular growing conditions (see Tables 4.1–4.3). During three trial years, the highest height of untreated rape growth-point was noted in 2005 (on average per four cultivars included in Scheme No 1 (J_R+J) – 35.3 mm). Height of untreated rape growth-point was noted lower with every succeeding trial year, and the lowest it was in 2007 (13.5 mm). In the year 2005, average height (22.5 mm) of the growth-point of plants treated with fungicide was higher than that of untreated plants in 2006 and 2007 (21.1 mm and 13.5 mm respectively). Such substantial decrease in the growth-point can be explained mostly by different meteorological conditions during the different autumns and also by the year-by-year decreasing sowing rate (see subsection 3.1), which could have affected some plant density changes. D. Becka et al. (2004) have reported that more leaves, less height of growth-point, and greater diameter of root-neck is the result of lower crop density, when plants have enough space and no strong intra-specific competition exists. From the three factors (cultivar, fungicide application, and growing conditions) analysed in our investigations, growing conditions of a specific year had the greatest impact on the height of growth-point (percentage of influence η=48%). According to Schemes Nos 1-2, a significant impact ($p<0.05$) of fungicide application in autumn was noted on the height of growth-point in all three trial years (see Table 4.1-4.2). Our experiments also showed a significant impact of the cultivar on the height of growth point. We found that the cultivar with the highest growth-point was hybrid 'Elixir' (see Table 4.1.) – the height of growth-point of this cultivar without fungicide treatment reached 48.1 mm in autumn 2005. Comparing all the used cultivars, we observed that hybrid-type cultivars tended to have a higher height of growth point (see Table 4.1).

Cultivar (Factor A)	Effect of fungicide treatment (Factor B) on plant biometrical parameters							
	Height of growth-point, mm		Root-neck diameter, mm		Number of leaves per plant		Root mass, g	
	C	J_R+J	C	J_R+J	C	J_R+J	C	J_R+J
Excalibur F1	26.0	18.0	8.72	9.06	8	10	6.21	6.68
Californium	17.0	14.7	7.27	7.27	7	8	3.68	4.05
Elixir F1	29.8	19.3	7.85	8.43	8	9	5.09	6.15
Falstaff	20.3	16.3	7.31	7.23	8	9	3.64	3.75
Average	23.3	17.1	7.79	8.00	8	9	4.66	5.16
$LSD_{0.05B}$ or p-value	1.74		p>0.05		0.47		0.50	

Table 4.1. The effect of the fungicide metconazole application (Scheme No 1) in autumn on some average 3-year (2005-2007) plant biometrical parameters of winter oilseed rape (C – check without fungicide treatment; J_R+J – fungicide treatment according to Scheme No 1 in autumn)

Application of fungicides as growth regulators in autumn can increase root-neck diameter (Miliuviene et al., 2004), but in our trial, on average per three years, root-neck diameter was not affected significantly ($p>0.05$, see Table 4.1) – there was only a slight tendency for the root-neck diameter to increase; however, this parameter was affected by the used cultivar and the year's conditions. Fungicide application effect on root-neck diameter was substantial in some individual years in respect of the applied fungicide, e.g., in 2007 (see Table 4.3). Similarly to the height of growth-point, also average root-neck diameter was strongly affected (percentage of influence η = 58%) by the conditions of a particular year (average root-neck diameter from Scheme No 1: 9.4 mm in 2005, 8.1 mm in 2006, and 6.2 mm in 2007).

Cultivar	Height of growth point, mm		
	C	J_R+J	F
Aviso	20.45	15.40	14.65
Falstaff	18.33	15.78	13.50
Average	19.39	15.59	14.08
$LSD_{0.05}$	4.09		

Table 4.2. The effect of fungicides metconazole and tebuconazole application on the height of growth-point in autumn 2006 (C – check without fungicide treatment; J_R+J – Scheme No 1 (metconazole); F – Scheme No 2 (tebuconazole))

Also average plant mass was affected strongly by the growing conditions of the trial year (on average per three years from Scheme No 1: 80.5 g in 2005, 32.4 g in 2006, and 25.4 g in 2007; percentage of year's influence – η = 77%). Application of fungicide as a growth regulator can help winter oilseed rape growers to control plant overgrowing. Trial results showed only a tendency ($p>0.05$) for plant mass to decrease after fungicide application. At the same time, a significant impact ($p<0.05$) of fungicide application on the fresh root mass was noted in autumn. A small but significant ($p<0.05$) increase in root mass was observed in all trial years and for all included oilseed rape cultivars (see Tables 4.1 and 4.3). Also a small root length increase was observed as a result of fungicide application. A significant impact ($p<0.05$) of fungicide application, according to Schemes Nos 1–2 in autumn, was noted on the number of leaves per plant in all trial years (Tables 4.1 and 4.3). However, the number of leaves per plant was optimal in respect of fungicide application for good wintering and sufficient branching in the next spring of winter oilseed rape in all trial years.

Cultivar	Effect of fungicide treatment (Factor B) on plant biometrical parameters								
	Root-neck diameter, mm			Number of leaves per plant			Root mass, g		
	C	J_R+J	F	C	J_R+J	F	C	J_R+J	F
Californium	6.54	7.44	7.11	8	10	9	3.04	4.21	3.42
Elixir F1	4.81	4.92	5.51	7	7	8	1.47	1.71	2.01
Average	5.67	6.18	6.31	8	9	8	2.26	2.96	2.72
$LSD_{0.05}$	0.49			0.87			0.39		

Table 4.3. The effect of fungicides metconazole and tebuconazole on some biometrical parameters of winter oilseed rape in autumn 2007 (C – check without fungicide treatment; J_R+J – Scheme No 1 (metconazole); F – Scheme No 2 (tebuconazole))

Our experiments gave us confidence that winter oilseed rape biometrical parameters can be influenced by application of both azole-group fungicides (metconazole and tebuconazole) in autumn. Those results gave motivation for discussion about fungicide application in autumn. In our trials, winter oilseed rape was sown at an optimal sowing time (close to 20 August), which did not contribute to plants' overgrowing very much; fungicide effect might be more marked when rape is sown too early (late July till approximately 10 August). Even from our results we can conclude that fungicide (from azole group) treatment is an effective tool for rape growth regulation in cases when growing conditions and the used cultivar favour rape overgrowing in autumn.

4.2 Disease incidence and fungicide effect on it

Sclerotinia stem rot, Phoma stem canker (also Phoma leaf spot in the sowing autumn), and Alternaria spot (leaf spot in the sowing autumn, and pod spot in the next summer) were noted in all the trial years.

Sclerotinia stem rot was reported as the most important and most harmful rape disease; however, there are data that development of this disease differs depending on site, field, and especially on a year. Different forecast and warning systems related with Sclerotinia stem rot exist. Dacom computer advice (Schemes Nos 9-10) and Swedish risk point system (Scheme No 8) were tested during investigations, and their efficacy was compared with the routine fungicide application at GS 65 (Scheme No 1). During three-year investigations, only one year (2007) was favourable for Sclerotinia stem rot development: incidence of the disease reached 14-15% depending on the cultivar, and efficacy of fungicide treatment was high (57-87%) in this year (Fig. 4.1). The results showed that fungicide application according to Scheme No 1 (such routine scheme is widely used by growers) and Dacom advice (Scheme No 9) was appropriate in the years with high occurrence of the disease.

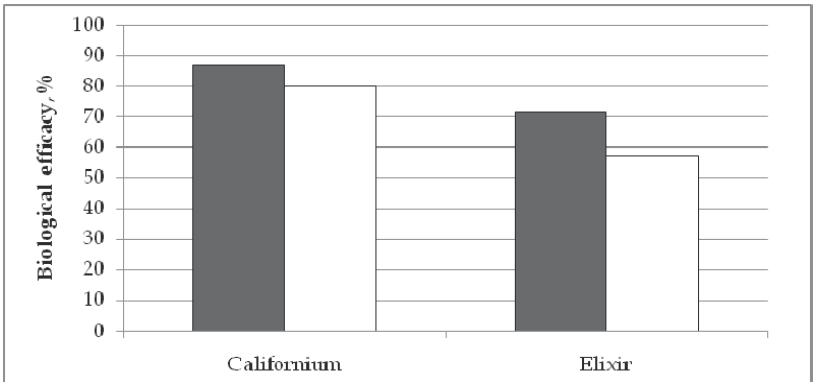

Fig. 4.1. Biological efficacy of fungicide application depending on the treatment scheme (■ – Scheme No 1: 0.5 L ha^{-1} metconazole, 90 g L^{-1},and □ – Scheme No 9: 0.7 L ha^{-1} metconazole, 90 g L^{-1}) and the cultivar, 2007

A similar routine scheme, applying fungicide boskalid, 500 g kg^{-1}, (Scheme No 5 at GS 65) and using cultivars 'Aviso' and 'Falstaf', gave similar results in 2007 (biological efficacy: 100% and 95% respectively). Besides, after treatment with boscalid, 500 g L^{-1}, rape stems visually seemed greener and healthier even during harvesting.

In 2006 and 2008, incidence of the disease did not exceed 3%, and fungicide treatments were not economically beneficial. Due to this, application of fungicides according to routine

schemes (No 1 in 2006 and 2008, and Nos 4–7 in 2008) was inefficient. There is large amount of data that routine fungicide application frequently is not reasonable (Dunker & Tiedemann, 2004; Young et al., 2007); results of our investigations confirmed unnecessary treatments also under conditions of Latvia, if meteorological conditions were not favourable for Sclerotinia stem rot development.

Unfortunately, Dacom recommendation also proved to be ineffective in the years 2006 and 2008. The amount of precipitation was insufficient for Sclerotinia stem rot development before and during winter oilseed rape flowering in 2006 and 2008; however, Dacom programme still gave advice to spray (Schemes Nos 9–10). As a result of co-operation with the creators of the programme, it was improved for the next seasons taking into account reference of our data.

Results of Swedish risk point system (Scheme No 8, started in 2008) were disputable: the system did not recommend fungicide application for cultivar 'Californium', but recommended it for cultivar 'Elixir' (having a later time of flowering), which later proved to be unnecessary. Germination of apothecia before and during rape flowering has to be a very important indicator which influences infection of rape with Sclerotinia stem rot. Germination of sclerotia started in 2008, but soil was too dry for the development of apothecia in many places in Latvia. We suggest that this indicator is uppermost if total amount of risk points is 40–50 (threshold value). The obtained results allowed to improve both systems; investigations were continued (in 2008/2009 and 2009/2010) to increase effectiveness of forecasts.

Phoma stem canker, caused by complex of *Leptosphaeria* spp., is a world-wide rape disease, causing serious losses in all regions. Recently, the increasing incidence of stem canker has been observed also in Latvia (Balodis et al., 2008). Incidence of *Phoma* leaf spot of up to 1% was noted in the sowing autumn of 2005, up to 8% – in 2006, and up to 6% – in 2007, but severity of the disease was still very low and mostly did not reach 1%. Leaf spot form is not harmful, and its impact on the yield was not observed. But autumn, as indicated by most of authors, is crucial time for further disease (as stem canker) development, when the pathogen from leaf spots grows and reaches petioles and stems. Fungicide application in autumn is important for Phoma stem canker control. The increasing area under oilseed rape, absence of high-yielding cultivars with high resistance against *Leptosphaeria* spp., and the tendency of growers to shorten crop rotation, leads to increase in the spread and harmfulness of stem canker (West et al., 1999). A similar situation (rapid increase in stem canker incidence during the last years) was observed in Lithuania (Brazauskiene et al., 2007).

In 2006 and 2007, we recorded the total incidence of stem canker (caused by *Leptosphaeria* spp.) directly after the harvest, as in the first years of investigations we were not certain about the presence of both shapes of stem canker in Latvia and we did not know how to diagnose them separately. In our investigations, the incidence of stem canker fluctuated from 48% to 86% in untreated plots. (Table 4.4). The obtained data clearly showed ineffectiveness of fungicide application in 2007, when incidence in treated plots was even higher (on average 89%; Table 4.4).

The only explanation for such high incidence of Phoma stem canker in the treated plots in 2007 was the presence also of Sclerotinia stem rot which was controlled by the fungicide better and the free place on stems was occupied by Phoma stem canker. In untreated plots, high occurrence of Sclerotinia was observed, and Phoma stem canker had less living space. Observations in 2007 also led to the idea that fungicide application schemes which are effective against Sclerotinia stem rot can be ineffective against Phoma stem canker in the years with high incidence of Sclerotinia.

Cultivar	Incidence, %			
	2006		2007	
	Check	J_R+J	Check	J_R+J
Excalibur	64	49	74	91
Californium	48	54	82	86
Elixir	50	35	86	91
Falstaf	74	55	76	88
Average	59	48	80	89

Table 4.4. The incidence of stem canker (caused by *Leptosphaeria* spp.) depending on cultivars and fungicide application: 0.5 L ha^{-1} metconazole, 90 g L^{-1}, at GS 14-16, and 0.5 L ha^{-1} metconazole, 90 g L^{-1}, at GS 65 in 2006 and 2007

Incidence of stem canker differs depending on years, regions, and fields. Virulence of the pathogen is the most important factor which determines the yield losses. Depending on pathogen species (now two distinct species, *L. maculans* and *L. biglobosa*, which have different virulence are determined) yield losses from 1% to 56% have been reported (Gugel&Petrie, 1992). Presence of *L. maculans* and *L. biglobosa* was approved also in Latvia during our investigations (Balodis et al., 2008). Existence of two species and inconsistent data about fungicide efficacy were the reason to evaluate not only incidence, but also severity of stem canker in the trial in 2008 (Fig. 4.2). Basal stem canker (mostly caused by *L. maculans*) was reported as the cause of more harmful damage to a plant if compared with upper stem lesions (mostly caused by *L. biglobosa*).

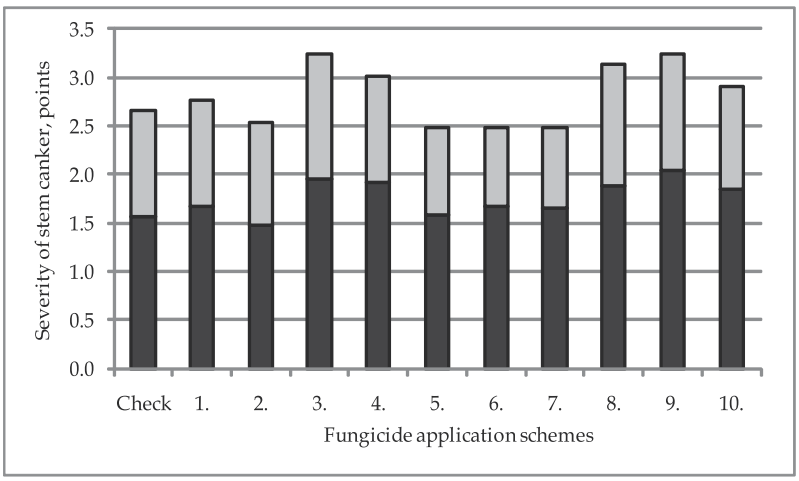

Fig. 4.2. Development of stem canker depending on fungicide application schemes (1 – J_R+J; 2 – F; 3 – J_p; 4 – C_{61}; 5 – C_{65}; 6 – J_p+C_{65}; 7 – Jp+J_{65}; 8 – C_{sign}; 9 – PP; 10 – PP_{mod}; ■ – upper stem canker; ■ – basal canker), cultivar 'Californium', 2008

Severity of stem canker fluctuated within 1.48–2.04 (basal canker) and 0.82–1.28 (upper stem canker) points (max possible is 4 points) for cultivar 'Californium'. Fungicide spraying did not cause a convincing decrease in the disease – only a slight tendency was observed for the

individual treatments (Schemes Nos 2, 6 and 7), where fungicide was applied at least once at GS 14-16 or 31-33, to cause a small reduction in Phoma stem canker severity.

Cultivar 'Elixir' was more susceptible against stem canker in our investigations. Severity of basal stem canker reached 2.56 points (1.9–2.56), on the upper stem part – 0.95–1.67 points. Fungicide application for 'Elixir' was as much ineffective as for cultivar 'Californium'; also the same tendency of some effect of Schemes Nos 2, 6 and 7 was observed.

Infection by ascospores is possible during all autumn period and also during the succeeding spring and summer under conditions of Latvia. Infection of leaves was low in autumn, and it might be possible that infection of stems does not occur in autumn like it is in countries with a milder climate (West et al., 2002). Leaf spot most frequently was observed on the oldest leaves which died during winter, and therefore did not influence development of stem canker. Very contradictory results have been found by other authors, for example, yield losses of 1-56% related to stem canker have been reported (Gugel&Petrie, 1992). There are different recommendations regarding the best time of fungicide application, e.g., J. R. Hood et al. have noted increase in leaf spotting between October and January. They observed that fungicide spray in October is more effective against leaf spots, but spraying in November decreases severity of canker and yield loss (Hood et al., 2007). Under conditions of Latvia, November usually is too late for fungicide application. Average air temperature in November mostly is below 5 °C (5 °C in 2005, 4.3 °C in 2006, and 1.2 °C in 2007). Further investigations which are necessary to find the best fungicide application scheme for decrease of Phoma stem canker have been started in 2009 and are being implemented at present, in 2010.

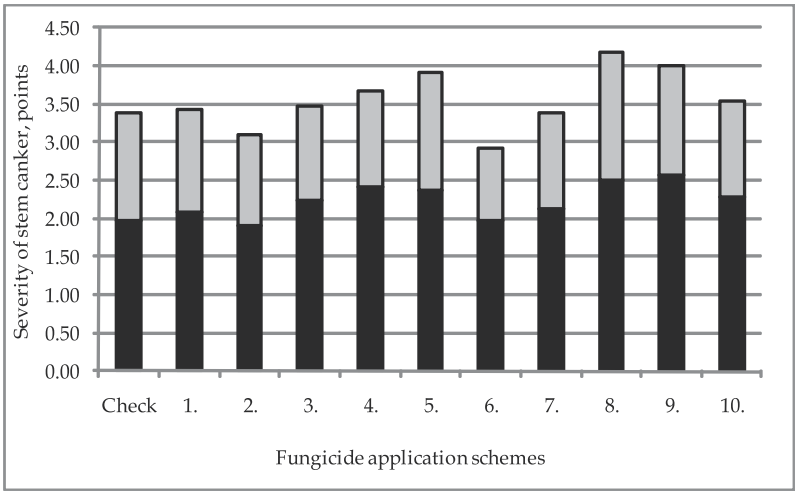

Fig. 4.3. Development of stem canker depending on fungicide application scheme (1 – J_R+J; 2 – F; 3 – J_p; 4 – C_{61}; 5 – C_{65}; 6 – J_p+C_{65}; 7 – Jp+J_{65}; 8 – C_{sign}; 9 – PP; 10 – PP_{mod}; ■ – upper stem canker; ■ – basal canker), cultivar 'Elixir', 2008

Alternaria spot is the most widespread disease in Latvia. The effect of fungicide application according to different schemes was observed during all trial years. Incidence of Alternaria leaf spot in the autumn of the sowing year fluctuated from 43% to 94% (mostly 70-80%) in untreated plots depending on the cultivar and the year (Table 4.5). The severity of Alternaria leaf spot did not exceed 2% in all trial years, which is a typical situation in agro-climatic

conditions of Latvia. O. Treikale (2006) has reported similar results on poor severity of the disease in autumn in Latvia. Fungicide biological efficacy was not stable and fluctuated (0-65%) depending on the year and the cultivar. Similar results were obtained, when metconazole was replaced with tebuconazole (Scheme No 2: F).

Development of Alternaria leaf spot in autumn is dependent on different factors, but mathematically the impact of fungicide application (ŋ%) on disease incidence was 69-74% during our investigations. Findings of Lithuanian researchers have demonstrated a more significant effect of fungicide application on the disease severity than on disease incidence (Petraitiene&Brazauskiene, 2007), but leaf spot severity lower than 2%, as in our trials, could not really influence the development of rape plants in autumn.

Alternaria spot affected also pods. Pod spot is reported as harmful disease in many regions of rape cultivation (Brazauskiene &Petraitiene, 2006). Development of the fungus from *Alternaria* spp. on siliques is equally influenced by the air temperature and moisture in July when winter rape silique ripening period occurs. In our trials, the incidence of pod spot reached only 7% in 2006, and 26% in 2008 when July was dry (17% of the month's long-term average rainfall in 2006, and 76% – in 2008).

Cultivar	Incidence of Alternaria spot on leaves, %					
	2005		2006		2007	
	Check	J_R+J	Check	J_R+J	Check	J_R+J
Excalibur	77	62	81	45	43	44
Californium	86	53	80	43	71	46
Elixir	91	77	79	38	78	42
Falstaf	94	60	83	45	79	28
Average	87	63	80	43	68	40

Table 4.5. The effect of fungicide application (Scheme No 1: 0.5 L ha^{-1} metconazole, 90 g L^{-1}, GS 14-16) on the incidence of Alternaria leaf spot in the sowing autumn depending on the year and the cultivar

Biological efficacy (22-84%) of fungicide application schemes differed depending on the year and the cultivar. Unfortunately, in 2007, which was more favourable for Alternaria pod spot development (136% of the month's long-term average rainfall in July), disease incidence was not evaluated. The obtained results are disputable, and more investigations are necessary to find relationship between development of Alternaria pod spot and its impact on the development of rape. Also influence of the disease on rape yield is still not clear.

4.3 Effect of fungicide application on the yield

Although fungicide application effect on the decrease of three main diseases was contradictory during the trial years, yield increase in plots where fungicides were applied was substantial in 2006 and 2007 (Table 4.6).

More favourable for the development of diseases was the year 2007, and accordingly higher efficacy of fungicide application on yield increase was observed in this year.

Fungicide application according to Scheme No 9 – Dacom advice – provided yield increase in 2007 when Sclerotinia stem rot was widespread (+0.71 t ha^{-1}), but in 2006, the yield in

sprayed plots was of the same level as in untreated check plots. Also other two schemes applied on cultivars 'Aviso' and 'Falstaf' in 2007 gave some average yield increase (No 2: tebuconazole, 250 g L^{-1}, at GS 14-16, gave +0.29 t ha^{-1}; No 5: boscalid, 500 g kg^{-1}, at GS 65, gave +0.63 t ha^{-1}), but the yield was of the same level as in untreated check plots when fungicide tebuconazole, 250 g L^{-1}, (Scheme No 3) at GS 31-33 was applied.

Cultivar	Yield, t ha^{-1}			
	2006		2007	
	Check	J_R+J	Check	J_R+J
Excalibur	6.48	6.80	6.19	6.86
Californium	5.36	5.21	5.17	5.90
Elixir	5.04	6.09	5.40	6.07
Falstaf	5.52	5.92	5.19	5.39
Average	5.60	6.01	5.45	6.07
$LSD_{0.05\ for\ fungicide\ treatment}$	0.31		0.20	

Table 4.6. The effect of fungicide application according to Scheme No 1 (J_R+J) on winter oilseed rape yield in 2006 and 2007

Contradictory results were obtained in the year 2008, when fungicide application by any scheme caused significant ($p<0.05$) yield increase by more than 1.0 t ha^{-1} for cultivar 'Elixir', but the yield of cultivar 'Californium' was of the same level as that of untreated check plot. Also other researchers (West et al., 2001) have reported yield increase depending on cultivars and years. Yield level of any crop is affected by many factors, and disease development and disease control effect are only two of them. But, of course, any rape grower hopes to control disease development by fungicide application and in such a way to increase the yield. A substantial and economical yield increase, if compared with untreated crop, is the main criterion for applying any activity in the field. Our results require continuation of trials in order more reliable data are achieved on fungicide application for the decrease of disease incidence and increase of oilseed rape yield.

5. Acknowledgements

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Disease Decision Support Systems: Their Impact on Disease Management and Durability of Fungicide Effectiveness

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1. Introduction

In modern agriculture, fungicides play a major role in maintaining crop health and reliable yields of products that meet the demand for high quality. Hence, fungicides represent a key component of most integrated disease management programs, and their effectiveness should be sustained as much as possible. However, fungicide effectiveness has been significantly affected by the development of resistance in some fungal pathogen populations. Moreover, environmental considerations are becoming increasingly important and, as a consequence, it is less acceptable to apply fungicides needlessly. In this chapter we intend to demonstrate that disease decision support systems could help reduce fungicide usage and as a consequence maintain the durability of fungicides effectiveness. This will be done using apple scab, caused by the fungal pathogen *Venturia inaequalis* as a model system. In most apple producing areas of the world, apple scab is the most important disease and its control depends almost exclusively on frequent use of fungicides; in some areas up to 75% of all pesticides used are applied to control apple scab. Apple scab is a complex disease that develops following two phases; a monocyclic phase caused by ascospores produced in pseudothecia originating from leaf lesions present the previous fall, and a polycyclic phase produced by conidia originating from lesions on leave and fruits. Various management tactics to manage apple scab were developed during the last half century. These tactics are either biologically/culturally or chemically based. However, in most IPM programs, the control of apple scab is based on the use of fungicides and these programs are developed to improve fungicide efficacy using procedures to estimate potential ascospore dose, weather-based models to estimate ascospore maturity, leaf growth and risk of infection periods. These tools were integrated into various disease decision support systems (DDDS). Almost all of the DDSS focus on management of primary infections (monocyclic phase) and much less attention has been paid to secondary infections (polycyclic phase) that develop during the summer, despite that up to 6-8 additional fungicide sprays are often used to manage

summer scab. This review discusses the basic approaches for managing apple scab and their effect on durability of fungicide effectiveness.

2. The paradigm and dilemma of disease management in modern agriculture

In most parts of the world, plant diseases induce loss of food crops. Therefore, diverse control measures were developed for suppression or management of plant pathogens. There is a general consensus in the scientific community to consider that sustainable disease management could be achieved only through the integration of several control measures including cultural, physical, genetic, biological and chemical measures. These control measures, however, differ in efficacy, duration of effectiveness and cost. In addition, the occurrence of plant pathogens and the severity of the diseases they cause often vary from year to year, location to location, from a production system to another… hence rational and cost-effective disease management necessitates the consideration of several factors, so reaching an informed and rational decision for disease management is a complex task. In addition to agronomic considerations, growers must deal with economic and social considerations which include the value of the crop, the tolerance level of the consumers to presence of diseases (or pathogens) and the consumer's concerns regarding pesticide use and residues on food products.

In practice, grower's decisions regarding disease management are generally based on risk. The factors that influence grower's perception of risk are mainly related to the value of the crop and the characteristics of the pathogen being considered. In intensive production systems (high-value crops) the economic threshold for disease damage is generally low. In other words, it is generally more profitable to apply a control measure than to tolerate even a low level of damage. On the contrary, in extensive production systems (low value crops), depending on disease pressure, it is not always cost-effective to apply a control measure. Another important factor related to disease management decision is the potential of the disease to cause damages. Some diseases are more destructive than others and under favourable conditions may develop rapidly eventually leading to complete destruction of the crop, for example potato late blight, caused by *Phytophthora infestans* or apple scab caused by *V. inaequalis*. Thus, from a crop production standpoint, the decision as to apply a disease control measure mainly depends on: the value of the crop, the type of disease and its potential to causes losses, and on the cost of control measures. Hence, chemical control is often perceived as the most effective control measure. However, the intensive and sometime solely use of fungicides to manage some diseases resulted in disease management practices that can harm human health as well as the environment. In addition, as the use of fungicides has increased, so has the incidence of resistance to certain classes of fungicides.

The dilemma of crop disease management in the 21th century could be summarized as follow: food crops must be produced in sufficiently large amount to avoid food shortage; it should be free of diseases to respond to the consumer demand and to be shipped over long distances or stored for prolonged period of time; crop production should be profitable for growers but not too expensive for the consumers; it should be within acceptable limits of pesticide residues and produced in such way that it will not harm the environment. Various disease management strategies were developed in response to some of these considerations including biological control, use of resistant cultivars and integrated disease management. However, disease management practices cannot be divorced from economics and farmers cannot implement disease management strategies that are not cost-effective. Hence, it is

expected that fungicides will continue to play a role in disease management programs, especially in intensive production systems. However, to maintain their effectiveness and to minimize their effect on human health and on the environment, they should be used in a rational and informed way. One approach to achieve this goal is to use disease decision support systems.

To illustrate the benefits of using DDSS to manage diseases with fungicide we shall use the example of apple scab. In most parts of the world, apples are produced in intensive mono-crop production systems. Apples are generally considered as high value crop because the costs per ha of implementing and maintaining an orchard is high. Apple tree is a perennial crop, hence several cultural control practices are impossible or difficult to implement, for example crop rotations. Apple scab is a typical disease for which growers are risk adverse because any failure in controlling infections on fruits will result in economic losses (MacHardy, 1996). Since almost a century, researches were conducted on *V. inaequalis* biology and ecology, on host-pathogen relationships, and on apple scab epidemiology and management, (Aylor, 1998; MacHardy, 1996). As a result, the disease has been adequately managed, but durable management practices have not yet been attained. Apple scab management is largely, if not exclusively, based on fungicide applications and up to recently, this approach was cost effective. In North America as well as in several countries, the market constraint for high-quality blemish-free fruits and the technical hitches of achieving control once the disease has become established in the orchard have led to the application of regular fungicide sprays, applied from bud-break to harvest (MacHardy, 1996). Such spray programs, though costly, have usually proved successful, reliable and simple for farm managers to implement. However, pesticides are often regarded as highly undesirable by consumers and there is an ongoing negative media campaign against them. As a consequence, several retailers have identified the occurrence of pesticide residues as being one of the prime concerns of consumers about fresh products. Moreover, with the rising costs of chemicals, and increasing resistance to fungicides in several pathogens' populations, such practices have become less acceptable. Methods of optimizing fungicide use are thus now being sought.

3. Understanding the pathogen: life cycle of *Venturia inaequalis*

Venturia inaequalis (Cke.) Wint. (*Spilocaea pomi*) (Ascomycetes, Pleosporales, Venturiaceae) causes apple scab, scurf, and black spot on apple (*Malus pumila*). Apple scab infections are mostly visible on leaves and fruits. On leaves lesions develop first as an area of lighter shade of green compared to the rest of the leaf (Fig. 1a). Rapidly, the lesions become covered with fungal mycelium and spores giving an olive-dark-green and velvety appearance to the lesion (Fig. 1b). Under severe infections, the entire leaf can be covered by lesions giving rise to a symptom called 'sheet scab' (Fig. 1c). When sheet scab is present it can lead to premature partial or complete tree defoliation. Lesions on fruits are generally smaller and become corky with age (Fig. 1d). When the fruits are infected early in the season, large lesions which deform the fruit and may cause premature fruit drop (Fig. 1e). Fruit infections that occur in late summer or early fall often lead to symptoms that are visible only after harvest, during storage. These lesions are small and this symptom is call 'pin point scab' (Fig. 1f).

In most parts of the world, apple scab epidemics are initiated by ascospores arising from sexual reproduction occurring the previous fall. There is one sexual infection cycle per year and the amount of ascospores depend on the amount of disease present in the orchard the

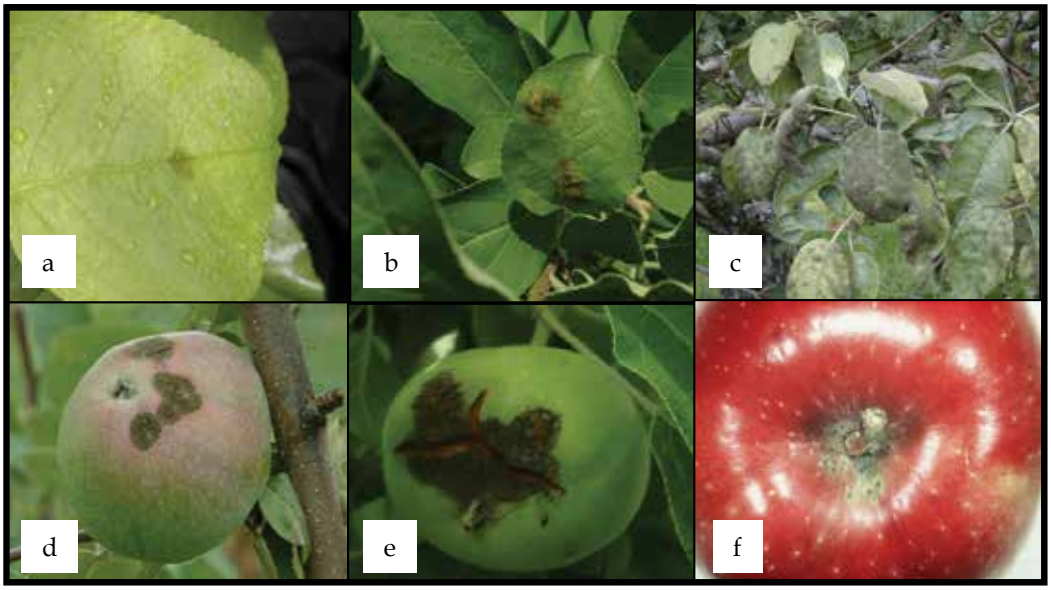

Fig. 1. Symptoms on apple leaves and fruits caused by *Venturia inaequalis*: Newly developed lesions on apple leaves (a); well developed lesions on leaves (b); symptoms of sheet scab (c); lesions on fruit (d); fruit deformation caused by early infections (e); pin point scab (f).

previous fall and the weather conditions during the winter. In apple production areas with mild winters, *V. inaequalis* can also overwinter as conidia arising from asexual reproduction. In these areas, conidial primary inoculum overwinters on wood and in bud scales (Beker et al., 1992). Nevertheless, in most areas where apple scab is a problem, the ascosporic inoculum is the main source of inoculum and plays a highly significant role in epidemic development. The complete life cycle of *V. inaequalis* is depicted in Figure 2. Briefly, the pathogen overwinters as pseudothecia (fruiting bodies) in infected fallen leaves on the orchard floor. In early spring, pseudothecia mature in response to increasing temperatures and to alternating wetting and drying of apple leaves litter. The primary infections are caused by ascospores released from mature pseudothecia and in general, first ascospores are released at the 'green tip' apple tree phonological stage. Ascospores are released during a period of 8 to 12 weeks with a peak around bloom. The ascospores are forcibly discharged form the pseudothecia in leaf litter, become airborne and wind dispersed. When the ascospores are deposited on apple leaves, the infection process (spore germination, penetration and colonization of leaf tissue) begins provided that there is a film of water on the leaf surface and that the air temperature is above 0^{o}C (Mills , 1944; Mills and Laplante, 1951). The infection efficiency is influenced by the temperature during the leaf wetness period; longer the wet period higher the infection efficiency (Mills and Laplante, 1951). Depending on leaf age, temperature and humidity, 9-21 days after the beginning of spore germination, typical olive-green, velvety lesions develop on the site of infection (Fig 1.). Conidial production begins shortly after lesions become visible, each scab lesions having a potential to produce up to 100,000 new conidia. Conidium productivity of individual lesion is influenced by cultivar, humidity, photoperiod and temperature (Dewdney et al., 2003). Newly produced conidia are wind and rain splash dispersed (Aylor, 1998). The conditions that influence conidial infections are similar to those influencing ascospore infections

(Hartman et al., 1999). As for the leaves, fruit infection efficiency is influenced by the cultivar, fruit age, inoculum dose, temperature and fruit wetness duration (Schwabe et al., 1984; Xu and Robinson, 2005). *V. inaequalis* life cycle is completed by sexual reproduction and initiation of new pseudothecia in late autumn (Fig 2.).

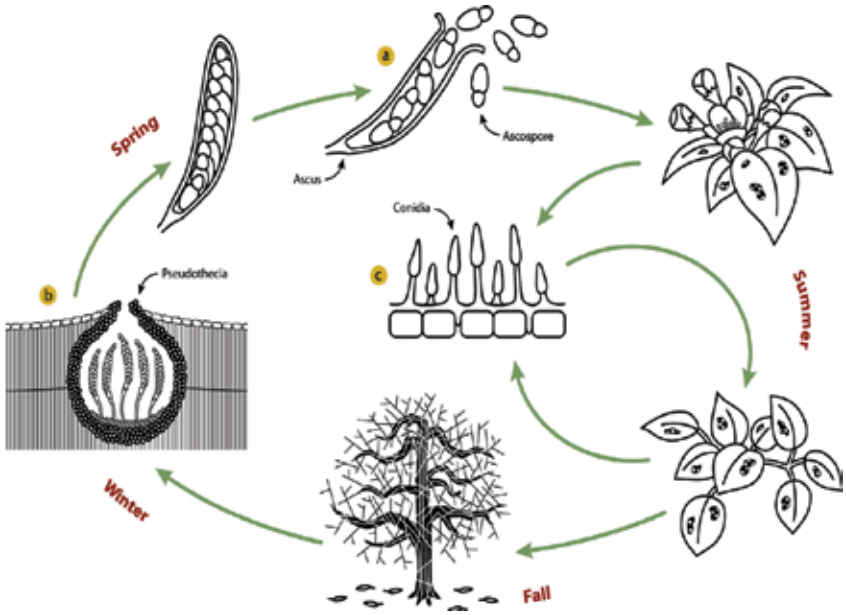

Fig. 2. Life cycle of *Venturia inaequalis* showing the ascospores (a) responsible for the primary infections produced in pseudothecia (b) and the conidia (c) responsible for the secondary infections.

4. Understanding the disease: epidemiology of apple scab

Understanding how plant pathogen populations increase over time is critical for developing appropriate management practices. Apple scab epidemics comprise three phases. The first phase corresponds to the overwintering period and is often referred as the inactive phase. The second phase, called the primary infections period, is caused by ascospores produced in overwintered pseudothecia (Fig. 2-a-b). This phase is monocyclic because no new ascospores are produced until the following spring. The second phase, called the secondary infections period, is caused by conidia produced on leaf and fruit lesions (Fig. 2c). This phase is polycyclic because new conidia are produced on new lesions until the end of the season. The quantity of initial ascosporic inoculum (Qa_0) is proportional to the quantity of disease the previous fall (Y_{max}) and is often referred as the potential ascospore dose (PAD). During the monocyclic phase the disease, severity will increase to reach a maximum denoted by Y'_{max} determined by the amount of ascosporic inoculum (Qa_0) and on suitability of weather for infection. In other words, the primary scab severity is limited by the amount of primary inoculum, higher is the quantity of ascospores, higher is the potential for primary infections (Fig. 3). During the polycyclic phase, the initial conidium inoculum is proportional to the amount of disease (Y'_{max}) at the end of the monocyclic phase ($Qc_0 = f[Y'_{max}]$) (Fig. 3). The

polycyclic epidemic may overlap monocyclic epidemics resulting in a rapidly progressing epidemic. These characteristics of apple scab epidemics are the foundations of most strategies to manage the disease. In theory, effective control of primary infections should result in low risk of secondary infection and hence allow for a more relaxed fungicide spray program during the summer. Thus, in most part of the world, the strategies to manage apple scab aim at controlling primary infections in order to avoid secondary infections and secondary inoculum build-up which will render management difficult and the objective of 1-2% fruit scab at harvest unattainable.

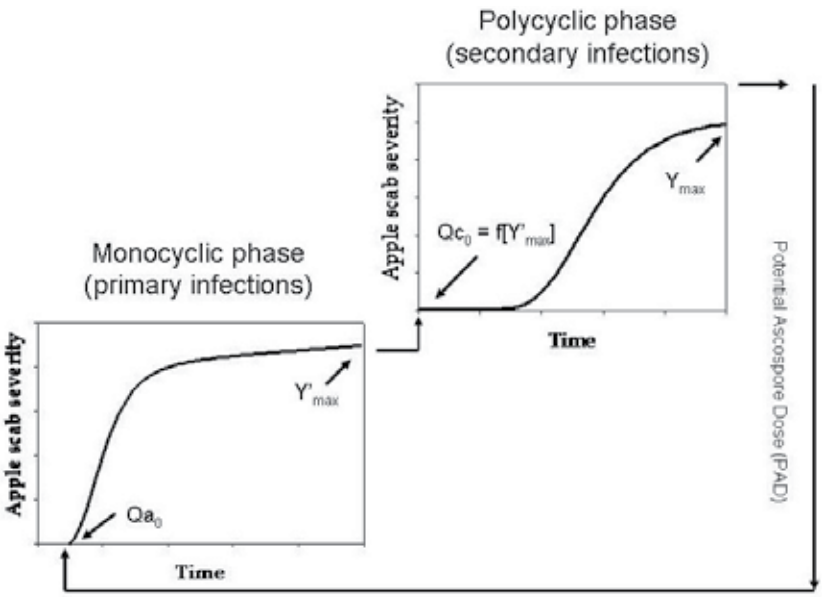

Fig. 3. Diagram describing the relationship between the monocyclic and polycyclic phase of the development of apple scab (*Venturia inaequalis*). Qa_0, is the quantity of ascosporic inoculum at the beginning of the primary infection period (monocyclic phase); Y'_{max}, the final disease severity at the end of the primary infection period, Qc_0, the quantity of conidial inoculum at the beginning of the secondary infection period (polycyclic phase); and Y_{max} the final disease severity at the end of the secondary infection period.

5. Development of disease decision support system (DDSS)

Even though, the term 'epidemic' is often used to describe a disease that develops sporadically and causes high level of damage, in this review we define epidemic as the dynamic of disease, explicitly, the change in the amount of disease with time. We defined the term 'model' as a simplified representation of a pathosystem. Botanical epidemiologists developed a large amount of models that could be used to make decisions regarding disease management and to improve our understanding of epidemic development. As a result, there is a variety of models that range from set of rules; mathematical equations; to dynamic simulation of epidemics. In botanical epidemiology an epidemic is the result of the interactions between a population of hosts (ex apple trees) and a pathogen population (ex. *V. inaequalis*). This interaction is constantly influenced by external factors such as climatic

conditions, cultural practices, aggressiveness of the pathogen, etc. (Table 1). These factors are related and interact together resulting in complex multilevel interactions denoted as a pathosystem. In some pathosystems, it is virtually impossible to model all interactions; hence the first step in modeling an epidemic is to identify the interactions that are essential for the progress of the epidemic.

Pathogen	Host	Environment
Abundance of pathogen (ascospore and conidial dose)	Susceptibility (quantitative or qualitative)	Temperature
Pathogenicity /aggressiveness	Single or mixed cultivars planting	Rainfall / Dew
Adaptability to host resistance or fungicides	Ontogenic resistance in leaves and fruits	Leaf wetness period
Dispersal efficiency (long and short distance)	Tree density, pruning,	Wind
Survival efficiency (winter survival)	Tree health	Soil properties
Reproductive fitness		Pesticide damage

Table 1. Example of factors that influence the dynamic of the apple scab pathosystem

The general approach in developing DDSS is to examine separately each important process in the pathogen life cycle and to model the factors which significantly influence the progress of this process. The resulting sub-models are depicted as diagrams and generally link together within a simulation program (Fig. 4). For apple scab, the pathosystem is defined as the general interaction between apple trees and *V. inaequalis*, the models representing the various process and sub-processes include the mathematical models relating weather conditions to overwintering of pseudothecia, maturation of pseudothecia, discharge of ascospores, dispersal of ascospores, leaf infections, spore germination, fungal colonization, sporulation, etc (Fig. 4).

6. Models describing the major sub-processes of apple scab epidemics

6.1 Models for estimating potential ascospore dose

As shown in Figure 3 and 4, the amount of primary inoculum is influenced by the amount of disease present the previous fall and to a less extent by the conditions during the winter months. Hence, MacHardy and coworkers developed the concept of Potential Ascospore Dose (PAD). Initially, the PAD was derived from lesion density, pseudothecial density, ascal density, leaf litter density, and the number of ascospores per ascus, and was expressed in ascospores per square meter of orchard floor (MacHardy, 1994; MacHardy 1996). Gadoury and MacHardy (1986) studied the relationship between PAD values and most of the variables used to calculate PAD values. They reported that the most important variables influencing variation in PAD values was the number of lesions/m^2 of apple leaf tissues and the percent leaves infected (scab incidence). From this work, it was concluded that the PAD could be derived from leaf scab incidence just prior to leaf fall. To facilitate the adoption of the PAD concept, a non-sequential sampling procedure was develop based on the relationship between leaf scab incidence on 600 extension shoots (10 shoots by 60 trees) and PAD values. However, the requirement to sample 600 shoots limited grower adoption of the

sampling procedure. As a result, a sequential sampling procedure was developed based on the analysis of 66 fall assessments of leaf scab (MacHardy et al., 1999; Reardon et al., 2005). This sampling procedure is use to classify orchards as "low risk" or "high risk" (Reardon et al., 2005).

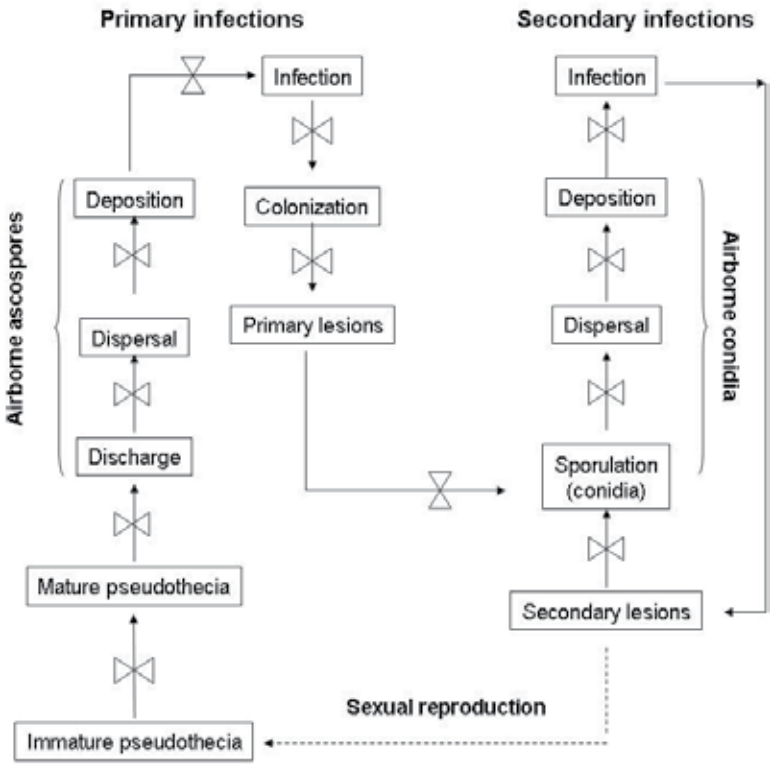

Fig. 4. Schematic representation of the relationships between the most significant sub-processes in apple scab epidemics

6.2 Models for estimating ascospore maturity

The monocyclic phase of apple scab development begins when the first ascospores are ejected and ends when the stock of ascospores is exhausted hence these biofixs represent the temporal boundary of the primary infection period. A widespread principle shared by most apple scab management programs is that the fungicide spray program could be ended or interval between fungicides applications increased when the seasonal stock of ascospores is exhausted. From a management standpoint, it is thus crucial to know or estimate accurately ascospore maturity and release. Ascospore maturity could be assessed by visual observations of pseudothecia and release by spore trapping. Work by Carisse et al. (2006) and Charest et al. (2002) on spatial distribution of airborne ascospores suggested that several traps would be required because of spatial heterogeneity in inoculum density and that the detection threshold may be lower than the density of airborne ascospores in a well-managed orchard. In addition, information of airborne ascospore concentration has no predictive value because by the time the information become available, infection already took place.

Hence, in practice, the period of availability of ascospores is predicted using models. The rate of ascospores maturation is mostly influenced by temperature; hence most models predicting ascospore availability are temperature–based. These models were developed to predict the first ascospore release (Proctor, 1982; James and Sutton, 1982) or the temporal dynamics of ascospore maturity (Gadoury and MacHardy, 1982; Rossi et al., 2000; Schwabe et al., 1989). The models most commonly used to predict ascospore availability use degree-day accumulation (Tbase 0°C) to predict proportion or percent of matured ascospores (Fig. 5).

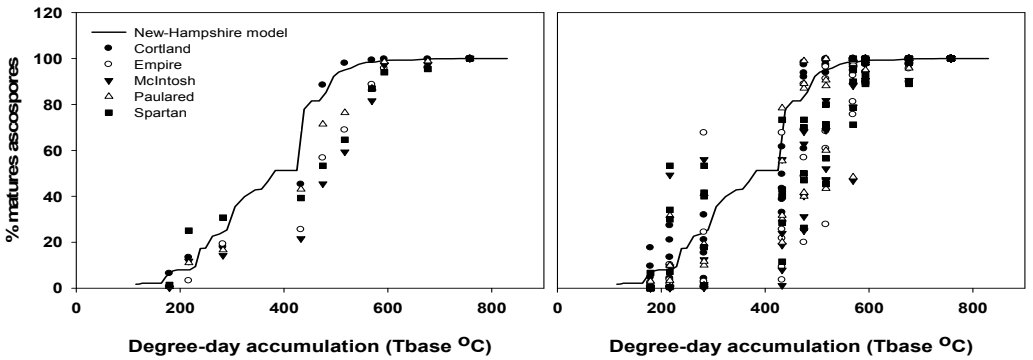

Fig. 5. Relationship between percent mature ascospores of *V. inaequalis* released from leaves of the cultivars Cortland, Empire, McIntosh, Paulared, and Spartan and degree-day accumulation since 50% silver tip (McIntosh). Percent mature ascospores are presented as average of 10 observations (left) and as individual observations (right). Lines represent the predicted values calculated from the model published by Gadoury and MacHardy, 1982.

6.3 Models to estimate risk of primary infections

Almost all DDSS developed for apple scab management are using the pioneer work of Mills and Mills and Laplante (Mills, 1944; Mills and Laplante, 1951). Before the Second World War, the only fungicides available for management of apple scab were sulphur and lime sulphur. To be efficient, sulphur must be applied before an infection event; the closest from the event the higher efficacy. Lime sulphur has some post infection activity provided that it is applied within few hours after the infection event. Hence tools were needed to determine the risk of infections. Mills (1944) analyzed available data on efficiency of primary infections as a function of duration of leaf wetness (hours) and temperature during the leaf wetness period (°C) and built a first table that relates the risk of infection to any combinations of leaf wetness duration at temperature ranging from 5 to 26°C. Mills and Laplante (1951) collected additional data during 20 years and concluded that minimum leaf wetness period required for infection was about 1.5 times longer than reported earlier. From his observations a table that depict the risk of *V. inaequalis* infections as a function a leaf wetness duration and temperature during the wet period (Fig. 6) was proposed. The initial version of this table commonly called the Mills' table was modified by several researchers to account for discontinuous leaf wetness period and conidial infections (Becker and Burr, 1994; Hartman et al., 1999; Schwabe, 1980; Sys and Soenen, 1970) and to extend the range of temperatures, mainly low temperatures, included in the table (Stensvand et al., 1997).

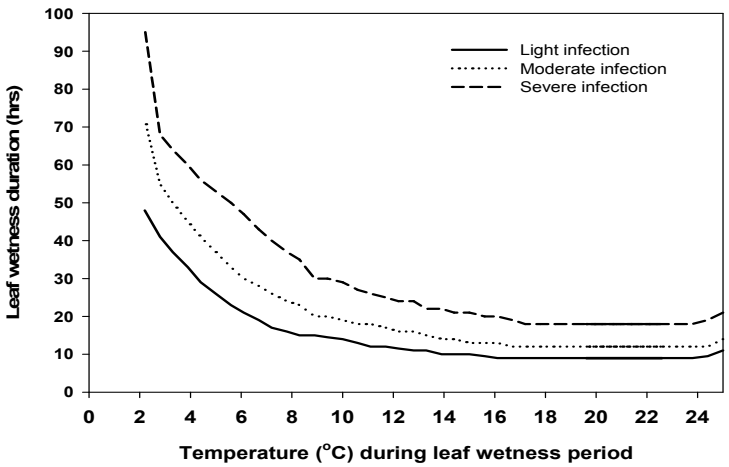

Fig. 6. Severity of infection by *V. inaequalis* as a function of temperature and surface wetness duration (hours) (Mills, 1944; Mills and Laplante, 1951).

These models form the foundation of most apple scab management programs which predominantly focus on management of primary infections (MacHardy, 1996). Much less attention has been paid to secondary infections, mainly because they are often assumed to depend largely on primary infections (Carisse et al., 2009; Holb et al., 2005). In the absence of management tools for summer scab, however, risk-averse growers may apply fungicides regularly, potentially resulting in an additional 6 to 8 sprays, whereas growers who are convinced that they have achieved adequate primary infection control may not spray enough during the summer, potentially resulting in yield losses (Holb et al., 2003; Holb et al., 2005; MacHardy, 1996). Nevertheless, despite the large amount of research that has been done on apple scab, the disease is still one of the most destructive and economically important disease in several apple production areas. Scab management may require 6 to 16 sprays annually depending on disease pressure, cultivar susceptibility, weather conditions, and marketing objectives (Reardon et al., 2005, Van der Sheer, 1992, Holb et al., 2005). Intensive use of fungicide in apple production has led to unstable control as fungicide resistance arose. The development of fungicide resistance is influenced by several factors such as the mode of action of the fungicide, the biology of the pathogen, and fungicide use pattern. Understanding how resistance develops, and how it can be managed is key to warrant sustainable disease control with fungicides.

7. Sustainability of fungicidal disease control

7.1 Fungicides used to manage apple scab

Fungicide used to manage apple scab could be divided into two types: protectant and penetrant. The protectant fungicides also called pre-infection fungicides are active on the plant surfaces where they stay after application. These fungicides do not penetrate into the plant; hence they can be washed off the plant and must be re-applied to new growth that develops after application. These fungicides, for example captan, are applied before

infections take place to inhibit the early stage of the infection process such as spore germination and penetration. Therefore protectant fungicides must be applied before the beginning of an infection, generally initiated by a rain event, or within 24 hours after the beginning of the infection. At a later stage of the infection process, the fungicide must penetrate through the outer surfaces of the apple leaves and fruits to reach *V. inaequalis* already grown inside the tissues. The penetrant fungicides, often referred as systemic or post-infection fungicides are absorbed into plants. These fungicides have different mobility into the plant. Some are locally systemic, moving on a short distance generally only within a few layers of plant cells, while others have a translaminar movement, moving from one side of a leaf to the other. Within a few hours of application, penetrant fungicides become rain fast. Several penetrant fungicides inhibit fungal growth and sporulation; hence they are effective when applied after spore penetration into the apple tissues. However, they have limited curative ability because they are effective only within the first 24- to 96-hour after fungal penetration into the plant.

Fungal pathogens including *V. inaequalis*, represent a community of different individuals with different sensitivities to fungicides. When the community is exposed to a fungicide, the surviving population is mostly constituted of individuals with reduced sensitivity that will better survive, reproduce and then increase their relative proportion in the population, treatment after treatment. At the individual level, in phytopathogenic fungi, reduced sensitivity is the result of a single or multiple changes (mutation, insertion or deletion) in the fungal genome, most often in the fungicide target genes. Reduced sensitivity to the fungicide is an ecological advantage under fungicide selection pressure; hence the proportion of less sensitive or resistant individuals will increase in sprayed fields if their fitness is not altered. The rate of increase in proportion of resistant individuals depends upon the initial frequency of resistance in the population, the biology of the fungus (population dynamics, number of cycles per season, migration, recombination due to the sexual stage…), the fungicide (mode of action, mainly single-site or multi-site, the resistance mechanism, possibly driven by a single or several genetic changes, and the fitness cost associated to the resistance mechanism) and the pattern of use of the fungicide (number and sequence of treatments, dose, anti-resistance strategies). Among these risk components, the number of genetic changes conferring the resistance is of major importance. When single genetic changes are involved, a rapid change toward resistance may arise (qualitative resistance), leading to a population that is largely resistant, and disease control failure. When multiple genetic alterations are involved, the change toward resistance develops gradually (quantitative resistance), leading to a reduced sensitivity of the entire population. The risk of resistance development in a fungal population is then a result of combination between the fungicide risk, the pathogen risk and the usage risk. For example, the QoI (strobilurin-based) fungicides are single site fungicides subject to single-change resistance within the target gene and hence are at high risk for resistance that may appear abruptly. Multi-site fungicides interfere with several metabolic processes of the fungus and hence several mutations affecting many sites in the fungus would be needed for resistance to develop, which is often deleterious for the fungus survival. Consequently, the risk of resistance to these fungicides is low. Typically, these fungicides are protectant and inhibit spore germination. Fungicides used to manage apple scab in Canada are described in Table 2.

FRAC CODE	Mode of action	Group name	Chemical group	Active ingredient
3	C14- demethylase in sterol biosynthesis (erg11/cyp51)	DMI-fungicides (DeMethylation inhibitors)	triazoles	flusilazole
3	C14- demethylase in sterol biosynthesis (erg11/cyp51)	DMI-fungicides (DeMethylation Inhibitors)	triazoles	myclobutanil
9	methionine biosynthesis (proposed) (cgs gene)	AP – fungicides (Anilino- Pyrimidines)	anilino-pyrimidines	cyprodinil
9	methionine biosynthesis (proposed) (cgs gene)	AP – fungicides (Anilino- Pyrimidines)	anilino-pyrimidines	pyrimethanil
11	complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)	QoI-fungicides (Quinone outside Inhibitors)	oximino acetates	kresoxim-methyl
11	complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)	QoI-fungicides (Quinone outside Inhibitors)	oximino acetates	trifloxystrobin
7 + 11	complex II: succinate-dehydrogenase + complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)	SDHI (Succinate dehydrogenase inhibitors) + QoI-fungicides (Quinone outside Inhibitors)	pyridine-carboxamides + methoxy-acrylates	boscalid + pyraclostrobin
29 + M	uncouplers of oxidative phosphorylation + multi-site contact activity	--	dinitrophenyl crotonates	dinocap + mancozeb
M	multi-site contact activity	Phthalimides	phthalimides	captane
U12	unknown mode of action	Guanidines	guanidines	dodine
M	multi-site contact activity	dithiocarbamates and relatives	dithio-carbamates	mancozeb
M	multi-site contact activity	dithiocarbamates and relatives	dithio-carbamates	metiram

Table 2. Description of the fungicides registered in Canada for management of apple scab (*Venturia inaequalis*).

Active ingredient	% active ingredient	Recommended dose range LOW to HIGH (kg or L/ha)		EIQ	FIELD EIQ range LOW to HIGH		Resistance risk
fFlusilazole	20	0.10	0.20	45.94	0.92	1.84	medium
myclobutanil	40	0.34	0.34	24.01	3.27	3.27	medium
cyprodinil	75	0.37	0.37	26.77	7.43	7.43	medium
pyrimethanil	40	0.75	1.00	12.67	3.80	5.07	medium
kresoxim-methyl	50	0.18	0.36	15.07	1.36	2.71	high
trifloxystrobin	50	0.14	0.18	29.78	2.08	2.61	high
boscalid + pyraclostrobin	25.2% boscalid + 12.8% pyraclostrobin	1.00	1.20	26.44 + 27.01	10.12	12.14	medium + high
dinocap + mancozeb	4.7% dinocap + 72% mancozeb	6.75	6.75	46.51	244.25	244.25	low / unknown - low
captane	50	3.00	6.00	15.77	23.66	47.31	low
dodine	65	1.08	3.25	23.45	16.46	49.54	Low to medium risk.
mancozeb	75	5.00	6.00	25.72	96.45	115.74	low
metiram	80	4.50	6.00	40.61	146.20	194.93	low

Table 3. Risk of resistance development and environmental impact of the fungicides registered in Canada for management of apple scab (*V. inaequalis*).
Environmental Impact Quotient (EIQ): The Environmental Impact Quotient (EIQ) has been used to classify toxicological data available on some common pesticides into a practical form for application usage by taking in consideration most of the environmental concern in agriculture. As stated by Kovach et al. (1992), each pesticide has an EIQ reflecting its impact on consumers, farm workers and a combination of different living organisms in the ecosystem. From this EIQ value, a field use rating can be calculated using a simple formula: Field EIQ = EIQ * % active ingredient * rate of application.

7.2 Resistance management strategies

Despite the availability of a large variety of fungicides, for most of them, there is a conflict between environment safety profile and risk for resistance (Table 3). Most of the fungicides at moderate to high risk for resistance including DMIs and QoIs have a good human and environment safety profile. On the contrary, multi-site fungicides which are at low risk for resistance generally have a bad environment safety profile (Table 3) (Schneider and Dickert, 1994). To maintain the efficacy of fungicides, it is important to implement fungicide resistance program, ideally before resistance becomes a problem. In practice, most growers do not know the level of fungicide resistance present in the pathogen's population. In

absence of knowledge on the level of resistance and of tools to accurately predict how fast resistance will develop, it is advisable to follow some anti-resistance measures such as use at-of risk fungicides only when needed ideally only at key times in the season, when disease pressure is low use fungicide at low risk for resistance. If at-risk fungicides must be used, they should be mixed with a fungicide at low risk, used only a few times per season and used in rotation with fungicide with a different mode of action. For the most part, evaluations of the effectiveness of these strategies have not been assessed scientifically. The merits of tank-mixing compared to alternating sprays have been debated. Some theorize that tank-mixing reduces selection pressure only when the partner fungicide is highly effective and good coverage is achieved. Alternating fungicides is thought to act by reducing the time of exposure. In practice, examples can be cited for the effectiveness of both approaches. Nevertheless, both practices are more effective when cultural practices are implemented to reduce disease pressure. To prolong the effectiveness of fungicides and usefulness to growers, implementation of resistance management strategies is crucial. However, monitoring resistance levels in pathogen populations is essential for assessing risk and evaluating the effectiveness of disease and anti-resistance management practices.

7.3 Monitoring fungicide resistance

Regardless of the method used, monitoring the sensitivity of fungicide resistance of fungal population is time consuming and costly. Methods for monitoring resistance are described in detail in FRAC monograph No 3. (Russel, 2004). In brief, these methods could be broadly grouped in biological and molecular methods. Biological methods are based on the detection of the baseline sensitivity difference of a sample from the wild type population (not exposed to the fungicide). These tests can be performed either on solid agar in Petri dishes with visual rating (germination, germ tube elongation and mycelia growth) or in liquid medium using a spectrophotometer or biomass weight (rating of mycelial growth

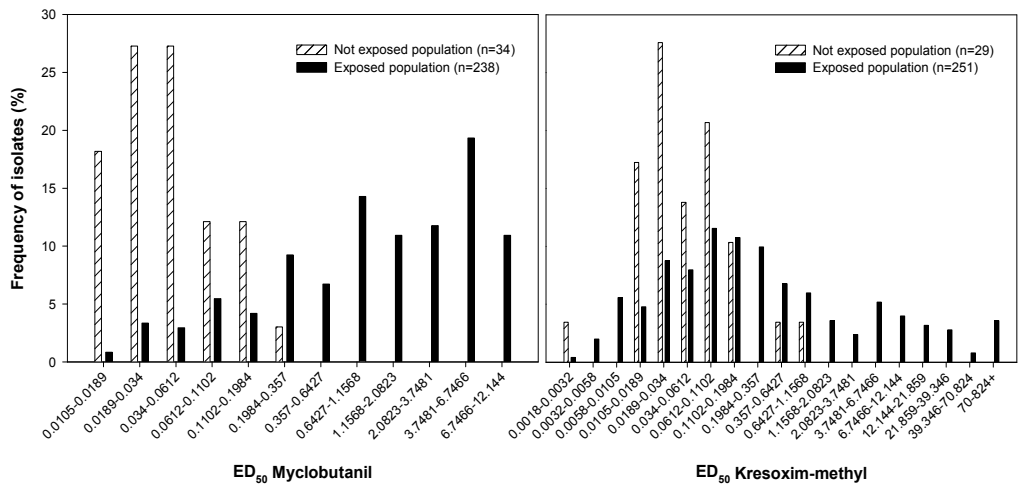

Fig. 7. Frequency distributions of ED_{50} values for myclobutanil and kresoxim-methyl of *V. inaequalis* in 2003 from orchards located in eastern Canada. ED_{50} values (in µg/ml) are based on mycelial radial growth on PDA amended with different doses of the fungicides (from Jobin and Carisse, 2007).

only). This method was widely used to study the status of resistance in *V. inaequalis* populations from various apple production areas (Carisse and Jobin, 2010; Carisse and Pelletier, 1994; Jobin and Carisse, 2007) (Fig. 7).

Molecular methods are based on the detection and/or quantification of the mutations in the fungal genes that confer the resistance to the fungicide. Depending on the type of resistance, single mutation, single nucleotide polymorphism (SNP), or more than one SNPs are involved. Even when only one mutation is detected in most of the resistant isolates, it may be not reliable to monitor resistance based on this single mutation without considering that some resistant isolates might have other mutations and that the proportion of these may increases as the population is exposed to the fungicide. Nevertheless, molecular methods have helped understand the mechanisms of fungicide resistance and develop fast method for detection or quantification of resistant pathogen genotypes. For *V. inaequalis*, molecular methods were developed only for resistances whose mechanism was fully identified, i.e. resistance to anti-microtubules in benzimidazoles (carbendazim, thiophanate-methyl) and inhibitors of mitochondrial complex III (strobilurins) (Table 4). Resistance to multisite inhibitors, DMIs, anilinopyrimidines and SDHIs are still monitored with biological tests. For benzimidazoles and QoIs, resistance was found to be determined by single mutation within the target genes (β-tubulin and cytochrome b, respectively) (Quello *et al*, 2010; Fontaine *et al*, 2008). These mutations were detected after partial PCR amplification of these genes and digestion of the PCR product by a restriction enzyme that recognizes the restriction site generated (or lost) by the mutation determining resistance (PCR-RFLP or CAPS test). Digestion patterns are scored on an electrophoresis gel and are a direct indicator of the presence of the mutation. This test was developed for mono-lesion isolates, in the case of benzimidazoles and for bulk population of spores in the case of QoIs. Moreover, an allele-specific PCR test (AS-PCR) was also developed to detect the G143A change within the cytochrome b of *V. inaequalis* (Fontaine *et al*, 2008). Roughly, PCR was achieved using primers differing by their 3' nucleotides, enabling to detect specifically either the susceptible or the resistant allele of the sample. This method was particularly adapted for bulk population samples because of its higher specificity. At last, when molecular and phenotypic data are not coherent, sequencing or pyrosequencing are largely used by researchers to characterize novel mutations within the target gene or to refute the hypothesis of target alteration as a possible resistance mechanism.

8. A DDSS that integrates the three components of the apple scab pathosystem: Pathogen-host-environment

The structure of DDSS for apple scab management varies from one simple model such as the Mills rules for infections, coupled with simple models such as ascospore maturity models up to more complex simulation models (Rossi et al., 2007). The simple rule-based DDSS may not be sufficient to make proper estimation of risk, while the complexity of some simulation models refrain growers from using them. In addition, the decision as to whether to spray also depends on considerations not related to the pathogen development among which the time elapsed since the last spray, amount of weathering of last fungicide spray, time to the next forecasted infection period, opportunity to apply the fungicide and amount of new foliage not protected by the last fungicide spray. They are only few DDSS that consider leaf growth as a risk factor even though, from a yield loss perspective, it is probably one of the most important factors. Considering that the main goal of most scab management program is to

Resistance mechanism	Resistance detection method	Reference
Benzimidazoles: Target alteration: 3 altered codons and 4 resistant alleles in the β-tubulin gene	*In-vitro* (single-spore isolates; mycelial growth) PCR-RFLP (single spore-isolates)	Quello et al, 2010
Anilinopyrimidines: Unknown	*In-planta* (bulk spores populations; apple seedlings)	FRAC http://www.frac.info
DMIs: Unknown Possibly target alteration	*In-vitro* (mono-lesion spore suspension; germination and germ-tube elongation) *In-planta* (bulk spores populations; apple seedlings)	Gao et al, 2009 Jobin and Carisse, 2007 FRAC http://www.frac.info
QoIs: Target alteration: G143A change in the cytochrome b change Unknown resistance mechanism (detoxification or activation of the alternative oxidase?)	*In-vitro* (bulk spores populations; germination, germ-tube elongation and mycelium growth) AS- PCR (bulk spore population) CAPS (bulk spore population)	FRAC http://www.frac.info Steinfeld et al, 2001 Jobin and Carisse, 2007 Fontaine et al, 2008

Table 4. Summary of the methods used to monitor resistance in *Venturia inaequalis* for different class of fungicides.

control foliar scab during the primary infection period in order to avoid secondary epidemic build up during the summer months, knowing how many leaves are protected is crucial.

Concept. The critical time for spraying fungicide against apple scab is during the peak of ascospore release and the period of rapid leaf emergence (Fig. 8). Currently, most apple scab management programs are ''pathogen oriented'' in other words based on the estimated amount of mature ascospores of *V. inaequalis* and on the predicted severity of ascospore infection (MacHardy 1996). However, another strategy more ''host oriented'' is to prevent ascospore infections by protecting new leaves (susceptible to *V. inaequalis*) with appropriate fungicides. Hence, we developed a new DDSS for assessing the day-to-day variation in risk of primary infection and weekly risk for secondary infections by *V. inaequalis.* The system comprises three components to time fungicide sprays against primary infections and one component to time fungicide sprays against secondary infections. The need for fungicide sprays are determined based on risk of primary infections estimated from 1) the number of unprotected leaves (new leaves since the last spray), 2) the availability of ascospores calculated from degree-day accumulation, expressed as low, or high availability of ascospores, and 3) infection risk based on Mills table, expressed as no, low, moderate and

high risk. The need for fungicide sprays during the summer is based on monitoring leaf scab incidence and using a threshold of 0.5 or 1.0% leaf scabbed (Carisse et al., 2009). Assessment of disease risk occurs daily, although hourly weather data are required to calculate degree-days and identify the beginning of rainfall event, duration of surface wetness and temperature.

Component 1. Estimation of daily leaf emergence. Because temperature has been reported to be the main factor affecting early season leaf emergence, cumulative degree-days were used as a predictor of leaf emergence (Carisse et al., 2008). Emergence of cluster and terminal apple leaves was monitored over several years for different rootstocks, cultivars and sites. The pattern of both cluster and terminal leaf emergence was not affected by rootstocks or cultivars, although variations were observed among years and sites. These patterns of leaf emergence on cluster and terminal shoots as a function of cumulative degree-days (Tbase 5°C) were described by the Richards and the Weibull models, respectively, (Carisse et al., 2008). In Eastern Canada, the first cluster leaves emerged from the end of April to early May, and then the number of leaves per cluster shoot increased rapidly to reach a plateau of about eight leaves per shoot in mid-May (Fig. 8). The pattern of leaf emergence is different for the terminal leaves. In Eastern Canada, the first leaves emerged during the first week of May and emergence of subsequent leaves was gradual and at a lower rate than for the cluster leaves until the end of June, which corresponded more or less to the end of the primary scab infection period (Fig. 8).

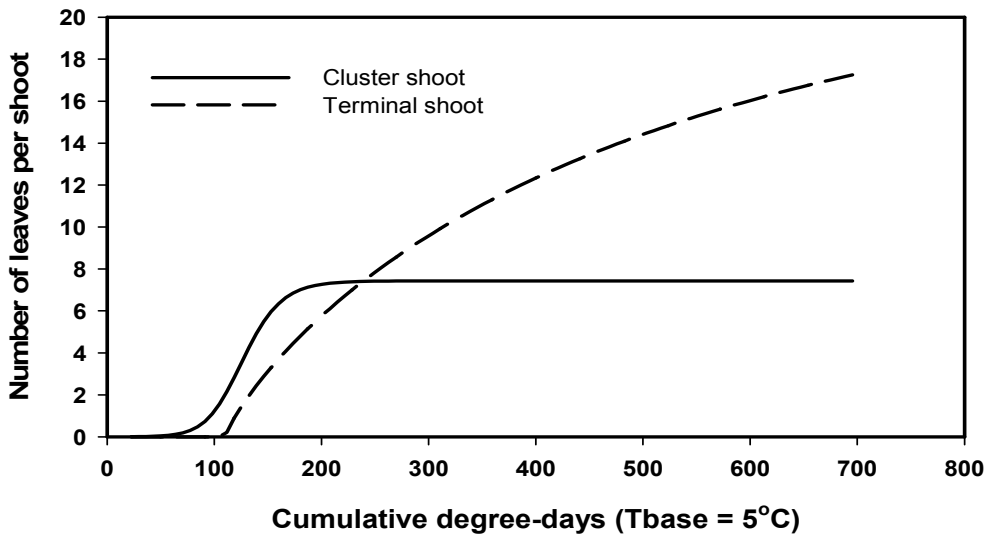

Fig. 8. Temporal progress of the number of leaves per cluster or terminal apple tree shoot as a function of accumulation of degree days (Tbase=5°C) since April 1st.

Component 2. Estimation of ascospore availability. The DDSS assume that there will always be mature ascospores at bud break and that the amount of primary inoculum (ascospores) in the overwintering leaves is low (well managed orchard with less than 5% leaf scab at harvest the previous fall) or high (poor control during the previous season and >5% leaf scab at harvest the previous fall). The rate at which ascospores mature in the overwintering leaves on the orchard floor is determined mainly by temperature (Gadoury

and MacHardy, 1982). In our DDSS, the biofix to begin accumulating DD is April 1st and the base temperature is 5°C. Regardless of the potential ascospore dose (PAD), ascospore maturation occurs in three phases. In the first stage, ascospores mature slowly because temperature is usually cooler, then maturation accelerates with increasing temperatures. Finally, the last ascospores mature until exhaustion of the stock in pseudothecia. In practice, there is so much variation in the proportion of mature ascospores between leaves and among cultivars (Fig.5.) (Carisse et al., 2006) that degree days were used to determine the period of availability of ascospores from about 50 to 350 CDD. At 350 the DDSS assume that over 95% the ascospore supply is depleted if sufficient rain events have occurred). This marks the end of primary infection season. When the CDD is between 50 and 100 or >300 the availability of ascospore was considered as low, while when the CDD are between 100 and 300 the availability was considered as high. Ascospore release is strongly light dependent (Brooke, 1969); hence the DDSS assume that only a small portion of available ascospores is released at night. Ascospores are not released to any significant extent during night hours in low inoculum orchards (MacHardy, 1998). In high inoculum orchards, although the percentage of ascospore released is small, the total number of ascospores released may cause significant primary infections. Hence in the DDSS ascospore release is triggered by a rain that begins during the day or at any time of the day in low and high primary inoculum orchards, respectively.

Component 3. Estimation of primary infection risk. Conditions suitable for the infection of leaf and fruit tissues by ascospores are identified using the surface wetness duration and temperature criteria of Mills & Laplante (1954), with the modification of MacHardy & Gadoury (1989). The algorithm outputs a continuous variable based on four wetness-based risk categories: nil, light, moderate and severe (Fig. 6). Since ascospore ejections during the night are low, it is possible to exclude nocturnal hours of leaf wetness (except in the case of a massive ejection period in a high inoculum orchard). When two wet events (rain) are interrupted by a non-wet period with high relative humidity (<85%) calculation is done on the entire period. On the other hand, if the interruption is dry (HR<85% and <24 hours), the two wet events are added without consideration for the dry period (Becker et al.,1994).

Component 4. Determining the need for fungicide sprays against secondary scab. Summer scab is managed based on a foliar scab action threshold of 0.005 scabbed leaves per shoot (0.5%) and classification of leaf scab incidence as above or below the threshold using the SSCP developed by Carisse et al. (2009). The first orchard classification is conducted in mid-June and the orchard is classified as above or below the action threshold of 0. 5% foliar scab or as in the "no decision" zone. The following criteria is used to time fungicide sprays: 1) if the orchard is classified as above the action threshold, a 7 to 14-day summer spray program is initiated and continued until harvest unless a prolonged dry period is forecasted, in which case the orchard is reassessed. Unless that reassessment classified the orchard as below the threshold, the summer spray program is maintained. 2) if the orchard is classified as below the action threshold, fungicide spray applications are postponed until the threshold is reached. If the action threshold of 0.5% foliar scab is not reached by mid-August, a less conservative threshold of 1.0% foliar scab could be used from then until harvest. 3) If the orchard is classified as in the "no decision" zone, sampling is repeated 7 to 14 days later depending on the frequency of rainy days. With the SSCP, a minimum of 50 and a maximum of 240 shoots per orchard section are sampled, and care must be taken to select shoots that were representative of the trees (i.e. shoots at the top, bottom, edges, and center of the canopy).

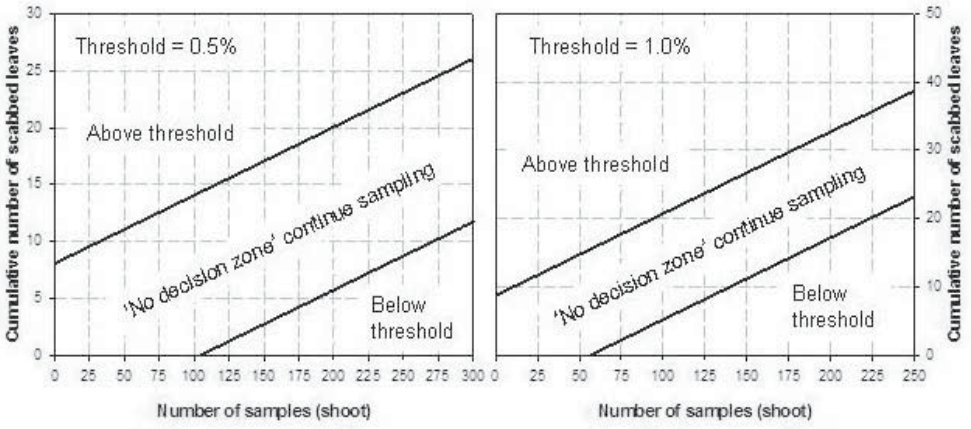

Fig. 9. Sequential sampling for classification plans for classifying scab incidence in apple orchards as above or below action thresholds of 0.005 (A) and 0.01 (B) scabbed leaves per shoot (from Carisse et al., 2009).

Orchard testing of the DDSS. The DDSS was tested in 2008, 2009, and 2010 at the Agriculture and Agri-Food Canada Experimental Farm, in Frelighsburg, Quebec, Canada. The DDSS was tested in a 0.72 ha orchard implemented in 2002 with a tree density of 1470 tree per ha and composed of six apple cultivars with various level of susceptibility to *V. inaequalis* (Dewdney et al. 2003): Spartan, Cortland, McIntosh, Paulared, Empire and Gingergold, grafted on M9, B9, M9, M9, EM26, B9 rootstocks, respectively. An additional block planted with the cultivar McIntosh grafted on M9 was used as a control and sprayed according to the standard practices. The DDSS was used to identifying the key time for fungicide applications based on the number of leaves unprotected (new leaves since the last spray) with an action threshold of 0.5 new leaves. The choice of fungicide was done according to the anti-resistance management approach describe previously. Briefly, at-risk fungicides were used only during period of high risk for disease development, each at-risk fungicide was not used more than twice per season and when possible tank-mixed with fungicide at low risk for resistance. Fungicides at high risk for the environment were used to control secondary infections only when the disease was above the action threshold. A few days before harvest, leaf scab incidence was estimated from a sample of 100 shoots randomly selected within each orchard section, and fruit scab incidence was estimated from 25 fruits on 10 trees selected at random within each section. The number of fungicide sprays and the seasonal environmental impact quotient (EIQ) was calculated for each cultivar (Kovach et al., 1992).

Over the three seasons of the trial, the incidence of scab and the weather conditions varied considerably. In 2008 and 2009, the weather conditions during the spring were highly favorable for apple scab development, while in 2010 rains events were sporadic. Nevertheless, for the three years, it was possible to maintain the disease under the economic threshold of 1% fruit scab at harvest. However, in 2009 leaf scab at harvest on the highly susceptible cultivar was above the threshold of 5% leaf scab, hence in 2010 it was treated as high inoculum orchards (MacHardy, 1998). Despite the high level of leaf scab in 2009, the incidence of leaf scab in 2010 was low, showing the importance of spring conditions in determining the severity of epidemics. The difference in weather conditions during both the

primary and secondary scab seasons demonstrated the value of using DDSS to assist in fungicide spray decisions. In 2008 and 2009, the DDSS enable better timing of fungicide sprays compared to the standard spray program resulting in better scab control with fewer sprays (Fig. 10). In 2010, a season with scab risk much lower, the DDSS resulted in large reduction in amount of fungicides used (Fig. 10). The DDSS provided information valuable in timing fungicide sprays. Overall, the DDSS enable a better scab control with minimum usage of at risk fungicides and much lower environmental impact of the spray program (Fig. 10) (Table 5).

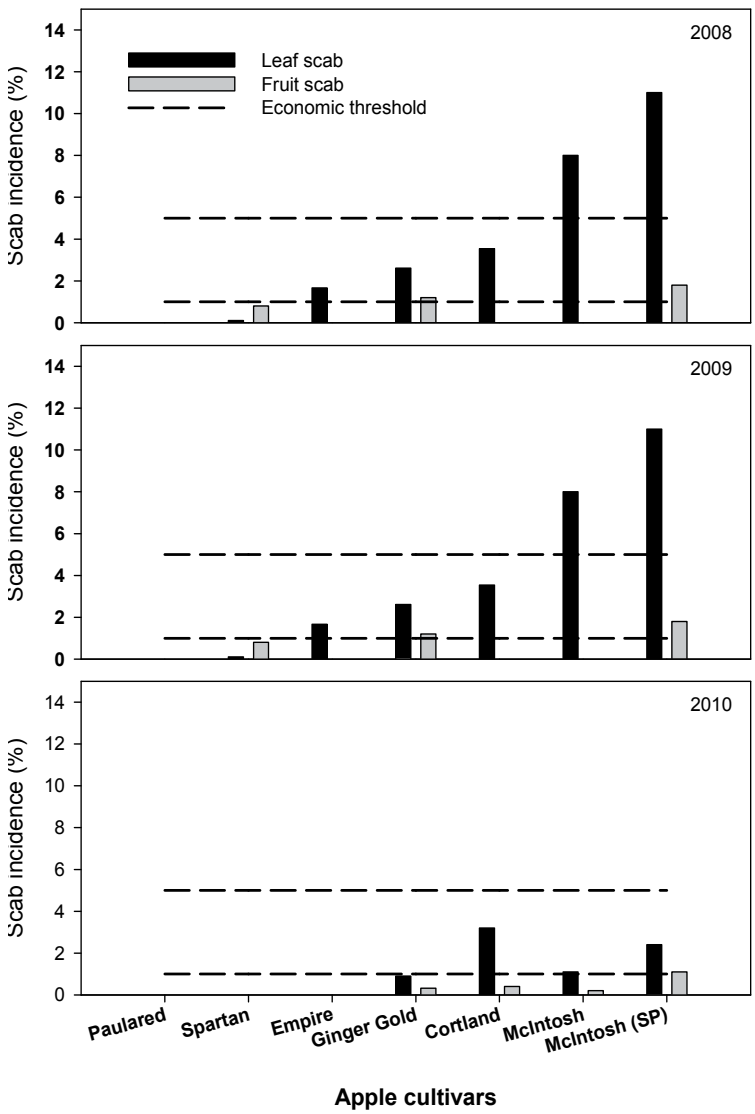

Fig. 10. Leaf and fruit apple scab incidence at harvest on cultivars with different susceptibility from orchards managed using the DDSS and compared to a standard fungicide spray program (McIntrosh-SP).

Cultivar	Number of at risk fungicides	Total number of applications	EIQ
Paulared	1.67	6.33	448.00
Spartan	2.67	9.33	657.42
Empire	2.67	10.33	746.05
Ginger Gold	3.00	11.00	746.05
Cortland	3.00	12.00	939.08
McIntosh	3.00	12.67	1019.83
McIntosh (SP)	4.67	15.67	1230.32

Table 5. Fungicide use pattern for apple cultivars with different level susceptibility to *V. inaequalis* managed using the DDSS and for the cultivar McIntosh managed using a standard spray program.

9. Conclusion

In this review we demonstrated the complexity of disease management decision making and the usefulness of DDSS in identifying the key time for applying fungicides. Much more information will be needed to demonstrate the long term effects of optimal use of fungicide on the development of resistance and hence on the durability of the fungicide effectiveness. Most of the DDSS available for management of apple scab are entirely weather-based. With our DDSS, growers are required to inspect their orchard for the presence of leaf and fruit scab. This may be regarded as time consuming but determining if the disease is above or below an action threshold is the foundation of integrated disease management. If we consider the long term value of optimal fungicide usage, it is certainly worth doing it. Ideally, fungicide resistance should also be monitored routinely. Once rapid and inexpensive methods become available, it will be possible to detect shift in *V. inaequalis* population and to adjust the fungicide use pattern accordingly.

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Part 2

Fungicide Resistance

10

The QoI Fungicides, the Rise and Fall of a Successful Class of Agricultural Fungicides

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1. Introduction

Fungal diseases represent a major threat to crops. To cope with this problem, growers use enormous amounts of chemicals. Fungicides have long been used to reduce crop losses. Chemical control of plant diseases started to develop in the latter half of the nineteenth century when recommendations regarding the use of fungicidal preparations based upon the active ingredients sulphur, lime and copper sulphate were made. Up until the 1940s, the arsenal of available fungicides was very small. Chemical-based disease control continued to rely predominantly upon such inorganic chemical preparations that were frequently prepared by the user. The Second World War was a period of great advances in the chemical industries of most countries. The post-war period thus saw the introduction of new chemistry and new compounds based on pre-war chemistry (e.g., dithiocarbamates and phthalimides). Virtually all components were protectants i.e., they needed to be present on the crop at the time that the fungal infection arrived; once the infection was established, they were useless. A notable addition to these was the introduction in 1955 of an antibiotic designed in Japan specifically for rice blast control, blasticidin S. This was the first evidence that a systemic product could be applied on a field scale (Russell, 2005).

The 1960s was a time of considerable expansion in chemical crop protection. The start of this period saw some consolidation of previous years with further introductions from established areas of chemistry. Compounds such as mancozeb (a dithiocarbamate) and captafol (a phthalimide) were introduced with great success. Nevertheless, the major achievement of this decade was the introduction of the systemic fungicides thiabendazol and benomyl, both characterised by a methyl benzimidazole carbamate (MBC) mode of action, which marked the advent of internal plant therapy. In addition, chlorothalonil (a phthalonitrile), carboxin and oxycarboxin (carboxanilides), ethirimol (2-aminopyrimidine) and the first members of the morpholine fungicides, dodemorph and tridemorph, were introduced at this time (Russell, 2005). However, the 1970s was possibly the most significant decade for advances in crop protection chemistry. At this time the agrochemical market was growing at a rate of approximately 6.3% per annum in real terms (Finney, 1988). Fosetyl-Al, a phosphonate fungicide with the unique property of being phloem mobile, was one of the major discoveries of the 1970s. It was followed by metalaxyl, the first of the phenylamides,

and propamocarb, a carbamate fungicide. Options for disease control were expanded in the 1980s through the introduction of several triazoles, the first members of the sterol demethylation inhibitor (DMI) class of fungicides and several additional members of the morpholine group (Russell, 2005).

Over the last twenty years, the standards of disease control have been further improved by the development of the strobilurins (Bartlett et al., 2002). They are a truly remarkable group with activity against all major fungal genera. They exhibit protectant, systemic and eradicant action. The first strobilurin fungicides to reach the market were azoxystrobin and kresoxim-methyl; the development of similar compounds soon followed. Collectively, they are called the QoI group, a term derived from their mode of action in binding at the Qo site of cytochrome *b*. The strobilurins quickly became one of the most important agricultural fungicides, accounting for over 20% of the global fungicide market within the first ten years of their commercial introduction. However, one of the apparent strengths of systemic fungicides, their high specific mode of action, also proved to be a serious weakness. Soon after their introduction, QoI resistant isolates of *Blumeria graminis* f. sp. *triciti* on wheat and *Plasmopara viticola* on vines were documented (Heaney et al., 2000). Since then, resistant strains of over thirty different plant pathogens have emerged. This fungicide group is considered to be high risk; thus, strict anti-resistance strategies including limiting the number of treatments and using mixtures or alternations, must be taken. In most cases, resistance resulted from modification of the cytochrome *b* target site. However, an increasing amount of experimental evidence has suggested that QoI resistance can occur via other mechanisms (Fernández-Ortuño et al., 2008b). This chapter reviews our current knowledge of this successful class of agricultural fungicides and the mechanisms of resistance to QoI fungicides in phytopathogenic fungi, furthermore, it discusses the implications of the emergence of resistance and for risk assessment, including aspects such as the fitness cost associated with QoI resistance.

2. Discovery and structure of the QoI fungicides

Strobilurins are natural substances produced mainly by basidiomycete wood-rooting fungi such as *Strobilurus tenacellus* (Pers. ex Fr.) Singer and *Oudemansiella mucida* (Schrad. ex Fr.) Hohn, or by the gliding bacterium *Myxococcus fulvus* (Bartlett et al., 2002). As such, their name derived from the genus *Strobilurus*. Strobilurin A, the first QoI molecule, was isolated from liquid cultures of *S. tenacellus* by Anke et al. (1977). Similar compounds were subsequently identified and named in the order of their discovery (i.e., strobilurin B, C, D and so on). These compounds were isolated by chromatographic means, and their molecular structures were identified by high-resolution mass spectrometry. Further spectroscopic analyses were performed to determine their molecular structures as described in the work of Schramm et al. (1978). In fungi, these products are biosynthesised from phenylalanine via the shikimic acid cycle (Balba, 2007). Structurally, the basic common feature of all natural strobilurins is the presence of a methyl (E)-3-methoxy-2-(5-phenylpenta-2,4-dienyl) acrylate moiety, linked to the rest of the molecule at the α-position. They have therefore been named *β*-methoxyacrylates or MOAs (Sauter, 2007). They vary only in the aromatic ring substitutions at positions 3 and 4 (Fig. 1).

These natural compounds break down rapidly in light and are therefore not reliable for disease control. However, knowledge of their structures and physical properties provided the starting point for independent research programmes within the chemical companies

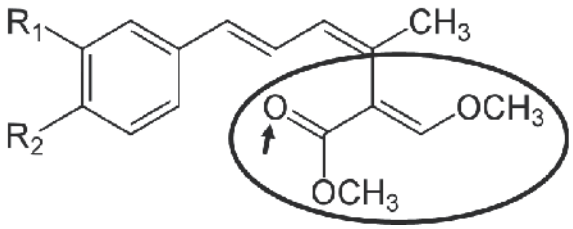

Fig. 1. The basic chemical structure of natural strobilurins. The *β*-methoxyacrylate moiety is highlighted with a circle and the carbonyl oxygen responsible for binding is indicated by an arrow. R_1 and R_2 represent radicals that are distinct in the different naturally-occurring strobilurins.

Syngenta and BASF. During the process of lead structure optimisation, thousands of QoI analogues were synthesised and tested before suitable photo-stable compounds with fungicidal activities were developed. Most of these attempts were focused on the modification of the α-substitution in the (E)-*β*-methoxyacrylate group. One of the important directions taken in the structural modifications was the replacement of the basic toxiphoric group of (E)-*β*-methoxyacrylate with a methoxyiminoacetate group. This modification was led by BASF. In 1992 the first QoI fungicides, azoxystrobin from Zeneca (now Syngenta) and kresoxim-methyl from BASF, were announced (Godwin et al., 1992; Ammermann et al., 1992). These fungicides were made commercially available in 1996 (Fig. 2).

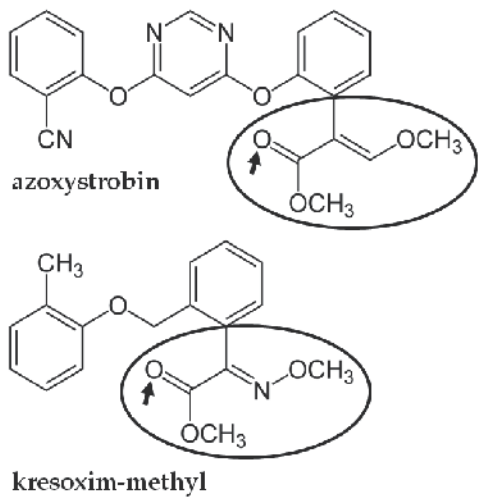

Fig. 2. The chemical structures of azoxystrobin and kresoxim-methyl. The (E)-*β*-methoxyacrylate group of azoxystrobin and the methoxyiminoacetate group of kresoxim-methyl are highlighted with circles and the carbonyl oxygen moieties responsible for binding are indicated by the arrow.

The development of similar compounds soon followed. A further modification was the replacement of the (E)-*β*-methoxyacrylate group with 2-methoxyiminoacetamide. This modification was marketed under the name metominostrobin. In addition to strobilurins, there are other compounds such as famoxadone and fenamidone (Fig. 3) that are chemically distinct from strobilurins but are part of the same QoI fungicide cross-resistance group.

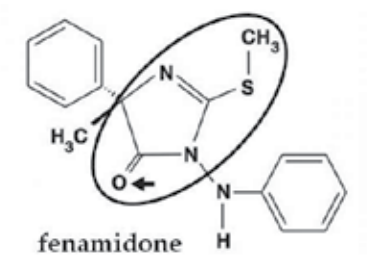

Fig. 3. The chemical structure of fenamidone. The toxophore moiety is highlighted with a circle and the carbonyl oxygen responsible for binding is indicated by an arrow.

Several products resulting from these development efforts are now commercially available. At present, based on structural similarities, eight chemical groups of Qo inhibitors can be distinguished, representing fifteen QoI active ingredients (Table 1). Commercial QoI fungicides are among the best-selling agricultural fungicides worldwide, and are used primarily as plant protectants against most major fungal and oomycete pathogens.

Classes	Fungicides	Current owner	First sales
Methoxyacrylates	Azoxystrobin	Syngenta	1996
	Picoxystrobin	Syngenta	2002
	Enestrobin	SS[a]	-
	Pyraoxystrobin	SRICI[b]	-
Oximinoacetates	Kresoxim-methyl	BASF	1996
	Trifloxystrobin	Bayer	1999
Oxazalidinediones	Famoxadone	DuPont	1997
Oximinoacetamides	Metominostrobin	Bayer	1999
	Dimoxystrobin	BASF	2004
	Orysastrobin	BASF	2006
Imidazolinones	Fenamidone	Bayer	2001
Methoxycarbamates	Pyraclostrobin	BASF	2002
	Pyrametostrobin	SRICI	-
Dihydrodioxazines	Fluoxastrobin	Bayer	2004
Benzylcarbamates	Pyribencarb	KCI[c]	-

[a]Sinochem Shanghai
[b]Shenyang Research Institute of Chemistry Industry
[c]Kumiai Chemical Industry

Table 1. The QoI fungicides.

3. Biochemical mode of action of QoI fungicides

The QoI fungicides display a single-site mode of action that was elucidated in 1981 by Becker et al. The fungicidal activity of QoI fungicides relies on their ability to inhibit mitochondrial respiration by binding at the Qo site (the outer, quinol oxidation site) of the cytochrome bc_1 enzyme complex (complex III). This inhibition blocks the transfer of electrons between cytochrome *b* and cytochrome c_1, leading to an energy deficiency in the fungal cells by halting the production of ATP (Fig. 4), and ultimately leading to fungal death. The QoI target, cytochrome bc_1, is an integral membrane protein complex essential for

fungal respiration. In eukaryotes it comprises 10 to 11 different polypeptides with a combined molecular mass of roughly 240 kDa, and operates as a structural and functional dimer. Cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein (ISP) form the catalytic core of the enzyme. The catalytic mechanism, called the Q-cycle, requires two distinct quinone-binding sites: Qo, the quinol oxidation site, and Qi, the quinone reduction site (Fisher & Meunier, 2008). The location of the quinol/quinone binding sites of bc_1, both of which are located within the cytochrome *b* subunit has been resolved by X-ray crystallography using bound inhibitors. Detailed information about interactions between bc_1 and inhibitors has since become available for several QoI fungicides. It is now known that, despite differences in binding between the different Qo inhibitors, their fit to the enzyme pocket is very similar (Esser et al., 2004). The toxophore is similar in all compounds and always contains a carbonyl oxygen moiety (Figs. 1, 2 and 3) that is thought to be responsible for binding to the enzyme.

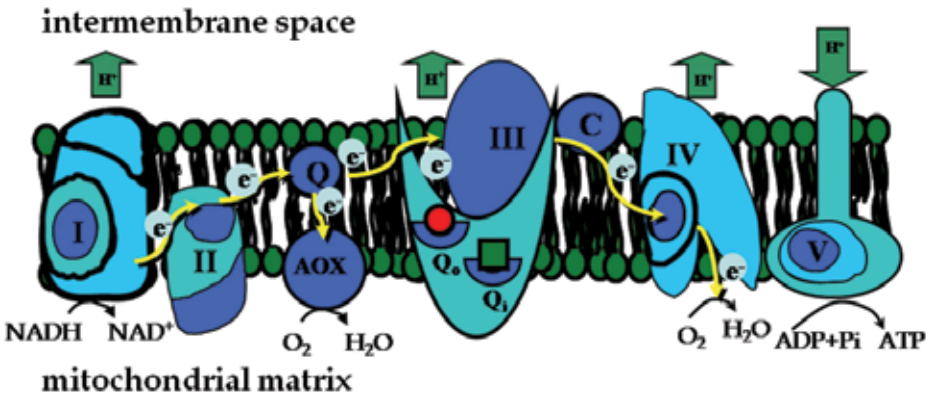

Fig. 4. Schematic representation of the mitochondrial electron transport system. I, II, III and IV are the different complexes of the transfer chain. V is the ATP synthase complex. Q is the ubiquinone pool and C is the peripheral protein cytochrome *c*. The yellow arrows inside the membrane indicate the direction of electron flow. The Qo and Qi binding sites of the cytochrome bc_1 enzyme complex (complex III) are delineated by a red circle and a green square representing Qo- and Qi-inhibitor molecules, respectively. In some fungi, inhibitors of the respiratory pathway induce the synthesis of alternative oxidase (AOX), an enzyme that diverts electrons at the ubiquinone pool (Q), but generates much less energy.

One important characteristic of the fungicidal action of this class of chemicals is their rapid activity. Studies performed using different QoIs have demonstrated that spore germination and zoospore motility are developmental stages of fungi and oomycetes that are particularly sensitive to QoI fungicides (Godwin et al., 1997; Leinhos et al., 1997). This can be explained by their biochemical mode of action, namely the disruption of energy production, with the consequence that they are particularly effective against these highly energy-demanding stages of development that are very critical for successful colonization of the plant (Fig. 5). The detailed understanding of the effects of QoIs on different stages of fungal development has been important for the optimisation of application timing to most effectively control of disease. QoIs are, therefore, best applied prior to infection or in the early stages of the disease cycle to capitalise on their potent effects as protectant or preventive fungicides (Bartlett et al., 2002).

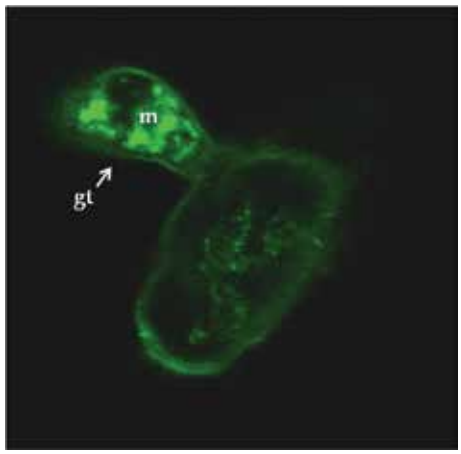

Fig. 5. Mitochondria in the germination tube of a phytopathogenic fungus. Mitochondria in germinating conidia of the cucurbit powdery mildew fungus *Podosphaera fusca* were labelled with MitoTracker Green FM (Molecular Probes). Green fluorescence indicates the presence of mitochondria (m) that are primarily located in the germination tube (gt). Germination is a physiological process that is highly dependent on energy; therefore, most of the conidial mitochondria are reallocated to the germination tube.

4. Agricultural use of QoI fungicides

Since their introduction, QoIs have become essential components of plant disease control programmes because their wide range of efficacy against many agriculturally important fungal diseases. QoIs have been registered in numerous countries for use on several different crops, including cereals, turf grass, grapevines, and a number of vegetables and ornamental plants. The individual active ingredients of QoIs have been the primary reason for their success, they are all characterised by one or more of the following attributes: broad-spectrum activity, control of isolates resistant to other fungicides, low use-rates and excellent yield and quality benefits (Bartlett et al., 2002). In a number of crops, QoIs have led to major changes in disease control programmes. For example, QoIs provided grapevine growers for the first time a single active ingredient to prevent both powdery (*Erysiphe necator*) and downy (*P. viticola*) mildews. In other crops such wheat and barley, QoIs exhibited increase yield and quality benefits over other classes of fungicides. QoIs have been important additions to the fungicide resistance-management armoury for many crops, particularly bananas. This class of fungicide has also proven particularly useful for the maintenance of protected horticultural crops; this was especially true in Europe where the number of available active ingredients is dwindling due to the high cost of maintaining a long list of registered uses. Furthermore, QoI fungicides are important not only as foliar-applied fungicides but also as seed-treatment applications and in-furrow treatments for soil-borne disease control (Bartlett et al., 2002).

All commercialised QoIs have been demonstrated to exhibit a broad spectrum of activity. Of particular importance is their activity against all four major groups of pathogens responsible for fungal-like diseases, namely Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes. However, QoIs vary in their levels of activity against the different plant diseases, and not all of them exhibit high levels of control of these four major groups of

plant pathogens (Bartlett et al., 2002). Among other plant diseases, QoIs are effective in the protection of crops against powdery mildews (Fig. 6), brown rusts and downy mildews (Balba, 2007). Moreover, QoIs may exert some negative effects against the sexual stages of plant pathogenic fungi and oomycetes. Azoxystrobin has been shown to inhibit the production of viable ascospores and oospores of grapevine powdery and downy mildews, respectively (Godwin & Cortesi, 1999; Vercesi et al., 2002); thus, this QoI reduces the evolutionary potential of these important pathogens of vineyards.

Fig. 6. Disease symptoms of cucurbit powdery mildew on zucchini leaves elicited by *Podosphaera fusca*. Powdery mildew diseases are major targets for QoI fungicides. Unfortunately, they exhibit a high rate of resistance development and many cases of QoI-resistant powdery mildew fungi have been documented.

QoI fungicides may exhibit additional yield and quality benefits. Of particular interest has been the consistently greater yield of several crops, including wheat and barley, that are subjected to strobilurin-based fungicide programmes. This has been termed the strobilurin "greening effect", a phenomenon that refers to delayed leaf senescence and an increased grain-filling period that results in an enhanced biomass and yield (Bartlett et al., 2002). Two hypotheses have been presented to explain this phenomenon. The first hypothesis is related to the effects of strobilurins on non-disease-related physiological processes such as chlorophyll and phytohormone biosynthesis, stomatal aperture, water consumption in addition to modulation of nitrate reductase, photosynthetic and plant antioxidant enzyme activities. The second theory is related to the strong preventive activity of strobilurins, preventing the germination of pathogenic, non-pathogenic and saprophytic fungi and thereby preventing the initiation of energy intensive host defence responses. However, neither hypothesis has been unequivocally proven to be responsible for this phenomenon. It is possible that elements of both hypotheses contribute to these "unexpectedly positive" yield benefits resulting from the use of strobilurins (Bartlett et al., 2002). Moreover, some strobilurins, such as pyraclostrobin, may have priming-inducing activities on plants (Herms et al., 2002). In the primed condition, plants are able to "recall" previous infections, root colonisations or chemical treatments. As a consequence, primed plants respond more rapidly and/or effectively when re-exposed to biotic or abiotic stress; such improved responses are frequently associated with enhanced disease resistance (Goellner & Conrath, 2008). Findings made using pyraclostrobin suggest that this compound, in addition to

exerting direct antifungal activity, may also protect plants by priming them for increased activation of stress-induced defence responses.
Unfortunately, the single site mode of action of QoI fungicides results in a high intrinsic risk of development of resistance to this group of fungicides. Strict anti-resistance strategies, including treatment limitations and the use of mixtures or alternating fungicides, are therefore required (McGrath, 2001). In Spain, for example, there are cucurbit-growing areas where the cucurbit powdery mildew pathogen *P. fusca* has developed high levels of resistance (up to 74% in Murcia) to QoI fungicides. In these areas, disease control based on these compounds is virtually ineffective; these high rates of resistance are presumably the result of frequent use of these popular fungicides by growers (Fernández-Ortuño et al., 2006).

5. Mechanisms of resistance to QoI fungicides

5.1 Mutations in the cytochrome *b* gene

The primary mechanism of QoI resistance is target site-based and involves mutations in the mitochondrial cytochrome *b* gene (*CYTB*), resulting in peptide sequence changes that prevent fungicide binding. Mutations that affect sensitivity to QoI fungicides have been found in two regions of *CYTB*, corresponding to amino acid positions 120-155 and 255-280 of the encoded protein. In the folded cytochrome b, these domains are close to each other and are important for ligand binding. Two amino acid substitutions, from glycine to alanine at position 143 (G143A) and from phenylalanine to leucine at position 129 (F129L), have been detected in the cytochrome *b* proteins of several phytopathogenic fungi and oomycetes that are resistant to Qo inhibitors (Gisi et al., 2002). Recently, an additional amino acid substitution, from glycine to arginine at position 137 (G137R), has also been linked to QoI resistance (Sierotzki et al., 2006). Isolates expressing F129L or G137R mutant proteins exhibit moderate (partial) resistance that is typically overcome by the recommended field levels of QoIs. In contrast, isolates expressing G143A mutant protein exhibit high (complete) levels of resistance that is always associated with the failure of QoI to control disease (Fig. 7). The G143A substitution has been detected in resistant isolates of more than twenty species, including phytopathogenic ascomycetes and oomycetes such as several powdery mildews and *Alternaria* species in addition to the major *Mycosphaerella* pathogens, *M. fijensis* and *M. graminicola* (for an updated list of plant pathogens in which the G143A substitution has been documented, please visit the web page of the Fungicide Resistance Action Committee, FRAC, QoI Working Group at www.frac.info). The G143A substitution is also present in strobilurin-producing basidiomycetes that are naturally resistant to these fungicides (Kraiczy et al., 1996).
Although many reports have attributed QoI resistance in plant pathogens to the G143A substitution, the role of this amino acid change in QoI resistance has been very difficult to investigate because the cytochrome *b* target is encoded by a mitochondrial gene. Consequently, functional genetic studies of this mutation have only been performed in *M. fijiensis* (Gisi et al., 2002). In light of the obstacles in investigating the role of *CYTB* mutations in QoI resistance using molecular approaches, most studies have focused on demonstrating a correlation between QoI resistance and the G143A substitution. However, this statistical approach has not always been as thorough as required due to the low number of resistant isolates analysed. Regardless, a correlation between QoI resistance and the G143A substitution has been clearly demonstrated in *Blumeria graminis* f. sp. *tritici*, *M. graminicola* and *Pyricularia grisea* (Fraaije et al., 2002; 2005; Kim et al., 2003).

Fig. 7. Cytochrome *b* and QoI resistance. Molecular model of the cytochrome *b* protein of *Saccharomyces cerevisiae*. A QoI fungicide molecule bound to the Qo site of the cytochrome bc_1 complex is depicted in green. The amino acids substitutions G143A and F129L that are responsible for QoI resistance are depicted in cyan and blue, respectively. Note how these amino acids interact with the fungicide molecule.

5.2 Alternative respiration

The second mechanism of resistance to QoI fungicides is mediated by the induction of an alternative, cyanide-resistant respiration that is sustained by alternative oxidase (AOX) (Wood & Hollomon, 2003). In this rescue mechanism, mitochondrial electron transfer is diverted by circumventing the inhibitory site of QoI in the cytochrome bc_1 complex (Fig. 4). However, the energy provided by alternative respiration only seems to counteract QoI effects *in vitro* but not *in planta*. Under field conditions, alternative respiration appears to have a limited impact on the protective activities of QoI fungicides for two main reasons. First, this pathway provides low levels of ATP that represent only 40% of the normal efficiency for energy conservation. This is due to the fact that complexes III and IV of the mitochondrial electron transport system are bypassed, and AOX lacks proton pumping activity. Consequently, processes that demand large amounts of energy and are critical steps for successful colonization of the plant, such as spore germination and host-penetration, are not supported. Second, plant antioxidants, such as flavones, are released during infection and interfere with the induction of alternative respiration by quenching reactive oxygen species that are necessary to induce the *AOX* gene and are generated by QoIs (Wood & Hollomon, 2003).

Despite the widely accepted minor role of AOX in QoI resistance, several reports using alternative oxidase-deficient mutants and specific inhibitors of this enzyme have revealed that alternative respiration also limits QoI effectiveness *in planta*, especially once the infection has been established (Olaya & Köller, 1999; Avila-Adame & Köller, 2003; Miguez et

al., 2004). A possible explanation is that a decreasing demand for energy efficiency during the later stages of the infection process, such as mycelial growth and sporulation, enables AOX to become more effective as an infection progresses (Wood & Hollomon, 2003). This is consistent with the lack of eradicant activity of QoI fungicides against many fungi; after visible symptoms have appeared, control is typically ineffective.
Moreover, alternative respiration may provide an opportunity for the selection of a point mutation in *CYTB* by decreasing levels of damaging reactive oxygen species and ensuring ATP synthesis. Both of these functions contribute to cell survival while selection increases the frequency of resistant mitochondria (Wood & Hollomon, 2003; Miguez et al., 2004). The QoI-targeted cytochrome *b* protein is encoded by mtDNA that is known to mutate at a higher frequency than nuclear DNA; this mutation frequency is increased by the accumulation of reactive oxygen species that result from the inhibition of the electron transport system by QoIs (Bohr & Anson, 1999). Under such circumstances, alternative respiration could represent an essential pathway for the transition from sensitivity to full resistance in phytopathogenic fungi in the presence of sub-lethal concentrations of QoI fungicides. Regardless, despite the lack of a clear-cut example in which AOX plays a role in field resistance to QoIs, the potential involvement of this enzyme in the development of QoI resistance should not be ignored (Wood & Hollomon, 2003).

5.3 Efflux transporters

Efflux transporters can enable fungi to survive exposure to toxic compounds, preventing the accumulation of compounds at toxic concentrations inside fungal cells. These membrane-bound proteins are known to provide protection against a wide range of natural toxic compounds and xenobiotics (Del Sorbo et al., 2000). The ATP-binding cassette (ABC) transporter family and the major facilitator superfamily (MFS) are the most important efflux pumps involved in the protection of fungi against fungicides (Fig. 8) (Stergiopoulos et al., 2003; De Waard et al., 2006). In agriculture, however, there are few obvious cases of multi-drug resistance to fungicides in plant pathogens. The first efflux transporter gene involved

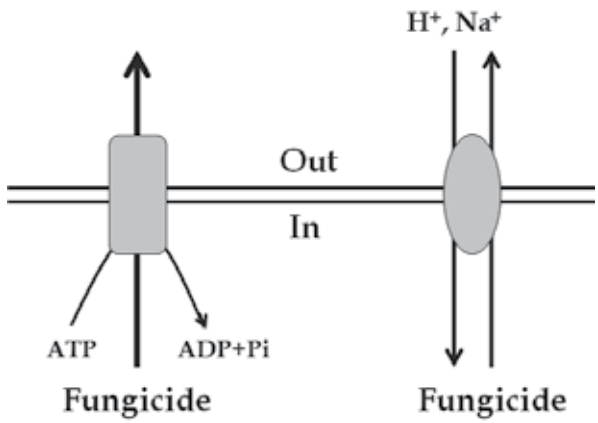

Fig. 8. Schematic representation of the two major classes of efflux transporters in fungi. ATP-binding cassette (ABC) transporters utilise the free energy of ATP hydrolysis to pump fungicides out of the cell. The transporters from the major facilitator superfamily (MFS) mediate the extrusion of fungicides in a coupled exchange with protons or sodium ions.

in QoI insensitivity to be reported in a plant pathogen was *MgMfs1*, a MFS transporter gene of *M. graminicola* (Roohparvar et al., 2007). Most natural isolates of *M. graminicola* resistant to strobilurins and over-expressing *MgMfs1* also contain the G143A substitution, suggesting that the contribution of *MgMfs1* to the QoI resistance in field strains should be small.
Prior to the isolation of *MgMfs1*, Reimann & Deising (2005) described an efflux transporter-mediated mechanism of resistance to QoI fungicides in field isolates of the wheat pathogen *Pyrenophora tritici-repentis*. The involvement of this type of mechanism in fungicide resistance was determined by using inhibitors of these membrane transporters, such as the hydroxyflavone derivative 2-(4-ethoxy-phenyl)-chromen-4-one, in combination with fungicides; such inhibitors shifted resistant isolates back to normal sensitivity levels, preventing infection of wheat leaves. In addition, those authors also reported the induction of efflux transporter activity under field conditions and after *in vitro* applications of sub-lethal doses of fungicides. Although data regarding resistance to QoI fungicides based on efflux transporters are scarce, the contribution of these energy-dependent mechanisms in the *in planta* adaptation to fungicides by phytopathogenic fungi should be seriously considered.

5.4 Unknown mechanisms

In recent studies, resistance to QoI fungicides based on the G143A substitution has been deemed unlikely to evolve in phytopathogenic basidiomycetes (such as *Puccinia* spp.) or in several ascomycetes (such as *Alternaria solani*, *Monilinia fructicola* and *Pyrenophora teres*) because they carry a type I intron immediately after this codon. Consequently, a nucleotide substitution in codon 143 of the *CYTB* gene would prevent the splicing of the intron and lead to a deficiency in cytochrome *b*; such a mutation would likely be lethal mutation (Grasso et al., 2006). Indeed, serious resistance problems have not developed among *Puccinia* species, despite the extensive use of QoI on crop hosts of this group of pathogens. Nevertheless, one isolate of *Puccinia horiana* resistant to QoI has been reported to have *CYTB* sequences identical to sensitive genotypes, including the type I intron and, therefore, without the G143A substitution (Grasso et al., 2006). The case of *Botrytis cinerea* is different; two types of *CYTB* gene were found in Japanese populations of the grey mould pathogen, one with three introns and the other with an additional intron (Bcbi-143/144 intron) inserted between the 143rd and 144th codons. All QoI-resistant isolates showed the G143A mutation whereas the isolates possessing the Bcbi-143/144 intron were QoI-sensitive (Banno et al., 2009). The possession of such an intron may guarantee a low risk of QoI resistance but it might not always be fixed in pathogen populations.
There are a number of isolates from different fungal pathogens such as the above mentioned *B. cinerea*, *M. fructicola*, *P. fusca, Podosphaera aphanis* and *Venturia inaequalis*, in which the G143A substitution has not been always presented or has occasionally explained the QoI-resistant phenotypes (Ishii et al., 2001; Fountaine et al., 2006; Fernández-Ortuño et al., 2008a; Ishii et al., 2009; Lesniak et al., 2009; Schnabel, 2010). Moreover, in *P. fusca* and *V. inaequalis* the role of alternative respiration in resistance has been ruled out, though the mechanisms responsible for QoI resistance remain to be characterised (Steinfeld et al., 2001; Fernández-Ortuño et al., 2008a). In *P. fusca*, considering the pattern of cross-resistance to different Qo inhibitors, the high levels of resistance of the resistant isolates, and the absence of consistent mutations in *CYTB*, a structural change in the Rieske protein, the other protein component of the Qo site, has been suggested as the most plausible explanation for QoI resistance (Fernández-Ortuño et al., 2008a). Experimental evidence regarding the role of the Rieske

protein in the resistance to QoI fungicides has not yet been reported, though its nature as a nuclear-encoded target makes it an ideal candidate to explain QoI resistance in pathogens in which *CYTB* mutations have not been found.

6. Evolution and fitness costs associated with QoI resistance

The fact that QoIs inhibit a target site that is encoded by a mitochondrial gene implies several important differences in the way resistance to these fungicides evolved within fungal populations. The inheritance of *CYTB*-based QoI resistance is non-Mendelian and results in a 0:1 segregation at the single progeny and a 1:1 segregation at the population level. After homoplasmic resistant individuals have emerged, the selection process for QoI resistance in populations is expected to follow mechanisms similar to those of nuclear-encoded resistance; in particular, the key to effective resistance is the transition process from hetero- to homoplasmic cells. The evolution of QoI resistance in a fungal population must be the combined result of recurrent mutations and the selection process imposed by the fungicide. However, how this mutation is selected from within a population of wild-type, sensitive mitochondria, is not clear (Gisi et al., 2002). Some reports have described that mitochondrial heteroplasmy with respect to the G143A mutation results in different levels of QoI resistance (Lessemann et al., 2007). The question of how many mutated mitochondria per cell are required to produce a full QoI-resistant phenotype has not yet been answered. Moreover, the dynamics of mitochondria and the status of heteroplasmy in the mitochondrial genome encoding the *CYTB* gene can cause instability of the mutated gene over time, making it difficult to precisely monitor QoI resistance using molecular methods (Ishii, 2010). Heteroplasmy of *CYTB* genes has been described in several plant pathogenic fungi such as *B. cinerea*, *P. leucotricha* and *V. inaequalis* (Zheng et al., 2000; Fountaine et al., 2007; Ishii et al., 2007; Lessemann et al., 2007). In these studies, it was clearly shown that the sensitive isolates of these pathogens exhibited a low frequency of the mutated *CYTB* gene.

Evolution of resistance is closely related to the fitness costs associated with fungicide resistance. If fitness costs are associated with fungicide resistance, the frequency of resistant isolates will decline in the absence of fungicide. On this basis, Ishii et al. (2002) performed studies with cucumber powdery and downy mildews; they found that under both laboratory and commercial greenhouse conditions, QoI-resistant isolates persisted for a few years following the withdrawal of the selection pressure imposed by the fungicide. The authors proved that the homoplasmic mutated sequences in the *CYTB* gene of QoI-resistant strains may gradually revert to heteroplasmic status to include the wild-type sequences in the absence of fungicidal selection pressure, and that the proportion of mutated sequences rapidly increases in the high copy numbers of heteroplasmic *CYTB* gene when fungi are exposed to strong selection pressure by QoI fungicides.

Despite its practical relevance, little is known about the fitness cost associated with QoI resistance conferred by the G143A substitution. Fitness penalties have been observed in QoI-resistant field isolates of *P. grisea* and *P. viticola*, and laboratory mutants of *Cercospora beticola* (Avila-Adame & Köller, 2003; Malandrakis et al., 2006). In contrast, fitness penalties are not apparent in QoI-resistant field isolates of *B. graminis* and *B. cinerea*, and laboratory mutants of *Penicillium digitatum* (Heaney et al., 2000; Chin et al., 2001; Banno et al., 2009; Zhang et al., 2009). To test the fitness costs associated with *CYTB*-based QoI resistance, Fisher & Meunier (2008) used *S. cerevisiae* as a model system. Specific residues in the Qo site of yeast cytochrome *b* were modified to obtain new forms that mimicked the Qo binding site of

several fungal plant pathogens and to study the impact of the introduction of the G143A substitution on bc_1 complex activity. As expected, the G143A substitution resulted in high levels of resistance to QoI fungicides. However, the G143A substitution also led to a slight reduction of bc_1 complex activity in most of the Qo site mimics (e.g. *P. fusca*) but not in *B. graminis* f. sp. *tritici*. Based on these observations in the yeast model, the authors suggested that the G143A substitution might affect the fitness of plant pathogens differentially. It has been also widely assumed that fitness costs are fixed. However, the cost of resistance to QoI fungicides seems to vary with environmental conditions, such as temperature; fitness costs are higher in growth conditions that are sub-optimal for the fungus, as shown for *B. graminis* and *M. graminicola* (Brown et al., 2006). Thus, although the G143A substitution can confer resistance to QoI fungicides, in the absence of such compounds it may also cause some degree of fitness penalty, because of the possible effect on mitochondrial respiration.

Virtually no data exist describing the fitness costs associated with other mechanisms involved in QoI resistance. For *B. cinerea*, the usefulness of QoI fungicides is very limited and restricted to certain crops. It is interesting to note that, in liquid cultures, *B. cinerea* produces high levels of AOX in the absence of any external stimulus; therefore, it may be better adapted for the loss of complexes III and IV of the mitochondrial electron transport chain than species in which AOX is only induced as a rescue mechanism (Wood & Hollomon, 2003). Moreover, for the MFS transporter gene of *M. graminicola MgMfs1* it has been suggested that the increased strobilurin efflux activity provided by this transporter may be a condition for normal fitness because the efflux may prevent strobilurin accumulation in fungal membranes, thus, safeguarding normal membrane function. Therefore, it would be interesting to analyse the fitness costs associated with the different mechanisms of QoI resistance in fungal plant pathogens where QoI fungicides are still in use. Such studies would likely provide relevant information that may be used for the rational design of anti-resistance strategies

7. Conclusions

The QoIs are an outstanding class of agricultural fungicides that exhibit excellent properties in a number of areas, including human and environmental safety. QoI fungicides have been extremely successful because of the benefits associated with their use and are clearly one of the most valuable classes of single-site fungicide discovered by the agrochemical industry. However, one of the apparent strengths of these fungicides, their high specific mode of action, is also a serious weakness. The group is considered a high risk one in terms of resistance development; therefore, strict anti-resistance strategies, including the limitation of treatments and the use of mixtures or alternating compounds, should be followed to maintain the high efficacy of this class of fungicides. Although their use is no longer recommend in some crops, such as cereals, in many other crops if recommended use-patterns are strictly followed, the dependence of crop protection on the QoIs is likely to continue for many years into the future. A single point mutation in the *CYTB* gene that causes a G143A substitution in cytochrome *b* confers resistance to QoIs in many plant pathogens. While there is an extensive literature that emphasises the contribution of the G143A substitution in QoI resistance, this fact has not been always clearly substantiated. Although important insights into the mechanisms influencing the evolution of *CYTB*-based QoI resistance have been gained in recent years, many basic questions remain. Most of the unanswered questions are related to the mitochondrial nature of the cytochrome *b* gene.

Interestingly, an increasing amount of experimental evidence attributes the phenomenon of QoI resistance to mechanisms that are distinct from cytochrome *b* mutations, such as alternative respiration, efflux transporters and other unknown mechanisms. Thus, the cause of QoI resistance in phytopathogenic fungi may be more complex than a simple point mutation, and the widely accepted role of cytochrome *b* in QoI resistance should be reevaluated. In the microbial world, there are examples of microbes that can develop resistance to a given antibiotic through different mechanisms, and this could also be the case for QoIs. Therefore, we should avoid making general assumptions and carefully clarify the molecular basis of QoI resistance for single pathogen species before developing rapid diagnostic methods that will help to maintain the high efficacy of this important class of agricultural fungicides for as long as possible.

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Fungicide Resistance in Cucurbit Powdery Mildew Fungi

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1. Introduction

Powdery mildew is the major cause of losses in production of cucurbits worldwide (Cohen et al., 2004; Křístková et al., 2009; Lebeda et al., 2007b; McCreight, 2006) (Fig. 1a,b and 2a,b). This disease is caused by two obligate biotrophic ectoparasites: *Golovinomyces cichoracearum s.l.* (*Gc*) (syn. *Erysiphe cichoracearum s.l.*) and *Podosphaera xanthii* (*Px*) (syn. *Sphaerotheca fuliginea*) (Křístková et al., 2009; Lebeda, 1983) (Fig. 3a,b and 4a,b). Their distribution and relative occurrence varies throughout the world (Bardin et al., 2009; Křístková et al., 2009; Pérez-García et al., 2009). For example, both *Px* and *Gc* are important in Central Europe whereas *Px* occurs almost exclusively in the USA.

Both cucurbit powdery mildew (CPM) species (*Gc* and *Px*) have high evolutionary potential and according to the terminology of McDonald & Linde (McDonald & Linde, 2002) could be considered as "risky" pathogens (Lebeda et al., 2007a). Pathogen populations with a high evolutionary potential are more likely to overcome plant genetic resistance and/or develop fungicide resistance than pathogen populations with a low evolutionary potential (Kuck & Rusell, 2006). CPM species are highly variable in their pathogenicity and virulence which is illustrated by the existence of a large number of different pathotypes and races (Jahn et al., 2002; Lebeda & Sedláková, 2006; McCreight, 2006).

Breeding of cucurbit crops for powdery mildew resistance has a long and successful history, with many resources of race-specific resistance now known in muskmelon (*Cucumis melo*) (McCreight, 2006). There is also excellent resistance in cucumbers (*Cucumis sativus*) (Jahn et al., 2002). Resistance has been bred in some cultivars of squash and pumpkin (*Cucurbita pepo*) and in gourds (*Cucurbita* spp.) (Jahn et al., 2002; Lebeda & Křístková, 1994). Degree of suppression achieved with resistant cultivars is variable, partly due to pathogen adaptation. Additionally, there is tremendous variation within the different cucurbit crops, and incorporating resistance into all horticultural types is an enormous task. Thus utilising plant disease resistance is not an option for managing CPM for all farmers. Furthermore, resistant cultivars do not always provide adequate suppression to be utilized as the sole management practice.

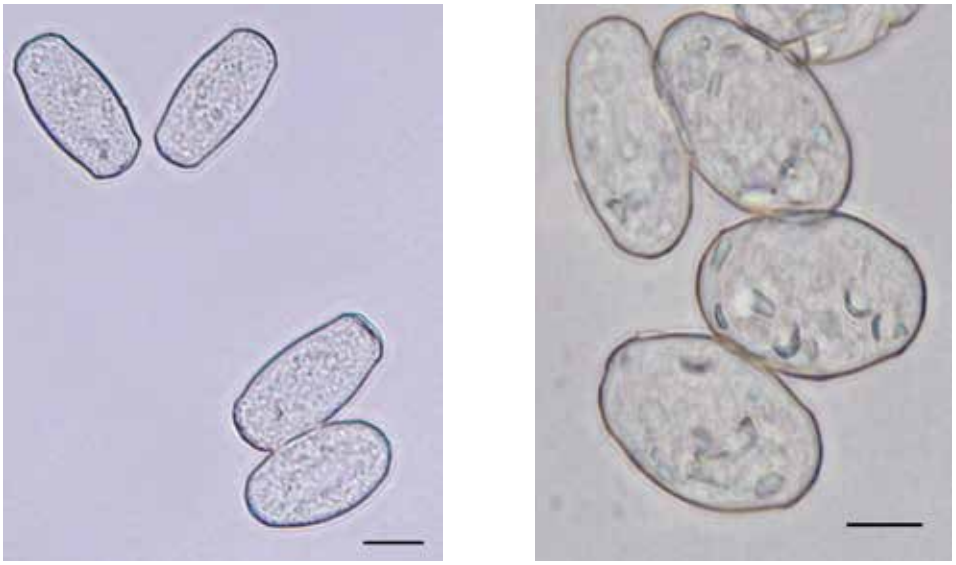
Application of fungicides continues to be the principal approach for managing powdery mildews around the world (Hollomon & Wheeler, 2002). This is due to the limitations of resistance and lack of other management practices. Systemic and translaminar fungicides are especially important for controlling CPM because they provide adequate protection of abaxial leaf surfaces, where conditions are more favorable for disease development than on adaxial surfaces (McGrath, 2001). These fungicides have specific single-site mode of action, thus they are active at one point of one metabolic pathway of the pathogen, and therefore are generally more at-risk for resistance development than other fungicides (McGrath, 2001). Several reports have been published about the appearance and increase of CPM populations (mainly *Px*) resistant to six groups of single-site inhibitors (benzimidazole, DMI, morpholine, hydroxypyrimidine, phosphorothiolate, QoI) (Hollomon & Wheeler, 2002; McGrath, 2001, 2006; Sedláková & Lebeda, 2008). On the other hand, contact fungicides have

1a 1b

Fig. 1. a,b. Symptoms of cucurbit powdery mildew developing under field conditions (a – limited infection, b – serious infection)

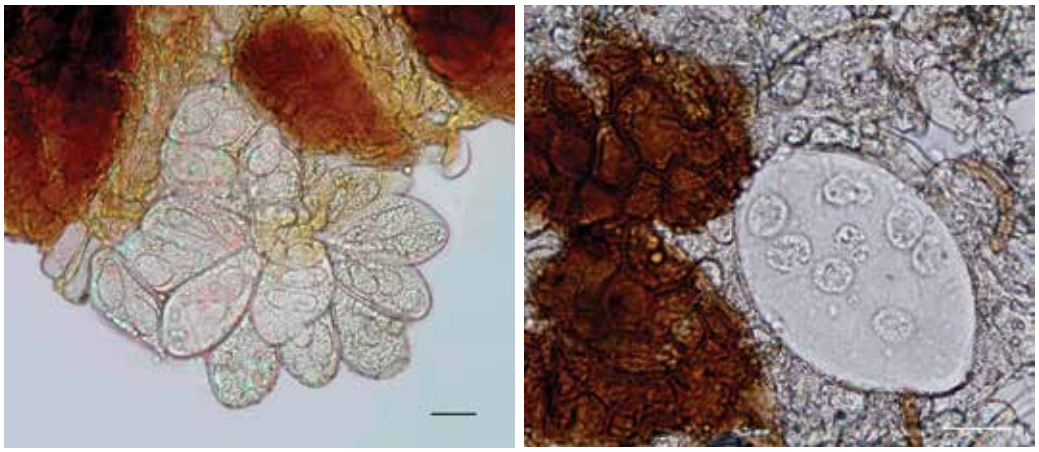
2a 2b

Fig. 2. a,b. *Cucurbita pepo* leaf with powdery mildew (a – adaxial leaf surface, b - abaxial leaf surface)



3a 3b

Fig. 3. a,b. The cucurbit powdery mildew fungi. a - conidia of *Golovinomyces cichoracearum* lacking fibrosin bodies, b – conidia of *Podosphaera xanthii* with fibrosin bodies (scale bar: 10 μm)



4a 4b

Fig. 4. a,b. Asci with ascospores of two powdery mildew fungi. a – *Golovinomyces cichoracearum* (7-15 pedicelate asci per chasmothecium each with two ascospores), b – *Podosphaera xanthii* (one ascus without pedicelus per chasmothecium containing eight ascospores) (scale bar: 20 μm)

low potential for resistance development because they are multi-site inhibitors (Kuck & Russell, 2006; McGrath, 2001); however, they are less important for controlling CPM because of their inherent lower efficacy due to inability to protect abaxial surfaces. To date, CPM resistance has been detected to only two groups of multi-site inhibitors (quinoxaline and

miscellaneous) (McGrath, 2001). Current recommendation for managing CPM and fungicide resistance is an integrated disease management program for CPM that entails using fungicides with different modes of action, including multi-site inhibitors, as well as, resistant cultivars, together with information from epidemiological studies and disease monitoring (Sedláková & Lebeda, 2008).

2. Fungicide resistance in cucurbit powdery mildew fungi – historical overview and recent status

CPM pathogens have demonstrated ability to develop resistance, often quickly, to fungicides prone to resistance due to their single-site mode of action (McGrath, 2001). With all at-risk fungicides, repeated use typically in commercial production has resulted in selection of strains with lower sensitivity or resistance to the fungicide then were present before use. Some strains are fully resistant and thus not affected by the fungicide (aka practical or field resistance). The history in the USA of the development of fungicides for CPM and of resistance provides an illustration of the potential of the CPM pathogens. A similar history has occurred in other countries. The dominant CPM pathogen in the USA is *Px*.

MBC (methyl benzimidazole carbamate) fungicides aka benzimidazoles (FRAC Group 1) were the first chemical class of fungicides with a single-site mode of action used for CPM (McGrath, 2001). Resistance developed very quickly to benomyl, the first fungicide in this group. In the USA, benomyl-resistant strains were detected in 1967, the first year of field evaluations at USA university facilities. This was the first documented case of resistance in the USA. At that time, global experiences with fungicide resistance were limited and thus the potential impact on control and the need for management were not recognized. Benomyl formulated as Benlate was registered in 1972 for commercial use on cucurbit crops in the USA. The first case of control failure in the field occurred the next year. Another MBC fungicide, thiophanate-methyl, formulated as Topsin M, is still labeled for CPM and thus available for use in production fields. It is not recommended because resistant strains continue to be found widely and commonly despite limited use of thiophanate-methyl for other diseases. Resistance to this group of fungicides is qualitative, thus pathogen strains are sensitive or fully resistant. And cross resistance occurs among the fungicides, thus resistant strains are insensitive to all fungicides in the group.

The next chemical class developed for CPM was the DMI (demethylation inhibitor) fungicides (FRAC Group 3) (McGrath, 2001). The first active ingredient in this group was triadimefon. It was registered for CPM in the USA in April 1984. The first reported control failure documented through university fungicide efficacy experiments occurred just two years later. Control failure became widespread during the early 1990s. Resistance to DMIs is quantitative, thus pathogen strains exhibit a range in sensitivity. While cross resistance exists among fungicides in this group, there are inherent differences in activity. The next DMI fungicide developed, myclobutanil, when used at a high concentration was effective in university experiments against pathogen strains fully resistant to triadimefon and where this fungicide was ineffective. Myclobutanil was granted registration in the USA in 2000. For two years prior to 2000 an emergency exemption from registration (FIFRA Section 18) for myclobutanil was granted in some states because neither benomyl nor triadimefon, the only mobile fungicides registered for this use at the time, were adequately effective due to resistance. The degree of DMI insensitivity in the CPM pathogen population continued to

shift during the 1990s. As a result, myclobutanil applied at its lowest label rate no longer controlled CPM as well as at the highest rate. USA federal (Section 3) registration was granted for another new DMI, triflumizole, in 2002. Subsequently, sensitivity to the DMI fungicides has remained fairly stable through 2009. Myclobutanil and triflumizole have provided effective control of CPM in most, but not all, field efficacy evaluations conducted over those years. None of the DMI fungicides developed recently, which are difenoconazole, tebuconazole, and metconazole, have exhibited greater inherent activity than the DMIs currently registered, unlike the situation with myclobutanil being substantially more active than triadimefon.

QoI (quinone outside inhibitor) fungicides (FRAC Group 11) were the next chemical class developed for CPM (McGrath, 2001). Azoxystrobin was registered in the USA in spring 1999. It could be used in some states in 1998 where an emergency exemption was granted. Additional QoIs were registered in fall 1999 (trifloxystrobin) and 2002 (pyraclostrobin). Resistance to QoIs was first detected in the USA in 2002 (McGrath & Shishkoff, 2003). Control failures were reported from several states throughout the USA; resistant strains were confirmed to be present in Georgia, North Carolina, Virginia, and New York. Impact on control was dramatic, with failure occurring where QoIs were highly effective the previous year, reflecting the qualitative nature of resistance to this group of fungicides. Resistant strains of CPM have been common in the USA based on bioassays conducted recently in several states. QoI fungicides are no longer recommended for CPM because resistant strains are common, they are fully resistant due to the qualitative nature of the resistance, and there is cross resistance among QoI fungicides. Resistance to QoI and also to MBC fungicides has been detected at the start of CPM development where tested. There continues to be selective pressure to maintain QoI resistance in the CPM pathogen population in the USA because the only fungicide available with a new active ingredient, boscalid, also contains a QoI fungicide.

Carboximide (FRAC Group 7) was the fourth chemical class of mobile fungicides at risk for resistance development available for managing CPM in the USA. The first product, which was registered in 2003, contained boscalid plus pyraclostrobin. Pathogen strains have exhibited a range in sensitivity to boscalid. Strains fully resistant to this fungicide were first detected in 2008 (McGrath, *unpublished*). These strains were able to tolerate label rates (500 ppm) in a leaf-disc bioassay (McGrath & Fox, 2010). Control failure in a fungicide evaluation in 2009 was associated with their presence (Wyenandt, *personal communication*). It is feasible but not known yet whether the new carboximides in development are sufficiently different chemically from boscalid that their efficacy will not be compromised due to cross resistance with boscalid.

Quinoline (FRAC Group 13) is the chemical class most recently to become available for use in the USA. Quinoxyfen was registered for use on melon in 2007 and on pumpkin and winter squash in 2009. It has been highly effective in university fungicide evaluations (e.g. McGrath & Fox, 2009).

There are a few additional fungicides in development with high inherent activity based on baseline sensitivity of *Px*.

Experience with resistance in CPM has revealed challenges in predicting resistance specifically and in managing resistance (McGrath, 2001). While it is well established that whether or not a fungicide has a risk of developing resistance can be predicted based on if it has single-site mode of action, it is not as straight forward to predict details such as how quickly resistance will develop, the type of resistance (qualitative or quantitative), degree of cross resistance, and how long a fungicide will continue to provide control of CPM after

resistance is detected. For example, when the first QoI fungicide was commercialized, it was predicted that relative risk for this group was low (compared to the benzimidazoles), it would take several years for resistance to develop, and it would be quantitative. Additionally, resistance was expected to develop first in *Px*. However, the risk proved to be high, qualitative resistance developed and quickly, with control failure occurring during the fourth year of commercial use for CPM, and resistance was detected prior to this in *Didymella bryoniae*. In Europe resistance was detected in just one year of use (Hollomon & Wheeler, 2002). In contrast, resistance has developed slowly in the DMI fungicides. Fungicides developed since the first one (triadimefon), which became ineffective due to resistance, have continued to provide some control of CPM. Another fungicide commercialized after the DMI fungicides (boscalid) appears to be becoming ineffective as the result of *Px* developing a very high level of resistance. Inherent activity of the newest DMI fungicides has not proven higher than older products as expected. Pathogen strains resistant to a fungicide have often exhibited resistance to unrelated fungicides (correlated resistance).

Managing resistance has been challenging partly due to lack of tools (McGrath, 2001). The general recommendation for a fungicide program to manage resistance is to alternate among at least two fungicides at risk for resistance development and to mix these with a contact fungicide that has low resistance risk. In the USA, there has rarely been a period when more than one at-risk fungicide was available for commercial use to which the CPM pathogen had not already exhibited development of insensitivity. Host plant resistance is the only other management practice for CPM to use in an integrated management program. While there is good potential utility of using resistant cultivars in an integrated program for managing fungicide resistance, it has limitations due to resistance not being incorporated into all horticultural types and pathogen ability to also evolve to overcome genetic resistance as well as fungicides.

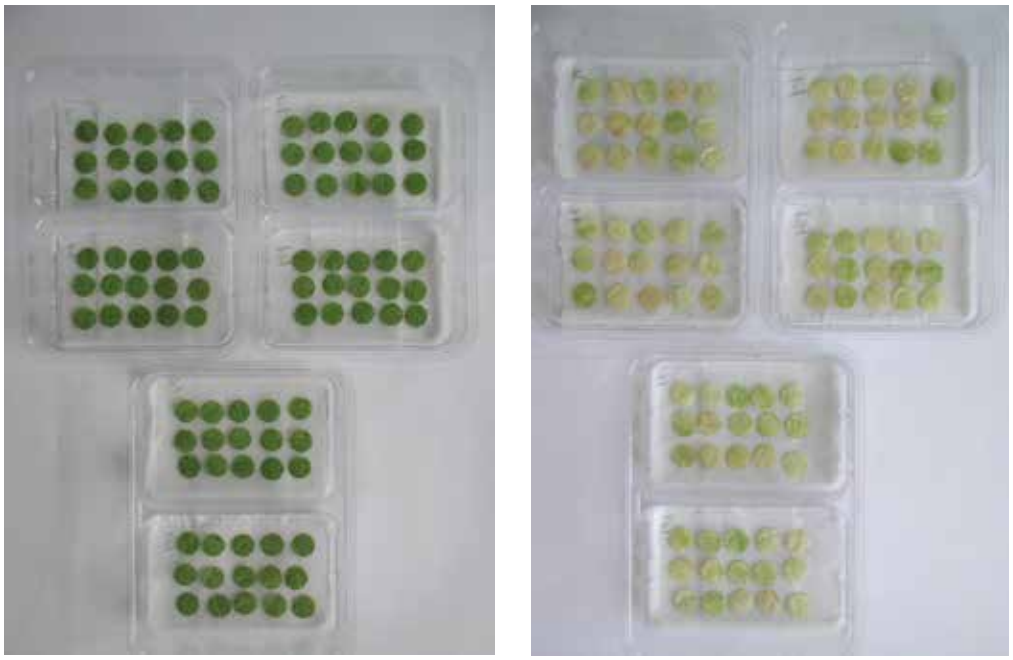
3. Methodology of fungicide resistance research

3.1 Laboratory approach

3.1.1 Leaf-disc bioassays for determining fungicide sensitivity of CPM isolates

A modified leaf-disc bioassay was developed for fungicide resistance screening in Czech CPM populations (Sedláková & Lebeda, 2004a,b, 2006, 2008) (Fig. 5a,b). All screened fungicides were tested at five concentrations (one recommended by the producer; two others below and above this). Treatment with distilled water served as the control. There were five leaf discs (15 mm in diameter) in three replicates for every concentration of each fungicide. Discs were placed into plastic boxes (190 × 140 × 65 mm) containing the fungicide solutions and soaked for 30 minutes. The discs were removed from the fungicide suspension and placed with the adaxial surfaces up on wet filter paper in plastic boxes with the septum (190 × 140 × 65 mm) lined with five layers of moistened cellulose cotton-wool and one layer of filter paper. There were five leaf discs of each of two different fungicide concentrations in each box and three boxes for each isolate. Boxes were open for approximately 1 h (at laboratory temperature in a sterile room) to allow the discs to dry. The discs were inoculated 24 h later by tapping spores off primary leaves of cucumber 'Stela F_1' which were covered with 3- to 4-day-old sporulating mycelium of the isolate of CPM to be tested. Incubation proceeded under the same conditions as for the maintenance of isolates (see 4.1.2).

A laboratory procedure for assessing sensitivity to fungicides was independently developed in NY (McGrath et al., 1996). *Cucurbita pepo* seedlings at the cotyledon stage were sprayed to coverage with fungicide solutions using atomizer bottles connected to an air compressor



5a 5b

Fig. 5. a,b. A modified leaf-disc method for fungicide resistance screening in cucurbit powdery mildew fungi, tested fungicide Topsin M 70 WP (active ingredient: thiophanate-methyl) (a – before inoculation, b – after 14 inoculation days, resistant isolate, profuse sporulation on leaf discs at every fungicide concentration tested)

operated at 30 psi. The fungicide concentrations used were identified through preliminary testing. Treated plants dried over night in a fume hood, then leaf discs were cut and placed with adaxial surface up on water agar in segmented Petri dishes. Discs were cut with a #9 cork borer. Up to six discs treated with the same fungicide concentration were placed in each section. Non-treated discs were placed in one of the four sections. Each disc was inoculated in its center by transferring spores of the isolate to be tested from a leaf culture. Clumps of spores were transferred using a disposable pipette whose tip had been melted in a Bunsen burner flame to form a fine, sealed tip suitable for selecting small groups of spores and for sterilizing in alcohol. Assay plates were incubated for about 10 days under constant light in the laboratory. This bioassay has been used with experimental fungicides to obtain baseline sensitivity data as well as with registered fungicides to investigate shifts in pathogen sensitivity.

3.1.2 Evaluations of the fungicide bioassays

Evaluations for the Czech bioassay were conducted 6-14 days after inoculation by using a 0-4 scale (0 = no sporulation, 1 = sporulating mycelium covering ≤ 25% of leaf disc surface, 2 = > 25% - ≤ 50%, 3 = > 50% - ≤ 75%, 4 = > 75%) (Lebeda, 1984, 1986). The total degree of infection for each isolate was expressed as a percentage of the maximum scores according to Towsend & Heuberger (1943): $P = \Sigma (n \cdot v) \cdot 100/x \cdot N$, where P = the total degree of infection, n = number of discs in every category of infection, v = the category of infection, x

= the maximum level of sporulation, N = the total number of evaluated discs. Three types of reactions were assigned: sensitive (degree of infection, DI = 0-10%); tolerant (DI = 10.1-34.9%); and resistant (DI ≥35%) (Fig. 6).

Fig. 6. Three types of assigned reactions: a – sensitive (degree of infection, DI = 0-10%), b – tolerant (DI = 10.1-34.9%), c – resistant (DI ≥ 35%)

The leaf-disc bioassay conducted in NY was assessed 9-14 days after inoculation (Fig. 7). Percent of each disc with visible powdery mildew was estimated. Ability of an isolate to produce conidia when growing on fungicide-treated leaf tissue, and thereby multiple, was considered an important measure of tolerance/resistance. An isolate was rated sensitive to the fungicide concentration on the disc if no conidia had formed, tolerant if there was growth on fewer than half of the discs, and resistant if there was growth on most of the discs. Where there was no pathogen growth evident with the unaided eye, the disc was examined with a dissecting microscope to ensure inoculum was present. For tolerant and resistant isolates, average percent CPM growth on the fungicide-treated discs was compared to the non-treated discs to determine whether growth was suppressed by the fungicide.

3.2 Field approach

An in-field seedling fungicide sensitivity bioassay was developed in NY to assess fungicide sensitivity of CPM pathogen populations in commercial production fields (Fig. 8 and 9). Seedlings were sprayed with various fungicides and concentrations, placed for at least 4 hours in fields where powdery mildew was developing, then kept in a greenhouse until symptoms developed about 10 days later (Fig 9). Severity was visually estimated on each leaf. Severity on treated seedlings was compared to non-treated ones to estimate frequency of the pathogen population able to tolerate each fungicide concentration tested.

Additionally, efficacy of individual fungicides at risk for resistance development was determined under field conditions in NY with naturally-occurring pathogen strains to assess whether resistance was affecting control. Each fungicide tested was applied weekly with a tractor-sprayer to fungicide plots in a replicated experiment (e.g. McGrath & Fox, 2009). Powdery mildew severity was also assessed weekly by rating severity on both surfaces of leaves.

These approaches are being utilized in some other states.

Fig. 7. Leaf-disc bioassay conducted in segmented Petri dish with sensitive reaction in section of dish on left (no growth of tested CPM isolate), tolerant reaction in upper section (limited growth on some discs), and resistant reaction on right (isolate growth on treated discs similar to growth on non-treated discs in lower plate section)

Fig. 8. Fungicide-treated *Cucurbita pepo* seedlings left with non-treated seedlings for at least 4 hours in a squash crop affected by powdery mildew for an in-field fungicide sensitivity bioassay

Fig. 9. Leaves from in-field bioassay seedlings eleven days after exposure to a cucurbit powdery mildew pathogen population (treatments clockwise from severely-affected non-treated control leaf at left: 50 ppm thiophanate-methyl, 80 ppm myclobutanil, 100 ppm boscalid, 1 ppm quinoxyfen, 80 ppm triflumizole, and 50 ppm trifloxystrobin)

4. Case studies of fungicide resistance in cucurbit powdery mildew fungi

4.1 Czech Republic (Central Europe)

4.1.1 Origin and characterization of cucurbit powdery mildew isolates

Occurrences of powdery mildews (*Golovinomyces cichoracearum* (*Gc*), *Podosphaera xanthii* (*Px*)) on cucurbits was monitored in the Czech Republic (CR) during 2001 to 2007. Each year, at least 100 locations were visited mostly in the main production areas, but also in areas with non-optimal climatic conditions for growing cucurbits (e.g. hilly areas) (Lebeda & Sedláková, 2004a, Lebeda et al., 2007a, 2009a). The timing of visits was focused on the main harvest period (August & first half of September). Severity of symptoms on host plants was evaluated on a 0–4 scale (Lebeda & Křístková, 1994). Whole leaves were collected. Discrete colonies of CPM on the leaves were selected for isolation. Before isolation, spores were microscopically examined in a 3% KOH solution (Lebeda, 1983) for species determination. Isolates were not obtained where a mixture of powdery mildew species were found.

A total of 196 isolates (130 *Gc,* 66 *Px*) originating from the Czech Republic and collected in the years 2001-2007 were screened for tolerance and/or resistance to the two frequently used fungicides (fenarimol and dinocap) and a fungicide ineffective due to resistance (benomyl). A total of 88 (52 *Gc*, 36 *Px)* CPM isolates from 2005-2007 were also tested for tolerance and/or resistance to thiophanate-methyl and 35 (21 *Gc*, 14 *Px*) CPM isolates from 2007 for tolerance and/or resistance to azoxystrobin. 179 CPM isolates originated from infected leaves of *Cucurbita pepo* and *C. maxima*, 13 were from *Cucumis sativus*, three from *C. melo* and one from *Cucurbita moschata* (Sedláková & Lebeda, 2010). All tested isolates were first screened for pathogenic variation (pathotypes, races) by a leaf-disc method (Bertrand et al., 1992; Lebeda, 1986). These isolates were characterized by using a differential set of six cucurbitaceous taxa (Bertrand et al., 1992) and found previously to belong to the various

pathotype groups (Lebeda & Sedláková, 2004a,b, unpublished data). Races were identified by using 11 differential genotypes of *Cucumis melo* (Bardin et al., 1999). Most isolates expressed medium or high pathogenicity (Lebeda et al., 2007a, Lebeda & Sedláková, 2006, Sedláková & Lebeda, 2010).

4.1.2 Pathogen isolation, multiplication and maintenance of isolates

The infected leaf samples collected from production fields were placed on wet filter paper in plastic containers (110 × 85 × 45 mm) in a mobile ice-box for transportation. Individual colonies from these leaves were used to establish CPM cultures. Conidia from pure cultures were transferred by tapping spores on to primary leaves of susceptible cucumber (*Cucumis sativus*) 'Stela F_1'. Isolates were cultured in plastic boxes (190 × 140 × 130 mm) in a growth chamber at 24°C/18°C day/night and 12 h photoperiod (Lebeda et al., 2010) (Fig. 10a,b).

10a

10b

Fig. 10. a, b. Maintenance of cucurbit powdery mildew (CPM) isolates (a – CPM isolates in plastic boxes, b – sporulation of CPM on cotyledons of *Cucumis sativus,* susceptible cv. 'Stela F_1'

Fig. 11. Maintenance of a cucurbit powdery mildew (CPM) isolate on cotyledon in 60-mm Petri dish containing 1.5% water agar

4.1.3 Plant material

Highly susceptible cucumber 'Stela F_1' was used for leaf discs. Plants were grown in mixed substrate (ratio of volume 2 : 1) containing mould and Florcom SB (horticultural substrate based on peat; produced by BB Com, s.r.o., Letohrad, Czech Republic) and under optimal growth conditions (25°C/15°C day/night, with daily watering and weekly fertilization by Kristalon Start (NU3 B.V., Vlaardingen, the Netherlands), 10 ml/10 l of H_2O) in the glasshouse and without any pesticide treatment (Fig. 12). Discs were cut with a cork borer from the leaves of 6- to 8-week-old plants (3- to 6-true-leaf stage) (Lebeda, 1986).

Fig. 12. Highly susceptible cucumber cv. ' Stela F_1' used for preparation of leaf discs

4.1.4 Fungicides and leaf-disc bioassay

Efficacy of four widely used fungicides was tested: fenarimol, formulated as Rubigan 12 EC, producer: Margarita International, Camercio e Servicios, Ltd., Funchual, Portugal; dinocap, formulated as Karathane LC, producer: Dow AgroSciences, Mozzanica, Italy; thiophanate-methyl, formulated as Topsin M 70 WP, producer: Nippon Soda Co. Ltd., Tokyo, Japan; azoxystrobin, formulated as Ortiva, producer: Syngenta Limited, Guildford, Great Britain). They are registered in the CR for field application to control CPM (Kužma, 2005; Minář, 2006, 2007). Fungicide benomyl, formulated as Fundazol 50 WP, producer: Chinosin Pharmaceutical Works Ltd. Budapest, Hungary, was included to serve as a resistant fungicide control. Fundazol is no longer effective and its registration has been cancelled in the CR (Kužma, 2004). These five fungicides are from different chemical groups and have specific features (FRAC 2010; Hollomon & Wheeler, 2002; McGrath, 2001, Table 1).

All mentioned fungicides were tested using a modified leaf-disc bioassay (Sedláková & Lebeda, 2004a,b, 2006, 2008) with five concentrations (one recommended by the producer;

plus two others below and above this) (Table 2, see section 3.1.1). Evaluations of the fungicide bioassay were conducted 6-14 days after inoculation by using a 0-4 scale (Lebeda, 1984, 1986, see section 3.1.2)

Target site and code	Group name	Chemical group	Common name	Source preparation used	Type of resistance	FRAC Code
G1: C14-demethylase in sterol biosynthesis	**DMI**-fungicides (**D**e**M**ethylation **I**nhibitors) (SBI: Class I)	Pyrimidines	Fenarimol	Rubigan 12 EC	Quantitative	3
C5: uncouplers of oxidative phosphorylation		Dinitrophenyl crotonates	Dinocap	Karathane LC	Quantitative	29
B1: β-tubuline assembly in mitosis	**MBC**-fungicides (**M**ethyl **B**enzimidazole **C**arbamates)	Benzimidazoles	Benomyl	Fundazol 50 WP	Qualitative	1
		Thiophanates	Thiophanate-methyl	Topsin M 70 WP		
C3: complex III: cytohrome bc1 (ubiquinol oxidase) at Qo site (*cyt b gene*)	**QoI**-fungicides (**Q**uinone **o**utsite **I**nhibitors)	Methoxy-acrylates	Azoxystrobin	Ortiva	Qualitative	11

*According to: FRAC Code List©2010, Hollomon & Wheeler (2002), Lebeda & Sedláková (2004b), McGrath (2001), Sedláková & Lebeda (2008), Tomlin (2003)

Table 1. Features of the fungicides* used for resistance screening

Fungicide	Concentration of fungicide (µg a.i./ml)/concentration of formulated product* (%)				
	1	2	3**	4	5
Fenarimol	9.6/0.008	18/0.015	36/0.03	72/0.06	144/0.12
Dinocap	28/0.008	52.5/0.015	105/0.03	210/0.06	420/0.12
Benomyl	62.5/0.0125	125/0.025	250/0.05	500/0.1	1000/0.2
Thiophanate-methyl	131.25/0.018	262.5/0.037	525/0.075	1050/0.15	2100/0.3
Azoxystrobin	125/0.05	250/0.1	500/0.2	1000/0.4	2000/0.8

*Formulated products used were Rubigan 12 EC for fenarimol, Karathane LC for dinocap, Fundazol 50 WP for benomyl, Topsin M 70 WP for thiophanate-methyl, and Ortiva for azoxystrobin.
**the concentration recommended by the producer

Table 2. Concentrations of fungicides tested

4.1.5 Results and discussion

Significant differences were observed in fungicide sensitivity of CPM isolates within and between the years 2001-2005 and 2006-2007. Resistant and/or tolerant isolates of both CPM species were detected in different locations (Lebeda et al., 2008, 2009b, 2010; Sedláková & Lebeda, 2004a,b, 2006, 2008, 2010; Sedláková et al., 2009) (Tables 3, 4, 5 and 6).

Fenarimol (Rubigan 12 EC) exhibited decreasing activity in bioassays in 2002 (only for *Gc*) and in 2005 (for both CPM species) (Table 3). Despite the increasing frequency of fenarimol-

tolerant strains, efficacy of fenarimol has remained sufficient to achieve control of CPM under field conditions. Detection of change in sensitive to fenarimol in Czech CPM populations reveals the potential for selection of fenarimol-insensitive strains. Fenarimol belongs to the DMI pyrimidine group of fungicides which have a specific, single-site mode of action that is active against only one point in one metabolic pathway in a pathogen, therefore it is recognized as being at risk of resistance development in CPM population (McGrath, 2001). The genetic structure of the pathogen population is determined mainly by windborne conidia from cucurbit crops in countries surrounding the CR as the main source of initial inoculum for annual reestablishment of the disease (Lebeda et al., 2010; McGrath, 2001; Sedláková & Lebeda, 2008). Overwintering as chasmothecia is considered to be rare (Křístková et al., 2003, 2009). Resistance to DMI fungicides is commonly referred to as "quantitative resistance" because it results from modification of several interacting genes and thus loss of effectiveness due to resistance can be regained by using higher rates or more frequent applications (McGrath, 2001). These facts combined with the use of other effective fungicides might be the explanation for the apparent disappearance of locally-developed resistance to fenarimol. The occurrence of fenarimol-resistant *Px* strains has been reported from Greece, Spain, Israel, Japan, Australia and the Netherlands (López-Ruiz et al., 2010; McGrath, 2001). Resistance to fenarimol is described also in some other pathogens, e.g. *Blumeria graminis* f.sp. *hordei* (Buchenauer et al., 1984) and *Uncinula necator* (Ypema et al., 1997). Cross-resistance of fenarimol-resistant *Px* strains to some other DMI fungicides was documented in the Netherlands and Israel (Scheppers, 1983; 1985), the United Kingdom (Kendall, 1986) and Spain (López-Ruiz et al., 2010).

Efficacy of fungicide (active ingredient), conc. µg a.i./ml^{-1} , ppm)						Total no. of isolates/frequency (%)		
Fenarimol						Σ	*Gc*	*Px*
C	9.6	18	36*	72	144			
+	-	-	-	-	-	153/78	97/63	56/37
+	(-)	-	-	-	-	17/9	16/94	1/6
+	+	-	-	-	-	3/1.5	3/100	-/-
+	(-)	(-)	-	-	-	8/4	4/50	4/50
+	(-)	(-)	(-)	-	-	6/3	5/83	1/17
+	+	(-)	-	-	-	3/1.5	2/67	1/33
+	+	(-)	(-)	-	-	3/1.5	3/100	-/-
+	+	+	(-)	-	-	1/0.5	-/-	1/100
+	+	+	(-)	(-)	-	2/1	-/-	2/100

Gc = Golovinomyces cichoracearum, Px = Podosphaera xanthii;
C = control (untreated by fungicide, characterized by profuse sporulation), *concentration recomended by the producer.
Reaction of CPM: - = sensitive reaction (no sporulation), (-) = tolerant reaction (limited sporulation), + = resistant reaction (profuse sporulation)
**acording to Lebeda et al. (2010), Sedláková & Lebeda (2008, 2010)

Table 3. Response of CPM populations in the years 2001-2007 to different concentrations of Rubigan 12 EC (active ingredient: fenarimol)**

Dinocap (Karathane LC) showed a similar decreasing activity as fenarimol during the years 2001-2005 (Table 4). This phenomenon could be interpreted as the persistence of strains with increasing levels of insensitivity, which were incorporated into the CPM populations by mutation, migration, and/or gene flow (McDonald & Linde, 2002). Elimination of these strains in the same manner as occurred for fenarimol (Ypema et al., 1997) could be a possible explanation for increased efficacy of dinocap in 2006 and 2007. This fungicide appears to still be highly effective for control of Czech CPM populations. Dinocap belongs to the multi-site activity contact fungicides with far lower risk of developing resistance than fungicides with single-site activity (Gisi, 2002; Kuck & Russell, 2006; McGrath, 2001). There are few reports of dinocap resistance worldwide and only for *Px*. It has been reported only from southern Spain, Japan and Taiwan (McGrath, 2001).

Efficacy of fungicide (active ingredient), conc. µg a.i./ml^{-1} , ppm)						Total no. of isolates/frequency (%)		
Dinocap						Σ	*Gc*	*Px*
C	28	52.5	105*	210	420			
+	-	-	-	-	-	150/76.5	99/66	51/34
+	(-)	-	-	-	-	17/9	9/53	8/47
+	(-)	(-)	-	-	-	16/8	13/81	3/19
+	(-)	(-)	(-)	-	-	10/5	8/80	2/20
+	+	-	-	-	-	2/1	1/50	1/50
+	+	(-)	-	-	-	1/0.5	-/-	1/100

Gc = Golovinomyces cichoracearum, Px = Podosphaera xanthii;
C = control (untreated by fungicide, characterized by profuse sporulation), *concentration recomended by the producer.
Reaction of CPM: - = sensitive reaction (no sporulation), (-) = tolerant reaction (limited sporulation), + = resistant reaction (profuse sporulation)
**according to Lebeda et al. (2010), Sedláková & Lebeda (2008, 2010)

Table 4. Response of CPM populations in the years 2001-2007 to different concentrations of Karathane LC (active ingredient: dinocap)**

Benomyl (Fundazol 50 WP) and **thiophanate-methyl** (Topsin M 70 WP) were both ineffective during our seven-year study (Table 5). The screened CPM populations were highly resistant to benomyl as well as to thiophanate-methyl. Both benomyl and thiophanate-methyl belong to benzimidazole fungicides to which there is a great risk of resistance developing (McGrath, 2001). Benzimidazole resistance is commonly referred as "qualitative resistance" because it results from modification of a single major gene and is seen as a complete loss of disease control that cannot be regained by using higher rates or more frequent fungicide applications (McGrath, 2001). The type of resistance combined with the fact that cross resistance occurs between these fungicides explains why benomyl and thiophanate-methyl have been ineffective in the CR. Benomyl registration was cancelled in the CR in 2004 (Kužma, 2004). Despite this, benomyl-resistant strains were common in Czech CPM populations during the years 2005-2007, thus documenting their ability to persist in pathogen populations in CR. Benzimidazole resistance is the most frequently mentioned form of resistance reported from the USA, Australia, the Netherlands and Japan (Brown, 2002; McGrath, 2001, 2006; McGrath & Shishkoff, 2001). Most reports pertain to

benomyl. Thiophanate-methyl has been mentioned in reports of resistance only from the USA (Matheron & Porchas, 2007; McGrath, 2001, 2005,). Occurrence of benzimidazole resistance has been reported in other pathogens, including *Didymella bryoniae* (Keinath et al., 1998 and *Uncinula necator* (Ypema et al., 1997).

Efficacy of fungicide (active ingredient), conc. µg a.i./ml^{-1} , ppm)						Total no. of isolates/frequency (%)		
Benomyl						Σ	*Gc*	*Px*
C	62.2	125	250*	500	1000			
+	(-)	-	-	-	-	1/0.5	1/100	-/-
+	(-)	(-)	-	-	-	4/2	3/75	1/25
+	(-)	(-)	(-)	(-)	-	1/0.5	-/-	1/100
+	(-)	(-)	(-)	(-)	(-)	3/1.5	2/67	1/33
+	+	(-)	(-)	(-)	(-)	2/1	2/100	-/-
+	+	+	(-)	-	-	1/0.5	1/100	-/-
+	+	+	(-)	(-)	(-)	3/1.5	2/67	1/33
+	+	+	+	(-)	-	2/1	1/50	1/50
+	+	+	+	(-)	(-)	7/3.5	4/57	3/43
+	+	+	+	+	(-)	11/6	9/82	2/18
+	+	+	+	+	+	161/82	105/65	56/35
Thiophanate-methyl								
C	131.25	262.5	525*	1050	2100			
+	(-)	(-)	(-)	(-)	(-)	1/1	1/100	-/-
+	+	+	(-)	(-)	-	2/2	1/50	1/50
+	+	+	+	-	-	2/2	-/-	2/100
+	+	+	+	(-)	-	6/7	1/17	5/83
+	+	+	+	(-)	(-)	7/8	6/86	1/14
+	+	+	+	+	(-)	9/10	7/78	2/22
+	+	+	+	+	+	61/70	36/59	25/41

Gc = Golovinomyces cichoracearum, Px = Podosphaera xanthii;
C = control (untreated by fungicide, characterized by profuse sporulation), *concentration recomended by the producer.
Reaction of CPM: - = sensitive reaction (no sporulation), (-) = tolerant reaction (limited sporulation), + = resistant reaction (profuse sporulation)
**according to Lebeda et al. (2010), Sedláková & Lebeda (2008, 2010)

Table 5. Response of CPM populations in the years 2001-2007 to different concentrations of Fundazol 50 WP (active ingredient : benomyl) and in 2005-2007 to Topsin M 70 WP (active ingredient: thiophanate-methyl)**

Azoxystrobin (Ortiva) exhibited decreasing efficacy in 2007 (Table 6). Even though 40% of CPM isolates were highly sensitive, most of the screened CPM isolates expressed a high level of tolerance or resistance to this fungicide. The screened Czech CPM population showed a high potential for developing resistance to azoxystrobin. Before this time, occurrence of azoxystrobin-resistant strains had not been reported from the CR, therefore results of our one-year study could be considered a base for future experiments. Azoxystrobin belongs to the strobilurin QoI fungicides which have a single-site mode of

action that binds to the subunit protein of cytochrome bc_1 complex of the electron transport chain located in the inner-mitochondrial membrane, thereby inhibiting fungal respiration (Sauter et al., 1995). It is generally known now that these site-specific fungicides have a high risk of resistance development in a pathogen population (McGrath, 2001). The type of resistance to strobilurins is qualitative (same as for benomyl) which means that individuals of the CPM pathogen are either highly sensitive to strobilurins or highly resistant (Ishii et al., 2001; McGrath & Shishkoff, 2003). This fact does not correspond to the situation in Czech CPM population in relation to azoxystrobin in the year 2007. This could reflect the structure of the CPM population in Central Europe, where *Gc* is propably the most important CPM pathogen on field cucurbits (Lebeda, 1983; Křístková et al., 2003, 2009) and CPMs are highly variable in their pathogenicity and virulence (Jahn et al., 2002; Lebeda & Sedláková, 2004 a,b, 2006; Sedláková & Lebeda, 2008, 2010). In many parts of eastern Asia and the northern Mediterranean area, strobilurin resistance in CPM has developed, sometimes within the first season of use (Hollomon & Wheeler, 2002). Resistance to azoxystrobin in *Px* was recorded from Spain (Fernández-Ortuño et al., 2006), Japan (Ishii et al., 2001) and the USA (McGrath & Shishkoff, 2003; McGrath, 2005, 2006). Reduced efficacy of azoxystrobin was also claimed in cucurbit downy mildew (*Pseudoperonospora cubensis* (Berk. & M. A. Curtis) Rostovzev.) in Japan (Ishii et al., 2001) and the USA. Cross-resistance among QoI fungicides has been documented with *E. graminis* f. sp. *tritici* and *Plasmopara viticola* (Heaney et al., 2000). For most of the plant pathogens in which QoI resistance has been described, resistance was conferred by a point mutation in cytochrome b (*cyt b*) gene leading to an amino acid change from glycine to alanine at position 143 (G143A) (Gisi et al., 2002). Based on recent published data, it is evident that field resistance to QoI fungicides in *Px* is not supported by typical mutations in the mitochondrial cytochrome *b* gene (Fernández-Ortuño et al., 2008).

Efficacy of fungicide (active ingredient), conc. µg a.i./ml^{-1} , ppm)						Total no. of isolates/frequency (%)		
Azoxystrobin						Σ	*Gc*	*Px*
C	125	250	500*	1000	2000			
+	-	-	-	-	-	14/40	11/79	3/21
+	(-)	-	-	-	-	6/17	1/17	5/83
+	(-)	(-)	-	-	-	1/3	-/-	1/100
+	(-)	(-)	(-)	(-)	(-)	1/3	-/-	1/100
+	+	-	-	-	-	1/3	1/100	-/-
+	+	+	(-)	(-)	-	1/3	1/100	-/-
+	+	+	+	+	+	11/31	7/64	4/36

Gc = Golovinomyces cichoracearum, Px = Podosphaera xanthii;
C = control (untreated by fungicide, characterized by profuse sporulation), *concentration recomended by the producer.
Reaction of CPM: - = sensitive reaction (no sporulation), (-) = tolerant reaction (limited sporulation), + = resistant reaction (profuse sporulation)
**according to Lebeda et al. (2010), Sedláková & Lebeda (2008, 2010)

Table 6. Response of CPM populations in the year 2007 to different concentrations of Ortiva (active ingredient: azoxystrobin)**

4.2 New York (northeastern USA)

4.2.1 Investigation of fungicide sensitivity in cucurbit powdery mildew populations

The in-field seedling fungicide sensitivity bioassay (see section 3.2) has been used to assess fungicide sensitivity of CPM pathogen populations most growing seasons on Long Island, NY. Spring-planted zucchini and summer squash crops (*Cucurbita pepo*) were used for the first bioassay conducted in a year because this is where powdery mildew starts to develop each season. Additional bioassays were conducted in main season crops and research plantings of jack-o-lantern pumpkin (*C. pepo*) to examine the impact of fungicide use on fungicide sensitivity.

Seedlings of pumpkin cv. 'Sorcerer' were started in a growth chamber, then transplanted to pots and grown in a greenhouse until the 1st to 4th true leaf stage. Their growing point and unexpanded leaves were removed just before treatment. Seedlings were treated with various doses of fungicides using a back-pack CO_2-pressurized sprayer equipped with a single nozzle boom operated at 40 psi. Treated seedlings were left overnight to dry. Then they were placed in fields amongst cucurbit plants with powdery mildew symptoms. Each group of seedlings had 1 treated seedling for each fungicide dose tested plus two non-treated seedlings. They were left for about 4 hours during the middle of the day to be exposed to the wind-dispersed spores of the CPM pathogen in the fields. Afterwards the seedling were kept in a greenhouse until symptoms of powdery mildew were visible, which took at least 10 days. Then severity (percent tissue with symptoms) on upper leaf surfaces was visually estimated for each true leaf. Frequency of pathogen strains in a field able to tolerate each fungicide dose was estimated by calculating the ratio of severity on fungicide-treated plants relative to non-treated plants.

4.2.2 Determination of fungicide sensitivity for cucurbit powdery mildew isolates

Isolates of *Px* were obtained from field-grown cucurbit plants for determining fungicide sensitivity of individual members of CPM populations with the leaf-disc bioassay developed in NY (see section 3.1.1). Leaves with discrete colonies of CPM on the abaxial surface were collected from commercial and research fields. In the laboratory leaves were cut to remove pieces with discrete colonies, which were placed on wet filter paper in Petri dishes, with abaxial surface facing upward, to incubate for at least one day to obtain ample conidia for transferring to cotyledons in culture plates (Fig. 11). Conidia were moved with the sealed pipette transfer tool. Cultures were incubated for 9-21 days under constant light in the laboratory before use in a bioassay or transfer to a new leaf. Optimum period was 11 days. Cultures could be held for a longer period before transferring had to be done, due to declining culture condition, when leaves used were in peak condition and conidia were transferred to one location rather than multiple locations to obtain ample quantity of conidia for bioassays. Pumpkin cv. 'Sorcerer' was grown to the cotyledon stage in 48-cell trays filled with Pro-mix in a growth chamber at 29°C/26°C day/night with 18-hr day and daily watering. Isolates were tested in successive bioassays with three fungicide concentrations each (see section 3.1.1).

4.2.3 Fungicides

The fungicides tested were:

thiophanate-methyl at 50 ppm (µg/ml) (formulated as Topsin M 70 WP®; FRAC Group 1; producer: Nippon Soda Co. Ltd., Tokyo, Japan);

trifloxystrobin at 50 ppm (Flint®; FRAC Group 11; producer: Bayer CropScience, Research Triangle Park, NC);
myclobutanil at 20, 40, 80, 100, 120 and 150 ppm (Nova 40W®; FRAC Group 3 fungicide; producer: Dow AgroSciences LLC, Indianapolis, IN);
triflumizole at 80, 100, 120 and 150 ppm (Procure 50WS®; FRAC Group 3; producer: Crompton Manufacturing Co., Inc., Middlebury, CT);
boscalid at 125, 150, 175, 200 and 500 ppm (Endura®; FRAC Group 7; producer: BASF Corporation, Research Triangle Park, NC); and
quinoxyfen at 1, 5, and 10 ppm (Quintec®; FRAC Group 13; producer: Dow AgroSciences LLC, Indianapolis, IN).
Endura was used rather than the fungicide with boscalid registered for this use, Pristine, because it also contains pyraclostrobin. All other fungicides used are registered and labeled for managing CPM in the USA. There are several FRAC Group 3 and 11 fungicides registered in the USA and labeled for CPM. Myclobutanil and trifloxystrobin were used as representatives for these groups, respectively. Since MBC and QoI resistance is qualitative only one concentration is needed for its detection. A range of concentrations was used for the other fungicides because resistance is quantitative. All concentrations listed were not included in each assay. The concentrations tested were usually selected based on previous results with the goal of having discriminatory concentrations that some isolates would be resistant to.

	Proportion of population fungicide tolerant [average (range)]				
Year	Thiophanate-methyl 50 ppm	Trifloxystrobin 50 ppm	Myclobutanil 80 ppm	Boscalid 175 ppm	Quinoxyfen 10 ppm
2006	74 (50-89)	30 (5-48)	62 (38-86)	ND	ND
2007	ND	70 (0-100)	71 (13-100)	14 (1-22)	0 (0-0)
2008	72 (18-100)	66 (21-100)	28 (7-46)	12 (6-21)	1 (0-2)
2009	59 (20-100)	80 (28-100)	21 (7-29)	20 (4-48)	2 (0-5)

ND = not determined.

Table 7. Fungicide sensitivity of *Podosphaera xanthii* populations in NY based on results from seedling bioassays conducted in spring crops early in disease development

	Proportion of population fungicide tolerant (average for all fields assayed)							
	Myclobutanil		Triflumizole		Boscalid		Quinoxyfen	
Date	100 ppm	120 ppm	100 ppm	120 ppm	125 ppm	175 ppm	1 ppm	5 ppm
8/10/07	20	7	55	31	21	16	38	1
8/23/07	38	13	15	6	45	14	18	2
9/7/07	4	0	0	0	23	11	4	3
10/2/07	3	0	0	0	20	13	ND	2

ND = not determined.

Table 8. Fungicide sensitivity of *Podosphaera xanthii* populations in NY based on results from seedling bioassays conducted in pumpkin crops during the 2007 season

	Proportion of population fungicide tolerant (average for all fields assayed)							
	Trifloxy-	Myclobutanil		Triflumizole	Boscalid		Quinoxyfen	
Date	strobin	80 ppm	120 ppm	80 ppm	100 ppm	175 ppm	1 ppm	5 ppm
8/6/08	9	2	1	2	15	14	7	2
8/12/08	42	6	4	7	44	24	25	4
8/21/08	ND	9	5	8	20	15	6	9
9/9/08	42	7	ND	7	22	15	4	4
9/30/08	16	14	ND	ND	7	ND	4	2

ND = not determined.

Table 9. Fungicide sensitivity of *Podosphaera xanthii* populations in NY based on results from seedling bioassays conducted in pumpkin crops during the 2008 season

	Proportion of population fungicide tolerant (average for all fields assayed)							
	Trifloxy-	Myclobutanil		Triflumizole	Boscalid		Quinoxyfen	
Date	strobin	80 ppm	120 ppm	80 ppm	50 ppm	175 ppm	1 ppm	10 ppm
9/3/09	77.4	4.1	0.4	4.6	17.3	10.9	6.3	0.5

ND = not determined.

Table 10. Fungicide sensitivity of *Podosphaera xanthii* populations in NY based on results from seedling bioassays conducted in pumpkin crops during the 2009 season

4.2.4 Results and discussion

Both bioassays proved to be useful tools for investigating fungicide resistance in CPM. The in-field seedling fungicide sensitivity bioassay conducted in spring-planted crops provided information quickly (11 days) about the CPM population that could be used to guide fungicide recommendations for main season crops. The leaf-disc bioassay provided precise information about the sensitivity of individuals in the population, but required a lot of labor and time to obtain. Both procedures were used to examine impact of fungicide use on pathogen sensitivity to fungicides.

A range in response from very sensitive to resistant was detected to the five fungicide chemical groups tested with the seedling bioassay conducted in spring crops in NY in 2006-2009 (Table 7). On average, greater than 50% of the CPM population was resistant to MBC (FRAC Group 1) fungicides. Resistance to QoI (FRAC Group 11) fungicides was also common most years. There evidently was a lot of variation among fields where the bioassay was conducted. During each production season there was little evidence of change in the pathogen population (Tables 8-10).

5. Conclusions and future prospects

1. Currently there are two fungi predominantly responsible for causing cucurbit powdery mildew (CPM). They are distributed worldwide and considered economically important on almost all commonly grown cucurbits (Křístková et al., 2009). *Podosphaera xanthii* is considered more common than *Golovinomyces cichoracearum* (McGrath & Thomas, 1996).

2. Protection of cucurbits against CPM is primarily accomplished with fungicides; resistant cultivars are not available in all horticultural types and host plant resistance is often used in combination with fungicides for CPM to achieve a high degree of control and to manage selection of pathogen races able to overcome genetic resistance (McGrath, 2001, 2006).
3. Fungicide resistance is known in both powdery mildew species (McGrath, 2001; Sedláková & Lebeda, 2008).
4. Based on published literature, there is very limited information about fungicide resistance/susceptibility of *Golovinomyces cichoracearum* in comparison with *Podosphaera xanthii* (McGrath, 2001; Sedláková & Lebeda, 2008). The dominant pathogen is considered to be *P. xanthii*. However, *G. cichoracearum* is widespread around the world in temperate regions, and probably is the most important CPM pathogen on field-grown cucurbits in Central Europe (Lebeda, 1983; Křístková et al., 2003; 2009).
5. During the last several decades, many new fungicides effective against CPM (e.g. Kuck & Russell, 2006; Tomlin, 2003) have been introduced to the market, providing superior control over the contact fungicides relied upon earlier (López-Ruiz et al., 2010; McGrath, 2006; McGrath & Shishkoff, 2003).
6. These new fungicides are mostly single-site inhibitors in a metabolic pathway of the pathogens and thus have a high risk of resistance developing to them (McGrath, 2001, 2005, 2006; McGrath & Shishkoff, 2001; Sedláková & Lebeda, 2008).
7. There have been reports of failure to control CPM with fungicides; some cases have been shown to be associated with resistance in this group of pathogens (McGrath, 2001, 2006; Sedláková & Lebeda, 2008).
8. Distribution and dynamics of fungicide resistance in CPM fungi in large growing areas or continents are not known. The goal of recent research has been to obtain comprehensive data about fungicide resistance pertaining to the geographic distribution of resistant pathogen strains, their spatial and temporal variability, and changes in the fungicide resistance status of the pathogen in Central Europe (Czech Republic). Temporal aspects of fungicide resistance and impact of fungicide use on pathogen sensitivity is being investigated in the USA (NY).
9. Various procedures, described in this chapter, have been developed and utilized to investigate fungicide resistance in CPM fungi in the laboratory and field.
10. Nevertheless, additional detailed studies on CPM fungi are needed focused on the mechanisms of fungicide resistance, its genetic background, epidemiology, and spatial and temporal changes to gain a better understanding of this phenomenon. More international cooperation and coordination are required for significant progress in this field, and for more efficient plant protection of cucurbits against both powdery mildew species.

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12

Phenotypic Analyses of Fenhexamid Resistant *Botrytis cinerea* Mutants

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1. Introduction

Grey mould caused by the fungus *Botrytis cinerea* Pers ex Fr. [anamorph of *Botryotinia fuckeliana* (de Bary) Whets] is a major disease of grapes (Elad et al. 2004; Keller et al. 2004). Although biological control of fungal growth is becoming popular on account of environmental concerns (Elmer & Reglinski 2006), generally *B. cinerea* infections are prevented by the application of fungicides. Fenhexamid, a hydroxanilide derivative, is one of the newly developed fungicides that exhibit strong inhibitory activity against *B. cinerea*, owing to its ability to inhibit 3-ketoreductase in the C4-demethylation enzyme complex during ergosterol biosynthesis (Rosslenbroich et al. 2000; Debieu et al. 2001). Fenhexamid is used in many European countries and is considered to be an effective fungicide against *B. cinerea* due to its unique mode of action (Rosslenbroich et al. 2000; Debieu et al. 2001; Baroffio et al. 2003). However, *B. cinerea* wild types exhibiting resistance to fenhexamid were detected shortly after its introduction (Leroux et al. 2002; Baroffio et al. 2003). In Japan, although fenhexamid was registered in 2000, it is not widely used and thus, the existence of fenhexamid resistance in *B. cinerea* has not been reported in Japan.

Molecular-based techniques have been developed to rapidly detect the sensitivity of fungi to several fungicides by taking advantage of point mutations in target genes (Oshima et al. 2002; Paplomatas et al. 2004; Banno et al. 2008, Furuya et al. 2009; Saito et al. 2009). A rapid method for the detection of fungicide resistance is necessary for viticulturists to better understand the incidence of resistance in *B. cinerea* populations in their vineyards. However, it is unknown whether molecular-based techniques for fungicide resistance detection could be applied to the study of fenhexamid resistance in *B. cinerea*. In fenhexamid-resistant field isolates of *B. cinerea*, Fillinger et al. (2008) found various point mutations leading to amino acid substitutions between 195 and 412 in the target protein, 3-ketoreductase, and revealed a relationship between high resistance to fenhexamid and the amino acid substitution at codon 412 in the protein. In the present study, in order to evaluate whether the detection of fenhexamid resistance with molecular-based techniques is possible and whether there are other point mutations in the target gene that are related to the resistance to fenhexamid, we generated 18 mutants showing resistance to fenhexamid by chemical mutagenesis and determined putative point mutations in the *erg27* gene that conferred resistance to fenhexamid.

2. Materials and methods

2.1 *B. cinerea* strains

Two wild type strains of *B. cinerea* (YW01 and YU0622) were collected from the experimental vineyard of the University of Yamanashi, Yamanashi, Japan in 2006. The two wild type strains were used to obtain fenhexamid-resistant mutants. The strains were maintained on potato dextrose agar (PDA, Difco, Detroit, MI) plates and incubated at 23°C until use.

2.2 MNNG treatment

B. cinerea mutation was performed as described by Ziogas et al. (2003) with slight modifications. Briefly, conidia were obtained from 2-week-old *B. cinerea* cultures on PDA plates by washing them with sterile water and filtering them through sterile mesh twice. Conidial concentration was determined with a hemocytometer. Conidial suspension, which contained approximately 10^7 conidia mL^{-1}, was agitated in 10 µg mL^{-1} N-methyl-N-nitro-nitrosoguanidine (MNNG) on a shaker at 23°C for 4 h in the dark and washed twice with sterile distilled water. The conidia were resuspended in water, spread on PDA plate containing 20 µg mL^{-1} fenhexamid and incubated at 23°C for 2 weeks. *B. cinerea* colonies were subsequently transferred and maintained on a PDA plates containing 2.5 µg mL^{-1} fenhexamid. In Total, 18 fenhexamid-resistant mutants were obtained by this method (hereafter referred to as YM01 to YM18).

2.3 Physiological characteristics of *B. cinerea* mutants

The two wild type strains and eighteen mutants, YM01 to YM18, were examined for mycelial growth, spore germination and sensitivity to osmotic pressure on PDA plates. Three 4-mm mycelial plugs for each strain were transferred to the centers of PDA plates (diameter 15 cm) for mycelial growth measurements. Six measurements of the colony diameter for each strain were taken after continuous incubation for 4 days at 23°C in the dark. Mean diameters were calculated with the diameter of plug being subtracted from the mean. Spore germination was determined by counting germinated spores using a microscope following incubation for 6 h on PDA plates in the dark. Sensitivity to osmotic pressure was assessed after incubation for 3 days on PDA plates containing 2.5% KCl as described by Ziogas et al. (2003). Data on mycelial growth, spore germination and sensitivity to osmotic pressure were subjected to analysis of variance using a Dunnett's multiple range test at $P = 0.05$.

2.4 Fungicide sensitivity tests

Fungicide sensitivity tests were performed for the wild type strains and eleven mutants, YM01, YM02, YM03, YM04, YM05, YM06, YM10, YM11, YM12, YM13, YM14. The fungicides used in this study were benzimidazole (thiophanate-methyl; Topjin M 70%; Nippon Soda, Tokyo, Japan), benomyl (Benleto 50%, Sumitomo, Tokyo, Japan), dicarboximide (procymidone; Sumirex 50%; Sumitomo, Tokyo, Japan), fenhexamid (Sigma-Aldrich), fludioxonil (Wako, Osaka, Japan), iprodione (lovral, Bayer CropScience, Tokyo, Japan) and N-phenylcarbamate (diethofencarb, Wako, Osaka, Japan). For each *B. cinerea* strain, mycelial disks (4 mm diameter) were excised from the leading edge growing actively on PDA plates

and transferred to PDA plates (diameter 9 cm) containing each fungicide at various concentrations. After incubation for 3 days at 23°C in the dark, four measurements of the diameters of the mycelial growth on PDA plate were taken from each of three replicates per treatment. Means were calculated with the diameter of the inoculated plug being subtracted from each mean. At least six concentrations each, three replications for each fungicide, were tested to calculate respective fungitoxicity curves. EC_{50} for each fungicide was determined using the dose-response curves after probit analysis.

Sensitivity to fenhexamid for two wild type strains and eighteen mutants of *B. cinerea* was determined by placing the mycelial plug obtained as described above, onto PDA plates (diameter 9 cm) containing 2.5 or 80 µg/µl of fenhexamid. Sensitivity of each strain to fenhexamid was classified as follows: sensitive (S) if there was no growth on the PDA plate containing fenhexamid at the lowest concentration; moderately resistant (MR) if there was growth on the PDA plate containing 2.5 µg/µl of fenhexamid but not at the higher concentration; and highly resistant (HR) if there was growth at all concentrations.

2.5 Virulence and fenhexamid resistance of *B. cinerea* strains on cucumber seedlings

The virulence of *B. cinerea* wild type strains and mutants was determined by examining symptom severity caused by each strain on cucumber seedlings as described by Ziogas et al. (2003) with slight modifications. Cucumber (*Cucumis sativus* cv. Tokiwajibai) seedlings growing in plastic pots for 8-10 days (two seedlings per 7 cm pot) were used at the cotyledon stage. Commercial fenhexamid (Password 50%, Bayer CropScience, Tokyo, Japan) was used for this experiment by dissolving it in sterile water to obtain the appropriate concentration of fenhexamid solution. The cotyledons of cucumber seedlings were sprayed with fenhexamid solution with a hand sprayer and the sprayed seedlings were kept at room temperature until the surfaces of the cotyledons were dry. The center of each cotyledon was punctured with a sterile needle and a 4-mm mycelial plug from the edge of the growing mycelia on PDA plates was placed on the wound. The inoculated plants were incubated at 23°C for 48 h in the dark. Infection incidence was scored by evaluating the lesion of each cotyledon according to the following indices: 0: no infection; 0.5: rot only under inoculum; 1: rot two times bigger than the plug; 2: rot three times bigger than the plug; 3: rot four times bigger than the plug; 4: rot more than five times bigger than the plug (Fig 1). Twenty cotyledons were used for each test.

2.6 DNA sequence of the *erg27* gene in *B. cinerea* mutants

To determine the position of the genetic mutations leading to fenhexamid resistance, we analyzed the DNA sequence of the *erg27* gene encoding 3-ketoreductase in the two wild types strains and the 18 mutants. Genomic DNA was extracted from mycelia according to the protocol described in a previous study (Saito et al. 2009). To determine the sequence of the *erg27* gene, four pairs of oligonucleotide primers were designed as follows: 3kr-F1 (5′- ATGGGATTACCACCATGGGA -3′) and 3kr-R1 (5′- TGCGGAATAAGTGGCGGTAC -3′), 3kr-F2(5′- TGGTCTCCGATTACCTGATG -3′) and 3kr-R2 (5′- TCGTGTGCGAGAACGTAATG -3′), 3kr-F3 (5′- CTTCAAAACAAGCCAAACCC -3′) and 3kr-R3 (5′- GGCTTCCCATCCATCTTACA -3′), and 3kr-F4 (5′- AATCGGAGGAAGAACCAGCA -3′) and 3kr-R4 (5′- TCATTTTTTAACCTTCAAAA -3′).

These four primer pairs amplify the positions between 1234 and 2920 of the *erg27* gene, corresponding to an entire open reading frame (accession no. AY220532) (Albertini et al. 2004). Ten µL of PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.2 mM of each dNTP (TAKARA, Ootsu, Japan), 2 µM of each primer pair, 1.5 U of *Taq* DNA polymerase (TAKARA), and 100 ng of *B. cinerea* DNA was dispensed into a microtube. PCR amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). After an initial incubation at 95ºC for 3 min, *B. cinerea* DNA was amplified for 35 cycles of 95ºC for 30 s; 65ºC for 30 s; and 72ºC for 40 s. The final extension was done at 72ºC for 7 min. The PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining under UV illumination. DNA sequencing of PCR products was performed using an ALFexpress DNA sequencer (GE Healthcare, Piscataway, NJ) with a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) according to the manufacturer's instructions.

2.7 Statistical analysis

Analyses were made with Ekuseru-Toukei 2006 (Social Survey Research Information Co. Ltd. Japan). Dunnett's multiple range test was used to assess the differences between mycelial growth rate, osmotic sensitivity and spore germination.

3. Results

3.1 Physiological characteristics of *B. cinerea* mutant strains

Eighteen fenhexamid-resistant mutants were obtained by MNNG treatment. All the mutants were derived from wild type strain YW01, except YM03 was derived from wild type strain YU0622. According to our classification of sensitivity to fenhexamid, the two wild type strains, YW01 and YU0622, were classified as sensitive strains (S). Five mutants YM03, YM07, YM08, YM10 and YM11 were classified as highly resistant (HR) and the remaining 13 mutants were classified as moderately resistant (MR) (Table 1). In order to investigate fitness parameters of fenhexamid resistant mutants, mycelial growth rate, sensitivity to osmotic pressure and spore germination were examined for the two wild type strains and eighteen mutants. For mycelial growth rate, seven mutant strains, YM07, YM09, YM11, YM14, YM15 YM16 and YM18, showed an extreme reduction in mycelial growth and were excluded from Dunnett's multiple range tests. Two mutant strains, YM04 and YM08, showed a significant reduction in mycelial growth rate when compared to that of wild type strain YW01 ($P < 0.01$) (Table 1). Three mutant strains, YM04, YM13 and YM17, showed a significant reduction in sensitivity to osmotic pressure and three strains, YM09, YM14 and YM16, in spore germination (Table 1).

3.2 Sensitivity to other fungicides

A study of the sensitivity of 11 representative mutants of *B. cinerea* to other fungicides in comparison with the two wild-type strains did not show any cross-resistance of fenhexamid with benzimidazole, benomyl, diethofencarb, fludioxonil, iprodione and procymidone (Table 2). However, seven mutants, YM01, YM04, YM05, YM10, YM11, YM12 and YM13, showed a higher EC_{50} value for benzimidazole than that of the two wild type strains (Table 2).

Strain	Sensitivity to fenhexamid [a]	Mycelial growth [b] (mean±SE[e])	Sensitivity to osmotic pressure [c] (mean±SE[e])	Spore germination [d] (mean±SE[e])
YW01	S	110.6±2.1[f]	94.5±1.2[f]	94.7±0.8[f]
YU0622	S	109.0±2.5	94.2±0.6	92.2±0.4
YM01	MR	105.4±1.5	95.7±1.2	92.5±1.2
YM02	MR	111.3±1.9	97.3±0.7	96.6±0.7
YM03	HR	109.3±1.5	94.7±1.0	93.7±1.6
YM04	MR	100.9±1.5**	82.7±1.5**	86.8±0.8
YM05	MR	106.1±1.2	93.5±1.4	92.5±0.9
YM06	MR	103.4±0.4	96.0±0.7	93.3±1.2
YM07	HR	18.8±4.0[g]	nd[h]	95.2±1.9
YM08	HR	97.0±1.4**	90.3±1.2	92.3±0.7
YM09	MR	38.4±1.8[g]	nd	65.7±2.8**
YM10	HR	107.0±0.7	94.3±0.9	92.7±2.5
YM11	HR	59.4±2.3[g]	nd	98.8±0.9
YM12	MR	110.4±1.1	97.7±0.8	95.9±1.7
YM13	MR	107.9±1.3	86.5±1.7**	92.5±1.8
YM14	MR	28.5±0.7[g]	nd	55.0±6.7**
YM15	MR	31.3±0.1[g]	nd	81.0±4.6
YM16	MR	5.8±0.6[g]	nd	61.8±6.7**
YM17	MR	116.6±0.5	89.0±0.9*	92.5±2.8
YM18	MR	27.4±2.0[g]	nd	94.6±3.1

Table 1. Characteristics of wild type and mutant strains of *B. cinerea*

[a] S: sensitive, MR: moderately resistant, HR: highly resistant.
[b] Mean colony diameter (mm) measurements after incubation for 4 days (n=4).
[c] Proportion (%) of mycelial growth in the presence of KCl (2.5%) after incubation for 3 days (n=4).
[d] Proportion (%) of germinated conidia after incubation for 6h (n=500).
[e] SE stands for standard error.
[f] Within columns, asterisks represent a significant difference when compared with wild type YW01, according to Dunnett's multiple range test (*P<0.05: **P<0.01).
[g] Data was excluded for Dunnett's multiple range test.
[h] not determined.

Strain	fenhexamid	benzimidazole	benomyl	diethofencarb	fludioxonil	iprodione	procymidone
YW01	0.046	999.05	184.52	0.914	0.029	0.206	0.463
YU0622	0.043	954.30	346.38	1.187	0.013	0.215	0.466
YM01	30.71	2066.36	169.11	0.973	0.041	0.305	0.531
YM02	13.40	1000.63	181.67	1.000	0.031	0.199	0.472
YM03	118.49	924.26	312.51	1.184	0.013	0.198	0.450
YM04	13.13	2873.76	302.27	1.280	0.012	0.319	0.543
YM05	5.84	1584.32	nd	0.758	0.037	nd	0.234
YM06	11.61	657.46	nd	0.843	0.036	nd	0.243
YM10	140.92	3420.99	nd	0.779	0.045	nd	0.234
YM11	170.60	2088.93	nd	1.169	0.028	nd	0.375
YM12	4.64	2130.84	nd	0.810	0.042	nd	0.236
YM13	3.45	3725.21	nd	0.999	0.042	nd	0.465
YM14	39.47	nd[a]	nd	nd	nd	nd	nd

Table 2. Fungicide EC_{50} values for wild type and mutant strains of *B. cinerea*.

[a] not determined.

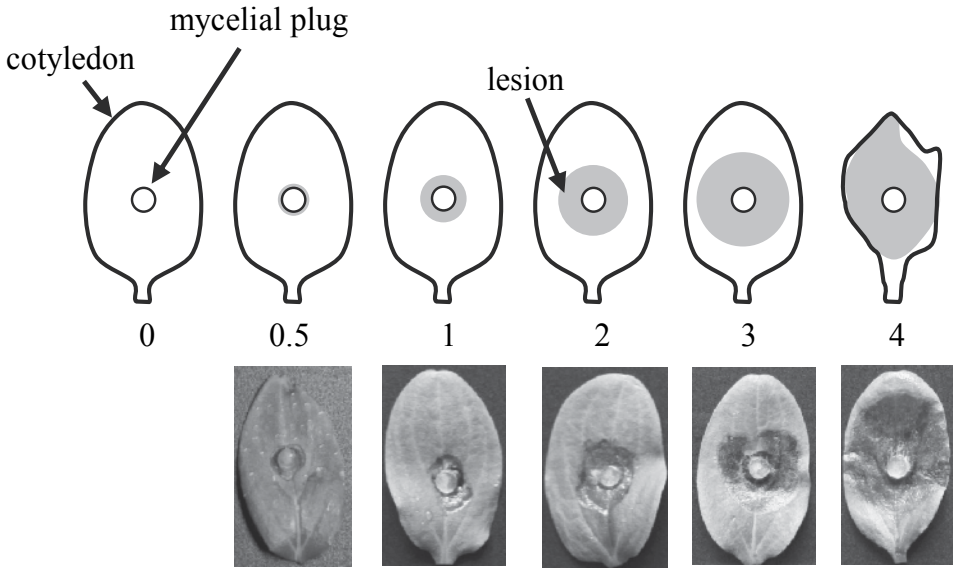

Fig. 1. Scoring key for virulence tests. Lesions on cotyledon of cucumber seedlings were scored according to their sizes. 0: no infection; 0.5: rot only under inoculum: 1: rot two times bigger than the plug; 2: rot three times bigger than the plug; 3: rot four times bigger than the plug; 4: rot more than five times bigger than the plug

3.3 Virulence and fenhexamid response of wild type and mutant strains on cucumber seedlings.

Virulence and fenhexamid response of wild type and 18 mutant strains were examined by monitoring the symptoms on the cotyledon of the cucumber seedlings, according to our evaluation criteria (Fig 1). Virulence studies revealed that seven mutant strains, YM07, YM08, YM14, YM15, YM16, YM17 and YM18, lose their ability considerably to infect cotyledons of cucumber plants (Table 3). However, the other 11 mutants showed virulence similar to the two wild type strains on water treated cotyledons of cucumber seedlings (Table 3). Fenhexamid was effective against the wild type strains but not against the 11 mutants which retain the ability to cause infection. In fact, these eleven mutants were not controlled by fenhexamid at the concentration of 1 000 µg mL^{-1} (Table 3). At the highest concentration of 10 000 µg mL^{-1}, the eleven mutants caused as much of an infection as wild type strains at the lowest concentration of 1 µg mL^{-1} (Table 3).

3.4 Sequence analysis of the *erg27* gene.

To determine the positions of the genetic mutation leading to fenhexamid resistance, we analyzed the DNA sequence of the *erg27* gene encoding 3-ketoreductase in the two wild type strains and 18 mutants. No amino acid substitution was detected in the two wild type strains except a deletion at codon 298 in YU0622 strain (Table 4). We also found the same deletion at codon 298 in YM03, which was derived from YU0622. Various point mutations in the sequenced regions of the *erg27* gene, corresponding to amino acid substitutions between 23 and 516 of the protein, were found in the 18 mutants (Table 4).

fungicide concentration (μ g mL^{-1})	YW01	YU0622	YM01	YM02	YM03	YM04	YM05	YM06	YM07	YM08	YM09	YM10	YM11	YM12	YM13	YM14	YM15	YM16	YM17	YM18
water	4	4	4	4	4	4	4	4	0.50	0.08	4	4	4	4	4	0.63	0.60	0.10	0.43	0
fenhexamid																				
1	3.90	3.83	4	4	4	4	4	4	0	0	4	4	4	4	4	0	0	0	0	0
10	3.58	3.24	4	4	4	4	4	4	0	0	4	4	4	4	4	nd	nd	nd	nd	0
100	2.20	2.07	4	4	4	4	4	4	0	0	4	4	4	4	4	nd	nd	nd	nd	0
1000	1.03	0.68	4	4	4	4	4	4	0	0	4	4	4	4	4	nd	nd	nd	nd	0
10000	0.23	0	3.68	3.21	4	3.86	3.68	3.21	nd[a]	nd	3.46	4	2.48	3.39	3.13	nd	nd	nd	nd	nd

Table 3. Virulence tests on cucumber seedlings with wild type and mutant strains of *B. cinerea*

[a] not determined.

		modified residues in 3-ketoreductase protein																						
strain	Fen[a]	G23	C53	T63	K73	V101	L102	H105	K117	K159	K168	L195	T273	P298	S310	K337	I397	I411	F412	H423	A452	Q495	C516
YW01	S																						
YU0622	S													-[b]									
YM04	MR	S																					
YM05	MR	S																					
YM18	MR	S																					
YM13	MR	S	R																				R
YM12	MR			I																			
YM06	MR				E																		
YM16	MR							Y				S						V					
YM09	MR								K											R	P		
YM17	MR									N			A		P								
YM01	MR																V						
YM02	MR																V						
YM14	MR																V						
YM15	MR																V					R	
YM03	HR													-[b]					V				
YM08	HR					A													I				
YM10	HR						Y				E					E			S				
YM07	HR																		S				
YM11	HR																		S				

Table 4. Resistant phenotypes and associated putative mutations in 3-ketoreductase protein.
a Sensitivity to fenhexamid. HR: highly resistant, MR: moderately resistant, and S: sensitive.
b Deletion of one amino acid.

Eleven mutants out of 18 have only one amino acid substitution in the target gene whereas 7 mutants have more than 2 nonsynonymous substitutions (Table 4). The glycine-to-serine mutation at position 23 and the isoleucine-to-valine mutation at position 397, respectively, were revealed to be common in four moderately resistant mutants (Table 4). At position 412, phenylalanine was substituted by isoleucine, valine or serine in the highly resistant mutants, YM03, YM07, YM08, YM10 and YM11 (Table 4). Interestingly, the mutation at position 412 was observed in five mutants that we classified in the previous section as HR (highly resistant).

4. Discussion

Eighteen fenhexamid resistant mutants were successfully obtained after chemical mutagenesis treatment. The result of the physiological characteristics of wild type strains and mutants showed that eleven mutants out of 18 had a fitness penalty, suggesting that the mutation leading to fenhexamid resistance or other unknown mutations would be responsible for some of physiological abnormalities of the mutants, including reduced mycelial growth, sensitivity to osmotic pressure and spore germination. De Guido et al. (2007) obtained 8 fenhexamid resistant mutants by UV-irradiated or unirradiated conidia plated on fenhexamid-containing medium and examined the physiological characteristics of these mutants in comparison with those of wild type strains. According to their results, three mutants out of eight were penalized in mycelial growth (De Guido et al. 2007). In another study, six fenhexamid mutants obtained by chemical mutagenesis did not show any penalties in mycelial growth and osmotic sensitivity but in spore germination (Ziogas et al. 2003). This variability in the data of some characteristics of fenhexamid resistant mutants might be due not only to the acquisition of the fenhexamid resistance but also other mutations in the genome. Although some physiological penalties observed in the mutants could be associated with the acquisition of fenhexamid resistance, we cannot rule out that these may be due to mutations in other part of the fungal genome. Further genetic studies with an appropriate microorganism would be required to elucidate the exact mechanisms responsible.

Virulence tests showed that seven mutants lost their ability to cause infection on cotyledons of cucumber plants. Although the low mycelial growth rate observed in some of these mutants would explain their loss of infection ability, we could not uncover the reason for loss of virulence in the other mutants, such as YM07 and YM17. In the wild type strains, the pathogenic symptoms on the cotyledons were gradually reduced as the concentration of fenhexamid was increased, whereas in eleven mutants, the pathogenic symptoms were constantly observed even when the concentration of fenhexamid reached 10000 μg mL^{-1}. This result suggested that regardless of the fenhexamid resistance level, fenhexamid-resistant *B. cinerea* strains may no longer be susceptible to fenhexamid in field conditions, as the concentration of fenhexamid used to spray vineyards is restricted to less than 750 μg mL^{-1} per application in Japan.

Although Ziogas et al. (2003) reported that the mutation that conferred resistance to fenhexamid was responsible for the increased sensitivity to fludioxonil and iprodione in *B. cinerea*, we didn't detect any cross-resistance relationships among fungicides tested in this study. This contradiction could be attributed to the occurrence of additional mutations in

the relevant gene that conferred tolerance to other fungicides. Resistant mutants were classified into two phenotypic classes in their response to fenhexamid (moderately and highly resistant). The existence of two levels of resistance suggests that distinct mutations in the same locus or in different loci might be involved.

Sequence analysis of the *erg27* gene encoding the target protein of fenhexamid was conducted for 2 wild type strains and 18 mutants. We found a deletion at codon 298 in one of the wild type strain, YU0622 and also in one of the mutants, YM03 which was derived from YU0622. However, this did not appear to have significant effect on virulence, physiological characteristics, or sensitivity to fungicides. Thus, the deletion at codon 298 did not affect the sensitivity to fenhexamid.

Notably five mutants classified as highly resistant, YM03, YM07, YM08, YM10 and YM11, have consistently a point mutation at codon 412. The substitution of phenylalanine at codon 412 with isoleucine, serine or valine has been reported in fenhexamid-resistant *B. cinerea* field isolates (Fillinger et al. 2008). Fillinger et al. (2008) also reported that the point mutation of phenylalanine at codon 412 is responsible for the high resistance of *B. cinerea* field isolates to fenhexamid. In addition, taking into consideration that only one point mutation at codon 412 was found in YM03, YM07 and YM011 in the *erg27* gene, the amino acid substitution at codon 412 might be responsible for high resistance to fenhexamid. In moderately resistant mutants, we detected multiple point mutations in the *erg27* gene, corresponding to amino acid substitutions between position 23 and 516 of the protein. Fillinger et al. (2008) found amino acid substitutions between position 195 and 400 of the protein in field isolates of *B. cinerea* that showed moderate resistance to fenhexamid. It is noteworthy that the moderately resistant mutants, YM04, YM15, and YM18, showed one point mutation at codon 23 where the putative NADPH binding site of the N-terminus of the protein is located (Albertini & Leroux 2004). The glycine-to-serine substitution at codon 23 in those three mutants is responsible for one of the important features of 3-ketoreductase in *B. cinerea*. The 3-ketoreductase gene is conserved in mammals and possesses a putative N-terminal NADP(H) binding site whose conserved structure is GXXXGXG (Peltoketo et al. 1999; Kallberg et al. 2004). In those three mutants, the glycine-to-serine substitution occurred at the second glycine in the site, resulting in GXXXSXG. Interestingly, the glycine at this position is not conserved among 3-ketoreductases in mammals (Peltoketo et al. 1999). Furthermore, in 3-ketoreductase of *Saccharomyces cerevisiae* and *Candida albicans*, asparagine is found at this position instead, resulting in GXXXNXG (Peltoketo et al. 1999). However, the precise mechanism by which the glycine-to-serine substitution at codon 23 of *B. cinerea* 3-ketoreductase induces fenhexamid resistance in *B. cinerea* remains to be determined. The glycine-to-serine substitution may modify the affinity of 3-ketoreductase to fenhexamid. Future studies employing genetic transformation analysis of wild type isolates having a modified *erg27* gene on the NADPH binding site may reveal the relationship between the function of the binding site and fenhexamid resistance in *B. cinerea*. Collectively, a great number of independent point mutation in the *erg27* gene, leading to amino acid substitutions, would be involved in the resistance to fenhexamid. Therefore, it would be impossible to develop molecular-based techniques to detect resistance to fenhexamid.

Ziogas et al. (2003) have reported that the fenhexamid-resistant mutation frequency in *B. cinerea* is high and thus, its inherent resistance risk would be considerable. In the present

study, our findings suggest that the point mutation at codon 412 may confer high resistance to fenhexamid, whereas other amino acid substitutions occurring in 3-ketoreductase might confer moderate resistance, demonstrating that various amino acid substitutions in 3-ketoreductase confer fenhexamid resistance to *B. cinerea*. This may be the reason why *B. cinerea* frequently acquires fenhexamid resistance in the field (Leroux et al. 2002; Barroffio et al. 2003; Ma & Michailides 2005; Esterio et al. 2007; Myresiotis et al. 2007; Kretschmer & Hahn 2008). Therefore, it would be important to use fenhexamid in carefully designed anti-resistance strategies to maintain its effectiveness.

5. Conclusion

Our results suggested that various point mutations in the *erg27* gene that encodes 3-ketoreductase might confer fenhexamid resistance to *B. cinerea*. Due to the various amino acid substitutions in the protein, currently available molecular-based techniques would not be applicable to the detection of fenhexamid resistance in *B. cinerea*. Finally, we need to account for the risk of emerging fenhexamid resistance in *B. cinerea* populations in grapevines.

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13

Utilization of Sweat Potato Starch Wastewater and Monosodium Glutamate Wastewater for Cultivation of an Anti-Fungal Biocontrol Agent *Paenibacillus Polymyxa*

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1. Introduction

Effluents from monosodium glutamate manufacturing plants possess a high strength of COD (10,000--30,000 mg/l), ammonium (15,000--25,000 mg/l), sulphate (15,000--30,000 mg/l) and very low pH (< 2) (Yang *et al.*, 2005). Effluents from sweat potato starch industry contains a high load of protein, pectin, and starchy materials with the COD of 10,000 - 35,000 mg/l (Mishra *et al.*, 2004). Due to the low pH, high concentration of COD, sufate and NH_3-N, the treatment of such wastewaters by conventional activated sludge processes consumes a lot of energy, resulting in high treatment costs (Bai *et al.*, 2004).

Phyllosphere bacteria often have a positive influence upon plant, where they may be involved in the fixation of nitrogen, promoting the growth of plants, or the control of plant pathogens. However, some high infectivity fungi may damage many economically important crops and trees, and bring a significant risk and safety concerns for the food supplies. Although chemical control agents have been used world widely, the biological control agents have attracted a great R&D interest because of their potential for long-term application as environmental friendly agents (Ten Hoopen and Krauss, 2006). Biocontrol agents have been used to protect plants against foliar diseases in several crops (Yuen and Schoneweis, 2007; Alvindia and Natsuaki, 2008; Perello *et al.*, 2007). However, many of them are poor competitors for leaf surface nutrients compared with indigenous phyllosphere microbes (Zhang *et al.*, 2008).

Strains of *Paenibacillus polymyxa* have been isolated from different soils (Ash *et al.*, 1993), rhizospheres and roots from plants cultivated all over the world, and many of them have been described as effective plant growth promoting rhizobacteria (Pichard *et al.*, 1995; Petersen *et al.*, 1996; Lorentz *et al.*, 2006). Untill now, *P. polymyxa* has been seldom discovered in the phyllosphere. In the present work, a strain of *P. polymyxa* EBL06 was isolated from wheat phyllosphere, which could restrain the growth of the filamentous fungi. *P. polymyxa* strains

have been proved to produce a wide variety of secondary metabolites, including different antibacterial and/or antifungal compounds (von der Weid *et al.*, 2000). Therefore, the antagonistic effect of these strains upon microbial growth suggests a potential application as biological control agents. The aims of this study were to characterize the newly isolated strain of *P. polymyxa* EBL06 and optimize the production of the bacterium using the sweat potato wastewater (SPW) and the monosodium glutamate wastewater (MGW).

2. Materials and methods

2.1 Isolation and *in vitro* antagonist assays

The experiment was conducted in a native field located at the Tongzhou near Beijing City, China. Wheat cultivars were planted in the field in October, 2006, watered and fertilized in accordance to local cultivation practices. Wheat phyllosphere microbes were collected in May, 2007 according to the methods described by Zhang *et al.* (2008). An anti-fungi bacterial strain EBL06 was isolated on Potato-dextrose Agar (PDA) medium Petri dish, and maintained on the PDA slant tubes. The antagonistic activity of the strain was evaluated by the method of confronting cultures with the filamentous fungi (Foldes *et al.*, 2000; Bai *et al.*, 2008), such as *Trichoderma harzimum*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium* spp., *Macrophom* spp., which was modified as the following. A point of filamentous fungi was inoculated onto the center of PDA Petri dish; three points of isolation were inoculated at 2 cm distance from the center of the Petri dish symmetrically. The Petri dish was then incubated at 28 ^{o}C for a few days. The isolated strain was considered to be antagonistic to the filamentous fungus if it restrains the fungi growth with inhibition zone (Fig. 1).

2.2 Resistance to antibiotics of the Bacteria

Resistance to antibiotics was determined using standard antibiotic disks. Inhibition diameters were recorded after 24 h of incubation at 30 ^{o}C under aerobic conditions. The classification of the strain, as sensitive, not sensitive or intermediate sensitive to the antibiotics, was done according to the inhibition diameters. Tests were performed in triplicate.

2.3 PCR amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene

The primer set 27F-1492R was used in PCR amplification of the 16S rRNA gene fragment of isolate EBL06 under that conditions as described by Kuklinsky *et al.* (2004). The 16S rDNA was sequenced by Shanghai Sangon Co. Ltd., China. Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (nucleotide blast). Similar 16S rRNA gene sequences, from previously cultured bacteria, were downloaded from GenBank and manually checked for ambiguous sites using bioedit 7.0.1 software. Alignments were then performed against the 16S rRNA gene sequence of isolate EBL06, where the pair-wise deletion option for gaps was employed. The alignment data were then used for neighbour-joining analysis with 1000 bootstrap replicates (MEGA version 4.0; Arizona State University, USA) (Li *et al.*, 2007).

2.4 Optimization of culture conditions

The SPW samples were collected from a sweat potato starch process waste stream in Changsha, China, mainly containing COD 16000 mg/l. The pH of SPW was 6.2; it was adjusted to 7.0 by NaOH when the SPW was used as the culture medium. The starch, pectin

and sugars in the SPW were used as the main carbon source throughout the investigation. The MGW samples were obtained from Henan Lianhua Monosodium Glutamate Co., Ltd., which located in Zhoukou, Henan Province, China. The MGW consisted of 1.20% total Kjeldahl-N, 1.17% NH_4^+, 4.06% SO_4^{2+} and 0.275% reducing sugar. Trace element components in the MGW were given as follows (mg/l): Ba, 0.932; Ca, 389; Co, 0.0123; Cu, 0.605; K, 259; Mg, 79.0; Pb, 0.588; Sr, 0.869; Zn, 1.80; Mn, 2.66; Fe, 4.28; Na, 794; Cr, 0.862; P, 81.4; V, 0.0082. The pH of raw MGW 1.5 and was adjusted to 6.7 by NaOH when the MGW was used as the culture medium. Ammonium in the MGW was used as the main nitrogen source.

The isolate EBL06 was grown on the PDA slants at 30 °C for 2 days. A single clone was inoculated into 250-ml flask containing 100 ml of the PDA medium at 30 °C for 24 h. This seed culture was used to initiate the growth in fermentation medium used in this study. The basic medium (BM) is composed of as follows (g/l): $MgSO_4 \cdot 7H_2O$ 0.10, KH_2PO_4 0.50, NaC1 0.50, and K_2HPO_4 1.50. The fermentation was conducted in 250 ml Erlenmeyer flasks containing 100 ml medium inoculated with 5 ml of seed culture. Unless otherwise was stated, the agitation rate and incubation temperature were 200 r/min and 30 °C, respectively.

All experiments were conducted in duplicate and the average values are reported. Key results were repeated three times to establish their validity.

3. Results

3.1 Morphology and antagonistic fungus activity of the isolate EBL06

Microscopic observation of the isolate EBL06 is a mesophilic, Gram-positive and motile bacterium, and cells are rod-shaped with peritrichous flagella in overnight culture in PD medium at 30 °C and 150 r/min using Light-microscophy. The cells are found singly, double, and chains. Colonies of the strain on PDA are slightly yellow, circular, smooth, convex, semi-transparent and 2--3 mm in diameter with an entire margin after incubation for 48 h at 30 °C.

EBL06 showed a significant antagonistic activity towards fungal species of *Cladosporium cucumerinum*, *Trichoderma harzimum*, *Botrytis cinerea*, *Fusarium* spp. and *Macrophom* spp. on PDA plates. Figure 1 shows two images of the test process.

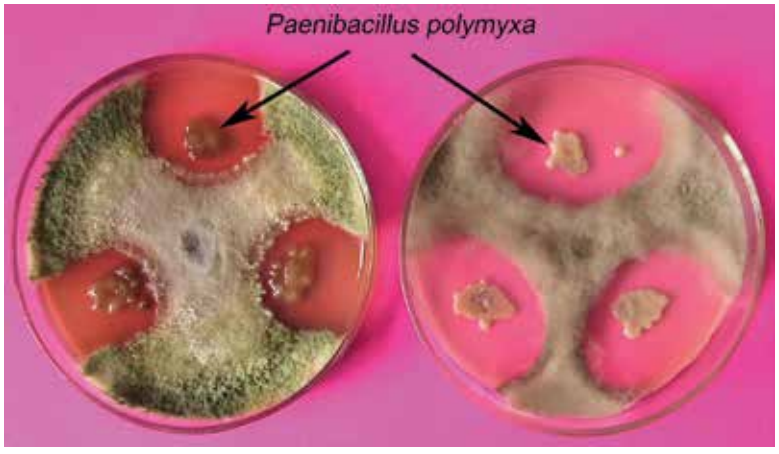

Fig. 1. Growth impact of the isolated EBL06 on the *Trichoderma harzimum* (left) and *Botrytis cinerea* (right) at the PDA medium.

3.2 Resistance to antibiotics of the isolate EBL06

The growth behaviour of the isolate EBL06 was studied in the presence of a range of antibiotics. The strain was susceptible to penicillin, streptomycin, kanamycin and tetracycline, and was weakly susceptible to chloramphenicol, and resistant to polymyxine and colistine.

3.3 Phylogenetic analysis

To analyze the phylogenetic position, the 16S rDNA sequence of the isolate EBL06 was determined, and a phylogenetic tree was constructed (Fig. 2). The sequence was deposited in the GenBank database under the accession number EF545556. The phylogenetic analysis indicated that the isolate EBL06 is most closely related to species of *P. polymyxa*.

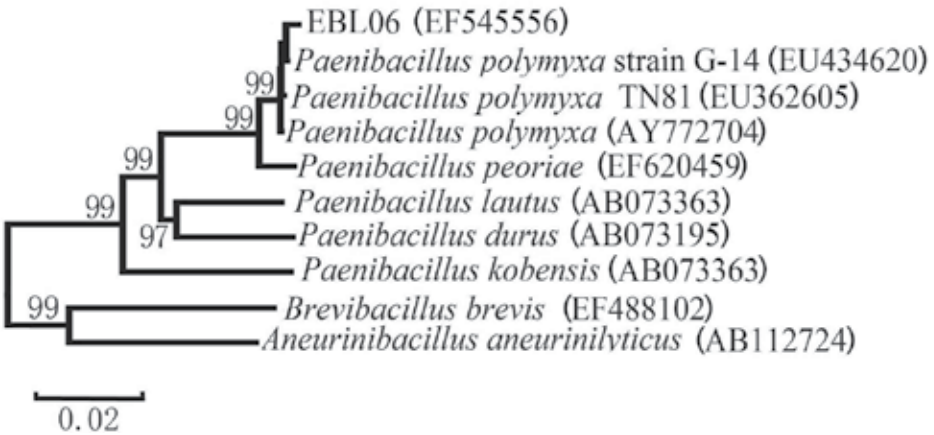

Fig. 2. Neighbor-joining trees showing the phylogenetic position of the isolate EBL06 and its related species based on 16S rRNA gene sequences. The GenBank accession number for each microorganism used in the analysis is shown after the species name. Bootstrap values (expressed as a percentage of 1000 replications) greater than 50% are shown at the branches.

3.4 Effect of carbon sources on the isolate EBL06 growth

The fermentation medium contained 10 ml MGW, 90 ml BM, and 2% each carbon sources, including sugar, D-glucose, soluble starch, and SPW (90 ml, the carbon source equal to 2 g starch, no BM). After inoculation with 2 ml of inoculum, the medium was incubated at 30 ^{o}C for 20 h. The effect of carbon sources on the production of the isolate EBL06 are presented in Fig. 3. It was found that SPW are the most suitable carbon source.

3.5 Effect of nitrogen sources on the isolate EBL06 growth

The fermentation medium contained 90 ml SPW as carbon source, 10 ml BM, and 0.5% different nitrogen sources, including corn steep liquor, potassium nitrate, ammonium sulfate and MGW (10 ml, the nitrogen source equal to 0.5 g $(NH_4)_2SO_4$, no BM). A control experiment was conducted without addition of nitrogen source. After inoculation with 2 ml of inoculum, the medium was incubated at 30 ^{o}C for 20 h. The results of impact of nitrogen sources on the production of the isolate EBL06 are presented in Fig. 4. It was found that corn steep liquor and MGW were the most efficient nitrogen sources for production of the strain. Comparing with the corn steep liquor, MGW are the most suitable nitrogen source due to the low cost.

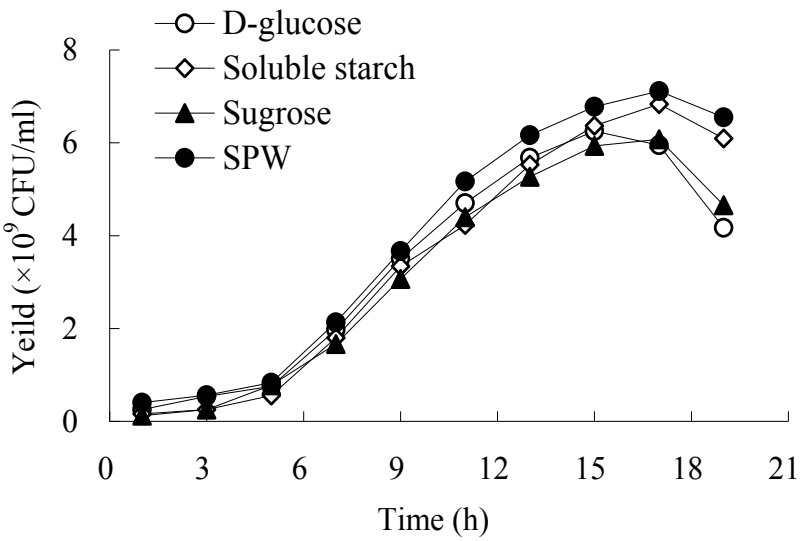

Fig. 3. Effect of different carbon sources on the isolate EBL06 growth.

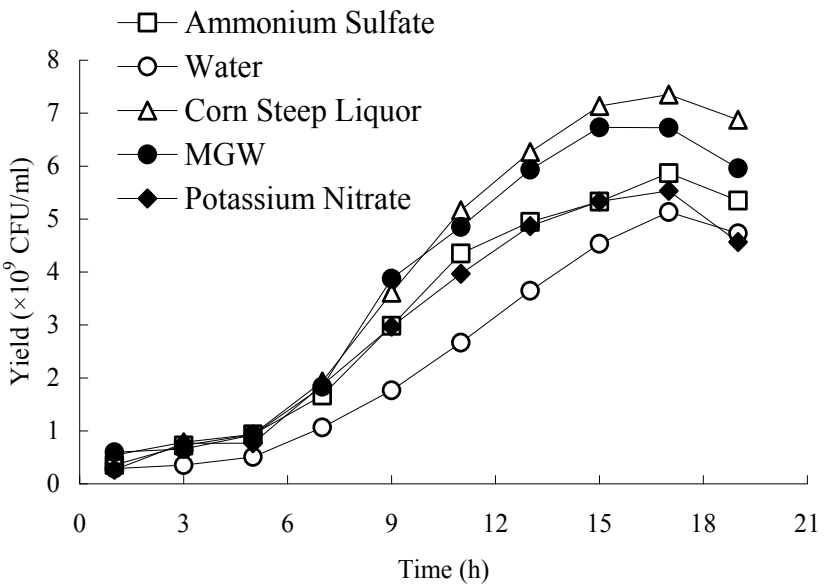

Fig. 4. Effect of different nitrogen sources on the isolate EBL06 growth.

3.6 Effect of pH on the isolate EBL06 growth

The fermentation medium contained 10 ml MGW, 90 ml SPW. After inoculation with 2 ml of inoculum, the medium was incubated at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 respectively. The

isolate EBL06 production at different pH is shown in Fig. 5. The highest yield could be given at pH 6.5--7.0 after 15 h fermentation. Consequently, pH 7.0 was selected in the following experiment.

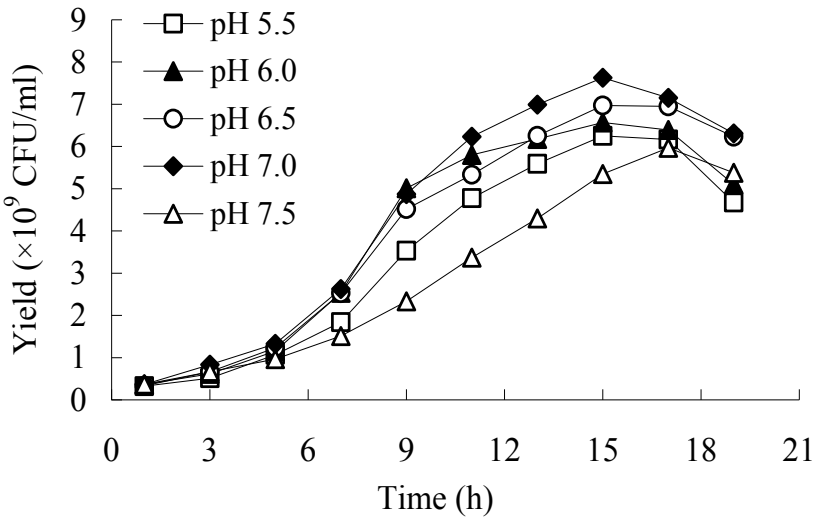

Fig. 5. Effect of initial culture medium pH on the isolate EBL06 growth.

3.7 Effect of culture temperature on the isolate EBL06 production

The fermentation medium contained 10 ml MGW, 90 ml SPW. After inoculation with 2 ml of inoculum, the medium was incubated at 24, 28, 32, 36 and 40 °C, respectively. The time courses of the isolate EBL06 production at different temperature are shown in Fig. 6. The maximum yield of 7.3×10^9 CFU/ml was reached at 32 °C after 15 h fermentation.

4. Disscussion

P. polymyxa endospore was reported to be resistant to desiccation, heat, and UV irradiation, and have excellent biochemical characteristics that allow for further formulation and commercialization procedures. Previous studies have shown that strains of *P. polymyxa* can produce different peptide antimicrobial substances (Rosado and Seldin, 1993; Piuri et al., 1998; Dijksterhuis et al., 1999; Seldin et al., 1999). The peptide metabolites are generally classified into two groups according to their antimicrobial activities. The first group includes the polypeptins, polymyxins, jolipeptin, gavaserin, and saltavalin, which showed antibacterial activity against both gram-negative and gram-positive bacteria. The second group consists of a single family of closely related peptides variously designated gatavalin, fusaricidins, all of which contain an unusual fatty acid side chain, 15-guanidino-3-hydroxypentadecanoic acid (Raza *et al*., 2009). As a phyllosphere isolate, *P. polymyxa* EBL06 could be suitable to survive in the phyllosphere conditions; the antagonistic fungi ability also give them a growth advantage in the competitive environment. *P. polymyxa* EBL06 showed a significant antagonistic activity towards all the filamentous fungi tested. It will be used as a potential biocontrol agent for protecting plant against fungal disease in further study.

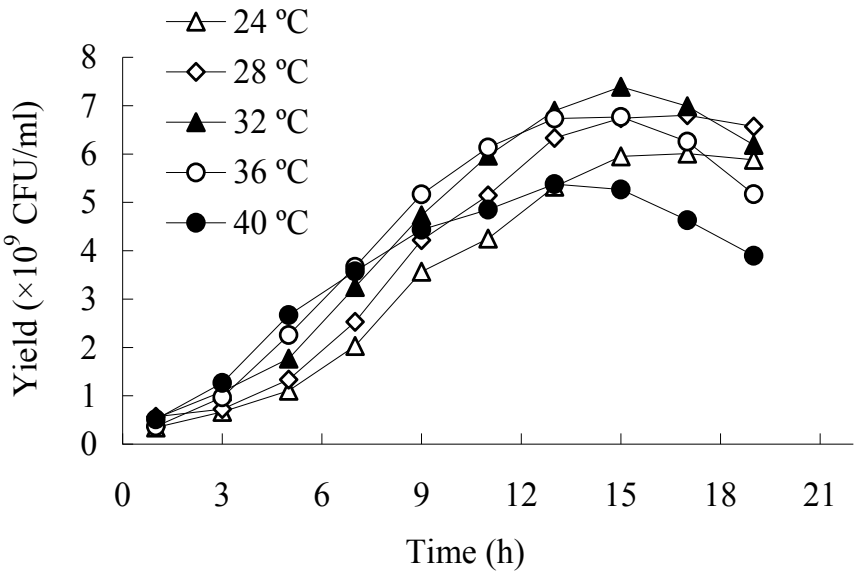

Fig. 6. Effect of culture temperature on the isolate EBL06 growth

MGW is one of the most intractable wastewater because of its high strength of COD, ammonium and sulphate (Yang *et al.*, 2005). SPW also contains high COD and nutrient loadings. A few studies have already focused on the utilization of such high organic loading wastewater as substrates or production media in fermentation processes (Bai *et al*., 2004; Mishra *et al.*, 2004; Huang *et al.,* 2005). As far as the authors are aware, no papers have been published on utilization of both MGW and SPW in one process. In the present study, the isolate EBL06 could use well the starch in the SPW as the main carbon source, and ammonium in the MGW as the main nitrogen source. In addition, the nutrient and trace element components in the wastewaters are also suitable for the isolate EBL06 growth. Compared with other culture media, the mixture of SPW and MGW is the most suitable for the biocontrol agent production from the economic and environmental point of view.

P. polymyxa has also been isolated from several places such as food, rhizosphere, poultry production environments, soils and most of them can restrain fungal pathogen growth (He *et al*., 2007; Raza *et al*., 2009; Svetoch *et al*., 2005). Further studies will investigate the anti-fungal pathogen activity of the isolate EBL06 both in phyllosphere and in rhizosphere by field trials. Further understanding of the survival strategy of the isolate EBL06 in phyllosphere might improve the efficiency of the biological treatments and also lead to enhanced yields of agricultural crops.

In conclusion, a newly isolated *P. polymyxa* EBL06 from wheat phyllosphere can be used as a nonchemical alternative biocontrol agent against plant disease caused by fungal pathogen. A novel process for economical production of *P. polymyxa* biocontrol agent was developed using MGW and SPW. It is feasible to develop a hybrid biotechnological process, integrating the production of environmental friendly biocontrol agent with treatment of intractable wastewater.

5. Acknowledgments

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Part 3

Fungicide and Environment

14

Environmental Risks of Fungicides Used in Horticultural Production Systems

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1. Introduction

Fungicide use is arguably the most important component of pest and disease management programs in horticultural production systems, particularly vine and orchard crops. This is because fungal diseases, such as downy mildew and botrytis, have the potential to destroy horticultural crops rendering them unsaleable. However, most fungal diseases are difficult to eradicate as, despite attempts at eradication, disease outbreaks can continue to occur across several growing seasons, often originating from spores that have lain dormant over winter. The devastating and lasting impact fungal diseases can have on horticultural crops has resulted in it being considered best practice to implement preventative agrochemical spray programs (McConnell et al., 2003). This often involves fortnightly fungicide applications during the growing season. However, the regular use of fungicides can potentially pose a risk to the environment, particularly if residues persist in the soil or migrate off-site and enter waterways (e.g. due to spray drift, run-off) (Kookana et al., 1998; Wightwick & Allinson, 2007; Kibria et al., 2010; Komarek et al., 2010). If this occurs it could lead to adverse impacts to the health of terrestrial and aquatic ecosystems. For instance, concerns have been raised over the long term use of copper-based fungicides, which can result in an accumulation of copper in the soil (Wightwick et al., 2008; Komarek et al., 2010). This in turn can have adverse effects on soil organisms (e.g. earthworms, microorganisms) and potentially pose a risk to the long-term fertility of the soil (Wightwick et al., 2008; Komarek et al., 2010).

To ensure the sustainability of horticultural production systems, a balance needs to be found between controlling fungal disease risks to crops and protecting terrestrial and aquatic ecosystems. Research into the potential environmental risks posed by fungicide use is needed so that evidence-based policy decisions can be made on the future management of fungicide use in horticultural crops. This need is driven by an increasing community expectation for governments and industry to implement measures to protect environmental

assets. In addition, from marketing perspectives, there is increasing pressure on agricultural industries to demonstrate their 'clean and green' credentials to address concerns from consumers and to maintain market access.

The risk to the environment posed by the use of fungicides in horticultural production systems has received relatively little attention compared to other types of agrochemicals, such as insecticides and herbicides. For instance, in North America the United States Geological Survey (USGS) has conducted detailed nation-wide surveys of agrochemical residues in surface and ground waters (Gillom et al., 2006). These surveys have produced a wealth of data on agrochemical residues in the environment which scientists and policy makers have used to make decisions on the environmental risks of agrochemicals. However, these large surveys appeared to give cursory consideration to fungicides, with just one fungicide out of 75 different agrochemicals included in the analytical screens (Gillom et al., 2006). Similarly, there is relatively little ecotoxicological data detailing concentrations causing adverse effects to organisms (e.g. effect concentration values, EC_{50}) for even the most widely used fungicides (Frampton et al., 2006; Maltby et al., 2009). Given the current lack of data on their environmental risks, future research needs to give greater consideration to fungicides. In part, this is because fungicides tend to be applied repeatedly over a specific period of the year, and so arguably pose a greater environmental risk than other types of agrochemicals, such as insecticides, which tend to be applied more sporadically to eradicate pest outbreaks when detected. Another important consideration is that there has been a continual evolution of new fungicidal compounds in a race to overcome fungicide resistance, improve the effectiveness of fungicides, and to reduce application rates. As more farmers seek to adopt newer fungicides there is a need to consider the costs and benefits of this adoption, as newer chemicals may not necessarily pose a lower environmental risk than those they replace. For instance, in recent years the use of organophosphate insecticides (e.g. chlorpyrifos) has been restricted in the USA due to the presence of unacceptably high concentrations in some waterways. Subsequently, the use of synthetic pyrethroids has increased, and these in turn are now presenting a sediment contamination issue (Banks et al., 2005; Weston et al., 2004). This is an example of where well intentioned changes to agrochemical use has led to a transfer in risks, rather than the intended reduction in risks.

This chapter provides an overview of the potential environmental risks posed by fungicide use in horticultural crops, including discussion of the main types of fungicides and patterns of use; and the environmental fate and toxicity of these fungicides. Fungicides are discussed within an ecological risk assessment context and perspectives given in relation to linking policy with science in managing ecological risks associated with fungicide use in horticultural crops. Throughout the chapter, fungicide use in Australian viticulture is presented as a case-study. The discussion focuses on risk to the soil environment, particularly in relation to copper-based fungicides, with secondary consideration given to risks to aquatic environments.

2. Types of fungicides and their usage patterns

There are currently 47 different fungicides (active ingredients) registered for use in vineyards in Australia (AWRI, 2010), representing 40% of the total number of agrochemicals registered for use in viticulture. Most of the fungicides registered for use in viticulture are used to control downy mildew (*Plasmopara viticola*) (21) and powdery mildew (*Uncinula necator*) (17). As is the case with most fungal diseases, it is considered best practice to

prevent grapevine disease outbreaks rather than to eradicate once infection has occurred, as reactive spraying is considered less effective (Nicholas et al., 1994; McConnell et al., 2003). Thus, preventative fungicide applications are widely considered to offer the best 'insurance' in protecting against fungal infection (Nicholas et al., 1994). In grapevines, this tends to involve applying around 4 – 6 preventative fungicide applications during periods of rapid foliage growth (i.e. spring/summer; Nicholas et al., 1994).

The fungicides registered for use in Australian viticulture can be categorised into 16 main chemical classes (Table 1), the majority of which are considered to be broad spectrum protectants. Copper-based compounds (to control downy mildew and other diseases) and sulfur (to control powdery mildew) have been in use since the mid to late 1800's and are still widely used (Russell, 2005). Copper (Cu) and sulfur (S) are also the only fungicides permitted for use in organic agriculture (OIECC, 2009). Historically, Cu was applied as a mixture of copper sulphate and lime (Bordeaux mixture), however copper oxychloride and copper hydroxide are now the main forms applied (Pietrzak & McPhail, 2004). Copper and S are contact fungicides with multi-site activity and have traditionally been applied at high rates (up to 50 kg/ha/yr) to achieve effective control. However, lower applications rates are now used. For instance, a recent survey of vineyards in 10 different regions of Australia found that the mean annual Cu usage across the regions was in the range of 5 – 13 kg/ha/yr (Wightwick et al., 2008). Likewise, in Australia, organic standards currently limit annual usage to 8 kg/ha (OIECC, 2009), with similar restrictions in place in other countries.

Synthetic organic fungicide compounds were first introduced during the 1940's, with the main class being the dithiocarbamates (e.g. mancozeb; Table 1). Like the Cu and S formulations, the dithiocarbamates are broad spectrum, contact fungicides with multi-site modes of action (Russell, 2005). During the 1970's, systemic fungicides with greater activity and more specific modes of action were introduced, and these are able to stop fungal development after infection has occurred (Russell, 2005). Significant classes of systemic fungicides are the triazoles (e.g. penconazole), strobilurins (e.g. trifloxystrobin) and phenylamides (e.g. metalaxyl, an effective eradicant). The strobilurins are highly efficacious, having both protective and eradicative action (Russell, 2005). As they are more effective, systemic fungicides can be applied at much lower rates than the older classes of fungicides. For instance, the typical application rates for the strobilurins and triazoles (0.13 to 0.25 kg/ha) are an order of magnitude lower than the dithiocarbamates (1.5 – 3.5 kg/ha) (Russell, 2005). There also many different 'natural' fungicide products available (e.g. beneficial micro-organisms, biosurfactants, phosphoric acid, clay preparations, cow's milk), however, in most cases their effectiveness has not been fully demonstrated (Van Zwieten et al., 2007).

Information on the quantity of individual (or classes of) fungicides being used within a watershed is useful for predicting the fate and risk of these fungicides in the environment. However, as highlighted in recent reviews, there is currently very little information available on the quantity of particular chemicals used in Australian agriculture (Kookana et al., 1998; Radcliffe, 2002; Wightwick & Allinson, 2007). In 2001, agrochemical usage data was collected in a major dairy and horticultural production region in northern Victoria, Australia (Kookana et al., 2003). This study found that 14 different fungicides (active ingredients) were in use, with the dithiocarbamates (45%) and copper-based fungicides (20%) accounting for the majority of the total fungicide used (based on amounts reported to be applied). Ziram (40%) and metriam (32%) accounted for most of the total quantity of dithiocarbamates used. Sulfur (11%), chlorothalonil (a chloronitrile) (11%), and

Chemical class (no. of active ingredients registered)	Range of physical-chemical properties[a]				
	Molecular weight	Water solubility (mg/L)	Log K_{ow}	Log K_{oc}	Half-life in soil (d)
Inorganic (10)					
copper compounds Cu sulphate, Cu oxychloride, Cu hydroxide, Cu ammonium acetate, Cu octonoate, Cu oxide	98 – 427	$<1.0 \times 10^{-5}$ - 2.9	NR	NR	NR
sulfur	32	Insoluble	NR	NR	NR
peroxyacetic acid, phosphorous acid, potassium bicarbonate	NR	NR	NR	NR	NR
Triazole (8)					
flusilazole, hexaconazole, myclobutanil, penconazole, tebuconazole, tetraconazole, triadimefon, triademenol	284 – 315	17 - 260	2.9 – 3.9	2.5 – 3.4	14 – 420
Dithiocarbamate (6)					
mancozeb, metriam, propineb, thiram, zineb, ziram	240 – 1089	1.0×10^{-5} – 65	-0.26 – 1.23	2.6 – 5.7	1 - 30
Carboximide (3)					
boscalid, iprodione, procymidone	274 – 330	4.5 – 13	3.0 – 3.1	2.8 – 3.2	7 – 200
Pyrimidine (3)					
cyprodinil, fenarimol, pyrimethanil	199 – 331	13 – 121	2.8 – 4.0	2.4 – 2.9	20 - 840
Strobilurin (3)					
azoxystrobin, pyraclostrobin, trifloxystrobin	403 – 408	0.6 – 6	2.5 – 4.5	3.4	7 - 70
Amide/amine (2)					
fenhexamide, spiroxamine	298 – 302	20	2.8 – 3.5	NR	< 1 – 64
Phenylamide (2)					
benalaxyl, metalaxyl	279 – 325	$37 - 7.1 \times 10^{3}$	3.5	1.7 – 3.5	70 - 77
Quinone/quinoline (2)					
dithianon, quinoxyfen	296 – 308	0.12 – 0.14	3.2 – 4.7	NR	123 – 494

Chemical class (no. of active ingredients registered)	Range of physical-chemical properties[a]				
	Molecular weight	Water solubility (mg/L)	Log K_{ow}	Log K_{oc}	Half-life in soil (d)
Anilide (1)					
fluzinam	465	0.07	3.6	NR	33 – 62
Benzamidazole (1)					
carbendazim	191	8	1.8	2.5	NR
Chloronitrile (1)					
chlorothalonil	266	0.6	2.6	3.1	30 - 90
Hydrazide (1)					
oxadixyl	278	NR	0.8	NR	60 - 90
Morpholine (1)					
dimethomorph	388	>50	NR	NR	NR
Phenylpyyrole (1)					
fludioxonil	248	3.9×10^{-4}	1.8	4.1	10 - 25
Phthlamide (1)					
captan	300	3.3	2.8	2.3	1 – 10

[a] *Log K_{ow} – octanol-water partition coefficient, Log K_{oc}, - soil sorption coefficient. NR – not reported Data from Tomlin, 2000; Wauchope, 2005; IUPAC, 2010.*

Table 1. Chemical classes of fungicides registered for use in Australian viticulture and their typical physical-chemical properties.

carboximides (iprodione and procymidone; 9%) were the other main classes of fungicides used (Kookana et al., 2003). In the United States the USGS conducted a nation wide study to estimate the annual agrochemical use intensity rates (mass per unit area of agricultural land) based on typical usage patterns from 1999 to 2004 (USGS, 2010). Analysing the available data it was found that sulfur accounted for over 90% of the total amount of fungicide used in grape production. This is consistent with the situation in northern Victoria, Australia, where excluding S, copper (54%) and dithiocarbamates (28%) accounted for the majority of total fungicide use (Fig. 1). Mancozeb accounted for the vast majority (over 70%) of the total amount of dithiocarbamates used; and the triazoles (predominately myclobutanil), pyrimidines (predominately cyprodinil) and strobilurins (predominately azoxystrobin and trifloxystrobin) were the other main fungicides used (USGS, 2010). Whilst this information is now several years old, it shows that many agriculturists in North America and Australia are still predominately using 'old' classes of fungicides, i.e. inorganic formulations (Cu and S) and dithiocarbamates. This is despite a range of newer more effective fungicides, such as the triazoles and strobilurins, being available in the market place for over 10 years. The situation elsewhere may be different. For instance, Gregoire et al. (2010) noted in a study of fungicide use from 2003 to 2006 in a viticultural catchment of France that strobilurins accounted for 36% of the total amount of synthetic organic fungicides applied.

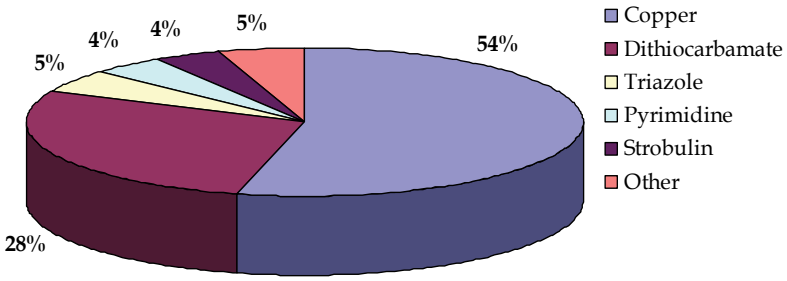

Fig. 1. Contribution (%) of the main fungicide classes, estimated to be used for grape production (excluding sulfur) in the United States, to the total amount of fungicides applied (based on data in USGS, 2010)

3. Environmental fate

Whilst a farmer's objective is to apply fungicides to the agricultural crop/plant, inevitably a proportion of the chemical spray will miss its target. Much of the lost chemical will enter the soil surface where it will persist for a period of time and potentially migrate off-site due to leaching and/or runoff. In addition, some of the agrochemicals applied on farm will migrate off-site due to aerial drift. Once agrochemicals have migrated off-site they can potentially enter nearby waterways and groundwater resources where they can cause adverse effects to aquatic organisms (Wightwick & Allinson, 2007). The fate and behaviour of agrochemicals, including fungicides, in the environment is influenced by the properties of the chemical (e.g. ability to bind to soil, susceptibility to degradation) and environmental factors (e.g. soil type, rainfall, topography, agricultural management practices). These environmental factors, in particular soil type, are widely varied, thus there are many scenarios to consider when assessing the potential for off-site migration and the persistence of agrochemicals in soil (Wightwick & Allinson, 2007; Arias-Estevez et al., 2008; Komarek et al., 2010). The next section provides an overview of the factors influencing the environmental fate of fungicides and current knowledge on their fate in the environment.

3.1 Persistence, accumulation and availability of fungicides in soil

The two most commonly used inorganic fungicides, Cu and S, are elements and thus do not breakdown in the environment. On the other hand, synthetic organic fungicide compounds do degrade via a number of abiotic and biotic mechanisms, including hydrolysis, volatilisation, oxidation, photolysis, ionisation and microbial degradation. It is generally considered that microbial degradation is the most significant degradation mechanism (Katayama et al., 2010). In the first instance, the persistence (or rate of breakdown) of organic fungicide compounds is related to their chemical structure (e.g. the size, strength of chemical bonds). However, persistence is also influenced by edaphic factors in the environment. Consequently, organic fungicide compounds persist for varying lengths of time in the environment, which is often expressed in terms of their expected half-life (Kookana et al., 1998; Arias-Estevez et al., 2008; Katayama et al., 2010).

Both inorganic and organic fungicide compounds can be strongly bound within soil (Gevao et al., 2000; Komarek et al., 2010). Cu, being a divalent cation, binds strongly (mostly by specific adsorption) to inorganic and organic material in the soil, and is known to

accumulate in agricultural soils (Wightwick et al., 2008; Komarek et al., 2010). The extent to which organic fungicide compounds are bound within soil is predominately related to their hydrophobicity as indicated by their log K_{ow} value (a measure of the partitioning of a chemical between octanol and water). Hydrophobicity is also indicated by the log K_{oc} value, or how readily the chemical adsorbs to soil organic matter. A higher log K_{ow} and/or log K_{oc} value indicates that the chemical will have a stronger binding affinity in soil (Gevao et al., 2000; Katayama et al., 2010). The extent to which a fungicide is bound within the soil influences the likelihood that the fungicide will accumulate in the soil. However, it is also related to their bioavailability, defined as "the degree to which chemicals present in soil may be absorbed or metabolised by humans or ecological receptors or are available for interaction with biological systems" (Harmsen, 2007). This is a particularly important consideration for Cu as it is generally considered that the vast majority of Cu in soil is so tightly bound that it is biologically inert (McLaughlin et al., 2000). In the first instance, bioavailability is influenced by the environmental availability of the chemical, defined as the fraction, physico-chemically driven by desorption processes, potentially available to be taken up by organisms (Harmsen, 2007).

The environmental availability of fungicides in soil is related to the extent and type of the adsorption and desorption processes, involving a complex series of soil chemical interactions with both mineral and organic phases in soil (McBride, 1981; Gevao et al., 2000; Komarek et al., 2010). Copper and other fungicides added to the soil partition into three separate pools (Fig. 2). The most available pool being the proportion of the fungicide that is soluble in the soil solution whilst the 'unavailable' pool is the proportion of fungicide very tightly bound within the soil and not easily released. The 'potentially available' pool is considered to be reactive (or labile), as biochemical processes associated with the decomposition of organic material and other dissolutive processes, can release bound fungicide thus making it available (Stevenson & Fitch, 1981; Katayama et al., 2010).

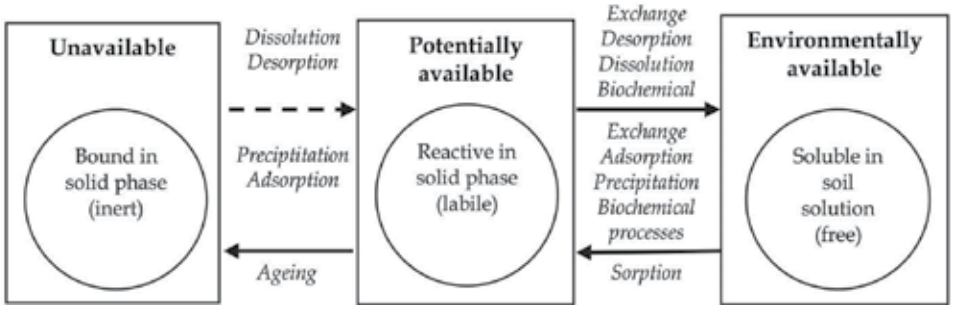

Fig. 2. Diagram illustrating the partitioning of fungicides into the unavailable, available and potentially available pools in soil (adapted from Harmsen, 2007)

A great deal is known about the soil chemical reactions influencing the adsorption of Cu (and other metals) in soil. This is discussed in detail in several reviews (e.g. Pickering, 1979; McBride, 1981; Stevenson & Fitch, 1981; Swift et al., 1995), with only an overview of the main soil-chemical reactions provided here. Copper binds strongly to negatively charged sites on inorganic mineral constituents in soil, with specific adsorption (covalent bonding) to iron (Fe), aluminium (Al), and manganese (Mn) oxides, and exchange on layer silicate clays (electrostatic association) (McBride, 1981). Specifically adsorbed copper is not easily released and is considered to be inert and not biologically available (McBride, 1981). The

environmentally available pool includes Cu which is soluble in the soil solution (as free ions and complexed with organic and inorganic ligands) as well as readily exchangeable forms of Cu. The potentially available or labile pool of Cu in soil is comprised predominately by Cu adsorbed to organic matter. Organically bound Cu has generally been found to represent 20 -50% of the total Cu in soil (Stevenson & Fitch, 1981; Pietrzak & McPhail, 2004; Strawn & Baker, 2008). It is important to note that Cu can become very tightly bound in soil due to the formation of three-way complexes with both mineral and organic components of the soil. Copper organically bound in this manner can be considered to be less reactive and associated with the inert pool of Cu in soil (Stevenson & Fitch, 1981; Boudescoque et al., 2007). In this context, it is known that the longer the organically sorbed Cu remains in the soil, the stronger the bond becomes with the soil solid phase. This 'ageing' effect results in the transfer of Cu from the labile 'potentially available' pool into the inert 'unavailable' pool, as adsorbed Cu becomes incorporated into the crystalline structure of soil minerals over time (McLaughlin, 2001; Ma et al., 2006).

Copper adsorption to the solid phase of the soil decreases with increasing soil acidity due to a decrease in the number of negatively charge sites, particular at pH < 5 (Sauve et al., 2000). The proportion of Cu present in the soil solution may also increase with increasing soil alkalinity (pH > 8) due to the formation of complexes with carbonate, hydroxy groups and dissolved organic matter, which reduces Cu sorption to soil solid phases (McBride, 1981; Burton et al., 2005; Fernandez-Calvino et al., 2008). A change in soil pH, particularly a lowering, can cause adsorbed Cu to be released (McLaughlin, 2001). This could occur due to the application of some fertilisers, soil amendments and agrochemicals. Soil pH is also important as it influences the speciation of the Cu in the soil solution (McBride, 1981, Sauve, 2000). In acid soils, soil solution Cu is present mainly as the free Cu^{2+} ion, whereas in alkaline soil the soluble Cu is present mostly as dissolved organic matter complexes (McBride, 1981; Burton et al., 2005; Fernandez-Calvino et al., 2008). It is important to note that the presence of other cationic metals/elements influences Cu adsorption due to competition for binding sites (McBride, 1981; Stevenson & Fitch, 1981). Soil organisms can release organically bound Cu as they utilise organic matter for nutrition and can also modify the chemistry of the surrounding soil; for example by changing pH, ionic strength, and macronutrient cation concentrations, and by excreting organic ligands (Peijnenburg & Jager, 2003).

Increases in the concentration of Cu in the surface soils of vineyards that have received regular inputs of copper fungicides have been reported from many different viticultural regions of the world (Table 2; Komarek et al., 2010). Typically 10 to 20 fold increases in Cu concentrations compared to untreated native soils have been reported in the soils of vineyards with long histories of copper fungicide use (Table 2; Wightwick et al., 2008; Komarek et al., 2010), with the highest reported concentrations in Brazil (up 3216 mg/kg total Cu; Mirlean et al., 2007). As would be expected the extent of Cu accumulation has been found to be influenced not just by the history of copper fungicide use (e.g. years of Cu use) but also soil properties which influence the retention of Cu in soil, such as organic carbon, cation exchange capacity (CEC), and pH (Morgan & Taylor, 2003; Wightwick et al., 2008; Komarek et al., 2010). A number of studies have also sought to characterise the environmental availability of the copper fungicide residues. Overall, the availability of Cu in soil is influenced firstly by the total Cu concentration, with pH, clay, CEC, organic matter being the next most important soil factors (Brun et al., 1998; Sauve et al., 2000; Gray & McClaren, 2006; Fernandez-Calvino et al., 2009; Wightwick et al., 2010). It is generally accepted that the 0.01 M calcium chloride ($CaCl_2$) extractable concentration provides a useful indication of the environmentally available

proportion of Cu in soil (Harmsen, 2007; Peijnenburg et al., 2007). The $CaCl_2$ extractable Cu concentrations of vineyard soils have been reported in several viticultural regions of the world, including Southern France (0.10 to 9.24 mg/kg, n = 22; Brun et al., 1998); Roujan region of France (1.00 to 39.0 mg/kg, n = 6; Chaignon et al., 2003); Slovenia (0.04 to 0.07 mg/kg, n = 22; Rusjan et al., 2007); Czech Republic (0.53 to 0.70 mg/kg, n = 5; Komarek et al., 2008); Australia (< 0.1 to 0.94 mg/kg, n = 100; Wightwick et al., 2010); and Brazil (0.20 to 5.00 mg/kg, n = 21; Mirlean et al., 2007). For the most part the $CaCl_2$ extractable Cu has represented < 1%, and often < 0.5%, of the total Cu concentration in the soil. Even in Brazil, where very high concentrations of accumulated Cu (1214 to 3216 mg/kg total Cu) have been reported in vineyard soils, the $CaCl_2$ extractable Cu only represented 0.1 to 0.9% of the total Cu (Mirlean et al., 2007). Considerably higher $CaCl_2$ extractable Cu concentrations (up to 30 mg/kg) have been reported in acidic vineyard soils. For example, Brun et al. (1998) found acidic vineyard soils (pH 4.5 to 5.5) to have much higher and greater variability in $CaCl_2$ Cu availabilities (0.4 to 9.24 mg/kg; 1.05 to 7.24% of total Cu) than neutral to alkaline soils (pH 6.5 to 8.6) (0.10 to 0.56 mg/kg; 0.13 to 0.54% total Cu). Similarly, relatively high concentrations of ammonium acetate (NH_4OAc) extractable Cu concentrations (which provides a similar indication of availability to $CaCl_2$ extractable Cu) were reported in a recent study of 170 acidic vineyard soils (mean pH 5.6 (4.0 – 7.9); mean NH_4OAc extractable Cu, 4.9 mg/kg (range, 0.1 – 30.3 mg/kg) (Fernandez-Calvino et al., 2009).

Country	Region	No. of vineyards surveyed	Total Cu (mg/kg)		Years of Cu use	Reference
			Mean	Range		
France	Bordeaux	20	458	305 - 845	NR	Delas (1981)
	Southern France	8	121	47 - 177	50 – 106	Brun et al. (1998)
	Roujan	13	161	75 - 398	NR	Chaignon et al. (2003)
Italy	Northern	NR	297	NR	NR	Deluisa et al. (1996)
	Southern	NR	75	NR	NR	
Germany		NR	1280	NR	NR	Tiller & Merry (1981)
Spain	North-West	20	144	40 - 301	>100	Arias et al. (2004)
Canada	Ontario	16	40	10 – 77	3 - 25	Frank et al. (1976)
Greece	Nemea	24	89	NR	>100	Vavoulidou et al. (2005)
Brazil	Southern	21	2198	1214 - 3216	100	Mirlean et al. (2007)
Croatia	Coastal Croatia	20	200	71 – 626	40 – 100	Vitanovic et al. (2010)
New Zealand	Nationwide	43	35	1– 259	1 – 100	Morgan & Taylor (2003)
Australia	Victoria	5	59	51 - 77	> 90	Pietrzak & McPhail (2004)
		9	90	9 - 229	20 - 30	
	Nation wide	35	73	24 – 159	40 – 100	Wightwick et al. (2008)
		63	53	6 – 223	1 - 38	

NR – not reported.

Table 2. Summary of copper concentrations reported in the surface soils of vineyards in viticultural regions of the world (adapted from Wightwick et al., 2008).

Understanding the adsorption of synthetic organic fungicides is complex due to the large number of different active ingredients registered for use, all of which have differing physico-chemical properties (Table 1). Moreover, the specific mechanisms controlling the adsorption of different organic chemical compounds are not as well understood (Gevao et al., 2000; Arias-Estevez et al., 2008; Katayama et al., 2010; Komarek et al., 2010). However, in general, it is expected that organic fungicides are adsorbed to organic matter, oxides and clay minerals. This can involve many mechanisms including cation exchange, hydrogen bonding, covalent bonding, van der Waals forces, charge-transfer, ligand exchange, and hydrophobic partitioning (Gevao et al., 2000). The importance of these various mechanisms differs depending on the physico-chemical properties of the chemical, however much of the adsorption is expected to occur with the organic components of the soils (Kookana et al., 1998; Gevao et al., 2000). Adsorption to clay minerals is an important process in soils with low organic matter contents (Gevao et al., 2000; Komarek et al., 2010). It is important to note that hydrophobic agrochemicals may also become adsorbed to dissolved organic matter, and thus remain environmentally available in the soil solution. Like Cu, sorbed fungicides can over time become more tightly bound in the soil due to 'ageing' or 'sequestering' processes, whereby the chemical diffuses into remote microsites within the soil matrix (Gevao et al., 2000; Katayama et al., 2010). Chemicals that are adsorbed in the soil in this manner are likely to persist for longer periods of time due to their decreased availability for microbial degradation (Gevao et al., 2000). Experimentally determined log K_{oc} values can be used to indicate how strongly chemicals adsorb to organic matter in the soil matrix. Based on published log K_{oc} values, most of the fungicides registered for use in Australian viticulture have a moderate to high tendency to adsorb to organic carbon, such as the phenylpyyroles, chlorothalonil, strobilurins, dithiocarbamates, and triazoles. Other fungicides have lower log K_{oc} values, such as captan, benzamidazoles, and pyrimidines (Table 1). However, it is important to note that these K_{oc} values tend to be determined using only one soil type, and it is known that the partitioning of chemicals with organic carbon can vary widely across different soil types (Kookana et al., 1998; Katayama et al., 2010). Like Cu, sorbed fungicide compounds can be released from soil binding sites and re-enter the soil solution. This release is most likely to occur for organically bound chemicals due to the activity of micro-organisms, with the release occurring very slowly for aged pesticides (Gevao et al., 2000).Typically, multiple agrochemicals are applied during a growing season, many or all of which can compete for available soil binding sites. This competitive binding can influence the availability of chemicals in soil. For example, Leistra & Matser (2005) reported that the presence of the fungicide carbendazim in soil reduced the adsorption of another fungicide iprodione by 70%. Elevated concentrations of Cu in soil can also react with synthetic organic fungicide compounds. Arias et al. (2006) reported that the adsorption of penconazole is greater in the presence of Cu, possibly due to the formation of Cu-penconazole complexes. Similarly, it has been reported that Cu can form complexes with dithiocarbamate fungicides which can inhibit their rate of degradation (Weissmahr & Sedlak, 2000).

Information on the typical half-lives of synthetic organic fungicides in soil is available (Table 1). Chemicals with a half-life less than 30 days are generally considered to have low persistence, between 30 – 100 days to have moderate persistence, and greater than 100 days to have high persistence (Komarek et al., 2010). The dithiocarbamates, amide/amines, phenylpyyroles, phthlamides and strobilurins generally have low persistence in soil; the

phenylamides, anilides, chloronitriles and hydrazides moderate persistence; and the quinones/quinolines high persistence. The persistence of some other classes of fungicides can not be so easily classified. Some triazoles, such as propiconazole (53 days) and triadimefon (14 – 60 days) have moderate persistence, whilst penconazole (133 – 343 days), myclobutanil (306 days) and flusilazole (420 days) have very high persistence. Similarly, with the carboximides, boscalid (200 days) and iprodione (84 days) have much higher persistence than procymidone (7 days). Of the pyrimidines, fenarimol (840 days) is more persistent than cyprodinil (20 – 60 days) and pyrimethanil (55 days). It is important to note however that the published half-lives provide an indication of typical persistence under specific conditions only, i.e. often this is based on laboratory studies undertaken under controlled conditions with a small number of different soil types. However, the persistence of chemicals can vary considerably under different conditions, for example laboratory derived half-lives for myclobutanil have been found to range from 164 – 515 days (IUPAC, 2010). Also synthetic organic fungicide compounds can be degraded to metabolites or transformation products, which may behave differently to their parent compounds. For example, the dithiocarbamate mancozeb is considered to have low persistence in soil (half-life 1 – 7 days), however it is rapidly degraded to ethylenethiourea (ETU) which not only can persist for 5 – 10 weeks but is more soluble than the parent compound (IUPAC, 2010; Komarek et al., 2010). Other dithiocarbamates, such as metriam and zineb, are also rapidly transformed to ETU. Significant metabolites have been identified for many of the other fungicides including the strobilurins, phthlamides, carboximides, pyrimidines and chloronitrile (IUPAC, 2010). In some instances the metabolite has greater persistence in soil whilst for other chemicals the parent compound is more persistent. For example, trifloxystrobin typically has a half-life in soil of seven days, whereas its metabolite ((E,E)-trifloxystrobin acid; EETFA)) has a half-life of up to 268 days (IUPAC, 2010). On the other hand, (E)-2-(2-[6-cyanophenoxy)-pyrimidin-4-yloxyl]-phenyl-3-methoxyacrylic acid, the metabolite of azoxystrobin, is less persistent (half-life 22 days) than the parent compound (half-life 70 to 180 days; IUPAC, 2010).

There have been few studies to determine the persistence and accumulation of synthetic organic fungicides in horticultural soils, with most of the studies from viticultural regions of Spain (Komarek et al., 2010). The largest of these studies by Bermudez-Cousa et al. (2007) measured the concentrations of cyprodinil, fludioxonil, folpet, metalaxyl, procymidone and penconazole in the soils of 20 vineyards over a two year period. No fungicides were detected in the majority of the soil samples collected, however all fungicides (except folpet) were detected in a number of samples. Procymidone (up to 1124 μg/kg) and metalaxyl (up to 1002 μg/kg) were detected at the highest concentrations, followed by cyprodinil (up to 462 μg/kg), penconazole (up to 411 μg/kg) and fludioxinil (up to 349 μg/kg). In general, the fungicides were only detected in the soils during the seasons that they were being applied (i.e. spring, summer). Whilst fungicides may persist in the soil from one season to the next, there was no clear evidence that they were accumulating in the soil (Bermudez-Cousa et al., 2007). This is perhaps not surprising given that the majority of synthetic organic fungicides are expected to have low to moderate persistence in soil. Similar results have been reported in the other smaller studies conducted in Spain (Komarek et al., 2010). For example, Rial-Otero et al. (2004) detected cyprodinil (260 μg/kg), fludoxinil (991 μg/kg), procymidone (20 μg/kg) and tebuconazole (12 μg/kg) in vineyard soils one month after the final treatment of the crop. Only fludioxinil was detected in the soil nine months later, however this was at a much lower concentration than originally detected (52 μg/kg; Rial-Otero et al., 2004).

3.2 Transport of fungicides from horticultural production systems

Agrochemicals can be transported from agricultural properties due to atmospheric transport (e.g. spray drift, volatilisation), surface run-off and leaching (infiltration through the soil profile) and potentially enter surface and ground waters (Wightwick & Allinson, 2007). The extent of spray drift is influenced largely by the method, type and set-up of the application equipment as well as the weather (i.e. wind) (MacGregor et al., 2004). Whilst the off-site transport via leaching and run-off is related to the adsorption of the chemical to the solid phase of the soil (Kookana et al., 1998). Chemicals dissolved or suspended in the soil pore water are most vulnerable to leaching though the soil profile. These chemically available chemicals can also be transported via surface run-off. However, chemicals strongly sorbed to soil particles are also prone to movement via particle bound surface run-off (Kookana et al., 1998). Given that Cu is strongly bound in surface soils, it is generally considered that Cu has a low likelihood of being transported via leaching but is susceptible to surface run-off of the soil particles to which it is bound (McBride, 1981). The mobility of synthetic organic fungicide compounds in soil can be assessed based on their published log K_{oc} values (Table 1). The majority of the synthetic organic fungicide compounds are likely to be relatively immobile in soil and unlikely to move via leaching. However, if they persist in the soil may move via the surface run-off of soil particles. Once again it is important to note that the published log K_{oc} values are based on a small number of soil types, and fungicides may be more mobile in some types of soils, e.g. particularly porous soils low in organic matter (Kookana et al., 1998; Arias-Estevez et al., 2008). It is also noted that some fungicide metabolites can have differing mobility in soil relative to their parent compound. For instance, whilst dithiocarbamates are considered to have low mobility in soil (log K_{oc} 2.6 – 5.7) the major metabolite ETU has relatively high mobility (log K_{oc} 1.85; IUPAC, 2010). Similarly, EETFA (log K_{oc} 2.08) is more mobile than its parent compound trifloxystrobin (log K_{oc} 3.38; IUPAC, 2010). It is also important to note that the leaching and surface run-off of chemicals can be accelerated if they are complexed to dissolved organic matter (colloids) in the soil pore water; this is termed 'colloid assisted transport' (Kookana et al., 1998; Komarek et al., 2010).

Over the past decade several studies have sought to determine the extent to which fungicides are transported from horticultural properties to nearby waterways. Ribolzi et al. (2002) and Fernandez-Calvino et al. (2008) reported significant amounts of Cu were transported from vineyard soils to nearby surface waters following storm events. The study of Fernandez-Calvino et al. (2008) reported that total Cu concentrations in sediments increased from 18 mg/kg at an upstream point in the catchment with no productive vineyards to 209 mg/kg at a downstream point of the catchment which represented the largest area of viticultural production. In both studies the readily available fraction of the Cu in sediment/suspended sediment was < 5% and often < 1% of the total Cu (Ribolzi et al., 2002; Fernandez-Calvino et al., 2008). Several recent studies have also studied the movement of synthetic organic fungicides by surface run-off in catchments dominated by viticultural/horticultural production (Hildebrandt et al., 2008; Gregoire et al., 2010; Rabiet et al., 2010). The largest of these studies involved monitoring concentrations of seven fungicides in surface water collected following 58 run-off events (over four years) in a wine-growing catchment in France (Gregoire et al., 2010). Over the four years the chemicals that were detected most frequently (% of samples detected) and at the highest concentrations (maximum concentration detected) were dimethomorph (57 - 98%; 0.66 to 5.7 μg/L), pyrimethanil (39 - 100%; 0.39 to 5.8 μg/L), and azoxystrobin (30 - 41%; 0.36 to 3.4 μg/L). For the most part tetraconazole and penconazole

were detected in less than 10% of the samples at concentrations less than 0.2 μg/L (Gregoire et al., 2010). In a similar study Rabiet et al. (2010), reported that concentrations of fungicides (azoxystrobin, carbendazim, dimethomorph, procymidone, tebuconazole) were found to be highest during the application season. Relatively high concentrations of dimethomorph (up to 3.4 μg/L), procymidone (up to 1.3 μg/L) and tebuconazole (up to 1.9 μg/L) were detected. Contrary to the study of Gregoire et al., 2010) azoxystrobin was detected at much lower concentrations (average of 0.08 μg/L; Rabiet et al., 2010). Hildebrandt et al. (2008) detected metalaxyl in both ground water (8 to 18% of samples; maximum concentration 0.25 to 0.36 μg/L) and surface water (4 to 14% of samples; maximum concentration 0.01 to 0.04 μg/L) in a catchment dominated by horticultural production. Little consideration has been given to assessing spray drift as a source of off-site fungicide movement, although Merli et al. (2010) found that despite up to 11 applications of quinoxyfen, very little of this fungicide was detected in sediments of a nearby surface ditch. Similarly in a study of pesticides in irrigation supply channels in a horticultural region in Australia, chlorothalonil, the only fungicide tested for, wasn't detected, despite spray drift being identified as a main source of local surface water contamination (Rose & Kibria, 2007).

4. Environmental toxicology

The presence and persistence of fungicides in agricultural soils can cause adverse effects to soil organisms, such as earthworms and micro-organisms, and the crucial functions these organisms are responsible for (e.g. the breakdown of organic matter, facilitating nutrient cycling). Thus, any negative impacts caused by fungicide residues can have lasting impacts on the fertility and health of agricultural soils. Likewise, fungicide residues, which make their way into surface and ground waters, have the potential to cause adverse effects to the structure (i.e. biodiversity) and functioning of aquatic ecosystems. The environmentally available proportion of fungicides in soil, sediment and water can cause toxic effects to organisms. However, not all of the fungicide will be absorbed by organisms (be environmentally bioavailable to) and react with toxic sites of action within exposed organisms (be toxicologically bioavailable or bioaccessible) (Fairbrother et al., 2007; Harmsen, 2007; Katayama et al., 2010). The environmental and toxicological bioavailability varies between different types (and species) of organisms, thus different organisms/species will have differing sensitivities to the various fungicides. The mechanisms controlling toxicity are complex. For instance the uptake of chemicals by organisms can be reduced if they are complexed with organic matter, as this impedes diffusion across biological membranes, and if cations such as H^+, Na^+, Ca^{2+} and Mg^{2+} compete at cell binding sites (Peijnenburg & Vijver, 2007; Katayama et al., 2010). Toxic effect is also related to the whether the chemical reaches a site of toxic action and the rate of detoxification and excretion (Peijnenburg & Vijver, 2007). Organisms can also develop tolerance to chemicals by using defence mechanisms such as regulating the rate at which the chemical is taken up, excreting compounds such as polysaccharides to complex the chemical thus impeding uptake, internal sequestration, and genetic adaptation (Bruins et al., 2000; Peijnenburg & Vijver, 2007). The likely ecotoxicological effect of chemicals can be indicated using controlled dose-response laboratory studies which report toxicity as an effect concentration (e.g. LC_{50} value, concentration causing mortality to 50% of the test organisms). Such ecotoxicological data can be used to indicate the likely effects of a chemical in the field and the relative potency of different chemicals. This section provides an overview of the known toxicities of Cu and

synthetic organic fungicides to terrestrial and aquatic organisms, based on published laboratory derived ecotoxicological effects data.

4.1 Ecotoxicological effects to terrestrial organisms

Considerable ecotoxicological data has been published describing the effects of Cu to soil organisms (Baath, 1989; Giller et al., 1998; Markich et al., 2002; Frampton et al., 2006; Jansch et al., 2007; IUPAC, 2010; US EPA, 2010). Lethal and sub-lethal (e.g. reproduction, growth, avoidance) toxicity data is reported for over 15 different macro-invertebrate species, with much of the data related to the composting worms *Eisenia fetida* and *E. andrei*. The majority of the ecotoxicological data for soil micro-organisms describes adverse effects to the microbial biomass and indicators of microbial activity, principally rates of respiration and nitrification (Baath, 1989; Giller et al., 1998). There is considerable variation in the reported toxicity values determined by different researchers and using different soils. For example, the reported reproductive EC_{50} for *E. fetida/E. andrei* varies from 8 to 927 mg/kg total Cu (mean 268 mg/kg; CV% 110). Even greater variation has been reported for measures of microbial activity. For example, a study using 19 different European soils found that the total Cu EC_{50} values for nitrification and respiration varied from <100 to >1000 mg/kg (Oorts et al., 2006b). On first in impression it would seem that much of the variation in the reported ecotoxicological data is due to effect concentrations being based on total Cu concentrations rather than the available fraction. However, several studies have reported that effect concentrations represented as the available fraction (e.g. $CaCl_2$ extractable, free Cu^{2+}) are even more variable (Oorts et al., 2006b; Broos et al., 2007; Criel et al., 2008). That said much of the variation in the reported ecotoxicological data can be explained by factors which influence the environmental availability of Cu, such as pH, CEC, clay and organic carbon content of the soil (Oorts et al., 2006b; Broos et al., 2007; Criel et al., 2008). Similarly, toxicity values determined using freshly spiked soils have been reported to be much lower than when using more realistic field aged soils, due to higher Cu availability (Scott-Fordsmand et al., 2000; Oorts et al., 2006a). This presents a significant challenge for ecological risk assessment as much of the ecotoxicological data on Cu has been generated using spiked soils. Using regression equations reported in the literature (Broos et al., 2007; Criel et al., 2008) the Cu concentration expected to cause an ecotoxicological effect (EC_{50}) to *E. fetida* reproduction, substrate induced respiration (SIR) and substrate induced nitrification (SIN) for vineyard soils in 10 different regions of Australia has been predicted (Table 3). Based on these models, accumulated Cu in Australian vineyard soils, (which has typically been reported in the range of 24 – 159 mg/kg total Cu (Table 1)), is unlikely to be causing adverse effects to rate of respiration and nitrification, but could potentially be causing adverse effects to earthworm reproduction.

It is difficult to compare the relative toxicity of Cu across different soil invertebrate species and microbial end points because of the high variability in the reported ecotoxicological data. However, soft bodied invertebrates (e.g. earthworm *Aporrectodea caliginosa*; mean LC_{50} 226 mg/kg total Cu) are generally more sensitive to Cu than hard bodied invertebrates (e.g. centipede *Lithobius mutabilis*; mean LC_{50} 1817 mg/kg total Cu). *Aporrectodea caliginosa* appears to be amongst the most sensitive invertebrate species (Frampton et al., 2006), which is of particular relevance as *Aporrectodea sp.* have been reported to be an abundant earthworm species present in vineyard soils (Thomson, 2006). Whilst soil micro-organisms have often been reported to be relatively tolerant to increased soil Cu concentrations, in some instances ecotoxicological effects have been reported at Cu concentrations that could realistically be present in the soils of vineyards (Baath, 1989, Giller et al., 1998). For instance,

Olayinka & Babalola (2001) reported that respiration and nitrogen mineralisation were significantly reduced at a total Cu concentration of 174 mg/kg. Similarly, Bogomolov et al. (1996) reported that litter decomposition was inhibited at total Cu concentration of 100 mg/kg. An increasing number of studies have also reported adverse effects to the activity of soil enzymes at relatively low Cu concentrations (Baath, 1989; Olayinka & Babalola, 2001; Kim et al., 2008). For example, Kim et al. (2008) showed that adverse effects on urease activity began at $CaCl_2$ extractable Cu concentrations less than 5 mg/kg. Interestingly, fungi seem to be relatively tolerant to Cu whilst nitrifying bacteria are particularly sensitive (Baath, 1989). Frampton et al. (2006) and Jansch et al. (2007) have estimated a lethal hazardous concentration to 5% (HC_5) for soil invertebrates to be 183 mg/kg total Cu (derived using available ecotoxological LC_{50} values) and a sub-lethal HC_5 value to soil organisms of 55 mg/kg total Cu (derived using EC_{50} values for invertebrates, microbial processes and plant species). At these HC_5 values it is assumed that 5% of all species/endpoints will be adversely affected; or, in other words, 5% of species/endpoints will have a value below the HC_5 concentration.

Region	Physical-chemical soil properties mean (range)[a]			Predicted EC_{50} (mg/kg total Cu) mean (range)[b]		
	pH	CEC ($cmol_c/kg$)	Clay (%)	*E. fetida* rep.	SIR	SIN
Murray Valley	8.7 (6.8 – 9.9)	17 (6.0 – 27)	22 (7.8 – 34)	353 (202 – 483)	1119 (811.5 – 1378)	2165 (1329 – 2663)
Southern Highlands	5.6 (5.1 – 6.3)	14 (5.1 – 21)	15 (10 – 20)	331 (183 – 418)	952.7 (870.7 – 1083)	813.1 (575.8 – 1097)
Riverland	7.7 (7.0 – 9.2)	23 (11 – 31)	28 (23 – 41)	439 (295 – 528)	1253 (1074 - 1546	1717 (1421 – 2370)
Barossa	7.2 (6.1 – 8.5)	14 (<0.1 – 37)	17 (6.0 – 34)	299 (9.44 – 583)	1011 (772.5 – 1395)	1487 (1023 – 2058)
southeast, SA	7.8 (7.2 – 8.6)	27 (11 – 36)	31 (18 – 36)	478 (288 – 573)	1346 (1260 – 1438)	1779 (1503 – 2102)
Riverina	6.6 (5.9 – 7.2)	21 (15 – 25)	33 (24 – 38)	418 (343 – 468)	1356 (1159 – 1481)	1252 (948.3 – 1498)
Mudgee	6.0 (5.6 – 6.4)	8.5 (1.5 – 23)	17 (11 – 29)	231 (88.4 – 436)	1016 (887.8 – 1277)	979.1 (808.0 – 1171)
Swan Valley	5.9 (5.1 – 7.0)	1.6 (0.1 – 5.0)	7.8 (4.6 – 12)	76.9 (18.5 – 207)	813.8 (742.5 – 867.0)	966.3 (577.9 – 1406)
Margaret River	5.9 (5.0 – 7.0)	9.0 (3.6 – 17)	9.8 (4.7 – 14)	250 (150 – 374)	856.8 (744.4 – 995.4)	936.4 (554.4 – 1422)
Tasmania	6.4 (5.7 – 7.7)	20 (3.6 – 52)	18 (4.9 – 43)	367 (149 – 713)	985.4 (749.6 – 1588)	1181 (857.0 – 1745)

[a] *Physical-chemical soil property data from Wightwick et al. (2008).*
[b] *E. fetida 28-day reproduction EC_{50} values predicted using regression equation in Criel et al. (2008); Substrate Inducted Respiration (SIR) and Substrate Induced Nitrification (SIN) EC_{50} values predicted using regression equations in Broos et al. (2007).*
n = 10 except for Southern Highlands and Mudgee (n = 9), Margaret River (n = 13), Tasmania (n = 7)

Table 3. The predicted ecotoxicological effects to earthworm reproduction and microbial activity (respiration and nitrification) for 10 viticultural regions of Australia.

A number of field studies have reported adverse effects of Cu fungicides on soil organisms. Paoletti et al. (1998) found that increases in total Cu concentration were correlated ($R^2 = 0.50$) with a decrease in earthworm biomass. A series of controlled field studies in South African vineyards found populations of the earthworms decreased significantly following a series of copper oxychloride spray applications (Maboeta et al., 2003; Eijsackers et al., 2005). Furthermore, a study in avocado orchards reported that accumulated Cu resulted in a decrease in earthworm activity and in decrease in the rate of litter decomposition (Van Zwieten, 2004). In relation to microbial activity, Wang et al. (2009) found a relationship between increased soil Cu concentration and decreased microbial biomass and phosphatase activity in the soils of apple orchards that had received copper-based fungicides for 5 to 45 years. Similarly, Merrington et al (2002) reported a 20% decrease in microbial biomass in avocado orchard soils with 280 – 345 mg/kg total Cu, compared with clean reference soils. Ranjard et al. (2006) found that applications of copper fungicides markedly changed the structure of bacterial and fungal communities in vineyard soils.

Compared to Cu, there is very little published data on the toxicity of the synthetic organic fungicide compounds to soil organisms. Most of the ecotoxicological data has been generated for the earthworm *E. fetida*. Like Cu, most of the fungicide compounds are moderately toxic to earthworms, with LC_{50} values typically in the range of 200 – 1000 mg/kg (Table 4). Carbendazim (LC_{50} 4 mg/kg) and triadimefon (LC_{50} 50 mg/kg) are the most toxic to *E. fetida* (Table 3) (IUPAC, 2010). However, toxicity data has been generated using a limited number of soils, so the variability in toxicity of the fungicides across different soils types is largely unknown. Where toxicity data has been reported from multiple studies, such as with captan (LC_{50} *E. fetida* <89 – 12121 mg/kg, n = 7), there is considerable variability in the reported effect concentrations (US EPA, 2010; Table 3). As

	Earthworm LC_{50} (mg/kg)	
Chemical class	**mean**	**range**
Sulfur	> 2000	
Triazole (e.g. myclobutanil)	394	50 – 1381
Dithiocarbamate (e.g. mancozeb)	720	140 - 1262
Carboximide (e.g. iprodione)	> 1000	
Pyrimidine (e.g. pyrimethanil)	252	192 - 313
Strobilurin (e.g. trifloxystrobin)	617	283 - > 1000
Amide/amine (e.g. spiroxamine)	> 1000	
Phenylamide (e.g. metalaxyl)	652	473 – 830
Quinone/quinoline (e.g. dithianon)	751	578 - > 923
Benzamidazole (carbendazim)	4	
Chloronitrile (chlorothalonil)	269	
Hydrazide (oxadixyl)	1000	
Morpholine (dimethomorph)	> 500	
Phenylpyyrole (fludioxinil)	1000	
Phthlamide (captan)	3639	< 89 - 12121

Data from IUPAC, 2010; US EPA 2010; Frampton et al., 2006.

Table 4. Summary of reported data on the acute lethal toxicity (LC_{50}) to earthworm (*Eisenia fetida)* for the different chemical classes of fungicides registered for use.

noted by Bunemann et al. (2006), some studies have shown synthetic organic fungicide compounds to have negative effects on soil organisms. For example, captan and chlorothalonil have been reported to cause reduced respiration, whilst metalaxyl reduced dehydrogenase activity (Bunemann et al., 2006). Carbendazim is also reported to have relatively high toxicity to invertebrates, causing an avoidance response in earthworms at soil concentrations of 10 mg/kg and estimated to have a lethal HC_5 value to soil invertebrates of 0.75 mg/kg (Bunemann et al., 2006; Frampton et al., 2006).

4.2 Ecotoxicological effects of fungicides on aquatic organisms

The effect of Cu on aquatic organisms has been very widely studied, and a large amount of ecotoxicological data is available, most of which relates to fish and invertebrates (Markich et al., 2002; US EPA, 2010). Copper is considered highly toxic to aquatic invertebrates, which is reflected in the Australian and New Zealand Water Quality Guidelines trigger value for Cu of 1.4 μg/L in water (for the protection of 95% of species; ANZECC & ARMCANZ, 2000). There is much less aquatic ecotoxicological data available for synthetic organic fungicides (Maltby et al., 2009; US EPA, 2010). The ANZECC water quality guidelines, and other jurisdictions around the world, do not provide water quality trigger values for any fungicide. The lack of ecotoxicological data for fungicides is surprising given their frequency of use and the fact that most do not have specific modes of action, thus are likely to be toxic to a wide range of organisms, not just fungi (Maltby et al., 2009).

A recent study collected aquatic ecotoxicological data for 42 different fungicides, including ~20 fungicides registered for use in horticulture in Australia (Maltby et al., 2009). This review found that chlorothalonil had the most data available (46 taxa) of all agricultural fungicides, and that most of the fungicide data related to invertebrates (67%). There was no suitable ecotoxicological data available for aquatic fungi or micro-organisms, despite these organisms likely sensitivity to fungicides, and the key role they play in aquatic ecosystems (e.g. decomposition, nutrient cycling; Maltby et al., 2009). In compiling the available ecotoxicological data, Maltby et al. (2009) estimated HC_5 concentrations for the 42 fungicides and compared their relative toxicity to fish, invertebrates and primary producers (e.g. plants). Table 5 summarises these values for the fungicides registered for use in Australian viticulture. Comparing the HC_5 values to the ANZECC & ARMCANZ (2000) water quality trigger value for Cu (1.4 μg/L), it can be seen that although ziram has a comparable low HC_5 value (1 μg/L), on the whole, synthetic organic fungicides appear to be much less toxic to aquatic organisms, particularly the triazoles and dithiocarbamates (Table 5). Fluzinam, carbendazim, and chlorothalonil have relatively high toxicity, whereas the strobilurins, primidines, and captan are moderately toxic (Table 5). The relative toxicity to the three main taxonomic groups (fish, invertebrates, primary producers) varied across the different fungicides. For example the dithiocarbamates and cyprodinil were most toxic to invertebrates, whilst the triazoles, dithanon and captan were least toxic to invertebrates. Fluzinam and carbendazim have relatively low toxicity to primary producers; chlorothalonil has relatively high toxicity to fish, whilst the strobilurins exhibit similar toxicities across the three taxonomic groups (Table 5).

As chemicals are normally present as mixtures in the environment, the issue of mixture toxicity is an important issue to address. This is perhaps particularly important for fungicides given that they are considered general biocides, capable of causing a toxic effect in organisms from different taxonomic groups (Maltby et al., 2009; Norgaard & Cedergreen,

2010). This is supported by a recent study which found that the pyrethroid insecticide α-cypermethrin was up to 12 times more toxic to the aquatic crustacean *Daphnia magna* in the presence of the fungicides prochloraz, epoxiconazole and propiconazole (Norgaard & Cedergreen, 2010). On the other hand, Cu has been reported to have an antagonistic effect on organic pesticide compounds, resulting in them being less toxic (Kungolos et al., 2009).

Chemical class	HC_5 (μg/L) mean (range)	Median EC_{50} value (μg/L) mean (range)		
		Fish	Invertebrate	Primary Producer
Triazole (myclobutanil, tebuconazole)	229 (220 – 238)	4419 (3137 – 5700)	1710 (720 – 2700)	1628 (1450 – 1805)
Dithiocarbamate (mancozeb, metriam, thiram, zineb, ziram)	51 (1 – 122)	40320 (12 – 180000)	664 (300 – 1125)	1391 (134 – 3250)
Pyrimidine (cyprodinil)	31	2410	660	2600
Strobilurin (azoxystrobin, trifloxystrobin)	25 (8 – 42)	367 (62 – 671)	157 (86 – 228)	235 (17 – 453)
Quinone (dithianon)	14	130	7000	90
Anilide (fluzinam)	8	89	97	1490
Benzamidazole (carbendazim)	8	225	237	27170
Chloronitrile (chlorothalonil)	6	38	183	783
Phenylpyyrole (fludioxonil)	63	740	370	485
Phthlamide (captan)	30	121	8400	320

Data from Maltby et al., 2009.

Table 5. Summary of estimated hazardous concentration (HC_5) values and effect concentrations to aquatic organisms for the different chemical classes of fungicides registered for use in Australian viticulture.

5. Assessment of ecological risks from fungicide use in horticultural production

The information presented on the fate, behaviour and toxicity of fungicides in the environment is useful for understanding the ecological risks posed by the use of fungicides in horticultural production systems. However, to meet the needs of risk managers/decision makers (e.g. policy officers, regulators) this information needs to be considered in a methodical manner that seeks to quantify and describe the risk(s), so that evidence-based decisions can be made. Risk managers/decision makers also require information on the level of confidence in assessments of risks, as they are governed by policies and regulations that often have little flexibility for "if" and "maybe" statements. The widely accepted ecological risk assessment (ERA) framework developed by the United States of America Environment Protection Agency (US EPA) provides structure and guidance for assessing ecological risks, such as those posed by agrochemical use (US EPA, 1998). The ERA framework involves four key steps: (1) problem formulation; (2) analysis (characterise the exposure and effects); (3) risk characterisation (analyse the degree of overlap between the exposure and effect); (4) risk management. In this section information on the fate, behaviour and toxicity of the fungicides registered for use in Australian viticulture is discussed in

relation to the ERA framework and how well this information contributes to describing the environmental risks posed. This is discussed in context of four of the main classes of fungicides used in Australia, these being the traditional copper-based and dithiocarbamates fungicides and the new triazole and strobilurin fungicides.

The reported data on physico-chemical properties (Table 1) can be used to rank the potential for chemicals to leach and persist in the soil. Over 50% of the fungicides registered for use in Australian viticulture theoretically have the potential to leach, including the widely used dithiocarbamates and triazoles. Comparatively, Cu and strobilurins are relatively immobile in soil (Table 6). Next to Cu, the triazole fungicides pose the greatest risks associated with persistence in soil having half-lives in soil of up to 400 days. The dithiocarbamates and strobilurins have much shorter half-lives (typically < 30 days), so are of less concern in relation to risks associated with soil persistence (i.e. toxicity to soil organisms, surface run-off). However, there is a degree of uncertainty around these 'crude' estimates of risk as the behaviour and persistence of chemicals can vary greatly in different soils (e.g. more mobile in sandy soils). Also ETU and EETFA are both more mobile than their parent compounds, which in turn may contribute to an increased risk of leaching (IUPAC, 2010).

	Copper-based	**Dithiocarbamates**	**Triazoles**	**Strobilurins**
Mobility in soil	Low	Medium to low	Medium to low	Low
Persistence in surface soils	High	Low	Moderate to high	Low to moderate
Field data on concentrations in soil	Good	None	Very limited	None
Field data on concentration in aquatic environments	Limited	None	Limited	Limited
Toxicity to earthworms	Moderate to low	Moderate to low	Moderate to low	Moderate to low
Toxicity to aquatic invertebrates	High	Low	Low	Moderate to low
Toxicity to fish	Moderate	Moderate to low	Low	Moderate to low
Toxicity to aquatic primary producers	Moderate	Low	Low	Low

Table 6. Summary of the relative behaviour and toxicity of four key classes of fungicides

A great deal is known about the ecotoxicological effects of Cu to terrestrial and aquatic organisms. However, there is a relatively high degree of uncertainty around the reported terrestrial ecotoxicological data due to the high variability in the data. Much of the data has been generated in the laboratory using freshly spiked soils and risk assessors need to be careful in applying this data as large discrepancies in ecotoxicological effect values have been found between freshly spiked and aged soils (more realistic of the real world). Due to the large degree of variability in reported ecotoxicological effects across different soil, risk assessors need to carefully decide which value to use for a particular species/endpoint. Using the wrong value could cause the risk to be greatly over, or arguably worse, under-estimated. Regression models have been developed to predict ecotoxicological effects in specific soil types (e.g. based on clay, CEC, pH; Oorts et al., 2006; Broos et al., 2007; Criel et

al., 2008). These enable ecotoxicological data to be normalised to a specific soil type prior to use in the risk assessment, thus reducing uncertainty. However, at present, predictive regression models have only been generated for a small number of species/endpoints (principally *E. fetida* reproduction, respiration and nitrification). To indicate the likely risk, hazardous concentration (HC_5) values have been estimated for soil organisms (Frampton et al., 2006; Jansch et al., 2007). There is, however, a degree of uncertainty around these HC_5 values as the ecotoxicological data was not normalised before use in the calculations. In Australia both water (1.4 µg/L, protection of 95% of species) and sediment (65 mg/kg, trigger value) guideline values have been derived for Cu for use in ERA (ANZECC & ARMCANZ, 2000). Like Cu, most of the fungicide compounds are moderately toxic to earthworms, including the triazoles, dithiocarbamates, and strobilurins (Table 6). However, toxicity data has been generated using a limited number of soils and species/endpoints (mostly *E. fetida*). So variability in toxicity across different soils and the relative sensitive of different species is largely unknown. Based on the aquatic HC_5 values published by Maltby et al. (2009) it appears that the synthetic organic fungicides are much less toxic than Cu to aquatic organisms, particularly the triazoles. The dithiocarbamates and strobilurins have similar toxicity, although the dithiocarbamates are considerably less toxic to fish (Table 5).

There is sufficient data on the concentrations of Cu in vineyard soils (both total and available) with which to compare to ecotoxicological effect data. In comparison, there is a paucity of field data on the persistence and concentrations of the synthetic organic fungicide compounds in vineyard soils, with no data for Australian vineyards and only a few studies worldwide (Komarek et al., 2010) (Table 6). Whilst these studies did not investigate dithiocarbamates or strobilurins, some triazoles (penconazole and tebuconazole) have been reported to persist in vineyard soils as expected. The more significant finding is that procymidone (up to 1124 µg/kg) and fludioxinil (up to 349 µg/kg) were detected at relatively high concentrations in vineyard soils, despite them being expected to have low persistence in soil. This re-enforces the inherent uncertainty in predictions of the behaviour and fate of chemicals in the environment and the need for field studies to form part of the ERA. No concentrations of fungicides have been reported in surface waters of viticultural/horticultural production regions of Australia, although a paucity of studies have been undertaken (Wightwick & Allinson, 2007). However, some studies in Europe have detected Cu and other synthetic organic fungicide compounds in aquatic environments within viticultural/horticultural catchments (Hildebrandt et al., 2008; Gregoire et al., 2010; Komarek et al., 2010; Rabiet et al., 2010).

The risk characterisation phase of ERA considers the exposure and effect characterisations to formulate a final decision on the risk(s) posed, or in other words the probability of an adverse effect. Ideally the risk characterisation should quantify and describe the nature of the risk and is typically carried out using hazard quotient and/or probabilistic risk assessment method (Urban & Cook, 1986; Solomon et al., 2000). Due to the lack of environmental concentrations and toxicity data on the fungicides used in viticultural production systems of Australia, it is currently not possible to characterise the risks for fungicides other than Cu with any degree of certainty. The data on Cu in vineyards soils in 10 different regions of Australia from the survey of Wightwick et al. (2008) can be compared to the reported hazardous concentration values (Frampton et al., 2006; Jansch et al., 2007). In doing this only one of the 98 vineyards exceeded the lethal HC_5 to soil invertebrates of 183 mg/kg total Cu, whilst 44% of the vineyards exceeded the sub-lethal HC_5 to soil organisms of 55 mg/kg total Cu. Although there is a relatively high degree of uncertainty around these

estimated HC_5 values, for instance the 95% confidence interval for the sub-lethal HC_5 ranges from 28 – 92 mg/kg total. Nonetheless it appears that at present the accumulation of Cu in Australian vineyard soils is unlikely to be causing lethal toxic effects to soil invertebrates in most situations but may be causing sub-lethal effects to invertebrates and microbial processes. Ecotoxicological data for invertebrates suggests that the earthworm *A. caliginosa* is the most sensitive species. This is of significance as it is also one of the most abundant earthworms in Australian vineyard soils, thus representing an important keystone species to protect. Despite the likely risks, at present there is currently insufficient exposure and effect data for which to even attempt to characterise the risks of the synthetic organic fungicides and to the other risk scenarios, such as the transport to surface waters and leaching to groundwater.

From a risk management perspective, the available information on the fate, behaviour and toxicity of fungicides can be used to indicate the relative risks posed by the different chemical classes. This is of importance as there is a trend for horticultural industries to move away from using copper-based and dithiocarbamates fungicides to the newer triazole and strobilurin fungicides. The increased use of dithiocarbamates and strobilurins over copper-based fungicides is likely to result in a decreased risk to soil organisms due to their much lower persistence in soil (Table 6). Furthermore, a move away from copper-based fungicides is likely to result in a decreased risk to aquatic organisms, particularly if dithiocarbamates and triazoles are used.

6. Policy perspectives on assessing and managing ecological risks associated with fungicide use

Scientific information on ecological risks associated with fungicide use needs to be fed back into the wider policy/regulatory frameworks and processes to enable effective management of these risks. This section provides perspectives on the needs and mechanisms for linking science with policy in the management of ecological risks associated with fungicide use.

6.1 Regulatory and policy framework for agricultural chemicals

At the international level, policy development in relation to chemicals dates back to the 1800's, with the St Petersburg Declaration, which marked the need for more information regarding chemicals (Selin, 2010). In 1992, Agenda 21 was adopted at a United National Conference on the Environment thus instigating significant developments in the management of chemicals. This included introducing scientific assessments (risk and hazard) to evaluate the environmental impact of chemicals. More specifically, instruments such as the Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade, and the Stockholm Convention on Persistent Organic Pollutants have been introduced to provide for a national decision-making process on the import and export of a range of chemicals including fungicides. Additionally, the Globally Harmonized System for the Classification and Labelling of Chemicals was introduced in 2006 to ensure physical hazards and toxicity information is made available to enhance protection of human health and the environment (UNEP, 2006). More recently, the Strategic Approach to International Chemicals Management 2006 (SAICM) was established, which aims to get the international community to work more co-operatively to protect health and the environment throughout the chemical life cycle. A positive step forward under the SAICM is the requirement for science based approaches to

be applied to new chemicals, existing chemicals, tools and strategies for assessment. Additionally, SAICM outlines the need for civil society and industry to work co-operatively together as well as the need for research into identifying the effects of chemicals to humans and the environment, including technologies to assess these impacts (UNEP, 2006).

The regulation of agricultural chemicals in Australia is complex. In summary, the national government, through the Australian Pesticide and Veterinary Medicines' Authority (APVMA), has responsibility for registration of chemical products through to the point of retail sale. The state and territory governments are responsible for controlling the use of these chemical products. Before registration of a chemical is approved by the APVMA, the chemical product is assessed for its likely impact on human health and the environment. This is a process whereby scientific and hazard assessments are used as part of the policy development aimed at assessing whether a chemical product is 'safe' to be used by industry and the general public, while considering impacts to trade, human health, the environment and animal welfare. The current framework underpinning policy on the management of agricultural chemicals (including fungicides) is focussed on assessing the 'input' standard. That is, understanding the impacts that chemicals have prior to registration and use. However, in Australia there appears to be little or no assessment of the 'performance' standard. For instance, there are essentially no structured ongoing monitoring programs in Australia to determine the presence, concentrations and long term impact of agricultural chemicals in the environment post registration (Wightwick & Allinson, 2007). This represents a significant lack of knowledge in relation to the potential long term impacts of fungicides in the natural environment. The regulatory framework in Australia does recognise that certain chemicals may pose a high risk and where identified individuals are required to undertake specific training and in some cases licensing to be able to use the chemical. From a policy perspective this places a greater emphasis on the need for assessing the 'performance' standards of these chemicals to ensure there is minimal or no impact to chemical users and the environment. At present the triggers for identifying high risk chemicals in Australia occurs in a somewhat ad-hoc fashion predominately being through the APVMA's adverse experience reporting system, those identified in other jurisdictions, and due to concerns raised by community groups.

In Australia, more needs to be undertaken at the national and local levels to not only implement international arrangements, but also further link science and policy through increased research into the longer term effects of chemicals and their presence in the environment (i.e. to assess the 'performance' standard of the chemical product), throughout the chemicals 'life cycle'. The information gathered from this type of research can be used to inform regulators and, where required, change the existing controls on a particular chemical product.

6.2 The pesticide policy debate

Communities are becoming more and more aware that the mis- or overuse of agrochemicals including pesticides may harm aquatic and terrestrial organisms, and potentially change the normal structure and function of ecosystems. As a result, the need to protect the natural environment has become part of the mainstream public policy debate. It is therefore important to understand the external impacts on primary production and the broader community resulting from the use of agricultural chemicals. The externalities and adverse effects of agrochemicals are said to be present when the utility to an individual depends not only on the goods and services the individuals purchase and consume but also on the activity of some

other individual(s), i.e. "where individual A's welfare depends on a range of goods and services which they consume (x 1, x 2, … , x n) as well as on some activity, Y, carried out by another individual, B" (Cullis & Jones, 1992). Moreover, the "distinguishing feature of an externality is that it is an interdependence that occurs outside of the price mechanism that is not compensated" (Cullis & Jones, 1992). The externalities relating to fungicides are where they enter the natural environment from a single or group of primary producers or other land managers, and affect other users of water, resulting in a loss of recreation amenity, drinking water quality/supply and income from commercial activities. These externalities can have adverse human health and environmental impacts. Fungicide pollution can occur: from producers to producers; producers to consumers and consumers to consumers. The most significant type of relationship is the producer-consumer interaction. This 'many to many' interaction is the dominant form for agrochemical pollution (Herath, 1998). Furthermore, fungicide pollution is not easily visible or detected, so the potential environmental risks do not evoke an immediate response, as it can take place over many years and may be as a result of many different land uses within a catchment. It tends to be only when a major incident occurs that the extent of these externalities is recognised. Consequently, the costs of agrochemicals are generally borne by the affected groups who, in the absence of a market, are not compensated by the polluters. Therefore, it is important that more research and assessment of the long term 'performance' standard of fungicides and chemicals in general, throughout their life cycle, is undertaken. This will ensure that there are no further repeats of problems caused by legacy chemicals, such as dichlorodiphenyltrichloroethane (DDT) (APVMA, 2010b), where it has taken a long period of time to fully understand the impact the chemical and its metabolites have had and are having on the natural environment.

It is clear that science needs to be closely linked to policy development and in turn policy needs to inform the science, to improve the management of ecological risks associated with agrochemical use throughout the supply chain (from manufacture to use). This process is referred to as an evidence based policy approach, or an approach that "helps people make well informed decisions about policies, programs and projects by putting the best available evidence from research at the heart of policy development and implementation" (Davies, 1999). Such an approach includes up to eight stages including identifying issues; policy analysis (i.e. using evidence to develop options and make decisions); identifying policy instruments (e.g. advocacy, monetary, legislative); consultation; co-ordination; decision making; implementation; and evaluation (Althaus et al., 2007). Evidence-based approaches differ significantly from opinion-based policy, which relies on selective use of evidence or untested views of individuals or groups, often established by an ideological position or prejudices. Worldwide evidence-based policy approaches have become a major part of many governments' policy and regulatory development as it enhances industry and community confidence in government decisions. The ongoing challenge for governments is to form stronger links between science and policy and to ensure policy assists in determining the science priorities, to ensure that fungicides are managed throughout the chemical life cycle to ensure producitvity continues whilst protecting human health and the environment.

Presently, science informs policy on the management of agrochemicals predominately through structured regulatory and policy frameworks that form part of pre-registration assessments. Policy also informs the direction of science where the community has pressured the regulator to undertake further review of the risks a chemical product may be having on the environment or to human health (whether that be real or perceived). Furthermore, in Australia, the APVMA has in place an adverse experience reporting system

and existing chemicals review program to identify priority risk chemicals. This provides a more structured process whereby policy makers can identify and direct scientific research needs, which is crucial given the large number of agricultural chemicals registered for use (i.e. currently in Australia there are over 2000 active ingredients registered for use). Scientific information for environmental risk assessments is then used by policy makers and regulators to assess whether a chemical product needs to be reviewed for registration amendments such as label changes, changes to the method of manufacture, suspension and/or cancellation of registration and approval (APVMA, 2010a). The insecticide DDT and more recently the fungicide quintozene are two contrasting examples of where science has informed the policy debate to instigate management actions to reduce environmental risks associated with agrochemical use.

DDT is a historical example where science and policy did not initially work cohesively enough to manage the ecological risk. DDT was first used in Australian agriculture during the 1950's as a highly effective broad-spectrum insecticide. Ecological and human health concerns about DDT were first raised in Australia during the 1960's. However, despite the known risks of DDT, its use was not phased out until the 1980's (APVMA, 2010b). This was largely due to ongoing economic debate around the benefits of DDT outweighing the disadvantages. The community and subsequent governments are still bearing the costs ('externalities') from the legacy of DDT due to slow uptake of scientific knowledge on the environmental risks of DDT by policy makers,. To avoid repeats of legacy chemicals such as DDT, it is clear that science and policy need to work together more closely and take a more proactive approach to understanding and managing environment risks.

In Australia, the recent suspension of the use of the fungicide quintozene (APVMA, 2010a) is an example of where science and policy have communicated more cohesively. Dioxins can be un-intentionally formed during the manufacture of some agricultural chemicals. This may present an environmental concern when the chemicals are used as dioxins are recognised as persistent pollutants of concern (Holt et al., 2010). In recognition of the potential risks, Australian researchers took a somewhat more proactive approach by analysing the concentration of dioxins in 27 different current use agrochemical formulations. This research highlighted that unacceptably high concentrations of dioxins were present in quintozene formulations (Holt et al., 2010). The scientists notified the APVMA of this concern in June 2009 and following results of confirmatory analysis the APVMA initiated regulatory action and suspended the use of quintozene in April 2010. Compared to the DDT example, this more cohesive and timely action between science and policy will help to avert further issues relating to the legacy of dioxins in the environment resulting from use of quintozene. The challenge is for science and policy to identify and action similar issues in a timely and structured manner.

In the future, the pesticide policy debate is likely to be complicated by the large projected increase in the human population, from 6.5 billion people in 2005 to 9 billion people in 2050 (United Nations, 2002). The predicted increase in population will place huge demands on natural resources on which primary production depends for food production. In order to increase food production, it is likely that there will be significant increases in the use of agricultural chemical, including fungicides, to meet this world demand. This will place even further pressure on science and policy to work together to effectively manage the competing pressures on controlling environmental and human health risk, meeting international and national legislative obligations, whilst ensuring pests and disease are controlled to guarantee crop quality and production.

7. Conclusions and future research needs

Fungicides are widely used in horticultural production systems to ensure crop quality and production. However, the use of such fungicides may cause adverse effects to terrestrial and aquatic ecosystems if fungicide residues persist in soil, or if they migrate off-site to surface and ground waters. Whilst a great deal is known about the fate, behaviour and toxicity of Cu in both terrestrial and aquatic environments, comparatively little information has been reported for the synthetic organic fungicide compounds. The accumulation of Cu in surface soils following the use of copper fungicides has been reported in many regions of the world, and there is evidence suggesting adverse effects to earthworms and soil micro-organisms. However, only a small number of studies have measured the persistence of synthetic organic fungicide compounds in the soils of horticultural properties, providing a limited knowledge base on which to assess the likely risks. Similarly, only a few studies have sought to investigate the off-site movement of fungicides and their presence in aquatic environments. Assessments of the behaviour and toxicity of fungicides in the environment are complicated by the environmental and biological availability, which varies greatly across different soils, sediment, and waters (i.e. large influence of pH, clay, organic matter). Using ecological risk assessment principles, it is currently not possible to characterise the environmental risks of fungicides with any degree of certainty. An exception being risks of Cu accumulation to soil organisms, but, even here there is a high degree of uncertainty due to large variability in ecotoxicological effect values across different soil types. The relative risks of different fungicides needs to be considered when making recommendations on the future management of fungicide use.

Current regulatory/policy frameworks both in Australia and worldwide focus on assessment of the 'input' standard of agricultural chemical products prior to their release onto the market. However, significantly more work is needed to assess the 'performance standard' of registered chemicals over the longer term to ensure the environment and human health are not adversely impacted throughout their life cycle. Stronger linkages between science and policy will ensure the more effective and timely assessment and management of ecological risks associated with agricultural chemical use.

Further research into the potential environmental risks posed by fungicide use is needed so that evidence-based policy decisions can be made on the future management of fungicide use in horticultural production systems. This research needs to focus on generating toxicity data for more species/endpoints, in particular aquatic fungi, microbial processes, and earthworms of agricultural relevance to improve the 'input' standard. Research also needs to focus on understanding the influence of differing soil, sediment and water properties and chemical mixtures on the behaviour and toxicity of fungicides in the environment. Field studies to determine environmental concentrations, in particular for synthetic organic fungicide compounds, are also of paramount importance in being able to assess actual environmental risks (i.e. 'performance' standard) throughout the chemicals life cycle. Further information on the types and amounts of fungicides being used is needed to focus this research on priority fungicides of concern.

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15

Benzimidazole Fungicides in Environmental Samples: Extraction and Determination Procedures

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1. Introduction

Because of the widespread use of agricultural pesticides for different applications, the pesticide residues may present a main source of pollution, which poses risks to plant, animal and human health. Benzimidazole fungicides (BFs) are the largest chemical family that have an imidazole ring containing both acidic and basic nitrogen atoms. They are used for prevention and treatment of parasitic infections in agriculture and aquaculture and are efficient at low doses as well as they inhibit the development of a wide variety of fungi. Some benzimidazoles have also found applications as pre- or post-harvest fungicides for control of a wide range of pathogens. They are either applied directly to the soil, or sprayed over crop fields (Wu et al., 2009). Most of these compounds persist in the environment after their application, with some even remaining for many years. This group includes thiabendazole analogues and benzimidazole carbamates.

Thiabendazole (TBZ) was the first benzimidazole to be marketed. After its introduction, a number of alternative benzimidazoles offering similar activity came on the market, such as parbendazole (PAR), cambendazole (CAM), mebendazole (MBZ), fuberidazole (FDZ) and oxibendazole (OXI). BFs possessing sulphide and sulphoxide functional groups were subsequently introduced, offering a wider spectrum of activity and efficacy. Albendazole (ABZ), fenbendazole (FBZ), triclabendazole (TCB) and oxfendazole (OFZ) have been used in the treatment of different stages of gastrointestinal nematodes. Luxabendazole (LUX) is another benzimidazole-sulphide used but is not licensed for use in the European Union. Netobimin (NETO) and febantel (FEB), which are the pro-drugs of ABZ and FBZ, respectively, have greater water solubility resulting in improved absorption and increased bioavailability. Similar probenzimidazoles have found widespread use as fungicidal agents, including benomyl (BNM) and thiophanate-methyl (TPM), which are precursors of carbendazim (MBC). Such modifications have given rise to new BFs with much slower rates of elimination, higher potencies and broader activity spectra.It is well established that MBC, the common stable

metabolite of BNM and TPM, is considered as the major fungitoxic principle of the benzimidazole precursor fungicides. Accordingly, regulatory limits for these fungicides are generally all expressed as MBC, the single measurement marker for the food safety or environmental impact of the total benzimidazole-containing residues (Danaher et al., 2007).

The octanol-water partition coefficient, K_{ow}, is an important property because it can provide an indication of the solubility of these residues in different solvents and give an indication of the elution conditions necessary for liquid chromatography. Most of these molecules have similar K_{ow} values, which are generally in the range 0.8–3.3. Under suitable conditions, molecules may be protonated (pK_a~5-6) or deprotonated (pK_a~12). Few pK_a values have been published but a summary of the experimental pK_a values and calculated octanol-water partition coefficients (K_{ow}) are listed in Table 1.

Although many public benefits have been realized by the use of benzimidazole compounds, their potential impact in both the environment and public health cannot be disregarded. Even if acute toxic effects of benzimidazole compounds are scarce due to their high lethal dose 50 values, several toxic effects have been associated to a chronic exposure to benzimidazole compounds, such as teratogenicity, congenic malformations, polyploidy, diarrhea, anemia, pulmonary edemas, or necrotic lymphoadenopathy.

On account of their extensive use, residual environmental impact and toxic effects at low levels, regulations have set maximum residue levels (MRLs) for benzimidazoles and their metabolites to ensure consumer safety with the range of 0.01–10 mg·kg^{-1}, depending on the fungicide–commodity combination (Plant Protection-Pesticide Residues-Regulation (EC) No. 396, 2005). In particular, for most benzimidazoles, the marker residue tolerance has recently been defined as the sum of a parent drug and/or its related metabolites (sum-MRL substances) instead of single compounds (Danaher et al., 2007).

Their massive use in the last years has led into their accumulation in the environment, thus contaminating the water streams. European Water Framework Directive (Directive 2006/11/CE 4) has established a maximum concentration level (MCL) of 0.1 µg·L^{-1} for most benzimidazole compounds present in natural waters, and a total concentration of all pesticides of 0.5 µg·L^{-1}. The need to determine low concentrations of these substances as well as matrix complexity means that analytical methods with high sensitivity, selectivity and resolution have to be applied to soil, sediment, water and other environmental samples. Elaborate sample preparation involving analyte isolation and enrichment is generally necessary before the final analysis, which is usually performed using gas chromatography (GC) or high-performance liquid chromatography (HPLC).

The selection of an adequate sample treatment protocol allowing to carry out a multiresidue determination of benzimidazole compounds is currently a challenge, due to their chemical properties. The development of highly sensitive methods for the multiresidue determination of benzimidazole compounds in environmental samples is desirable, and for that purpose a preconcentration step and appropriate instrumental techniques are usually required. In this sense, extensive sample cleanup or preconcentration procedures might be applied to the determination of a wide variety of benzimidazole compounds, and the use of highly selective detection methods (i.e. tandem mass spectrometry) is usually required for multiresidue analysis.

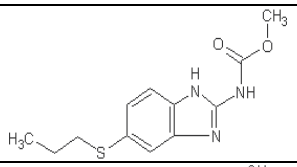
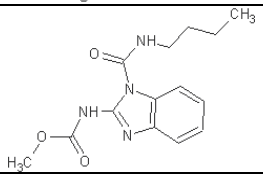
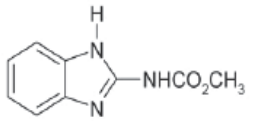
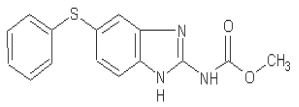
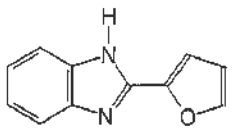
A large amount of effort has been invested in the past few decades to develop and validate analytical methodologies to quantify benzimidazole compounds and their metabolites in environmental samples at concentration levels below the legislated MCLs.

In this chapter some methodologies for the determination of BFs in environmental samples are presented. Its scope is the coverage of the main aspects which are involved in their determination in environmental matrices: sample handling, extraction/preconcentration and determination. We will conclude with a general conclusion and notes on future perspectives.

2. Environmental liquid samples preparation

Contamination of natural waters by pesticides is one of the main environmental problems around the world because of the common use, persistence, bioaccumulation and toxicity of these pollutants. Determining the degree of ground and surface water contamination by these compounds is one of the fundamental aims of environmental analytical laboratories. To reach the low limits of detection is necessary the use of sensitive multi-residue methods for detecting and identifying these compounds, with the fewest number of intermediate step.

Sample preparation is one of the most important steps in a whole analytical process. The objective of the sample preparation is not only to isolate the target analytes from the samples, thus reducing or even eliminating the interferences originally present in the sample, but also simultaneously to concentrate the analytes to facilitate their determinations at low levels. Sample preparation gradually becomes a major part of analysis, capable of taking up to 80% of the total time of a complete analysis process. Different steps in the process, extraction, clean-up and detection play a key factor in the safety and accuracy of analysis.

Name	Chemical structure	K_{oc}	pK_{a1}	pK_{a2}
Albendazole (ABZ)		2.2-2.92	5.54	13.11
Benomyl (BNM)		1.4	4.48	
Carbendazim (MBC)		1.29-1.69	5.52	13.09
Fenbendazole (FBZ)		3.07-4.01	5.12	12.72
Fuberidazole (FDZ)		2.71	4.0	

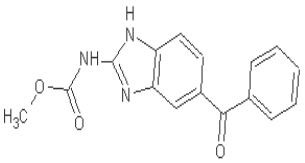
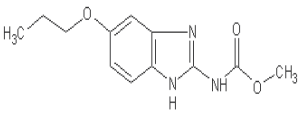
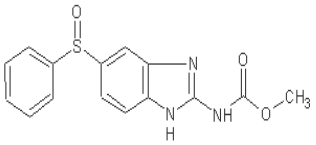
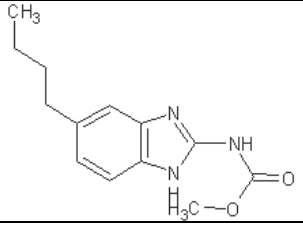
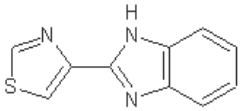
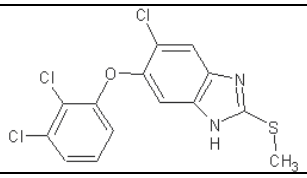
Name	Chemical structure	K_{oc}	pK_{a1}	pK_{a2}
Mebendazole (MBZ)		2.44-2.52	4.13	11.79
Oxibendazole (OXI)		1.86-2.63	6.26	13.78
Oxfendazole (OFZ)		1.88-2.13	4.13	11.79
Parbendazole (PAR)		1.86-2.63	5.99	13.53
Thiabendazole (TBZ)		1.58-1.76	5.82	12.79
Triclabendazole (TCB)		4.90-6.66	5.31	12.91

Table 1. Chemical structures and calculated properties of some benzimidazole fungicides (BFs) (*Danaher et al., 2007)*

Because the low concentration levels in environmental waters, an extraction and preconcentration step is usually required. First extraction method was liquid–liquid extraction (LLE) with conventional organic solvent (Blanchflower et al. 1994; Fernández et al., 2001). However, LLE suffers from the disadvantages of being time-consuming, expensive and requiring large volumes of both samples and toxicity. Other methods, like solid phase extraction (SPE) (Picón et al., 2000; Garrido et al., 2003; Moral et al., 2006), on-line supported liquid membrane (SLME), microporous membrane liquid–liquid extraction (MMLLE) (Sandahl et al., 2000), cloud point extraction (CPE) (Halko et al., 2004) and solid phase microextraction (SPME) (López Monzón et al., 2007) have been proposed to extract and concentrate BFs from aquatic environments.

In recent years, solid phase extraction (SPE) has widely been applied for the analysis of different pesticides in water samples, owing to the high enrichment factors achievable by this methodology using the high breakthrough volumes of materials such as C_{18} (Picón et al., 2000; Zamora et al., 2003; Garrido Frenich et al., 2003), polystyrenedivinylbenzene (Guenu et al., 1996), ethylvinylbenzene-divinylbenzene (JunkerBuchheit, 1996), polystyrene (Shimamura et al., 1998) and active carbon (Jeannot et al., 2000). In this sense, Guenu et al. used precolumns packed with PS-DVB sorbents for the on-line determination of very polar pesticides, including carbendazim (Guenu et al., 1996). The evaluation of the PS-DVB sorbent was first carried out by measuring the recoveries using off-line extraction with a 20 mg PS-DVB cartridge and percolating drinking and river water both spiked with 0.1 $\mu g \cdot L^{-1}$ of each pesticide Limits of detections (LODs) were at the 0.05-0.3 $\mu g \cdot L^{-1}$ level in surface waters for different polar pesticides.

Other authors used C_{18} extraction cartridges for extracting carbendazim, fuberidazole and thiabendazole from water samples obtaining LODs between 0.001 to 0.125 $\mu g \cdot L^{-1}$. Satisfactory predictions ranging from 102 to 114% for carbendazim, 96 to 115% for fuberidazole and 90 to 107% for thiabendazole were sufficiently low to determine pesticide residues in water samples (Picón et al., 2000; Garrido Frenich et al., 2003). SPE requires large volumes of sample (>200mL) and organic solvent (12–50 mL) and BFs losses occur during the evaporation of the extracts (Guenu et al., 1996).

Recent developments in SPE field are mainly related to the use of new sorbent materials. Molecularly imprinted polymers (MIPs) have proven to be a very valuable technique for selective solid-phase extraction of the template molecule and structurally related compounds. The inherent selectivity of the molecular recognition of these materials allows a high degree of sample clean-up to be achieved (Baghianni et al., 2006; Pichón et al., 2006). Additionally, MIPs have also been used for sample enrichment for the determination of a wide range of analytes. MIPs have successfully been applied as highly selective sorbents for the extraction of benzimidazole compounds in an organic media (de Prada et al. 2007; Cacho et al., 2008; Turiel et al., 2005). However, coelution of the different benzimidazole compounds has been described when using these imprinted polymers as selective stationary phases (Cacho et al., 2009). For that, development of molecularly imprinted solid-phase extraction (MISPE) procedure permit the enrichment of benzimidazoles fungicides, based on an on-line sample enrichment of water samples by means of an imprinted polymer, synthesized by precipitation polymerization using thiabendazole as template molecule, methacrylic acid as functional monomer, and divinylbenzene as cross-linker. Initial experiments carried out by solid-phase extraction on cartridges demonstrated a clear imprint effect for thiabendazole, as well as the ability of the imprinted polymer to selectively rebind several benzimidazole compounds. The developed methodology has been applied to the quantification of thiabendazole, carbendazim, and benomyl in river, tap and well water samples within a single analytical run at concentration levels below the legislated maximum concentration levels. In this sense, obtained detection limits were of 2.3–5.7 $ng \cdot L^{-1}$ for the analysis of benzimidazole fungicides in different water matrices. Recoveries obtained for the determination of benzimidazole fungicides in spiked samples ranged from 87% to 95%, with relative standard deviations (RSD s) below 5% in all cases (Zamora et al, 2009).

López-Monzón et al. (2007) established a suitable and sensitive method for simultaneous determination of BFs (carbendazim, benomyl, fuberidazole and thiabendazole) in water samples using solid phase microextraction (SPME). Authors evaluated the efficiency of extraction of these compounds on different kinds of fibres and optimized several SPME

conditions: extraction time, ionic strength, extraction temperature and desorption time. The optimized SPME procedure was used for extraction and determination of these compounds in different environmental water samples (sea, sewage, and ground waters). A Carboxen-polydimethylsiloxane (CAR-PDMS) fibre was the optimum coating for extraction of these targets. Obtained recoveries ranged from 80.6 to 119.6 with RSDs below 9% and limits of detection between 0.03 and 1.3 ng mL^{-1} for the different analytes. A chromatogram of this process is shown in Figure 1.

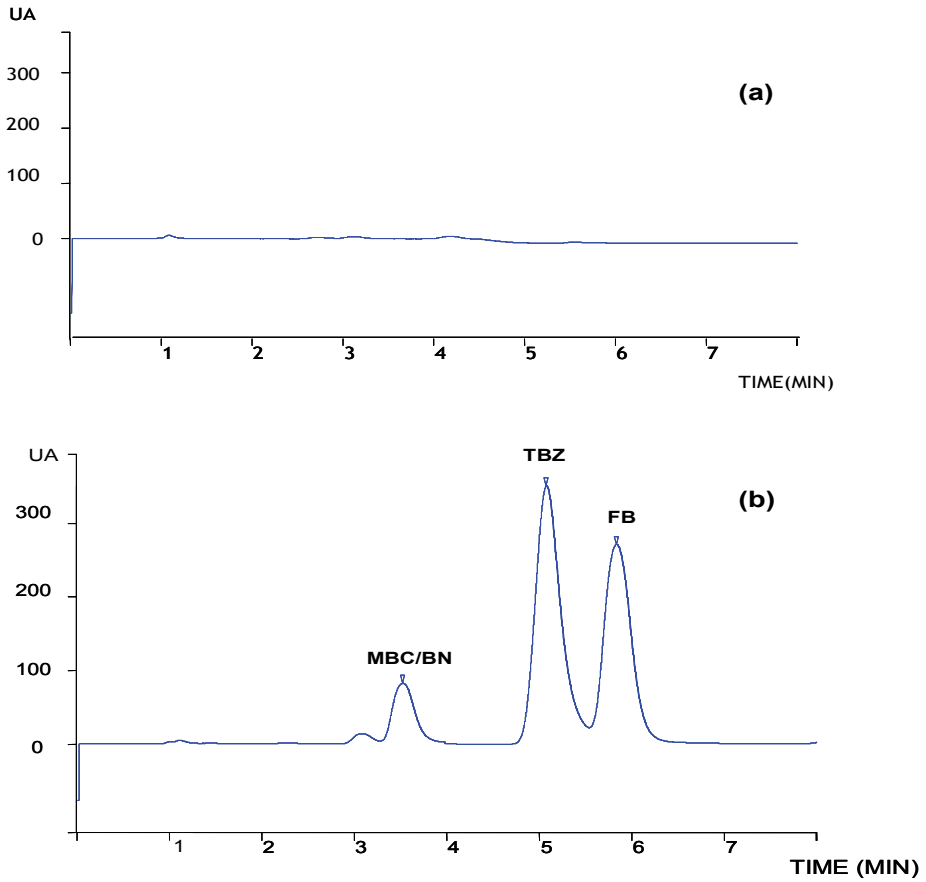


Fig. 1. Obtained chromatogram for a blank sample (a) and for the extract of benzimidazole fungicides from a spiked seawater sample (b) under optimum SPME extraction (López-Monzón et al., 2007).

Extraction process like microporous membrane liquid–liquid extraction (MMLLE) and supported liquid membrane extraction (SLME) have been demonstrated to be efficient for sample preparation. MMLLE is a two-phase aqueous–organic solvent system and SLM a three phase aqueous–organic solvent–aqueous system, which leads to more selective extraction. All of them require lower amount of sample (4–20 mL) and organic solvent. An inconvenient of these procedures is time-consuming although enrichment rates of 0.6 times min^{-1} by SLME and 2.7 times min^{-1} by MMLLE have been reported (Sandahl et al., 2000) and SPME takes more than 1 h to obtain a concentration factor of 80 (López Monzón et al., 2007).

Other pre-treatment method to extract and preconcentrate BFs in liquid samples is cloud-point extraction (CPE). The cloud point phenomenon occurs when micellar solutions of non-ionic or zwitterionic surfactants are heated above certain temperature, referred to as cloud point temperature, and they become turbid. At the cloud point, the surfactant solution undergoes phase separation into a surfactant-rich liquid phase and an almost micelle-free dilute solution whose concentration is equal to or lower than the critical micelle concentration. Obtained small volume of the surfactant-rich phase permitting extraction schemes to be designed allows us to preconcentrate and extract the analytes in one step, prior to liquid chromatographic analysis. CPE using non-ionic surfactant such as POLE and Genapol X-080 provides good extraction efficiency of different BFs in environmental liquid samples (Halko et al., 2004). The limit of detection (LOD) were 6 ng mL^{-1} for carbendazim, ng mL^{-1} benomyl, 0.15 ng mL^{-1} for thiabendazole and 0.01 ng mL^{-1} for fuberidazole in both surfactants. Obtained recoveries in spiked water samples ranged from 68% to 94% for Genapol and from 68% to 96% for POLE.

Other techniques like liquid phase microextraction, LPME (a small amount of a water-immiscible solvent and an aqueous phase containing the analytes of interest) have emerged as an attractive alternative for sample preparations because of its simplicity, effectiveness, low cost, minimum use of solvents and excellent sample cleanup ability. Different configurations of this technique have recently emerged, including static LPME, dynamic LPME, single-drop LPME and hollow fiber-based liquid-phase microextraction, HF-LPME (extracting phase is placed inside of a porous hydrophobic hollow fiber) (Psillakis et al.,2002; Psillakis et al., 2003; Rasmussen et al, 2004; Pedersen-Bjergaard et al., 2005). However, several disadvantages, such as the instability of liquid drop in single-drop LPME, air bubbles formation in HF-LPME, long analysis time and relatively low precisions, are often encountered for such techniques. Very recently, a novel microextraction technique, named dispersive liquid–liquid microextraction (DLLME), based on dispersion of tiny droplets of the extraction solvent within the aqueous solution has been developed by Assadi and co-workers (Rezaee et al., 2006; Berijani et al., 2006; Fattahi et al., 2007). DLLME is a miniaturized LLE that uses microliter volumes of the extraction solvent. This method was applied to extract carbendazim and thiabendazole in water and soil samples using 80.0 μL of $CHCl_3$ as extraction solvent (Wu et al., 2009). Obtained limits of detection for MBC and TBZ were 0.5 and 1.0 ng·mL^{-1} for liquid samples and 1.0 and 1.6 ng·g^{-1} in solid samples, respectively.

Studies on the use of surfactant-coated mineral oxides columns for SPE have demonstrated these new sorbent materials to be a promising tool for the extraction/preconcentration of organic compounds in a wide polarity range (Merino et al., 2004; Moral et al., 2005). Adsorption of ionic surfactants on mineral oxides is a cooperative process; firstly, a monolayer of surfactant (i.e. hemimicelles) is formed with the surfactant head-group facing towards the oxide surface and its hydrocarbon tail-groups protrude into solution, interacting laterally between them. After that, surfactant adsorption occurs through hydrophobic interactions between hydrocarbon tail-groups, which results in the formation of discrete surface aggregates termed admicelles. Because of the amphiphilic character of surfactants, admicelles are aggregates in which there are regions of different polarity, acidity, etc. This feature makes these aggregates extremely versatile extractants because of the number of interactions they can establish with analytes. This type of process have been used for carbendazim , thiabendazole and fuberidazole preconcentration in water samples (Moral et al., 2006). Recently, Moral et al. (2009) have used supramolecular solvents based

on vesicles of decanoic acid as a good extractant of benzimidazolic fungicides from natural waters. The high concentration of decanoic acid in the extractant phase and the capability of solubilisation of the vesicles permited the favourable partition of analytes from environmental water using a quite low volume of supramolecular solvent (100 μL). Actual concentration factors around 150–200 are easily obtained using a single-step extraction without the need of solvent evaporation. Equilibrium conditions are rapidly established, the whole extraction takes about 20 min and several samples can be simultaneously extracted. The proposed method provided detection limits for the determination of CBZ, TBZ and FBZ in natural waters of 32, 4 and 0.1 ng·L^{-1}, respectively, and a precision, expressed as relative standard deviation of 5.5% for CBZ, 4.0% for TBZ and 2.5% for FBZ.

3. Environmental solid samples preparation

Analytical methods for the determination of BFs in soil are scarce in the available scientific literature. Traditional methods employ large volumes of solvents under aggressive shaking and/or temperature conditions. The most frequently used methods for the extraction of organic compounds from soils are Soxhlet or ultrasonic extraction. The newer extraction techniques, such as supercritical fluid extraction (SFE) (Van der Velde et al., 1992; Snyder et al, 1993; Ling and Liao, 1996), pressurized liquid extraction (PLE) (Mogadati et al., 1999) and microwave-assisted extraction (MAE), are very attractive because they are faster, use much smaller amounts of solvents and are environmentally friendly techniques.

SFE uses a solvent in its supercritical state. This technique is attractive because it is fast, uses small amounts of solvent and commercially available SFE systems allow sample extraction in unattended operations. PLE is done in a closed-vessel at elevated temperatures and pressures. The higher temperature at which the extraction is conducted increases the capacity of the solvent to solubilise the analyte, and the higher pressure increases the diffusion rate into the pores of the matrix, thus facilitating the mass transfer of the analyte into the extracting solvent.

A method based on the sonication of soil samples placed in small columns (sonication-assisted extraction in small columns, SAESC) has been developed for the rapid and sensitive analysis of herbicides and insecticides in soil (Sánchez-Brunete et al., 1998; Castro et al., 2001). In 2002, Sánchez-Brunete et al. published a method for the simultaneous determination of fungicides in soil. The method is simple and uses low volumes of ethyl acetate as extracting solvent, reducing the human exposure to toxic solvents and the environmental impact of the analytical procedure although they did not include BFs in their work (Sánchez-Brunete et al., 2002).

In the last few years, there has been an increase in the number of procedures using microwave energy to extract organic compounds from environmental matrices. Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvent in contact with a sample in order to obtain partition of analytes from the sample matrix into the solvent (Shu et al., 2003; Ramil Criado et al., 2003). In comparison with other conventional methodologies, such as Soxhlet extraction, MAE requires less energy, shorter analysis periods and the use of smaller solvent volume of analysis. Coscollá et al. (2009) developed a confirmatory and rapid procedure for extraction of different pesticides, including thiabendazole and carbendazim, in PM 2.5 particles by MAE using ethyl acetate. Recoveries ranged from 72 to 109% and the limit of quantification (LOQ) of 6.5 pg·m^{-3} were obtained for this method.

The use of micellar media as alternative to conventional organic solvents in the MAE procedure could offer important advantages such as safety, simplicity, lower toxicity, lower cost and greater compatibility with the aqueous organic mobile phase in the liquid chromatography (LC) separation process. This combination, called microwave-assisted micellar extraction (MAME) has been applied to the extraction of different compounds from solid matrices (Padrón-Sanz et al., 2002; Eiguren Fernández et al., 2001; Mahugo-Santana et al., 2004; Padrón Sanz et al., 2005). Among the advantages of the MAME are reduced volumes of extractant, low cost and reduced toxicity in comparison to organic solvents. Moreover, MAME reduce analysis time compared with conventional Soxhlet extraction.
In this sense, a MAME procedure for benomyl (BNM), carbendazim (MBC), thiabendazole (TBZ) and fuberidazole (FDZ) in soil samples was published by Halko et al. (2006). MAME extraction is influenced by different factors which must be controlled in order to obtain satisfactory results. The surfactant concentration, pH of solution, irradiation power and extraction time were studied and optimized using a factorial design. MAME using non-ionic surfactants, such as POLE and Genapol X-080 provides good extraction efficiency (between 71-105%) of the studied fungicides and LODs between 0.02-0.6 $\mu g \cdot g^{-1}$ for FDZ and TBZ and 25-30 $\mu g \cdot g^{-1}$ for MBC/BNM. MAME is an alternative not only to classical extraction such as Soxhlet extraction, but also to MAE.

4. Detection and determination methods

Numerous analytical methods have been reported for quantitation of benzimidazole fungicides in different matrices based on spectrophotometry (Chiba, 1977), fluorimetry (Zamora et al. 2003; Cuesta et al. 2003), phosphorimetry (Salinas y et al. 2005), electrochemical (Huebra et al. 2000), enzyme-linked immuno-sorbent assay (Itak et al. 1993), gas chromatography-mass spectrometry (Lesueur et al. 2008), although is routinely carried out by high performance liquid chromatography coupled with ultraviolet (Melo et al. 2005; Singh et al. 2007; Prousalis et al. 2004), fluorescence (Moral et al. 2006; Hu et al. 2008), and mass spectrometry (Radisic et al. 2009) detectors. These methods have many advantages of high sensitivity and accuracy and some of them have been adopted by regulatory agencies. In environmental samples, flow injection analysis (FIA) coupled with chemiluminescence (CL) is a well established technique for the ultra-trace analysis of a variety of compounds in diverse matrices using various CL reagents including benzimidazole fungicides (Fletcher et al. 2001). FIA-CL method for the determination of carbendazim has been reported (Liao and Xie 2006), which is based on the enhancement of CL reaction of luminol and hydrogen peroxide (H_2O_2) by a carbendazim in sodium hydroxide–sodium dihydrogen phosphate medium (pH 12.6). This method was applied to the determination of carbendazim in tap-water samples with a linear range of 0.02–2 mg mL^{-1} and a limit of detection of 7.24 ng mL^{-1}. Recently, a simple FIA-CL method for the determination of BFs (fuberidazole, benomyl, and carbendazim) was developed because CL emission was observed when the reaction of Cu^{+2} and H_2O_2 was carried out in an alkaline Na_2CO_3 solution without CL reagent. On the addition of a trace amount of BFs to a mixture of Cu^{+2}–H_2O_2–Na_2CO_3, CL emission was enhanced (Waseem et al. 2007, Waasem et al., 2010).
Gas chromatography (GC) determination of BFs is difficult because their thermal instability do not permit their analysis directly unless they are derived into thermally stable derivatives. However, TBZ and TCB are sufficiently volatile to allow their determination by GC without derivatisation. Some reseachers have found GC coupled to mass spectrometry

useful for confirmation of the presence of benzimidazole residues. However, GC–MS procedures usually require derivatisation of residues to induce volatility and allow the generation of suitable MS spectra for confirmatory analysis and, as a result, have been largely been replaced by LC–MS/MS. Some quantitative GC methods have been developed by some researchers to determine the presence of benzimidazole fungicides in different matrices including soils (Castro et al., 2001; Leuseur et al., 2008).

4.1 Liquid chromatography based detection systems

4.1.1 Detection by UV and fluorescence.

Spectrometric methods are suitable for quantitation of high levels of benzimidazole residues in different matrices. However, when quantitation of benzimidazoles in the low $\mu g \cdot kg^{-1}$ range and greater selectivity is required, chromatographic separation of residues prior to spectrometric detection is generally required.

To analyze the growing number of modern pesticides and their degradation products, which are generally thermolabile, polar and non-volatile, the analytical methods employing gas chromatography in combination with specific detectors or coupled with mass spectrometry (MS) are not reliable without a time-consuming derivatization step, which itself can generate interferences. Consequently, liquid chromatography (LC) combined with diode array detection (DAD) has been used increasingly in recent years as a complementary method for pesticide analysis.

Benzimidazole fungicides like albendazole (ABZ), benomyl (BNM), carbendazim (MBC), fenbendazole (FBZ), fuberidazole (FDZ) and thiabendazole (TBZ) have been used from Cacho et al. (2009) to optimise a MISPE method coupled to HPLC-UV. These compounds were monitored at 200 and 240 nm. LODs obtained were between 0.03-0.09 $ng \cdot mL^{-1}$ depending of the compound. The obtained detection limits were below the legislated MCL in all cases.

Zamora et al. (2009) coupled the molecularly imprinted solid-phase extraction (MISPE) procedure to a HPLC-UV system to determine benomyl (BNM), carbendazim (MBC) and thiabendazole (TBZ) in river, tap and well waters. LODs obtained were between 2.5-5.0 $ng \cdot L^{-1}$ depending of the studied compound.

A number of researchers have developed different methods for determination of benzimidazole residues using HPLC coupled to fluorescence detection. Fluorescence detection offer sensitivity and selectivity to methods, potentially reducing the need for extensive sample clean-up.

Moral et al. (2006) used supramolecular sorbents for the extraction/preconcentration of BFs: carbendazim (CBZ), thiabendazole (TBZ) and fuberidazole (FDZ), and to evaluate their applicability for the analysis of natural waters. Same authors optimized supramolecular solvent–based extraction and the fluorescence measurements were performed at 286/320 (time = 0–9 min) and 300/350 nm (time 9–15 min) excitation/emission wavelengths, respectively. These wavelengths were selected on the basis of the excitation and emission spectra obtained for each of the target analytes solubilized in the mobile phase. Obtained detection limits were 0.032 $ng\ mL^{-1}$ for CBZ, 0.004 $ng \cdot mL^{-1}$ for TBZ and 0.0001 $ng\ mL^{-1}$ for FBZ (Moral et al., 2009).

Wu et al. (2009) used a HPLC-fluorescence detection for the determination of carbendazim (MBC) and thiabendazole (TBZ) in water and soil samples obtaining good results. For water samples, LODs were 0.5 $ng \cdot mL^{-1}$ for MBC and 1.0 $ng \cdot mL^{-1}$ for TBZ. In the case of the soil samples, LODs were 1.0 $ng \cdot g^{-1}$ for MBC and 1.6 $ng \cdot g^{-1}$ for TBZ.

LC-fluorescence could be applied as an alternative technique to allow determination and confirmation of selected benzimidazole residues. However, not all benzimidazole residues fluoresce naturally. For that, other methods like LC–MS for determination and confirmation of BFs will be reviewed for environmental samples.

4.1.2 Detection by mass-spectrometry.

In the last years, liquid chromatography–mass spectrometry (LC–MS) techniques have advanced in their sensitivity, specificity and reliability. Progress is mostly due to development of hyphenated LC–tandem MS techniques, which are today the methods of choice for the determination of trace organic analytes in environmental samples. Swift growth in the use of LC–MS/MS for the analysis of organic contaminants in environmental matrices has been compelled by the need for high-quality data on their occurrence in the environment at very low concentration levels (Petrovic et al., 2010). Application of tandem mass spectrometry (MS/MS) has resulted in improved determination and confirmation of polar compounds in different environmental matrices.

LC should preferably be applied for the analysis of organic micropollutants only when using tandem MS because this combination is able to produce fragment ions that are necessary for the explicit identification of the analytes (Reddersen & Heberer 2003). LC–MS/MS allows separation and detection of compounds having the same molecular mass but different product ions, even if they co-elute. MS/MS detection is therefore preferred for increased analytical sensitivity and selectivity in complex water matrices (Díaz-Cruz & Barceló 2005). The most commonly used LC–MS/MS interfaces are atmospheric pressure ionization technologies, such as ESI and atmospheric pressure chemical ionization (APCI). ESI is well suited for the analysis of polar compounds, whereas APCI is very effective in the analysis of medium-polarity and low-polarity substances (Radjenovic et al. 2007). Optimization of MS parameters, including cone voltage and collision energy, is performed via flow injection analysis for each compound of interest (Gros et al. 2006; Baugros et al. 2008).

As discussed above, the majority of the analytical procedures published for determining benzimidazoles have been developed based on high performance liquid chromatography (HPLC) because of the polar nature (zwitterion) and thermal lability. In recent years, HPLC-based method combined with sensitive mass spectrometric detection (LC–MS) or versatile tandem mass spectrometry (LC–MS/MS) amenable to multiple reaction monitoring (MRM) has become the analytical tool of choice for simultaneous sensitive quantification and confirmation of a wide range of target fungicides and the structurally related metabolites in complex matrices (Wang et al., 2007; Economu et al., 2009).

Balizs (1999) described the determination of benzimidazole residues including TBZ, ABZ, MBZ, FDZ, OFZ and OXI using LC-MS and LC-MS/MS optimizing, initially, by direct infusion of a standard solution of each benzimidazole. After that, BFs residues were separated using ammonium acetate and acetic acid in the mobile phase to produce protonated molecular ions. Obtained mass spectra were simple and consisted of a few fragments of the protonated molecular ion $[M+H]^+$. Guo et al. (2010) included BNM, MBC and FDZ to develop their multi-residue procedure for effective extraction of BFs for analysis by LC-MS/MS. Table 2 shows the individual precursor to product ion transitions specific to different analytes including precursor ion (Q_1) and product ion (Q_3) obtained for these authors. These methods were applied in muscle, eggs and agricultural products. Balizs (1999) obtained detection limits between 3 and 20 $\mu g \cdot kg^{-1}$. Gou et al. (2001) improved these

LODs because they used a QuEChERs process (salting-out liquid-liquid partitioning extraction followed by dispersive solid phase extraction), obtaining values between 0.15-0.30 ng $\cdot g^{-1}$.

Compound	Q_1	Q_3
ABZ	266	233
BNM	291	192
MBC	192	132
FBZ	300	268
FDZ	185	92
MBZ	296	264
OXI	250	176.1
OFZ	316.2	159.1
TCB	359	343
TBZ	202	131

Table 2. Typical ions of the BFs in LC-MS and LC-MS/MS (*from Balizs (1999) and Gou et al. (2010)).*

To our knowledge, there not papers exclusively related with determination of BFs in environmental samples using LC-MS/MS. However, there are some published multiresidue methods that include benzimidazole compounds. For example, Jeannot et al. (2000) used LC-MS for the multi-residue analysis of pesticides in surface waters among those included CBZ. Limit of detection for CBZ was 6 ng $\cdot L^{-1}$.

It is important to remember that the analysis is complicated by the instability of several benzimidazoles during analytical processes. The accepted methods have been well documented for measuring BNM or/and tiophanate-methyl as MBC or other breakdown products (Di Mucio et al., 1995; Singh et al., 2007), which entails a complete or quantitative conversion of the parent molecule to its stable metabolite prior to analysis. The major disadvantage of such a total residue approach is the conversion into other transformation products, which might also be marker species already present as their natural occurrence in the sample (Danaher et al., 2007). To overcome this problem, successive efforts have been made in developing methodology for direct determination of BNM or other benzimidazoles like tiophanate-methyl in the intact form, by addition of decomposition retardants (Chiba et al., 1977), or by virtue of the enhanced stabilities in strongly acidic aqueous media or at low temperatures (García Reyes et al., 2004; Cacho et al., 2009).

4.2 Other methods

Bioassays have been used to detect benzimidazole residues in food but are more routinely used to evaluate the potency of anthelmintic substances. In the other hand, immunochemical technologies offer a number of advantages in environmental analysis (e.g., reduced time of analysis, low limits of detection (LODs) with sensitivity in many cases comparable to conventional chromatographic techniques, high throughput of samples, cost-effective detection, and adaptability to field use). However, one of the most important advantages is their possible automation that can be adapted to work remotely, on-line or at-site (Farré et al., 2007).

Within the most representative immunochemical technologies applied to environmental analysis are enzyme-based immunoassays which usually use a change in color or emission

of light to measure the concentration of the analyte. This technique offers numerous advantages over other immuno-techniques because their signal is amplified by forming a great number of product molecules and they are widely used, especially those based on heterogeneous conditions, such as enzyme-linked immunosorbent assays (ELISAs). Although there have been a number of significant applications of ELISA technology in the area of benzimidazole analysis in biological matrices, there not significant works related with environmental samples. However, Thomas et al. (1996) developed a method that incorporates many of the advantages of immunochemical techniques into a format more suitable for routine analytical laboratories. It is high-performance immunoaffinity chromatography (HPIAC). In HPIAC, the antibody is immobilized onto a high-performance liquid chromatographic support and used as an affinity ligand to extract the target analyte from an aqueous sample injected onto the HPIAC column. Any material not specifically recognized by the antibody passes through the column to waste, while the target analyte remains bound to the immobilized antibody until the mobile phase conditions are changed to disrupt the antibody analyte interaction. The analyte is subsequently eluted and detected. These authors described the application of HPIAC coupled on-line with either HPLC-DAD or HPLC-MS to determine the fungicide carbendazim in water samples and they compared the obtained results with the ELISA technique. LODs were better with HPIAC methods (0.025 ng·mL^{-1} for HPLC-MS and 0.075 ng·mL^{-1} for HPLC-DAD) than ELISA method (0.10 ng·mL^{-1}).

Table 3 presents a summary of the most representative methods to extract and determine BFs in terms of the sample matrix and analytical parameters.

Analytes	Samples	Extraction	Determination tecnique	Analytical Parameters	Ref.
CBZ	soil	-	HPLC-UV/Vis	RSDs: 4.5% LODs: 0.3 µg·mL^{-1}	Huebra et al. (2000)
CBZ	water	SLM, MMLLE	HPLC-UV	RSDs: < 5% LODs: 0.1 – 0.5 µgL^{-1}	Sandahl et al. (2000)
CBZ, FDZ, TBZ	water	SPE	cross-section fluorimetry	Recovery: 90-120% RSDs: 1.5 – 4.8 % LODs: 0.017 – 0.29 ng·mL^{-1}	Zamora et al. (2000)
CBZ, FDZ, TBZ	water	SPE	spectrofluorimetry	Recovery: 91-115% RSDs: 0.02-4.0 % LODs: 0.1-100 µg·L^{-1}	Garrido et al. (2002)
CBZ, FDZ, TBZ	water	SPE	luminescence spectrometer	Recovery: 97-109% RSDs: 3.3-6.4% LODs: 0.001-0.13 µg·L^{-1}	Zamora et al. (2002)
CBZ, BNM, FDZ, TBZ	water	CPE	HPLC-Fluorescence	Recovery: 70-96 % LODs: 0.01-6 ng·mL^{-1}	Halko et al. (2004)

Analytes	Samples	Extraction	Determination tecnique	Analytical Parameters	Ref.
FDZ	water	-	fluorescence spectrophosphorimeter	RSDs: 3.1% LODs: 95 ng ·L^{-1}	Salinas et al. (2005)
CBZ, BNM, FDZ, TBZ	soil	MAME	HPLC-Fluorescence	Recovery: 70-105 % LODs: 0.02-0.06 µg ·g^{-1} 25-30 µg ·g^{-1} (MBC/BNM)	Halko et al. (2006)
CBZ	soil	ultrasonic solvent extraction, QuECheRS, PLE	GC-MS HPLC-MS/MS	RSDs: <20 % LODs: 0.01 ng ·g^{-1}	Lesueur et al. (2007)
CBZ, BNM, FDZ, TBZ	water	SPME	HPLC-Fluorescence	RSDs: <9.0 % LODs: 0.03-1.3 ng ·mL^{-1}	López Monzón et al. (2007)
ABZ, BNM, FBZ, FDZ, CBZ, TBZ	spiked river, tap and well water	MISPE	HPLC-DAD	Recovery: 89-105% RSDs: 2.1-6.7% LODs: 0.03-0.09 µg ·L^{-1}	Cacho et al. (2009)
CBZ, TBZ	water, soil	LLME	HPLC-Fluorescence	Recovery: 50.8-70.9% RSDs: 3.5 -6.8% LODs: 0.5–1.0 µg ·L^{-1}; 1.0–1.6 ng ·g^{-1}	Wu et al. (2009)
CBZ, FDZ, TBZ	river, underground water	supramolecular solid-phase extraction	HPLC-Fluorescence	RSDs: 2-6 % LODs: 0.1-32 ng ·L^{-1}	Moral et al. (2009)
BNM, CBZ, TBZ	water	MISPE online	HPLC-DAD	Recovery: 87-95% RSDs: <5% LODs: 2.3–5.7 ng ·L^{-1}	Zamora et al. (2009)

Abbreviations: RSD: Relative standard deviation, LOD: limit of detection, LC: liquid chromatography, GC: Gas chromatography, HPLC: high performance liquid chromatography, UV: Ultraviolet visible detection, PDA: Photodiodearray detection, MS: mass spectrometry, APCI: atmospheric pressure chemical ionisation, MIP: molecularly imprinted polymer, MISPE: Molecularly imprinted solid-phase extraction, ELISA: enzyme linked immunoassays, SPE: solid phase extraction; LLME: liquid-liquid microextraction; SPME: solid phase microextraction; MAME: Micellar assisted microwave extraction; CPE: cloud point extraction.

Table 3. Most representative methods to determine benzimidazole fungicides (BFs) in environmental samples.

5. Conclusions and future perspectives.

This chapter describes the most relevant aspects which are involved in the determination of benzimidazole fungicides (BFs) in environmental samples. It is well known that sample preparation is one of the most critical steps in the determination of trace pollutants in different environmental matrices. In order to improve the quality of the analytical methods used, sample preparation techniques should be improved for this proposal. On the other hand, although some of the techniques presented in this overview are known and have been applied (HPLC with UV and/or fluoresence detection) to determine BFs in environmental samples, trends in this field should develop multi-residue methodology that allows the determination of the complete range of benzimidazole fungicides (BFs) in different environmental matrices in a single analysis. In this sense, advanced analytical methods have been developed and optimized with the aim of improving precision and sensitivity. LC–MS and LC-MS/MS have found more widespread application in environmental analysis offering more sensitive detection and increased confidence in reporting results. It should be desirable the development of specific methods for BFs in environmental samples using LC–MS/MS combined with new sample treatments to apply to real environmental samples. Alternatively, HPLC coupled to UV and fluorescence detection in series may offer a low cost to LC–MS/MS and may be particularly effective in the optimization of sample treatments.

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16

Propiconazole Toxicity on the Non-Target Organism, the Arbuscular Mycorrhizal Fungus, *Glomus irregulare*

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that colonize the roots of most terrestrial plants. Indeed, 80% of vegetal species realize this symbiosis (Bonfante and Perotto, 1995). Plants generally benefit from this AMF association through increased plant nutrient uptake, plant growth and survival rates (Smith and Read, 2008). The symbiotic association may also increase host plant resistance/tolerance against biotic (Hol and Cook, 2005; Akhtar and Siddiqui, 2008) and abiotic stresses, including salinity, drought and pollution (Gerdemann, 1968; Franco-Ramirez et al., 2007; Giri et al., 2007; Sudova et al., 2007; Cartmill et al., 2008; Debiane et al., 2008, 2009; Campagnac et al., 2010).

The functioning of AMF may be impaired by cultural practices such as fungicides application (Sukarno et al., 2006). Unfortunately, the use of fungicides is generalized in modern agriculture for the control of fungal diseases. Most of fungicides act directly on essential fungal functions such as respiration, lipid synthesis or cell division (Leroux, 2003). Consequently, they can exhibit undesirable effects on non-target organisms. Among the fungicides, the Sterol Biosynthesis Inhibitor (SBI) family is one of the most used in agriculture (Hewitt, 1998). Four main classes can be distinguished according to their action target site: (i) squalene epoxidation (e.g. naftifine, terbinafine, tolnaftate), (ii) Δ14 demethylation or DMIs (e.g. imazalil, prochloraz, triadimenol, propiconazole), (iii) Δ14-reduction and/or Δ8→ Δ7 isomerisation (e.g. fenpropidine, fenpropimorph, tridemorph), (iiii) C4 demethylation (e.g. fenhexamid) (Leroux, 2003).

Several studies carried out on SBI fungicide impact on mycorrhizal plants showed contradictory results on the plant growth, on AM fungal development and on the symbiosis functioning (Dodd and Jeffries, 1989; Von Alten et al., 1993; Schweiger and Jacobsen, 1998; Kjoller and Rosendahl, 2000; Schweiger et al., 2001). The use of different experimental procedure in the reported studies (plant species, culture conditions, fungicide formulation, application methods…) did not allow easy comparison with the results obtained and led to some difficulties to give clear conclusion concerning the SBI fungicides effect on AMF (Sancholle et al., 2001).

Monoxenic culture technique has improved noticeably the understanding of the symbiosis (Declerck et al., 2005). The *in vitro* cultures allow non-destructive observations of AMF (Fortin et al., 2002), they are standardized and reproducible method enabling comparisons of various molecules impact on AMF (Debiane et al., 2008, 2009; Hillis et al., 2008; Zocco et al., 2008; Campagnac et al., 2010). In addition, thanks to these monoxenic cultures, big amounts of biological material free of contaminant microorganisms can be obtained.

Zocco et al. (2008) and Campagnac et al. (2008, 2009, 2010) studied the effects of two SBI fungicides, i.e. fenpropimorph and fenhexamid on AMF and root development. The fungitoxicity of fenpropimorph on the development of *Glomus intraradices* was shown by a reduced fungal development, a decrease of sterol content and the increase of a precursor, the squalene suggesting inhibition of an unusual target enzyme in AMF, the squalene epoxidase (Campagnac et al., 2009). Fenpropimorph was also underscored by the significant reduction in root growth and appeared to be due to (i) the replacement of the normal Δ5-sterols by unusual sterols: 9β,19-cyclopropylsterols, Δ8,14-stérols, Δ8-sterols and Δ7-sterols (ii) and the induction of an oxidative stress (lipid peroxidation, antioxidant enzyme activities). However no impact on the sterol profile and root development was detected with fenhexamid (Campagnac et al., 2008), while a significant oxidative stress was highlighted in roots (Campagnac et al., 2010).

Indeed, when plants are subjected to environmental stresses, as SBI fungicides, reactive oxygen species (ROS) are produced, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), alkoxy radical (RO) and the singlet oxygen ($^1O_2$) (Elstner, 1982). The ROS may initiate destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids (Herbinger et al., 2002). As a consequence, higher plants induce efficient antioxidant systems to protect them against oxidative injury (Asada, 1999). The antioxidant systems consist of antioxidant enzymes including superoxide dismustase, peroxidase (POD), catalase, glutathione reductase and non-enzymatic antioxidants including ascorbate and glutathione which are designed to minimize the concentrations of $O_2^{\cdot-}$ and H_2O_2. Nevertheless, antioxidant capacity is dependant on the severity of the stress, on the species and/or on the stage of development (Dat et al., 2000). One of the most damaging oxidative effects is the peroxidation of membrane lipids, which results in the concomitant production of malondialdehyde (MDA), a secondary end product of polyunsaturated fatty acids (FA) oxidation (Hodges et al., 1999; Cho and Park, 2000; Jouili and El Ferjani, 2003). As a response to environmental stress, cells can modify their membrane lipid composition in order to maintain optimal physical properties (Thompson, 1992). The regulation of the lipid composition and the adjustment of the unsaturation level of membrane FA are extremely important to deal with pollutant toxicity and make a contribution to plant survival (Thompson, 1992; Chaffai et al., 2005; Bidar et al., 2008). Modifications in the properties of cellular membranes occur to ensure the function of processes that take within them, and lead to improve growth involving to plant adaptation. Alteration in membrane phospholipids (PL) caused by lipid peroxidation which led to high MDA level is a useful biological marker to highlight the occurrence of oxidative stress conditions (Gallego et al., 1996; Hodges et al., 1999). Whereas a number of studies concerned plant oxidative stress, no study was carried out on the oxidative stress induced by environmental abiotic stress as SBI fungicides on AMF, a beneficial fungus.

In the present work, propiconazole, a SBI belonging to the second group of fungicides (DMIs) and commonly used against mildews and rusts on cereals, was studied.

Propiconazole is a fungicide that inhibits the biosynthesis of ergosterol leading to a decrease capacity of the fungus to maintain normal membrane processes (Köller, 1992). Indeed, in target-fungus, as *Nectria haematococca*, growth inhibition observed with SBI fungicides was correlated with ergosterol biosynthesis inhibition, which led to ergosterol decrease and the accumulation of abnormal or precursor sterols (Debieu et al., 1998). On *Taphrina deformans*, a phytopathogenic fungus, growth inhibition results from an insufficient amount of functional sterol, accumulation of C-14 methyl sterol, or perhaps a combination of both responses to treatment with such an inhibitor (i.e. propiconazole) (Weete et al., 1985).
As propiconazole is a persistent fungicide in environment (half-life estimated to about to 214 days), repeated pulverisations of this SBI could provoke its accumulation in soils (Bromilow et al., 1999). All azole derivative fungicides are directed against cytochrome P450 enzymes. They can have multiple inhibition sites such as the inhibition of sterol formation by blocking 14α-demethylation, gibberellin-biosynthesis or brassinosteroid biosynthesis. Indeed these different metabolims which have many steps thought to be catalyzed by cytochrome P450 monooxygenases (Lupetti et al., 2002; Sekimata et al., 2002). Unfortunately, it could also affect non-target organisms such as plants. Propiconazole phytotoxicity was observed on several occasions (He et al., 1995; Hanson et al., 2003; Meksem, 2007). It seemed that SBI induce perturbations to the hormonal level (gibberellins synthesis) (Rademacher, 1991a,b) and to the photosynthesis level (Gopi et al., 2005). Inhibiting root development, propiconazole could thus have an indirect impact on mycorrhizal fungal development. However, little is known about its potential effect on non-target plant-beneficial fungi such as AMF.
This work was thus focused on investigation of the SBI molecule, propiconazole, toxicity on *Glomus irregulare* development. Moreover, whereas the studies on SBI effect on fungi concerned usually sterol metabolism, oxidative stress was less commonly studied. It is why, we used the monoxenic cultures in order to link up the direct impact of propiconazole, on the AMF *Glomus irregulare* development with the oxidative stress by analysing FA, PL, their associated FA (PLFA) compositions and contents and by measuring malondialdehyde (MDA) production as well as peroxidase (POD) specific activity.

2. Experimental

2.1 Root and fungal growth conditions

The inoculum consisted of Ri T-DNA-transformed chicory roots (*Cichorium intybus* L.) colonized by the AMF *Glomus irregulare*. The AMF used was *Glomus sp.* DAOM 197198 formerly identified as *Glomus intraradices* Schenck and Smith and presently reclassified in a clade that contains the recently described species *Glomus irregulare* Blaszk., Wubet, Renker, and Buscot (Stockinger et al., 2009; Sokolski et al., 2010).
Monoxenic cultures were grown in mono-compartmental Petri dishes (9 cm) on medium MSR (Declerck et al., (1998) modified from Strullu and Romand (1986)), solidified with 0.25% (w:v) gellan gel (Phytagel: Sigma, St Louis, MO, USA) without fungicide (control) or amended with different concentrations of propiconazole. A piece of mycorrhizal transformed chicory root was added on medium and the dishes were placed at 27°C in the dark in an inverted position.
Glomus irregulare cultures were established in bi-compartmental Petri dishes (9 cm) with a watertight plastic wall separating the root compartment (RC) from the fungal compartment (FC) (St Arnaud et al., 1996). RC was filled with 25 mL MSR medium without propiconazole and a piece of mycorrhizal transformed chicory roots was added on medium. After 3 weeks,

FC was filled with 30 mL of MSR medium with or without fungicide (control). This compartment was kept root free (by cutting) and used to investigate the treatment effect on extraradical hyphae development in FC. The dishes were also incubated at 27°C in the dark in an inverted position.
For the spore germination test, 50 dishes (5.5 cm diameter) containing 15 mL of MSR medium with or without fungicide (control) were prepared. Spores were extracted from an agar piece containing *Glomus irregulare* spores. This piece of agar was fine-cut to separate spores from hyphae and to facilitate gelose dissolution with Tris buffer (Tris-HCl 50 mM, pH 7.5, EDTA 10 mM). Spores were taken and poured in each 50 dish by treatment (one spore by dish) and dishes were incubated as previously during 30 days.

2.2 Propiconazole treatment

Propiconazole (technical grade) was supplied by Syngenta (Swiss). To prepare fungicide-enriched medium, active matter of propiconazole was dissolved in acetone (0.5 $mL.L^{-1}$ medium), and added to sterilized (121°C for 15 min) MSR medium (80°C) in order to obtain final concentrations of 0.02; 0.2 and 2 $mg.L^{-1}$ of SBI fungicide. The bottles were agitated by hand and the medium was poured into standard mono or bi-compartmental Petri dish (9 cm). Control treatment, containing MSR medium without propiconazole, was supplemented with the same volume of acetone for the mono-compartmental and the FC of the bi-compartmental Petri dishes.

2.3 Fungal growth evaluation

2.3.1 Spore germination

Spore germination on MSR medium with or without (control) propiconazole at 0.02; 0.2 and 2 $mg.L^{-1}$ was followed with low power microscope at 10–40x magnification at 2, 4, 6, 8, 15, 22 and 30 days, to determine the germination percentage of *Glomus irregulare* spores for each treatment. Spores are considered as germinated when a germ tube appears.
Germination rate = Germinated spore number / Total spore number * 100.

2.3.2 Colonization rate

The medium of colonized root cultivated in mono-compartmental Petri dish was solubilized in 1 vol of Tris buffer (Tris–HCl 50 mM, pH 7.5, EDTA 10 mM). The roots were collected by filtration, rinsed with sterile water, cleared in KOH 10% for 1h at 70°C and stained with Trypan Blue (Trypan Blue 0,5 g in 32:32:467 of water:glycerol:lactic acid (v:v:v) for 1h at 70°C (Phillips and Hayman, 1970) to quantify root colonization by *Glomus irregulare*, with the method of McGonigle et al. (1990).

2.3.3 Hyphal length

After 9 weeks of incubation in the mono-compartmental Petri dish, *Glomus irregulare* hyphal lengths were measured under a low power microscope at 10-40x magnification using a gridline intersects technique described by Declerck et al. (2001) and data were integrated using Newman's formula (1966): $L = (\Pi * N * A) / (2 * H)$ (L= hyphal length; N= intersection number; A= gridline surface; H= gridline length sum)
With a little gridline, germ tube resulted from germinated spores were measured as previously after 30 days of incubation.

2.3.4 Spore number

After 9 weeks of incubation with chicory roots, number of spores of *Glomus irregulare* was determined by addition of the number of spores in each 1 cm grid of the gridline used for hyphal length.

2.4 Fatty acid (FA) extraction and analysis

After incubation, *Glomus irregulare* medium was solubilised under agitation in 1 vol Tris buffer (Tris–HCl 50 mM, pH 7.5, EDTA 10 mM) and the fungal mycelium was collected by filtration on a 53 µm sieve, rinsed with sterile water and lyophilized for 48h. Lipids extraction was performed on 3 to 15 mg *Glomus irregulare* dry weight. The material was saponified with 3 mL of 6% (w:v) in methanolic KOH at 70°C for 2h. After addition of 1 vol of distilled water, the saponifiable fractions were extracted three times with 5 vol of hexane and evaporated under N_2. FA were methylated using 3 mL of BF_3:methanol (14%) at 70°C for 3 min, and reaction was stopped in ice. FA methyl esters were extracted three times with 5 vol of hexane after the addition of 1 mL of distilled water. These extracts were evaporated under N_2 and transferred to chromatography vials. FA methyl esters were analysed as described in Campagnac et al. (2010) using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionisation detector (Norwalk, CT, USA) and a ECTM- 1000 (Alltech Associates Inc., Deerfield, IL, USA) capillary column (30 m x 0.53 mm i.d.) with hydrogen as carrier gas (3.6 mL.min^{-1}). The temperature programme included a fast rise from 50°C to 150°C at 15°C.min^{-1} and then a rise from 150°C to 220°C at 5°C.min^{-1}. FA were quantified using heptadecanoic acid methyl ester (C17:0) as an internal standard. Their identification relied on the retention times of a wide range of standards (Sigma-Aldrich).

2.5 Phospholipid (PL) extraction and analysis

A second part of fungal samples collected was used to extract PL. PL extraction was carried out as described by Avalli and Contarini (2005). Lyophilized *Glomus irregulare* samples (4 to 14 mg) were dissolved in 20 mL of dichloromethane:methanol (2:1, v:v) at 75°C during 2h to collect all lipid fractions. After filtration and concentration under N_2, lipid fractions were collected in 2 mL of chloroform and applied to Solid Phase Extraction (SPE) cartridges. A silica gel bonded column (GracePure 3 mL volume, 500 mg sorbents, Grace Davidson Discovery Sciences, Alltech, Deerfield, USA) was used. After conditioning with 6 mL of hexane and 3 mL of chloroform:2-propanol (2:1, v:v), lipid fractions were added on the column. Lipid fractions were eluted with 6 mL of the precedent solvent. FA fraction was eluted first with 6 mL of 2% acetic acid in diethyl ether in order to separate them from the PL fractions. Total PL were then collected using two different conditions: (1) with 6 mL of methanol to collect the first part of PL contained in the sample and (2) with 6 mL of chloroform:methanol:H_2O (3:5:2, v:v:v) to recover the rest of phosphatidylcholine which is not totally eluted in the first condition. The recovered fraction was dried under N_2 and was re-dissolved in 0.2 mL of chloroform. A first part (0.1 mL) was injected into HPLC system, the second part (0.1 mL) was collected to extract phospholipid fatty acids (PLFA).

HPLC-ELSD analysis was carried out using an HPLC Waters 600 Controller (Meadows Instrumentation Inc, Bristol, UK) instrument with an automatic injector. A silica analytical column (150 mm x 3.0 mm, i.d. 3 µm) (Alltech, Deerfield, USA) was used. The chromatographic separation was carried out using a linear tertiary gradient according to the following scheme: *t*0 min: 58%A, 40%B and 2%C; *t*7 min: 52%A, 40%B and 8%C and finally *t*22 min: 58%A, 40%B and 2%C. Total chromatography run time 27 min per sample. Eluent

A consisted of 100% isopropanol, eluent B of 100% hexane and eluent C 100% H_2O. The flow rate of the eluent was 1.5 mL.min^{-1}. An Alltech (Deerfield, USA) model 3300 ELSD was used; the pressure of N_2 was maintained at 6 bars and the drift tube temperature was set at 40°C.

2.6 Phospholipid fatty acid (PLFA) extraction and analysis

PLFA collected previously (0.1 mL) were extracted according to the method described above for the total FA extraction and analysis.

2.7 Determination of malondialdehyde (MDA) concentration

A third part of fungal samples collected was used to determine MDA production. To prepare crude cell-free extracts, frozen samples (8 to 30 mg) were ground in mortar with liquid nitrogen. Samples were then suspended in 1 mL of phosphate buffered saline (10 mM). After centrifugation (3 min/10000g), supernatants were divided in 250 µL-aliquots and supplemented with (2,6 di-tert-butyl-4-methylphenol at 2.5 g.L^{-1} ethanol) to determine MDA, peroxidase and proteins. A high performance liquid chromatography MDA assay was used to evaluate the MDA production as described by Debiane et al. (2008). Tetraethoxypropane was used as the standard, and thiobarbituric acid (TBA) as the reagent. 100 µL of either standard solutions or methanol extracts were injected in the HPLC system and the MDA-TBA adducts were detected.

2.8 Determination of peroxidase (POD) activity

One of 250 µL-aliquots collected previously was used to quantify peroxidase (POD) activity. It was measured in supernatants using a commercial available reagent kit as the method described by Mitchell et al. (1994).

2.9 Statistical analysis

Data (spore germination, hyphal length, sporulation, colonization rate, FA contents and percentage, MDA concentration and POD activity) from different concentrations of propiconazole (0; 0.02; 0.2 and 2 mg.L^{-1}) exposed fungus were compared. The means were obtained from five replicates. ANOVA was carried out using Statgraphics release 5.1 (Manugistic, Inc., Rockville, MD, USA). The method used to discriminate between the means was the LSD test ($p<0.05$). Levene's test of variance homogeneity was checked before the use of the multiple comparison procedure. Data of colonization percentages and data of FA percentages were converted to arcsine values before the analysis of ANOVA and LSD test.

3. Results

3.1 Propiconazole decreased drastically *Glomus irregulare* development

3.1.1 Impact of propiconazole on *Glomus irregulare* spore germination

The impact of propiconazole on *Glomus irregulare* spore germination was measured at 2, 4, 6, 8, 15, 22 and 30 days in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}). The obtained spore germination kinetics are presented in Fig. 1. At 2 mg.L^{-1} propiconazole concentration, spore germination was drastically decreased. The reduction was estimated to about 37%, by comparison to the control. Whereas, in the absence of propiconazole (control), maximum of *Glomus irregulare* germination (92%) was reached at day 15, in the presence of 2 mg.L^{-1} of propiconazole, only 58% germination was obtained at the end of the experiment (after 30 days).

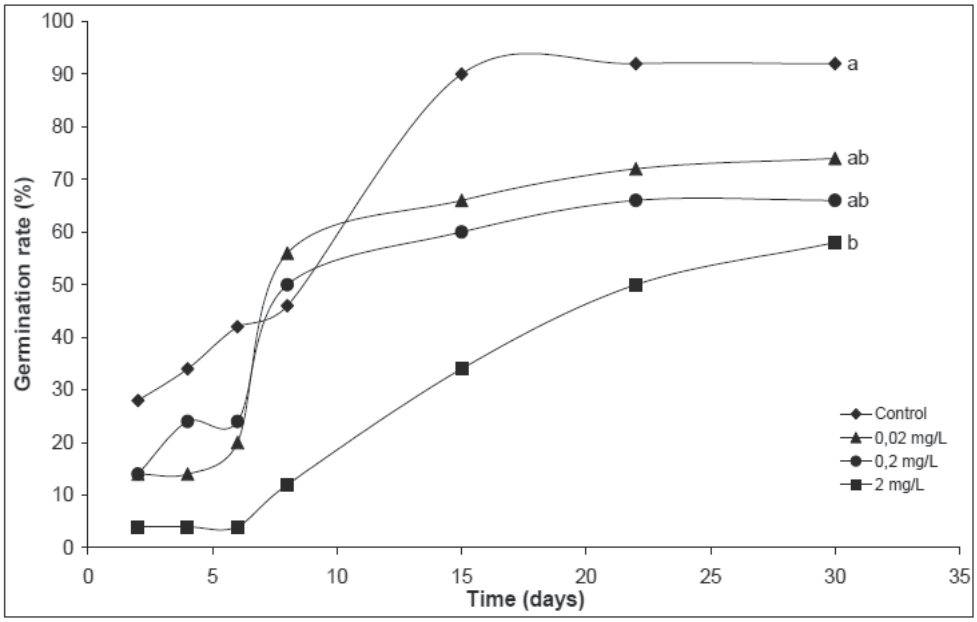

Fig. 1. Spore germination of *Glomus irregulare* at 2, 4, 6, 8, 15, 22 and 30 days in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}). Data are presented as means. The means were obtained from 50 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

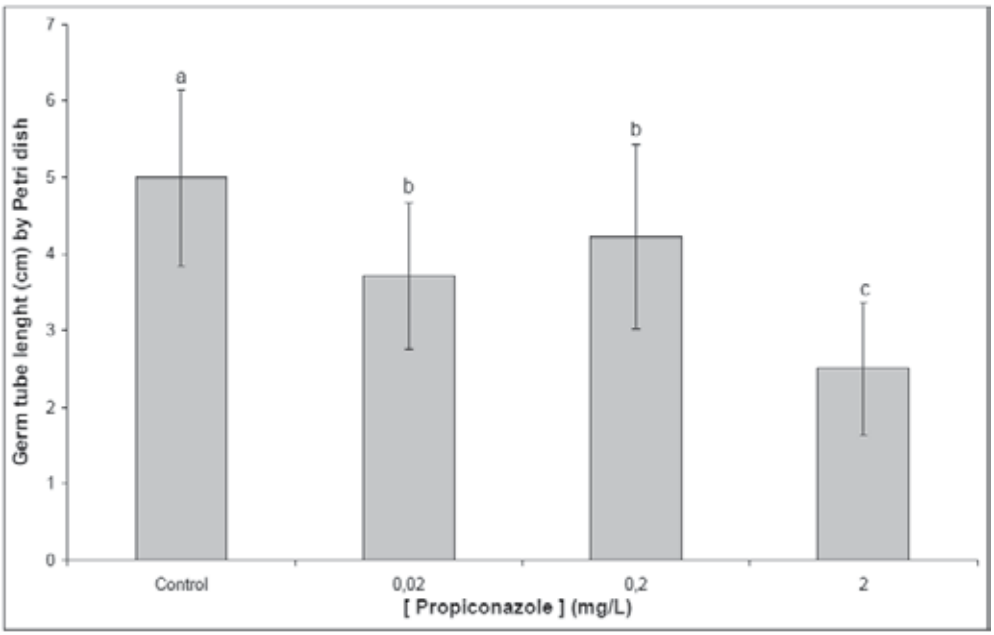

Fig. 2. Germ tube length of *Glomus irregulare* spore after 30 days of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}).

Data are presented as means ± SD. The means were obtained from 22 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

Germ tube lengths of germinative spore grown after 30 days, in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 $mg.L^{-1}$) are shown in Fig. 2. The length of germ tube which germinated in the absence of propiconazole reached 5 cm. Propiconazole reduced significantly germinative hyphal length from the concentration of 0.02 $mg.L^{-1}$. A significant decrease in the germ tube length was observed at the highest concentration of the fungicide and was estimated to about 3 cm.

3.1.2 Impact of propiconazole on root colonization by *Glomus irregulare*

The chicory root colonization by *Glomus irregulare* after 9 weeks of incubation in the absence (control) and in the presence of increasing propiconazole concentrations (0.02; 0.2 and 2 $mg.L^{-1}$) is shown in Fig. 3. The percentage of mycorrhization in chicory roots by *Glomus irregulare* grown without fungicide (control) reached 75% for total colonization, 34% for arbuscules and 36% for vesicles. The total colonization of the chicory roots was significantly decreased in the presence of propiconazole as compared to the control. They were estimated about 59% and 40% respectively at 0.2 and 2 $mg.L^{-1}$. The arbuscules, also, were significantly reduced by the fungicide. The decreases were about 48% and 68% as compared to the

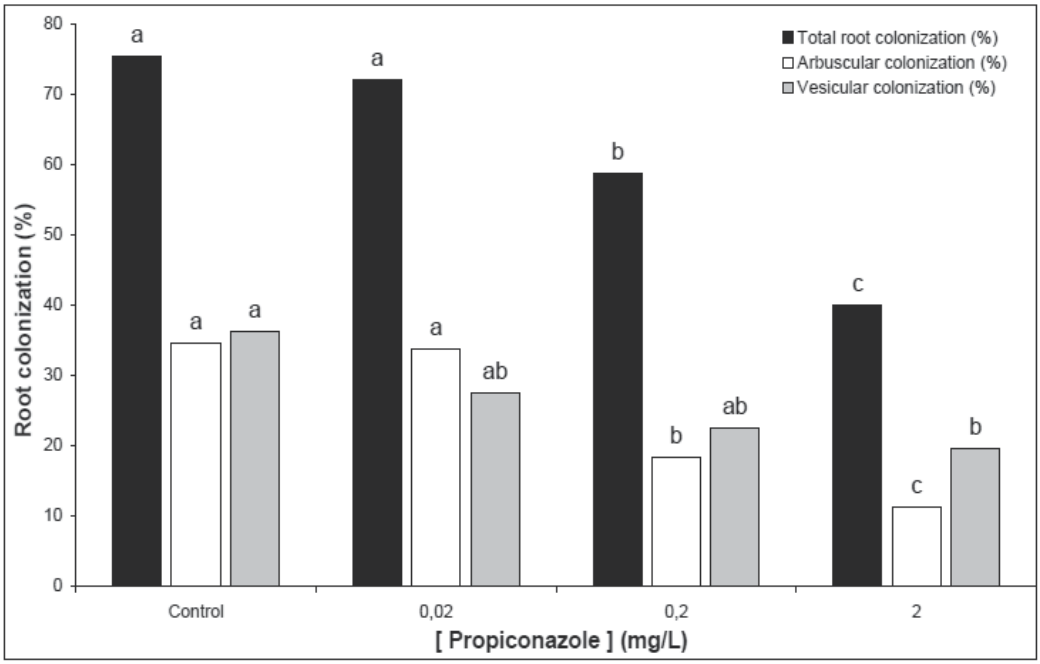

Fig. 3. Chicory root colonization by *Glomus irregulare* (total, arbuscular and vesicular colonization) after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 $mg.L^{-1}$) in mono-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

control in the presence of 0.2 and 2 mg.L^{-1} of propiconazole respectively. Concerning vesicles, significant decrease was observed at the highest concentration of propiconazole (2 mg.L^{-1}) which reach only 19%.

3.1.3 Impact of propiconazole on extraradical hyphae lengths and sporulation of *Glomus irregulare*

The impact of increasing propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) on extraradical hyphae lengths and *Glomus irregulare* spore formation is presented in Fig. 4. In the absence of propiconazole (control), the hyphal length reached more than 860 cm. It decreased significantly on media supplemented with propiconazole from 0.2 mg.L^{-1}.

The hyphal length reached only 397 and 118 cm in the presence of 0.2 and 2 mg.L^{-1} propiconazole respectively. *Glomus irregulare* sporulation decreased significantly from 0.2 mg.L^{-1}. Reduction was about 29% as compared to the control. At the highest propiconazole concentration (2 mg.L^{-1}), the spore number was drastically reduced and was estimated only to 35 spores by dish, whereas the control reached 2901 spores.

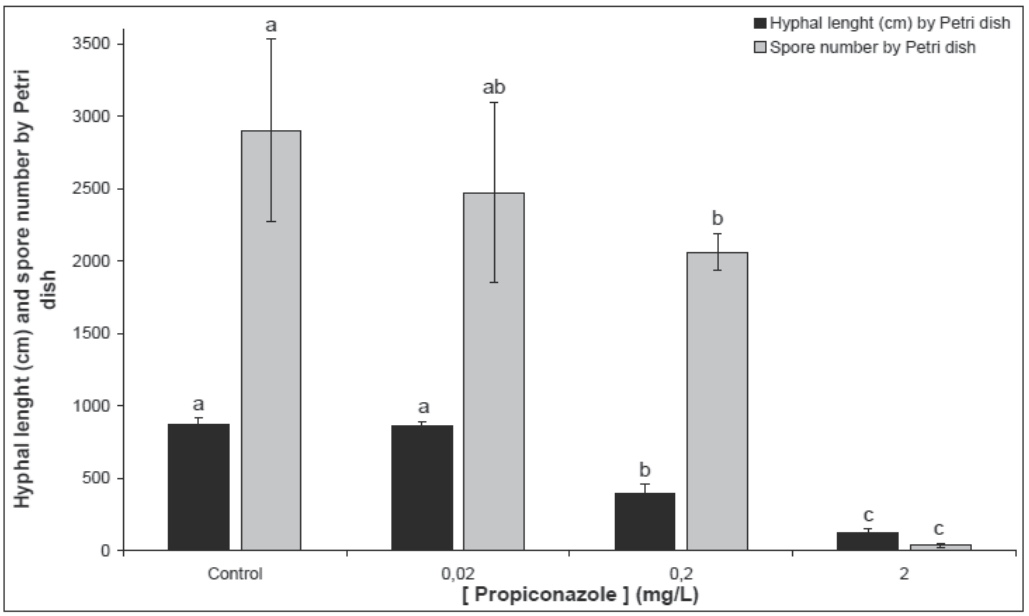

Fig. 4. Hyphal length and sporulation of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in mono-compartmental Petri dish. Data are presented as means. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

3.2 Propiconazole disturbed *Glomus irregulare* FA content

Glomus irregulare FA compositions and contents, when growing without and with increasing propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}) in bi-compartmental Petri dish, are shown in Table 1. It was found that the distribution of *Glomus irregulare* FA

Fatty acids (FA)	Control		[Propiconazole] (mg.L^{-1})							
			0.02		0.2		2			
	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%
C16:0	44.27 ± 4.16^{a}	12^{a}	46.97 ± 4.87^{a}	12^{a}	47.57 ± 7.74^{a}	12^{a}	42.98 ± 6.56^{a}	11^{a}
C16:1ω5	283.83 ± 32.49^{a}	76^{a}	300.03 ± 39.92^{a}	76^{a}	296.11 ± 49.26^{a}	74^{a}	289.02 ± 40.75^{a}	76^{a}
C18:0	0.15 ± 0.02^{a}	0.04^{a}	0.13 ± 0.03^{a}	0.03^{a}	0.2 ± 0.04^{a}	0.05^{a}	0.29 ± 0.1^{a}	0.07^{a}
C18:1	18.7 ± 2.34^{a}	5^{a}	20.15 ± 2.46^{a,b}	5^{a}	25.53 ± 4.22^{b}	6^{a}	22.87 ± 2.97^{ab}	6^{a}
C18:2	2.42 ± 0.31^{a}	0.6^{a}	2.47 ± 0.27^{a}	0.6^{a}	2.6 ± 0.43^{a}	0.6^{a}	2.51 ± 0.4^{a}	0.6^{a}
C19:0	3.09 ± 0.44^{a}	0.8^{a}	3.3 ± 0.43^{a}	0.8^{a}	3.54 ± 0.59^{a}	0.9^{a}	3.55 ± 0.5^{a}	0.9^{a}
C18:3	0.13 ± 0.02^{a}	0.03^{a}	0.14 ± 0.03^{a}	0.04^{a}	0.16 ± 0.03^{a,b}	0.04^{a}	0.19 ± 0.03^{b}	0.05^{a}
C20:1	0.42 ± 0.05^{a}	0.1^{a}	0.46 ± 0.07^{a}	0.1^{a}	0.42 ± 0.15^{a}	0.1^{a}	0.41 ± 0.09^{a}	0.1^{a}
C20:2	0.53 ± 0.11^{a}	0.1^{a}	0.56 ± 0.07^{a}	0.1^{a}	0.6 ± 0.12^{a}	0.1^{a}	0.85 ± 0.29^{a}	0.2^{a}
C21:0	8.24 ± 1.06^{a}	2^{a}	8.86 ± 1.08^{a}	2^{a}	9.29 ± 1.39^{a}	2^{a}	9.15 ± 1.2^{a}	2^{a}
C20:3	2.6 ± 0.39^{a}	0.7^{a}	2.67 ± 0.35^{a}	0.7^{a}	2.69 ± 0.52^{a}	0.7^{a}	2.73 ± 0.35^{a}	0.7^{a}
C20:5	1.1 ± 0.16^{a}	0.3^{a}	1.22 ± 0.22^{a}	0.3^{a}	1.4 ± 0.39^{a}	0.3^{a}	1.17 ± 0.21^{a}	0.3
C22:0	4.39 ± 0.61^{a}	1^{a}	4.81 ± 0.61^{a}	1^{a}	4.95 ± 0.87^{a}	1^{a}	4.98 ± 0.8^{a}	1^{a}
C22:1	0.82 ± 0.09^{a}	0.2^{a}	0.82 ± 0.25^{a}	0.2^{a}	0.96 ± 0.48^{a}	0.2^{a}	1.03 ± 0.22^{a}	0.3^{a}
C22:2	0.14 ± 0.04^{a}	0.04^{a}	0.16 ± 0.02^{a}	0.04^{a}	0.3 ± 0.11^{b}	0.07^{a}	0.24 ± 0.08^{ab}	0.06^{a}
Total FA (mg.g^{-1} dry weight)	370.83 ± 38.56^{a}		392.78 ± 50.09^{a}		396.05 ± 64.82^{a}		381.98 ± 53.75^{a}	
Saturated/unsaturated FA	0.19^{a}		0.19^{a}		0.2^{a}		0.19^{a}	

Table 1. Fatty acid (FA) composition and content of *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish.
Data are presented as means ± SD. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

ranged from C16:0 to C22:2 with three major compounds (C16:0, C16:1ω5 and C18:1). *Glomus irregulare* FA profile was not affected by the presence of the SBI fungicide propiconazole. The total FA contents and the ratio saturated/unsaturated FA of *Glomus irregulare* were similar when the AMF was grown in propiconazole supplemented medium or not. However, the amounts of unsaturated FA were modified by the treatment. The FA C18:1 and C22:2 increased significantly by about 36% and 114% respectively from 0.2 mg.L^{-1} of propiconazole and the FA C18:0, C18:3 and C20:2 increased at the highest concentration (2 mg.L^{-1}) by about 93%, 46% and 60% respectively.

3.3 Propiconazole affected phosphatidylcholine content of *Glomus irregulare* and its associated FA

Phospholipid content analysis of *Glomus irregulare* after 9 weeks of incubation showed that the presence of propiconazole at the concentration of 2 mg.L^{-1} increased significantly phosphatidylcholine quantity by about 207% as compared to the control (Fig. 5.).

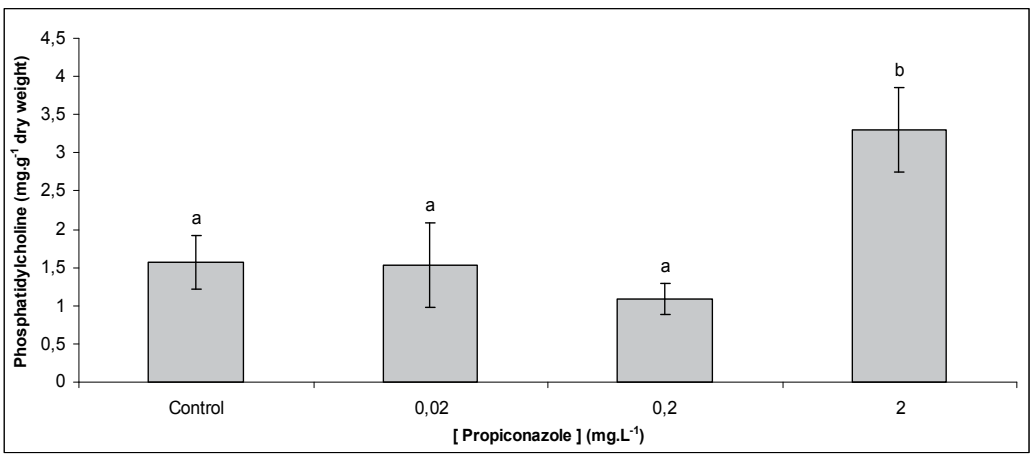

Fig. 5. Phosphatidylcholine content of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± standard error. The means were obtained from 5 replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$).

Glomus irregulare phospholipid fatty acids (PLFA) compositions and contents, when growing without and with increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}) in bi-compartmental Petri dish, are shown in Table 2. Six PLFA were detected: C16:0 as the major constituent, C16:1ω5, C18:0, C18:1, C18:3 and C20:2. No significant differences were found in the proportion of each PLFA as compared to the control. But the ratio saturated/unsaturated PLFA and the quantity of each PLFA increased at the highest propiconazole concentration (2 mg.L^{-1}), except for the PLFA C16:1ω5, C20:2 and C18:1 which was not detected at this concentration. In addition, the quantity of total PLFA increased at the highest propiconazole concentration (2 mg.L^{-1}).

	Control		[Propiconazole] (mg.L^{-1})					
			0.02		0.2		2	
Phospholipid fatty acids (PLFA)	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%	mg.g^{-1} dry weight	%
C16:0	0.49 ± 0.12^{a}	54^{a}	0.47 ± 0.12^{a}	51^{a}	0.47 ± 0.1^{a}	51^{a}	1.23 ± 0.43^{b}	57^{a}
C16:1ω5	0.07 ± 0.02^{a}	8^{a}	0.1 ± 0.05^{a}	10^{a}	0.14 ± 0.09^{a}	14^{a}	0.14 ± 0.06^{a}	6^{a}
C18:0	0.11 ± 0.03^{a}	12^{a}	0.12 ± 0.05^{a}	12^{a}	0.11 ± 0.01^{a}	12^{a}	0.34 ± 0.18^{b}	16^{a}
C18:1	0.03 ± 0.03^{a}	3^{a}	0.06 ± 0.06^{a}	5^{a}	0.02 ± 0.01^{a}	3^{a}	n.d.	
C18:3	0.12 ± 0.02^{a}	14^{a}	0.09 ± 0.03^{a}	10^{a}	0.12 ± 0.04^{a}	13^{a}	0.25 ± 0.05^{b}	12^{a}
C20:2	0.09 ± 0.08^{a}	9^{a}	0.14 ± 0.09^{a}	12^{a}	0.06 ± 0.04^{a}	7^{a}	0.21 ± 0.13^{a}	9^{a}
Total PLFA (mg.g^{-1} dry weight)	0.91 ± 0.0.24^{a}		0.98 ± 0.35^{a}		0.92 ± 0.23^{a}		2.17 ± 0.82^{b}	
Saturated/ unsaturated PLFA	1.93^{a}		1.51^{a}		1.71^{a}		2.45^{b}	

Table 2. Phospholipid fatty acids (PLFA) composition and content of *Glomus irregulare*, after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from 5 replicates. different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.05$). n.d.: not detected.

3.4 Propiconazole induced oxidative stress in *Glomus irregulare*

Malondialdehyde (MDA) production (a lipid peroxidation biomarker) and peroxidase (POD) specific activities (an anti-oxidant enzyme) in *Glomus irregulare*, when growing in the absence (control) and in the presence of increasing concentrations of propiconazole (0.02; 0.2 and 2 mg.L^{-1}) are shown in Table 3. Significant increases in the MDA content were pointed

[Propiconazole] (mg.L^{-1})	MDA (µmol.g^{-1} of protein)	POD activity (nKat.g^{-1} of protein)
Control	0.775 ± 0.224^{a}	2.8E-08 ± 1.8E-08^{a}
0.02	1.334 ± 0.19^{ab}	2.4E-08 ± 1.5E-08^{ab}
0.2	1.611 ± 0.674^{b}	2.2E-08 ± 1.6E-06^{ab}
2	1.559 ± 0.583^{b}	5.7E-09 ± 2.7E-09^{b}

Table 3. Concentration of malondialdehyde (MDA) and peroxidase (POD) activities in *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different propiconazole concentrations (0.02; 0.2 and 2 mg.L^{-1}), in bi-compartmental Petri dish. Data are presented as means ± SD. The means were obtained from five replicates. Different letters indicate significant differences between increasing concentrations of propiconazole, as determined by ANOVA followed by a multiple range test (LSD) ($p < 0.02$).

out in *Glomus irregulare* treated with propiconazole at 0.2 and 2 $mg.L^{-1}$. These rises were estimated to about 108% and 101% respectively.
In the absence of propiconazole, POD specific activities were estimated to about $2.8E^{-08}$ $nKat.g^{-1}$ of protein. It decreased significantly nearly 80% at the highest concentration of propiconazole, to reach $5.7E^{-09}$ $nKat.g^{-1}$ of protein.

4. Discussion

In the present work, we have pointed out the toxicity of the SBI molecule, propiconazole, on the non-target fungus, *Glomus irregulare.* The study focused on the effect of the fungicide on the FA, PL and PLFA compositions and contents, lipid peroxidation (evaluated in term of MDA content), and antioxidant enzyme activities (evaluated through the determination of POD activity), in relation with the AMF development.
Glomus irregulare development has been shown to be negatively impacted by increasing propiconazole concentrations (0.02, 0.2 and 2 $mg.L^{-1}$). Drastic reductions have been observed in the main steps of the AMF life cycle (germination, hyphal elongation, root colonization, extra-radical hyphae development and spore production). Contradictory effects of different propiconazole formulations applied on AMF colonized plants grown in pots were observed. No negative impacts on AMF development were reported by Nemec (1985), Hetrick et al. (1988), Plenchette and Perrin (1992), Von Alten (1993) and Kjoller and Rosendahl (2000). However inhibition of plant colonization and spore germination were described (Dodd and Jeffries, 1989; Plenchette and Perrin, 1992). The diversity of experimental procedures of these studies (plant species, growth conditions, fungicide formulations, application methods of fungicides, micro-organisms) present led to some difficulties to compare results, and to conclude on the impact of SBI on AMF (Sancholle et al., 2001). In our study, we used monoxenic cultures which are easily reproducible and used by different authors in order to evaluate toxicity of fungicides on AMF (Wan et al., 1998; Campagnac et al., 2008, 2009; Zocco et al., 2008). Inhibitory effects on fungal development of two other SBI fungicides i.e. fenpropimorph and fenhexamid was obtained in this way (Campagnac et al., 2008, 2009; Zocco et al., 2008).
Inhibition of spore germination could induce negative consequences on root colonization and fungi surviving. Moreover, as the main role of the mycorrhizal symbiosis is to improve the uptake of soluble mineral elements as phosphorus and nitrogen, present in soil in lower concentrations, the depletion of the AM colonization, in particular the arbuscular colonization in the presence of propiconazole, as shown in our data, could thus have a negative impact on plant water and mineral nutrition and on plant health. The negative propiconazole effect on root colonization and phosphate uptake by the AMF were reported by Dodd and Jeffries (1989) and Hetrick et al. (1988) respectively. Besides fungicides, many studies reported negative effects on *Glomus sp.* development of various pollutants (i.e., polycyclic aromatic hydrocarbons and heavy metal) and other abiotic stresses (i.e., salinity) (Schützendübel and Polle, 2002; Verdin et al., 2006; He et al., 2007; Hildebrandt et al., 2007; Debiane et al., 2008, 2009).
In order to explain propiconazole toxicity on AMF development, changes in the lipids, especially FA, PL, PLFA and the lipid peroxidation biomarker MDA, were investigated in *Glomus irregulare* grown under propiconazole treatment.

The composition of *Glomus irregulare* FA is ranged from C16:0 to C22:2 with three main compounds C16:1ω5 as major FA, C16:0 and C18:1. This FA profile is in agreement with previous studies carried out on different *Glomus sp.* (Gaspar et al., 1994; Fontaine et al., 2001; Sancholle et al., 2001; Grandmougin-Ferjani et al., 2005). Whereas propiconazole did not affect the AMF total FA contents, FA composition and the proportion of each FA, significant increases of *Glomus irregulare* unsaturated FA C18:1, C18:3, C20:2, C22:2 contents were observed while the treatment was applied as compared to the control.

In addition, our data showed a drastic increase of phosphatidylcholine at the highest propiconazole concentration. This result is in accordance with the study of Weete et al. (1985) which reported an increase in PL plasma membrane of the pathogenic fungus, *Taphrina deformans* in presence of propiconazole at a concentration which inhibited its growth by 50%. Moreover, in presence of propiconazole at 2 $mg.L^{-1}$, PLFA analysis highlighted significant increases in the total quantity due mainly to C16:0, C18:0 and C18:3 increases. Similar disturbances in PL and in PLFA quantities were also described in plant under salinity stress (Parti et al., 2003; Elkahoui et al., 2004). Parti et al. (2003) explained these lipid changes by mean of adaptations which increase the ability of the plant to endure salinity. Thus, increases of phosphatidylcholine and PLFA quantities observed in *Glomus irregulare* grown under propiconazole treatment could indicate some possible adaptations of the AMF under the SBI fungicide stress in order to try to maintain its membrane integrity and fluidity compatible with an optimal membrane functionality. Indeed, our results showed that the saturated/unsaturated FA ratio was more important in the presence of propiconazole at 2 $mg.L^{-1}$. This ratio saturated/unsaturated FA increase suggested a modification in membrane composition and is in agreement with the study of Benyagoub et al. (1996) which reported an increase in membrane fluidity on *Fusarium oxysporum* when exposed to an antifungal compound. In the same way, Kohli et al. (2002) found an increase of membrane fluidity in *Candida albicans* treated with azoles.

The disturbance in the unsaturated FA, especially the polyunsaturated C18:3, in the presence of propiconazole reminds an induction of lipid peroxidation. Indeed, our results pointed out concomitant increases of MDA production in *Glomus irregulare* under propiconazole treatment suggesting an oxidative stress. The disruption in polyunsaturated FA levels may therefore be related to the direct reaction of oxygen-free radicals with unsaturated lipids. These observations are in accordance with previous studies, which reported that abiotic stresses such as pollution, drought, salinity and heat induced an oxidative stress leading to MDA production by plant cells (Sinha et al., 2005; Bidar et al., 2007; He et al., 2007; Debiane et al., 2008, 2009; Yamauchi et al., 2008). In fact, the oxidative stress arising from abiotic stress exposure could generate ROS (Apel and Hirt, 2004), which can interact with polyunsaturated FA to generate aldehydes of which MDA is the main one (Esterbauer et al., 1991). The increase of MDA content in the AMF in presence of propiconazole, suggested an oxidative stress, which can be involved in mediating compositional membrane disruption, demonstrated in our conditions by important increases in phosphatidylcholine and its associated FA.

Little is known about ROS scavenging systems in AMF. To date only genes encoding three proteins putatively involved in ROS homeostasis have been identified and characterized in AMF: a CuZn superoxide dismutase in *Gigaspora margarita* (Lanfranco et al., 2005) and six genes putatively encoding glutathione S-transferases (GST) (Waschke et al., 2006) and a

metallothionein (González-Guerrero et al., 2007) in *Glomus intraradices*. Although the metallothionein was potentially involved in metal chelation, it was also shown to be involved in ROS scavenging, an activity that results from the capability of their thiolate groups to be reversibly oxidized (González-Guerrero et al., 2007). Recently, Benabdellah et al. (2009), provided the first evidence for the existence of a functional glutaredoxin (GintGRX1) in the AM fungus *Glomus intraradices*, a multifunctional protein with oxidoreductase, peroxidase and GST activity. Their findings also indicated that GintGRX1 might play a role in oxidative stress protection in the AM fungus. Antioxidant enzymes are important components in preventive oxidative stress. In the present study, POD specific activity was found to be inhibited at the highest concentration of propiconazole (2 $mg.L^{-1}$) indicating that the AMF antioxidant capacity were reduced when *Glomus irregulare* was grown on media containing propiconazole. Whereas fungicides as fenpropimorph and propiconazole induced an increase in plant POD activity (Jaleel et al., 2008; Campagnac et al., 2010), indicating an enhancement of free radicals under fungicide stress, our study showed a depletion of the antioxidant enzymes production in *Glomus irregulare* under propiconazole treatment. These results indicated that propiconazole stress could have a negative effect on proteins such as POD and that could thus damage the ROS scavenging by antioxidant enzymes.

To our knowledge, this is the first study which concerns the direct impact of propiconazole on the FA metabolism of the AMF *Glomus irregulare* in relation with its development. Taken in their whole, our data suggest that the drastic decrease of *Glomus irregulare* development observed could be linked to lipid (total FA, PL and PLFA) metabolism perturbation and to the toxicity of MDA accumulation. MDA production originated from the peroxidation of membrane lipids which could affect membrane functionality and consequently its function in nutrient uptake, exchanges, signal transmission and membrane enzyme regulation. According to these results, there will be probably a relationship between fungicide toxicity, the production of ROS under fungicide stress, lipid peroxidation, and membrane function disturbance, very probably due to an alteration of the membrane composition. It could be interesting, in a future work, to consider the target-lipid classes that also constitute the membrane (i.e. sterols). Moreover, a study of lipid rafts, functional microdomain of plasma membrane could be particularly interesting.

In conclusion, this study presented herein has contributed not only to investigate the toxicity of agricultural chemicals on AMF but also can provide a useful approach in soil ecotoxicology studies and risk assessment. In addition, the data highlighted the importance of investigating for side effects of pesticides on non-target soil organisms and demonstrated the emergency of using sustainable alternative method to control plant diseases. This work further emphasized the interest of *in vitro* cultures to investigate the mechanisms behind the impact of disease control molecules on the non-target AM fungal symbionts.

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Part 4

Fungicide Toxicology

17

Fungicides and Their Effects on Animals

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1. Introduction

Fungicides are pesticides that specifically inhibit or kill fungi underlying diseases important to man. Understanding mechanisms of fungicide action and toxicity is important because humans and domesticated animals encounter these pesticides through a wide variety of applications. In agriculture, fungicides are used to protect tubers, fruits and vegetables during storage or are applied directly to ornamental plants, trees, field crops, cereals and turf grasses (Gupta & Aggarwal, 2007). In industry, numerous fungicides are used to protect products during shipment, suppress mildews that attack painted surfaces, preserve wood, control fungal growth in paper pulps, and protect household carpet and fabric (Osweiler et al., 1985). In veterinary medicine, fungicides are commonly an antibacterial/antiseptic treatment for foot rot disease but, some fungicides (e.g., copper sulphate), are also used a molluscicide to repel and kill slugs and snails, (Ortolani et al., 2004). Another example of dual use of fungicides is the treatment by the widely used agricultural fungicide thiabendazole against intestinal parasites in both human and veterinary medicine (Lorgue et al., 1996).

Each year, livestock are accidentally poisoned by fungicides applied to grains, potatoes, or other agricultural materials. Unfortunately, most toxicity data are from model laboratory animals (e.g., rats, mice, rabbits) and offer little information on livestock and pets. Therefore, it is valuable to be aware of several generalizations for fungicide toxicity. While these generalizations may serve as useful guidelines, always obtain detailed information for a specific fungicide wherever possible. In general, newer classes of fungicides have low-to-moderate toxicity (Gupta & Aggarwal, 2007). Because mechanisms of action and metabolic clearance differ among fungicides, specific reproductive, teratogenic, mutagenic, carcinogenic effects or patterns of organ toxicity may manifest according to the poison ingested (Hayes & Laws, 1990; U.S. Environmental Protection Agency, 1999). Additionally, some animals may be more susceptible to poisoning than others due to their physiology or behaviour. For example, some fungicides (e.g., copper sulphate, thiram, chlorothalonil and captan) have especially toxic effects on fish (Pimentel, 1971; Lorgue et al., 1996; Tomlin, 2000), and bees (Hardley & Kidd, 1983). In the past, each spring, wild birds (e.g., pigeons, pheasants) were poisoned by mercurial fungicides in fields sown with treated seeds (Bartik, 1981). The types of fungicides used in agriculture and food processing and storage range from those of relatively low toxicity to those, which can be lethal to animals. Guidelines for use and generally low toxicity make poisoning in animal uncommon. However, fungicides frequently used around the home constitute a major hazard to pets and livestock due to

accidents, carelessness, or deliberate misuse (Osweiler et al., 1985; Gupta & Aggarwal, 2007; Oruc et al., 2009). For example, fungicides have caused systemic poisoning in animals such as sheep (Garcia-Fernández et al., 1996; Ortolani et al., 2004; Oruc et al., 2009), poultry (Guitart et al., 1996 & 1999), and humans (Israeli et al., 1983; Kintz et al., 1997; Chodorowski, 2003; Kayacan et al., 2007; Calvert et al., 2008; Mortazavi & Jafari-Javid, 2009). Incorrect application and failure to use protective gear while applying fungicides are probably responsible for a disproportionate number of irritant injuries to skin and mucous membranes, as well as dermal sensitization. Fungicides are often used in combinations with other pesticides and carriers or solvents which, in combination, may be more toxic than estimated for any one of the compounds (Osweiler et al., 1985).
In France, Lorgue et al. (1996) reported that pesticides are the most common cause of animal poisoning (45.5%), with fungicides accounting for 6.1% of all pesticides. The two most commonly involved species are dogs and cattle. In 2003, 992 cases involving dogs and cats were confirmed as poisoning in France, and fungicides caused 2.8% of all poisonings (Barbier, 2005). Acute fungicide poisonings was 4.4% in 129 poisoning cases in Greece (Berny et al., 2009). In Italy, poisoning related with fungicides account was 8.1% of pesticides in pet poisonings (Caloni et al., 2004).
This chapter describes widely used fungicides and their toxicity in animals.

2. Toxicity category and LD_{50}/ LC_{50} values

Pesticides, including fungicides, are categorized on the basis of their relative acute toxicity (LD_{50} or LC_{50} values). Pesticides that are classified as highly toxic (Toxicity Category I) on the basis of either oral, dermal, or inhalation toxicity must have the signal words DANGER and POISON printed in red with a skull and crossbones symbol prominently displayed on the front panel of the package label. The acute (single dosage) oral LD_{50} for pesticide products in this group ranges from a trace amount to 50 mg/kg. Some pesticide products have just the signal word DANGER, which tells you nothing about the acute toxicity, just that the product can cause severe eye damage or severe skin irritation. Pesticide products considered moderately toxic (Toxicity Category II) must have the signal word WARNING displayed on the product label. In this category, the acute oral LD_{50} ranges from 50 to 500 mg/kg. Pesticide products classified as either slightly toxic or relatively nontoxic (Toxicity Categories III and IV) are required to have the signal word CAUTION on the pesticide label. Acute oral LD_{50} values in this group are greater than 500 mg/kg. Table 1 summarizes the LD_{50} and LC_{50} values for each route of exposure for the four toxicity categories and their associated signal word (Code of Federal Regulations, 2010).
LD_{50} is the dosage at which one-half of the test animals are killed. Usually rats are tested, although mice or rabbits may be used. LD_{50} is measured in milligrams of chemical being tested per kilogram of animal (mg/kg). One part per million (ppm) is equal to one mg/kg. LD_{50} is usually determined for the technical material rather than the formulated product. The higher the LD_{50}, the less toxic the material. LC_{50} is the lethal concentration at which 50% of test animals would be killed. Chemicals may be tested by mouth (oral), by skin (dermal), or inhalation. World Health Organisation (WHO) recommends standardized categories (levels of hazard) of major chemical classes of technical grade fungicides with representative examples of LD_{50} values in rats (Table 2) (International Programme on Chemical Safety, 2002; Gupta, P.K. & Aggarwal, M., 2007).

Routes of Exposure	Toxicity Category			
	I	II	III	IV
Oral LD_{50}	Up to and including 50 mg/kg	50-500 mg/kg	500-5,000 mg/kg	>5,000 mg/kg
Inhalation LC_{50}	Up to and including 0.2 mg/l	0.2-2 mg/l	2-20 mg/l	>20 mg/l
Dermal LD_{50}	Up to and including 200 mg/kg	200-2,000 mg/kg	2,000-20,000 mg/kg	>20,000 mg/kg
Eye Effects	Corrosive corneal opacity not reversible within 7 days	Corneal opacity reversible within 7 days; irritation persisting for 7 days	No corneal opacity; irritation reversible within 7 days	No irritation
Skin Effects	Corrosive	Severe irritation at 72 hours	Modarate irritation at 72 hours	Mild or slight irritation at 72 hours
Signal Word	DANGER POISON	WARNING	CAUTION	CAUTION

Table 1. Toxicity categories for active ingredients (Adapted from Code of Federal Regulations, 2010)

3. Fungicides

Below, structural classes of fungicide are listed in order of their adverse effects:

I. Benzimidazoles
II. Cadmium Compounds
III. Carbamic Acid Derivates
    a. Dithiocarbamates
    b. Ethylene Bis Dithiocarbamates (EBDC Compounds)
IV. Copper Compounds
V. Halogenated Substituted Monocyclic Aromatics (Substituted Benzenes)
VI. Organomercury Compounds
VII. Phthalimides (Chloroalkylthiodicarboximides)

I. Benzimidazoles

The major benzimidazole fungicides include benomyl, carbendazim, fuberidazole and thiophanate-methyl and thiabendazole. Benomyl and carbendazim have low toxicity, whereas fuberidazole has moderate toxicity (Gupta & Aggarwal, 2007). Both benomyl and carbendazim are well absorbed after oral exposure (80-85%) but poorly absorbed after dermal exposure (1-2%) in rat, mice, hamster and dogs. The major pathway of clearance is the urinary elimination in rats and mice but in dogs the majority of the dose is eliminated via faeces. In animals, benomyl is converted into carbendazim through the loss of the n-butylcarbamyl side-chain prior to further metabolism (Gardiner et al., 1974).

Benomyl

Benomyl commonly used systemic fungicide is used for a wide range of diseases on fruits, nuts, vegetables, field crops, turf and ornamentals. Available commercially in the form of a wettable powder, 50% w/w. Chronic feeding in dogs at 2500 ppm results in impaired liver

Chemical class	Category	LD_{50} (mg/kg, bw)	Chemical class	Category	LD_{50} (mg/kg, bw)
Halogenated substituted monocyclic aromatics			Fuberidazole	II	336
Chlorothalonil	U	>10,000	*Conazoles*		
Tecnazene	U	>10,000	Cyproconazole	III	1020
Dicloran	U	4000	Diniconazole	III	639
HCB	Ia	[d]10,000	Etridiazole	III	2000
Quintozene	U	>10,000	Hexaconazole	U	2180
Dinocap	III	980	Penconazole	U	2120
Dichlorophen	III	1250	Triadimefon	III	602
PCP	Ib	[c]80	Triadimenol	III	900
Chloroneb	O	-	Azaconazole	II	308
			Bromuconazole	II	365
Chloroalkylthiodicarboximides					
Captan	U	9000	Propiconazole	II	1520
Captafol	Ia	5000	Tetraconazole (oil)	II	1031
Folpet	U	>10,000	Imazalil	II	320
Anilinopyrimidines			*Morpholines*		
Mepanipyrim	U	>5000	Dodemorph (liquid)	U	4500
Pyrimethanil	U	4150	Fenpropimorph (oil)	U	3515
Cyprodinil	III	>2000	Tridemorph	II	650
Carbamic acid derivatives			*Amides*		
Ferbam	U	>10,000	Fenhaxamid	U	>5000
Thiram	III	560	Benalaxyl	U	[c]4200
Ziram	III	1400	Metalaxy	III	670
Propamocarp	U	8600	Flutolanil	U	>10,000
Maneb	U	6750	Tolyfluanid	U	>5000
Mancozeb	U	>8000	Dichlofluanid	U	>5000
Zineb	U	>5000	*Others*		
Nabam	II	395			
Metiram	U	>10,000	Cycloheximide	O	-
Benzimidazoles			Fludioxonil	U	>5000
Benomyl	U	>10,000	Dimethomorph	U	>5000
Thiophanate-methyl	U	>6000	Trifloxystobin	U	>5000
Carbendazim	U	>10,000	Fenpyroximate	II	245
Thiabendazole	U	3330			

Ia: extremely hazardous; Ib: highly hazardous; II: moderately hazardous; III: slightly hazardous
U: unlike to present acute hazard in normal use; O: obsolete or discontinued; c: the variability is reflected in the prefix "c" and dermal in the prefix "d" before LD_{50} values; bw: body weight.

Table 2. World Health Organisation hazardous classification oftechnical grade fungicides. Representative LD_{50} values are provided for rats (Complied from IPCS, 2002; Gupta & Aggarwal, 2007).

function and cirrhosis. The chronic no-effect level in dogs was 500 ppm. Embryotoxic and teratogenic effects were seen in rats incubated with 125, 250 or 500 mg/kg (Osweiler et al., 1985). There is a very low toxicity in animals, but mildly toxic to fish (Lorgue et al., 1996). Although the molecule contains a carbamate grouping, benomyl is not a cholinesterase inhibitor. There is no specific treatment for benomyl poisoning in animals. Symptomatic treatment is applied to promote excretion (Lorgue et al., 1996).

Carbendazim

Carbendazim is also known as MCAB, BCM, or MCB. It is a systemic fungicide used on fruits, vegetables, field crops, ornamentals and turf (Osweiler et al., 1985). It has low toxicity and available commercially in the form of a wettable powder and concentrated suspensions (Lorgue et al., 1996). There is no specific treatment for carbendazim poisoning in animals. Symptomatic treatment is applied to promote excretion (Lorgue et al., 1996).

Fuberidazole

Fuberidazole is available as crystalline powder, and has moderate toxicity (Gupta & Aggarwal, 2007). Possible fuberidazole poisoning in young pheasants has been reported (Laing, 2001).

Thiophanate-methyl

Thiophanate-methyl is also referred to as TM. It is systemic fungicide used on fruits, vegetables, field crops and nuts (Osweiler et al., 1985). It has very low toxicity and is available commercially in the form of a wettable powder and concentrated suspensions. In rats, oral administration causes tremors, lacrimation, nasal discharge, tonic colonic convulsions. The compound causes reduced respiratory rate, prostration and mydriasis in dogs (Lorgue et al., 1996).

Thiabendazole

This is systemic fungicide used on fruits, trees and vegetables (Osweiler et al., 1985). It is also used as anthelmintic in veterinary and human medicine. Cattle are more sensitive than the other domestic animals for adverse effects of thiabendazole (Lorgue et al., 1996).

II. Cadmium Compounds

Cadmium salts have been used as systemic fungicide on turf and the bark of orchard trees. They were formulated as solution and emulsions. Cadmium chloride may contain 12.3% elemental cadmium. Cadmium succinate may contain 29% elemental cadmium (Osweiler et al., 1985). Cadmium sebacate is combined with thiram and potassium chromate as broad-spectrum fungicide. Cadmium chloride has also a mixture with thiram. In many countries, cadmium fungicides have been discontinued due to their toxic effects.

III. Carbamic Acid Derivates

The carbamic acid class of fungicides includes dithiocarbamates and ethylene bis dithiocarbamates (EBDC) compounds. In general, carbamic acid derivates have low or moderate acute toxicity by the oral, dermal and respiratory routes, except nabam (Gupta & Aggarwal, 2007). Carbamic acid derivate fungicides, such as EBDCs, are only partially absorbed, then rapidly metabolized and excreted with no evidence of long-term bioaccumulation. Absorption of oral doses is rapid and is excreted within 24 hour with about half eliminated in the urine and half in the faeces. Their common metabolite is ethylenethiourea, and only low-level residues of ethylenethiourea are found in tissues particularly in the thyroid (Gupta & Aggarwal, 2007).

a. Dithiocarbamates

Ferbam

Ferbam is ferric dimethyldithiocarbamate (Osweiler et al., 1985). It is formulated as flowable and wettable powders, and used widely on fruit and nut trees, apples, vegetables, tobacco,

and home gardens. In acute studies, ferbam has low acute toxicity (Toxicity Category III) via the oral and dermal routes, and moderate (Toxicity Category II) acute toxicity via the inhalation route of exposure. In longer-term studies, ferbam is toxic to the liver, kidneys, and lungs (U.S. Environmental Protection Agency, 2005a).

Thiram

The full chemical name for thiram is tetramethylthiuram disulphide. It is used as a fungicide, seed protectant, animal repellent, rubber accelerator, and bacteriostat in soap (Osweiler et al., 1985). At high doses it acts as a repellent to birds, rabbits, rodents, and deer in fields and orchards. Thiram is available as dust, wettable powder, water suspension formulations, and in mixtures with other fungicides. It has been used in the treatment of human scabies, as a sunscreen, and as a bacteriostat in medicated soaps and certain antiseptic sprays. Another important source of thiram for environmental contamination is the degradation of the two widely used ethylene bisdithiocarbamate fungicides, ferbam and ziram (Dalvi, 1988).
Thiram is moderately toxic by ingestion, but it is highly toxic if inhaled. Hepatotoxicity is one of many toxic effects of thiram in exposed workers and test animals. Typical symptoms include as liver enlargement and dysfunction, hepatitis, degenerative changes, and focal necrosis (Hasegawa et al., 1988; Maita et al. 1991). Clinical signs in thiram poisoning are anorexia, listless behavior, dyspnea, convulsions, and death due to cardiac arrest (Kaya & Bilgili, 1998). It is metabolized in the body to toxic metabolites dimethyldithiocarbamate and carbon disulfide. Although these compounds have been shown to inhibit hepatic microsomal enzymes (Dalvi & Deoras, 1986). Levels of thiram ranging from 100-500 ppm in the food rations of hens, quail and partridges inhibit egg laying (Lorgue et al., 1996). An outbreak of thiram poisoning on Spanish poultry farms was reported. Thiram-contaminated poultry feed caused soft egg shells, depressed growth and leg abnormalities in about 1 million birds. Corn was the source of the contamination that previously treated with thiram (Guitart et al., 1996). Thiram is toxic to fish, and LC_{50} (48 h) was found 0.1-0.2 mg/l in fish (Lorgue et al., 1996).

Ziram

Ziram is zinc dimethyldithiocarbamate, also known as methyl cymate (Osweiler et al., 1985). It is formulated as flowable and wettable powders, and used widely on fruit and nut trees, apples, vegetables, tobacco. Ziram is used in the United Kingdom as a bird and animal repellent (e.g. against bullfinches, rabbits, hares, deer). At levels of 500 ppm in the diet, ziram inhibits egg laying quail and partridges (Lorgue et al., 1996). Ziram is more toxic than ferbam and thiram in adult fowl (Rasul & Howell, 1974).

b. Ethylene Bis Dithiocarbamates (EBDC Compounds)

Mancozeb

Mancozeb contains zinc and manganese ethylene bis dithiocarbamate. While related to maneb and zineb it is a distinct chemical (Osweiler et al., 1985). It is formulated as a dust and as wettable and liquid flowable powders. Available in mixtures with other compounds (e.g. benaxyl, maneb, carbendazim or cymoxanil, zineb). The compound has low toxicity to animals, and is not toxic to fish (Lorgue et al., 1996).

Maneb

Maneb is manganese ethylene bis dithiocarbamate, and is extensively used in agriculture. It is available commercially in the form of wettable powders, for dusting, and as a soluble concentrate. It has low toxicity to animals, but toxic to fish (Lorgue et al., 1996).

Metiram

Metiram is a member of the ethylene bis dithiocarbamate group of fungicides, which includes the related active ingredients mancozeb and maneb. It is used on apples, potatoes, and ornamental plants (leatherleaf ferns) in nurseries and greenhouses. Thyroid effects observed in subchronic studies in rats include increased thyroid weights, increased thyroid stimulating hormone (TSH) and decreased T4 (serum thyroxin) values (U.S. Environmental Protection Agency, 2005b).

Nabam

Nabam is disodium ethylene-1,2 bis dithiocarbamate and toxic doses may result in nerve damage (Osweiler et al., 1985). It is used as soil fungicide, and available commercially in liquid form for mixing with water for irrigation (Lorgue et al., 1996).

Zineb

Zineb is zinc ethylene bis dithiocarbamate, and used for many edible crops. Available commercially in the form of wettable powders and for dry dusting, and also in combination with ferbam, mancozeb and maneb fungicides. It has low toxicity. In cases of zineb poisoning in sheep, the animals present with characteristic yellow, watery diarrhea (Lorgue et al., 1996).

IV. Copper Compounds

There are number of fungicides that contain copper. Copper acetate was the first commercial copper fungicide. Bordeaux mixture is an old fungicide that is mixture of hydrated lime and copper sulphate. Some formulations also may include lead arsenate. Copper carbonate is also known as malachite. Copper hydroxide is cupric hydroxide. Copper containing formulations are also used as wood preservatives. Copper naphthenate is used treat wood and fabrics. Copper compounds may contain basic sulphates, oxychlorides, or oxides. Copper oxides are both cuprous oxide and cupric oxide. Copper sulphate is used as a fungicide and algaecide (Osweiler et al., 1985). Poisonings related with copper containing fungicides are important to livestock (Oruc et. al., 2009) and pets (Albo & Nebbia, 2004) especially sheep due to contaminated forage and feeds, and careless (Oruc et. al., 2009).

Copper sulphate

Copper sulphate is also known as bluestone, blue vitriol, blue copperas. The chemical name is cupric sulphate pentahydrate (Osweiler et al., 1985). It is used to control bacterial and fungal disease of fruit, vegetable, nut and field crops. Some of the disease that is controlled by this fungicide includes mildew, life spots, blights and apple scab. It is used in combination with lime and water as a protective fungicide, referred to as Bordeaux mixture, for leaf application and seed treatment. It is also used as an algaecide, an herbicide in irrigation and municipal water treatment systems, and as molluscicide, a material used to repel and kill slugs and snails (U.S. Environmental Protection Agency, 1986). Copper sulphate is available in dust, wettable powders, and fluid concentrates form.

Copper sulphate lead to acute or chronic copper poisoning in animals. Sheep are affected most often, although other species are also susceptible to copper (Cu) overdose. Chronic Cu toxicosis occurs in sheep when animals are fed diets over weeks or months that are marginally high in copper content (15–20 mg/kg, dry weight) with low concentrations of molybdenum (Zervas et al., 1990; Lorgue et al., 1996). Systemic poisonings have been reported in sheep (Ortolani et al., 2003; Ortolani et al., 2004; Roubies et al., 2008; Oruc et. al.,

2009). Poisonings with copper sulphate are also reported in dog and cats. For both species, fungicides account for nearly one-fourth of the calls of the agrochemical class and the majority of these (43% for dogs and 52% for cats) are related to the accidental ingestion of copper sulphate (Albo & Nebbia, 2004). Rabbits can also be poisoned with copper sulphate (Vinlove et al., 1992).

Clinical features of acute poisonings includes salivation, vomiting, watery diarrhoea that is grey-green in colour and often haemorrhagic, painful, severe colic and gastrointestinal effects. Neurological effects include convulsions, followed by paralysis, and decubitus. Death may occur within several hours to several days after ingestion. There is no specific antidote. Symptomatic care include gastrointestinal demulcents, adsorbents (activated vegetable charcoal), cardiorespiratory stimulants and treatments to control convulsions (xylazine, diazepam), if necessary. Laboratory investigation of copper carries out in contents of the stomach and/or intestine (Lorgue et al., 1996). In chronic poisonings, sheep and cattle are most affected species, and copper is hepatotoxic. Clinical signs are bright yellow mucous membranes, yellow-coloured skin, haematuria, bloody nasal discharge, anaemia, anorexia, occasional convulsions, edema of the head and neck (presenting several hours before the onset of jaundice). In general, death occurs within a few hours of presentation of these symptoms (Lorgue et al., 1996; Oruc et. al., 2009). Once jaundice is evident, treatment is unlikely to be effective. Before jaundice, dimercaprol (BAL), 2-4 mg/kg, by intramuscular route, twice in the first 24 hours, then two to four times in the next 48 hours should be administered. Another treatment includes EDTA (sodium calcium edentate 25% w/v), 40-50 mg/kg by intravenous route, given in one to two doses daily for 2-3 days. Ammonium molybdenate (50-500 mg/day per animal) and sodium thiosulphate (0.3-1 g/day per animal) may be administrated orally in the diet for 10 to 15 days. Laboratory investigations show accumulation in liver, kidney, feeds, and hay (Lorgue et al., 1996).

Copper sulphate less threat to birds than to other animals (Tucker & Crabtree, 1970). It is also toxic to fish and other aquatic invertebrates, such as crab, shrimp and oysters as well as earthworms in soil (Clayton & Clayton, 1981). Bees are endangered by strong, water-based compounds, such as a Bordeaux mixture of copper sulphate, lime and water (Hartley & Kidd, 1983).

V. Halogenated Substituted Monocyclic Aromatics (Substituted Benzenes)

This class of chemicals includes such as chloroneb, chlorothalonil, dicloran, hexachlorobenzene and pentachlorophenol.

Chloroneb

Chloroneb is used for seed treatment and on foliage, and is supplied as wettable powder for treatment of soil and seed. It has a low toxicity (Gupta & Aggarwal, 2007).

Chlorothalonil

Chlorothalonil is tetrachloroisophthalonitrile and is widely used in both agriculture and the household. Chlorothalonil's acute toxicity through ingestion is low; however, chlorothalonil is much more toxic when inhaled. In laboratory tests, chlorothalonil causes kidney damage, mild anaemia, liver damage, embryo loss during pregnancy, oxidative DNA damage (damage to the cell's genetic material), and cancers of the kidney and forestomach. Most of these effects have been observed in several test species. It is classified as a "probable human carcinogen" by the U.S. Environmental Protection Agency (Cox, 1997). It causes irreversible and severe ocular lesion in rabbits. Signs of toxicity include decreased body weight and

decreased haematological parameters and increased absolute kidney weight (Gupta & Aggarwal, 2007). Absorption of chlorothalonil from the gastrointestinal tract is of the order of 30-32% of the administered dose. At least 80% of the administered dose is excreted in faeces within 96 hour. Highest concentrations are observed in the kidneys, approximately 0.1% of the dose (Parsons, 2001).

Chlorothalonil is very highly toxic to fish, and concentrations as low as 2 parts per billion (ppb) can cause gill damage and anaemia. It is also toxic to shrimp, frogs, beneficial microorganisms, and earthworms. In plants it causes a variety of effects, including reductions in yield. Chlorothalonil is contaminated with the carcinogen hexachlorobenzene. Its major breakdown product is about thirty times more acutely toxic than chlorothalonil itself and is more persistent in soil (Cox, 1997).

Dicloran

Dicloran (DCNA) is pre- and post-harvest fungicide formulated as a dust, wettable powder and liquid. The major pre-harvest crop uses include celery and lettuce; the major post-harvest use is on sweet potatoes. It has a low acute toxicity in mammals by oral basis, and the target organs for dicloran include the kidney, liver, spleen and hematopoietic system, particularly red blood cells. Dicloran is toxic to birds, but has low toxicity effects for fish and aquatic invertebrates (U.S. Environmental Protection Agency, 2006).

Hexachlorobenzene

Principal formulations of hexachlorobenzene are dust and powders. In animals, liver enlargement is accompanied by changes in the drug-metabolizing enzymes, neurological changes, reduced weight gain, immunosuppression, teratogenic and carcinogenic effects (Renner, 1981). It possesses all the properties of chemical stability, slow degradation and biotrasformation, environmental persistence, bioaccumulation in adipose tissue and organs containing a high content of lipid membranes (Ecobichon, 2001).

Pentachlorophenol

Pentachlorophenol or its salts have been used as herbicides, bactericides, fungicides, molluscacides and insecticides, and it is also referred to as penta or PCP. The majority (90%) of pentachlorophenol is used as a wood preservative. Pentachlorophenol in high doses to pregnant animals is embriyotoxic and fetotoxic, but is not teratogenic (Osweiler et al., 1985).

Pentachlorophenol acts at the cellular level to uncouple oxidative phosphorylation, the target enzyme being Na^+, K^+-ATPase. Oxygen consumption is increased, while adenosine triphosphatase (ATP) formation is decreased. This leads to depletion of energy reserves (Eaton & Gallagher, 1997).

Pentachlorophenol is rapidly absorbed from the skin, digestive tract and lung. It is irritating to mucous membranes, the respiratory tract and the skin. In mild intoxications there is muscular weakness, anorexia and lethargy. Moderate toxicities results in accelerated respiration, hyperpyrexia, hyperglycaemia, glycosuria, sweating, and dehydration. Lethal intoxications result in the previous symptoms plus cardiac and muscular collapse and death with a rapid onset of rigor mortis (Osweiler et al., 1985; Sanli, 2002). Chronic toxicity results in anaemia. Acute lethal intoxications occur with blood levels of 100 ppm. There is no specific treatment. It has been suggested and, in some human cases, successful to use large volumes of balanced fluids to help flush the pentachlorophenol through the kidney (Osweiler et al., 1985).

VI. Organomercury Compounds

Mercury exists in a variety of organic and inorganic forms. The replacement of commercial mercurial compounds, including antiseptics (eg, mercurochrome), diuretics, and fungicides by other agents has decreased the likelihood of mercurial toxicosis; however, the possibility of exposure to environmental sources of organic methylmercury still exists (Aiello & Mays, 1998). Adverse effects of organic mercury are important to animal and human healthy. Inorganic mercury is converted to the organic alkyl forms, methylmercury and ethylmercury, by microorganisms in the sediment of rivers, lakes, and seas. Marine organisms (e.g., bivalves and fish) accumulate the most toxic form, methylmercury, and shellfish and fish must be monitored for contamination. There are reports of commercial cat food causing severe neurologic disturbances in cats fed an exclusive tuna diet for 7-11 months. The organic mercurials are absorbed via all routes and bioaccumulate in the brain and to some extent in the kidneys and muscle. Aryl mercurials (eg, phenylmercury fungicide) are slightly less toxic and less prone to bioaccumulation. Animals poisoned by organic mercury exhibit CNS stimulation and locomotor abnormalities after a lengthy latent period (weeks). Signs may include blindness, excitation, abnormal behavior and chewing, incoordination, and convulsions. Cats show hindleg rigidity, hypermetria, cerebellar ataxia, and tremors. Mercury is also a mutagen, teratogen, and a carcinogen, and is embryocidal. Differential diagnoses include conditions with tremors and ataxia as predominant signs, such as ingestion of other metals and insecticides and cerebellar lesions due to trauma or feline parvovirus (Aiello & Mays, 1998).

The mechanism of mercurial fungicides is to inhibit sulfhydryl group of enzymes involved in the transfer of amino acids across the blood brain barrier and then interfere with protein synthesis. Organomercurials can also release some mercury ions in the body, but their toxicity is not believed to be a primary action of mercury ions (Sandhu &Brar, 2000).

The first known cases of human poisoning from methylmercury-contaminated fish were reported in Japan, on the island of Kyushu, around Minamata Bay. Between 1953 and 1970, more than 121 poisonings were reported in this area, with 46 deaths recorded (Eyl, 1971).

By the mid-1970s, dietary toxicity thresholds and adverse effects of methylmercury were described for game birds and mink, and considerable information on mercury concentrations in tissues of wildlife had been generated in field biomonitoring studies (Thompson, 1996).

The aryl organomercurials, methyl or ethyl mercury chloride are poorly excreted and tend to accumulate in muscle, brain and other tissues, while the aryl organomercurials, phenyl mercury is more readily excreted via the kidney and less likely to accumulate in brain and muscles (Gupta & Aggarwal, 2007).

Neurologic signs may be irreversible once they develop. Chelating therapy with dimercaprol (3 mg/kg body wt, intramuscular, every 4 hr for the first 2 days, four times on the third day, and two times for the next 10 days or until recovery is complete) has been beneficial. When available, the water soluble, less toxic analogy of dimercaprol is the chelator of choice for organic mercury poisoning. Penicillamine (15-50 mg/kg, peros) may be used only after the gut is free of ingested mercury and renal function has been established (Aiello & Mays, 1998).

VII. Phthalimides (Chloroalkylthiodicarboximides)

This class of chemicals contains broad-spectrum fungicides such as captan, captafol and folpet. They are usually non-toxic to mammals (Gupta & Aggarwal, 2007).

Captan

Captan is a chloroalkylthio fungicide that belongs to the dicarboximide chemical family. Captan is used on a variety of crops as post-harvest fruit dips and seed treatment. It is also

used for indoor non-food uses and ornamental sites. Captan can be formulated as an emulsifiable concentrate, flowable concentrate, ready-to-use liquid, liquid soluble concentrate, solid, water dispersible granules, wettable powder, and dust (U.S. Environmental Protection Agency, 1999). Available also in combination with other fungicides (Lorgue et al., 1996).

Sheep and cattle are susceptible to captan poisoning. Sheep died after a single 250 mg/kg oral dose of captan. Signs of overexposure to captan include hypothermia, listlessness, depression, diarrhea, weight loss, anorexia, and increased water consumption in animals (Edwards et al., 1991; Lorgue et al., 1996).

Captan is rapidly degraded to 1,2,3,6-tetrahydrophthalimide (THPI) and thiophosgene in the stomach before reaching the duodenum. THPI has a half-life of 1-4 and thiophosgene is detoxified by reaction with cysteine or glutathione and is rapidly excreted. No captan is detected in blood or urine. Therefore, this compounds or even thiophosgene would survive long enough to reach systemic targets such as the liver, uterus and testes. Due to rapid elimination, meat, milk or eggs from livestock/poultry would be devoid of the parent materials (Kriger & Thongsinthusak, 1993).

Captan is highly to very highly toxic to fish such as bluegill sunfish, fathead minnow, brook trout, coho salmon, harlequin fish and brown trout. Captan is relatively non-toxic to honey bees, with a contact LD50 of >10 µg/bee (U.S. Environmental Protection Agency, 1999).

There is no specific treatment for captan poisoning in animals. Symptomatic treatment is applied (Lorgue et al., 1996; Sener, 2000).

Captafol

Captafol is used on fruits, vegetables, cereals and as a seed protectant. It is also used as wood preservative (Osweiler et al., 1985). Captafol is available in the form of concentrated suspensions. Cattle and fish are susceptible to captafol poisoning (Lorgue et al., 1996).

Folpet

The other name is "folpel" that used in France (Lorgue et al., 1996). It is used on fruits, vegetables and ornamental plants. It is also used in paints and plastics for fungal control (Osweiler et al., 1985). Folpet is formulated as a wettable powder or as a concentrated suspension. Although it has low toxicity, the most affected animals are cattle and poultry (Lorgue et al., 1996).

4. Conclusion

Fungicides vary enormously in their potential for causing toxic effects in animals. The main hazard to animals from fungicides is likely to arise from their use in agriculture and garden. Fungicides can lead to acute or chronic poisoning in animals, although fungicides have low to moderate toxicity for animals. Some livestock poisoning cases result from accidental overdosing or careless use of fungicide for treatment in the animals. In general, there is no specific treatment for fungicides poisoning in domesticated animals and humans. Some fungicides also have adverse effects on wildlife, such as birds, honey bees, fish and aquatic invertebrates. Fungicides are currently and, undoubtedly, will continue to be widely used in agriculture. Therefore, we should use fungicides on plants, seeds and trees carefully (i.e., according to manufacturer instructions and wearing appropriate protective gear) and we should monitor our agricultural products and domesticated animal foods for fungicide contamination.

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18

Introduction and Toxicology of Fungicides

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1. Introduction

Fungicides are either chemicals or biological agents that inhibit the growth of fungi or fungal spores. Modern fungicides do not kill fungi, they simply inhibit growth for a period of days or weeks. Fungi can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. Fungicides are used both in agriculture and to fight fungal infections in animals. Chemicals used to control oomycetes, which are not fungi, are also referred to as fungicides as oomycetes use the same mechanisms as fungi to infect plants (Latijnhouwers et al., 2000).

Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue, & only protect the plant where the spray is deposited; translaminar fungicides redistribute the fungicide from the upper, sprayed leaf surface to the lower, unsprayed surface; systemic fungicides are taken up & redistributed through the xylem vessels to the upper parts of the plant. New leaf growth is protected for a short period.

Most fungicides that can be bought retail are sold in a liquid form. The most common active ingredient is sulfur, present at 0.08% in weaker concentrates, and as high as 0.5% for more potent fungicides. Fungicides in powdered form are usually around 90% sulfur and are very toxic. Other active ingredients in fungicides include neem oil, rosemary oil, jojoba oil, and the bacterium *Bacillus subtilis*.

Fungicide residues have been found on food for human consumption, mostly from postharvest treatments (Brooks & Roberts, 1999).

Some fungicides are dangerous to human health, such as vinclozolin, which has now been removed from use (Hrelia, 1996), FCX and DFB that are used as pesticides to control pests and they have many side effects on natural non-target organisms (Rouabhi et al., 2009). In this chapter, we will develop the fungicides and their toxicity on biological and ecological systems.

2. Classification of fungicides

Different authors have differing classification systems according to chemical structure, which somewhat complicates and confuses both the presentation and the discussion of fungicides. Several classification systems based on structure appear more of a web organization than a rationalized listing. In addition to classification by chemical structural grouping, fungicides can be categorized agriculturally and horticulturally according to the

mode of application (use). According to the origin of fungicides, we can classify them in two major groups of fungicides

1. Biologically based fungicides (biofungicides): Contain living microorganisms (bacteria, fungi) that are antagonistic to the pathogens that cause turf disease. Examples: Ecoguard contains *Bacillus licheniformis*; Bio-Trek 22G contains *Trichoderma harzianum*. In the case of a biofungicide, the Latin name of the microbe that it contains is the generic name of the fungicide.
2. Chemically based fungicides: Synthesized from organic and inorganic chemicals, most of the fungicides that are sold throughout the world are chemically-based. They can be recognized according to similarities in three groups:

2.1 Chemical structure

There are 29 generic names (active ingredients) associated with turf grass fungicides, shown in the table1.

1.	propiconazole	2.	triadimefon
3.	myclobutanil	4.	fenarimol
5.	triticonazole	6.	tetraconazole
7.	fluoxastrobin	8.	trifloxystrobin
9.	azoxystrobin	10.	pyraclostrobin
11.	flutolanil	12.	boscalid
13.	polyoxin D	14.	thiophanate-methyl
15.	iprodione	16.	vinclozolin
17.	mefenoxam	18.	propamocarb
19.	fosetyl aluminum	20.	phosphonate
21.	quintozene	22.	chloroneb
23.	ethazole	24.	mancozeb
25.	thiram	26.	hydrogen dioxide
27.	chlorothalonil	28.	fludioxonil
29.	cyazofamid	30.	Biofungicides

Table 1. Fungicides generic names according to their chemical structure (Burpee, 2006).

These 29 names represent 16 groups that have similar chemical structures (table 2). It is important to know which fungicides are chemically related to one another. For example, you should know that azoxystrobin, trifloxistrobin, and pyraclostrobin are chemically related to each other. However, they differ chemically from a fungicide, such as propiconazole, which is in a different chemical group. Because (i) all fungicides in a chemical group generally, control the same diseases. For example, the strobilurin fungicides provide good to excellent control of anthracnose, brown patch, gray leaf spot and summer patch. If you have purchased one strobilurin fungicide for control of these diseases, it is probably not necessary to purchase another. (ii) Since all fungicides in a chemical group control the same diseases, it does not make sense to tank-mix fungicides that represent a common chemical group in order to expand the scope of control. For example, to control anthracnose and dollar spot, tank-mixing two strobilurin fungicides will not work well because the strobilurins provide poor to weak control of dollar spot. (iii) If a pathogen develops resistance to one fungicide in a chemical group, the pathogen is usually resistant to all fungicides in that particular group. In Georgia,

the fungi that cause dollar spot, Pythium blight and anthracnose have developed resistance to fungicides in one or more chemical groups.

Generic Names	Chemical Group	
propiconazole triadimefon myclobutanil triticonazole tetraconazole	triazoles	DMI (demethylation Inhibitors fungicides)
fenarimol	pyrimidines	
fluoxastrobin trifloxystrobin azoxystrobin pyraclostrobin	strobilurins	
polyoxin D	polyoxins	
thiophanate-methyl	benzimidazoles	
iprodione vinclozolin	dicarboxamides	
mefenoxam	phenylamides	
propamocarb	carbamates	
fosetyl aluminum phosphonate	phosphonates	
mancozeb thiram	dithiocarbamates	
quintozene chloroneb ethazole	aromatic hydrocarbons	
hydrogen dioxide	peroxides	
chlorothalonil	nitriles	
fludioxonil	phenylpyrolles	
cyanofamid	cyanoimidazole	
flutolanil boscalid	carboxamides	
Ecoguard Sonata Soilguard	Biofungicides	

Table 2. Chemical groups of fungicides according the Generic names (Burpee, 2006).

2.2 Topical activity

Fungicides can be placed into one of four groups based topical activity:

2.2.1 Contact fungicides

Contact fungicides act only on plant surfaces. They are not absorbed by leaves, stems or roots and cannot inhibit fungal development inside plants. Example: dithiocarbamates, nitriles, aromatic hydrocarbons, peroxides, phenylpyrolles, cyanoimidazoles.

2.2.2 Localized penetrants

Localized penetrant fungicides are absorbed by leaves and move short distances within a treated leaf, they do not move from one leaf to another and they are not absorbed by roots. These fungicides inhibit fungi on treated plant surfaces and inside treated leaves. Example: dicarboximides, strobilurins (except azoxystrobin and fluoxastrobin)

2.2.3 Acropetal penetrants

Acropetal penetrants can penetrate plants through roots, shoots and leaves. These fungicides are absorbed by the xylem and move upward (acropetally) in plants. Acropetal penetrants inhibit fungi on and in treated plant surfaces and inside plant parts that lie above the treated surface. Example: benzimidazoles, triazoles, pyrimidines, carboximides, acylalanines, plus the strobilurins azoxystrobin and fluoxastrobin.

2.2.4 Systemic fungicides

Systemic fungicides are the only fungicides that are absorbed into xylem and phloem and moves up and down in plants. These fungicides inhibit fungi on and in treated plant surfaces and inside plant parts that lie above or below the treated surfaces. Example: phosphonates.

2.3 Mode of action

The body or thallus of most fungi exists as microscopic tubes called hyphae (Fig. 1)

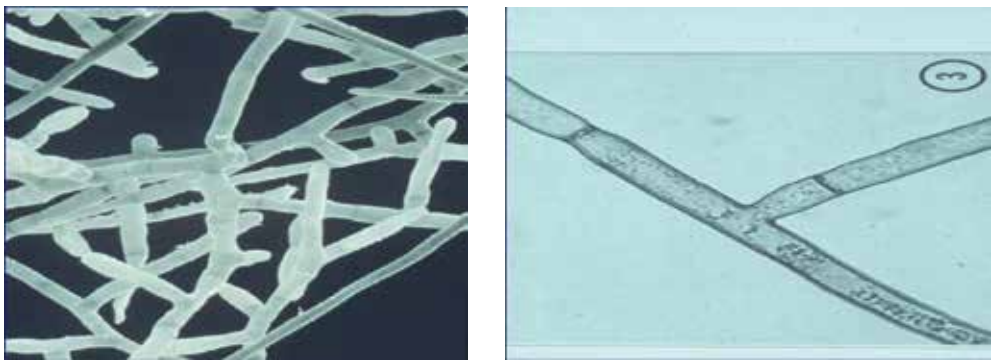

Fig. 1. Hyphae of a fungi (Burpee, 2006)

A fungal cell contains many of the same organelles as other eukaryotes (Fig.2).

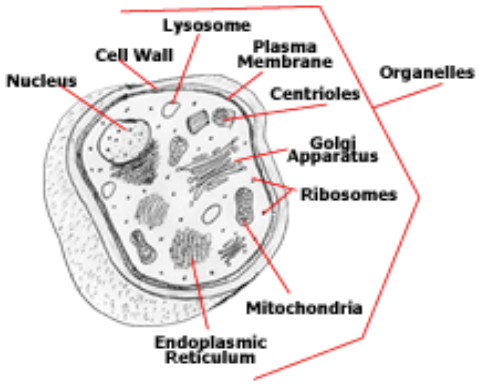

Fig. 2. Fungal cell with organelles (Foster and Smith, 2010)

Fungicides can be divided into 2 groups based on mode of action in fungal cells:

a. **Site-specific inhibitors:** Site-specific inhibitors target individual sites within the fungal cell (Fig. 3).

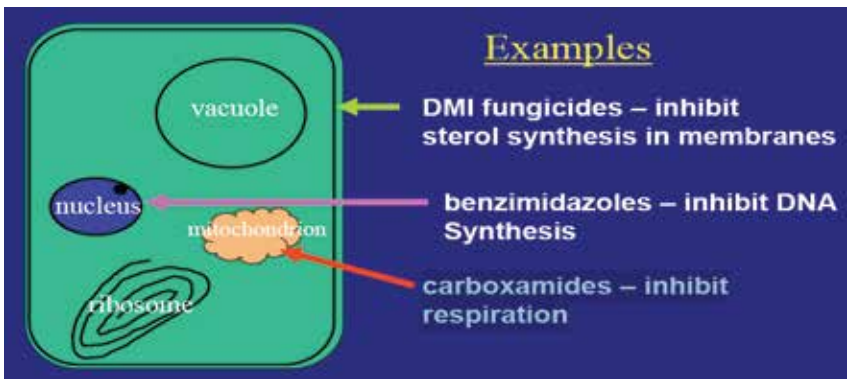

Fig. 3. Site-Specific Inhibitors. DMI: demethylation inhibitors fungicides (Burpee, 2006).

b. **Multi-site inhibitors**

Multisite inhibitors target many different sites in each fungal cell (fig. 4)

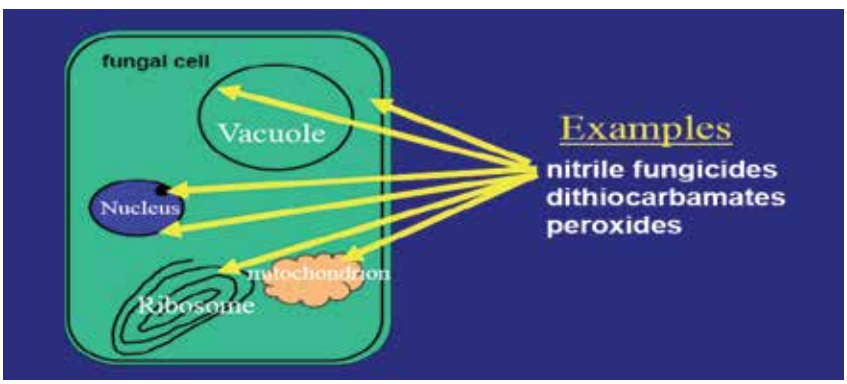

Fig. 4. Multi-site Inhibitors (Burpee, 2006).

3. Toxicology of fungicides

In general, fungicides are of low to moderate mammalian toxicology, although they are believed to have a higher overall incidence than other pesticides to cause developmental toxicology and oncogenesis (Costa, 1997). It has, for example, been estimated that more than 80 per cent of all oncogenic risk from the use of pesticides comes from a few fungicides (NAS, 1987). However, fungicides usually are responsible for only a small proportion of pesticide-related deaths, and account for only about 5 per cent or less of human pesticide exposures reported to Poison Control Centers (Blondell, 1997; Hayes and Vaughn, 1977; Litovitz et al., 1994). It has been noted that since fungi differ significantly in morphology and physiology from other forms of life, they may be successfully combated by compounds of low toxicity to other organisms, notably mammals (Edwards et al., 1991). However, since the mechanism of injury to pathogenic fungi may be different to that for injury to mammalian systems, it is possible that the two properties may co-exist in a given fungicide molecule (Marrs and Ballantyne, 2004).
It has been noted (Phillips, 2001) that the ideal fungicide should have the following characteristics: (a) low mammalian toxicity, (b) low ecotoxicity, (c) low phytotoxicity, (d) high penetration rates for spores and mycelia, and (e) limited biodegradation on the plant surface. Many fungicides combine several of these characteristics but few approach optimum for all of them.

3.1 Triazoles

This chemical family of fungicides, introduced in the 1980s, consists of numerous members: difenoconazole, fenbuconazole, myclobutanil, propiconazole, tebuconazole, tetraconazole, triadimefon, and triticonazole. They are important tools against diseases of turfgrasses, vegetables, citrus, field crops and ornamental plants. Homeowner products are available for use as well, and may be readily obtained at garden and nursery retail centers. They are applied as foliar sprays and seed treatments, but are diverse in use, as they may be applied as protectant or curative treatments. If applied as a curative treatment, triazole applications must be made early in the fungal infection process. Once the fungus begins to produce spores on an infected plant, the triazoles are not effective. Although the triazoles do not have the degree of systemic movement of many herbicides, they are xylem-mobile. They are readily taken up by leaves and move within the leaf. The triazoles are very specific in their mode of action – they inhibit the biosynthesis of sterol, a critical component for the integrity of fungal cell membranes. Because their site of action is very specific, there are resistance concerns (Fishel, 2005).

3.1.1 Toxicology

By the oral route of exposure, these triazoles would be considered as having low toxicity. Inhalation of dusts can cause irritation of the nose, lungs, and throat. For mycobutanil, in animals, effects were reported on the following organs: testes, adrenal gland, kidney, and thyroid. Mycobutanil did not cause cancer or birth defects; only doses that caused significant toxicity to parent animals caused reproductive effects on laboratory animals. Increased incidence of liver tumors at extremely high doses was reported in laboratory studies involving male mice who had been exposed to propiconazole or tebuconazole. There were no reproductive, developmental or chronic effects reported with either propiconazole or tebuconazole. Additionally, tebuconazole is considered to not cause any mutagenic or

genotoxic effects; however, EPA has classified it as a "possible human carcinogen" because of the liver effects seen with mice. The main concern with triadimefon is its potential to cause birth defects, although data suggest that in humans such effects would occur only at moderate to high doses of exposure. Ecologically, the main concern with the triazoles is with fish and other aquatic organisms. Their labels will carry statements expressing this concern in the Environmental Hazards section. Of this pesticide family, only difenoconazole is considered to be highly toxic to fish. Most of the triazoles are considered to be practically nontoxic to birds and bees. Mammalian toxicities for the triazole fungicides are shown in Table 3. Table 4 lists the toxicities to wildlife by the common name of the pesticide (Fishel, 2005).

Common name	Rat oral LD 50	Rabbit dermal LD50
Difenoconazole	1,453	2,010
Fenbuconazole	>2,000	>5,000 (rat)
Mycobutanil	1,600	>5,000 (rat)
Propiconazole	1,517	>4,000
Tebuconazole	1,700	>2,000
Triadimefon	569	2,000
Triticonazole	>2,000	>2,000

Table 3. Triazole fungicide mammalian toxicities (mg/kg of body weight).

Common name	Bird acute oral LD 50 (mg/kg)*	Fish (ppm)**	Bee***
Difenoconazole	PNT	HT	PNT
Fenbuconazole	ST	PNT	PNT
Mycobutanil	PNT	MT	PNT
Propiconazole	PNT	MT	PNT
Tebuconazole	PNT	MT	PNT
Triadimefon	PNT – ST	ST	PNT
Triticonazole	PNT	ST	PNT

Table 4. Triazole fungicide wildlife toxicity ranges.

*Bird LD 50: Practically nontoxic (PNT) = >2,000; slightly toxic (ST) = 501 – 2,000; moderately toxic (MT) = 51 – 500; highly toxic (HT) = 10 – 50; very highly toxic (VHT) =<10.

**Fish LC 50: PNT = >100; ST = 10 – 100; MT = 1 – 10; HT = 0.1–1 ; VHT = <0.1.

***Bee: HT = highly toxic (kills upon contact as well as residues); MT = moderately toxic (kills if applied over bees); PNT = relatively nontoxic (relatively few precautions necessary).

3.1.1.1 Toxicology of an Example of the family "Cyproconazole"

The primary dissipation routes of Cyproconazole (Fig. 5) in surface soil are microbial degradation and plant uptake. Soil photolysis (breakdown by sunlight) and volatilization are not significant routes of degradation. The major breakdown product (degradate, metabolite) of cyproconazole is further broken down to intermediate metabolites, carbon dioxide and bound material. There has been no evidence that Cyproconazole accumulates in the soil.

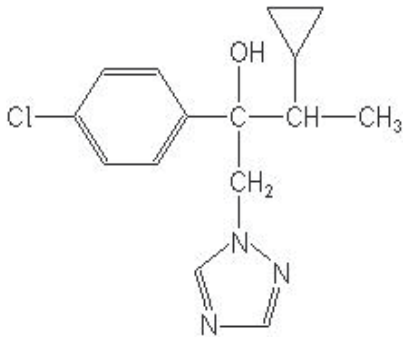

Fig. 5. Cyproconazole

Toxicology

Cyproconazole is of low risk to birds, mammals, bees and other non-target terrestrial organisms. Cyproconazole is moderately to slightly toxic to most aquatic organisms, and because of the low use rates the exposure potential is low and hence poses minimal risk of adverse effects (Envirofacts, 2005).

Oral LD50s are 1020mg/kg for male rats, 1333 mg/kg for female rats, 200 mg for male mice, and 218 mg/kg for female mice. The percutaneous rabbit LD50 is >2000 mg/kg. The rat 4-h inhalation is LC50 > 5.65 mg/L. The major plant residue is Cyproconazole. There is moderately rapid soil degradation; DT50 is about 3 months. Avian acute oral LD50 for Japanese quail is 150 mg/kg. Eight-day dietary LC50s are 816mg/kg (diet) for Japanese quail and 1197 mg/kg (diet) for mallard duck. Aquatic organism 96-h LC50 toxicity values include 18.9 mg=L for carp, 19 mg/L for trout, and 21 mg/L for bluegill sunfish. In Daphnia the 48-h LC50 is 26 mg/L. For bees, the contact LD50 is >0.1 mg/bee and the peroral LD50 is >1mg/bee (Marrs and Ballantyne, 2004).

Plants absorb cyproconazole and rapidly degrade it to multiple metabolites. Studies have shown that cyproconazole residues may exist at harvest, but the levels are insignificant and well under the safety margins for human and environmental risks as established by regulatory authorities in many countries, including the US EPA (Envirofacts, 2005).

3.2 Aromatics hydrocarbons fungicides

Major fungicides in this group include chlorothalonil, tecnazine, chloroneb, dichloran, hexachlorobenzene, quintozene, pentachlorophenol, and sodium pentachlorophenate. Many of these are, or metabolized to, uncouplers of oxidative phosphorylation. This can lead to excessive heat production, hyperpyrexia, liver damage, and corneal opacities.

3.2.1 Toxicology of an example of the family "Chloroneb"

Chloroneb (Fig. 6) is a broad spectrum systemic fungicide taken up by the roots, and used on various fruit and vegetable crops as a wet or dry application powder or dust. Mechanism of fungal toxicity may be related to inhibition of DNA polymerization (Phillips, 2001). Principal use in Soil systemic and supplemental seed treatment forseedling diseases of beans, sugar beets, turf and soybeans. Excellent against damping-off (Harding, 1979-80). Used for the treatment of turfgrass to control snow mold (Typhula) and Pythium blight (Meister et al., 1994).

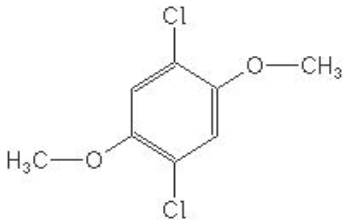

Fig. 6. Chloroneb

Toxicology

a. **Acute Toxicity**

Dermal: LD50 = >5000 mg/kg (rabbit). A 50% aqueous suspension of the 65% caused no irritation to guinea-pigs and repeated applications did not result in skin sensitization (Worthing, 1979).

Oral: LD50 = >11,000 mg/kg (rat) (Wothing, 1979)

b. **Environmental considerations**

Hazardous to fish and wildlife. Nonphytotoxic when used as directed. Does not leach from the soil (Harding, 1979-80). The material was not toxic to bluegill Sunfish at 4,200 ppm in the 48-hour exposure.

3.3 Dithiocarbamate fungicides

The dithiocarbamate fungicides: ferbam, mancozeb, maneb, nabam, thiram, zineb and ziram were evaluated at the Joint FAO/WHO Meeting in 1967. Although the biochemical data were limited, temporary acceptable daily intakes (ADI's) were established for all of these compounds, but it was pointed out that these ADI's are to be applicable to the parent compounds only (FAO/WHO, 1968).

3.3.1 Toxicology of an example of the family "Thiram"

Thiram (fig. 7) is a dimethyl dithiocarbamate compound used as a fungicide to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport. Thiram is also used as a seed protectant and to protect fruit, vegetable, ornamental, and turf crops from a variety of fungal diseases. In addition, it is used as an animal repellent to protect fruit trees and ornamentals from damage by rabbits, rodents, and deer. Thiram is available as dust, flowable, wettable powder, water dispersible granules, and water suspension formulations, and in mixtures with other fungicides. Thiram has been used in the treatment of human scabies, as a sunscreen, and as a bactericide applied directly to the skin or incorporated into soap.

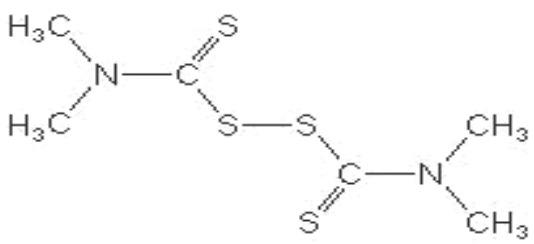

Fig. 7. Thiram.

Toxicology

a. **Acute Toxicity**

Thiram is slightly toxic by ingestion and inhalation, but it is moderately toxic by dermal absorption. Acute exposure in humans may cause headaches, dizziness, fatigue, nausea, diarrhea, and other gastrointestinal complaints. In rats and mice, large doses of thiram produced muscle incoordination, hyperactivity followed by inactivity, loss of muscular tone, labored breathing, and convulsions. Most animals died within 2 to 7 days. Thiram is irritating to the eyes, skin, and respiratory tract. It is a skin sensitizer. Symptoms of acute inhalation exposure to thiram include itching, scratchy throat, hoarseness, sneezing, coughing, inflammation of the nose or throat, bronchitis, dizzines, headache, fatigue, nausea, diarrhea, and other gastrointestinal complaints. Persons with chronic respiratory or skin disease are at increased risk from exposure to thiram (U.S. National Library of Medicine, 1995). Ingestion of thiram and alcohol together may cause stomach pains, nausea, vomiting, headache, slight fever, and possible dermatitis. Workers exposed to thiram during application or mixing operations within 24 hours of moderate alcohol consumption have been hospitalized with symptoms. The 4-hour inhalation LC50 for thiram is greater than 500 mg/L in rats. Reported oral LD50 values for thiram are 620 to over 1900 mg/kg in rats; 1500 to 2000 mg/kg in mice; and 210 mg/kg in rabbits (Edwards et al., 1991; Kidd and James, 1991). The dermal LD50 is greater than 1000 mg/kg in rabbits (U.S. National Library of Medicine, 1995) and in rats (Edwards et al., 1991; Kidd and James, 1991).

b. **Chronic toxicity**

Symptoms of chronic exposure to thiram in humans include drowsiness, confusion, loss of sex drive, incoordination, slurred speech, and weakness, in addition to those due to acute exposure. Repeated or prolonged exposure to thiram can also cause allergic reactions such as dermatitis, watery eyes, sensitivity to light, and conjunctivitis (Edwards et al., 1991). Except for the occurrence of allergic reactions, harmful chronic effects from thiram have been observed in test animals only at very high doses. In one study, a dietary dose of 125 mg/kg/day thiram was fatal to all rats within 17 weeks. Oral doses of about 49 mg/kg/day to rats for 2 years produced weakness, muscle incoordination, and paralysis of the hind legs. Rats fed 52 to 67 mg/kg/day for 80 weeks exhibited hair loss, and paralysis and atrophy of the hind legs. Symptoms of muscle incoordination and paralysis from thiram poisoning have been shown to be associated with degeneration of nerves in the lower lumbar and pelvic regions. Day-old white leghorn chicks fed 30 and 60 ppm for 6 weeks exhibited bone malformations. At doses of about 10% of the LD50 for 15 days, thiram reduced blood platelet and white blood cell counts, suppressed blood formation, and slowed blood coagulation in rabbits (Edwards et al., 1991).

c. **Organ toxicity**

Studies have shown evidence of damage to the liver by thiram in the form of decreased liver enzyme activity and increased liver weight (Edwards et al., 1991). Thiram may also cause damage to the nervous system, blood, and kidneys (U.S. National Library of Medicine, 1995).

d. **Ecological effects**

Effects on birds: Thiram is practically nontoxic to birds. The reported dietary LC50 of thiram in Japanese quail is greater than 5000 ppm (Hill and Camardese, 1986). Reported dietary LC50 values in pheasants and mallard ducks are 2800 ppm and 673 ppm, respectively (Hudson et al., 1984). The LD50 for the compound in red-winged blackbirds is greater than 100 mg/kg (Kidd and James, 1991).

Effects on aquatic organisms: Thiram is highly toxic to fish (U.S. National Library of Medicine, 1995). The LC50 for the compound is 0.23 mg/L in bluegill sunfish, 0.13 mg/L in trout, and 4 mg/L in carp (Mayer and Ellersieck, 1986). Thiram is not expected to bioconcentrate in aquatic organisms (Howard, 1989).

3.4 Benzimidazoles fungicides

This class is confused since the individual classes are closely related. Sometimes, however, the benzimidazoles are classified separately but in an overlapping manner, creating confusion. They are nitrogen heterocyclic compounds, with parent structures of thiabendazole and/or benzimidazole. Included in this overall group are benomyl, thiabendazole, thiophanate, thiophanate–methyl, mebendazole, carbedazim, imazalil, and fuberidazole. Benomyl, carbendazim, thiophanate, and thiophanate–methyl are sometimes referred to (and classified) as benzimidazoles carbamates. Many of these fungicides inhibit mitochondrial fumarate reductase, reduce glucose transport, and uncouple oxidative phosphorylation. Inhibition of microtubule polymerization by binding to ┐-tubulin is a primary action, and specific high affinity binding to host α-tubulin occurs at significantly lower concentrations than mammalian protein binding (Phillips, 2001).

3.4.1 Toxicology of an example of the family "Benomyl"

Benomyl was first reported as a fungicide in 1968 and introduced onto the UK market in 1971 by the US Company Du Pont (Tomlin, 1994). It is a systemic benzimidazole fungicide that is selectively toxic to microorganisms and to invertebrates, especially earthworms (Extoxnet, 1994).

Benomyl and its main metabolite carbendazim bind to microtubules (an essential structure of all cells) and therefore interfere with cell functions such as cell division and intracellular transportation. The selective toxicity of benomyl as a fungicide is possibly due to its heightened effect on fungal rather than mammalian microtubules (WHO/PCS, 1994).

Benomyl is used as a pre-harvest systemic fungicide, and as a post-harvest dip or dust. It combats a wide range of fungal diseases of arable and vegetable crops, apples, soft fruit, nuts, ornamentals, mushrooms, lettuce, tomatoes and turf. It is also available widely for amenity and amateur garden use (Whitehead, 1996).

Toxicology

a. **Acute Toxicity**

Benomyl is of such a low acute toxicity to mammals that it has been impossible or impractical to administer doses large enough to establish an LD50. It therefore has an arbitrary LD50 that is 'greater than 10,000 mg/kg/day for rats'. However, skin irritation may occur with workers exposed to benomyl (Extoxnet, 1994). It is a mild to moderate eye irritant and is a skin sensitizer. Florists, mushroom pickers and flower growers have reported allergic reactions to benomyl (MAFF, 1992).

In 1992, benomyl exposure caused adverse occupational health effects (headaches, diarrhoea and sexual dysfunction) in agricultural workers in Florida (Agrow, 1992).

b. **Chronic toxicity**

In a laboratory study, dogs fed benomyl in their diets for three months developed no major toxic effects but did show evidence of altered liver function at the highest dose (150 mg/kg). With longer exposure, more severe liver damage occurred including cirrhosis after two years (Extoxnet, 1994).

c. **Carcinogenic effects**

The US Environmental Protection Agency classified benomyl as a possible human carcinogen (Office of Pesticide Programs, 1996). There is an element of doubt in this classification because carcinogenic studies have produced conflicting results. A two year experimental mouse study has shown it probably caused an increase in liver tumors. The Ministry of Agriculture Fisheries and Food (MAFF) takes the view that this was bought about by the hepatoxic effect of benomyl (MAFF, 1992).

d. **Reproductive effects**

Tests on laboratory animals have shown benomyl can have an effect on reproduction. In one rat study, where the mothers were fed 1,000 mg/kg/day for four months, the offspring showed a decrease in viability and fertility (WHO, 1993). In studies to investigate the effects of benomyl on male reproductive performance, fertility was reduced at all dose levels tested. In another study, a no-effect level of 15mg/kg/day was established based on testicular abnormalities (MAFF, 1992).

Permanent reductions occurred in the size of testes and male accessory glands in 100 day-old offspring from female laboratory rats receiving 31.2 mg benomyl/kg body weight per day. Rats developed a reduced sperm activity following acute inhalation exposure, acute and sub-chronic oral exposure. The same effect occurred in dogs following a single four-hour inhalation exposure (MAFF, 1992).

e. **Environment**

Benomyl binds strongly to soil and does not dissolve in water largely. When applied to turf, it has a half-life of three to six months, and when applied to bare soil the half-life is six to 12 months (Extoxnet, 1994).

3.5 Piperazines fungicides

3.5.1 Triforine toxicology

Triforine (fig. 8) is a piperazine derivative used as a systemic fungicide with protectant, eradicant and curative characteristics. It is used for control of powdery mildew, rusts, black rot and scab on cereals, fruit, ornamentals, and vegetables (Royal Society of Chemistry, 1983; Worthing, 1983). Triforine is also active against storage diseases of fruit and suppresses red spider mite activity (Worthing, 1983). Because of its low hazard to beneficial insects, triforine may be used in Integrated Pest Management (IPM) programs. Triforine comes in emulsifiable concentrates, liquid seed treatments, and wettable powder formulations. Triforine is miscible with common insecticides and herbicides in the recommended manner of use (Royal Society of Chemistry, 1983).

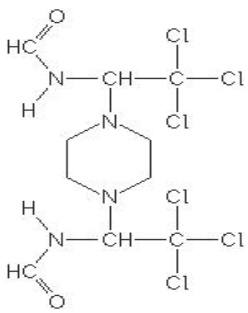

Fig. 8. Triforine

Toxicological effects

a. **Acute toxicity**

Triforine and the formulated product Saprol have a low acute and dermal toxicity and have a moderate acute inhalation toxicity. The acute oral LD50 for triforine in rats is greater than 16,000 mg/kg body weight. The acute percutaneous LD50 for rats is greater than 10,000 mg/kg. Acute dermal LD50 for rats is greater than 10,000 mg/kg body weight. The one-hour acute inhalation LC50 for triforine in rats is greater than 4.5 mg/l air (Worthing, 1983). This compound is rapidly absorbed and metabolized by the rat (OHS Database, 1994). The acute oral LD50 for the formulated product Saprol in rats is 5,273 mg/kg body weight. The acute dermal LD50 for Saprol in rats is 4,186 mg/kg body weight. The acute inhalation LC50 for Saprol in rats is greater than 5,288 mg/m3. Saprol is considered an irritant to the skin. The acute oral LD50 for triforine in mice is greater than 6,000 mg/kg; and greater than 2,000 mg/kg in dogs. The acute dermal LD50 for rabbits is greater than 10,000 mg/kg body weight (Thomson, 1990).

b. **Chronic toxicity**

In two-year feeding studies, the No-effect-level (NEL) for triforine in dogs was 100 mg/kg diet and 625 mg/kg diet for rats (Worthing, 1983).

Reproductive Effects

A decreased number of fetuses and an increased number of resorptions were observed in a study of pregnant rats fed triforine at a dietary level of 1,600 mg/kg (OHS Database, 1994). The formulated product Saprol does not affect reproduction and development. In another developmental study, rabbits were fed doses of 0, 5, 25 and 125 mg/kg/day of triforine. The maternal No-observable-effect-level (NOEL) was 5 mg/kg/day; the maternal Lowest-effect-level (LEL) was 25 mg/kg/day, rabbits exhibited reduced food intake and loss of body weight. The fetotoxic NOEL was 5 mg/kg/day; the fetotoxic LEL was 25 mg/kg/day, decreased average relative weight was observed (U.S Environmental Protection Agency, 1993).

Teratogenic Effects

In a developmental study, rabbits were fed doses of 0, 5, 25 and 125 mg/kg/day of triforine. The teratogenic NOEL was greater than 125 mg/kg/day. The formulated product Saprol is not considered a teratogen (U.S Environmental Protection Agency, 1993).

Mutagenic Effects

The formulated product Saprol is not considered a mutagen.

Carcinogenic Effects

In short and long-term studies of the formulated product Saprol, no irreversible or carcinogenic effects were observed.

c. **Ecological effects**

Effects on Birds

The acute oral LD50 for triforine in bobwhite quail is greater than 5,000 mg/kg (Worthing, 1983). The formulated product Saprol is practically non-toxic to birds by acute oral exposure and only slightly toxic by dietary exposure. The acute oral LD50 for Saprol in bobwhite quail is greater than 5,000 mg/kg. The dietary LC50 for bobwhite quail is 1,850 ppm in the diet. Mallard ducks had a dietary LC50 of greater than 4,640 ppm in the diet.

Effects on Aquatic Organisms

At 50 mg/l in water, there are no signs of poisoning in *Lebistes reticulatus*. Rainbow trout and bluegill sunfish tolerate 1,000 mg/l in water for 96 hours without symptoms (Royal Society of Chemistry, 1983). The 96- hour LC50 for rainbow trout and bluegill sunfish is greater than 1,000 mg/l (Worthing, 1983). The formulated product Saprol is of low hazard to fish and aquatic invertebrates. Both rainbow trout and bluegill sunfish had a 96-hour LC50 of greater than 500 mg/l. The aquatic invertebrate *Daphnia* (water flea) had a 48-hour EC50 of greater than 25 mg/l. Saprol was also noted to be of low hazard to *Scenedesmus subspicatus* (aquatic algae). The 96-hour EC50 was greater than 380 mg/l.

Effects on Other Animals (Nontarget species)

No toxic effect was observed in honeybees at less than or equal to 1,000 mg/kg diet (Worthing, 1983). Triforine and the formulated product Saprol are considered of low hazard to honeybees and to the predatory mite *Typhlodromus pyrii*. It is also of low hazard to earthworms at recommended dose rates (Meister et al., 1994).

3.6 Aliphatic aldehydes Fungicides

Several aliphatic aldehydes are used as fungicides, amongst them formaldehyde and formaldehyde releasers, which have been considered in detail elsewhere (Feinman, 1988).

3.6.1 Toxicology of Acrolein

Acrolein reacts with SH groups. It is formulated as a liquid (fig. 9).

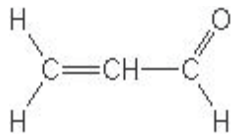

Fig. 9. Acrolein.

Toxicology

a. **Acute toxicity**

The rat oral LD50 is 29mg=kg and the mouse oral LD50 is 13.9mg=kg (males) and 17.7mg=kg (females). The percutaneous rabbit LD50 is 231mg=kg. By inhalation the rat 1-h LC50 is 65mg=m3 (males) and 60mg=m3 (females): the rat 4-h LC50 is 18.5mg=m3 (males) and 22mg=m3 (females). The rat 30-min LC50 is 131 ppm and the 10-min LC50 is 355 ppm (Ballantyne et al., 1989).

b. **Short-term and subchronic toxicology**

Hamsters, rats, and rabbits were exposed to acrolein vapor at 0, 0.4, 1.4, and 4.9 ppm for 6 h/day for 5 days/week for 13 weeks. At 4.9 ppm there was mortality, ocular and nasal irritation, depression of growth, and inflammation, necrosis, hyperplasia, and metaplasia of the respiratory tract epithelium. A no-effect concentration was not established for the rat (Feron et al., 1978).

c. **Chronic toxicology**

Sprague-Dawley rats were given acrolein by gavage at daily dosages of 0, 0.05, 0.5, and 2.5mg/kg up to 102 weeks. The only effects noted were decreased serum creatinine kinase and increased early cumulative mortality. There were no significant increases in neoplastic or non-neoplastic histopathology (Parent et al., 1992a).

Carcinogenicity

Given by intraperitoneal injection to male Fischer 344 rats, Acrolein had an initiating activity for urinary bladder carcinogenesis (Cohen et al., 1992).

Reproductive toxicology

Male and female rats were incubated and given 70 daily doses of Acrolein at 0, 1, 3, or 6mk=kg. The F0 generation was assigned to a 21-day period of co-habitation and dosing of females continued through co-habitation, gestation, and lactation. F1 generation pups were similarly treated. In general, reproductive indices were unaffected, with the exception of reduced pup weights in the F1 generation at the high dose. Gastric lesions were consistently found in the high dose and some mid-dose animals; erosions of the glandular mucosa and hyperplasia=hyperkeratosis of the fore stomach were the most frequent lesions. Relative to the controls, mortality and body weight gain decreases were noted for high dosage animals (Parent et al., 1992b).

3.7 Biological Fungicides (Biofungicides)

Biofungicides are microorganisms (microbial pesticides) and naturally occurring substances that control diseases (biochemical pesticides) that are approved for organic production. Biofungicides are widely used by organic vegetable growers to control selected foliar and soilborne diseases of vegetable crops (see Table 5). Biofungicides can be applied as a stand-alone treatment to control a target disease, provided the application is made before the disease starts (Francis and Keinath, 2010). In the case of a biofungicide, the Latin name of the microbe that it contains is the generic name of the fungicide.

3.6.1 Toxicology of EcoGuard

EcoGuard is a concentrated suspension of spores of Bacillus licheniformis SB3086 that has been found effective as a natural inhibitor of a variety of agronomically important fungal diseases - particularly dollar spot and anthracnose. EcoGuard allows you to control the disease and significantly improve overall turf quality at the same time. The activity of EcoGuard is due to the synthesis of powerful anti-fungal compounds that inhibit fungal growth. As a primary benefit, EcoGuard can be used just like other fungicides. As a secondary benefit, when EcoGuard is integrated with conventional turf management practices, you will see a noticeable and often dramatic improvement in the health and vigor of your turf. In addition, the turf will also recover more quickly from diseases with improved color and increased density in the damaged areas (Novozymes Biologicals Inc., 2007).

B. licheniformis SB3086 is a naturally occurring, ubiquitous bacterium originally isolated from United States farm soil. Consequently, the United States Environmental Protection Agency (USEPA) required limited data for federal registration of EcoGuardTM Biofungicide. The data from acute toxicity/pathogenicity studies on the active ingredient indicate that Bacillus licheniformis SB3086 is not toxic, infective or pathogenic via the oral or inhalation routes of exposure (tested at 1 x 108 Colony Forming Units (CFU) per animal), or via intravenous injection (tested at 1x10' CFU/animal). The end product was not very acutely toxic via oral, dermal, or inhalation routes of exposure. It was also not irritating to the eyes (tested on rabbits) or a dermal sensitizer (tested on guinea pigs), but was a slight dermal irritant (tested on rabbits).

Product	Active Ingredient	Disease	Treatment Site
Ballad	*Bacillus pumilus*	Several (Foliar)	Foliar
Bio-Save	*Pseudomonas syringe*	Post-harvest	Irish and sweet potatoes in storage
Contans	*Coniothyrium minitans*	White Mold	Soil applied
Kodiak	*Bacillus subtilis*	*Pythium, Rhizoctonia, Fusarium*	Seed treatment, beans only
Mycostop	*Streptomyces griseoviridis*	Several	Greenhouse; Soil applied
Regalia	Plant extract	Powdery mildew	Foliar
RootShield Granules, RootShield WP	*Trichoderma harzianum*	*Pythium, Rhizoctonia, Fusarium*	Soil applied
Serenade	*Bacillus subtilis*	Powdery mildew, other foliar diseases	Foliar
T22-HC	*Trichoderma harzianum*	*Pythium, Rhizoctonia, Fusarium*	Soil applied
Surround	Kaolin	Powdery mildew	Foliar
Trilogy	Neem Oil	Powdery mildew	Foliar
Actinovate AG	*Streptomyces lydicus*	*Fusarium, Rhizoctonia, Pythium, Phytophthora*	Soil applied Foliar
SoilGard	*Gliocladium virens*	Damping off	Greenhouse-transplants, soil applied

Table 5. List of biofungicides (biological) used to control selected vegetable crop diseases (Francis and Keinath, 2010)

The USEPA waived the requirement for subchronic, chronic, developmental, reproductive toxicity, genotoxicity and oncogenicity studies for federal registration of EcoGuardTM Biofungicide. Instead, the USEPA used reports from the scientific literature to evaluate this product. The data from these reports suggest that *B. licheniformis* is occasionally associated

with infections in individuals who have significant preexisting health problems such as severe immune system depression, cancer or trauma. In addition, it has been associated with reproductive failures (spontaneous abortions and inflammation of the placenta) in cattle, sheep and swine, usually in association with the ingestion of moldy hay. A search of the toxicological literature did not find any additional significant information on *B. licheniformis*.
A quantitative worker risk assessment was not provided in the registration package. However, *B. licheniformis* has been used in various industrial fermentation processes for a number of years and, according to the registrant, no pathogenicity, toxicity or hypersensitivity has been reported among these workers. Given the use pattern of the EcoGuardTM product, exposure of applicators would likely be less than that of fermentation workers. Also, the product label requires applicators and other handlers to wear a long-sleeved shirt, long pants, and shoes plus socks. In addition, handlers must also use a non-powered air purifying NIOSH approved respirator.
The limited toxicity data required to support the federal registration of EcoGuardTM Biofungicide indicate that this product is not very toxic following acute exposures. The active ingredient B. licheniformis also appears to have a low degree of pathogenicity and infectivity to animals and humans. Although there are no animal study data on longer-term exposure, significant risks to workers or the general public from EcoGuardTM use are not expected given the use pattern and the required personal protective equipment. The required personal protective equipment also should protect against the slight dermal irritation that EcoGuardTM may cause.

Ecological Risk

B. licheniformis SB3086 is not toxic, infective, or pathogenic to mammals when administered orally, by inhalation, direct tracheal injection, dermally, and by intravenous injection. A significant decrease in weight gain in young mallard ducks was observed when administered EcoGuardTM formulation at a rate of approximately 1.0 ml/kg body weight. The effect was attributed to formulation ingredients other than the active ingredients. B. licheniformis SB3086 appears not to be toxic, infective, or pathogenic to mallards; there were no mortalities.
The 30-day EcoGuardTM rainbow trout LC50 is greater than 1.1 xl06 CFU/ml which is roughly I I 7X (times) the expected environmental concentration (EEC) when the maximum application rate is applied directly to the surface of six-inch deep water body. The daphnia No Observable Adverse Effect Concentration (NOAEC) is 120X the EEC. There was no sign of infection or pathogenicity in either study.
The EcoGuardTM formulation had no effect on honeybee larva when exposed to *B. licheniformis* SB3086 at 1.6 x106 CFU/ml, roughly 2/3 full strength formulation, in their diet. No adverse behavioral or developmental abnormalities were observed in emerged adult honeybees that had been exposed as larva. All marine/estuarine organism and nontarget plant testing was waived by the USEPA.
Bacillus licheniformis is a ubiquitous soil organism. While numbers of *B. licheniformis* in soil are unknown and likely vary from soil to soil, the total number of Bacillus organisms is estimated to be 10' CFU/g soil. The added soil density of *B. licheniformis* from the proposed use rates would be 0.42% to 1.5%. This is a very small proportion of the naturally occurring bacilli in soil and is not expected to add substantially to the effects of the normally occurring Bacillus populations. No adverse effects to fish or wildlife resources are expected from use of EcoGuardTM Biofungicide when used as labeled. There are reports in the literature of B.

licheniformis being a sporadic mammal pathogen. *B. licheniformis* diseases appear to be limited to cows, sheep, and swine as very unusual events associated with the ingestion of moldy hay. Wild ruminants exposure to the combination of conditions seemingly implicated in livestock disease should be minimal. The IBA contained in EcoGuardTM poses no risk to fish or wildlife resources: The IBA concentration in EcoGuardTM is very low, application at the maximum label rate results in an IBA application rate of roughly 30 milligrams/acre (Serafini, 2003).

4. Conclusion

It is to note that this study showed a less or more toxicity of all category of chemical fungicides, contrarily, to biofungicides that showed a little or note side effects on human and ecosystems.

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19

Interactions of Fungicides and Pesticides with Specific Enzymes

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1. Introduction

This chapter concerns influences of fungicides and pesticides on specific enzymes of various living systems. There is a growing interest on enzyme systems and environmental factors affecting them in the field of biochemistry and molecular biology. As known, fungicides, pesticides and other chemicals can enter rain water, food, irrigation water or rivers in many cases, and may be hazardous for living systems. Many chemical substances including fungicides, pesticides, drugs and metal ions influence metabolism at very low concentrations by altering enzyme activities and disrupting physiological balances. Many biocides are known to interfere with a number of processes as they have neurotoxic, hematotoxic, genotoxic, hepatic and renal effects on vertebrates (Hayes, 1990; Pretty & Hine, 2005; Ecobichon, 1996; WHO, 1967; Eisler, 1996). Although there are numerous examples of applications of widely used pesticides and fungicides, little is known about their effects on specific enzymes in organisms and there is a serious lack of data and information on exposures, effects and biological evaluation that connect them. Whereas the effects of several factors on enzyme levels and activity is reasonably well appreciated, the effects of xenobiotic exposure on specific enzyme systems have not received substantial review yet. Relevant xenobiotics are derived from pharmaceutical, nutraceutical and environmental exposure, and many of the mechanisms involved are highly complex in nature, not easily predictable from existing *in vitro* tests and do not always predict well from *in vivo* animal models. After a detailed review of enzymes, fungicides and pesticides, a framework for considering the different levels of direct and indirect modulation by xenobiotics is developed herein, and areas that still require further investigation are highlighted. It is anticipated that this chapter may help explain some of the variation in levels of specific enzymes, guide the direction of long-term drug/nutraceutical safety trials, and stimulate ideas for future research.

2. Enzymes

2.1 Description

Enzymes are proteins which catalyze biochemical reactions in high yields. The molecules at the beginning of the enzymatic process are called substrates, and the enzyme converts them

into different molecules, called the products. Enzymes are required in almost all processes in a biological cell at significant rates. Enzymes are very selective for their substrates and speed up only a few reactions from among many possibilities. Thus, the set of enzymes made in a cell determines which metabolic pathways occur in the cell. Enzymes lower the activation energy of a reaction like all catalysts, thus they dramatically increase the reaction rates. Most enzyme reaction rates are much faster (millions of times) than those of un-catalyzed reactions. Since enzymes are catalysts, they are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Enzymes are known to catalyze about 4,000 biochemical reactions (Bairoch, 2000). The macromolecular components of almost all enzymes are composed of proteins, except for a class of RNA modifying catalysts known as ribozymes. Many enzymes consist of a protein and a non-protein (called the cofactor). The proteins in enzymes are usually globular. The intra- and intermolecular bonds that hold proteins in their secondary and tertiary structures are disrupted by changes in temperature and pH. This affects shapes and so the catalytic activity of an enzyme is pH and temperature sensitive. Enzymes are found in all tissues and fluids of the body. Intracellular enzymes catalyze the reactions of metabolic pathways. Plasma membrane enzymes regulate catalysis within cells in response to extracellular signals, and enzymes of the circulatory system are responsible for regulating the clotting of blood. Almost every significant life process is dependent on enzyme activity. Enzymes are classified on the basis of their composition. Those composed wholly of proteins are known as simple enzymes in contrast to complex enzymes, which are composed of protein plus a relatively small organic molecule. Complex enzymes are also known as holoenzymes. In this terminology the protein component is known as the apoenzyme, while the non-protein component is known as the coenzyme or prosthetic group where prosthetic group describes a complex in which the small organic molecule is bound to the apoenzyme by covalent bonds; when the binding between the apoenzyme and non-protein components is non-covalent, the small organic molecule is called a coenzyme. Many prosthetic groups and coenzymes are water-soluble derivatives of vitamins. Although enzymes are highly specific for the kind of reaction they catalyze, the same is not always true of substrates they attack. Generally, enzymes having broad substrate specificity are most active against one particular substrate.

2.2 Enzymatic catalysis

Catalysis of biochemical reactions in the cell is vital because reaction rates of the uncatalysed reactions are much lower. The mechanism of enzymatic catalysis and other types of chemical catalysis are in principle similar. The enzyme reduces the energy required to reach the highest energy transition state of the reaction by providing an alternative reaction route and by stabilizing intermediates. The reduction of activation energy (Ea) increases the number of reactant molecules with enough energy to reach the activation energy and form the product. In order for a reaction to occur, reactant molecules must contain sufficient energy to cross a potential energy barrier, the activation energy. All molecules possess varying amounts of energy depending, for example, on their recent collision history but, generally, only a few have sufficient energy for reaction. The lower the potential energy barrier to reaction, the more reactants have sufficient energy and, hence, the faster the reaction will occur (Bender, 1964). All catalysts, including enzymes, function by forming a transition state, with the reactants, of lower free energy than would be found in the uncatalysed reaction. Even quite modest reductions in this potential energy barrier may

produce large increases in the rate of reaction. There are a lot of mechanisms by which activation energy decrease may be achieved. The most important of these involves the enzyme initially binding the substrate(s), in the correct orientation to react, close to the catalytic groups on the active enzyme complex and any other substrates (Chaplin, 1986). In this way the binding energy is used partially in order to reduce the contribution of the considerable activation entropy, due to the loss of the reactants' (and catalytic groups') translational and rotational entropy, towards the total activation energy. Other contributing factors are the introduction of strain into the reactants, provision of an alternative reactive pathway and the desolvation of reacting and catalysing ionic groups. The energies available to enzymes for binding their substrates are determined primarily by the complementarity of structures. The specificity depends upon minimal steric repulsion, the absence of unsolvated or unpaired charges, and the presence of sufficient hydrogen bonds. These binding energies are capable of being quite large. However, enzymes do not use this potential binding energy simply in order to bind the substrate(s) and form stable long-lasting complexes. If this were to be the case, the formation of the transition state between enzyme-substrate and enzyme-product would involve an extremely large free energy change due to the breaking of these strong binding forces, and the rate of formation of products would be very slow. They must use this binding energy for reducing the free energy of the transition state. This is generally achieved by increasing the binding to the transition state rather than the reactants and, in the process, introducing an energetic strain into the system and allowing more favourable interactions between the enzyme's catalytic groups and the reactants. A description of several ways enzyme action may be affected as follows.

Salt concentration: If the salt concentration is close to zero, the charged amino acid side chains of the enzyme molecules will attract each other. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions will occur, and again the enzyme will precipitate. An intermediate salt concentration such as that of human blood (0.9%) or cytoplasm is the optimum for many enzymes.

pH: pH is a logarithmic scale that measures the acidity or H^+ concentration in a solution. The scale runs from 0 to 14 with 0 being highest in acidity and 14 lowest. When the pH is in the range of 0-7, a solution is said to be acidic; if the pH is around 7, the solution is neutral; and if the pH is in the range of 7-14, the solution is basic. Amino acid side chains contain groups such as -COOR and $-NH_2$ that readily gain or lose H^+ ions. As the pH is lowered an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^+ ions and eventually lose its active shape. Many enzymes perform optimumly in the neutral pH range and are denatured at either an extremely high or low pH. Some enzymes, such as pepsin, which acts in the human stomach where the pH is very low, have a low pH optimum.

Temperature: Generally, chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme-catalyzed reaction is raised still further, a temperature optimum is reached; above this value the kinetic energy of the enzyme and water molecules is so great that the conformation of the enzyme molecules is disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of changing the conformation of more

and more enzyme molecules. Many proteins are denatured by temperatures around 40-50 ^{0}C, but some are still active at 70-80 ^{0}C, and a few even withstand boiling.

Modulators: Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction it is an activator, and if it decreases the reaction rate it is an inhibitor. These molecules can regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the -S-S- bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change its shape or block it. Many well-known poisons such as potassium cyanide and curare are enzyme inhibitors that interfere with the active site of critical enzymes.

2.3 Enzyme inhibition

A number of substances may cause a reduction in the rate of an enzyme catalysed reaction. Some of these are non-specific protein denaturants. Others, which generally act in a fairly specific manner, are known as inhibitors. Loss of activity may be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the timescale of interest. Many drugs and poisons are enzyme inhibitors. If the inhibited enzyme is totally inactive, irreversible inhibition behaves as a time-dependent loss of enzyme concentration, in other cases, involving incomplete inactivation, there may be time-dependent changes in both Km and Vmax. More important for most enzyme-catalysed processes is the effect of reversible inhibitors. These are generally discussed in terms of a simple extension to the Michaelis-Menten reaction scheme (Michaelis & Menten, 1913).

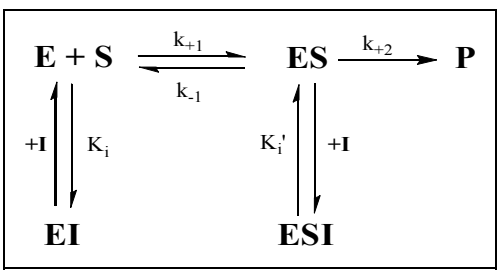


Fig. 1. Reaction scheme E: enzyme, S: substrate, I: inhibitor, P: product

I represents the reversible inhibitor and the inhibitory (dissociation) constants K_i and K_i' are given by

$$K_i = \frac{[E][I]}{[EI]} \qquad K_i' = \frac{[ES][I]}{[ESI]}$$

For the present purposes, it is assumed that neither EI nor ESI may react to form product. Equilibrium between EI and ESI is allowed, but makes no net contribution to the rate equation as it must be equivalent to the equilibrium established through:

$$EI + S \rightleftharpoons E + S + I \rightleftharpoons ES + I \rightleftharpoons ESI$$

In competitive inhibition, K_i' is much greater than the total inhibitor concentration and the ESI complex is not formed. This occurs when both the substrate and inhibitor compete for binding to the active site of the enzyme. The inhibition is most noticeable at low substrate concentrations but can be overcome at sufficiently high substrate concentrations as the Vmax remains unaffected. Normally the competitive inhibitor bears some structural similarity to the substrate, and often is a reaction product (product inhibition), which may cause a substantial loss of productivity when high degrees of conversion are required. A similar effect is observed with competing substrates, quite a common state of affairs in industrial conversions, and especially relevant to macromolecular hydrolyses where a number of different substrates may coexist, all with different kinetic parameters. The reaction involving two co-substrates may be modelled by the scheme (Cornish-Bowden, 1974).

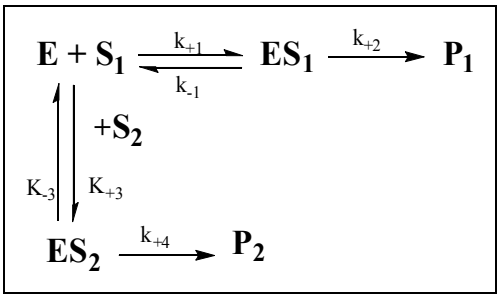


Fig. 2. Reaction scheme for two substrates

Both substrates compete for the same catalytic site and, therefore, their binding is mutually exclusive and they behave as competitive inhibitors of each others reactions. In, uncompetitive inhibition K_i is much greater than the total inhibitor concentration and the EI complex is not formed. This occurs when the inhibitor binds to a site which only becomes available after the substrate (S_1) has bound to the active site of the enzyme (Cornish-Bowden, 1976). This inhibition is most commonly encountered in multi-substrate reactions where the inhibitor is competitive with respect to one substrate (e.g. S_2) but uncompetitive with respect to another (Cornish-Bowden et al., 1978) (e.g. S_1), where the reaction scheme may be represented by:

$$E + S_1 \rightleftharpoons ES_1 + S_2 \rightleftharpoons ES_1S_2 \longrightarrow \text{product}$$

$$E + S_1 \rightleftharpoons ES_1 + I \rightleftharpoons ES_1I \not\longrightarrow$$

The inhibition is most noticeable at high substrate concentrations (i.e. S_1 in the scheme above) and cannot be overcome as both the V_{max} and K_m are equally reduced (Cornish-Bowden & Endrenyi, 1986). A special case of uncompetitive inhibition is substrate inhibition which occurs at high substrate concentrations in about 20% of all known enzymes (e.g. invertase is inhibited by sucrose). It is primarily caused by more than one substrate molecule binding to an active site meant for just one, often by different parts of the substrate molecules binding to different subsites within the substrate binding site (Crompton & Waley, 1986; Fersht, 1985). If the resultant complex is inactive this type of inhibition causes a reduction in the rate of reaction, at high substrate concentrations. It may be modelled by the following scheme:

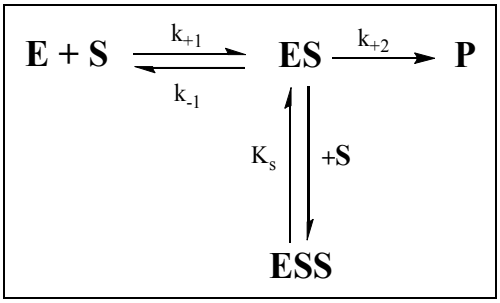


Fig. 3. Reaction scheme for high substrate concentrations

In noncompetitive inhibition, both the EI and ESI complexes are formed equally well (i.e. K_i equals K_i'). This occurs when the inhibitor binds at a site away from the substrate binding site, causing a reduction in the catalytic rate. It is quite rarely found as a special case of mixed inhibition (Henley & Sadana, 1985; Hill et al., 1977; Koshland, 1962). The fractional inhibition is identical at all substrate concentrations and cannot be overcome by increasing substrate concentration due to the reduction in Vmax.

3. Fungicides

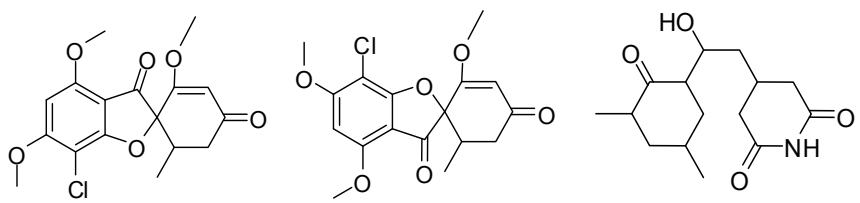
3.1 Description

Fungicides are chemical compounds or biological organisms used to kill or inhibit fungal spores or fungi which can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. They are used both in agriculture and to fight fungal infections in animals. Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue, only protect the plant where the spray is deposited; translaminar fungicides redistribute the fungicide from the upper, sprayed leaf surface to the lower, unsprayed surface; systemic fungicides are taken up and redistributed through the xylem vessels to the upper parts of the plant. New leaf growth is protected for a short period. Most fungicides that can be bought retail are sold in a liquid form. The most common active ingredient is sulfur, present at 0.08% in weaker concentrates, and as high as 0.5% for more potent fungicides. Fungicides in powdered form are usually around 90% sulfur and are very toxic. Other active ingredients in fungicides include neem oil, rosemary oil, jojoba oil, and the bacterium Bacillus subtilis. Fungicide residues have been found on food for human consumption, mostly from post-harvest treatments (Brooks & Roberts, 1999). Some fungicides are dangerous to human health.

3.2 Structure activity relationship

The advent of organic fungicides was an important milestone in the quest for antifungal compounds capable of exerting a selective effect without damaging the host plant. Selectivity may depend upon differences in biochemistry, cytology, or on differential accumulation. The criticality of the latter process is obvious from the narrow division between fungitoxicity and phytotoxicity in many classes of organic compounds. Protectant fungicides often act through selective accumulation within the pathogen, and many are potentially toxic to both fungal and plant cells, but the latter are protected by the cuticle which acts as a barrier to the passage of foreign chemicals. However, damage can still arise by exceeding a recommended dosage rate or through abnormal climatic conditions

favouring increased absorption. The organic chemist designing candidate fungicides, is also able to achieve a selective margin between fungitoxicity and phytotoxicity by structural modifications affecting partition through barriers such as cuticular and cytoplasmic membranes, by altering molecular size and shape or the lipophilic / hydrophilic balance or hydrogen-bonding capacity. The multiplicity of structural and conformational arrangements of groups of key atoms which are possible in organic compounds has led to important advances in antifungal specificity. This is nowhere more true than with systemic fungicides where it has been variously attributed to structural features of the entire molecule, or to some biologically active centre interacting with specific receptors. Differential fungitoxicity between stereoisomers is known. In griseofulvin, one of the first compounds shown to have systemic antifungal activity (Crowdy et al., 1955), the stereochemistry is critical . Of four isomers resulting from asymmetric centres at carbon atoms 2 and 6', only griseofulvin is active, and the racemic form has only half of the activity of (+)-griseofulvin. The diastereoisomer known as epi(+)-griseofulvin (II) and all transformation products therefrom are inactive.

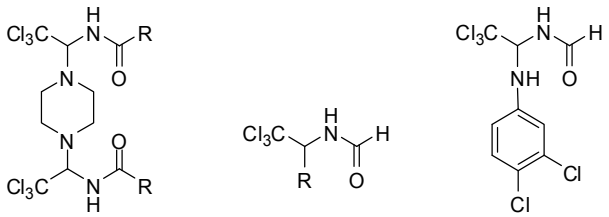


I II III

Fig. 4. Structures of the stereoisomers

Cycloheximide (III) has four asymmetric centres (Fig. 4) (at C-2, C-4, C-6 and C-2'), the compound produced by Streptomyces griseus being 2,6-trans. Probably the simplest example of selective fungitoxicity is that of secbutylamine which has a narrow antifungal spectrum involving a few Penicillium spp. (Eckert et al., 1975), The (-) isomer is considerably more active than the (+) isomer, a fact which cannot be related to selective accumulation by sensitive fungi, and it seems that this selectivity is due to factors closely associated with the mitochondria or pyruvic dehydrogenase which is the site of action. Reasons for the contrasting selectivity of structural isomers are not always obvious. In addition to differing partition parameters, stereochemical or hydrogen-bonding factors are almost certainly involved; additionally special features of the molecule involving particular reactions such as hydrolysis and oxidation are often of considerable importance. Hydrogen bonding is certainly involved in the striking differences that are shown by substituted 2,4- and 2,6-dinitrophenols. Almost without exception the former tend to have high intra- and inter-molecular hydrogen-bonding strengths leading to greater cuticular and epidermal penetration and greater consequent risk of phytotoxicity, whilst the 2,6-isomers are exclusively retained by cuticular waxes. The influence of chemical structure manipulation on biological activity has a fascination for the synthetic organic chemists. Interest in substituted formamides was stimulated after the systemic fungicidal activity of triforine (IV, R=H) was discovered (Ost et al., 1969). Carter et al. (1972) showed that the piperazine moiety was not essential for systemic activity and found that the compound (V, R=methoxy) controlled E. graminis when applied to roots although it was poorly fungistatic or

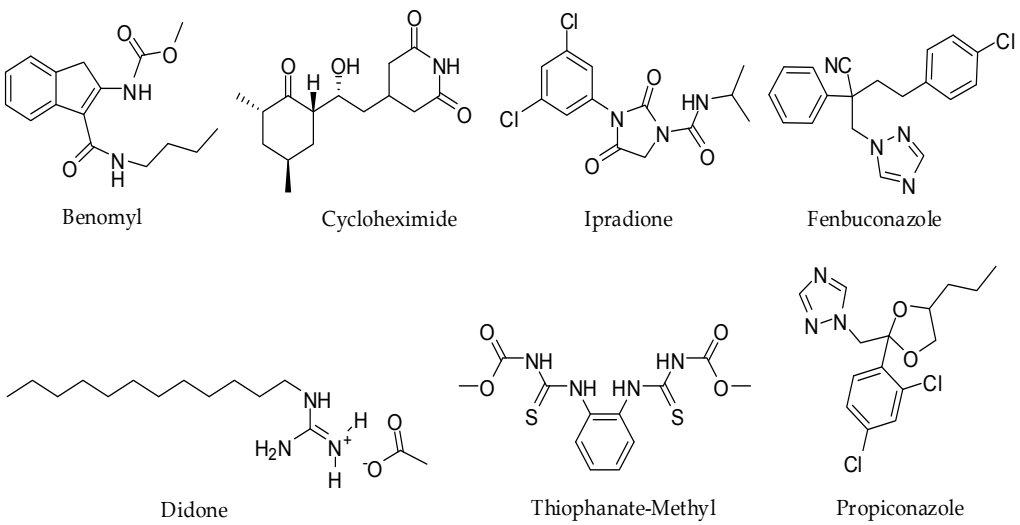
protectant. They subsequently tested about a hundred related compounds and found that the alkoxy and alkylamino analogues (VI, R=alkoxy or alkylamino) were more active systemically than the corresponding alkylthio compounds, and that activity appeared to be greater with the C_3 and C_4 members than with the methyl and ethyl analogues (Fig. 5).



IV V VI

Fig. 5. Structures of the analogues

The work of Brown and Woodcock (1975) revealed further structural specificities. Thus the basic structure (XVI, R=H) was inactive in both leaf-spray and root-drench tests against E. graminis on barley (EDs0 >400 x I0 "s M) but the phytotoxicity at the leaf tips of root-drenched plants suggested that translocation was not prevented. The tribromomethyl analogue exhibited some activity in the root-drench test which suggested that the trihalomethyl group contributed to fungitoxicity by way of lipophilic or inductive-electronic effects rather than through specific receptors. In general their results indicated little correlation between activity in leaf-spray and root-drench tests, thus emphasising that these modes of application had different structural requirements. One structural feature common to the triforine and chloraniformethan molecules that seems significant is the imino group. That the imino hydrogen atom does not appear to be critical , however, seems likely from the comparisons, although steric and conformational factors could account for inactivity . The structures of some widely used fungicides are provided below (Fig. 6, Fig.7).



Benomyl Cycloheximide Ipradione Fenbuconazole

Didone Thiophanate-Methyl Propiconazole

Fig. 6. Structures of some widely used fungicides

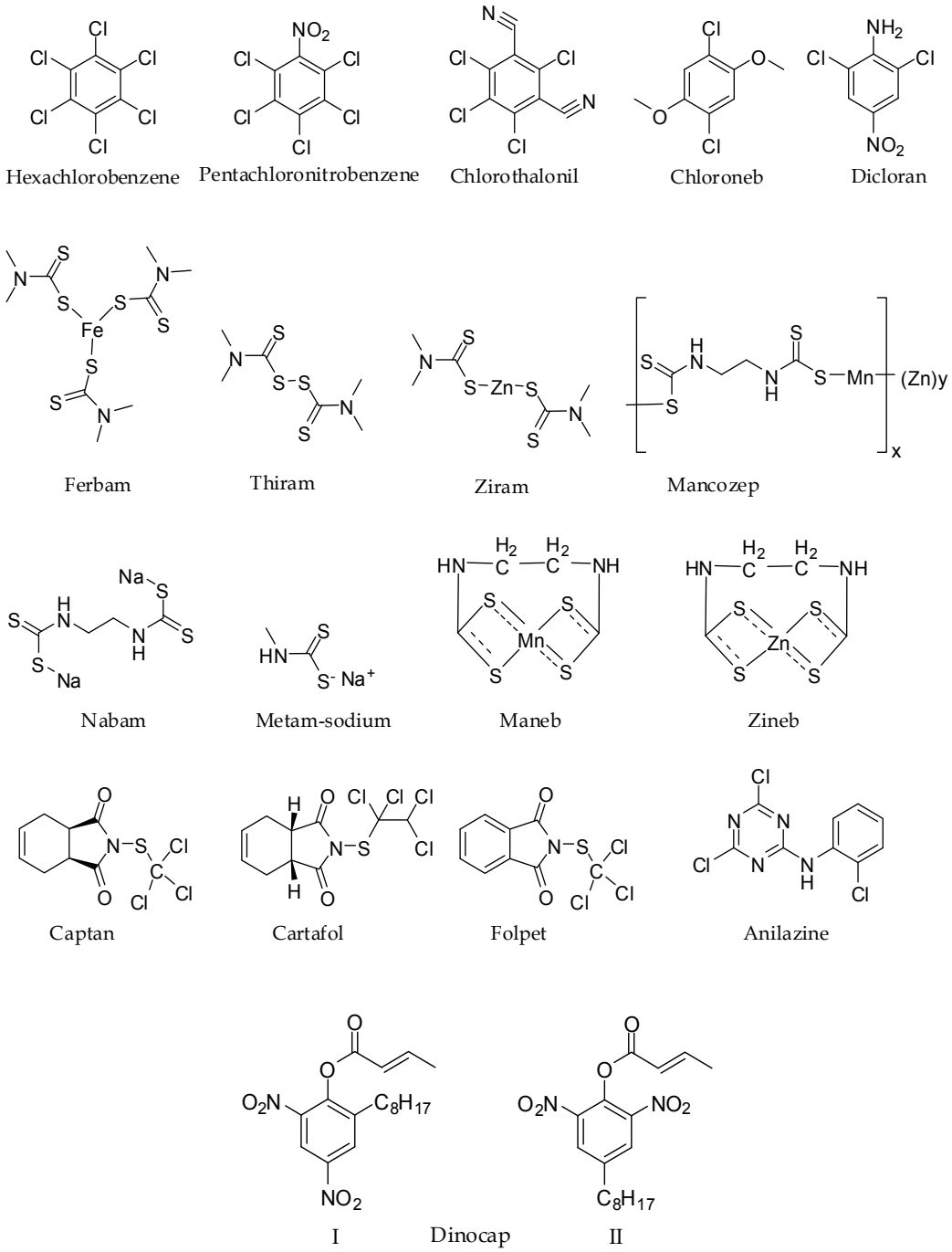


Fig. 7. Structures of some widely used fungicides

4. Pesticides

4.1 Description

Pesticides are substances or mixtures of substances used for preventing, destroying, repelling or mitigating any pest. A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. Pests include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes, and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance.

4.2 Structure activity relationship

Pesticides can be grouped according to chemical structure. Pesticides with similar structures have similar characteristics and usually have a similar mode of action. Most pesticide active ingredients are either inorganic or organic pesticides. From a scientific view, inorganic pesticides do not contain carbon and are usually derived from mineral ores extracted from the earth. Examples of inorganic pesticides include copper sulphate, ferrous sulphate, copper and sulphur. Organic pesticides contain carbon in their chemical structure. Most organic compounds are created from various compounds, but a few are extracted from plant material and are called 'botanicals'. Examples of organic pesticides include: captan, pyrethrin, and glyphosate. Organic pesticides with similar structures are grouped into families of chemicals. Prominent insecticide families incorporates organochlorines, organophosphates, and carbamates. Organochlorine hydrocarbons (e.g. DDT) could be separated into dichlorodiphenylethanes, cyclodiene compounds, and other related compounds. They operate by disrupting the sodium/potassium balance of the nerve fiber, forcing the nerve to transmit continuously. Their toxicities vary greatly, but they have been phased out because of their persistence and potential to bioaccumulate (Kamrin, 1997) Organophosphate and carbamates largely replaced organochlorines. Both function through inhibiting acetylcholinesterase enzyme, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis. Organophosphates are quite toxic to vertebrates, and have in some cases been replaced by less toxic carbamates (Kamrin, 1997). Thiocarbamate and dithiocarbamates are subclasses of carbamates. Prominent families of herbicides include phenoxy and benzoic acid herbicides (e.g. 2,4-D), triazines (e.g. atrazine), ureas (e.g. diuron), and Chloroacetanilides (e.g. alachlor). Phenoxy compounds tend to selectively kill broadleaved weeds rather than grasses. The phenoxy and benzoic acid herbicides function similar to plant growth hormones, and grow cells without normal cell division, crushing the plants nutrient transport system Triazines interfere with photsynthesis (Kamrin, 1997). The structures of some widely used pesticides are provided below (Fig. 8).

5. Interactions of fungicides and pesticides with enzymes

5.1 Modulation of the enzyme activity

Many chemicals affect the activity of specifif enzymes both *in vitro* and *in vivo* (Coban et al., 2008). For instance, medical drugs (Alici et al., 2008; Ekinci et al., 2007a), metal ions (Ekinci et al., 2007b; Tekman et al., 2008), pesticides and fungicides (Senturk et al., 2009; Ceyhun et al., 2010a) generally inhibit the enzymes at very low concentrations (Ekinci & Beydemir, 2010a). These inhibitions could be very dangerous in some cases (Gulcin et al., 2008; Ekinci

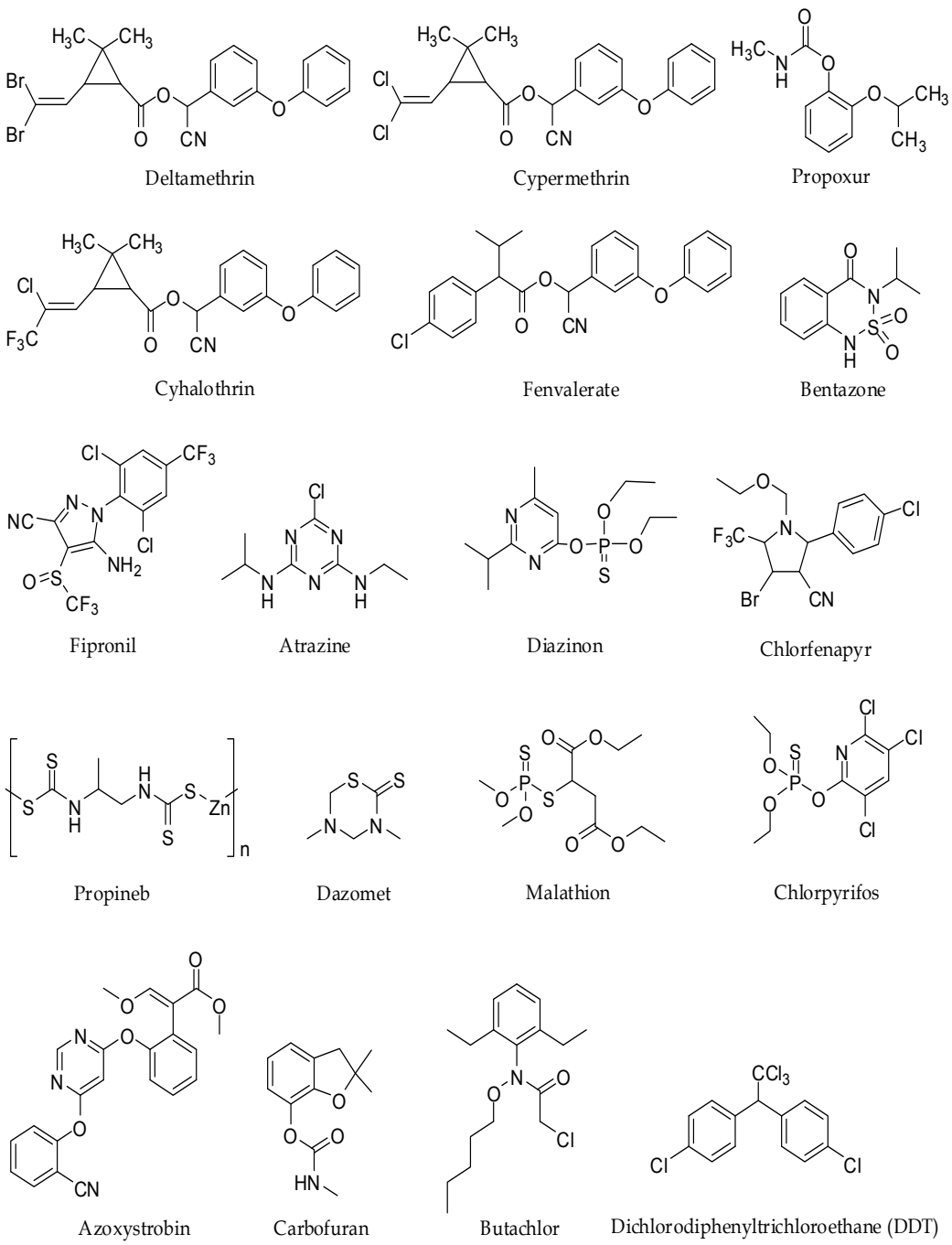


Fig. 8. Structures of some widely used pesticides

& Beydemir 2009a,b) whereas some enzyme inhibitors could be used for the treatment of several diseases (Bayram et al., 2008; Senturk et al., 2009a,b; Coban et al., 2009; Ekinci et al., 2010; Alp et al., 2010). Although investigation of the alterations in the activity of several enzymes have gained considerable attention over the past years (Coban et al., 2007; Ciftci et al., 2008), there is still a deep need of understanding the influences of pesticides and fungicides on specific enzyme systems. We have recently investigated the inhibitory effects of mancozeb, cypermethrin, deltamethrin and dinocap on the pH regulatory enzyme carbonic anhydrase (CA) from rainbow trout (Ekinci & Beydemir, 2010b).The physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-; therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis. In addition, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system (CNS) and inner ear. Our findings indicated that pesticides and fungicides dose-dependently decreased *in vitro* carbonic anhydrase activity at micromolar concentrations and that deltamethrin, dinocap, mancozeb and cypermethrin are potent inhibitors for fish carbonic anhydrase enzymes, and might cause undesirable effects with uncontrolled usage by disrupting acid-base regulation as well as salt transport in freshwater or seawater adapted fish. Our results showed that deltamethrin interestingly has a much lower IC_{50} value than cypermethrin, which has a Cl atom instead of a Br. Carbonic anhydrase has Zn^{+2} ion in its active site and it is assumed that electronegative atoms in the inhibitors coordinate to the zinc site at low concentrations. Thus, we concluded that carbonic anyhdrase is very susceptible to alterations in electronegativity of interacting groups. Because deltamethrin was the most powerful inhibitor in *in vitro* experiments, we used it for *in vivo* tests in different doses (0.25 μg/L, 1 μg/L and 2.5 μg/L) and the activities were measured at different time intervals (6^{th},12^{th},24^{th} and 48^{th} hours) for the CA enzymes in rainbow trout tissues (muscle, liver, kidney). For each tissue, inhibition values were calculated and compared with each other. Consequently, deltamethrin inhibited the CA enzymes of rainbow trout tissues with the rank order of muscle > kidney > liver. The pesticides and fungicides were determined to inhibit the CA enzymes of rainbow trout tissues at very low concentrations. The lowest inhibition effect was observed on liver carbonic anhydrase enzyme and we therefore proposed that the inhibitory impact of deltamethrin might be reduced by detoxification enzymes in the liver because detoxification occurs mainly in liver for all living systems (Ekinci & Beydemir, 2010b).

In another study, we aimed to determine the alterations in enzymatic activity of fish antioxidant metabolism in response to deltamethrin administration (Ceyhun et al., 2010b). To this end, three different deltamethrin concentrations (0.25, 1.0, 2.5 μg/L) were administrated to rainbow trout (*Oncorhynchus mykiss*) for different time intervals (6, 12, 24, 48 and 72 h) in order to observe the influences of the pesticide on the activities of glutathione reductase, glucose-6-phosphate dehydrogenase, 6-ghosphogluconate dehydrogenase. Glucose-6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells (such as erythrocytes) by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione in these cells that helps protect the red blood cells against oxidative damage. (Corpas et al., 1998). Glucose-6-phosphate dehydrogenase deficiency is an X-linked recessive hereditary disease. Individuals with the disease may exhibit nonimmune hemolytic anemia in response to a number of causes, most commonly infection or exposure to certain medications or

chemicals. G6PD deficiency is the most common human enzyme defect (Frank, 2005). 6-Phosphogluconate dehydrogenase (6PGD) is the third enzyme of the pentose phosphate metabolic pathway, catalyzing the conversion of 6-PGA (6-phosphogluconate) to D-riboluse-5-phosphate in the presence of $NADP^+$. The reaction, catalyzed by 6PGD, yields NADPH, which protects the cell against oxidant agents by producing reduced glutathione (GSH) (Bianchi et al., 2001; Lehninger et al., 2000). Glutathione reductase (GR; NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2), a flavoprotein, is an important enzyme which catalizes convertion of oxidized glutathione into reduced glutathione. The enzyme uses NADPH as electron donor for the reduction of GSSG. GR enables several vital functions of the cell such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis by maintaining a high ratio of GSH/GSSG (Schirmer et al., 1989; Rendón et al., 2004). We observed that the activities of the enzymes decreased with increasing deltamethrin concentrations and exposure time. The pesticide had greater inhibitory effect on gill enzymes than on muscle, liver and kidney enzymes.

Many environmental pollutants including fungicides and pesticides are capable of inducing oxidative stress in aquatic animals. Oxidative stress occurs as a result of the effect of xenobiotics causing disturbances in antioxidant enzyme systems and, as a result, the oxidative stress resulting from the production of reactive oxygen species (ROS) has gained considerable interest in the field of ecotoxicology (Kappus, 1987; Lemaire et al., 1996). The induction of antioxidant expression by the fungicides and pesticides reflects the activation of defense mechanisms in organisms to counteract ROS toxicity. Antioxidant enzymes, such as GR, G6PD and 6PGD, have major direct or indirect effects on antioxidant systems and they are useful biomarkers because they are involved in regenerating reduced glutathione (GSH) from glutathione disulfide (GSSG). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are indirect antioxidant enzymes in the pentose phosphate pathway and responsible for NADPH production. Because fish tend to adapt to oxidative conditions when exposed to pesticides, fungicides or other pollutants, relatively high levels of GR, G6PD and 6PGD enzymes are expressed in the muscle, liver, kidney, and gills of fish (Stephensen et al., 2000). Nevertheless, because of complex interactions and interrelationships among individual components, the physiological role of these enzymes in the cells is poorly understood. On the other hand, inhibited activity of enzymes caused by exposure to fungicide or pesticide may be due to several reasons; first is production of O_2^- (Bagnasco et al., 2000), second is direct action of fungicides and pesticides on the synthesis of the enzyme (Bainy et al., 1993; Oruç & Uner, 2000), and finally through direct inhibition of enzyme activity both *in vivo* and *in vitro*.

Teisseire and Vernet (2001) showed that the specific activities of enzymes of the Halliwell–Asada pathway, namely ascorbate peroxidase and glutathione reductase, increased after 24 h of exposure to folpet, reaching 155 and 273% of the control level at 96 h, respectively. A fast induction of glutathione S-transferase activity was observed after 6 h of folpet exposure. They were unable to discern whether glutathione S-transferase was involved in folpet metabolism or in peroxide scavenging. The fungicide was also found to stimulate activities of two H_2O_2-scavenging enzymes, catalase and pyrogallol peroxidase. They found the stimulation of catalase was rapid (as early as 12 h after exposure) and strong, since the activity was 252% of the control after 48 h of exposure. According to their data, induction of pyrogallol peroxidase was less important; although it reached 66% at 96 h. The fungicide did not affect guaiacol peroxidase activity. As suggested by the simultaneous and significant

induction (55 to 173%) of antioxidative enzyme defenses of L. minor, generation of reactive oxygen species by the fungicide and involvement of oxidative stress was proposed as a possible mechanism in the phytotoxicity of folpet (Teisseire & Vernet, 2001).
Wu and Tiedemann (2002) reported that two modern fungicides, a strobilurin, azoxystrobin (AZO), and a triazole, epoxiconazole (EPO), as foliar spray on spring barley (Hordeum vulgare L. cv. Scarlett) 3 days prior to fumigation with injurious doses of ozone (150–250 ppb; 5 days; 7 h/day) induced a 50–60% protection against ozone injury on leaves. Fungicide treatments of barley plants at growth stage (GS) 32 significantly increased the total leaf soluble protein content. Additionally, activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were increased by both fungicides at maximal rates of 16, 75, 51 and 144%, respectively. Guiacol-peroxidase (POX) activity was elevated by 50–110% only in AZO treated plants, while this effect was lacking after treatments with EPO. This coincided with elevated levels of hydrogen peroxide (H_2O_2) only in EPO and not in AZO treated plants. The enhancement of the plant antioxidant system by the two fungicides significantly reduced the level of superoxide (O_2^-) in leaves. Fumigation of barley plants for 4 days with non-injurious ozone doses (120–150 ppb, 7 h/day) markedly and immediately stimulated O_2^- accumulation in leaves, while H_2O_2 was increased only after the third day of fumigation. Therefore, O_2^- itself, or as precursor of more toxic oxyradicals, appears to be more indicative for ozone-induced leaf damage than H_2O_2. Ozone also induced significant increases in the activity of antioxidant enzymes (SOD, POX and CAT) after 2 days of fumigation in fungicide untreated plants, while after 4 days of fumigation these enzymes declined to a level lower than in unfumigated plants, due to oxidative degradation of leaf proteins. This was the first report demonstrating marked enhancement of plant antioxidant enzymes and enhanced scavenging of potentially harmful O_2^- by fungicides as a mechanism of protecting plants against noxious oxidative stress from the environment (Wu & Tiedemann, 2002).
Kara and Çelik (1997) investigated the effects of benlate, penncozep, bayleton, cupravit and dithane on human serum enzymes, myocardial creatine kinase (CK-MB), amylase, creatine kinase (CK), aspartate amino transferase (AST), serum glutamyl pruvic transferase (SGPT), alkaline phosphatase (ALK-P), δ-glutamyl transferase (GGT-P) and lactate dehydrogenase (LDH), *in vitro*. They reported that bayleton inhibited only SGPT and it was ineffective on the other seven enzymes. Benlate, penncozep, cupravit and dithane inhibited some enzymes, but activated the others. Benlate was the strongest inhibitor for CK-MB, cupravit for amylase, dithane for ALK-P, penncozep for CK, AST, SGPT and GGT-P. No inhibition was occurred in LDH. Of the fungicides they tested, the most effective one was penncozep whereas the least effective was Bayleton. The most inhibition was shown in SGPT and CK. They considered cupravit as an activator rather than inhibitor (Kara & Çelik, 1997).

5.2 Impact on gene expression

The levels and localization of expression of specific genes is very important for metabolism (Cankaya et al., 2007). In addition to altering enzyme activity, pesticides and fungicides have also strong impacts on expression of several proteins. We demonstrated that deltamethrin causes a significant elevation in the mRNA levels of stress related protein Hsp 70 (heat shock protein 70) in rainbow trout muscles (Ceyhun et al., 2010b). These stress proteins comprise a set of abundant and inducible proteins involved in the protection and

repair of the cell against stress and harmful conditions (Sanders, 1993), therefore, they are very useful biomarkers that have been used to monitor the impact of environmental factors on various animal species, including fish (Lewis et al., 1999). Elevated levels of various heat shock proteins have been measured in tissues of fish exposed to environmental contaminants, such as heavy metals (Williams et al., 1996; Duffy et al., 1999), industrial effluents (Janz et al., 1997; Vijayan et al., 1998), and polycyclic aromatic hydrocarbons (Vijayan et al., 1997; Vijayan et al., 1998). Thus, we evidenced the stress causing effect of deltamethrin.

We have very recently reported the acute and long term influences of deltamethrin on the expression of insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) and growth hormone (GH-I) in rainbow trout muscles (Aksakal et al., 2010). We treated rainbow trouts with different concentrations of deltamethrin (0.25 μg/L, 1 μg/L, 2.5 μg/L) and observed the alterations in mRNA expression levels of IGF-I, IGF-II and GH-I at different time intervals (at 6th, 12th, 24th, 48th, 72th hours and 30th day). The mRNA levels significantly decreased with increasing deltamethrin concentrations thus we demonstrated that deltamethrin has a powerfull impact on the expression of IGF-I, IGF-II and GH-I in rainbow trout which might cause undesirable outcomes not only in growth, but also in development and reproduction. There are similar studies in the literature. Nieves-Puigdoller et al. 2007 reported that exposure to hexazinone (HEX) and atrazine (ATZ), highly mobile and widely used herbicides along rivers in the United States, reduced feeding after 10 days of exposure and had an impaired growth rate in Atlantic salmon. They stated that HEX and ATZ at 10μg l^{-1} exposure had no effect on plasma levels of cortisol, growth hormone (GH), insulin growth factor I (IGF-I). Eder et al. (2008) demonstrated that treatment of juvenile Chinook salmon with chlorpyrifos (CP) and esfenvalerate (EV) led to significantly decreased IGF-I transcription in spleen on days 20 and 60, whereas a short-term increase was seen after CP exposure (day 4). The impact of commonly used pesticides, endosulfan and deltamethrin, on the molecular stress level in black tiger shrimp Penaeus monodon, was investigated using classical oxidative stress biomarkers, protein carbonylation profiles, and levels of heat shock proteins. Results showed that 4 days exposure to 0.1 μg L−1 deltamethrin significantly ($p < 0.05$) increased lipid peroxidation (LPO) level in gills (Dorts et al., 2009).

We also examined whether metallothionein-A (MT-A), metallothionein-B (MT-B) and cytochrome P450 1A (CYP 1A) expressions are induced in response to pesticide administration. For this purpose, we produced muscle metallothionein-A, metallothionein-B and cytochrome P450 1A cDNAs and used quantitative RT-PCR to assay mRNAs in rainbow trout exposed to acute and long-term deltamethrin administration. We observed that deltamethrin exposure significantly ($p<0.05$) increased the expression levels of Cyp1A, MT-A and MT-B in time and dose dependent manner. Polycyclic and halogenated aromatic hydrocarbons (PAHs and HAHs) can enhance the generation of reactive oxygen species (ROS) by inducing cytochrome P450 1A (CYP 1A) *in vivo* and *in vitro*. Metallothionein has been recognised as a useful biomarker for quantifying exposure to heavy metal pollution. Each molecule readily chelates up to seven metal ions through the formation of thiolate bonds with the cysteine residues. It is induced by heavy metals (Cherian & Nordberg, 1983) and its role in the sequestration and detoxification of heavy metals is widely accepted (Vallee, 1979). Metallothionein has been assayed in a range of animals, including many

aquatic organisms, in various tissues. Recently, increased interest has been directed towards the mRNA levels in addition to the levels of the protein itself (Hayes et al., 2004). Although MTs have been widely utilized to identify specific responses to heavy metal pollution, there is now a body of evidence demonstrating that in vertebrates (mammals and fish) MT synthesis is stimulated by different endogenous and exogenous agents (Kägi & Schäffer, 1988), e.g. glucocorticoid hormones, various kinds of stres (cold, heat, extreme exercise), cytokines and in particular compounds leading to production of reactive oxygen species (ROS) (Dalton et al., 1994). Therefore, in mammals and fish, not only inorganic pollutants such as heavy metals but also organic contaminants may activate MT neosynthesis (Wormser & Calp, 1988; Sato et al., 1989; Baumann et al., 1991; Pedrajas et al., 1995). Our study supported the theory that deltamethrin causes a great amount of oxidative stress such that induction of the CYP 1A increases generation of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and hydrogen peroxide, and MT synthesis is stimulated by different endogenous and exogenous agents in particular compounds leading to production of reactive oxygen species (ROS). Our study also contributed to determination of pesticide pollution impact in the freshwater environment and identification of novel inducers of such genes in addition to well known agents (Unpublished data).

6. Conclusion

It is unfeasible to forbid the use of fungicides and pesticides against harmful fungi and pests because of product loss today. Fungicides kill or inhibit fungi or fungal spores which can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. In addition to agriculture, fungicides are also used to fight fungal infections in animals. Similarly, pesticides are used against any pest including insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance. Although there are benefits to the use of fungicides and pesticides, there are also drawbacks, such as potential toxicity to humans and other animals. It is clear from above discussion that some fungicides and pesticides have useful effects on specific organisms, whereas others have significantly hazardous influences. Therefore, the impacts of fungicides and pesticides must be well defined in order to use the best agents in terms of greater effectiveness and less side effects. It is critically important to explore further interactions of biocides in order to detect compounds with different mechanism of action profiles as compared to dangerous ones, and to find novel applications for the usage of these widespread fungicides and pesticides. On the other hand, due to the complexity and immensity of world-wide pollution, there is a compelling need to develop rapid and sensitive screening methods for monitoring the effects and presence of fungicides and pesticides.

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Neurotoxic Effects of Triazole Fungicides on Nigrostriatal Dopaminergic Neurotransmission

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1. Introduction

Azole or conazole fungicides represent a large group of substances widely used agriculturally for the protection of crop plants and pharmaceutically in the treatment of various fungal diseases. They are synthetic compounds that can be classified into *imidazole* or *triazole*, depending on the number of nitrogen atoms in the five-membered ring. Some fungicides from the imidazoles group include, among others, the ketoconazole, miconazole and clotrimazole. Within the group of triazoles we can cite the itraconazole, fluconazole, flusinazole, triadimefon and flutriafol.

In turn, the triazole can be divided into two groups: *triazole antifungal drugs*, that include the fluconazole, voriconazole, isavuconazole, itraconazole, etc., and *triazole plant protection* fungicides, with tebuconazole, triadimefon, triadimenol, paclobutazol and flutriafol as the fungicides most commonly used. Structural formulas of triazole ring and some triazole fungicides are shown in the Fig. 1.

Azole fungicides exert their antifungal activity binding the half of the azole ring to the heme protein and by subsequent inhibition of cytochrome P450 51 (Cyp51), the enzyme that facilitates the 14-alpha-demethylation of lanosterol to ergosterol in mushrooms. Ergosterol is a component of fungal cell membranes, serving the same function that cholesterol serves in animal cells. Ergosterol is necessary to maintain the membrane fluidity and the integrity of the wall of fungal cells (Ghannoum & Rice, 1999).

Triazole fungicides have great importance in agriculture and medicine, being commonly used in different ways and in large quantities throughout the world. The intensive use of these compounds can generate a lot of residues that may potentially lead to substantial environmental contamination. So, triazole residues or triazole metabolites may occur in the environment and should be considered as a risk from food, drinking water and non-occupational exposure.

Despite this large scale use and the risk of exposure of human populations, there are few studies on potential toxic effects of this group of pesticides on biological systems. Most available data are published by regulatory agencies (e.g., Food and Agriculture Organization, Environmental Protection Agency), and the toxicity evaluations have been performed only according to regulatory submission requirements.

In this way, some data available by Environmental Protection Agency - USA (EPA, 2006), show that after an oral administration, the triazole fungicides are quickly absorbed and widely distributed in all evaluated tissues. After this absorption and distribution, the

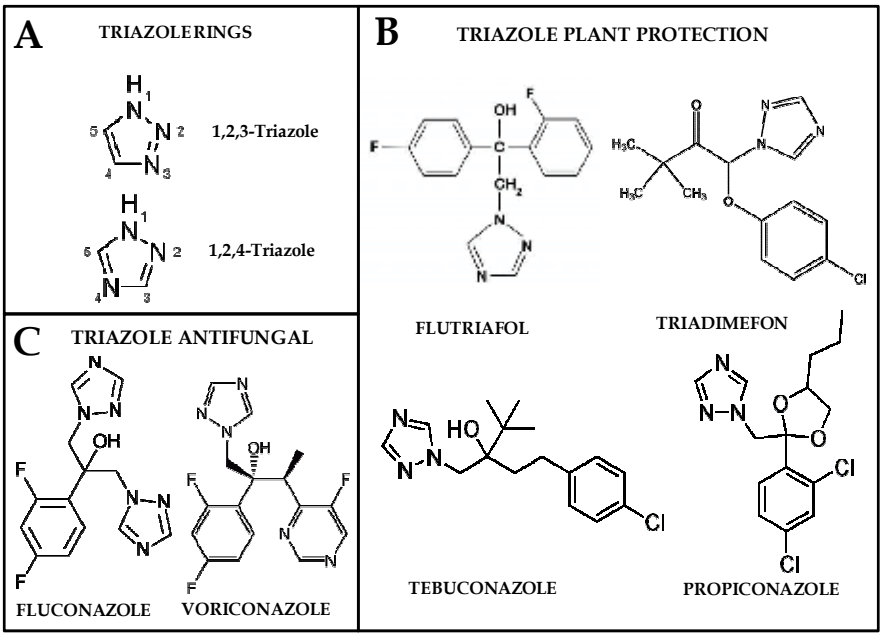

Fig. 1. Structural formulas of some triazole fungicides. A) The two isomers of triazole ring, which characterizes this group. B) and C) Examples of triazole plant protection fungicides and triazole antifungal drugs, respectively.

triazoles are metabolized giving origin to a variety of compounds whose toxic effects are currently being studied. Also according to the EPA (2006), among these compounds, the 1,2,4-triazole, triazole alanine and triazole acetic acid are the most common metabolites of the triazoles found in rat and mouse tissues. Excretion occurs mostly via urine, largely as unchanged parent (80-95%). With an estimated half-life of 8-10 hours, excretion is largely completed within 48 hours of administration of a single dose.

Studies published by EPA (2006) propose that triazoles present various deleterious effects on mammalian biological systems, especially on the nervous system. So, the EPA report shows evidences that exposure to triazoles, in general, causes neurotoxicity, including: neuropathological lesions in rat and mouse brain; neuropathological lesions in rat peripheral nervous system; and decreases in brain weight in several studies in both rats and mice.

However, as described above, in spite of their large use, comprehensive data assessing the effects of triazoles economically relevant on mammalian biological systems, mainly on nervous system, have been quite limited, and in the case of certain triazole fungicides (e.g., flutriafol), almost completely lacking. Because of this, in this chapter we propose to review some effects and possible mechanisms of action of two important triazoles: triadimefon and flutriafol.

Flutriafol ([RS]-2,4'-difluoro-α-[1H-1,2,4-triazol-1-ylmethyl] benzhydryl alcohol) is an economically important agricultural chemical that has proved its effectiveness in controlling several diseases affecting a wide range of crops. It is extremely persistent in the environment and it is accumulated in soil after repeated annual applications. Its residues also present high potential of mobility in the soil.

Flutriafol would likely also be a groundwater contaminant (EPA, 1991). Although the final destination and the behavior of this fungicide in the water have not been precisely evaluated, its use in large quantities and their application on cereals, could be indicate a probably water contamination. This contamination could occur due to the leaching process, taking this pesticide to the interior of the watercourses.

The toxicological effects of flutriafol on biological systems are little described. However, some studies in rats have associated its exposure to a decrease in body weight, ocular damages, decrease in fetal bone formation, hepatotoxicity with alterations of liver volume and hypertrophy, respiratory system irritation, and the suspicion of possible reproductive toxicity with a decrease in female fertility, since it is also considered to be an endocrine disruptor (Zarn et al., 2003).

Despite the fact that the acute oral LD_{50} of flutriafol has been established about 1200 mg/kg in rats, some works also demonstrate that sub-acute administration of low doses of flutriafol (10, 50, and 125 mg/kg) to pregnant rats produces a significant dose-related reduction of fetal ossification in the treated groups. At the dose of 10 mg/kg, incomplete ossification of some skull bones can be noted, and at doses of 50 and 125 mg/kg, the incidence of fetuses with extra ribs is increased (PSD, 1996). Such data could indicate that exposure to low concentrations of flutriafol can cause adverse effects in rats, although neurotoxic effects have not been described until recently.

Another important triazole fungicide, from a toxicological point of view, is the triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)-2-butanone], a broad spectrum, systemic triazole fungicide registered for use on fruits and grains.

Triadimefon is remarkable for the neurobehavioral effects that it induces in rodents. So, it was observed that exposition to low doses of triadimefon (50-100 mg/kg) increased the frequency of locomotion and rearing in rats. Also, exposition to higher doses (200 mg/kg) of triadimefon induced highly stereotyped behaviors and self-mutilation (Crofton et al., 1988, 1989; Perkins et al., 1991; Moser et al., 1995; Walker et al., 1990). It is known that changes in motor activity may occur as a result of neurochemical changes, specifically in the dopaminergic neurotransmission in the nigro-striatal pathway. So, to verify the hypothesis that the triadimefon-induced behavioral effects can be due to an action on dopaminergic system, Crofton et al. (1989) evaluated the effects of combined treatment of triadimefon with either an inhibitor of dopamine synthesis or a dopamine vesicle depletor (reserpine). These authors observed that reserpine partially blocked the increases in motor activity produced by triadimefon, confirming that the fungicide produces its effects acting on the dopaminergic terminal.

Those were the initial studies that confirmed the effects of triadimefon on the dopaminergic system in rodents. Currently, the stimulatory effect on motor behavior of this fungicide has been well characterized and it is well known that this effect is produced by neurochemical changes in dopaminergic neurotransmission in the nigro-striatal pathway.

Based on this information, and considering the commercial importance of the triazole fungicides flutriafol and triadimefon, its persistence in the environment and the correlation between exposure to triazole fungicides and dopaminergic system alterations, the objective of this chapter was to review the effects of these fungicides on the dopaminergic nigro-striatal system. The first part of this chapter includes a review of the physiology of dopaminergic neurotransmission in rat striatum. The second part of this review deals with the *in vitro* and *in vivo* effects of both fungicides on dopaminergic neurotransmission.

2. Dopaminergic neurotransmission

Dopamine is a neurotransmitter related to cognition, motor functions, motivation and reward, emotions, attention, and learning. In the nigrostriatal system, dopamine has a fundamental function in the control of complex movements. Because this, the functions performed by dopamine in the striatum are of great clinical importance and several neurological diseases like Parkinson and Huntington occur as a result of dysfunction in this region. For example, if there is a sharp decline in dopamine levels or its actions are blocked, a syndrome with hypokinesia and muscle rigidity, characteristic of Parkinson's disease, is observed. This is produced because dopaminergic neurons, a smaller proportion of brain neurons, have a large functional significance.

2.1 Synthesis of dopamine

From a biochemical point of view, dopamine is a biogenic amine which belongs to the group of catecholamines. Dopamine is synthesized in the terminal of dopaminergic neurons from tyrosine, a non-essential amino acid, which may come from the diet or synthesized in the liver from phenylalanine. The blood tyrosine reaches the brain through the blood-brain barrier using a specific transport system for neutral amino acids.

Dopamine is synthesized in two enzymatic steps shown in the Fig. 2: the first step is the hydroxylation of tyrosine to form L-dihydroxyphenylalanine (L-DOPA) by the action of cytosolic enzyme tyrosine hydroxylase (EC 1.14.16.2). This stage is the limiting step in the biosynthetic pathway of catecholamines, so that tyrosine hydroxylase is subject to strict control by end product inhibition. The next step in the synthesis is the decarboxylation of L-DOPA to form dopamine by the action of the enzyme DOPA-decarboxylase (EC 4.1.1.28). This enzyme is not a limiting factor in the biosynthesis of catecholamines and it is not specific for the L-DOPA but can decarboxylate other amino acids.

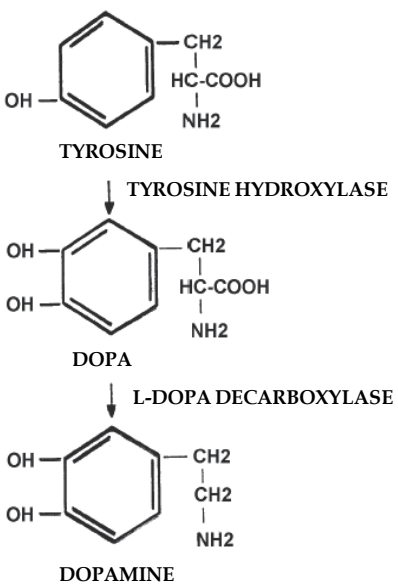

Fig. 2. Scheme of the dopamine biosynthesis.

2.2 Release of dopamine

After its synthesis, dopamine can be stored in synaptic vesicles in the dopaminergic axon terminal. Storage of dopamine is done through a contra-transport mechanism by which dopamine is specifically transported using a membrane transporter into synaptic vesicles following a hydrogen gradient generated by an ATPase.

However, not all dopamine is stored in synaptic vesicles into the dopaminergic terminal. Several experimental evidences indicate the existence of two pools of intracellular dopamine: a *vesicular pool*, consisting mainly of newly synthesized dopamine and a *cytoplasmic pool*, consisting of reuptaked dopamine and newly synthesized dopamine that is not stored in vesicles.

When an action potential reaches the dopaminergic terminal, the change in the membrane potential opens voltage-dependent calcium channels and the entry of these ions into the terminal. The calcium ions induce the migration of the synaptic vesicles to the active zones, its anchorage to the terminal membrane, and the exocytotic dopamine release. Dopamine can also be released by a calcium-independent process, through the dopamine transporter (DAT). After its release, dopamine diffuses into the synaptic space and interacts with specific receptors that can be pre- or post-synaptic.

After the release of dopamine to the synaptic cleft and its interaction with specific receptors, the action of the neurotransmitter may be finished by different mechanisms, as shown in Fig. 3.

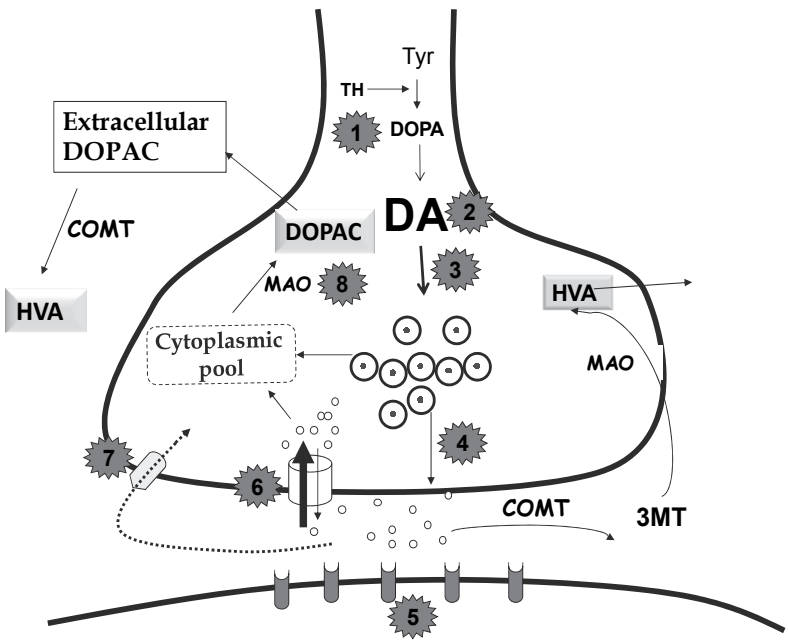

Fig. 3. Dopaminergic synapse. Tyrosine hydroxylase (TH) converts tyrosine to DOPA (1) and DOPA decarboxylase converts DOPA into dopamine (2). Dopamine can be stored in synaptic vesicles (3) and then released (4). Once released, dopamine can bind to post-synaptic receptors (5), be metabolized, reuptaked (6) or join autoreceptors (7). Within the axon terminal, dopamine may be metabolized by mitochondrial monoamine oxydase (MAO).

2.3 Reuptake of dopamine: the dopamine transporter (DAT)

Once released into the cleft synaptic, the synaptic actions of the dopamine can be finalized through three main mechanisms: 1) reuptake or transport of dopamine back to inside of the presynaptic terminal; 2) enzymatic breakdown (see next section) and; 3) diffusion into the cleft synaptic.

The reuptake of dopamine is realized directly by transport of this substance back into the axon terminal together with sodium and chloride ions. This reuptake is carried through the presynaptic membrane by a membrane transporter, the DAT.

The DAT is a glycoprotein of 58-77 kDa with 12 transmembrane domains and a large extracellular loop with N and C terminals to the cytosolic side. This protein can be divided into three regions according to their functional roles. The first five transmembrane domains are related to functions common to all carriers of the same type as, for example, the ionic dependence of transport.

Under certain conditions (for example, altering the composition of the extracellular medium), the DAT can reverse the direction of dopamine transport, going to release dopamine to the extracellular medium rather than reuptake it.

2.3 Catabolism of dopamine

The enzymatic breakdown (catabolism) of dopamine is achieved through the action of two enzymes: monoamine oxydase (MAO) and catechol-O-methyl-transferase (COMT), which differ in their activity and location (Fig. 4).

MAO is an intraneuronal enzyme located in the external mitochondrial membrane and it acts on reuptaked dopamine. COMT is an extraneuronal enzyme that acts on released dopamine.

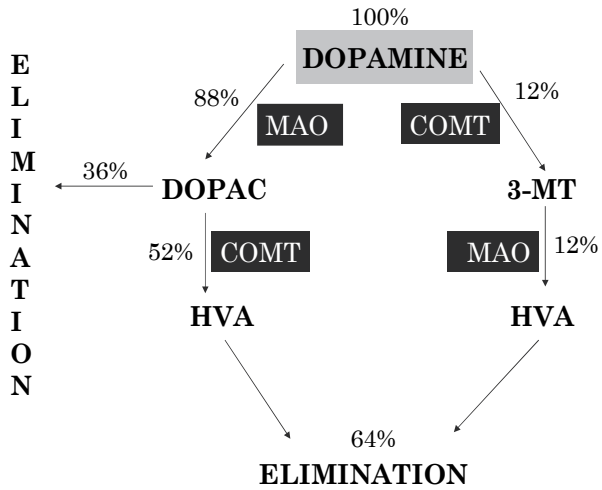

Fig. 4. Scheme of the catabolism (degradation) of dopamine. The numbers indicate the transformation rates in percentage (Adapted from Westerink, 1985).

In presence of molecular oxygen, MAO coverts dopamine into their corresponding aldehydes. The aldehydes generated are rapidly oxidized, producing acidic metabolites, as

the dihydroxyphenylacetic acid (DOPAC), or reduced, resulting in neutral metabolites as methoxyhydroxyphenylethanol (MOPET) and dihydroxyphenylethanol (DOPET). There are two MAO isoenzymes: MAO-A, mainly involved in catecholamine metabolism, and MAO-B, related to the metabolism of another neurotransmitter, the serotonin.
COMT catalyzes reactions of methylation of catechols and acidic and neutral metabolites, transforming most of the dopamine released in 3-methoxytyramine (3-MT). In turn, 3-MT is subsequently converted into homovanillic acid (HVA) by the action of MAO. HVA can also be produced from DOPAC by the action of COMT.
Under *in vivo* normal conditions, the majority of dopamine is degraded to its acidic metabolites DOPAC and HVA. Taking into account the site of DOPAC and HVA synthesis, we could consider DOPAC as an index of intraneuronal degradation of dopamine, while HVA would be an index of extraneuronal degradation. However, as HVA also is produced from DOPAC, it could be considered as a secondary metabolite of dopamine.
The ability to measure levels of dopamine and its metabolites provides an index of activity of both enzymes and of dopamine neurons in general.

3. Effects of triazole fungicides on nigrostriatal dopaminergic system

It has been described that triazole fungicides triadimefon (and its metabolite triadimenol) and flutriafol produce changes in dopaminergic neurotransmission (Walker & Mailman, 1996; Gagnaire & Micillino, 2006; Santana et al., 2009). As previously described, the effects produced by triazole fungicides on the dopaminergic system began to be described at the end of 1980s and early 1990s, with initial observations of neurotoxic effects of triadimefon on the motor activity. Several posterior studies, demonstrated that these behavioral effects produced by triadimefon were produced by changes in dopaminergic neurotransmission. In this session we will show some experimental results that demonstrate the effects and possible mechanisms of action of flutriafol and triadimefon on the nigro-striatal dopaminergic system of rodents.

3.1 Behavioral and *in vitro* effects of triadimefon

Triadimefon increases locomotion and induces stereotyped behavior in rodents (Crofton et al., 1988, Moser & MacPhail, 1989). Some possible targets for the alterations in dopaminergic neurotransmission induced by triadimefon are the mechanisms of synthesis, release, reuptake, and degradation of dopamine in nerve endings.
To determine the possible neurochemical mechanism of action of tiradimefon to induce behavioral change, Crofton et al. (1989) evaluated the effects of combined treatment of triadimefon with either a tyrosine hydroxylase inhibitor (D,L-alpha-methyl-p-tyrosine methyl ester, alpha MPT) or a depletor of catecholamine stores (reserpine). These authors observed that alpha-MPT did not block the increased motor activity produced by triadimefon, while reserpine reversed this effect. Based in this result, the authors suggested that increased motor activity produced by triadimefon was not mediated through release of newly synthesized catecholamines, but rather on dopamine released or reuptaked.
It has also been shown that the hyperactivity induced by triadimefon could be mediated through an action of this fungicide on dopamine post-synaptic receptors. So, dopamine D1 (SCH23390) and D2 (remoxipride) antagonists blocked, in different proportions,

triadimefon-induced locomotion (MacPhail et al., 1993). However, Walker et al. (1990) demonstrated that triadimefon does not bind directly to these dopamine receptors, being probably the activation of dopamine system produced in an indirect form. These authors also hypothesize that triadimefon could be act in a similar form to psychomotor stimulants such cocaine, amphetamine and methylphenidate, that exert their behavioral effects by increasing the concentration of dopamine in the synaptic cleft. To test this hypothesis Walker and Mailman (1996) examined the effects of triadimefon on dopamine uptake, dopamine efflux, and binding to the DAT.

Walker and Mailman (1996) observed that both triadimefon, and its main metabolite triadimenol, inhibited the *in vitro* uptake of [^{3}H]dopamine in striatal synaptosomes from rat striatum, increasing the concentrations of this neurotransmitter in the extracellular medium. The authors also observed that the two fungicides appear to act in a similar form to other well-characterized inhibitors of dopamine uptake bounding with high affinity to the DAT (Walker & Mailman, 1996). These data about the mechanism of action of triadimefon and triadimenol suggest that in high concentrations (high levels of exposure) these fungicides can produce similar effects to those caused by psychomotor stimulants.

Psychomotor stimulants (e.g. cocaine, amphetamine) are known to cause behavioral sensitization, a phenomenon defined as a progressive increase in the locomotor or stereotypic response to drug treatment (development phase) with an even further enhancement of this response following a drug challenge after a withdrawal period (expression phase). The effects produced by triadimefon on the dopaminergic system seem to indicate that exposure to this fungicide could lead to behavioral sensitization.

The possibility that exposition to triadimefon could lead to behavioral sensitization, was tested in a long-term study by Reeves et al. (2003; 2004). In these works, the authors observed that intermittent intraperitoneal injections of triadimefon appear to induce behavioral sensitization in mice. This behavioral effect was associated with neurochemical long-term changes in both the mesolimbic and nigrostriatal dopaminergic system.

3.2 *In vivo* effects of triadimefon and flutriafol on dopamine release from rat striatum

The data described in prevous section refer to effects of triadimefon observed in *in vitro* experimental preparations, such as cell cultures and isolated preparations of nervous tissue, among others. In the present section we will describe the effects of triadimefon and flutriafol on *in vivo* release of dopamine in the striatum of rats. The data reviewed here were obtained by microdialysis technique which allows monitoring the *in vivo* release of dopamine in awake and freely-moving rats using cerebral microdialysis (see Bergquist et al., 2002, for review).

The microdialysis technique coupled to high affinity liquid chromatography with electrochemical detection (HPLC-EC) was used by Gagnaire and Micillino (2006), to examine the effects of administration of triadimefon on *in vivo* dopamine release from rat striatum. These researchers observed that systemic administration of this fungicide produced a gradual and sustained increase in striatal dopamine levels. In this study, the authors report that i.p. administration of 100 or 200 mg/kg of triadimefon increased dopamine extracellular levels to 170 and 398%, respectively, measured by *in vivo* brain microdialysis (Fig. 5A).

The effects of flutriafol on *in vivo* dopamine release were studied by Santana et al. (2009). These authors demonstrated that *in situ* administration of different concentrations of this fungicide into rat striatum also induced changes in dopaminergic neurotransmission. So, intrastriatal administration of 1, 6 and 12 mM flutriafol through the microdialysis probe produced significant concentration-dependent increases in dopamine levels to 218 ± 51%, 1376 ± 245% and 3093 ± 345%, respectively (Fig. 5B).

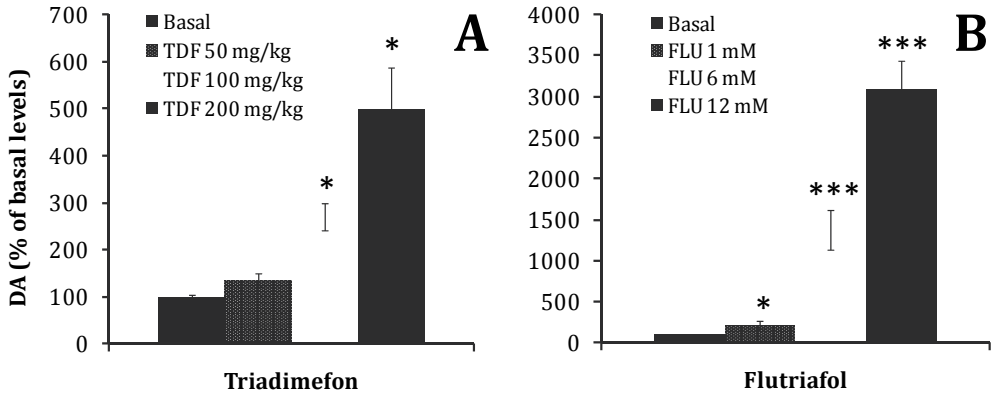

Fig. 5. Maximal increases (mean ± SEM) in dopamine levels in striatal microdialysis samples taken after systemic administration of triadimefon 50, 100 or 200 mg/kg (A) and intrastriatal infusion of flutriafol 1, 6 or 12 mM (B). The maximal increases produced by triadimefon and flutriafol were observed at 240 and 40 min after the beginning of infusion, respectively. The results (n=4- 8/group) were expressed as a percentage of the basal levels (100%). Basal levels were considered as the mean of substance concentrations in the three samples before triadimefon (or vehicle) or flutriafol perfusion. Significant differences: *P<0.05; *P<0.01; and *P<0.001 with respect to the control group or basal (Source: Gagnaire & Micilino, 2006; Santana et al., 2009).

So, administration of triadimefon and flutriafol produced significant dose-dependent increases on *in vivo* dopamine release from rat striatum. In accordance with these results, at least two general mechanisms can be proposed to explain the increases in extracellular DA levels observed: triadimefon and flutriafol could be implicated in inducing the neurotransmitter release from synaptic vesicles or producing dopamine reuptake inhibition. To check which mechanism would be implicated in inducing dopamine release by these fungicides, different drugs with known actions were used on dopaminergic terminals, as well as modified perfusion medium administered together with the fungicides into the striatum.

To evaluate the mechanism by what triadimefon induces *in vivo* dopamine release, Gagnaire and Micilino (2006) used the following experimental approach: a) compared the effects of this fungicide to those of amphetamine, a releaser of dopamine from its storage sites; b) investigated whether the increase in the dopamine levels induced by triadimefon was sensitive to tetrodotoxin (TTX), a blocker of voltage sensitive sodium channel and; c) compared the release dopamine profile with that of GBR 12909, a classical inhibitor of DAT. The results obtained are summarized in the Fig. 6.

Systemic administration of triadimefon 200 mg/kg induced significant increases in extracellular dopamine levels which reached 741% of basal levels. When triadimefon was administered to TTX (5 μM) pretreated rats, it had no effect on the dopamine release, i.e., TTX completely inhibited the increase in dopamine induced by triadimefon. On the other hand, Gagnaire and Micilino (2006) observed that systemic administration of amphetamine (2 mg/kg) produced a sharp increase in dopamine release which was not inhibited by intrastriatal infusion of TTX (Fig. 6). Finally, in the same study, these authors compared the dopamine release profile which that of GBR 12909. This DAT inhibitor was systemically administered (10 mg/kg) and its effects on the extracellular dopamine levels were evaluated in experimental conditions similar to those for the study of the effects of triadimefon. In the same way that the triadimefon, the acute treatment with GBR 12909 produced a gradual and sustained increase in extracellular dopamine levels which reached 356% of the control values (Gagnaire & Micilino, 2006).

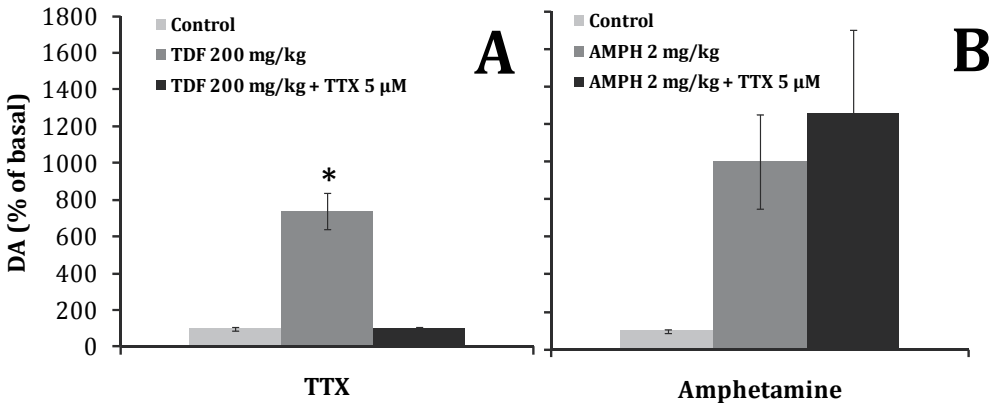

Fig. 6. Representative histograms of possible mechanism of action of triadimefon on the *in vivo* dopamine release. The samples of dopamine were obtained by *in vivo* brain microdialysis and extracellular levels were quantified by HPLC-EC. Triadimefon (200 mg/kg) or amphetamine (2 mg/kg) was administered via i.p. 90 min after the beginning of the perfusion of TTX. A) Administration of triadimefon induced a maximal increase of 741% on dopamine extracellular levels and TTX completely inhibited this increase in dopamine release. B) Administration of amphetamine induced a maximal increase of 1000% on dopamine extracellular levels; when TTX was administered in amphetamine pretreated animals the maximal increase in dopamine levels was of 1260%. There was no statistically significant difference between the two groups. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%) of control group. Basal levels were considered as the mean of dopamine concentrations in the three samples before treatment administration. Significant differences were *P<0.05, respect to the basal levels (Source: Gagnaire & Micilino, 2006).

Taken together, these results by Gagnaire and Micilino (2006) show that the triadimefon-induced dopamine release from rat striatum appear to be action potential dependent since infusion of TTX completely inhibited this increase. The results also suggest that triadimefon does not act as a dopamine releaser like amphetamine and appear to act in a similar way to GBR 12909, since the two substances induced increases in dopamine release with similar profiles.

In other study Santana et al. (2009) investigated the neurochemical mechanism by what flutriafol produce increases in extracellular dopamine levels in striatum of rats. To evaluate if flutriafol-induced dopamine release was due to an increased dopamine exocytotic release and/or a change in the activity of DAT, Santana et al. (2009) investigated the effects of flutriafol under Ca^{++}-free or Na^{+}-free conditions, and after pretreatment with reserpine and TTX. The results are summarized in the Fig. 7.

The involvement of vesicular release in the increase of striatal dopamine levels induced by flutriafol was investigated by measuring the effect of coadministration of 6 mM of this fungicide, diluted in a Ca^{++} or Na^{+}-free Ringer medium, on dopamine extracellular levels. When flutriafol was perfused in either Ca^{++}-free or Na^{+}-free Ringer, the dopamine levels reduced 92% and 70%, respectively, when compared with the effect of flutriafol only (Fig. 7). These results showed that the striatal output of dopamine induced by flutriafol were Ca^{++}- and Na^{+}-dependent, although the lack of these ions in the medium did not completely block the dopamine release induced by flutriafol. Perfusion of flutriafol in TTX-treated or reserpine-pretreated animals, reduced the levels of dopamine by 73% and 86%, respectively, also compared with effect of flutriafol infusion (Fig. 7). These results seem to indicate that dopamine release evoked by flutriafol is dependent of dopamine stored in the synaptic vesicles and on depolarization mediated by voltage-sensitive sodium channels.

In another set of experiments, Santana et al. (2009) studied the involvement of the DAT in the flutriafol-induced dopamine release by measuring the effect of coadministration of 6 mM flutriafol and nomifensine on dopamine release. When nomifensine or flutriafol were administered, the dopamine release increased over 16 and 14 times with respect to the basal levels, respectively (Fig. 8). This effect of nomifensine is due to the fact that it acts as an inhibitor of the dopamine uptake (Meiergerd & Schenk, 1994; Wieczorek & Kruk, 1994), increasing the extracellular dopamine levels. When flutriafol was coinfused with nomifensine, the increase was over 32 times, showing an additive effect between flutriafol and nomifensine. This addictive effect could mean that both substances act through different mechanisms at the dopaminergic terminal: nomifensine acting on DAT and flutriafol acting on exocytotic release, corroborating the results of the other experimental groups.

In this way, we can observe that the neurochemical mechanism of action of triadimefon and flutriafol to induce *in vivo* dopamine release from rat striatum appear to be different. So, opposed to the data described for triadimefon, the results obtained by Santana et al. (2009) showed that flutriafol-induced dopamine release *in vivo* is not due to an action of this fungicide on DAT, but through an exocytotic release. Considering the difference observed in the biochemical mechanisms of action of two pesticides belonging to the same class, some comparisons between these two studies can be considered. There is a great difference in the chemical structures between the compounds. So, triadimefon might have in its structure a site able to link to DAT, thus changing the transporter activity.

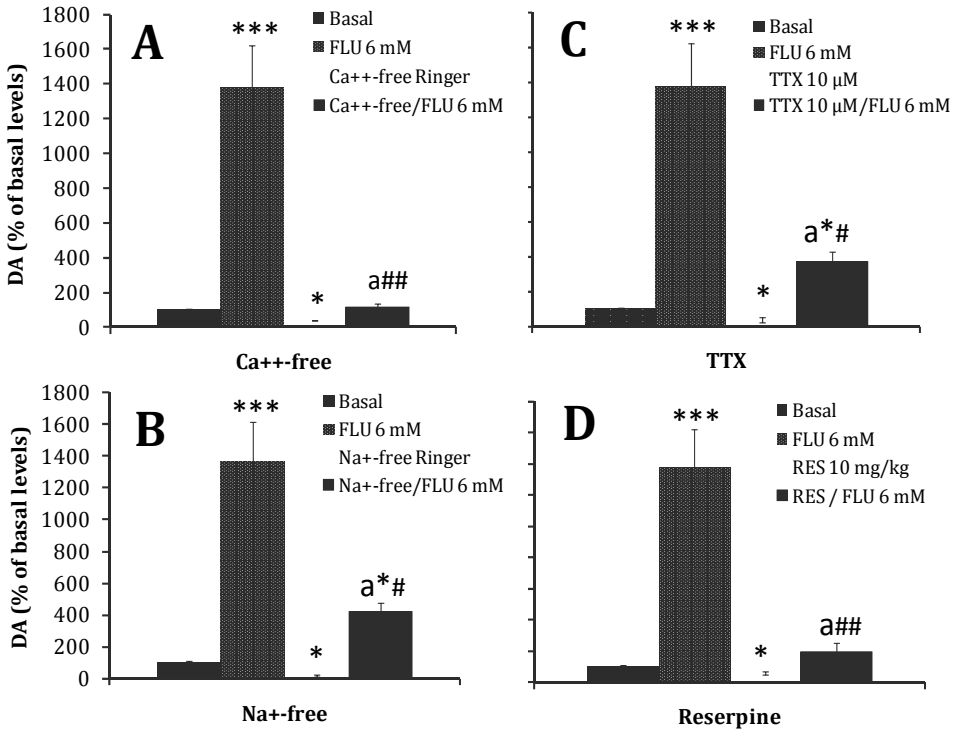

Fig. 7. Representative histograms of mechanism of action of flutriafol on the *in vivo* dopamine release. The samples of dopamine were obtained by *in vivo* brain microdialysis and extracellular levels were quantified by HPLC-EC. Intrastriatal infusion of 6 mM flutriafol during 1 h induced a maximal increase of 1376 ± 245% on dopamine extracellular levels. To determine the mechanism of action, flutriafol was administered together with TTX, reserpine (RES) or in a Na^+-free and Ca^{++}-free Ringer solution. These different treatment, administered during 1 h, produced significant decreases in dopamine release in striatum: TTX (10 µM): 37±15%; Na^+-free Ringer solution: 20±7%; Ca^{++}-free Ringer: 40±1%; and reserpine (10 mg/kg, i.p., administered 1 h before the beginning of experiment): 58±7% of decrease respect to basal levels. A) When flutriafol was infused in the Ca^{++}-free Ringer solution the maximal increase in dopamine level observed was 113 ± 25%, relative to basal levels; B) Administration of flutriafol diluted in the Na^+-free Ringer solution increased dopamine levels to 418 ± 57%, respect to basal; C) In TTX pretreated animals the administration of flutriafol induced an increase of 368 ± 63%, respect to basal and; D) Intrastriatal infusion of flutriafol in reserpine pretreated animals produced a maximal increase of 191 ± 63%, of basal. These increases observed were 92%, 70%, 73%, and 87% lower than that observed for flutriafol in non-pretreated rats, respectively. The values specified represent the maximum increases observed 30 min after the start of the flutriafol infusion. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine concentrations in the three samples before treatment administration. Significant differences were *P<0.05, **P<0.01, and ***P<0.001, respect to the basal levels, and #P<0.05, ##P<0.01, respect to 6 mM flutriafol control group (Source: Santana et al., 2009).

Another important fact, which would explain the discrepancy between these results, is the difference in the experimental design used in both studies. Gaignaire and Micillino (2006) compared the dopamine release profile induced by i.p. administration of triadimefon with those obtained from different compounds administered into the striatum, while in study of Santana et al. (2009) all the drugs (including flutriafol) and modified mediums were administered *in situ* through microdialysis probe, according to the classically described experimental protocols for this technique.

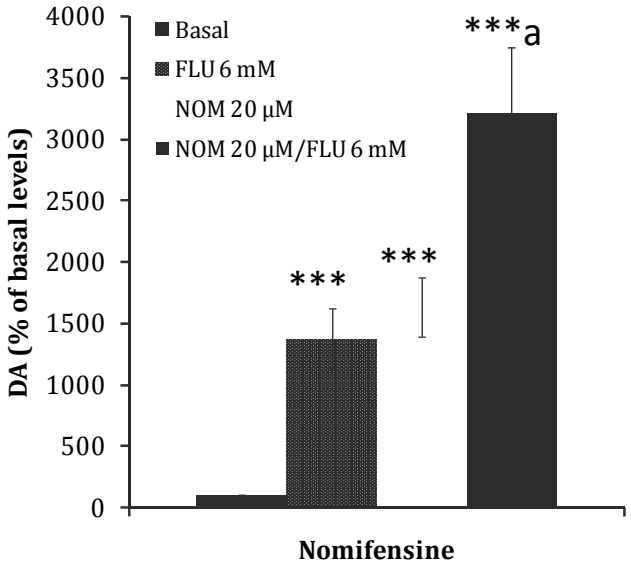

Fig. 8. Representative histograms of the effects of 6 mM flutriafol infusion in nomifensine (NOM) pretreated rats on the dopamine extracellular levels from rat striatum. The results are shown as the mean ± S.E.M., expressed as a percentage of basal levels (100%). Basal levels were considered as the mean of dopamine concentrations in the three samples under treatment administration. Significant differences were *P<0.05, **P<0.01, and ***P<0.001, respect to the basal levels and ^{a}P<0.05, respect to 6 mM flutriafol control group.

Additionally, Gagnaire and Micillino (2006) reported that intrastriatal administration of TTX in triadimefon i.p. pretreated animals produced an increase in dopamine release that was significantly lower than those observed when the pesticide was administered in non

pretreated animals, similar by the results obtained by Santana et al. (2009). Nevertheless, the former authors only affirm that the fungicide does not act as a stimulant of dopamine release, but it would promote an increase in extracellular dopamine levels caused by inhibition of DAT. Gagnaire and Micillino (2006) also observed that the effect produced by triadimefon resembles that one provoked by amphetamine (with a similar release profile), indicating that this fungicide would either block dopamine uptake or induce vesicular release from the storage sites, without the occurrence of a TTX-provoked inhibition.

7. Conclusion

Triadimefon and flutriafol are two economically important fungicides that belongs the class of triazole, an economically important group of fungicides largely used in agriculture to plant protection and in the treatment of fungal diseases. Because its intensive use, these compounds represent a potential risk for the contamination of environment and human populations. Despite this potential risk, there are little studies about toxicological effects of these fungicides on the biological systems of mammals. The triazole fungicide more studied is the triadimefon, being its neurotoxic effects on nervous system is well characterized. Triadimefon increases locomotion and induces stereotyped behavior in rodents; these effects are associated with increased dopamine turnover in nigrostriatal and mesolimbic brain dopamine pathways. Additionally, triadimefon exposure may generate behavioral sensitization, an effect also mediated by the dopaminergic system. More recently, was demonstrated that systemic administration of different doses of triadimefon to rats produced a gradual and sustained increase on *in vivo* dopamine release from striatum, that was dose-dependent. The effects of other triazol, the flutriafol, on *in vivo* dopamine release in rat striatum also were studied. Just as triadimefon, also flutriafol, administered *in situ*, induced concentration-dependent dopamine release from striatum. However, although triadimefon and flutriafol belong to the same chemical group, their mechanisms of action appear to be different. In the case of triadimefon, the increases in the extracellular dopamine levels possibly occur through an inhibition of DAT while flutriafol induces dopamine release via a vesicular, Ca^{++}, Na^{+} and TTX-dependent mechanism, being independent of DAT.

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21

Influence of Fungicide Residues in Wine Quality.

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1. Introduction

The cultivation of grapes may be regarded as the oldest and most relevant food sector of the world from 6000 years ago as well as the enology. During the 70s, the world's wine production was growing reaching 10.2 million Ha. Since the 80s, the planted area has decreased due to the excess of wine in the world market and the consequent eradication of vineyards has happened (-21%) reaching a total area of 7.92 million Ha in 2001 and keeping constant, though with small fluctuations in recent years (OIV, 2006). An estimated 57% of grape world production is used for winemaking, considering an average annual consumption of about 40 l of wine per person.

The vast acreage used by vineyards and climatic conditions have led to a suitable habitat for the development of large number of pests, not only responsible for the decrease in crop yield but even for the total loss itself. Control of pests and diseases of the vine is one of the pillars that must underpin profitable wine production. It should be firstly pointed out Lepidoptera *(Lobesia botrana* and *Clysia ambigüella)* and secondly the cryptogamic ones, especially *powdery* and *mildew*, and finally rot, especially the acidic and noble ones, among the major pests (insects, mites and nematodes) and diseases (fungi) affecting the cultivation of the vine (Coscollá, 1992; MAPA, 1992; Carrero, 1996).

The defense of the wine producer against this situation sometimes results in abuses which produce the presence of pesticide residues in grapes as consequence and eventually in must and wine, though the type of wine-making and its stages may influence on their disappearance. In modern agriculture, considering that organic farming is in continuous expansion, one can hardly do without the use of synthetic plant protection products to ensure a regular and substantial production of quality.

2. Effects of pesticide residues.

As a direct result of pesticide treatments carried out in viticulture, not to mention the possible environmental pollution now, we can cite three effects of their use:

- Residues in grapes, musts and wines.
- Influence on fermentation and organoleptic characteristics of wine.
- Health and hygienic quality and toxicological effect on the consumer.

2.1 Residue presence in grapes, must and wine.

The occurrence of residues of fungicides, as direct result of treatments with plant protection products during the growing season and especially between veraison and ripening of the vine, depends on such diverse factors as: products, formulation and dosage of treatment used, time between product application and harvesting, product safety time and climatological factors (sunshine, rain, etc.) (CIBA, 1993; Coscollá, 1993; Celik et al., 1995; Pimentel, 1997; Montemurro et al., 2002; Whitmyre et al., 2004).

Firstly and before entering fully into the issue of fungicide residues in wines, it is imperative to note the great development achieved by the analytical methodology in recent years which have helped to reach high sensitivities using macro and micro procedures *on- line,* solid phase micro extractions, introduction of new methods of extraction as QuEChERS, etc. (Oliva et al., 1999a; Payá et al., 2007). In terms of analytical techniques, gas chromatography (GC) and high pressure liquid chromatography (HPLC) coupled to mass detectors (MS) lead to safe and reliable results. In this field, it is also important the required implementation of quality criteria in both trials (UNE/ISO 17025 and SANCO Guidelines) and supervised experiences (GLP) for quality assurance and technical competence of laboratories undertaking pesticide residue analysis. To cite papers aimed at the development of methodologies for multi-residue determination of fungicides in grapes, must and wine by GC and selective HPLC coupled to confirmation by MS would be almost endless in this area (Navarro et al., 2000b; Oliva et al., 2000a; Agüera, et al., 2004; Anastassiades et al., 2007; Payá et al., 2007).

Experimental data available show that if pesticides are used as indicated by manufacturers, no residues higher than those set by law at the time of harvest should appear. When harvested grapes are used in wine-making, they are transported to the winery where the elaboration of wine starts by oenotechnological processes such as crushing, draining, maceration, pressing, must racking, alcoholic fermentation, racking, clarification, stabilization and filtration. In this sense, the type of wine and the correct use of oenotechnological processes can decisively influence the disappearance or elimination of pesticide residues.

An extensive review of literature shows that there are substantial losses of pesticide residues in the transition from grape to must and from this to wine. It is important to note the influence of the dissipation rate in the crop on the possible presence of residues in the wine. Moreover, oenological processes as crushing, pressing, racking, clarification and filtration are important factors in the disappearance of fungicide residues in wine. Finally, the technique of wine-making -with or without maceration-, the addition of tannins, cryomaceration, etc. also influence the disappearance or reduction of fungicide residues (Garcia & Xirau, 1994; Navarro et al., 1997; Cabras et al., 1997; Flori et al., 2000; Angioni et al., 2003; Ruediger et al., 2005; Oliva et al., 2006, 2007a & 2007b).

Thus, some studies can serve as examples in which it was confirmed that the residues of fungicides such as pyrimethanil ones in grapes were kept in must and wine but reduced to 50% for tebuconazole, though fludioxinil and kresoxim methyl residues reduce gave reductions of 50% in must and almost eliminated in the finished wine (Figure 1) (Cabras & Angioni, 2000a).

Some authors note that the crushing of grapes does not affect the disappearance of fungicides such as azoxystrobin, cyprodinil, fludioxonil, pyrimethanil and quinoxyfen (Cabras et al., 2000b; Fernández et al., 2005). After pressing, the residues of penconazole, fenarimol or vinclozolin appeared predominantly linked to the lees. However, there are fungicides such as metalaxyl that remained in a high percentage in the must due to its high solubility in water-alcohol solutions (Navarro et al., 1999).

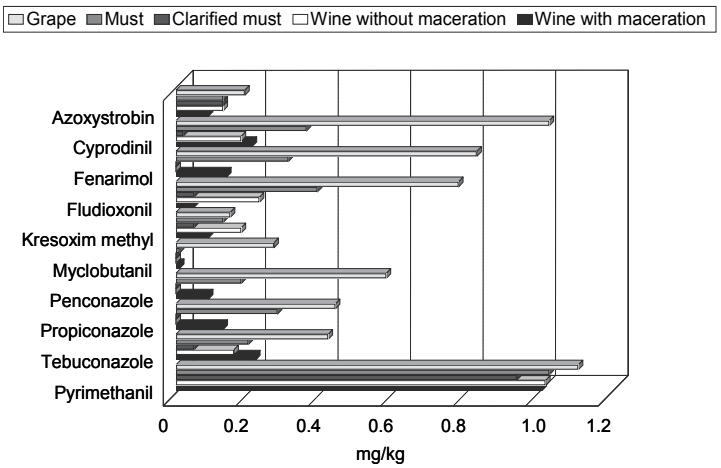

Fig. 1. Influence of the oenotechnologycal processes on the disappearance of fungicide residues

Kresoxim methyl and tetraconazole - newly marketed fungicides in viticulture- left no detectable residues in grapes after 21 days of application. Others, such as azoxystrobin, mepanipyrim and fluazinam did appear in grapes, though their residue levels decreased in must and much more in those obtained in vinifications by maceration (0.12 compared to 0.20 ppm of azoxystrobin in must with and without maceration, respectively) (Cabras et al., 1998). For treatment with quinoxyfen -very novel fungicide in powdery control, concentrations of 0.38 mg/kg were detected in grapes harvested after the application of the product (below the MRL established in Italy; 0.5 mg/kg). Fourteen days later, the residue levels had decreased to 0.09 ppm. After the vintage pressing, only 45% of the residues present in the berries were found in must. When this was subjected to centrifugation, this operation removed 8% of the level and then no residue detection was possible in the wines at the end of the fermentation process (Cabras et al., 2000b).

Moreover, the racking is a process of great impact on the disappearance of some pesticides as they can be washed and separated by lees. Also, stabilization processes such as clarification and filtration of wine can cause the disappearance of the quantities of residues present in them. The use of clarifying agents such as gelatin, bentonite, activated carbon, caseinates, etc. can drag the residues of the pesticides that due to their physical-chemical characteristics are not solved in the liquid phase. Similarly, the use of certain media such as nylon filters may significantly influence the disappearance of some pesticides (Cabras et al., 1997; Fernández et al., 2005b; Oliva et al., 2007a & 2007b).

For example, when removing the faeces suspended in must either by dynamic (centrifugation) or statically (with or without clarifying agents) 90% of sulfur and 70% of phthalimides or 40% of the dicarboximidics fungicides were lost, while water soluble products remained in the must. We must consider that the water solubility of most pesticides is low. Other products such as benzimidazols were neither eliminated in the previous process, but they disappeared when using bentonite as clarifying agent. Finally, the filtration of wine before bottling has some effect in the elimination of residues, though this is minimal (Navarro, 2000c; Soleas & Goldberg., 2000; Ruediger et al., 2004).

Comparing the evolution of residues when the wine-making is made by traditional procedures against carbonic maceration, there was greater concentration remaining in the

latter process possibly because the grapes remain intact for longer time and then no transference of pesticide residues from the berries to the liquid phase occur and neither degradation reactions. However, the residual quantities after pressing were significantly less in the must from carbonic maceration.

Studying the disappearance of fungicides such as benalaxyl, metalaxyl, cyproconazole, fenarimol, penconazole, vinclozolin and mancozeb in traditional wine-making process compared to that made by carbonic maceration, it appeared that the remaining residues in the wines made by this technique were among five to ten times lower than those determined in the obtained by the traditional process. The product with the lowest rate of dissipation was metalaxyl (Navarro et al., 2000a).

In the case of traditional wine-making, remaining percentages ranged from 67 to 95% (for fenarimol and metalaxyl, respectively) during the maceration process. The first significant decline in residual levels occurred in the pressing being reduced to average values of 30% compared to the initial level in pomace, noting that the pesticide which showed higher disappearance was mancozeb. However, changes were minor in fermentation. In the lees, remaining levels were between 8-17%. Finally, clarification and filtration collaborated in the disappearance of residues (Navarro et al., 1999).

In the study of the disappearance of some fungicides on Tempranillo grape from La Rioja throughout the winemaking process, we observed the different behavior presented by the fungicides tested (Table I). Thus, procymidone and vinclozolin behaved similarly in the process of maceration and fermentation; carbendazim was not removed and dichlofluanid disappeared in about 70-80%.

Process	Procymidone	Vinclozolin	Iprodione	Carbendazim	Dichlofluanid
Maceration, fermentation and pressing	15%	12%	30-40%	--	70-80%
Racking	25%	45%	18-20%	30-40%	20%
Clarification	12%	13%	20%	25-30%	100%
Total	**52%**	**70%**	**70-80%**	**55-70%**	**100%**

Table I. Decrease of residues in Tempranillo grapes.

During the operations of racking and clarification, the differences were much smaller noting that dichlofluanid disappeared completely the clarification. Losses of the residues of these fungicides ranged from 50 to 100% during the winemaking process (Santos, 1997).

We can find a concrete example of the disappearance of residues during the wine production examining the study of the elimination of fenhexamid -widely used fungicide- when applied in conditions of GAP (Good Agricultural Practices) and CAP (Critical Agricultural Practices). Figures 2 and 3 show the results obtained (Barba et al., 2009b).

As we can see, the fungicide was removed by 85%during the production influencing the oenotechnological processes more than the initial concentration.

The evolution of the residue levels of four fungicides (cyprodinil, fludioxonil, pyrimethanil and quinoxyfen) during elaboration of three types of wine with maceration (traditional red wine, carbonic maceration red wine and red wine of long maceration and prefermentation at low temperature) and two types of wine without maceration (pink and white) has been studied. The disappearance curves of each fungicide have been analyzed during the period

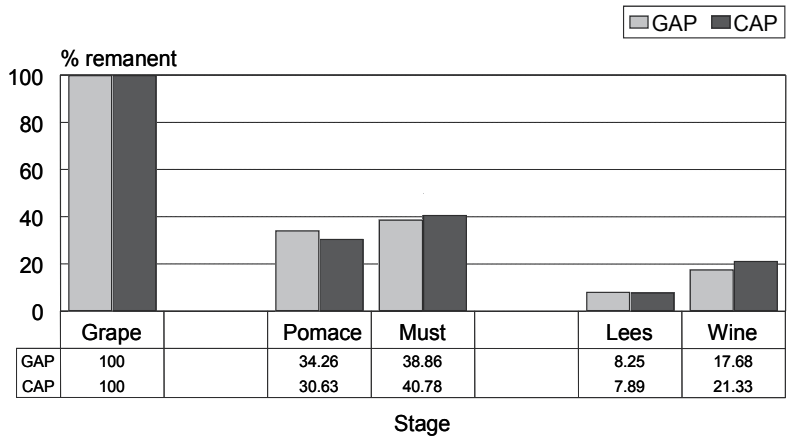

Fig. 2. Fenhexamid dissipation during the wine production.

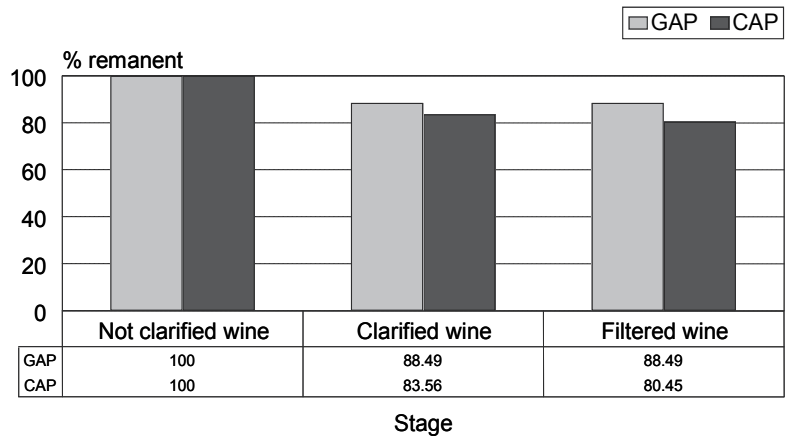

Fig. 3. Influence of clarification and filtration process in fenhexamid dissipation during the wine production.

of time of each wine-making process (21 days) and during the different steps involved in the oenological elaborations. Fludioxonil was the fastest fungicide to reduce its residual levels during the time and steps of wine-making procedures without maceration, while pyrimethanil was the lowest in practically all the cases (with and without maceration). During carbonic maceration wine-making, the decay constant of cyprodinil was greater than that of the rest pesticides in all assays (time and steps) [Fernández et al., 2005a].

The removal of recent-use fungicide (famoxadone fenhexamid, fluquinconazole, kresoxim-methyl, quinoxyfen and trifloxystrobin) can vary between 40-100% during the process of making Monastrell red wines.

Another study investigated the influence of two yeasts (*Saccharomyces cerevisiae* and *Kloeckera apiculata*) and two lactic bacteria (*Leuconostoc oenos* and *Lactobacillus plantarum*) on the degradation of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil and tretraconazole) during alcoholic and malolactic fermentation. The results indicated that degradation occurs only during alcoholic fermentation for the fungicide pyrimethanil (20-40% decreases in the concentration of control at 10 days of fermentation.)

For the other five fungicides, the alcoholic fermentation did not produce a deletion by degradation or adsorption in yeast. Neither the bacteria studied showed degradative effect on fungicide during malolactic fermentation (Cabras et al., 1999).
There is also evidence that the copper content in the grapes and subsequent transfer to must and wine is influenced by the number of applications, the period between application and harvest and the amount of copper applied. 18% samples exceeded the MRL established (20 mg/kg) of all the wine samples analyzed in 16 Italian wineries. Therefore, it is required to set harvest time within 20 to 40-50 days. In addition, this study showed that the copper content did not depend on the strategy of pest control (conventional, integrated or organic). Of the three above factors, the most important is the amount of copper applied, since it implies about 50% of the final concentrations in grapes and wine (García-Esparza et al., 2006).
Other researchers studied the possible effect of fermentation processes on the levels of total arsenic and inorganic species [As (III) and As (V)] and organic (arsonic monomethyl acid [MMAA] and dimethyl arsinic acid [DnaA]) in 45 wines of southern Spain. The total arsenic levels were very similar for the different types of wines studied. Values in the wine samples analyzed varied between 2.1 and 14.6 mg/l. The results suggested that consumption of these wines do not have a significant contribution to the diet of total and inorganic arsenic for a moderate drinker (Herce-Pagliai et al., 2002).
Regarding the influence of storage time and temperature of wine, one study proposed the creation of an equation based on kinetic analysis that allows us to predict the concentration of ethyl-carbamate in wine after storage time at given temperature. So, you can predict the time of consumption when ethyl-carbamate levels do not exceed certain concentrations, which may lead to chronic toxicity to the consumer (Hasnip et al., 2004).
Studies on the behavior of fenhexamid in grapes during wine-making and the effect of the microflora on the alcoholic and malolactic fermentation for the degradation of fenhexamid showed that the elimination of this fungicide in the wine depends if the process is made with or without maceration. So, when there is no maceration, there is removal of 49%, while in case of maceration is 62%. Therefore, the presence of skin in contact with the must for a time produced an increase in the elimination of the fungicide studied. No effect attributed to yeast or bacteria is observed for fenhexamid degradation in wine-making (Cabras et al., 2001).
When we studied the elimination of famoxadone during wine-making, we found that there was 100% removal of the fungicide in vinification with and without maceration. Thus, levels lower than the limit of quantification (0.05 mg/kg) was achieved in wines from grapes harvested at levels close to the MRL (2 m/kg) (De Melo Abreu et al., 2006).
Removing spiroxamine is affected by the performance or not of maceration. So, when wine-making does not include maceration, removal is between 20-27%. While, removal increased to 45-62% in wines obtained with maceration. Transfer factor from grape to must was 0.55, so that half of the fungicide is removed together with the solid parts of the grape. In the case of wine to grapes, it was 0.26. In general, the elimination of residues of spiroxamine ranged 23-56%, depending on the winemaking technique used. No levels above 10% of the MRL existed in wine after treatment under GAP, so there was no toxicological risk in wines made from grapes treated with spiroxamine (Tsiropoulos et al., 2005).

2.2 Influence on the fermentation and organoleptic characteristics of wine.

Organoleptic characteristics of a wine can be considered as the result of a balance of aromatic substances and flavoring elements that compose it and that are responsible for

governing the harmony between the smell and taste of wine. It is therefore extremely important to control the acid fraction (it depends on many wine properties and phenomena that take place inside), density and color (appearance, astringency and structure).

Influence on the general parameters.

To test the influence of the presence of pesticide residues on wine main features, general parameters were compared. They were: density, total acidity, volatile acidity, alcohol content and fermentation time in wines made from control grapes (with no phitosanitary treatment), traditionally cultivated grapes (typical treatments from the area of cultivation) and grapes treated with vinclozolin, fenarimol, mancozeb, metalaxyl and penconazole. In all cases, the evolution of fermentation was correct, but had no stops fermentation. The values of alcoholic grade in all wines were within the minimum values required in the DO of the area. The values and evolution of the acid fraction did not endanger its preservation or the balance of its constitution (Oliva et al., 1998a).

In another study of harvested grapes treated with the fungicide pyrimethanil, cyprodinil (Switch), fludioxonil (Switch), azoxystrobin, kresoxim methyl and quinoxyfen, vinifications were made with maceration at 5 °C for four days and six days at 25 °C. When alcoholic fermentation was over, racking and stabilization by clarification and filtration were performed and then bottled. Determinations of the general parameters indicated in Table II were carried out in the bottled wine.

Regarding the acid fraction, it must be noted that no significant differences with respect to the control wine for the total acidity values existed in winemaking in the presence of kresoxim methyl. The same applies to the pH in the case of wine made with residues of azoxystrobin. In contrast, only wine-making in case of residues of pyrimethanil differs significantly from the control for the volatile acidity. The same happens for the alcoholic grade (Fernández et al., 2001).

	Control	Quinoxyfen (a)	Kresoxim (b)	Azoxystrobin (c)	Swicth* (d)	Pyrimethanil (e)	DS $p \leq 0.05$
TA	7.58±0.28	7.19±0.07	7.26±0.30	7.13±0.02	7.15±0.18	7.23±0.07	**a,c,d,e**
VA	0.040±0.000	0.040±0.000	0.043±0.006	0.040±0.000	0.040±0.000	0.060±0.010	**e**
pH	3.38±0.02	3.453±0.006	3.45±0.06	3.413±0.006	3.456±0.006	3.493±0.006	**a,b,d,e**
AG	12.26±0.06	12.40±0.20	12.66±0.28	12.80±0.65	12.40±0.30	13.43±0.40	**e**
CI	19.27±0.58	15.16±1.26	15.34±0.99	16.15±1.09	15.97±2.75	15.26±0.22	**a,b,c,d,e**
H	0.45±0.01	0.46±0.01	0.47±0.02	0.47±0.01	0.46±0.01	0.47±0.01	**b,c,e**
TP	2.45±0.32	1.96±0.08	1.81±0.25	2.12±0.16	1.80±0.16	1.51±0.10	**a,b,d,e**
FI	48.40±1.05	38.57±4.54	40.50±2.86	42.97±2.56	38.57±4.76	33.80±1.60	**a,b,d,e**
TPI	83.47±2.45	58.67±4.92	67.97±2.61	71.67±3.70	59.47±7.71	53.10±0.20	**a,b,c,d,e**
H_c	19.10±0.10	23.43±1.70	23.93±0.35	23.34±0.74	24.20±2.76	22.10±1.11	**a,b,c,d,e,**
S	5.26±0.02	4.87±0.54	4.17±0.10	4.33±0.13	4.27±0.50	4.66±0.32	**b,c,d,e**

TA: total acidity (g/l tartaric acid); **VA**: volatil acidity (g/l acetic acid); **pH**: pH unites; **AG**: alcoholic grade (% v/v ethanol); **CI**: Color intensity; **H**: Hue; **TP**: Total poliphenols totales (g/l galic acid); **FI**: Folín index; **TPI**: Total poliphenols index; **H_c**: Hue CIELab; **S**: Saturation; **DS**: Degree of significance; * Cyprodinil and fludioxonil mix.

Table II. Influence of some fungicides in several parameters in wine.

For all parameters responsible for the color, marked differences are seen in the wines produced in the presence of pyrimethanil. For the remaining wines, these differences are not so marked.
Considering that the higher or lower value of color intensity is strongly influenced by the degree of ripeness of the grapes, the maceration time and temperature to which it is made and if we consider that these factors have been the same in all wine-makings, it appears that the presence of the tested fungicides has influenced the diffusion of phenolic compounds from the skin to the must during maceration.
To test the influence of the fungicides myclobutanil and dichlofluanid on the production of rosé wines from Monastrell grapes, a study adding two doses (1 and 5 ppm) to the must pressed and determining later the general parameters was carried out. The results showed that although there were no stops in fermentation, the highest dose of diclofuanide did cause a delay of five days. Statistical analysis of data showed significant differences in almost all parameters, except for volatile acidity, residual density (differences in analytical significance), which indicates that all winemaking reached the end of fermentation without residual sugar and therefore not re-fermentations would not occur in the bottle, nor influenced the concentration of acetic acid in wine (Oliva et al., 1999c).
Studies performed in La Rioja (Spain) in grape Tempranillo (Rioja) treated with two doses of carbendazim, dichlofluanid, iprodione, procymidone and vinclozolin for three crops showed no significant differences in the general parameters of density, pH and total acidity. The presence of procymidone and vinclozolin decreased the concentration of malic, total polyphenols were higher in the double dose of all treatments, the red color showed higher values at double dose, but there were no differences in the shade or hue (Santos, 1997). The sensory evaluation qualifies wines as well or very well, though specific observations on development of unpleasant tastes and not certain odors were made in wines with double dose, especially in the case of carbendazim. Dichlofluanid brings smell of ethyl acetate in double doses and some appreciations in single doses.

Influence on the viability of yeast.

Among the many factors that influence the evolution of yeast flora during fermentation, the presence of pesticide residues can be highlighted by its importance as these may alter the biochemical pathways of fermentation due to its effect on synthesis reactions or inhibition of respiration or fermentation. As a result, there may be a gradual decline of the viability of population and braking of the yeast fermentation, which could lead to a complete stop of the process in severe cases (Girond et al., 1989; Frezier & Dubordieu, 1991; Doignon & Rozes, 1992; Hatzidimitrou et al., 1997; Oliva et al., 2000b; García, 2002).
It was found that sulfur has no negative action on yeast in the case of inorganic fungicides, but it may cause development of off-flavors in wine (at high concentrations). On the contrary, copper significantly inhibits the growth of *Saccharomyces cerevisiae* at concentrations of 10 ppm.
Organic products with marked fungicide character derived from sulphonamides (dichlofluanid) or phthalimides (folpet and captafol) are particularly harmful to certain strains of yeasts (*Hanseniaspora uvarum, Saccharomyces cerevisiae and Saccharomyces bayanus*) (Oliva et al., 1999c). Benzimidazole, carbendazim and thiophanate methyl have no effect on yeast (only at very high doses). In contrast, benomyl can be active even at low concentration. Dichlofluanid is the only fungicide whose use requires caution from the point of view of influence on the yeast flora (Santos, 1997).

Triazoles as hexaconazole, penconazole and tetraconazole do not cause any alteration on fermentation kinetics but biocide effects were observed early in their presence at different doses, which causes small slowdowns at the beginning of fermentation (Cabras et al., 1999; Oliva et al., 2000b).

The yeast flora typified carried out in grapes, must and wine obtained by the addition of active dry yeast (LSA) to vegetable material treated with famoxadone fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen showed the absence of selective effect of all. No differences were observed between species or in the rates of appearance. There is also evidence that the presence of these fungicides do not affect the count levels of yeast in the grapes (even in more adverse treatment conditions) (Table III) or during the fermentation process, taking samples and conducting the count to 1, 5, 12 and 20 days since the start of fermentation (Table IV) (Oliva et al., 2007c).

Unites	Control	Quinoxyfen		Kresoxim		Famoxadone		Trifloxystrobin		Fluquinconazole		Fenhexamid	
		GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP
Log CFU/g	2.5	2.5	3.2	3.7	3.3	3.4	3.7	5.2	3.7	3.3	3.6	3.5	5.9
Log CFU/cm²	1.9	1.8	3.8	3.0	2.6	2.8	3.0	4.5	3.0	2.6	3.0	2.9	5.2

Table III. Yeast count on the grape surface.

Days	Control	Quinoxyifen		Kresoxim		Famoxadone		Trifloxystrobin		Fluquinconazole		Fenhexamid	
		GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP
1	2.6	2.5	4.5	3.7	3.3	3.5	3.8	5.2	3.8	3.3	3.7	3.6	6.0
5	7.8	7.9	9.5	7.6	9.3	7.5	9.5	7.6	9.5	7.4	9.2	7.6	9.5
12	6.9	7.7	9.4	8.1	8.7	8.0	8.7	7.3	9.0	7.6	9.2	8.3	8.5
20	5.6	6.7	8.5	8.8	7.5	8.2	7.8	7.5	7.0	7.2	6.9	8.1	5.2

Table IV. Evolution of yeast during fermentation.

Some authors reported the influence of certain fungicides on the fermentation kinetics of *Saccharomyces cerevisiae*, especially triazole and imidazole.

In trials of new compounds of non-systemic fungicides (fludioxonil and fenilpirrol), some anilinipirimidinas (cyprodinil and pyrimethanil) and new active substances similar in structure to natural strobirulines (azoxystrobin and kresoxim methyl) has been found not to significantly affect development of fermentation in winemaking targets of Airen variety, but more specifically to influence some characteristics of the finished wine (García, 2002).

The influence of pesticides on the malolactic fermentation, not very studied, seems to be not significant. Although, some researchers showed fungicides such as mancozeb, methyl metiram, cymoxamil, dichlofluanid, vinclozolin and iprodione may have depressant effect of this process (Cabras et al., 1999 & 2001).

We have evaluated the *in vitro* inhibitory effect of fungicides the famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen on growth of *Saccharomyces cerevisiae, Hanseniaspora uvarum, Dekkera bruxellensis*, and *Torulaspora Zygosacharomyces delbrueckii rouxii*, which are yeast flora normally present in initial natural flora of grape and wine. The effect was measured by determining the inhibition halos of different yeast inoculated on the medium GPYA (Oliva et al., 2009b).

The effect of plant protection products tested on different yeast was mixed but generally not apparent inhibition of growth on the surface was appreciated. Only, an inhibitory effect on

growth of *H. uvarum* in the presence of kresoxim methyl is clearly shown at a concentration of 400 ppm (concentration resulting from using twice the recommended application) (Figure 4).

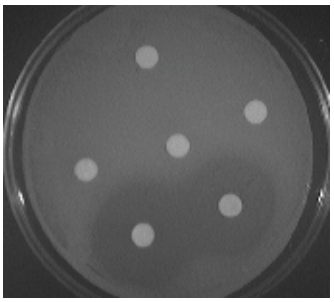

Fig. 4. Inhibition halos of Stroby (400 ppm of kresoxim methyl) on *H. Uvarum*

This effect is manifested by the appearance of a halo of inhibition of 1cm radius. This product does not show any effect on the rest of yeasts included in the study. This same species along with *S. cerevisiae* is slightly inhibited by commercial grade WG Flint (trifloxystrobin 50%) tested at concentration of 300 ppm, though the inhibition zone is less than 1 mm. These results indicate the absence of inhibitory effects of the plant protection products studied on the normal flora in the process of winemaking. The values tested were well above the legislated MRLs by Spain and the EU for wine grapes.

Influence on the aromatic fraction.

Although the descent in the aromas of wine has been studied extensively, various factors such as grape variety, cultural practice, climate, processing, processes, etc., there are few studies on the possible influence of pesticide residues. This is because its presence in grapes can cause a range of effects, often undesirable, and can alter the quality of wine produced by introducing bad tastes and smells into the must and wine.

In this sense, it was found the influence of some fungicides on the aromatic composition of wine, observing significant differences between the levels of major volatile -ethyl acetate, methanol, isobutanol and diethylacetal- and the wines considered classics in terms of phytosanitary treatments -three standard treatments in the area- compared with a control wine (Table V) (Oliva et al., 1999b).

The amounts of methanol are slightly higher than the control wine but double for the classic one. Specifically in the case of fenarimol, the level reaches 107 mg/l (compared to 54.5 in controls), which may be due to the increased activity of the enzyme pectin methyl esterase in his presence. The concentration of isobutanol in wines is due to the assimilation of the aminoacid valine -his predecessor- by yeast and this is a possible cause of the low level found in the classic wine due to their treatments. Remaining levels of other alcohols is normal, compared with the values given by different researchers (Santos et al., 1997; Aubert et al., 1997 & 1998).

For the minor volatile -acids, esters, alcohols and aldehydes-, no significant differences were found between the values of isobutyric and isovaleric acids (bacterial activity rates, poor quality factor). So, both pesticides studied did not affect bacterial activity. The levels of hexanoic acid, octanoic and decanoic acids showed significant differences in the classic wine and also in those treated with fenarimol, vinclozolin and penconazole (the high concentration of hexanoic acid may be due to the increase in the amount of yeast in a more

intense fermentation stage). In addition, only significant differences were found for isoamyl acetate, hexyl acetate, ethyl decanoate, ethyl acetate and phenyl ethyl dodecanoato among the fifteen selected esters, though their levels were normal. The latter together with ethyl hexanoate had strong influence on the aromatic profile of young wines.

Aromatic compounds	**Classic**	**Fenarimol**	**Mancozeb**	**Metalaxyl**	**Penconazole**	**Vinclozolin**
Major						
Ethyl acetate	◙					
Diethyl acetal		◙	◙	◙		◙
Methanol		◙				
Isobutanol	◙					
Minoritary						
Hexanoic acid	◙	◙			◙	◙
Heptanoic acid	◙					
Decanoic acid	◙		◙	◙		
Isoamyl acetate		◙				◙
Hexyl acetate	◙			◙	◙	
Ethyl decanoate	◙	◙			◙	
2 Phenyl ethyl acetate	◙				◙	
Ethyl dodecanoate		◙				
3-ol-1-octene	◙	◙	◙	◙	◙	
2-ethyl-1-hexanol	◙	◙				
1-octanol					◙	

Table V. Influence of some fungicides on the aromatic composition of wine.

On the other hand, only 1-octanol and 1-octen-3-ol differed significantly in quantity among the sixteen alcohols analyzed, but if they are present at high concentrations, it can be considered as a defect. Finally, no differences were found in the aldehydes examined and values were normal.

There are references of some pesticides in the family of the triazoles (penconazole, hexaconazole, fluquinconazole, etc.) They produce alterations in the synthesis of sterols and composition of the ester fraction in the aroma of wines (Aubert et al., 1997 & 1998; Oliva et al., 1999b).

Another study on the effects of three fungicide residues (cyprodinil, fludioxonil and pyrimethanil) on the aromatic composition (acids, alcohols and esters) of *Vitis vinifera* white wines (Airén var.) inoculated with three *Saccharomyces cerevisiae.* strains (*syn bayanus, cerevisiae* and *syn uvarum*) show that the addition of the three fungicides at different doses (1 and 5 mg/L) produces significant differences in the acidic fraction of the aroma, especially in the essays inoculated with *Saccharomyces cerevisiae,* although the final contents do not exceed the perception thresholds. The lower quality wines, according to isomeric alcohol content (Z-3-hexen-1-ol and 3-methylthiopropan-1-ol), are those obtained by inoculation with *Saccharomyces cerevisiae* (*syn bayanus*) and addition of cyprodinil. The addition of fungicides in the essays inoculated with *Saccharomyces cerevisiae* (*syn bayanus*) produces an

increase in the ethyl acetate and isoamyl acetate content, which causes a decrease in the sensorial quality of the wine obtained (García et al., 2004).
In the studies already cited -held in La Rioja on Tempranillo grapes-, it was found that the wines made from grapes treated not even reached the minimum of the reference dose (36-350 mg/l) of methanol from the hydrolysis of pectins of the grape but without importance in the aroma though it has because of its toxic effect. All wines met that acetaldehyde, produced in the first phase as an intermediate product of fermentation of yeast metabolism from pyruvate, remained at the lowest possible levels (6-190 mg/l). Wines with dichlofluanid and sub-products of formulations of procymidone and iprodione presented amyl alcohols -formed in yeast- over 180 mg/l (appearance of plant sensations), though there were significant differences between samples. These higher alcohols -more than two carbons- were transferred to the medium and its variations were due to the fermentation process. Their normal ranges are 150-500 mg/l but the effect is negative at levels above 300 mg/l communicating unpleasant herbaceous feelings. The wines of the experience had ethyl acetate levels within the range of acceptable quality, except the sample with dichlofluanid which exceeded the threshold of perception (over 150 mg/l). This ester is the most abundant and high content comes from the accidental development of oxidative yeasts which do not change the volatile acidity or bacteria that oxidize ethanol to acetic conferring elevated hardness and roughness, adhesive and chopped sensations in wine. Nor were there significant differences in the formation of fatty acid ethyl esters (responsible for fruity and floral sensations), though the wine had half the concentration of diclofuanide. No significant differences were found in the total content of acetate and methyl acetate. Not so with the acetates responsible for fruity and flower smell of wines such as isoamyl acetate (principal responsible for the fruity aroma), hexyl acetate and 2-phenylethyl acetate. The wine with dichlofluanid did not contain hexyl acetate or 2-phenylethyl and did not reach the level of perception of isoamyl. The total fatty acid content of more than five carbons were similar to those found in the literature, so these fungicides have little influence over them, except for dichlofluanid that presented the lowest values (Santos, 1997).
Also, the effect of new fungicides (famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, quinoxyfen and trifloxystrobin) residues on the aroma composition of Monastrell red wines shown that all fungicides treatments significantly affect the wine aroma composition, though it does not necessary indicate changes on the sensorial profile as the variation range do not overpass the olfactory threshold of each compound. The most affected group of volatile, in terms of active principle or treatment, were the acetate and acids indicating that all fungicides may have some influence on the yeast activity while alcoholic fermentation takes place. It is important to point out that quinoxyfen and trifloxystrobin do not affect the volatile terpenoids. Kresoxim methyl and fenhexamid active principles have the lowest effect on the aroma composition while fluquinconazole and trifloxystrobin principles were the most reactive (Oliva et al., 2008).

Influence on the acid fraction.

Many oenological properties or aspects are influenced by the acidity of the must and wine. Among them, we may cite the development and metabolism of microorganisms, the wine's color, precipitation of tartaric salts and especially the organoleptic properties.
The acid fraction of wine is also affected by the presence of residues of certain fungicides (fenarimol, metalaxyl, mancozeb and penconazole). In a experiment with different wine-makings -some with treatments, others as control and others from traditional farming-, it

was found that the evolution of total acidity was similar in relative terms during the production for all the tests, but their initial values were different and the final values guaranteed conservation and proper balance in its constitution. The evolution of the volatile acidity was different for the various tests, noting that the final value of volatile acidity decreased more than the control and that there was a dramatic decrease in the case of fenarimol (no justification found) (Oliva et al., 1998b).

Moreover, other studies have shown that residues dichlofluanid at high doses causes acidification of the wine (Oliva et al., 1999c; Santos, 1997). Also, white wines (var. Airén) fermented in the presence of the fungicides azoxystrobin, cyprodinil, fludioxonil, kresoxim methyl, pyrimethanil and quinoxyfen, showed that they cause decreases in the final content of the different acids in most cases (García, 2002).

We have studied the influence of the residues of famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen (fungicides widely used in vineyards) could have on the content of organic acids (citric, malic, succinic and tartaric) in Monastrell wines. Made two different treatments in the grape -at harvest time and other at critical conditions-, the statistical study indicated that there were significant differences for famoxadone and kresoxim methyl compared for malic acid in GAP, while those treated with fluquinconazole and quinoxyfen for citric acid in critical conditions, fenhexamid fluquinconazole and for succinic acid in both conditions and finally treated with quinoxyfen for tartaric acid in critical conditions. For trials where significant differences were obtained, an increase in the levels of malic and citric acids and a decrease of succinic acid and tartaric acid content were obtained. The final values of the acids studied were typical levels of quality wines, so we can conclude that the residues of fungicides do not affect the final quality of wines despite the differences found (Oliva et al., 2009c).

In studying the effect of the presence of azoxystrobin, quinoxyfen and kresoxim methyl on the final content of organic acids (citric, lactic, succinic and tartaric acids in white Airen wines) inoculated with different strains of *Saccharomyces*, it was observed that the absolute values of organic acids were within the optimum range for quality wines, except tartaric which was too low. From the analytical standpoint, the yeast most affected by the presence of residues of fungicides was *Saccharomyces cerevisiae syn uvarum*, causing declines of citric, lactic and tartaric acid but increases of succinic acid with respect to the control. Therefore and despite the influence of the residues of these fungicides, they do not significantly influence on the organoleptic quality of the finished wine, as they also produce decreases of the final contents of those acids (García, 2002).

Influence on the color and antioxidant activity.

The phenolic compounds are of great importance in oenology, as they are considered the origin of color and astringency (tannins) and have nutritional and pharmacological interest. The factors that influence their content in wine may be soil-climatological, genetic, cultural and oenological ones (presence of pesticide residues).

In relation to color, studying the influence of fenarimol, mancozeb, metalaxyl, vinclozolin and penconazole, there were no significant differences in the intensity of color and tone but does in case of saturation with respect to the control wine in the presence of fenarimol and penconazole (Oliva et al., 1999d).

By studying the influence of the fungicides azoxystrobin, cyprodinil (Switch), fludioxonil (Switch), kresoxim methyl, pyrimethanil and quinoxyfen in the final color of wine, significant differences were found between the vinification of the treated and control grapes.

As it can be seen in the data obtained and presented in Table VI, there were significant differences between the control wine and those made in the presence of residues of fungicides for all parameters except for the total content in anthocyanins and the ortodiphenols. These differences were most pronounced for the wines produced in the presence of pyrimethanil (Fernández et al., 2001).

	Control	Quinoxyfen (a)	Kresoxim (b)	Azoxystrobin (c)	Swicth* (d)	Pyrimethanil (e)	DS p ≤ 0.05
CI	19.27±0.58	15.16±1.26	15.34±0.99	16.15±1.09	15.97±2.75	15.26±0.22	a,b,c,d,e
H	0.45±0.01	0.46±0.01	0.47±0.02	0.47±0.01	0.46±0.01	0.47±0.01	b,c,e
TP	2.45±0.32	1.96±0.08	1.81±0.25	2.12±0.16	1.80±0.16	1.51±0.10	a,b,d,e
FI	48.40±1.05	38.57±4.54	40.50±2.86	42.97±2.56	38.57±4.76	33.80±1.60	a,b,d,e
TPI	83.47±2.45	58.67±4.92	67.97±2.61	71.67±3.70	59.47±7.71	53.10±0.20	a,b,c,d,e
Cat.	257.9±5.1	233.8±24.7	203.2±8.5	221.7±31.9	213.9±23.9	174.0±17.6	b,d,e
Ant.	445.9±29.5	410.4±32.9	385.0±34.6	392.4±40.2	425.2±52.5	421.3±12.6	NS
Tan.	361.9±12.9	309.3±72.5	281.1±17.5	304.2±26.1	298.4±44.1	254.2±8.7	b,e
%M	48.27±3.19	47.52±3.49	47.92±1.50	46.95±0.55	38.23±2.14	41.15±1.84	d,e
%RP	33.58±1.80	38.50±3.44	36.00±0.88	36.44±0.72	44.07±1.61	44.34±2.74	a,d,e
%BP	18.13±1.46	14.65±0.95	16.08±0.73	16.60±1.04	17.69±0.71	14.51±1.05	a,b,e
OD	12.53±3.41	10.28±0.37	9.68±2.24	12.77±1.73	12.31±0.13	11.51±1.51	NS
H_c	19.10±0.10	23.43±1.70	23.93±0.35	23.34±0.74	24.20±2.76	22.10±1.11	a,b,c,d,e,
S	5.26±0.02	4.87±0.54	4.17±0.10	4.33±0.13	4.27±0.50	4.66±0.32	b,c,d,e

CI: Color intensity; **H**: Hue; **TP**: Total polyphenols (g/l galic acid); **FI**: Folín index; **TPI**: Total polyphenols index; **Cat**: Catechins (mg/l D-catechin); **Ant**: Totals anthocyanins (mg/l anthocyanins); **Tan**: Tannins (mg/l Tannic acid); **%M**: Monomers; **%RP**: Red polymers; **%BP**: Brown polymers; **OD**: ortodiphenols (mg/l D-catechin); **H_c**: Hue CIELab; **S**: Saturation; **DS**: Degree of significance; **NS**: Not significant; * Ciprodinil and fludioxonil mix.

Table VI. Influence of some fungicides in the final color of wine

The color intensity is the main element of trial in the visual phase of sensory analysis. The greater or lesser value for this parameter is strongly influenced by the degree of ripeness of the grapes, the maceration time and temperature to which it is made. If we consider that these factors have been the same at all wine-makings, it is conceivable that the presence of studied fungicide residues during the production has helped to reduce the rate of diffusion of the phenolic compounds from the skin into the must during the period of maceration.

The absolute value of total polyphenols index is indicative of the ability of the wine to undergo parenting. We observed great difference between the total polyphenols index for the control wine and those obtained in the presence of pesticide residues in this study.

When we study the phenolic composition of these wines, statistical analysis of the data showed that there were significant differences between the control wine and the remaining ones (Oliva et al., 2005) (Tables VII and VIII).

Regarding the phenolic compounds of low molecular weight (Table VII), it should be noted that there were significant differences between the control wine and those obtained in the presence of residues of fungicides for all compounds studied, except for tyrosol.

In conclusion, the wines made from grapes treated with fungicides presented significant differences for most compounds. Fungicides that influence phenolic compounds were azoxystrobin, kresoxim methyl and pyrimethanil. The minor influence was produced by quinoxyfen. The final contents of all compounds studied were within the values given in the literature.

Compound	Control	Kresoxim (a)	Quinoxyfen (b)	Azoxystrobin (c)	Switch* (d)	Pyrimethanil (e)	DS p ≤ 0.05
Gálic acid	19.08±1.81	13.88±1.16	14.10±3.16	14.08±3.27	12.60±2.19	6.19±0.55	a,b,c,d,e
Tyrosol	27.52±9.29	21.41±6.26	32.05±3.94	24.38±3.33	32.22±8.95	29.78±6.44	-
4-hid.benz. acid	10.34±1.05	5.32±2.31	6.16±2.43	6.87±0.36	4.95±1.06	2.58±1.78	a,b,c,d,e
Vainillic acid	4.23±1.78	4.18±1.26	3.28±1.00	7.93±2.24	3.50±1.88	3.67±1.57	c
Catechin	29.28±11.96	10.25±3.16	12.43±5.57	9.26±3.20	10.12±4.01	14.44±3.88	a,c,d,e
Siringic acid	13.48±1.66	11.07±0.75	10.52±3.25	12.14±2.26	8.63±1.22	7.43±2.53	a,d,e
4-cumaric acid	3.81±2.63	1.65±0.17	2.08±0.40	1.86±0.84	1.66±0.48	2.53±2.09	a,d,e
Ferulic acid	1.39±0.28	4.75±0.62	5.92±0.48	0.89±0.09	3.36±0.76	0.81±0.42	a,b,c,d,e
Tr.-resveratrol	0.52±0.08	1.24±0.34	1.97±0.14	1.74±0.12	1.63±0.14	1.42±0.53	a,b,c,d,e

Table VII. Phenolic compounds of low molecular weight (mg/l) in wines (mean ± DS).

Compound	Control	Kresoxim (a)	Quinixyfen (b)	Azoxystrobin (c)	Switch* (d)	Pyrimethanil (e)	DS p ≤ 0.05
Delfinidin-3-gl.	0.62±0.08	0.66±0.19	0.52±0.07	0.31±0.08	0.82±0.23	0.88±0.35	c
Cianidin-3-gl.	0.26±0.03	0.26±0.06	0.29±0.06	0.26±0.08	0.31±0.05	0.35±0.09	e
Petunidin-3-gl.	1.79±0.18	1.94±0.38	1.78±0.24	0.99±0.32	2.46±0.53	2.60±0.71	c
Peonidin-3-gl.	0.94±0.05	1.11±0.11	0.98±0.13	0.85±0.17	1.26±0.25	1.86±0.42	a,e
Malvidin-3-gl.	13.17±0.64	13.22±1.56	10.83±1.42	7.88±1.63	13.46±1.97	15.91±3.03	a,b,c

Table VIII. Anthocyanins (mg/l) in wines (mean ± DS).

The phenolic composition of wines with different intensity can be altered by the presence of residues of certain fungicides. Thus, in a study conducting microvinifications with spontaneous fermentation by yeast added (LSA) from grapes treated individually with famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen in critical conditions (same day of harvest), we determined the total amount of anthocyanins, hydroxycinnamic acids, flavonols and trans-resveratrol. No significant differences were found for these compounds among the witnesses with spontaneous fermentation with added yeast. Once the statistical study performed, significant differences were found in the anthocyanin content of grapes treated for famoxadone, fenhexamid and trifloxystrobin; for hidoxicinamic acid in the case of treatments with famoxadone fluquinconazole, kresoxim methyl and trifloxystrobin and differences appeared for trans-resveratrol for all fungicides except quinoxyfen and finally for flavonols in the case of famoxadone and kresoxim methyl. In addition, there was a decrease in the content when these differences occur for the four compounds (Barba et al., 2009a).

When measuring the antioxidant activity of wines, it was found that fungicides did not produce a decrease in the antioxidant activity of wines, both in treatment at pre-harvest

interval as in the case of the most unfavorable conditions (White: 7.19; Treated at pre-harvest interval: 6.45 and Unfavorable: 10.06 Tolox mmol/l). It was noted that the presence of famoxadone, kresoxim methyl and quinoxyfen increased the antioxidant activity directly related to their levels in grapes (Oliva et al., 2009a).
Finally, one can deduce that the presence of some of the studied fungicides may cause a loss of co-pigmentation, lower color stability and decrease in antioxidant capacity. However, the contents analyzed were in the normal range of Monastrell red wines and therefore we should not discourage the use of these fungicides.

2.3 Hygienic-sanitary quality and toxicity for consumer.

If the oenotechnological processes are not performed or done incorrectly, some of the residues in the juice can pass to the end wine and stay for longer or shorter time, leading to poor health and hygiene quality of the finished product.
The presence of residues of fungicides in wine greatly concerns the consumer. Therefore, both the European Union and individual countries are conducting studies to determine transfer factors in the process of making wine and also checking the safety of residues remaining in the finished wine. Currently, there is no established maximum residue limits (MRLs) for specific wines, though there are several countries that provide these values for wine grapes.
We highlight the importance of fungicides as a complement to the establishment of transfer factors, since they can generate harmful effects on humans as toxic. Its bioavailability in the body depends on its toxicological kinetics: absorption, distribution, metabolism and elimination, so it is interesting to study this. These processes are influenced by both external factors related to exposure patterns and the chemical (type of employment, environmental temperature, type of pesticide, frequency, intensity and duration of exposure, etc.) and factors inherent to the individual (age, gender, genetic endowment, health, nutritional status, lifestyle, major route of absorption, etc.) (Fait & Colosio, 1998; Bollinger et al., 2005).
Bioavailability is essentially a pharmaceutical term which refers to the portion of the dose of a drug administered exogenously that reaches the organ or tissue in which it carries out its action. As determining the concentration in tissue is too invasive, the value of the concentration in plasma is accepted. This concept is used to quantify the degree to which a substance is used by the body. Today, the scope of the study of the bioavailability of a xenobiotic has spread to other areas and so there are many studies on nutrient and some pollutants. This value is implied by the ingestion toxicity studies performed in experimental animals in the registration of a substance for pesticide residues, but the substance is applied in pure form -unmixed with food- and has been amply demonstrated in the pharmaceutical field that the presence of food can significantly reduce or even increase the bioavailability of a xenobiotic to the body. Therefore, the study of the bioavailability of a residue embedded in the medium that reaches the body is essential to determine the dose that actually enters it. The results published by our research group have been the first to provide this in food, but we do not include data on them since this was studied for insecticides.

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Do Cytochrome P450 Enzymes Contribute to the Metabolism of Xenobiotics in Human?

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1. Introduction

The cytochromes P450 (CYP) comprise a large multigene family of hemethiolate proteins which are of considerable importance in the metabolism of xenobiotics and endobiotics. CYP enzymes in humans as well as in other species have been intensively studied during recent years (Pelkonen et al., 2008; Turpeinen et al., 2007). It is possible to characterize metabolic reactions and routes, metabolic interactions, and to assign which CYP is involved in the metabolism of a certain xenobiotic by different *in vitro* approaches (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Hodgson and Rose, 2007a). Risk assessment needs reliable scientific information and one source of information is the characterization of the metabolic fate and toxicokinetics of compounds. Toxicokinetics refers to the movement of a xenobiotic into, through, and out of the body and is divided into several processes including absorption, distribution, metabolism, and excretion (ADME). Metabolism is one of the most important factors that can affect the overall toxic profile of a pesticide. During metabolism, the chemical is first biotransformed by phase I enzymes, usually by the cytochrome P450 (CYP) enzyme system, and then conjugated to a more soluble and excretable form by phase II conjugating enzyme systems (Guengerich & Shimada, 1991). In general, these enzymatic reactions are beneficial in that they help eliminate foreign compounds. Sometimes, however, these enzymes transform an otherwise harmless substance into a reactive form – a phenomenon known as metabolic activation (Guengerich & Shimada, 1991).Exposure to pesticides is a global challenge to risk assessment (Alavanja et al., 2004; Maroni et al., 2006). On a world-wide basis, acute pesticide poisoning is an important cause of morbidity and mortality. In an extrapolation, WHO/UNEP estimated that more than 3 million people were hospitalized for pesticide poisoning every year and that 220 000 died; it particularly noted that two-thirds of hospitalizations and the majority of deaths were attributable to intentional self-poisoning rather than to occupational or accidental poisoning (Konradsen et al., 2005; WHO/UNEP, 1990). Humans are inevitably exposed to pesticides in a variety of ways: at different dose levels and for varying periods of time (Boobis et al., 2008; Ellenhorns et al., 1997).

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2. CYPs - the human xenobiotic-metabolizing enzymes

CYPs are found in high concentrations in the liver, but are present in a variety of other tissues, including lungs (Lawton et al., 1990), kidneys ((Hjelle et al., 1986; Tremaine et al., 1985), the gastrointestinal tract (Dutcher & Boyd, 1979; Peters and Kremers, 1989), nasal mucosa (Adams et al., 1991; Eriksson and Brittebo, 1991), skin (Khan et al., 1989) and brain tissue (Bergh & Strobel, 1992; Dhawan et al., 1990). CYPs are categorized into families and subfamilies by their sequence similarities. Humans have 18 families of cytochrome P450 genes and 44 subfamilies. The enzymes are thus identified by a number denoting the family, a letter denoting the subfamily and a number identifying the specific member of the subfamily. The example given below explains the system of nomenclature followed (Fig. 1).

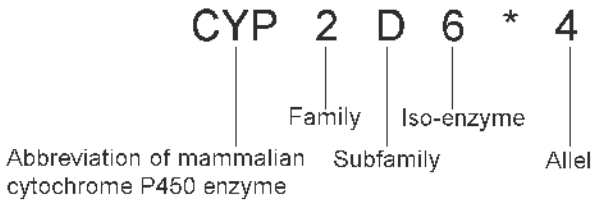


Fig. 1. An example of the nomenclature of the cytochrome P450 enzymes (modified from (Wijnen et al., 2007)).

The website http://drnelson.utmem.edu/CytochromeP450.html contains more detailed classification related to the cytochrome P450 metabolizing enzymes. The CYP enzymes in families 1-3 (Fig. 2) are active in the metabolism of a wide variety of xenobiotics including drugs (Pelkonen et al., 2008; Pelkonen et al., 2005; Rendic & Di Carlo, 1997).

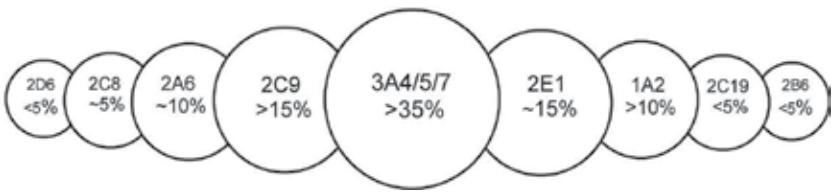


Fig. 2. Relative abundance of individual CYP forms in the liver (modified from (Pelkonen et al., 2008)).

2.1 CYP1A subfamily

CYP1A1 and CYP1A2 are members of the CYP1A subfamily. CYP1A1 is a major extrahepatic CYP enzyme and its level of expression in human liver is very low (Pelkonen et al., 2008; Guengerich & Shimada, 1991; Ding & Kaminsky, 2003; Edwards et al., 1998; McKinnon et al., 1991; Pasanen & Pelkonen, 1994; Raunio et al., 1995). In humans, CYP1A2 is expressed mainly in liver and in lower levels in lung along with CYP1A1 (Liu et al., 2003; Wei et al., 2001; Wei et al., 2002). CYP1A2 represents about 10% of total CYP enzymes in the human liver (Pelkonen & Breimer, 1994; Shimada et al., 1994). CYP1A2 enzyme levels in the human liver display some variability between individuals (Shimada et al., 1994).

2.2 CYP2A subfamily

The human CYP2A subfamily contains three genes i.e. CYP2A6, CYP2A7, and CYP2A13, and two pseudogenes (Hoffman et al., 1995; Honkakoski & Negishi, 1997; Pedro et al., 1995).

CYP2A6 represents 10% of the total CYP content in liver (Pelkonen et al., 2008; Yun et al., 1991). CYP2A6 enzyme activity in the human liver displays a relatively large variability between individuals, and some Japanese are known to lack the functional protein completely (Pelkonen et al., 2008; Pelkonen et al., 2000; Shimada et al., 1996).

2.3 CYP2B subfamily

CYP2B6 represents approximately 1-10% of the total hepatic CYPs. A notable interindividual variability in the expression of CYP2B6 has been reported (Code et al., 1997; Faucette et al., 2000; Lang et al., 2001; Stresser & Kupfer, 1999; Yamano et al., 1989). CYP2B6 has a high polymorphic expression and it is affected by genotype and gender.

2.4 CYP2C subfamily

The CYP2C subfamily has four active members, namely 2C8, 2C9, 2C18 and 2C19. CYP2Cs are the second most abundant CYP proteins in human liver and the CYP2C subfamily consists of three members, comprising about 20 % of the total P450 enzymes. In humans, CYP2C9 is the main CYP2C, followed by CYP2C8 and CYP2C19, while CYP2C18 is not expressed in liver (Pelkonen et al., 2008; Edwards et al., 1998; Shimada et al., 1994; Gray et al., 1995; Richardson et al., 1997). CYP2C9 is a major CYP2C isoform in the human liver, and it is one of several CYP2C genes clustered in a 500kb region on the proximal 10q24 chromosomal region (Gray et al., 1995; Goldstein and de Morais, 1994). In Caucasian populations, the frequencies of the two variant alleles, CYP2C9*2 and CYP2C9*3, range from 7% to 19% (Furuya et al., 1995; Ingelman-Sundberg et al., 1999; Miners & Birkett, 1998; Stubbins et al., 1996; Sullivan-Klose et al., 1996; Yasar et al., 1999). CYP2C19, another member of the CYP2C enzyme family, represents approximately 5% of the total hepatic CYPs and metabolizes drugs that are amides or weak bases with two hydrogen bond acceptors (Pelkonen et al., 2008; Lewis, 2004; Musana & Wilke, 2005). Poor metabolizers with low CYP2C19 activity represent 3 to 5% of Caucasians and African-Americans, and 12 to 23% of most Asian populations (Goldstein, 2001).

2.5 CYP2D subfamily

CYP2D6 represents 1 to 5% of the total CYP, and approximately 3.5 and 5-10% of the Caucasian population are ultra-rapid and poor metabolize's for this enzyme, respectively. The CYP2D6 gene is clearly the most polymorphic of all known cytochrome P450s; more than 75 polymorphisms have been identified. Four alleles account for > 95% of the functional variation observed in the general population (Pelkonen et al., 2008; Shimada et al., 1994; Musana & Wilke, 2005; Al, Omari, A., & Murry, 2007; Ingelman-Sundberg, 2004; Zanger et al., 2004).

2.6 CYP2E subfamily

Only one gene belonging to this subfamily, namely CYP2E1, has been identified (Nelson et al., 1996; Nelson et al., 2004). CYP2E1 is one of the most abundant hepatic CYPs, represents 15% of the total P450 and it is also expressed in lung and brain (Pelkonen et al., 2008; Raunio et al., 1995).

2.7 CYP3A subfamily

In humans, the CYP3A subfamily contains three functional proteins, CYP3A4, CYP3A5, and CYP3A7, and one pseudoprotein, CYP3A34. The human CYP3 family constitutes approximately 30 % of total hepatic P450 and is estimated to mediate the metabolism of

around 50% of prescribed drugs as well as a variety of environmental chemicals and other xenobiotics. Because of the large number of drugs metabolized by CYP3A4, it frequently plays a role in a number of drug-drug interactions (Pelkonen et al., 2008; Shimada et al., 1994; Musana & Wilke, 2005; Bertz & Granneman, 1997; Domanski et al., 2001; Imaoka et al., 1996; Rostami-Hodjegan & Tucker, 2007).

CYP3A4 is the major form of P450 expressed in human liver. It is also the major P450 expressed in human gastrointestinal tract, and intestinal metabolism of CYP3A4 substrate can contribute significantly to first-pass elimination of orally ingested xenobiotics (Guengerich, 1995; Guengerich, 1999). X-ray crystallography studies demonstrated that CYP3A4 has a very large and flexible active site, allowing it to oxidize either large substrates such as erythromycin and cyclosporine or multiple smaller ligands (Scott & Halpert, 2005; Tang & Stearns, 2001).

CYP3A5 is a minor polymorphic CYP isoform in human liver in addition to the intestine (Lin et al., 2002; Paine et al., 1997) and kidney (Haehner et al., 1996). Functional CYP3A5 is expressed in approximately 20% of Caucasians and about 67% of African-Americans (Kuehl et al., 2001). CYP3A5 may have a significant role in drug metabolism particularly in populations expressing high levels of CYP3A5 and/or on co-medications known to inhibit CYP3A4 (Soars et al., 2006).

Expression of CYP3A7 protein is mainly confined to fetal and newborn livers, although in rare cases CYP3A7 mRNA has been detected in adults (Hakkola et al., 2001; Kitada & Kamataki, 1994; Schuetz et al., 1994).

3. Xenobiotic biotransformation

Xenobiotic biotransformation is the process by which lipophilic foreign compounds are metabolized through enzymatic catalysis to hydrophilic metabolites that are eliminated directly or after conjugation with endogenous cofactors via renal or biliary excretion. These metabolic enzymes are divided into two groups, Phase I and Phase II enzymes (Rendic & Di Carlo, 1997; Oesch et al., 2000).

Phase I products are not usually eliminated rapidly, but undergo a subsequent reaction in which an endogenous substrate such as glucuronic acid, sulfuric acid, acetic acid, or an amino acid combines with the newly established functional group to form a highly polar conjugate to make them more easily excreted. Sulfation, glucuronidation and glutathione conjugation are the most prevalent classes of phase II metabolism, which may occur directly on the parent compounds that contain appropriate structural motifs, or, as is usually the case, on functional groups added or exposed by Phase I oxidation (LeBlanc & Dauterman, 2001; Rose & Hodgson, 2004; Zamek-Gliszczynski et al., 2006).

3.1 In vitro and human-derived techniques for testing xenobiotic metabolism

In order to study the metabolism and interactions of pesticides in humans we have to rely upon *in vitro* and human-derived techniques. *In vitro* systems have become an integral part of drug metabolism studies as well as throughout the drug discovery process and in academic research (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Lin & Lu, 1997). *In vitro* approaches to predict human clearance have become more frequent with the increase in the availability of human-derived materials (Skett et al., 1995). All models have certain advantages and disadvantages, but the common advantage to these approaches is the reduction of the complexity of the study system. However, the use of *in vitro* models is always a compromise between convince and relevance (Pelkonen et al., 2005; Brandon et al.,

2003; Pelkonen & Turpeinen, 2007; Pelkonen & Turpeinen, 2007). An overview of different *in vitro* models and their advantages and disadvantages are collected in Table 1.

Enzyme sources	Availability	Advantages	Disadvantages
Liver homogenates[a]	Relatively good. Commercially available. Human liver samples obtained under proper ethical permission.	Contains basically all hepatic enzymes.	Liver architecture lost. Cofactors are necessary.
Microsomes[a]	Relatively good, from transplantations or commercial sources.	Contains important rate-limiting enzymes. Inexpensive technique. Easy storage. Study of individual, gender-, and species-specific biotransformation.	Contains only CYP and UGTs. Requires strictly specific substrates and inhibitors or antibodies. Cofactor addition necessary.
cDNA-expressed individual CYPs[b]	Commercially available	The role of individual CYPs in the metabolism can be easily studied. Different genotypes. High enzyme activities.	The effect of only one enzyme at a time can be evaluated. Problems in extrapolation to HLM and *in vivo*.
Primary hepatocytes[c,d]	Difficult to obtain, relatively healthy tissue needed. Commercially available	Contains the whole complement of CYPs cellularly integrated. The induction effect can be studied. Well established and characterized. Transporters still present and operational.	Requires specific techniques and well established procedures. The levels of many CYPs decrease rapidly during cultivation. Cell damage during isolation.
Liver slices[e]	Difficult to obtain, fresh tissue needed.	Contains the whole complement of CYPs and cell-cell connections. The induction, morphology and interindividual variation can be studied.	Requires specific techniques and well established procedures
Immortalized cell lines[f]	Available upon request, not many characterized cell lines exist.	Non-limited source of enzymes. Easy to culture. Relatively stable enzyme expression level. The induction effect can be studied.	The expression of most CYPs is poor.

[a] (Kremers, 1999); [b] (Rodrigues, 1999); [c] (Guillouzo, 1995); [d] (Gomez-Lechon et al., 2004); [e] (Olinga et al., 1998); [f] (Allen et al., 2005).

Table 1. An overview of different in vitro models and their advantages and disadvantages (modified from (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Brandon et al., 2003; Pelkonen & Turpeinen, 2007)).

3.2 In vitro characterization of the metabolism and metabolic interactions of xenobiotics

The aim of *in vitro* characterization is to produce relevant and useful information on metabolism and interactions to anticipate, and even to predict, what happens in man. Each *in vitro* model has its own set of advantages and disadvantages as they range from simple to more complex systems: individual enzymes, subcellular fractions, cellular systems, liver slices and whole organ, respectively (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Brandon et al., 2003). To understand some of the factors related to xenobiotic metabolism that can influence the achievement of these aims, there are several important points to consider such as (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Pelkonen & Turpeinen, 2007; Hodgson & Rose, 2005):

- Determination of the metabolic stability of the compound
- Identification of reactive metabolites
- Evaluation of the variation between species
- Identification of human CYPs and their isoforms involved in the activation or detoxification
- Evaluation of the variation between individuals
- Identification of individuals and subpopulations at increased risk
- Overall improvement of the process of human risk assessment

An overview of different in vitro studies for the characterization of metabolism and metabolic interactions of xenobiotics are collected in Table 2.

In vitro test	*In vitro* model	Parameters	Extrapolations
Metabolic stability	Microsomes Homogenates Cells Slices	Disappearance of the parent molecule or appearance of (main) metabolites	Intrinsic clearance Interindividual variability Interspecies differences
Metabolite identification and quantitation	Microsomes Homogenates Cells Slices	Tentative identification by (e.g.) LC/TOF-MS	Metabolic routes Semi-quantitative Interspecies differences
Identification of metabolizing enzymes	Microsomes with inhibitors or inhibitory antibodies Recombinant CYPs Hepatocytes	Relative ability of enzymes to metabolize a compound	Prediction of effects of various genetic, environmental and pathological factors Interindividual variability
Enzyme inhibition	Microsomes Recombinant enzymes Hepatocytes	Inhibition of specific model substrate	Potential interactions
Enzyme induction	Cells Slices Permanent cell lines	Induction of CYP model activities or mRNA	Induction potential of a substance

Table 2. In vitro studies for the characterization of the metabolism and metabolic interactions of xenobiotics (modified from (Pelkonen et al., 2005; Pelkonen and Raunio, 2005)).

4. The contribution of CYPs to the metabolism of xenobiotics in human

4.1 CYP1A subfamily

The catalytic activities of CYP1A2 have been reviewed by Pelkonen *et al.* (Pelkonen et al., 2008). CYP1A2 has a major role in the metabolism of many important chemicals such as caffeine (Butler et al., 1989; Tassaneeyakul et al., 1992), phenacetin (Sesardic et al., 1990; Venkatakrishnan et al., 1998), theophylline (Sarkar & Jackson, 1994; Tjia et al., 1996), clozapine (Fang et al., 1998), melatonin (Facciolá et al., 2001; von Bahr et al., 2000), and tizanidine (Granfors et al., 2004a; Granfors et al., 2004b). CYP1A1 is a major enzyme in the metabolism of a number of insecticides and herbicides (Lang et al., 1997; Tang et al., 2002; Abass et al., 2010; Abass et al., 2007c). CYP1A2 mediates herbicides (Lang et al., 1997; Abass et al., 2007c; Nagahori et al., 2000), insecticides (Tang et al., 2002; Stresser & Kupfer, 1998; Foxenberg et al., 2007; Mutch & Williams, 2006), and pyrethroids metabolism (Scollon et al., 2009).

4.2 CYP2A subfamily

It has been shown that CYP2A6 has a major role in the metabolism of nicotine *in vitro* and *in vivo* (Kitagawa et al., 1999; Messina et al., 1997; Nakajima et al., 1996a; Nakajima et al., 1996b; Yamazaki et al., 1999) and in the activation of aflatoxin B1 (Yun et al., 1991; Salonpää et al., 1993). More substrates and inhibitors currently known to be metabolized by or to interact with CYP2A6 *in vitro* and *in vivo* have been summarized by Pelkonen and co-workers (Pelkonen et al., 2008; Pelkonen et al., 2000). CYP2A6 participates in the metabolism of quite a few pesticides such as carbaryl, imidacloprid, DEET, carbosulfan and diuron (Tang et al., 2002; Abass et al., 2010; Abass et al., 2007c; Schulz-Jander and Casida, 2002; Usmani et al., 2002).

4.3 CYP2B subfamily

CYP2B6 is known to metabolize a large number of substrates including drugs, pesticides and environmental chemicals, many of which have been described in detail in reviews (see e.g. (Ekins & Wrighton, 1999; Hodgson & Rose, 2007b; Turpeinen et al., 2006)). Several clinically used drugs such as cyclophosphamide, bupropion, S-mephenytoin, diazepam, ifosamide and efavirenz are metabolized in part by CYP2B6 (Granvil et al., 1999; Haas et al., 2004; Huang et al., 2000; Jinno et al., 2003; Roy et al., 1999b; Roy et al., 1999a). CYP2B6 appears to activate and detoxify a number of precarcinogens (Code et al., 1997; Smith et al., 2003). CYP2B6 plays a major role in pesticides metabolism. CYP2B6 mediates herbicides N-dealkoxylation (Coleman et al., 2000); organophosphate insecticides desulfuration (Foxenberg et al., 2007; Mutch & Williams, 2006; Buratti et al., 2005; Leoni et al., 2008; Sams et al., 2000; Tang et al., 2001); orghanochlorine and carbamate insecticides sulfoxidation (Abass et al., 2010; Casabar et al., 2006; Lee et al., 2006); fungicide metalaxyl *O*-demethylation and lactone formation (Abass et al., 2007b).

4.4 CYP2C subfamily

CYP2C8 meditates amodiaquine N-deethylation, which is the selective marker activity, paclitaxel 6α-hydroxylation and cerivastatin demethylation (Li et al., 2002; Rahman et al., 1994). A few insecticides are mainly metabolized by CYP2C8 such as parathion, deltamethrin, esfenvalerate, and β-cyfluthrin (Mutch & Williams, 2006; Scollon et al., 2009; Mutch et al., 2003; Godin et al., 2007).

CYP2C9 is responsible for the metabolism of the S-isomer of warfarin (Rettie et al., 1992). CYP2C9 also metabolizes tolbutamide, the selective marker, glipizide, fluvastatin,

phenytoin, several non-steroidal anti-inflammatory agents and many other drug groups (Miners & Birkett, 1998; Doecke et al., 1991; Kirchheiner & Brockmoller, 2005; Rettie and Jones, 2005). CYP2C9 is found to be involved in the metabolism of pesticides such as pyrethroid insecticides (Scollon et al., 2009; Godin et al., 2007), as well as organophosphorus insecticides (Leoni et al., 2008; Usmani et al., 2004).

CYP2C19 participates in the metabolism of many commonly used drugs including the antiepileptics phenytoin and mephenytoin (Bajpai et al., 1996; Komatsu et al., 2000; Tsao et al., 2001; Wrighton et al., 1993)(Bajpai *et al.* 1996, Komatsu *et al.* 2000, Tsao *et al.* 2001, Wrighton *et al.* 1993), selective serotonin receptor inhibitors citalopram and sertraline (Kobayashi et al., 1997; von Moltke et al., 2001), the psychoactive drugs amitriptyline (Venkatakrishnan et al., 1998; Jiang et al., 2002) and diazepam, among others (Jung et al., 1997). Among the substrates of CYP2C19 are several widely used pesticides such as the phosphorothioate insecticides (Foxenberg et al., 2007; Mutch & Williams, 2006; Leoni et al., 2008; Tang et al., 2001; Usmani et al., 2004; Buratti et al., 2002; Kappers et al., 2001), as well as the pyrethroid insecticides (Scollon et al., 2009; Godin et al., 2007).

4.5 CYP2D subfamily

CYP2D6 metabolizes approximately 20 % of all commonly prescribed drugs *in vivo* (Brockmöller *et al.* 2000). For example, CYP2D6 contributes to the metabolism of betablockers metoprolol and timolol (Johnson & Burlew 1996, Volotinen *et al.* 2007) and the psychotropic agents amitriptyline and haloperidol (Coutts *et al.* 1997, Fang *et al.* 1997, Fang *et al.* 2001, Halling *et al.* 2008, Someya *et al.* 2003). Dextromethrophan *O*-demethylation is the most used *in vitro* model reaction for CYP2D6 activity (Kronbach *et al.* 1987, Park *et al.* 1984). Known pesticide substrates for CYP2D6 include phosphorothioate insecticides (Mutch *et al.* 2003, Mutch & Williams 2006, Sams *et al.* 2000, Usmani *et al.* 2004b) as well as (Johnson and Burlew, 1996; Volotinen et al., 2007) carbamate insecticide (Tang et al., 2002). CYP2D6 is also involved in the N-dealkylation of the atrazine and diuron herbicides (Lang et al., 1997; Abass et al., 2007c).

4.6 CYP2E subfamily

The metabolism of very few clinically important drugs such as paracetamol, caffeine, acetaminophen, enflurane and halothane seems to be mediated to some extent by CYP2E1 (Gu et al., 1992; Lee et al., 1996; Raucy et al., 1993; Thummel et al., 1993). Chlorzoxazone is probably the most used *in vitro* model substrate for CYP2E1 activity (Peter et al., 1990). Few pesticides have been reported to be metabolized at least in part by human CYP2E1 such as atrazine, carbaryl, parathion, imidacloprid and diuron (Lang et al., 1997; Tang et al., 2002; Abass et al., 2007c; Mutch & Williams, 2006; Schulz-Jander & Casida, 2002; Mutch et al., 2003).

4.7 CYP3A subfamily

CYP3A4 participates in the metabolism of several clinically important drugs such as triazolam, simvastatin, atorvastatin, and quinidine (Rendic & Di Carlo, 1997; Bertz & Granneman, 1997). Detailed characteristics of several CYP3A4 substrates and inhibitors were summarized recently by Liu *et al.* (Liu et al., 2007). The known pesticides mainly metabolized by CYP3A4 belong to several chemical groups such as, carbamate, phosphorothioate, chlorinated cyclodiene and neonicotinoid insecticides (Tang et al., 2002; Abass et al., 2010; Mutch & Williams, 2006; Schulz-Jander & Casida, 2002; Buratti et al., 2005;

Sams et al., 2000; Tang et al., 2001; Casabar et al., 2006; Lee et al., 2006; Mutch et al., 2003; Usmani et al., 2004; Buratti et al., 2002; Buratti et al., 2003; Buratti & Testai, 2007; Butler and Murray, 1997), herbicides (Abass et al., 2007c; Coleman et al., 2000), fungicides (Abass et al., 2007b; Abass et al., 2009; Mazur et al., 2007), and organotin biocide (Ohhira et al., 2006).
CYP3A5 mediates midazolam, alprazolam and mifepristone metabolism (Christopher Gorski et al., 1994; Galetin et al., 2004; Hirota et al., 2001; Huang et al., 2004; Khan et al., 2002; Williams et al., 2002). Alprazolam has been suggested as a selective probe for CYP3A5 (Galetin et al., 2004). The metabolism of a number of organophoshate and pyrethroid insecticides has been reported to be mediated by CYP3A5 (Mutch & Williams, 2006; Mutch et al., 2003; Godin et al., 2007).
CYP3A7 has similar catalytic properties compared with other CYP3A enzymes, including testosterone 6β-hydroxylation (Kitada et al., 1985; Kitada et al., 1987; Kitada et al., 1991).

5. The impact of modern analytical techniques in xenobiotics metabolism

5.1 Mass spectrometric methods in metabolism studies.

Traditionally metabolism studies were performed using gas chromatography-mass spectrometry (GC-MS). Because metabolites are usually polar molecules with high molecular masses, they have to be derivatised before measurement. After derivatisation the measured analyte is not anymore original metabolite but a less polar compound which is possible to vaporise and use in GC. Derivatisation can cause errors to the measurements and it is usually the most time and labour demanding phase which causes extra costs (Sheehan, 2002). In metabolism studies biggest problem of GC-MS is its lower sensitivity compared with modern mass spectrometric methods. Nevertheless GC-MS is still a useful method also in metabolism studies especially with thermally stable volatile compounds.
Nowadays the primary method used in metabolism studies is liquid chromatogram-mass spectrometry (LC-MS). Liquid chromatography is an old technique to separate polar compounds in liquid phase. However, it took quite a long time to develop a reliable technique to connect LC to the mass spectrometer, because the eluent solvent has to be vaporized before actual MS measurements which are performed in high vacuum. However after the introduction of electrospray ionisation (ESI) development has been very rapid during last 20 years and the performance of instruments has steadily improved. Usually ESI is the best choice for the ionization of polar metabolites but there are also other common ionisation methods like APCI (atmospheric pressure chemical ionisation) and APPI (atmospheric pressure photo-ionisation), which can be used to ionise less polar compounds. ESI can be run either in positive or negative modes and the best choice is dependent on the specific analyte. During ionisation hydrogen is either combined with the analyte to produce $[M+H]^+$ ion or broken away to produce $[M-H]^-$ ion, which can be accelerated within electric field. In the same time usually also other adducts, like sodium and potassium adducts, are formed. Sometimes other adducts can cause problems or decrease the sensitivity of the method. In addition other compounds that elute at same time from LC flow can reduce or block totally the ionisation of the analyte and cause errors to the measurements. This phenomenon is called ion suppression and it is quite common in ESI (Jessome & Volmer, 2006).
The most useful sample handling procedure to be used in metabolism studies with LC-MS is protein precipitation. It is performed easily by addition of organic solvent, either methanol or acetonitrile, to the samples. Samples are mixed and centrifuged to get clear supernatant. Usually after protein precipitation samples are clean enough to be analyzed directly, but

also other sample handling method may be needed with samples containing a lot of lipids or salts (Rossi, 2002). Different extraction methods, like SPE (solid phase extraction) or liquid-liquid extraction are then a better choice. However they are more expensive and time consuming methods.

Already HPLC (high performance liquid chromatography) is able to separate metabolites directly without any modifications. Compared to the GC, the resolution of HPLC is quite poor. Because mass spectrometric methods can measure compounds coming to the instrument at the same time, this has not been so big a problem. During the last five years liquid chromatography has improved considerably after introduction of ultra performance liquid chromatography (UPLC). UPLC instruments can work in higher operation pressures (up to 15.000 psi) which makes possible to use smaller particles and diameters in columns and to improve resolution, speed and sensitivity of the method. A typical run in UPLC can be just 5 minutes to analyse several different compounds.

Mass spectrometry is a superior method in the metabolism studies because of its high sensitivity. Although mass spectrometry is usually understood as one concept, it actually consist of several different types of instruments and techniques. Different types of instruments have specific advantages and consequently each individual type suits best for certain kind(s) of measurements. In the identification of metabolites time of flight mass spectrometry (TOF) is the best option. It can detect all ionized compounds simultaneously which improve the sensitivity compared to scanning instruments (Fountain, 2002). With help of the TOF instruments accurate mass of the analyte (±5 ppm) can be measured and elemental composition can be calculated. Modern instruments can easily reach 1 ppm mass accuracy and use isotope patterns of analytes to solve the right elemental composition with few potential possibilities. This kind of identification can be used to find different metabolites in samples, because masses of potential metabolites can usually be calculated before measurements. There are also softwares, such as Metabolynx (Waters Corp., Milford, MA, USA), which can search potential metabolites automatically from mass chromatograms and help a lot in data processing.

Additional structural information can be achieved with help of Q-TOF or triple quadrupole instruments. Measured analytes can be decomposed by collision with gas molecules (CID, collision induced dissociation) to produce fragment ions. In Q-TOF instruments accurate mass of fragment ions can be also measured to resolve molecular masses of fragments. In most cases fragmentation produces information about location of possible biotransformations. Because fragmentation is compound-specific, fragments can be used for identification purposes if they are known from previous measurements. However fragmentation is not as universal as in EI-ionisation (electron ionisation) of GC-MS instruments because it is partly instrument specific. With ion trap instruments even produced fragment ion can be selected and collided again to produce new smaller fragment ions. To resolve the structure of a metabolite completely other methods like nuclear magnetic resonance (NMR) or x-ray crystallography are usually required.

Knowledge about fragmentation of the analyte is useful also in quantitative measurements. Quantifications are usually performed in triple quadrupole instruments, where fragmentation can be used to increase selectivity of the measurements. The mode of the measurement is called multiple reaction monitoring (MRM), because several compounds can be measured simultaneously. In triple quadrupole instruments the first quadrupole selects the measured analyte, the second one decomposes it and the third passes the formed fragment to the detector. Because fragmentation is specific to every analyte, only right one is

measured even if compounds with the same molecular mass come to the instrument at the same time. This kind of high selectivity makes also possible to measure several compounds at the same time even when they are not separated in liquid chromatography. In triple quadrupole instruments dynamic range is usually at least 5 orders of magnitude what is enough for quantification purposes. Earlier TOF instruments were saturated at quite low concentrations and could not be used for quantification purposes. Modern TOF instruments however can be used for quantification at least to a certain extent.

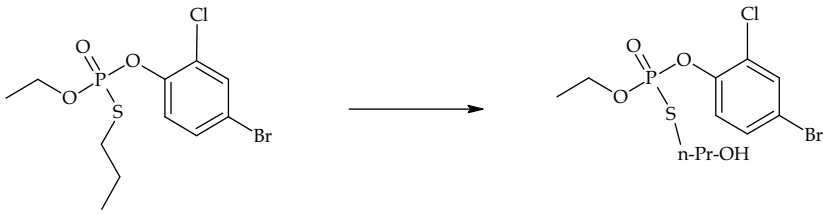
Newest technological addition to mass spectrometry is ion mobility. Ion mobility is a small gas filled drift tube in instruments, which ions travel through within electric field. Drift tube will separate compounds based on their shape and size in addition to mass and charge as in conventional instruments. Ion mobility is quite an old technique but just recently it has been combined with commercial mass spectrometers like Synapt HDMS (Waters Corp., Milford, MA, USA) (Kanu et al., 2008). Ion mobility can be used to clean important analytes from sample matrix and to separate very similar compounds like isomers from each other. Because technique is so new, its real practical significance in metabolism studies is still unclear.

Figure 3 presents a practical example about mass spectrometric measurements of the pesticide profenofos and its metabolite hydroxypropylprofenofos (Abass et al., 2007a). Accurate mass measurements were performed by Micromass LCT-TOF (Micromass, Altrincham, UK) using leucine enkephalin ($[M+H]^+$ at m/z 556.2771) as a lock mass compound. Error in accurate mass measurements of hydroxypropylprofenofos was 4.3 mDa. Fragmentations of hydroxypropylprofenofos were determined by Micromass Quattro II triple quadrupole instruments. In the first fragmentation hydroxypropylprofenofos loses ethanol to produce fragment of m/z = 343 Da. In the second step propanol is released to produce fragment of 285 Da. Difference in molecular masses of these two fragments indicates that hydroxylation has to be located in S-propyl moiety of the metabolite. Finally quantifications were performed in multiple reaction monitoring mode (MRM) of triple quadrupole instruments. Quadrupole 1 passes only hydroxypropylprofenofos (molecular mass 389 Da) or compounds with the same molecular mass. After quadrupole 1 hydroxypropylprofenofos will fragment in collision cell with help of argon gas and collision energy (CE= 20 eV) to produce a specific fragment of m/z 343 Da. In the final step only fragment 343 will pass quadrupole 3 and its amount is determined in the detector of the instrument. After calibration of the instrument with reference standards, the real amount of hydroxypropylprofenofos can be determined.

5.2 Nuclear Magnetic Resonance spectrometry in the metabolism studies.

Nuclear Magnetic Resonance spectroscopy (NMR) is a powerful analytical tool in studies of solid, gaseous and liquid samples. The versatility of the technique and the long relaxation times of the nuclear spins allow for probing various different properties of the samples. An even normal, simple one-dimensional spectrum contains valuable information about the sample concentration, electron distributions of the molecule, spatial proximities of different chemical sites and electrostatic connectivities between different nuclei of the molecule. The full potential of NMR can be unleashed by going into higher dimensional NMR spectroscopy. In typical two-, or three-dimensional NMR spectra one can probe spatial proximities of various nuclei, do diffusion separated spectroscopy, probe for heteronuclear connectivities multiple bonds away, or characterize intermolecular dipolar interactions at the protein-ligand complex interface.

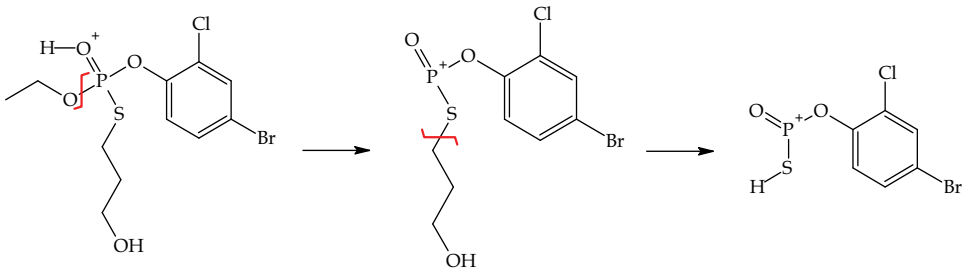
Accurate mass measurement of hydroxypropylprofenofos by time-of-flight mass spectrometer:



LC/MS-ESI+, MH^+=373Da
Accurate mass: 372.9477, Calc. mass: 372.9430
Profenofos
O-(4-bromo-2-chlorophenyl)-O-ethyl-S-propyl phosphorothioate

LC/MS-ESI+, MH^+=389Da
Accurate mass: 388.9422, Calc. mass: 388.9379
Hydroxypropylprofenofos
O-(4-bromo-2-chlorophenyl)-O-ethyl-S-hydroxy -propyl phosphorothioate

The fragmentation of hydroxypropylprofenofos by triple quadrupole mass spectrometer:



m/z = 389 Da m/z = 343 Da m/z = 285 Da

The quantification of hydroxypropylprofenofos by triple quadrupole mass spectrometer:

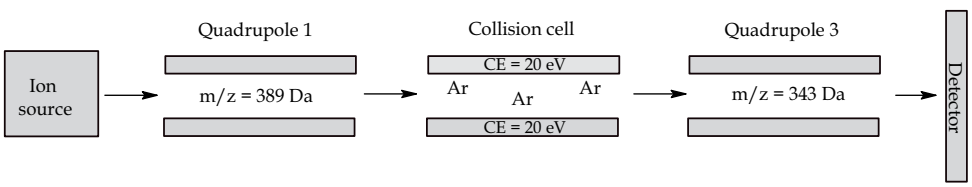


Fig. 3. Mass spectrometric measurements, accurate mass, fragmentations and quantifications, performed to study hydroxylation of profenofos in human hepatic subcellular fractions.

The biggest drawback of the NMR spectroscopy is its inherently low sensitivity, because the observed signal arises from the population difference of spin states. This population difference follows Boltzmann distribution and is quite low even at reasonably high magnetic fields used at modern NMR spectrometers. As the experiments are performed close to room or physiological temperature, there is only population difference of about 10 spins for every million spins in the sample.

Two recent sensitivity enhancing methods are cryogenic cooling of the probe-head electronics and miniaturization of the sample size. Equipment for both of these have been

commercially available now for several years and when combined the resulting cryo-microprobe would give up to 15 fold increase in sensitivity compared to regular room temperature probe head (Kovacs et al., 2005). Several articles have recently been published where full NMR analyses of complex natural products have been made using only nanomoles of material (would be equal to 1 μg if molecular mass is 1000) (Dalisay & Molinski, 2009; Choi et al., 2010a; Choi et al., 2010b; Djukovic et al., 2008).

If one wishes the acquisition parameters of NMR experiment can be set to provide quantitative spectrum. Typically the delay between individual transitions needs to be lengthened to allow full relaxation of spins before next transition. In simplest form the integral values of the individual resonances in spectrum give information of how many equivalent spins are present. Whenever there are modifications in chemical structure the change in integral provides valuables information on the chemical site of the modification (Holzgrabe, 2010).

The Chemical shift is a sensitive measure of the electronic surrounding of individual nuclei of a molecule. Even smallest changes in the chemical structure can cause peaks to resonate at slightly different frequency at the chemical shift range. Addition of the electronegative substituents to the molecule changes the chemical shifts of the resonances of the nearby nuclei. In favorable case the change in chemical shift can be observed for several nucleus many bonds away from the origin of modification site.

The signal fine structure, the splittings caused by spin-spin couplings, provides additional sensitive measure of the topology of the nucleus in the molecule. Change in number of nearby nucleus or even just a conformational change can be detected as a change observed coupling pattern caused by the spin-spin coupling.

In metabolic studies NMR spectroscopy is best utilized when used as a complementary technique to the mass spectrometric techniques. For instance the position isomerism studies are often quite tricky or even impossible to solve by mass spectroscopy e.g. what is the substitution pattern of the aromatic ring or which carbon of the aliphatic chain was hydroxylated. These questions can occasionally be answered in minutes by single ^{1}H NMR spectrum. Of course more challenging structural questions take longer and might require acquisition of several multi-dimensional data sets.

For the illustration of the position isomerism detection powers of NMR spectroscopy several simulated NMR spectra of the profenofos and the hydroxypropylprofenofos are displayed in figure 4. On the left are the aromatic signals of the profenofos and the corresponding spectrum if the bromine was in ortho position to the chlorine. The difference in signal positions and splitting patterns is clear. On the right are spectra of the propyl moiety of the profenofos and the hydroxypropyl moiety of the hydroxypropylprofenofos where the hydroxylation has occurred on terminal carbon 3 or in carbon 2.

6. Conclusion

The cytochrome P450 (CYP) superfamily comprises a broad class of phase I oxidative enzymes that catalyze many hepatic metabolic processes. Human CYPs have broad substrate specificity and enzymes in families 1-3 function mostly in the metabolism of a wide variety of xenobiotics. In human liver, CYP3A4 is found in the highest abundance and it metabolizes the greatest number of drugs and a very large number of other xenobiotics. CYP enzymes in humans as well as in other species have been intensively studied during recent years. It is now possible to characterize metabolism, metabolic interactions and to

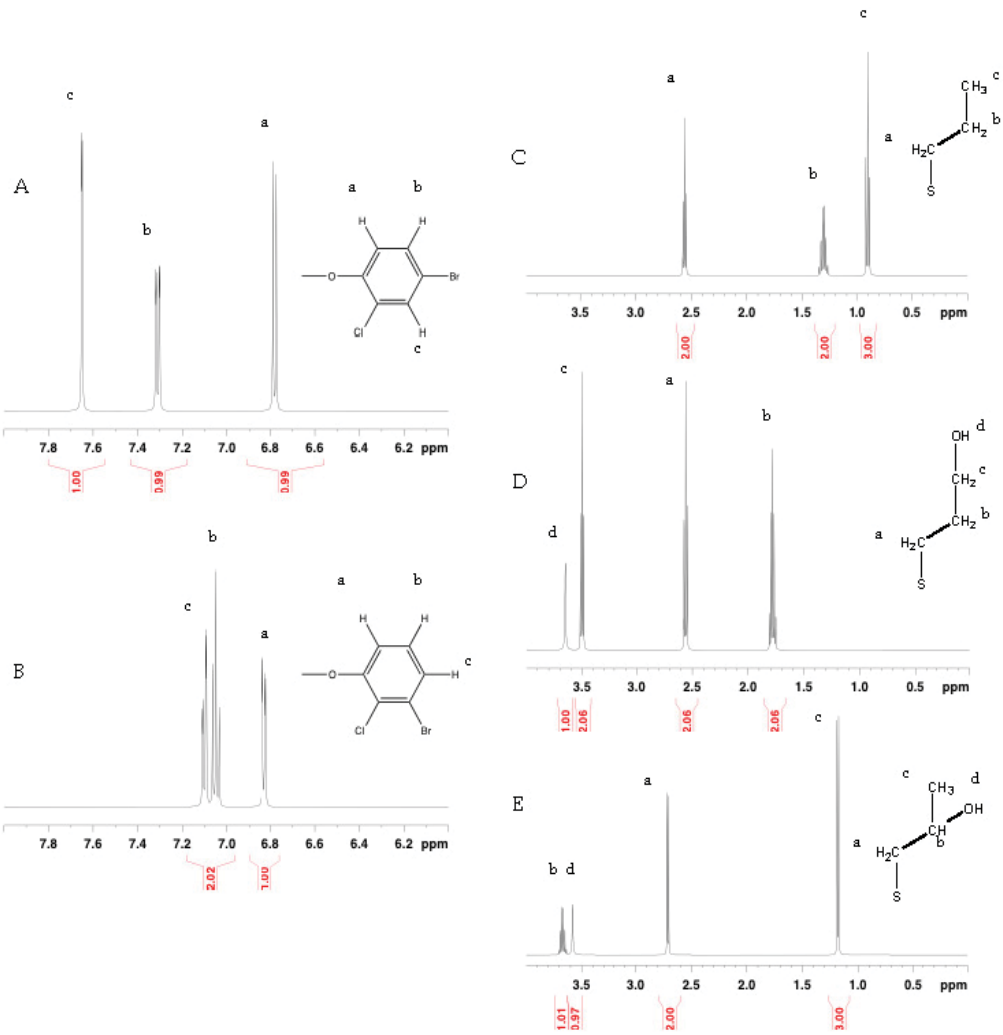


Fig. 4. An example of the effect of small changes in molecular structure to the outlook of ^{1}H NMR spectrum illustrated by 5 simulated NMR spectra. The values used for chemical shifts and coupling constants are only approximate and are presented for illustration purposes only. The values of signal integration are displayed below the frequency scale.

determine which P450 is involved in the metabolism of a certain xenobiotic by different in vitro approaches. The toxicity of many types of pesticides is mediated by enzymatic biotransformation reactions in the body. Recently, a number of papers have been published on the activity of human P450s involved in the metabolism of pesticides and these activities may result in activation and/or detoxification reactions.

The aim of in vitro characterization is to produce relevant and useful information on metabolism and interactions to predict what happens in vivo in human. To understand some of the factors related to xenobiotics, including pesticides, metabolism that can influence the achievement of these aims, there are several important points to consider such as metabolic stability, metabolic routes and fractional proportions, metabolizing enzymes

and potential interactions. In this review we described the human xenobiotic- metabolizing enzymes CYPs system; briefly illustrate in vitro human-derived techniques for studying xenobiotic metabolism and in vitro characterization of metabolic characteristics; review the role of CYPs in the metabolism of xenobiotics, including drugs and pesticides, in human in vitro; and finally describe the impact of modern analytical techniques in xenobiotics metabolism.

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Part 5

Fungicide Development

23

Neural Computation Methods in the Determination of Fungicides

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1. Introduction

Fungicides are a specific type of pesticides that control diseases produced by fungi, inhibiting specifically or directly killing these parasite organisms. It has been used during more than forty years for the protection of harvests and farming lands.

There are several treatments with fungicides: protector or preventative from the germination of the spores and follow up infection, or curing or eradicating, when mycelium has been formed and must be controlled. Treatments are applied in the soil and on stored vegetable products, seeds or plants. In this case two types of fungicides are classified: a) those with contact: unable to penetrate the inside of the vegetable and control epiphytic fungi, and b) those that are systemic and control endophyte fungi.

The large variety of fungicides that exist makes a classification absolutely necessary. We find different taxonomies that can be studied not only for their structural aspects, but such as chemical composition, and also in action modes. The guidelines that regulate the managed use and classification of these are defined in Spanish, European and American Environmental Agencies.

The use of chemical products in farming activities has produced important benefits in agricultural production, increasing profitability of harvests while simultaneously raising the quality levels of the food products. Nevertheless, there are other considerations with regard to these benefits that result in the systematic destruction of parasites, that affect the health of the plants, animals and human health, and require consideration of the interaction of the different chemical main components with animal species and with humans themselves (Rivas, 2004). To begin with, the form of the administración of fungicides favors their accumulation in the sediments and in drainage waters. Also, in cases where plaguicides have been used indiscriminately, the species of plagues have become resistent and difficult to control.

The main source of exposition of the general population to fungicides is through food, a fact that has forced the establishment of regulations of its maximum daily ingestion allowance. There are studies that relate the exposure to pesticides with damaging effects on human health: neurological damage, hormonal and reproductive disorders, dermatological or carcinogenic reactions (Alavanja, 2004).

Thus, in European countries the residues from plaguicides in commercial foods are subject to international standards and are strictly monitored. As a result, it is necessary to develop technologies that allow the treatment of residues and polluted environments. However the development of new methods with precise and sensitive detection are also essential, especially those that detect, quantify and control those damaging compounds for the ecosystem and as a consequence for human health.

Some of the most commonly used instrumental techniques for the detection of these compounds is found in thin layer chromatography (TLC), gas chromatography (GC) (Vassilakis, 1998) high performance liquid chromatography (HPLC) (Halko, 2006), gas chromatography - mass spectrometry (GC-MS) (Martínez Vidal, 2000) and high performance liquid chromatography – mass spectrometry (HPLC-MS) (Taguchi, 1998). The characteristics and properties of these substances make use of specified techniques more difficult, or perhaps even necessary to use preprocessing and/or a combination of techniques. Due to the unstable character of some of the pesticides it is not easy to perform GC analysis. In this case HPLC is an appropriate technique for its determination. A similar and very common step is to carry out some type of derivatization of the compounds, such as in the case where they are transformed into fluorescent compounds (Vassilakis, 1998). These pre-treatments require a large consumption of dissolvents and reactants in addition to the cost, the time and the analytical complexity of the cited techniques (Chiron, 1995).

Other developed chemical methods are spectrophotometric methods (Sastry, 1987), however one of its main drawbacks is the degree of overlap in the spectra, which complicates the analysis of complex mixtures of these compounds. The cynetic methodology based on the difference of reaction velocities is another effective way to analyze several analytes at the same time and has improved with the use of chemometric procedures. Some of the most commonly used are those obtained from the application of multivariate methods (PLS, PCR, CLS) and the analysis of variance ANOVA(Galeano-Diaz, 1997).

Chemometric methods are an important alternative to the purely instrumental ones (Olivieri, 2008), finding a high application of the same in the determination, not only of fungicides but also of pesticides in general, applying one or a combination with the instrumental ones or the neural ones. Among these we point out the MCR-ALS method (multivariate curve resolution-alternating- least-squares) which has been applied to the study of the contamination of sediments and waters and the measurement of air quality (Salau, 1997) and parallel factor analysis or PARAFAC (Bro, 1997). Other methods needing mention are those based on partial least squares approach (PLS) in the setting of environmental analysis (Piccirilli, 2006). Principal component analysis or PCA is a multivariate statistical method commonly used in data analysis (Jolliffe, 1986) but with some limitations and attempts to solve models based on these same methods such as UNMIX (Henry, 2003) and the PMF (positive matrix factorization) (Paatero, 1994). Both have been adopted by the United States Environmental Protection Agency (U.S. EPA) because they are robust methods to measure air quality. Alternative methods have also been developed based on tri-linear and multi-linear models such as TUCKER-3 (Tucker, 1966) and the ME (Paatero, 1999). Shao-Hua Zhu (Zhu, 2007) use fluorescence detection combined with chemometrics for the detection of Carbendazim in banana samples. They specifically use the excitation-emission matrix (EEM) fluorescence spectrum and three second order calibration methods: PARAFAC, SWATLD and APTLD, obtaining satisfactory results in the quantification of the fungicides in complex samples.

QSAR (quantitative structureactivity relationships) data analysis (quantitative structure activity relationships) describes the mathematical relationship between the structural properties of a series of chemical compounds and their possible toxicological activities, hence it is very useful in the determination of toxicity of synthetic fungicides.
A complementary approach to the cited methods, with high capacity and efficiency, which tackles the identification of fungicides, and pesticides in general, in complex samples is one based on a different computational approach to symbolic computation, as is the case with neural computation. The basic processing element of this computational approach is found in artificial neural networks (ANNs). ANNs can be defined as a cognitive information processing structure (massively parallel dynamical system) based upon models of the brain function. They are composed of highly interconnected computational elements with graph topology. Its most appealing property is its learning capability. Its behaviour emerges from structural changes driven by local learning rules, with generalization capability (Suárez Araujo, 1999)(García Báez, 2010).
The suitability of ANNs has been extensively demonstrated for its use in a wide variety of applications where real-time data analysis and information extraction are required in different settings related to pesticides in general and fungicides specifically, such as chemistry, environmental and agriculture fields (Yang, 1997). Applications in cited papers include those of Suárez Araujo and García Báez (Suárez Araujo, 2006)(Suárez Araujo, 2009)(García Báez, 2010) in which they identify fungicides in mixtures of up to three and four different fungicides using ANNs and neural network ensembles. Research by Guiberteau and collaborators have solved ternary mixtures of pesticides with similar molecular structure (Guiberteau, 2001), Baoxin Li (Li, 2006) identify three organophosphorated pesticides, Istamboulie solves insecticides (Georges, 2009), and Wan Chuanhao (Wan, 2000), who proposes the use of neural computation to identify pesticides from the family of carbamates, or the developments by Yongnian Ni and collaborators (Ni, 2004), that describe the RBF-ANN method and the PC-RBF-ANN method as the best at detecting mixtures of three pesticides of this same family. On the other hand, work by Karl-Heinz Ott (Karl-Heinz, 2003) shows an example of neural networks that determine the action mode for a large number of herbicides.
In this chapter, a study of neural computation has been presented where theory and architecture of paradigmatic supervised and unsupervised ANNs, and a new hierarchical unsupervised modular adaptive neural network (HUMANN) is reported. The neural network ensemble approach is introduced. Finally, we will discuss the ability of ANNs and a neural network ensemble to address this issue, describing the outcomes of implementations of such approaches for the determination of fungicides in different kind of samples.

2. Neural computation

Researchers from different and varied fields have studied the neural processing and control of biological systems and have attempted to develop synthetic systems, that is artificial systems, which possess similar capacities (Suárez Araujo, 1996), as well as formulating theories regarding how computation in biological systems really occurs. The first ANN model is attributed to Warren McCulloch and Walter Pitts and had universal computational capacities, a formal neural network, based on knowledge that was available on nervous system functions (McCulloch, 1943). Since then there have been a series of advances that have placed neural computation as a key element in the solution of a great variety of

problems in a wide domain of applications and as a capable computational paradigm for the advanced knowledge in the function and structure of the brain as well as its computation style.
Neural computation can be understood as parallel, distributed, and adaptive computing carried out by three dimensional modular architectures organized in layers and made up of a large number of processing elements, with a high degree of connectivity, with a learning capacity that may be supervised or not (Suárez Araujo, 1996). Its main structure of information processing is artificial neural network, where the adjective neural suggests that they are inspired in biological neural networks. ANNs study and use some strategies from the methods used by biological neurons to process information. Neural computation is just one of many different computational models that have been defined throughout history, where ANNs are considered as another approach to computational problems (García Báez, 2005).

2.1 Artificial Neural Networks

ANNs can be defined as large, parallel and interconnected networks that are made up of simple elements (generally adaptative) and with a hierarchical organization that attempt to interact with the objects of the real world by imitating the biological nervous system (Kohonen, 1989). ANNs found their inspiration in biological neural networks. Thus, there is a biological feel for the models and systems based on ANNs or neural computation. Nevertheless, the task of generating an ANN comparable to the biological neural network is not easy at all, since the human brain has on order 10^{11} neurons and each one of these receives an average of 10^3 to 10^4 connections, all of which are integrated to obtain a unique output with a complex structure of connections, not completely determined, providing a high and complex processing capacity. If we compare biological neurons to the logical gates in silicon, they are smaller but need a longer time to generate output, even though there is a lack of slantedness by the high number of neurons that exist as well as the large capacity of interconnectivity and work in parallel that these possess. From a computational perspective there is much research to do before obtaining a solid method such as the biological one to implement our neural models. Given that the human brain is much more complex in order of magnitude than any existing ANN, it is impossible to approach this process capacity, even with today´s technology.
The general framework of an ANN includes eight components (Rumelhart, 1986): a *set of processing elements* (*units* or *neurons*), each one self-contained, with local memory; *activation state* of each processing element; *output function* for each processing element; *connectivity pattern* between the processing units, where each connection has an associated *synaptic weight*; *propagation rule* or *network function*, to propagate the activity patterns based on the connections network; *activation rule* or *activation function*, to combine the inputs that arrive to a unit with the actual state of the unit and can produce a new activation level in it; *learning rule*, through which it can modify the connectivity patterns based on experience and *representation of the environment*, there are two information settings, one local and another global.
Following this framework we characterize ANNs in the three following levels: *connectivity topology* (covering the neural structure), *neurodynamics* and *learning*.
Connectivity topology is an essential part of the neural structure of an ANN and indicates the shape in which the different processing elements of a network are interconnected amongst themselves (Hecht Neilsen, 1990). The structural organization of the processing

elements that make up the ANN normally uses layers, taking into account the individual units that form it with similar characteristics. There are ANNs with a flat neural structure, with only one layer of processing elements (the input layer is excluded), also called *single-layer* ANNs. In general form, ANNs can have different degrees of depth, which is given by the number of layers that make it up and are referred to as *multi-layer* networks. In this last case we can identify: the *input layer*, where the elements that are not usually considered as making up a layer, since they do not carry out any of their processing, but instead simply distribute the input information to the rest of the processing elements with which they connect; the *output layer*, which represents the units that provide the output of the network and the *hidden layers*, all of those that generate the connectivity of the network between the input layer and the output layer without direct contact with the environment.

The connectivity density of ANNs as well as its direction, where direction is understood to be the information flow direction between two interconnected processing units, namely inter-layers and intra-layers, create a structural taxonomy of the ANNs (Simpson, 1990). Hence we can find networks with total connectivity, where all of the units of the network are connected with each other, *full-connected* networks and the dispersed or partial connectivity, where each neuron is connected to only one set of neurons of the network, called *partial-connected*. This full or partial connectivity can also refer to the relations that are established between the connections of the two layers. One particular case of this last category is *one-to-one* connectivity. With respect to the direction of connections between the layers we have *feed-forward* connected networks, where the connections go from the input to the output, *recurrent* networks, where connections can exist that go from earlier layers (*backwards*) or other networks where the connections tie neighboring units from their own layer, which are called *lateral connections*.

Neurodynamics cover of the local information processing that the units carry out. This process is given by the local computation model of the neuron, Fig. 1. Mathematically this is expressed with the so-called network function *net* that integrates the inputs **x** and the corresponding synaptic weights **w** (normally by means of a weighted sum, equation (1), when working with a linear computation model, McCulloch-Pitts model (McCulloch, 1943), although non-linear models also exist (Suárez Araujo, 1997)).

$$net_i(\mathbf{x}) = \sum_j w_{ij} x_j \tag{1}$$

The activation function f_{act} (normally a non-linear function of any type), acting on the function value of the network which allows the dynamics of the activation states of the units and finally an output function f_{out} to be obtained, that are applied to the activation state of the unit provides the output values y_i of the units. It is also possible to store local values inside the processing units, θ_i, that can be used in the calculation of the outputs, which is known as local memory:

$$y_i = f_{out}\left(f_{act}\left(net_i(\mathbf{x}) - \theta_i\right)\right) \tag{2}$$

Finally, an essential and differentiating part of ANNs is learning. We can define learning as the capacity of a system of absorbing information from the environment, without a need for the system to be programmed externally. Learning in ANNs follows a compound model using two stages: *load mode* (learning) and *retrieving mode* (execution). Load mode is where

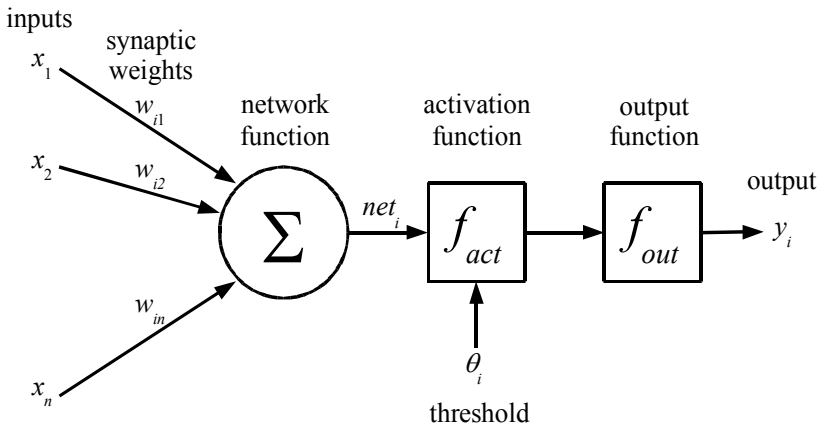


Fig. 1. General Building-Block of an Artificial Neuron

the learning takes place. Starting with the data that are received in the setting the ANNs are capable of processing them and storing the extracted information from them, in their synaptic connections. On the other hand, the retrieving mode allows responses to be obtained based on established questions from the setting, by means of adequate processing using the knowledge that has been acquired by the ANNs.

The learning processes produce changes in the network in order to try and achieve a new way to respond more efficiently to the specifies task. These changes can be gathered by modifications in the connectivity topology and/or by modifications in the synaptic weights:

$$\tau \frac{d\mathbf{w}}{dt} = -\frac{\partial}{\partial \mathbf{w}} R\left(\mathbf{x}(t), \mathbf{d}(t), \mathbf{w}(t)\right), \tag{3}$$

where the function R is called the *instantaneous learning potential* and **d** can or not be present, depend on kind of learning, and is defined as the *teacher signal*, and the synaptic weight vector **w** changes in the direction of decreasing R (Amari, 1990). We can reformulate and simplify the equation (3) for discrete case as:

$$w_{ij}(t+1) = w_{ij}(t) + \Delta w_{ij}(t). \tag{4}$$

In general terms, the ANNs carry out the learning process by itself based on a set of training data sets, which is called learning based on examples, in these cases learning algorithms are used, which are iteratively ordered to update the weighted values and/or necessary topological changes. As opposed to neurodynamics, the performance of non-local processes in the definition of the learning algorithms is allowed. There is also a dependent designer modality, which can establish the needed changes by means of an appropriate formulation to solve the problem.

With regard to the type of learning that can be computed we identify two types of different learning experiences (Rumelhart, 1986). *Associative learning*, where the experience learns to produce a pattern of specific activation in a set of units when another specific pattern occurs in another set of units. It allows, consequently, to map arbitrary activation patterns in the input, into other activation patterns into another set of units, normally output, and *Feature detection*, where the processing units learn to answer when faced with *interesting patterns* in

their input. It is the basis for the development of characteristic detectors and consequently the basis for knowledge representation in ANNs.

Based on the guided learning process we can identify three different paradigms (Haykin, 1994):

- *Supervised learning*: for each input pattern to the network there is a signal (correct response for it) which serves as a guide for the adjustments in the synaptic weights
- *Reinforcement learning*: with the response of the network given an input it facilitates a scalar evaluation of the response. It indicates whether it is correct or incorrect.
- *Unsupervised learning (self-organized learning)*: The network does not receive any tutoring, but has to organize its output based on the redundancy and structures that can be detected in the input.

The entire learning process, independent of its type and according to the taxonomy carried out entails a way in which the synaptic weights are modified and updated. This way consists in what we call *learning rules*, which are mathematically expressed, according to the type of systems (continuous or discrete), and by means of differential equations or difference equations. When dealing with the different types of rules we can speak of another classification for learning processes, where we identify four different types (Haykin, 1994)(Jain, 1996):

- *Hebbian*: It can be considered as a learning support, biological as well as computational, by constructing the basis of many follow up learning rules, essentially unsupervised ones. It is included in the coincidence learning category. In neurocomputation the Hebbian rule has its origin in a mathematical point of view the affirmations stated by Donald Hebb in 1949 (Hebb, 1949) based on neurobiological observations.
- *Competitive*: this type also has high neurobiological bases since its experiments have shown their use in the formation of topographic maps in the brain and in the orientation of cell nerves that are sensitive from the striate cortex. It is based on a competition process among all units to assign the exclusive representation in the face of a group of input patterns
- *Error correction*: focuses on rules based on error correction attempt to correct the error that is produced in the network when comparing the desired output with the actual output from the network. It is normally applied in the supervised paradigm. Biological plausibility is not so evident as in the earlier cases
- *Energy optimization*: one of the best known of these is the *Bolzmann learning*, and is characterized by its use of an energy function, determined by the states of the individual neurons, which attempts to be optimized

ANNs have many architecture properties and functionalities that make it especially appropriate to tackle highly complex problems based on behaviour, non-linear, etc. in real time, among the most relevant are (Haykin, 1994) *Generalization*, *Treatment of Contextual Information*, *Fault Tolerance*, *Evidential Response* and *Uniformity of Analysis and Design*. In general, the applications which have been most useful to ANNs are characterized by their capacity to handle the following tasks (Haykin, 1994)(Jain, 1996): *Pattern Classification*, *Function Approximation*, *Associative Memory*, *Prediction*, *Control* and *Optimization*.

Finally we must consider the capacities of the ANNs saturated in its structural organization. Two types of structural organization are generally considered: *monolithic* and *modular.* Monolithic organization, where ANNs are frequently considered as *black-boxes,* is often used, and not only with the level of construction of models such as an application level. This structural organization begins to present serious difficulties as the neural networks grow, or

when the applications become more complex. In specific cases, the complex problems, the efficiency usually decays until a point where it is impossible to solve using monolithic systems. The term *modular neural network* is used to identify many different types of neural structures, in general it can even be said that any network that is not monolithic is normally considered to be a modular one (Rojas, 1996). Likewise, in the literature we can find other terms that form part, include, or are related to modular neural networks, such as *multiple neural networks, neural ensembles, mixture of experts*, *hybrid systems* or *multi-sensorial fusion* (Gallinari, 1995)(Haykin, 1994).

The main idea underlying neural modular networks is the possibility of solving complex problems in a simpler, more flexible, and faster way. This objective is achieved based on the construction of parts or modules, that would probably be neural networks, and at the same time are simpler and smaller that an equivalent monolithic network. This restructuring allows the task learning to be carried out by each module, normally in a simpler way than the global task and the different training that can be carried out in an independent and parallel way (García Báez, 2005).

2.2 Neural architectures

2.2.1 Simple perceptron

The perceptron model (Rosenblatt, 1961) reflects the beginnings of machine pattern recognition. It is a feed-forward single-layer architecture with an input layer, which are made up of setting sensors, and an output layer, with responses sent by the network. Hidden layers are not needed. Its neurodynamics are made up of a network function that uses the classical weight summation for inputs, equation (1), and a step, bipolar or binary, equation (5), for the activation function, with a transition point which is determined by a threshold value θ_i stored locally in the neuron.

$$y_i = \begin{cases} 0 & \text{if } net_i < \theta_i \\ 1 & \text{if } net_i \geq \theta_i \end{cases} \tag{5}$$

The learning model that it follows is capable of adapting its weights and thresholds by means of a supervised paradigm using the so-called *perceptron rule*, based on the correction of the produced error in the output layer:

$$\Delta w_{ij} = a\left(x_i - d_i\right)x_j \tag{6}$$

where α is the learning rate and d_i is the desired output.

One of the advantages of this model is that it uses the *Perceptron Convergence Theorem* which guarantees the learning convergence in finite time and that the architecture always allows the solution to be represented. Precisely it is in this capacity of representation where the greatest limitation of the model resides, for instance in (Minsky, 1969) they discuss its inability to solve non linear separable problems, for example in the case of the exclusive-or problem (XOR).

2.2.2 Backpropagation

One way to overcome the representation limitations mentioned in the Simple Perceptron is through the use of Multi-layer Perceptrons (MLP). A MLP constitutes a topology with one or several hidden layers, and feed-forward connections among its successive layers, either in a

total or partial way. In order to represent any boolean function it is necessary that some of the neurons use non linear activation functions (thresholding function or sigmoid function, equation (7)), maintaining the rest of the neurodynamics the same as in the Simple Perceptron.

$$f_{act}(net,\theta) = \left(1 + e^{-net+\theta}\right)^{-1} \tag{7}$$

The most popular algorithm for training MLPs is *backpropagation* (Werbos, 1974). It is based on a supervised correction of the squared error generated in the output layer using a gradient descent method. This method forces the used activation functions to be differentiable and monotonic. It begins with the output layer and adjusts the weights of the connections that are affected there, producing a backpropagation of errors of the previous layer to occur which successively corrects the weights until reaching the first hidden layer.

$$\Delta w_{ij} = a\delta_i x_j \tag{8}$$

$$\delta_i = \begin{cases} f'_{act}(net_i,\theta_i)(d_i - x_i) & \text{if } i \in Output \\ f'_{act}(net_i,\theta_i)\sum_k \delta_k w_{ki} & \text{otherwise} \end{cases} \tag{9}$$

Some of the most noteworthy problems are studied in backpropagation, since the descent gradient descent does not insure reaching the global minimum error, as opposed to the Simple Perceptron (Minsky, 1969). Many variations of backpropagation have been proposed to over this obstacle, such as generalization, learning speed and fault tolerance.

2.2.3 Simple competitive learning

Together with Hebbian learning, competitive learning is one of the main approaches of unsupervised learning. It also makes up the basis for most neural systems with unsupervised pattern recognition. It uses one type of neuron called the *winner-take-all* or *grandmother cells* (Hertz, 1991) that compete against each other to act as the triggers. Consequently, the idea is to cluster or categorize the input data, that is, similar input will be classified as belonging to the same category and will trigger the same neuron.

Its single-layer architecture presents an input layer that has a full-connectivity with the output layer by means *excitatory connections* (weights greater than or equal to zero). The output layer simultaneously presents *inhibitory lateral connections* (weights less than or equal to zero) among neural neighbors as well as excitatory self-connections. Said connections are those that facilitate the competitive process while searching for the winning neuron from maximum activation.

Their neurodynamics in practice are usually simplified by carrying out the weighted sum of the inputs and the neuron with the largest value in it, considering it the winning unit, sending it in one of its output while the remaining units would send a zero, equation (10). It is also possible to substitute the weighted sum by euclidean distance, in such a case the winning unit will be that which is closest (higher similarity) to the input vector.

$$y_i = \begin{cases} 1 & \text{if } i = \arg\max_k (net_k) \\ 0 & \text{otherwise} \end{cases} \tag{10}$$

There are several alternatives with respect to the learning rule to use (Hertz, 1991). One of these is the so-called *standard competitive learning rule*, which tries to move the incident weights in the winning neuron directly towards the input pattern.

$$\Delta w_{ij} = \begin{cases} \alpha\left(x_i - w_{ij}\right) & \text{if } i = \arg\max_k\left(net_k\right) \\ 0 & \text{otherwise} \end{cases} \tag{11}$$

This model presents some problems which are difficult to resolve, amongst others we point out the selection of the appropriate number of neurons in the output layer, the potential presence of *dead units* that never end up being winners, the sensitivity with regard to the initial values that are assigned to the weights and the possible need to normalize inputs and/or weights.

2.2.4 Radial Basis Function Network

Radial Basis Function Network (RBFN) architecture can be considered from the point of view of function approximation as a system capable of obtaining the appropriate parameters for a linear combination of basic localized functions that are adjusted to a specific desired function (Musavi, 1992). It uses a modular architecture made up of a two layer set with feed-forward connections between its successive layers, where the first of these is totally connected with the inputs.

The hidden layer use radial basis functions, usually Gaussian kernels, as an activation function, equation (13), hence its name. The weights of the incident connections in each neuron of the hidden layer or *prototypes* makes up a point in the input space of the network. The network function in this layer is to obtain the distance between the input and each prototype, equation (12). Consequently the hidden neurons will be activated in the next inputs to its prototype, thus generating hyperspheres in the input space, which is different from the hyperplanes generated by other activation functions. The output layer makes up a Simple Perceptron or similar linear network that implements a linear combination of these radial basis functions.

$$net_i\left(\mathbf{x}\right) = \left(\sum_j \left(x_j - w_{ij}\right)^2\right)^{1/2} \tag{12}$$

$$f_{act}\left(net, \sigma\right) = e^{\frac{-net^2}{2\sigma^2}} \tag{13}$$

It follows a hybrid learning, where the hidden layer is adjusted with Simple Competitive Learning and the output layer by means of the perceptron rule or the backpropagation rule.

Among the advantages of this model is the faster convergence than the MLP with backpropagation. On the other hand it usually needs a greater number of hidden neurons than the others, which produces a slower retrieving mode.

2.2.5 Kohonen's Self-Organizing Maps

Self-Organizing Maps (SOMs) (Kohonen, 1989)(Kohonen, 1997) describe the idea that topographic maps, such as those that exist in the cortex of highly developed animal brains,

extract the features of the input space preserving its topology. On one hand they combine characteristics of competitive systems, quantifying the input space in different regions represented by a specific number of output neurons. On the other hand, they maintain a neighborhood relationship between the units of the output space, that is, two neighboring neurons will represent close regions in the input space. Thus these methods will generate a discrete map, possibly with reduced dimensionality that the input space, and will preserve the existing topology in this one. Among the most commonly used methods we find the Kohonen SOMs, although there is a great diversity in their variations. These exhibit a similar topology than Simple Competitive Learning, but the output layer is organized in an *m*-dimensional space in agreement with the form that we desire for the map, and the most common is a two dimensional matrix.
The neurodynamics that follow the Kohonen SOMs accept the same possibilities as those in Simple Competitive Learning. There is also unsupervised and competitive training paradigm which follows. The main variations are seen in the modification of the synaptic weights, equation (14), which not only affects the winning neuron but also to a lesser degree the set of neurons in the winners neighborhood *N*, and consequently being able to generate topological relations. The neighborhood relationship between nodes is normally given by a hexagonal or squared type lattice, whose size decreases during the training period.

$$\Delta w_{ij} = \begin{cases} \alpha\left(x_i - w_{ij}\right) & \text{if } i \in N\left(\arg\max_k\left(net_k\right)\right) \\ 0 & \text{otherwise} \end{cases} \tag{14}$$

Their uses range from the projection of multivariate data, density approximation to clustering, having been successfully applied in a multitude of fields (Kohonen, 1997). This method acquired the problems previously mentioned of Simple Competitive Learning, added by the fact that a larger number of parameters that can be adjusted are available.

2.2.6 HUMANN

HUMANN (García Báez, 2003) is a modular neural network that can implement the general approach of the classification process, which has three stages: a) feature extraction, b) template generation, c) discrimination (labeling), in a transparent and efficient way. HUMANN uses a multi-layer neural structure with three modules and with different neurodynamics, connectivity topologies and learning rules, Fig. 2.
The first neural module of our HUMANN is a Kohonen's s SOM module. The second module is the Tolerance layer. It is the main module responsible for the robustness of HUMANN against noise. Its topology is a two-dimensional array which has the same dimension as the Kohonen layer and a one-to-one interconnection scheme with that previous layer. The main objective of this layer is to compare the fitting between the input patterns and the Kohonen detectors. If the goodness of the fit is not sufficient the pattern is regarded as an outlier and is discarded. The weights of this layer are responsible for storing the mean ($\mathbf{w}^0$) and standard deviation ($\mathbf{w}^1$) of the fits between the inputs and the Kohonen detectors when this neuron is the winner. This is a new concept called the *Tolerance margin*, equation (15). The goodness of the representation of a pattern by a detector will be a function of the ratio of the scalar product or euclidean distance between both of them and the Tolerance margin of the detector. The needed learning rule to obtain the weights of the global variance in the degree of the pairing, is based on a differential equation that

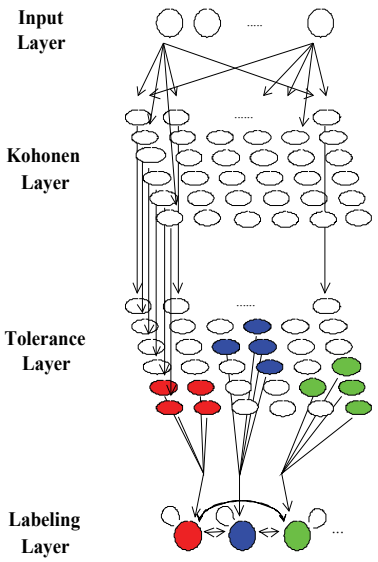


Fig. 2. HUMANN architecture

converges towards said average, to which must be added a decay term to make the finalt inputs to the system more relevant, in addition to avoiding possible pernicious effects of artifacts or outliers patterns, equations (16) and (17).

The labeling module (García Báez, 2003) implements the discrimination task. It maps the outputs of a neural assembly belongs to the Tolerance layer which have been activated by a category, into different clusters represented by labeling units. This module exhibits a full-connection topology and a dynamic dimension, which is fitted to the number of clusters detected in the data set. In addition it presents a group of lateral connection that connect each unit with all of the units in this layer, including itself, to this type of connection and is called *silent synapses*, since they do not directly influence the output of this layer, as opposed to the rest of the connections that we will call active connections.

$$y_i = \begin{cases} 0 & \text{if } x_i \geq (w_i^0 - \lambda w_i^1) \vee w_i^1 = 0 \\ 1 - \dfrac{(w_i^0 - x_i)}{\lambda w_i^1} & \text{otherwise} \end{cases} \tag{15}$$

$$\Delta w_i^0 = \begin{cases} \alpha\left(x_i - w_i^0\right) - \beta w_i^0 & \text{if } i = \arg\max_k\left(x_k\right) \\ -\beta w_i^0 & \text{otherwise} \end{cases} \tag{16}$$

$$\Delta w_i^1 = \begin{cases} \alpha\left(\left|x_i - w_i^0\right| - w_i^1\right) - \beta w_i^1 & \text{if } i = \arg\max_k\left(x_k\right) \\ -\beta w_i^1 & \text{otherwise} \end{cases} \tag{17}$$

The adaptive character of HUMANN is essentially an embodiment in the labeling module, because of its dynamic dimension (García Báez, 2001)(García Báez, 2003). This characteristic

is implemented by two neuronal mechanisms, a) neuronal elimination, b) neuronal generation. They perform the refinement processes in the neuronal circuits, and they are present in the human brain. Silent synapses are in charge of controlling the dimension of the output layer, which is dynamic and adjusts to the number of detected clusters in the set of training data. In this way, the dimension increases when a new cluster is detected and the cluster can be reduced when the patterns belong to a class that are poorly classified by two or more different units, or in the case when, upon detecting units that are practically not activated by any pattern. Thus, we can consider this system as an *ontogenic network* (Fritzke, 1997), able to adapt its dimension to the characteristics of its environment. The neurodynamics of this layer consist in an activation function that inhibits the output in the case where this value does not exceed a specific trigger threshold:

$$y_i = \begin{cases} net_i & \text{if } net_i \geq \rho \\ 0 & \text{otherwise} \end{cases} \tag{18}$$

In the case where no one exceeds the threshold value, all of the units will have an output value of 0, which indicates that the pattern could not be classified. If this type of event is occurs in the learning stage it will trigger the generation process of a new unit, which has its biological counterpart in the *neurogenesis process* (Alvarez Buylla, 1990), with the following increase in the output layer dimension of this module. This would indicate that an input pattern has arrived that has not been considered an outlier and no unit in the labeled (cluster) layer actually exists that it represents. The general training rule used for the adjustment of the active weights $\mathbf{w}^{act}$ would try to achieve that the winning neuron obtained as an output would have a value close to ,1, for this the weights are updated that lead to the winning unit, leaving the remaining ones unmodified, that is, a competitive learning, equation (19). The weights of the silent connections $\mathbf{w}^{sil}$ are symmetric and try to represent the existing time correlation between both units when one of them is the winner. In this way, if one wins against another it is normally activated in a simultaneous way, the weight of its connection is kept at a high level, while if the opposite occurs the connection will have values approximately equal to zero, that is, a Hebbian learning, equation (20).

$$\Delta w_{ij}^{act} = \begin{cases} \alpha\left(1 - net_i\right)x_j & \text{if } i = \arg\max_k\left(net_k\right) \wedge \left(net_i \geq \rho\right) \\ 0 & \text{otherwise} \end{cases} \tag{19}$$

$$\Delta w_{ij}^{sil} = \Delta w_{ji}^{sil} = \begin{cases} \alpha\, net_i\left(net_j - w_{ji}^{sil}\right) & \text{if}\left(i = \arg\max_k\left(net_k\right) \wedge \left(net_i \geq \rho\right)\right) \vee j = i \\ 0 & \text{otherwise} \end{cases} \tag{20}$$

If a group of two or more units from the labeling layer are connected with weighted values that exceed a threshold value, indicate that normally these units are being treated as activated before the same patterns and indicates which units are represented *coincidental clusters.* This event will trigger a process of neural reorganization that will reduce the dimension of this layer. This way, each set of units of coincidental clusters be transformed into a unique unit, producing a *synaptic reorganization*.

HUMANN is available in variants in order to process data with outlier noise and generate not exclusive clusterings, where the input can belong to several clusters at the same time. It

is scalable, hence it is adequate to use with large data sets, allowing clusters with arbitrary shapes and those with high tolerances to be found.

3. Neural Network Ensembles

The ANNs generate relationships between the inputs and outputs from a training set. It will be able to have several network configurations close to the optimal one, according to the initial conditions of the network and the ones typical of the environment. These configurations correspond to different ways of forming generalizations about the patterns inherent in the training set. As each network makes generalization errors on different subsets of the input space, it is possible to argue that the collective decision produced by the complete set, or a *screened* subset, of networks, with an appropriate collective decision strategy, is less likely to be in error than the decision made by any of the individual networks (Hansen, 1990). This has given rise to the use of groups of neural networks, in a trial to improve the accuracy and the generalization skills of them. In analogy with physical theory, it has been referred to the set of neural networks used as an *ensemble*, *neural network ensemble* (NNE) (Hansen, 1990).

Being able to tackle complex tasks in an efficient way has been another proof feature of the NNE approach. A NNE combines a set of neural networks which learn to subdivide the task and thereby solve it more efficiently and elegantly. In a sense, the NNE follows a divide-and-conquer approach by dividing the data space into smaller and easier-to learn partitions, where each ANN learns only one of the simpler partitions. The underlying complex decision boundary can then be approximated by an appropriate combination of different ANNs. NNEs are also very appropriate in applications where large volumes of data must be analyzed. It is necessary partitioning the data into smaller subsets, training different ANNs with different partitions of data, and combining their outputs using an intelligent combination rule. The situation of having too little data can also be handled using ensemble systems. (Polikar, 2007). Data fusion scheme, where several sets of data are obtained from various sources, is another scenario for using NNE. This last case is the typical one concerning with the subject studied in this chapter.

A NNE offers several advantages over a monolithic ANN: It can perform more complex tasks than any of its components. It is more robust than a monolithic neural network. It can produce a reduction of variance and increase in confidence of the decision, and can show graceful performance degradation in situations where only a subset of neural networks in the ensemble are performing correctly (Liu, 2003).

This ensemble based approach has shown to be very useful for a broad range of applications and under a variety of scenarios, (Polikar, 2007) essentially in data analysis and automated decision making applications. The idea of designing ensemble learning systems can be traced back to as early as 1958 (Liu, et al., 2003) and 1979 with Dasarathy and Sheela's paper (Polikar, 2006). Then, and since the early 1990s, algorithms based on similar ideas have been developed, appearing often in the literature under various other names, such as ensemble systems (Polikar, 2007), classifier fusion (Kuncheva, 2001) committees of neural networks (Drucker, 1994), mixture of experts, (Jacobs, 1991),(Jordan, 1994); boosting and bagging methods (Schapire, 1990),(Drucker, 1993) among others.The paradigms of these approaches differ from each other with respect to the procedure used for generating ensemble members, and/or the strategy for combining them. Or even some differences, from statistical point of view, can be found (Opitz, 1996).

Two strategies are needed to build an ensemble system:
a) Strategy for generating the ensemble members. This must seek to improve ensemble´s diversity. The other main stone in order for an ensemble to generalize well is the accuracy. Brown et al. (Brown, 2005) suggest that two ANNs are diverse if they make different errors on the same data points/inputs. Accuracy refers to how good the learning model is in comparison to random guessing on a new input (Brown, 2005). Two common methods to maintain the diversity within an ensemble are bagging and boosting, and its successor *AdaBoost* (Polikar, 2006)(Polikar, 2007). This group of techniques belongs to the sequential training methods of designing NNEs (Liu, 2003). The bagging method will randomly generate a new training set with an uniform distribution for each network member from the original data set (Polikar, 2006). The boosting approach (Schapire, 1990), resamples the data set with a non-uniform distribution for each ensemble member. The whole idea of boosting and bagging is to improve the performance by creating some weak and biased classifiers (Dam, 2008).
There exists some NNE where the training data is inherently resampled by classifier conditions, as the neural-based learning classifier system, NLCS, (Dam, 2008) or HUMANN based ensembles (Suárez Araujo, 2009)(García Báez, 2010). These NNEs implicitly maintain diversity in the population without using bagging or boosting. The stacked generalization (SG) and mixture of experts (ME) use different designing strategy to bagging or boosting techniques as well (Polikar, 2006)(Polikar, 2007). They generate two levels of ensemble members. A first level where individual classifiers are experts in some portion of the feature space. A second level classifier, which is used for assigning weights for the consecutive combiner, which is usually not a classifier, in the ME, and as a meta classifier for final decision in the SG (Polikar, 2006). These last ensemble schemes belong to the simultaneous training methods (Liu, 2003).
b) Combination strategy. It is necessary to combine the outputs of individual ANNs that make up the ensemble in such a way that the correct decisions are amplified, and incorrect ones are cancelled out. Two taxonomies can be considered, a) trainable vs. non-trainable combination strategies, b) combination strategies that apply to class labels vs. to class-specific continuous outputs.
In trainable combination rules, the parameters of the combiner, *weights*, are determined through a separate training algorithm, an example is the EM algorithm used by the ME model. There is no separate training involved in non-trainable rules, since the parameters become immediately available as the classifiers are generated. Weighted majority voting is an example of such non-trainable schemes (Polikar, 2006). In the second grouping several choices are available. For combining class labels we have majority voting, weighted majority voting, behavior knowledge space (BKS), and borda count schemes. For combining continuous outputs we can find some other schemes such as, algebraic combiners, decision templates and dempster-shafer based combination (Polikar, 2006).
Whereas there is no single ensemble generation algorithm or combination rule that is universally better than others, all of the approaches discussed above have been shown to be effective on a wide range of real world and benchmark datasets, provided that the classifiers can be made as diverse as possible. In the absence of any other prior information, the best ones are usually the simplest and least complicated ones that can learn the underlying data distribution (Polikar, 2006). Such an approach, does not guarantee the optimal performance, (Roli, 2002). For a small number of classifiers optimal ensembles can be found exhaustively, but the burden of exponential complexity of such search limits its practical applicability for

larger systems (Ruta, 2005). An appropriate design of NNE is where selection and fusion are recurrently applied to a population of best combinations of classifiers. rather than the individual best (Ruta, 2005).

4. Application of neural computation approach to identify fungicides

This section is dedicated to the application of the neural computation approach to face the resolution of difficult multi-component systems (overlapping) of fungicides with fluorescence detection. We present a study of automated decision making systems based on ANNs, data fusion and NNE to identify benzimidazoles fungicides (BFs). All these systems are modular neural computation systems, with a structure of pre-processing and processing stage, where their main element is the HUMANN neural architecture. This ANN is an unsupervised neural architecture which performs blind clustering. Because of this it can identify, in an automatic way, the different existing compounds in mixture samples without prior knowledge of which (and how many) of theses possible compounds can be found in the analysed sample. HUMANN has shown to be also suitable for identification of organochlorinated compounds with environmental interest (García Báez, 2003)(Suárez Araujo, 2003).

4.1 HUMANN-based systems to identify benzimidazoles fungicides with fluorescence detection

There are three HUMANN-based systems to identify the BFs Carbendazim 99.7% (methyl (1H-benzimidazol-2-yl) carbamate) (MBC), Benomyl 99.3% (methyl 1-(butylcarbomayl) benzimidazole-2-yl carbamate) (BM), Thiobendazole 99.6% (2-(4-thiazol)benzimidazole) (TBZ) and Furberidazole 99.6% (2-(2 furanyl)-1h-benzimidazole) (FB): a) HUMANN-based simple detector system, (Suárez Araujo, 2006), b) Multi-input HUMANN-based system, c) HUMANN-ensemble system (Suárez Araujo, 2009).

To gather the used data sets for each one of these HUMANN-based systems, an experiment design with chemical and computational requirements was performed (Suárez Araujo, 2009). These data sets, Table 1, are formed by emission, excitation and synchronous fluorescence spectra of each BFs, in six different concentrations, Fig. 3, which are adapted to the luminescent features of each compound. There were made mixtures for each one of them, with one, two, three and four compounds to obtain spectra at optimum, mean and median fluorescence features. To guarantee the measurements and define the margins of error in measurements, all the spectra were repeated three times.

The three HUMANN-based systems presented in the section share a common part, the feature extraction stage. This stage is implemented by the pre-processing module.

During the learning process, ANNs create internal representations of the characteristics of the training pattern set. The goal of the pre-processing stage is to prepare the information environment of HUMANN in such a way that it can adequately extract the information required. The pre-processing task constructs a set of feature vectors of the real signals which will be used in the processing stage. In the analysis of real fluorescence spectra two stages are followed: 1. modelling of the spectra. 2. Determination of the features vector which corresponds to the fluorescence spectrum model of mixture (García Báez, 2005)(Suárez Araujo, 2009).

	Characteristics	Benzimidazole family
	Compounds	4: Benomyl (BM), Carbendazim (MBC), Fuberidazole (FB), Thiabendazole (TBZ)
	Concentrations / compound:	6 (1 to 6)
	BM:	Interval = 250-1500 µg/l, Δc = 250 µg/l
	MBC:	Interval = 250-1500 µg/l, Δc = 250 µg/l
	FB:	Interval = 25-150 µg/l, Δc = 25 µg/l
	TBZ:	Interval = 2.5-15 µg/l, Δc = 2.5 µg/l
	Types of spectra:	4 synchronous, 1 excitation, 1 emission
	Mean / TBZ optimum (ΔΔ1):	Δλ = 47 nm, Interval = 200-400 nm
	Median (ΔΔ2):	Δλ = 53 nm, Interval = 200-400 nm
	BM/MBC optimum (ΔΔ3):	Δλ = 59 nm, Interval = 200-400 nm
	FB optimum (ΔΔ4):	Δλ = 29 nm, Interval = 200-400 nm
	Mean (λ_{em}):	λ_{em} = 327 nm, Interval = 200-315 nm
	Mean/Median (λ_{ex}):	λ_{ex} = 277 nm, Interval = 300-400 nm
Individual compounds	**Number of samples**	24 (4 compounds x 6 concentrations)
	Number of spectra / sample	3 per type = 18
	Number of spectra of compounds	72 per type = 432
Mixtures	**Number of mixtures**	100
	Number of spectra / mixture	3 per type = 18
	Number of spectra of mixtures	300 per type = 1800

Table 1. General characteristics of the sets of data of the benzimidazole fungicides. (From Chemical &Environmental Analysis Research Group (ULPGC))

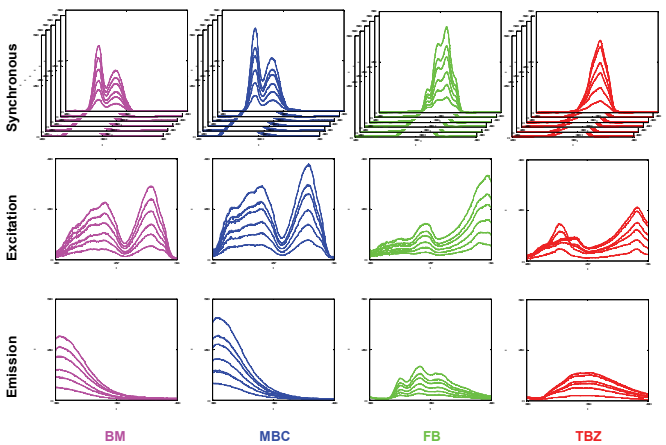


Fig. 3. Set of spectra of solutions of compounds belonging to the family of benzimidazole at different concentrations

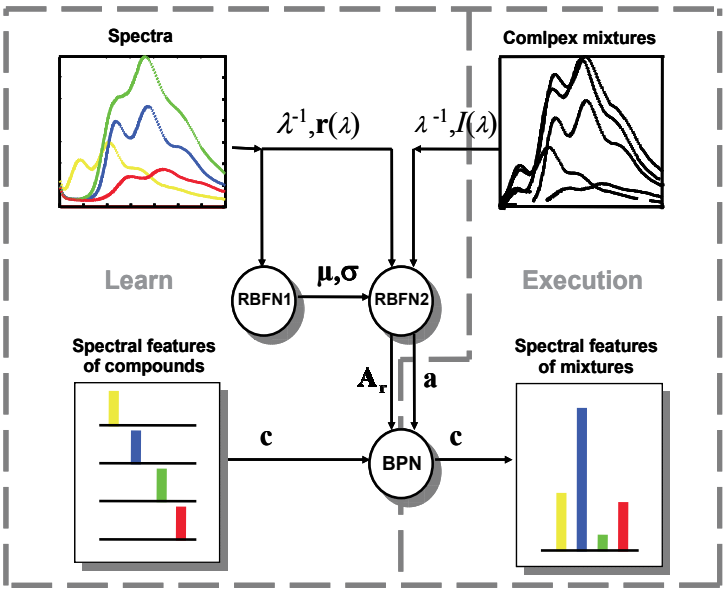


Fig. 4. Scheme of pre-processing stage

Using as basis the developments and later experimental studies made by Lloyd and Evett (Lloyd, 1977) and Cabaniss (Cabaniss, 1991) the fluorescence spectra can be modelled by a Gaussian distribution of intensity versus reciprocal wavelength (frequency). All spectra used in our developments have been previously normalized to the unit. The spectrum can be represented as equation:

$$I(\lambda) \approx a \cdot \mathbf{gaus}\left(\lambda^{-1}\right). \tag{21}$$

We will work with a linear approximation for the mixture model, such that the spectrum of a mixture will be represented by a linear combination of reference spectra. Reference spectra are the spectra of the compounds which can be identified in a mixture (García Báez, 2005)(Suárez Araujo, 2009):

$$I(\lambda) \approx c \cdot r(\lambda) = \sum_i c_i r_i(\lambda) \;\; I(\lambda) \approx c \cdot A_r \cdot \mathbf{gaus}\left(\lambda^{-1}\right) \tag{22}$$

where $\mathbf{r}(\lambda)$ are the reference spectra and $\mathbf{c}$ is a vector with the contributions of each of the spectra of $\mathbf{r}(\lambda)$ in the mixture. $\mathbf{c}$ is then a vector which characterises a mixture and which is ideal for use as a feature vector for HUMANN-based systems. Spectral representation via Gaussian distribution will be carried out using RBFNs, and the approximation of concentration coefficients vector ($\mathbf{c}$) through a backpropagation network. Our pre-processing module is therefore made up of a complex neural structure of RBFNs + BPN, Fig. 4 (García Báez, 2005)(Suárez Araujo, 2009). The feature vectors finally obtained will be the input data to the processing stage for each HUMANN-based system studied at the last part of this section.

a) HUMANN-based simple detector system. It is the HUMANN, Fig. 2, version for simultaneous classifications. It will allow the firing of several labelling neurons

simultaneously. Each mixture (input pattern) will therefore be classified as belonging to several classes. In the output layer, the necessary adjustments of parameters have been made during learning process to generate 3 neurons corresponding to the three categories of BFs, which have been defined as BM/MBC, FB and TBZ, to be detected. These classes have been defined because of high BM and MBC spectral correlation. The training set is represented by conventional (emission and excitation) and synchronous fluorescence spectra (Suárez Araujo, 2006), Fig. 3.

To evaluate the overall efficiency of the used systems, we defined a function E of mixture error with respect to the categories detected in any one mixture:

$$E_{FN} = \frac{NCND}{NCIM},\ E_{FP} = \frac{NCBD}{NCIM},\ E = E_{FN} + E_{FP} \tag{23}$$

where $NCIM$ is the total number of categories in the mixture, $NCND$ is the number of categories non detected, $NCBD$ is the number of categories bad detected, and E_{FN} is the false negative error and E_{FP} the false positive error in order to evaluate the sensitivity and specificity.

Highly satisfactory results for fluorescence detection of these fungicides, using mixtures of up to four, have been obtained, being observed that the most sensitive to BFs determination type of spectra are the synchronous ones, Fig. 6(a). This will be the type of spectra which will constitute the information environment of the two next neural architectures, which improve the obtained results by a single HUMANN, Fig. 6 and Fig. 7.

b) Multi-input HUMANN-based system. One of the systems proposed for the processing module is a multi-input system based on HUMANN (HUMANN1–4). This system uses simultaneously the feature vectors (dimension 3) of four types of synchronous spectra explained in Table 1, as an input, Fig. 5(a). Via this multi-fluorescence spectra system, HUMANN has information from the luminescent sources whose Δλ has been tuned to the optimum values for each compound to be detected. The dimension of the optimum SOM layer for this system has been found to be 3x3.

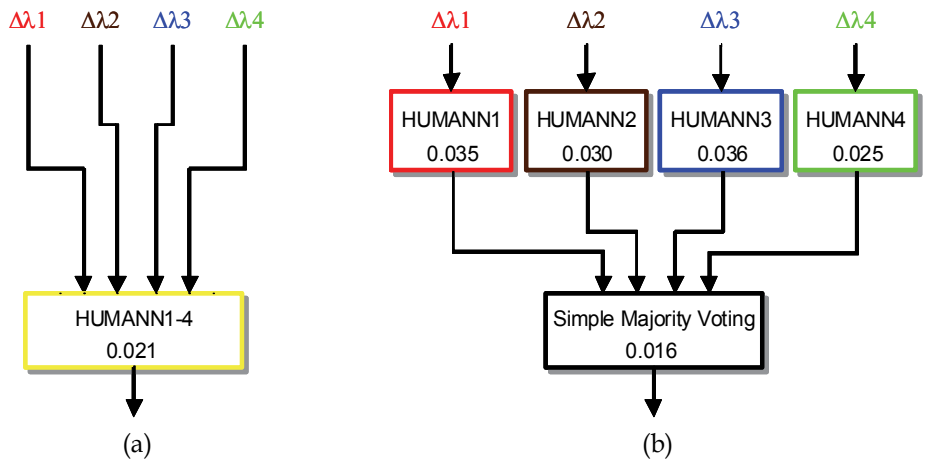
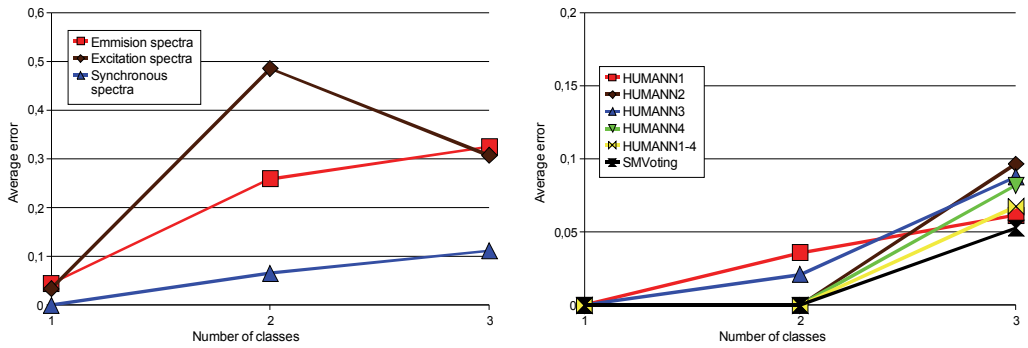
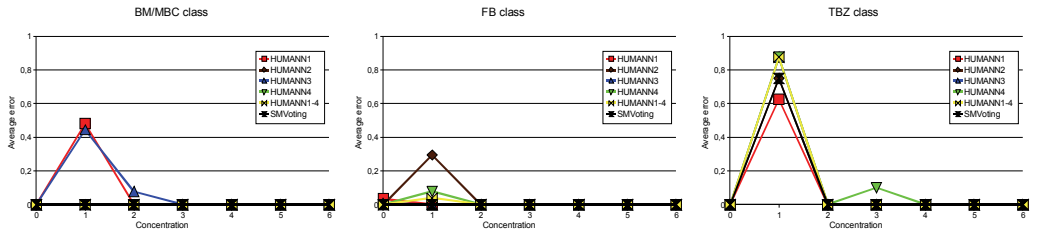


Fig. 5. HUMANN1-4 scheme (a). HUMANN-ensemble scheme (b)



(a) (b)

Fig. 6. Influence of the number of categories from the mixture in the average of the errors of the mixtures. Average error for each type of spectra (a). Average error for each HUMANN-based system (b)



(a) (b) (c)

Fig. 7. Influence of the concentration of the analytes BM/MBC (a), FB (b) and TBZ (c) in the mixture over the average of the errors of the mixtures. SMVoting is HUMANN-ensemble system with a simple majority voting combination strategy. The concentration "0" means absence of the analyte

c) HUMANN-ensemble system. The last automated decision making system to determine pesticides in a difficult multi-component system presented, is a NNE (HUMANN-ensemble). It will improve the obtained results with simple HUMANN fungicide detectors. The main aspect in the strategy for generating the ensemble members is diversity. The diversity of HUMANN ensemble depends on two aspects, correlation of HUMANN performance, which is a pair-wise measure of diversity, and the networks should have their errors in different parts of the input space. This effect is reached using four HUMANN architectures as ensemble members. Each one is working in a different local region of input space, which will be determined by the fluorescence characteristics of synchronous spectra: HUMANN1, HUMANN2, HUMANN3 and HUMANN4 respectively for the types of spectra $\Delta\lambda_1$, $\Delta\lambda_2$, $\Delta\lambda_3$ and $\Delta\lambda_4$ (Table 1). Each one of these HUMANNs has the same architecture of HUMANN1-4. Finally, a combination strategy is needed in order to obtain an overall NNE. This strategy must be such that the correct decisions may be amplified and the incorrect decisions cancelled or decreased (Polikar, 2006). Our HUMANN-ensemble system uses a non-trainable combination strategy that applies labels to class, the simple majority voting (SMV), Fig. 5(b). Each module polls a vote for each category, indicating whether it considers that the said category is present in the mixture or not. A later module is

responsible for the overall count, considering whether the compound belongs to one category or another, depending on whether more than half of the HUMANN modules consider this category is present in the mixture. The combination of the outputs of several ensemble members does not guarantee a superior performance to the best ensemble module, but it clearly reduces the risk of making a particularly poor selection (Polikar, 2006).
In modules of Fig. 5(b) we show the average value of the mixture errors over all the mixtures studied, taking into account the various systems deployed. As it can be seen, the most precise simple detectors are HUMANN4 and HUMANN2. Once again, HUMANN1-4 is more precise than any of the simple detectors, with a margin of *E* error 0.004 less than HUMANN4. HUMANN-ensemble is superior even to HUMANN1-4, with its margin of *E* error 0.005 less than the latter and 0.009 less than HUMANN4. Very successful results have then been obtained with the proposed neural computation systems having a high specificity and a good sensitivity in detection of benzimidazole fungicides. Furthermore, these systems are suitable for studying such figures of merit as precision, sensitivity and limit of detection of the method, for each of the fungicides, Fig. 7. The studied systems are also capable to indicate the existence of some other analyte different to BF in the analysed sample, or to face the resolution of multi-analyte mixtures (whatever analyte, even a clean sample). In this case, it is needed to use an appropriate training set in which there exists spectral characteristics of possible compounds that can be present in the difficult multi-analyte systems analysed. This is easily possible because of high adaptive character of HUMANN, concretely; this skill is embodied by the neurogenesis mechanism (García Báez, 2005).

5. Conclusion

This chapter is dedicated to the area of qualitative and quantitative chemical analysis of fungicides using neural computation methods. The study of this area will allow introducing the intelligent environmental monitoring area.
We have showed that the determination of fungicides is essentially carried out using chromatographic techniques combined with specific detection schemes. However, these methodologies are relatively expensive, time consuming and often require laborious sample treatment before analysis. There are other efficient alternatives to overcome these limitations, the chemometry methods.
A complementary approach to face these problems is using a computational model which has been biologically inspired, "the artificial neural network". This subject is the focus of the chapter. In this chapter we describe the resolution of difficult multi-component systems (overlapping) of fungicides, using ANNs, and also data fusion and NNE.
We present a general study of neural computation. This study covers theory and architecture of the main, and most used for chemical data processing, classical supervised and unsupervised ANNs. A new hierarchical unsupervised modular adaptive neural network (HUMANN) is also reported as very important ANNs in the identification of fungicides. The NNE has been analysed as a successful technique where outputs of a set of separately trained neural networks are combined to form one unified prediction. We have shown it to be a very appropriate approach to the scope of our application domain, noisy, outliers and overlapping data distributions.
Finally, this chapter presents the ability of ANN and NNE, to address the determination of benzimidazoles fungicides in complex mixtures, describing the outcomes of three implementations of such approaches: HUMANN-based simple detector system, multi-input

HUMANN-based system (HUMANN1-4), and HUMANN-ensemble. It has been demonstrated that these systems are very appropriate methods to face the extremely restricted scope of application of fluorescence spectrometry in the analysis of multi-component mixtures. This can be seen in the highly satisfactory results for fluorescence detection of these compounds of environmental interest, using mixtures of up to four different fungicides per mixture, and without prior knowledge of which (and how many) of these possible compounds can be found in the analysed sample. The comparative studies conclude the HUMANN-ensemble system provides better performance than simple detector system. This confirms the advantage to extract complementary pieces of information from different or/and diverse data sources.

The study presented in this chapter can be an important contribution in the environmental analytical chemistry field. This importance is based on that these neural computational methods use only spectral fluorescence data, are very simple, fast and economic method for monitoring of the environment. Furthermore these developments can consist on the first steps forward designing of an on line intelligent environmental monitoring system.

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24

Research and Development of Macrocyclic Compounds as Fungicides

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1. Introduction

"The latest economical development in agriculture calls for an increasing demand for food and feed on optimized plant production. If we are to intensify crop production, the efficient control of plant disease is essential. At present the most reliable means of doing this is by the use of fungicides" (Dehne, 2007). Unfortunately, resistance to these fungicides has always been observed, thus, leading to the continuing need of further research to discover new classes of fungicides, especially those with novel modes of action. One strategy for discovering new fungicides is to mimick the chemistry of biologically active natural products. Naturally occurring macrocyclic compounds, especially macrolactam and macrolactone have attracted considerable interest of chemists and the natural products community because they display a diverse range of biological activity including pesticidal activity. Several examples include avermectins, a group of 16-membered ring lactones with anthelmintic, insecticidal and acaricidal activities (Fisher, 1990 and Green, 1991), spinosyns, a group of 12-membered ring lactones with insecticidal activity (Sparks *et al*, 1998; Crouse *et al*, 2001), epilachnene, a kind of structurally novel azamacrolide with antifeedant activity (Attygalle *et al*, 1993), pyrenophorol, a macrodiolide with herbicidal activity (Kastanise and Chrysayi-Tokousbalides, 2000), and maltophilin, a novel macrolactam, produced by strains of *Stenotrophomonas maltophilia* R3089 isolated from the rhizosphere of rape plants (*Brassica napus L.*), which exhibited biological activity against a broad spectrum of fungi (Jakobi *et al*, 1996).

This chapter will describe the discovery of fungicidal novel lead compounds, especially those macrocyclic compounds which have relative simple structure and can be synthesized easily from readily available raw materials, by mimicking the structure of natural occurring macrolactone and macrolactam, and the development of the compounds with high fungicidal activity.

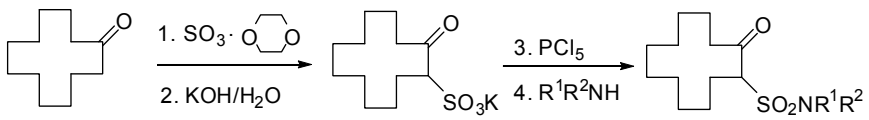
2. Research of the cyclododecanone derivatives

Cyclododecanone is an important intermediate in the organic synthesis and can be prepared from cyclododecatriene which can easily be produced in the large scale from petrochemical product butadiene (Wilke & Muller, 1958 and Weber *et al*, 1965). In this section, synthesis and fungicidal activity of several series of cyclododecanone derivatives are described.

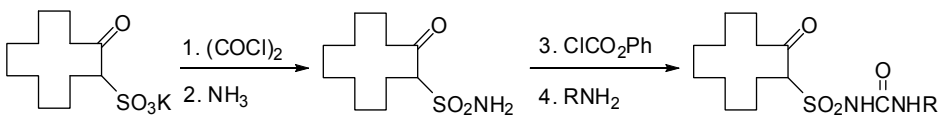
2.1 Synthesis and fungicidal activity of 2-oxocyclododecylsulfonamides (3) and 2-oxocyclododecylsulfonylureas (5)

Wang and Wang (1997) reported the synthesis and fungicidal activity of 2-oxocyclododecylsulfonamides (**3**). Compounds **3** were synthesized by amination of 2-oxocyclododecylsulfonic acid chloride, obtained from the readily available cyclododecanone (**1**) via 2-oxocyclododecylsulfonate (**Scheme 1**).

Bioassay showed that compounds **3** are active against *G. zeae* (*Gibberella zeae* Petch). Among them, compounds only having one substituent on the nitrogen of sulfonic acid amide group are more active than those having two substituents on the nitrogen of sulfonic acid amide group. This may mean the importance of hydrogen-bonding donor in the structure. In addition, QSAR study (CoMFA) (Xie *et al*, 1999) showed that 2-oxocyclododecylsulfonylureas (**5**) may have higher predicted fungicidal activity although some types of sulfonylureas are high efficient chemical herbicides. Thus, a series of compounds **5** were synthesized (Li *et al*, 2005) (**Scheme 2**).

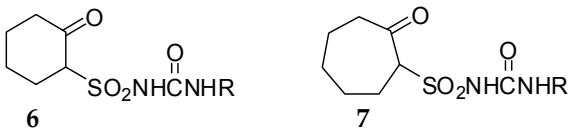


Scheme 1. Synthetic route of 2-oxocyclododecylsulfonamides (**3**)



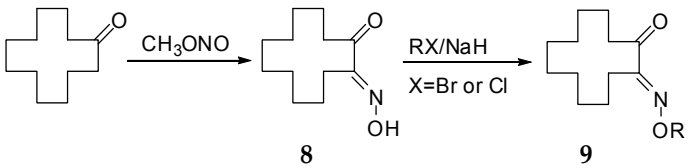
Scheme 2. Synthetic route of 2-oxocyclododecylsulfonylureas (**5**)

Compound **2** was allowed to react with oxalyl chloride to give corresponding sulfonyl chloride, which was converted into sulfonamide (**4**) using NH_3. The reaction of **4** with phenyl chloroformate and amines successively afforded desired compounds **5**. Bioassay showed that compounds **5** exhibited some fungicidal activity against *G. zeae* but do not fully accord with the prediction of CoMFA. The fungicidal activity of a representative compounds, N-(2,5-dichlorophenyl)-N′-(2-oxocyclododecylsulfonyl)urea against seven fungi [(*G. zeae*, *B. cinerea* (*Botrytis cinerea* Pers), *C. orbiculare* (*Colletotrichum orbiculare* Arx), *P. aphanidermatum* (*Pythium aphanidermatum* Fitzp), *F. oxysporum* (*Fusarium oxysporum* Schl.f.sp Vasinfectum), *R. solani* (*Rhizoctonia solani* Kuhn), and *V. dahliae* (*Verticillium dahliae* Kled)] was further evaluated. The results showed that it has better fungicidal activity against *C. orbiculare* and *P. aphanidermatum* than the commercial fungicide carbendazim. In addition, corresponding 2-oxocyclohexylsulfonylureas (**6**) and 2-oxocycloheptylsulfonylureas (**7**) were also synthesized and their fungicidal activity against *G. zeae* was evaluated. The result showed that compounds containing 12-membered ring (**5**) are more active than those containing 6- or 7-membered ring (**6**, **7**), which indicated that 2-oxocyclododecyl may be an active group showing pesticidal activity and is worth to pay attention to in research and development of novel pesticides.



2.2 Synthesis and fungicidal activity of (*E*)-α-oxocyclododecanone oxime ethers (9)

Li *et al* (2006) reported the synthesis and fungicidal activity of (*E*)-α-oxocyclododecanone oxime ethers (**9**). As shown in **Scheme 3**, compounds **9** were synthesized by oximation of cyclododecanone followed by etherification. Configuration of α-oxocyclododecanone oxime (**8**) was determined to be *E* isomer via the Beckmann reaction which gives 11-cyanoundodecanoic acid (Hou *et al*, 1999). Its *E* configuration was further confirmed by single crystal X-ray diffraction analysis of a representative of compound **9** (R = 4-$FC_6H_4CH_2$) (Li *et al*, 2006).

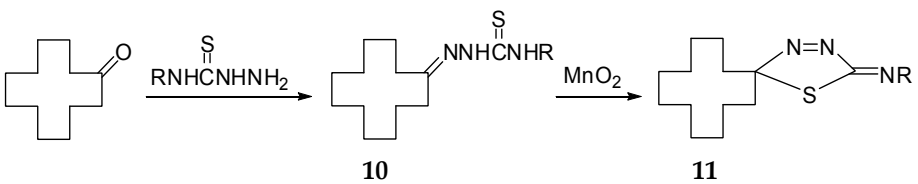


Scheme 3. Synthetic route of (*E*)-α-oxocyclododecanone oxime ethers (**9**)

Bioassay showed that most of compounds **9** present good fungicidal activity against *R. solani*, *C. cucumerinum (Cladosporium cucumerinum* Ell.et Arthur), *C. orbiculare*, *B. cinerea*, *F. oxysporum*, and *P. asparagi (Phomopsis asparagi* Bubak). Although their activity against *R. solani, C. cucumerinum, B. cinerea, F. oxysporum* and *P. asparagi* are lower than commercial fungicides carbendazim, but the activity of individual compound (e.g. R=$CH_2CO_2CH_3$) against *C. orbiculare* is better than carbendazim.

2.3 Synthesis and fungicidal activity of 2-(1,11-undecylidene)-5-substituted imino-Δ^3-1,3,4-thiadiazolines (11)

Zhang *et al* (2001) and Chen *et al* (2002) reported the synthesis and fungicidal activity of 2-(1,11-undecylidene)-5-substituted imino-Δ^3-1,3,4-thiadiazolines (**11**).



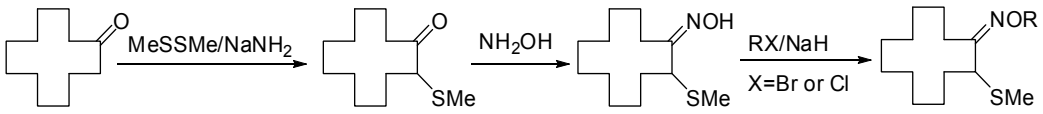
Scheme 4. Synthetic route of 2-(1,11-undecylidene)-5-substituted imino-Δ^3-1,3,4-thiadiazolines (**11**)

As shown in **Scheme 4**, Compounds **11** were synthesized by oxidative cyclisation of N-substituted cyclododecanone thiosemicarbazones (**10**), prepared by condensation reaction of cyclododecanone and *N*-substituted thiosemicarbazide, on treatment with manganese dioxide. The conformation of compounds **11** was analyzed by NMR, molecular mechanic calculation and X-ray diffraction study (Wang *et al*, 2002). Bioassay showed that most of

compounds **11** have some fungicidal activity against *R. solani* and *V. dahlide* and individual compound exhibit good activity against *R. solani.*

Synthesis and fungicidal activity of α–methylthiocyclododecanone oxime ethers (14)

Song *et al* (2005) reported the synthesis and fungicidal activity of α–methylthiocyclododecanone oxime ethers (**14**). As shown in **Scheme 5**, compounds **14** were synthesized by alkylation of α–methylthiocyclododecanone oxime, which was prepared by methylthiolation of cyclododecanone followed by oximation.

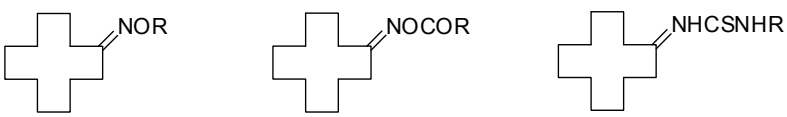


12 **13** **14**

Scheme 5. Synthetic route of α–methylthiocyclododecanone oxime ethers (**14**)

Bioassay showed that some of compounds **14** have good fungicidal activity against *R. solani and B. cinerea.*

Apart from mentioned above compounds (compounds **3**, **5**, **9**, **11** and **14**) carrying two substituents on the cyclododecane ring (carbonyl group and side chain, and thiadiazoline ring of compounds **11** may be considered as two substituents on the spiro carbon atom), monosubstituted cyclododecane derivatives, for example, **15**, **16** and **17**, were synthesized and were found to be completely ineffective as fungicides (Huang *et al*, 2007) (**Figure 1**). It is suggested that the coexistence of two polarizable groups on the cyclododecane ring is necessary for fungicidal activity of the cyclododecanone class of compounds. The results showed that it should be very useful for designing new classes of macrocyclic fungicides, especially those with novel modes of action.



15 **16** **17**

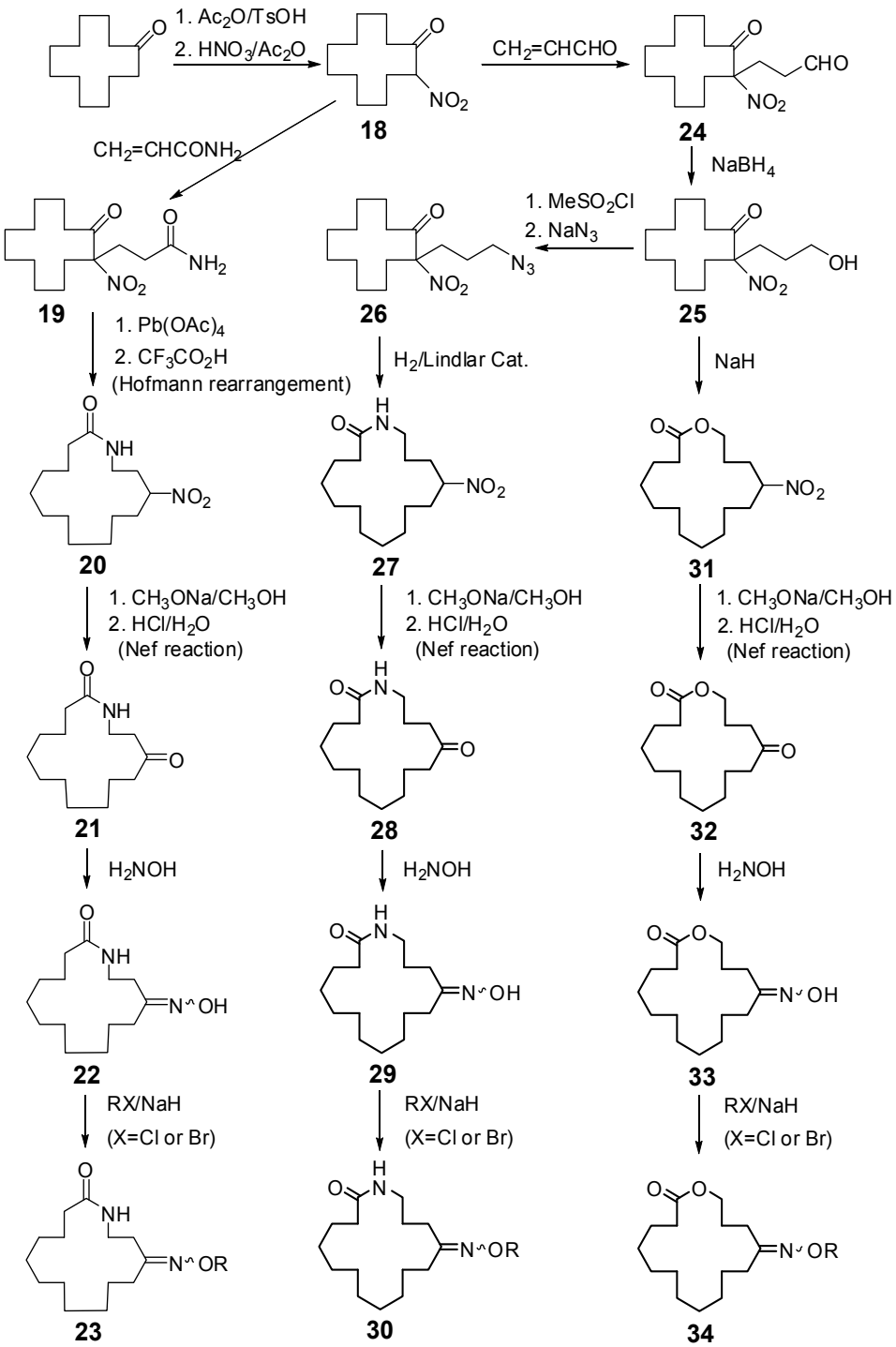
Fig. 1. Chemical structure of several monosubstituted cyclododecane derivatives

3. Research of macrolactam and macrolactone derivatives

In this section, the improvement of the fungicidal activity of the compounds with higher fungicidal activity (compounds **3**, **9** and **11**) in section 1 through structural derivation was described. The approach is to replace the cyclododecane ring in the cyclododecanone derivatives by macrolactam or macrolactone rings.

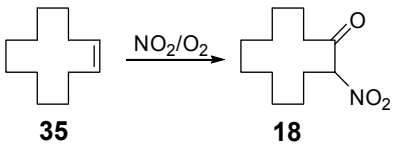
3.1 Synthesis and fungicidal activity of macrolactams and macrolactones with an oxime ether side chain (23, 30 and 34)

The improvement of the fungicidal activity of compounds **9** through the replacement of cyclododecane ring by macrolactam or macrolactone rings was described (Huang *et al*, 2007). Compounds 12-alkoxyiminotetradecanlactam (**23**), 12-alkoxyiminopentadecanlactam (**30**) and 12-alkoxyiminopentadecanlactone (**34**) were designed.



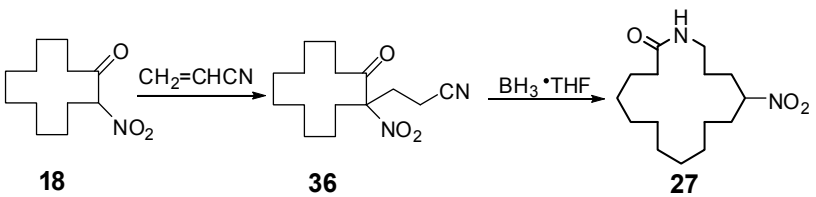
Scheme 6. Synthetic route of macrolactams and macrolactones with an oxime ether side chain

Compounds **23**, **30** and **34** were synthesized by oximation of compound **21**, **28** and **32** followed by etherification respectively. The compound **21** was synthesized from 2-nitrocyclododecanone (**18**), prepared from cyclododecanone, via Michael addition to acrylamide followed by Hofmann rearrangement, ring enlargement and Nef reaction (Pan and Wang, 1993; Jia *et al*, 2007). Compound **28** was synthesized from compound **18** via Michael addition to acrolein, selective reduction of aldehyde group followed by the conversion of hydroxyl group to amino group, ring enlargement and Nef reaction (Huang *et al*, 2004). Compound **32** was synthesized from compound **25** by ring enlargement and Nef reaction (Zhang *et al*, 2003) (**Scheme 6**).
Compound **18** was also synthesized from cyclododecene (**35**) by nitrooxidation (Fang *et al*, 2007) (**Scheme 7**).



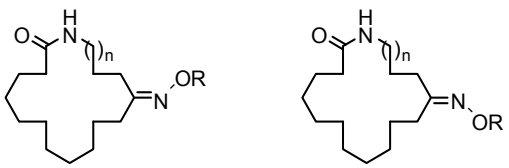
Scheme 7. Synthetic route of 2-nitrocyclododecanone (**18**)

Compound **27** was also synthesized from compound **18** via Michael addition to acrylnitrile followed by selective reduction of nitrile group, ring enlargement (Hou *et al,* 2006) (**Scheme 8**).



Scheme 8. Synthetic route of 12-nitro-1,15-pentadecanlactam (**27**)

There exist two isomers (*Z* and *E* isomers) for compounds **23**, **30** and **34**. The *Z* and *E* isomers of compounds **23** and **30** were isolated by column chromatography (**Figure 2**), but the two isomers of compounds **34** could not be separated due to the insufficient polarity difference in their structures.



n=0 for compounds **23**, n=1 for compounds **30**

Fig. 2. *Z* and *E* isomers of compounds **23** and **30**

As shown in **Table 1**, compounds **23** have fair to good fungicidal activity against *R. solani*. In general, the following structure-activity relationship in compounds **23** was observed: (1) The compounds with C3-C4 straight chain alkyl and benzyl without any substituent have better

fungicidal activity; (2) *E* isomers are more active than *Z* isomers, especially the compounds **23a** (*E*), **23c** (*E*), and **23f** (*E*), the EC_{50} values of which were 9.11, 7.21 and 7.24 μg/mL respectively, displayed higher fungicidal activity than corresponding *Z* isomers. The replacement of tetradecanlactam ring with pentadecanlactam ring (compounds **30**) results in significantly improved fungicidal activity. For example the EC_{50} values of the compounds **30a** (*E*), **30c** (*E*), **30c** (*Z*), **30h** (*Z*) were 3.62, 2.34, 3.97 and 2.34 μg/mL respectively. The pentadecanlactone derivatives (**34**) have somewhat improved fungicidal activity against *R. solani* than that of **23**, but less active than **30**. Namely, in the order of **23**, **34** and **30**, the compounds have a gradual increase of fungicidal activity. The results confirmed the original judgment in section 1: macrocyclic compounds with two polarizable groups on the ring may have certain fungicidal activity. It can also be seen from **Table 1**, all of tetradecanlactam and pentadecanlactam derivatives containing two polarizable groups –CONH- and =N-O- on the ring (**23** and **30**), and pentadecanlactone derivatives containing –COO- and =N-O- on the ring (**34**) displayed fair to excellent fungicidal activity against *R. solani*. In the macrocyclic derivatives with two polarizable groups on the ring, the compounds in which there is a three methylene distance ($CH_2CH_2CH_2$) between the two polarizable groups (**30**, **34**) are more active than those in which there is a two methylene distance (CH_2CH_2) between the two polarizable groups (**23**). The fact that compounds **30** have higher fungicidal activity than compounds **34** indicates that the macrocyclic derivatives with a hydrogen-bonding acceptor (here is =N-O-) and a hydrogen-bonding donor (here is -CONH-) have the best fungicidal activity among the macrocyclic derivatives with two polarizable groups and a three methylenes distance between these groups.

R	Compds No.	EC_{50} (μg /mL)	Compds No.	EC_{50} (μg /mL)	Compds No.*	EC_{50} (μg /mL)
$CH_3CH_2CH_2$	**23a** (Z)	22.10	**30a** (Z)	13.14	**34**a	8.08
	23a (E)	9.11	**30a** (E)	3.62		
CH_2=$CHCH_2$	**23b** (Z)	44.03	**30b** (Z)	45.61	**34**b	12.76
	23b (E)	21.76	**30b** (E)	15.92		
$CH_3(CH_2)_2CH_2$	**23c** (Z)	55.10	**30c** (Z)	3.97	**34**c	13.36
	23c (E)	7.21	**30c** (E)	2.34		
CH_3 $(CH_2)_5CH_2$	**23d** (Z)	209.43	**30d** (Z)	19.25	**34**d	9.68
	23d (E)	50.35	**30d** (E)	27.88		
CH_3 $(CH_2)_{14}CH_2$	**23e** (Z)	135.04	**30e** (Z)	47.75	**34**e	40.39
	23e (E)	127.22	**30e** (E)	51.48		
C_6H_5 CH_2	**23f** (Z)	18.15	**30f** (Z)	5.84	**34**f	45.13
	23f (E)	7.24	**30f** (E)	8.28		
4-$ClC_6H_4CH_2$	**23g** (Z)	97.25	**30g** (Z)	68.37	**34**g	19.61
	23g (E)	84.79	**30g** (E)	164.28		
2-Cl-6-$FC_6H_3CH_2$	**23h** (Z)	1734.74	**30h** (Z)	2.34	**34**h	51.27
	23h (E)	42.99	**30h** (E)	6.21		

* A mixture of *Z* and *E* isomers

Table 1. Fungicidal activity of compounds **23, 30** and **34** against *R. Solani*

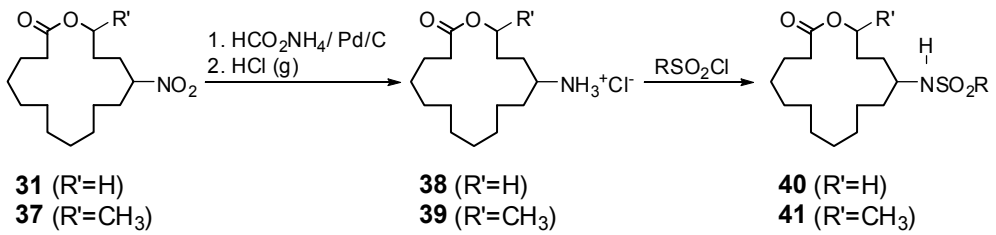
Furthermore, compound **30a** (a mixture of *Z* and *E* isomers) was selected as a representative to evaluated its fungicidal spectrum. The result is listed in **Table 2**. It can be seen from **Table 2** that compound **30a** has a broad spectrum of fungicidal activities, and especially has excellent fungicidal activities against *A. kikuchiana* (*Alternaria kikuchiana*), *P. physaleos* (*Phyllospicpa physaleos* Sacc), *R. solani* and *B. cinerea*. The EC_{50} values were 1.2, 1.9, 4.6 and 8.6 μg/mL respectively.

Pathogen	EC_{50} (μg/mL)
Pear black spot (*Alternaria kikuchiana*)	1.2
Tomoto southern blight (*Phyllospicpa physaleos Sacc*)	1.9
Cotton rhizoctonia rot (*Rhizoctonia solani Kuhn*)	4.6
Cucumber grey mold (*Botrytis cinerea Pers*)	8.6
Asparagus stem blight (*Phomopsis asparagi Sacc*)	12.0
Apple ring spot (*Physalospora piricola Nose*)	13.8
Cotton verticillium wilt (*Vercicillium alboatrum Reinke et Berthold*)	19.3
Cucumber anthracnose (*Colletotrichum Lagenarium*)	23.9
Cotton fusarium wilt (*Fusarium vasinfectum Atkimson*)	29.0
Cotton damping-off (*Pythium aphanibermatum (Eds.) Fipzp*)	33.3
Tomoto early blight (*Alternaria solani Jones et Grout*)	44.2
Tomoto leaf mold (*Cladosporium fulvum Cooke*)	53.1
Peppers fruit rot (*Phytophthora capsici Len*)	57.8

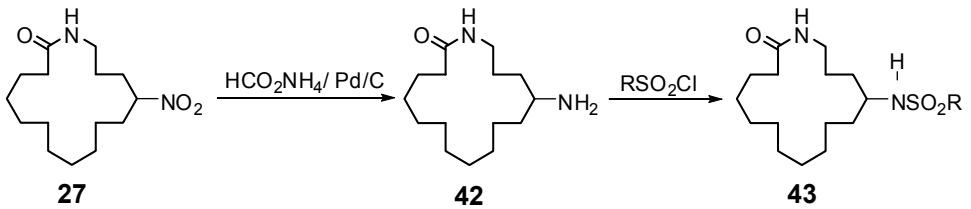
Table 2. Fungicidal spectrum of **30a** (a mixture of *Z* and *E* isomer)

3.2 Synthesis and fungicidal activity of macrolactones and macrolactams with a sulfonamide side chain (40, 41, 43 and 47)

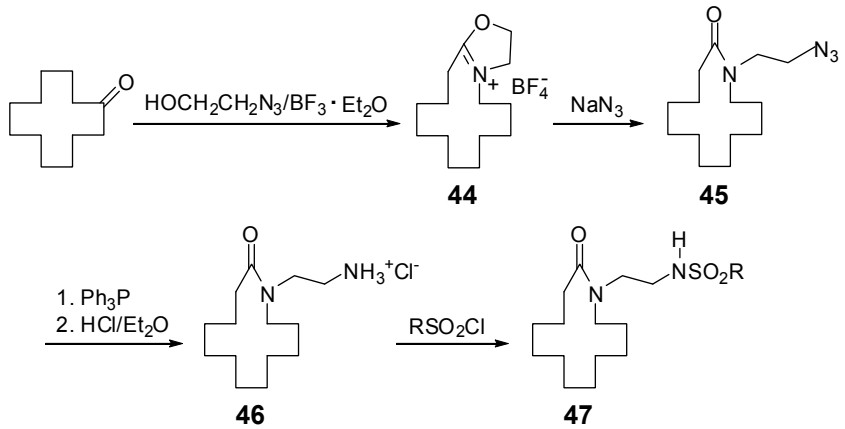
Based on the result obtained in section 3.1 and the structural feature of compounds **3** with certain fungicidal activity, Zhu *et al* (2008) introduced a sulfonamide group into pentadecanlactone, designing a series of novel 12-alkylsulfonamido-1,15-pentadecanlactones (**40**), which retain a hydrogen-bonding acceptor (here, it is –CO-O-) and a hydrogen-bonding donor (here, it is –NH-SO_2-) on the large ring and still have a three methylenes distance between two polarizable groups, and expect that the compounds have a better fungicidal activity than compounds **30** or comparable fungicidal activity with compounds **30**. In order to investigate whether the rule on the relationship between the activity and hydrogen-bonding has a general suitability to the macrocyclic compounds, further structural derivation on the compounds **40** was carried out: (a) A methyl group was introduced at C15 position and the 12-alkylsulfonamido-15-methyl-1,15- pentadecanlactones (**41**) were designed. (b) The lactone ring was replaced by lactam ring and the 12-alkylsulfonamido-1,15-pentadecanlactams (**43**) were designed. (c) The sulfonamide group was transferred to the terminal of side chain and still kept a suitable distance between the two polarizable groups, and the *N*-(alkylsulfonamidoethyl)-1, 12-dodecanlactams (**47**) were designed.



Scheme 9. Synthetic route of macrolactones with a sulfonamide side chain (**40** and **41**)



Scheme 10. Synthetic route of macrolactams with a sulfonamide side chain (**43**)



Scheme 11. Synthetic route of macrolactams with a sulfonamidoethyl side chain (**47**)

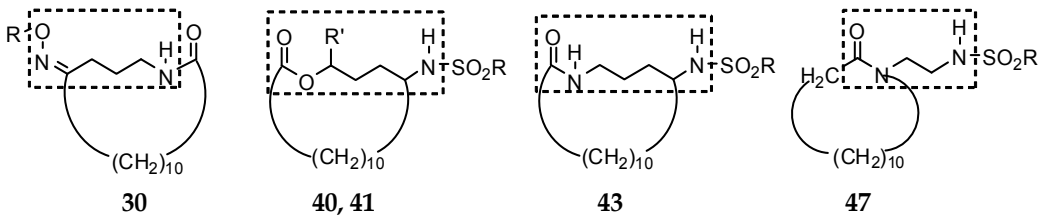
Compounds **40** and **41** were synthesized from intermediates **31** and **37** respectively by transfer hydrogenation using ammonium formate and palladium on carbon followed by sulfonylation with alkylsulfonyl chloride (**Scheme 9**). The compound **37** could be synthesized from nitrocyclododecanone (**18**) according to the method of synthesizing compound **31** just using methyl vinyl ketone instead of acrolein. Compounds **43** were synthesized from intermediate **27** according to the method of synthesizing compounds **40** (**Scheme 10**). The synthetic route of compounds **47** is shown in **Scheme 11**. Schmidt reaction (Li, 2006) of cyclododecanone with 2-azidoethanol followed by treating with sodium azide (Gracias *et al*, 1996; Gracias *et al*, 1997) give *N*-(2-azidoethyl)dodecanlactam (**45**), which was reduced and sulfonylated to afford compounds **47**.

As shown in **Table 3**, compounds **40**, **41**, **43** and **47** displayed fair to excellent fungicidal activity against *R. solani* and have a gradual increase of fungicidal activity in the order of **41**, **43**, **47**, and **40**. Compounds **40** displayed well to excellent activity except individual compound **40k**. Among them, compounds **40a**, **40b** and **40c**, the EC_{50} values of which were 2.4, 3.7 and 3.3 μg/mL respectively, displayed excellent fungicidal activity and were comparable with compounds **30**.

R	Compds No.	EC_{50} (μg/mL)	Compds No.	EC_{50} (μg/mL)	Compds No.	EC_{50} (μg/mL)	Compds No.	EC_{50} (μg/mL)
C_6H_5	**40a**	2.4	**41a**	51.3	**43a**	55.3	**47a**	11.6
4-MeC_6H_4	**40b**	3.7	**41b**	57.0	**43b**	13.4	**47b**	8.8
4-ClC_6H_4	**40c**	3.3	**41c**	69.6	**43c**	19.6	**47c**	18.7
3-$O_2NC_6H_4$	**40d**	18.7	**41d**	86.0	**43d**	27.6	**47d**	10.7
2,5-$Cl_2C_6H_3$	**40e**	28.4	**41e**	30.0	**43e**	30.4	**47e**	5.3
4-FC_6H_4	**40f**	4.5	**41f**	25.1	**43f**	36.7		
2-ClC_6H_4	**40g**	25.0	**41g**	37.9	**43g**	59.5		
2-$O_2NC_6H_4$	**40h**	22.3	**41h**	93.3	**43h**	21.5		
2-($MeCO_2$)C_6H_4	**40i**	20.9	**41i**	53.4	**43i**	12.8		
	**40j**	5.1	**41j**	20.3	**43j**	18.3		
$C_6H_5CH_2$	**40k**	52.4	**41k**	28.2	**43k**	8.1		
CH_3	**40l**	12.2	**41l**	56.4	**43l**	45.1		

Table 3. Fungicidal activity of compounds **40, 41, 43** and **47** against *R. solani*

As mentioned above the designed idea of compounds **40** was originated from compounds **30**. In view of the hydrogen-bonding acceptor and hydrogen-bonding donor, compounds **40** and **30** (**Figure 2**) have similar structural characteristics although compounds **40** are macrolactones with a sulfonamide side chain and compounds **30** are macrolactams with an oxime ether side chain. The results showed that the rule on the relationship between the fungicidal activity and hydrogen-bonding has a general suitability to the macrocyclic compounds. Compounds **47** have somewhat lower fungicidal activity against *R. solani* than that of compounds **40**. Although the former is 13-membered lactam derivatives and latter 16-membered lactone derivatives, they are similar in the chemical structure (active moiety is similar and all of lipophilic moiety are ten to eleven methylenes of large rings). The difference is that greater part of active moiety of compounds **47** is out of large ring as the skeleton of side chain which is flexible instead of rigid. The flexible characteristics of active moiety reduce the reactivity of molecule in combination with target enzyme. This may be the reason why the fungicidal activity of compounds **47** is somewhat lower than that of compounds **40**.



30 **40, 41** **43** **47**

Fig. 3. Comparison of structures of compounds **30**, **40**, **41**, **43** and **47**. The structures of active moiety of compounds in the square frames are similar.

Compounds **41** have a much lower fungicidal activity against *R. solani* (the EC_{50} values of all compounds **41** are larger than 20 μg/mL) than that of compounds **40**. However, their difference in chemical structure is only that there is a methyl group on the C15 for compounds **41** and none but hydrogen atom on the C15 for compounds **40**, which indicated that methyl group plays an inhibitory role to the fungicidal activity. May be the existence of methyl group with a great volume between two polarizable groups will interferes in the interaction of pesticide molecules with target enzyme as shown in **Figure 3**. In the molecule of compounds **43**, carbonyl group of amide can play the role as hydrogen-bonding acceptor, therefore they have good fungicidal activity, but the active hydrogen on the nitrogen atom adjacent carbonyl would interfere in the interaction of pesticide molecules and target enzyme. This may be the reason why fungicidal activity of compounds **43** is lower than compounds **40**.

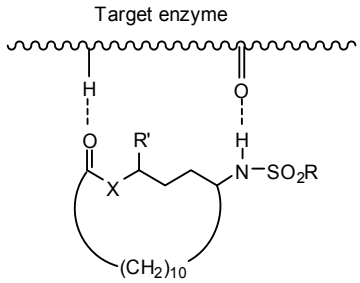


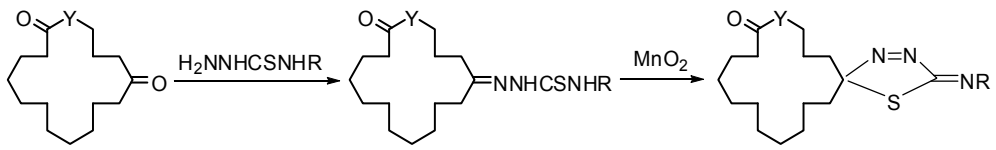
Fig. 4. Sketch map of the interaction of compounds **40**, **41** and **43** with target enzyme: Pesticide molecules combine with target enzyme by hydrogen-bondings. Interaction of pesticide molecule with target enzyme is interfered by methyl group for compounds **41** (R' is methyl group and X is O), and the interaction is interfered by active hydrogen on nitrogen atom for compounds **43** (X is NH and R' is H).

3.3 Synthesis and fungicidal activity of spiro-compounds containing macrolactam (macrolactone) and thiadiazoline rings (50, 51)

The result, from sections 3.1 and 3.2, indicated that it may be an effective approach to improve the bioactivity of cyclododecane derivatives to replace cyclododecane ring by macrolactam and macrolactone rings. Li *et al* (2010) designed and synthesized a series of spiro-compounds containing macrolactam and thiadiazoline rings (**50**), which retain a hydrogen-bonding donor (CO-NH) by replacing the cyclododecane ring of compounds **11** using macrolactam. For comparison, several spiro-compounds containing macrolactone and thiadiazoline rings (**51**) were also designed and synthesized.

Compounds **50** and **51** were synthesized by oxidative cyclisation of intermediates **48** and **49**, prepared by condensation reaction of 12-oxo-1,15-pentadecanlactam or 12-oxo-1,15-pentadecanlactone and *N*-substituted thiosemicarbazide, on treatment with manganese dioxide (**Scheme 12**)

Bioassay showed that compounds **50** have much better fungicidal activity than that of compounds **51** (**Table 3**). Compounds **50** have fair to excellent fungicidal activity against five fungi. However, compounds **51** have only poor fungicidal activity and the EC_{50} values of almost all compounds are greater than 30 μg/mL. It is worth notice that compound **50f** showed excellent fungicidal activity against *P. oryzae,* which is an important fungal pathogen causing serious damage to rice production in China.



28, 32 **48, 49** **50, 51**

28, **48** and **50**, Y=NH; **32**, **49** and **51**, Y=O

Scheme 12. Synthetic route of spiro-compounds containing macrolactam (macrolactone) and thiadiazoline rings (**50**, **51**)

X-ray diffraction analysis of two representative compounds (**50f**, R= p-ClC_6H_4; **51c**, R= o-BrC_6H_4) showed that their large ring skeleton can be described as [333133] and [33343] conformation respectively (CCDC 739298; Li *et al*, 2007). The conformation of the large ring skeleton of two compounds is somewhat different from each other. However, they should be similar in the solution due to the dynamic equilibrium (**Fig. 4**). Namely, in view of the conformation, compounds **50** and **51** have similar structural characteristics (**Fig. 5**). However, there are a hydrogen-bonding donor (-CONH-) and a hydrogen-bonding acceptor (N=N double bond of diazoline ring) on the large ring of compounds **50** and there are two hydrogen-bonding acceptor (-COO- and N=N double bond of diazoline ring) without hydrogen-bonding donor on the large ring of compounds **51**.

R	Compds No.	*B. cinerea*	*S. sclerotiorum*	*R. solani*	*P. asparagi*	*P. oryzae*
C_6H_5	**50a**	5.01	9.92	42.08	14.18	41.51
o-$CH_3C_6H_4$	**50b**	4.21	375.43	124.79	73.79	78.43
m-$CH_3C_6H_4$	**50c**	6.42	4.42	30.72	9.67	63.18
p-$CH_3C_6H_4$	**50d**	25.10	34.08	71.83	14.52	138.76
o-ClC_6H_4	**50e**	14.89	241.56	31.80	24.48	41.51
p-ClC_6H_4	**50f**	18.67	48.18	39.52	101.63	0.054
o-BrC_6H_4	**50g**	19.86	533.29	56.13	60.32	57.59
o-$CH_3OC_6H_4$	**50h**	21.85	21.09	37.89	517.04	397.84
p-$CH_3OC_6H_4$	**50i**	6.47	87.23	93.00	20.56	57.85
2,3-$(CH_3)_2C_6H_3$	**50j**	22.61	36.53	22.38	14.85	199.48
2,4-$(CH_3)_2C_6H_3$	**50k**	9.63	55.71	26.75	5.26	30.92
2,5-$(CH_3)_2C_6H_3$	**50l**	18.58	34.30	5.62	16.42	86.61
3,4-$Cl_2C_6H_3$	**50m**	34.23	15.03	36.82	9.02	0.72
2,5-$Cl_2C_6H_3$	**50n**	29.56	109.29	41.77	221.73	1.11
α-naphythyl	**50o**	15.73	767.24	17.80	46.15	195.80
benzyl	**50p**	14.44	8.32	23.49	21.33	116.85
o-ClC_6H_4	**51a**	43.81	169.20	159.06	116.49	211.36
p-ClC_6H_4	**51b**	49.95	59.91	126.32	42.48	338.37
o-BrC_6H_4	**51c**	36.42	204.57	207.69	53.55	246.10
p-BrC_6H_4	**51d**	49.88	38.30	17.10	29.21	338.37
2,5-$(CH_3)_2C_6H_3$	**51e**	126.29	162.84	421.80	208.41	3123.76

Table 4. Fungicidal activity of compounds **50** and **51** against five fungi (EC_{50}, μg/mL)

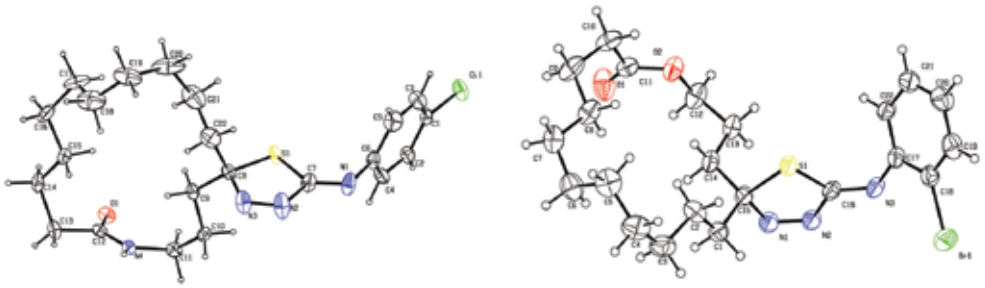


Fig. 5. The crystal structure of compound **50f** (left) and **51c** (right)

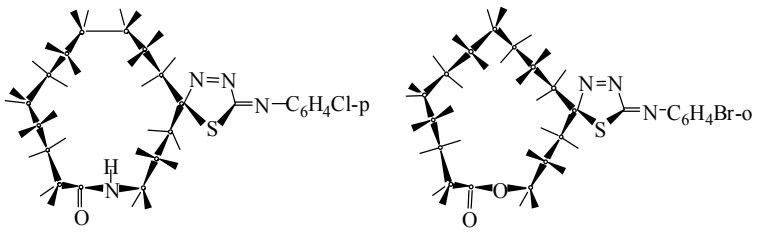
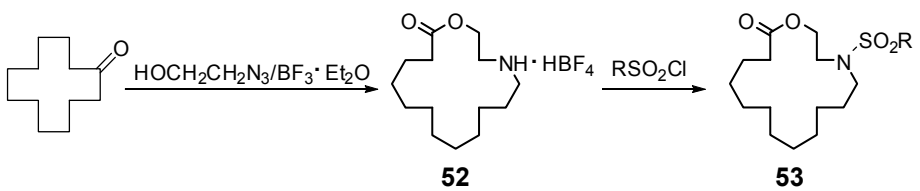


Fig. 6. The conformation of **50f** (left) and **51c** (right)

The results above showed that the presence of hydrogen-bonding donor to the fungicidal activity of macrocyclic compounds is very important and the rule on the relationship between the fungicidal activity and hydrogen-bonding has a general suitability to the macrocyclic compounds.

3.4 Synthesis and fungicidal activity of 16-oxo-1-oxa-4-azoniacyclohexadecan-4-ium tetrafluoroborate

Under the guidance of the rule on the relationship between the fungicidal activity and hydrogen-bonding, and following the principle of the simplicity of structure and the ease of synthesis, Dong *et al* (2008) designed and synthesized a novel azamacrolactone with a hydrogen donor (NH) and a hydrogen acceptor (C=O) on the ring and two methylene groups between these two polarizable groups, 16-oxo-1-oxa-4-azoniacyclohexadecan-4-ium tetrafluoroborate (**52**). And for further verifying the role of hydrogen donor, several sulfonyl-derivatives (**53**) of **52** with only one polarizable group (C=O) were also synthesized. Compound **52** was synthesized by intramolecular Schmidt reaction (Li, 2006) of cyclododecanone with 2-azidoethanol. And sulfonylation of **52** afford compounds **53** (**Scheme 13**).



Scheme 13. Synthetic route of 16-oxo-1-oxa-4-azoniacyclohexadecan-4-ium tetrafluoroborate (**52**) and its sulfonyl derivatives (**53**)

The fungicidal activity of compounds **52** and **53** against six fungus species in vitro was evaluated. The result showed that compound **52** has good fungicidal activity against these fungi, especially has excellent fungicidal activity against *R. solani* and is much better than compounds **53** which indicate that the presence of hydrogen-bonding donor to the fungicidal activity of macrocyclic compounds is indeed very important. In view of the ease of synthesis and the high fungicidal activity against *R. solani,* compound **52** will be expected to develop as a useful fungicide (see next section).

R	Compds No.	Inhibition rate (%)					
		Rhizoctonia solani Kuhn	*Fulvia fulva* (Cooke) Ciferri	*Colletotrichum orbiculare* (Berk. et Mont.) Arx	*Verticillium dahliae* Kleb.	*Sclerotinia sclerotiorum* (Lib.) de Bary	*Alterneria kikuchiarna* Tanaka
	52	96.0	89.3	73.2	86.9	85.7	85.7
C_6H_5	**53a**	0.0	63.6	56.3	61.9	61.3	66.6
p-MeC_6H_4	**53b**	0.0	50.1	46.4	50.7	39.5	52.5
o-Cl C_6H_4	**53c**	0.0	59.3	43.8	50.7	49.3	55.6
p-Cl C_6H_4	**53d**	0.0	45.0	0.0	44.5	39.5	55.6
o-$O_2NC_6H_4$	**53e**	0.0	45.0	43.8	34.6	36.0	52.5
m-$O_2NC_6H_4$	**53f**	0.0	45.0	35.4	50.7	46.2	46.2
p-$O_2NC_6H_4$	**53g**	7.5	45.0	29.6	44.5	39.5	55.6
p-(MeCO-NH)C_6H_4	**53h**	27.1	39.8	32.5	41.3	32.4	32.4

Table 5**.** Inhibition rate of the compounds **52** and **53** against six fungus species at 50 μg/mL

4. Development of the compounds with high fungicidal activity.

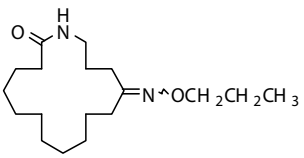
In this section, the development of two compounds (**30a** and **52**) with high fungicidal activity will be described.

4.1 The development of compound 30a

As described in section 3.1, 12-propoxyimino-1,15-pentadecanlactam (**30a**) (**Fig. 5**) exhibited a broad spectrum of fungicidal activity and especially excellent fungicidal activity against *A. kikuchiana*, *P. physaleos, R. solani* and *B. cinerea in vitro*. The EC_{50} values were 1.2, 1.9, 4.6 and 8.6 μg mL^{-1} respectively.

The diseases caused by *A. kikuchiana* and *P. physaleos* are not common in China. However, *R. solani* is an important agricultural fungus which causes serious decrease in yield especially in major cotton-growing regions in China (Chen *et al*, 1998; Gong *et al*, 2004; Deng *et al*, 2006), and grey mould caused by *B. cinerea* has become one of the most widely distributed diseases in vegetable (cucumber, tomato, calabash, eggplant, onion *etc*) and fruit (apple, grape, strawberry *etc*) growing regions and seriously affects crop production in China (Zhang *et al*, 2005; Xu *et al*, 2005; Chen *et al*, 2006; Wang *et al*, 2006).

In this section, the development of compound **30a** as a fungicide controlling cotton sheath blight (*R. solani*) and vegetable (cucumber and tomato) grey mould (*B. cinerea*) through pot culture and field efficacy trials, its dynamic distribution in cotton plants, and its toxicology was described (Huang *et al*, 2009. Original code name of **30a** was **7B3**)



30a

Fig. 7. Chemical structure of compound **30a**

4.1.1 Control effect of 30a against *R. solani*

Compound **30a** showed good control against *R. solani* on cucumber in the pot culture test (**Table 6**). The control effect of **30a** reached 53.5% at a rate of 315 g ai ha^{-1}, and was better than commercial fungicide thiram at a rate of 450 g ai ha^{-1} (42.1%). The control effect of **30a** reached 73.6% at a rate of 504 g ai ha^{-1} and is much better than thiram at a rate of 450 g ai ha^{-1}.

Treatment	Dosage, g ai ha^{-1}	Control effect (%)
30a 28% WP	168	39.0
30a 28% WP	315	53.5
30a 28% WP	504	73.6
thiram 50% WP	450	42.1

Table 6. Control effect of **30a** against *R. solani* on cucumber in pot culture test (Soil drenching)

In two-year two-place field efficacy trials, the control effect of **30a** against *R. solani* on cotton reached 64~92% at a rate of 140 g ai ha^{-1} and was better than or comparable to commercial fungicide carbendazin at the same rate (**Table 7**).

Treatment	Dosage		Control effect (%)	
	g, WP/1 kg seed	g, ai ha^{-1}	First year	Second year
Site: Jinan, Shandong Province, China				
30a 25% WP	4	93.3	88.9	65.3
30a 25% WP	6	140	91.5	74.7
carbendazin 50% WP	3	140	82.1	58.3
Site: Yuncheng, Shanxi Province, China				
30a 25% WP	4	93.3	69.2	61.9
30a 25% WP	6	140	72.7	64.4
carbendazin 50% WP	3	140	73.1	63.3

Table 7. Control effect of **30a** against *R. solani* on cotton in field efficacy trials (Seed dressing)

4.1.2 Control effect of 30a against *B. cinerea*

The control effect of **30a** against *B. cinerea* on tomato in the pot culture test reached 85.8% at a rate of 315 g ai ha^{-1} and 87.5% at a rate of 504 g ai ha^{-1} respectively, which is not as good as commercial fungicide huimeike [a mixed preparation of diethofencarb and chlorothalonil (1:1.8, w/w)], but is comparable to iprodione (**Table 8**). However, in a one-year one-place field efficacy trial (**Table 9**) **30a** displayed excellent fungicidal activity against *B. cinerea* on

cucumber. The control effect reached 95.1% at a rate of 450 g ai ha^{-1}, and was comparable to huimeike (control effect was 96.1% at a rate of 504 g ai ha^{-1}).

Treatment	Dosage, g ai ha^{-1}	Control effect (%)
30a 28% WP	168	80.7
30a 28% WP	315	85.8
30a 28% WP	504	87.5
huimeike 28% WP	315	96.6
Iprodione 50% WP	563	90.4

Table 8. Control effect of **30a** against *B. cinerea* on tomato in pot culture test (foliar spraying)

Treatment	Dosage, g, ai ha^{-1}	Control effect (%)
30a 25% WP	281	89.5
30a 25% WP	450	95.1
huimeike 28% WP	504	96.1

Table 9. Field efficacy trials of **30a** against *B. cinerea* on cucumber (Foliar spraying)

4.1.3 Dynamic distribution of 30a in cotton plant

The study showed that the concentration of **30a** reached 54.44 μg g^{-1} in roots of cotton plants 4 h after treatment with 500 μg mL^{-1} of **30a** (**Table 10**), which showed that **30a** can penetrate through the epidermis of the root and be absorbed by cotton plants. The content of **30a** in roots, stems and leaves all increased with extension of treatment time and the concentration of **30a** reached 137.05, 5.12 and 2.53 μg g^{-1} respectively, 48 h after treatment. However, the concentration of **30a** in roots was almost 27 and 54 times of the concentration in stems and leaves respectively, which revealed that **30a** has almost no acropetal translocation and systemic activity in cotton plant. The results provide a theoretical basis for an application method of **30a** for soil treatment or seed coating treatment.

Treatment Time (h)	Content of **30a** in the different parts of cotton plants (μg g^{-1})		
	root	stem	leave
4	54.44 ± 1.64	0.19 ± 12.30	0.04 ± 5.52
8	66.69 ± 2.81	0.87 ± 1.91	0.11 ± 2.52
16	96.99 ± 1.29	2.71 ± 2.08	0.33 ± 4.87
24	98.02 ± 2.08	4.27 ± 2.93	0.89 ± 2.48
36	116.25 ± 2.13	8.95 ± 6.84	2.93 ± 6.50
48	137.05 ± 4.39	5.12 ± 3.84	2.53 ± 5.80

Table 10. Distribution of **30a** in cotton plants when the chemical applied as root treatment

4.1.4 Toxicological test of 30a

The results of toxicological tests indicated that **30a** was a low toxicological compound （LD_{50} > 5000 mg kg^{-1} for acute oral and LD_{50} > 2000 mg kg^{-1} for acute dermal） based on classification standard procedure of People's Republic of China (People's Republic of China, 1995). The teratogenesis, mutagenesis and carcinogenesis tests were negative; therefore **30a** is safe for human being (**Table 11**).

Tested item	Results
Acute oral (Rats) (LD_{50} ,mg /kg^{-1})	> 5000
Acute dermal (Rats) (LD_{50} ,mg /kg^{-1})	> 2000
Eye irritation (Rabbits)	slight irritation
Skin irritation (Rabbits)	not irritation
Mutagenesis	negative
Teratogenesis	negative
Carcinogenesis	negative

Table 11. Toxicological profile of **30a**

4.1.5 Conclusion

30a has very weak systemic activity and is suitable for controlling cotton sheath blight with soil treatment or seed coating treatment. The field efficacy trials showed that the control effect of **30a** against *R. solani* on cotton is better or comparable to carbendazin and against *B. cinerea* on cucumber is comparable to huimeike. Therefore, **30a** may be expected to be further developed as a practical fungicide due to the high control effect, low toxicological properties and novel structure. However, the disadvantage is its long synthetic route from cyclododecanone (see **Scheme 6**).

4.2 The development of compound 52

As decribed in section 3.4, compound **52** (16-oxo-1-oxa-4-azoniacyclohexadecan-4-ium tetrafluoroborate) against six fungi (*R. solani* and so on) has good fungicidal activity, especially has excellent fungicidal activity against *R. solani*, an important agricultural fungus in China.

In this section, the development of compound **52** as a fungicide controlling cotton sheath blight (*R. solani*) through pot culture and field efficacy trials, and its toxicology was described (Dong *et al*, 2008. Original code name of **52** was **2a** or **D1**)

4.2.1 Control effect of 52 against *R. solani* on cotton

The results of potted test (**Table 12**) and field efficacy trials showed that compound **52** against *R. solani* on cotton was more active than commercial fungicide carbendazim.

Treatment	Dosage		Control effect (%)
	g, WP/1 kg seed	g, ai ha^{-1}	
52, 25% WP	2	47	81.8
52, 25% WP	3	70	87.2
Carbendazim, 50% WP	3	140	79.2

Table 12. Control effect of **52** against *R. solani* on cotton in pot culture test (seed dressing)

Treatment	Dosage		Control effect (%)	
	g, WP/1 kg seed	g, ai ha^{-1}	First year	Second year
52, 25% WP	4	93.3	55.8	48.9
52, 25% WP	6	140	61.9	63.6
carbendazin 50% WP	3	140	49.6	53.4

Table 13. Control effect of **52** against *R. solani* on cotton in field efficacy trials (seed dressing, site: Yuncheng, Shanxi Province, China)

4.2.2 Toxicological test of 52

The results of toxicological tests indicated that **52** was a low toxicological compound (LD_{50} > 3160 mg kg^{-1} for acute oral and LD_{50} > 2000 mg kg^{-1} for acute dermal)based on classification standard procedure of People's Republic of China (People's Republic of China, 1995). The mutagenesis, teratogenesis and carcinogenesis tests were negative; therefore **52** is safe for human being (**Table 14**).

Tested item	results
Acute oral (Rats) (LD_{50}, mg/kg^{-1})	> 3160 (male) > 3160 (female)
Acute dermal (Rats)(LD_{50}, mg/kg^{-1})	> 2000mg kg^{-1} (male) > 2000mg kg^{-1} (female)
Eye irritation (Rabbits)	slight irritation
Skin irritation (Rabbits)	not irritation
Mutagenesis	negative
Teratogenesis*	negative
Carcinogenesis*	negative

Table 14. Toxicological profile of **52**
*Unpublished results

4.2.3 Conclusion

The bioassay showed that compound **52** has excellent fungicidal activity against *R. solani* than commercial fungicide carbendazim. In addition, low toxicological property, short synthetic route and green synthetic technology with high atom economy (all of the atoms of raw materials including boron trifluoride were almost fully utilized except one nitrogen molecule and part of boron atoms lost, see section 3.4), indicated that compound **52** may be expected to further develop as a useful fungicide.

5. The biochemical mode of action of compounds 30a and 52 against *R. solani*

In this section, effects of compounds **30a** and **52** on ultrastructure of hyphae, cell membrane and respiration of mycelia cell suspension were described (Yan *et al*, 2010).

5.1 Effect of 30a and 52 on morphology and ultrastructure transformation of *R. solani*

Mycelia of *R. solani* grew smoothly along the surface of culture media without **30a** or **52**, and the shape of the whole colony appeared to be radiated from its central point. The fringe of

the colony was round and regular. However, the growth of mycelia was seriously depressed when it was cultured in the media with 50 μg mL^{-1} **30a** or **52**. The fringe of the colony was concavo-convex, irregular, and is not as smooth and regular as that of control mycelia.
SEM images indicated that the mycelial grew well in control media (Mu *et al*, 2006; Butler and Bracker, 1970) and it was of low density, fresh and had a fine structure, and most of the mycelia ramification occurred in right angle (**Fig. 8 A, D**). However, there were similar morphology changes in mycelia of *R. solani* when mycelia were cultured in culture media with **30a** or **52** of 50 μg mL^{-1}. Mycelia grew abnormally with comparatively high density of colony, the ramification was not in right angle any more, the distance between ramifications decreased and some mycelia were entangled each other (**Fig. 8 B, C**). In the presence of **30a** or **52**, the surface of mycelia was rough (**Fig. 8 B, E**) and mycelia were irregularly ramified and formed irregular ramification or abnormal configuration producing a "beaded" morphology with some parts of mycelia contracted and some parts swelled (**Fig. 8 C**).

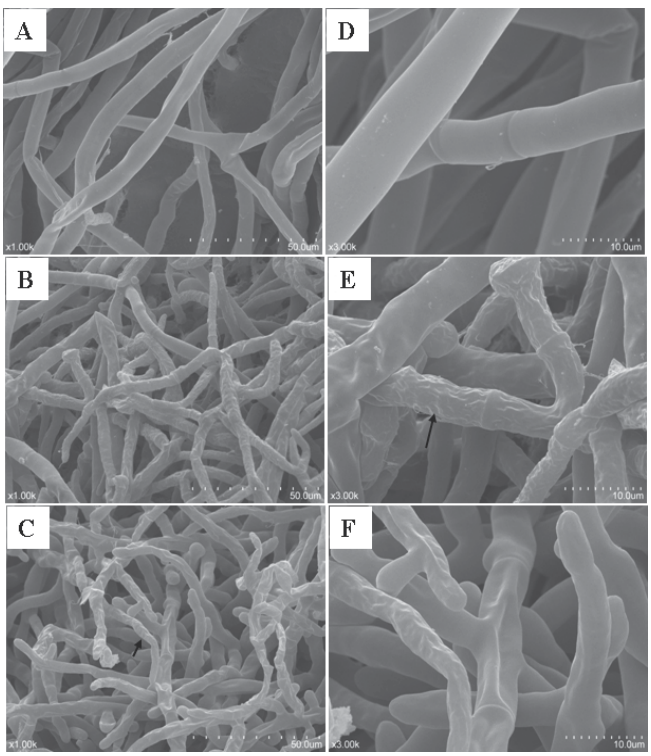


Fig. 8. Scanning electron micrographs of the hyphae from the colony of *R. solani* (A, D) 1000 (A) and 3000 (D) sections of *R. solani* hyphae grown on PDA medium in the absence of **30a** or **52** (control) (the mycelium was low density, fresh and had a fine structure); (B, E) 1000 (B) and 3000 (E) sections of *R. solani* hyphae grown on PDA medium containing 50 μg mL^{-1} **30a** (the mycelium was comparatively high density of colony and the surface of mycelium was rough (arrowhead)); (C, F) 1000 (C) and 3000 (F) sections of *R. solani* hyphae grown on PDA medium containing 50 μg mL^{-1} **52** (the amount of ramification increased and formed irregular ramification or abnormal configuration ("beaded" morphology) on the mycelium tip (arrowhead)). Bars: (A, B, C) 50.0 μm; (D, E, F) 10.0 μm

R. solani mycelial tips (5 mm) from the margin of actively growing colony on PDA medium were examined by TEM (**Fig. 9**). The cell walls and septa of the hyphae from the untreated control were uniform (**Fig. 9 B**, **C**). There were abundant organelles in cytoplasm such as vacuole (V), mitochondria (M) and lipid body (L) (**Fig. 9 A**). The dolipore septa and septal pore caps (SPCs) were obviously visible in control mycelia (**Fig. 9 D**). Following fungicides treatment, different ultrastructural modifications occurred in the hyphae (**Fig. 9 G**, **H** ultrastructure treated with **30a**; **Fig. 9 E**, **F**, **I**, **J** ultrastructure treated with **52**). The cell walls of the hyphae became considerably thicker following exposure to either **30a** or **52** (**Fig. 9 F**, **G**). The walls of the septa were also abnormally thickened (**Fig. 9 I**).

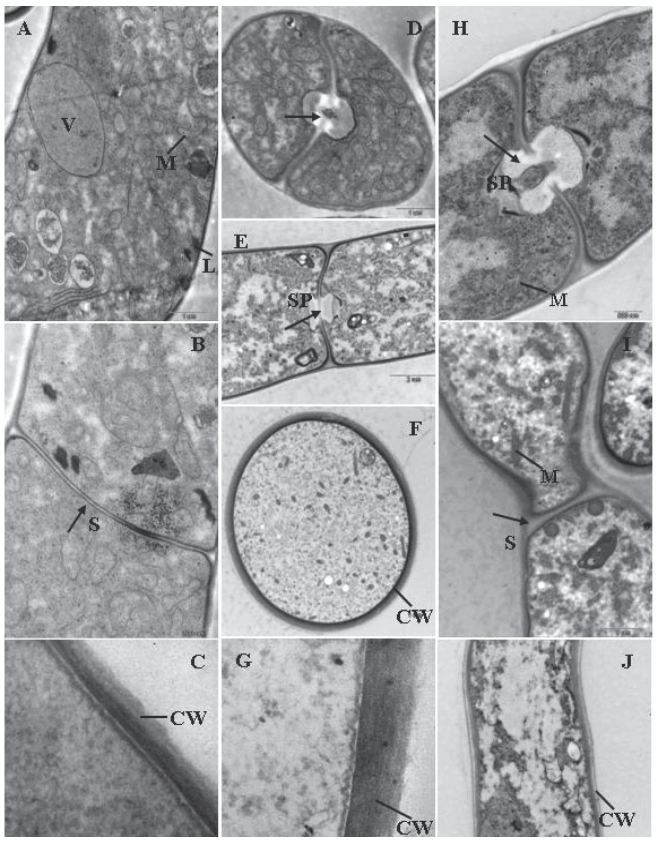


Fig. 9. Transmission electron micrographs of *R. solani* hyphae: (A, B, C, D) TEM of the hyphae of *R. solani* in the untreated control ((A) longitudinal of control hyphae; many organelles were observed such as vacuole (V), mitochondria (M) and lipid body (L); (B) uniform spectra (S) of control hyphae; (C) cell wall of control hyphae; (D) transverse of control hyphae and septal pore caps was visible); (E, F, I, J) TEM of the hyphae of *R. solani* treated with 50 µg mL^{-1} **52** ((E) longitudinal of **52**-treated hyphae (the septal pore caps disappeared); (F) transverse of **52**-treated hyphae (cell wall thickening); (I) longitudinal of **52**-treated hyphae (septum of hyphae thickening); (J) longitudinal of **52**-treated hyphae (cell wall thickening)); (G, H) TEM of the hyphae of *R. solani* treated with 50 µg mL^{-1} **30a** ((G) cell wall thickening of **30a**-treated hyphae; (H) longitudinal of **30a**-treated hyphae (the septal pore caps were almost unaffected)). Bars: (A, D, E, F, I, J) 1.0 µm; (B, H) 500 nm; (C, G) 100 nm.

Under the treatment of **30a** and **52**, the organelles became disorganized and decreased in the hyphae cytoplasm (**Fig. 9 F**, **G**). Another striking characteristics was the disappearance of septal pore caps of **52**-treated hyphae (**Fig. 9 E**), while the septal pore caps was almost not affected in **30a**-treated hyphae (**Fig. 9 H**).

SEM and TEM observations revealed that growth inhibition of *R. solani* as a response to **30a** and **52** was accompanied with marked morphological and cytological changes, including irregular ramification and a "beaded" morphology, excessive branching, irregular thickening of hyphae cell walls and necrosis or degeneration of hyphae cytoplasm. These changes were very similar to those occurring in some other fungi treated with chitosan and antibiotics, which inhibited fungi cell wall (Vesentini *et al*, 2007; Debono & Gordee, 1994). The cell wall of fungi is a sturdy structure providing physical protection and osmotic support, which is considered as that complex of macromolecules with chitin, glucan and mannose interconnected by covalent bonds. Hyphae growth, branching, cell fusion and other morphogenetic events all depended on a balance between decomposition and extension of the hyphae wall, as well as on synthesis and incorporation of new wall material (Wessels, 1993; Kang *et al*, 2001). In the present study, the hyphae walls of *R. solani* were thickened irregularly and the excessive branching of the hyphae, which were very similar to the phenomena induced by EBI (ergosterol biosynthesis inhibitor) fungicides (Kang *et al*, 2001) although there are great differences in chemical structure between **30a** or **52** and EBI fungicides. So it was also assumed that the thickness of cell wall and excessive branching might result from the changes of the activity of enzymes involved in wall synthesis. However, another phenomenon observed in TEM study was the increasing of thickness of septa and disappearance of septal pore caps of **52** treated hyphae. More studies should be done to interpret whether the phenomenon was in relation to cell wall associated enzymes.

5.2 The effect of compounds 30a and 52 on cell membrane of *R. solani*

The effect of **30a** and **52** on cell membrane was examined by measuring electrical conductivity of mycelia suspension. The conductivity of mycelia suspension treated with **30a**, **52** and triadimefon all increased extremely comparing with the conductivity of control mycelia along with all the time of treatment. **52** induced more significant electrolyte leakage from hyphae than **30a**, and similar change with triadimefon. Thus it was proposed that **30a** and **52** were all related to the impairment of cell membrane.

Electrolyte leakage was used as an indicator of cell membrane permeability of hyphae exposed to various fungicides. The alteration of conductivity induced by **30a** and **52** resembled with the alteration caused by triadimefon, one of a class of azole compounds which can inhibit ergosterol biosynthesis and damage the permeability of cell membrane (Yoshida *et al*, 1990). These results indicated that both **30a** and **52** caused damage to mycelia cell membrane system, induced electrolyte leakage from the cell, and as a result, the conductivity of solution was increased. Moreover, the presumption can also interpret the morphological and cytological alterations of the hyphae. Sterols are required for growth and reproduction of eukaryotic organisms and serve as architectural components of membranes. Thus, the thickening of hyphae walls was most likely to be associated with biochemical changes in the plasmalemma induced by **30a** and **52**. In addition, the phenomenon which **52** induced more significant grade in the morphological and cytological alterations and conductivity alteration than **30a** probably can be explained by the difference of solubility between **52** and **30a**, because **52** was a tetrafluoroborate possessing better solubility in water than **30a**.

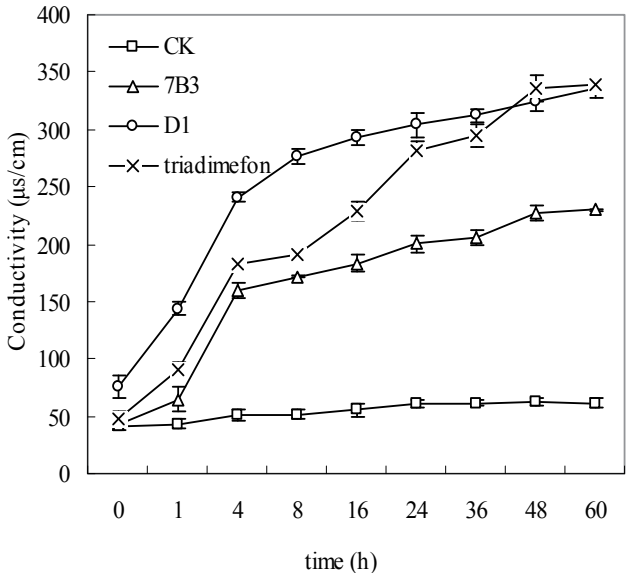


Fig. 10. Electrolyate leakage from *R. solani* suspensions during different time exposure to different fungicides. CK (□), **30a** (Δ), **52** (○), triadimefon (×). The conductivity of the solutions was measured using DDS-11C model conductivity detector at different treatment time after the addition of 1g fresh mycelia into 50 μg mL^{-1} **30a**, **52** and triadimefon solution, respectively.

5.3 Effect on respiration of intact mycelia

As shown in **Table 15**, both **30a** and **52** almost did not affect oxygen consumption of intact mycelia while azoxystrobin, a respiration inhibitor, exhibited the strong effect on oxygen consumption of intact mycelia. These results proved that either **30a** or **52** was not a respiration inhibitor and indicated that they did not disturb the energy generation system of *R. solani*.

Inhibitor	Concentration (μg mL^{-1})	R_0 (μmol O_2 g^{-1} min^{-1})	R_1 (μmol O_2 g^{-1} min^{-1})	Inhibition rate (%)
30a	10	27.03 ± 1.39	26.30 ± 0.85	3.39 ± 0.62
	100	27.03 ± 1.39	23.95 ± 7.49	12.05 ± 5.50
52	10	27.03 ± 1.39	26.77 ± 1.39	1.67 ± 1.02
	100	27.03 ± 1.39	26.41 ± 2.45	3.00 ± 1.80
azoxystrobin	10	27.03 ± 1.39	4.12 ± 1.80	84.86 ± 1.33

Table 15. Respiratory inhibition of intact mycelia of *R. solani* by **30a** and **52**

5.4 Conclusion

Both **30a** and **52** caused marked changes of hyphae with a "beaded" morphology, excessive branching, irregular thickening of hyphae cell walls and necrosis or degeneration of hyphae

cytoplasm and electrolyte leakage of membrane. In addition, neither **30a** nor **52** affected the respiration of mycelia. These results suggested that **30a** and **52** had the similar mode of action against *R. solani* relating to the impairment of biosynthesis of cell wall or membrane,

6. References

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CCDC 739298 contains supplementary crystallographic data for compound 50f. These data can be obtained free of charge from the CCDC, 12 Union Road, Cambridge CB2 1EZ,U.K. Telephone (44) 01223 762910; e-mail deposit@ccdc.cam.ac.uk

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25

Two-Component Signaling System in Filamentous Fungi and the Mode of Action of Dicarboximide and Phenylpyrrole Fungicides

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1. Introduction

Elucidating the mode of action of fungicides and the mechanism of fungicide resistance is a promising scientific approach to plant protection. However, the fungicidal mode of action or the mechanism of fungicide resistance has not been elucidated for all new fungicides. Furthermore, the mode of the action has not been fully elucidated for some fungicides that have long been on the market. Dicarboximide fungicides, one of the classes of fungicides dealt with in this paper, have also commercially available for a long time. Several theories have been proposed regarding their mode of action, but their bona fide fungicidal mechanism has not been fully elucidated.

Fungi, including phytopathogenic fungi, usually develop thalli and their cells are directly exposed to the environment. The cells inevitably experience several kinds of environmental stresses throughout their life cycles. These stresses include water activity (osmotic) stress and oxidative stress caused by the host response in a host-parasite interaction. To sense and respond to these stresses, fungi possess signal transduction systems and adaptation mechanisms. In the last 10 years, information obtained in the field of genome science on budding yeast (*Saccharomyces cerevisiae*, a leading model organism in fungal science) has enabled us to elucidate signal transduction systems and adaptation mechanisms in filamentous fungi, and further progress led to the clarification of the mode of action of dicarboximide and phenylpyrrole fungicides. These fungicides are closely involved in an osmotic signaling system in filamentous fungi. The fungicides now constitute an essential tool for studying this system. And as a result, this system has attracted great attention as a target of new antifungal agents. Furthermore, in some fungi, this system is involved in the pathogenicity of their hosts. In this paper, we introduce researches on the mode of action of these fungicides, which lead to the identification of the osmotic signaling system in pathogenic filamentous fungi, and related findings.

2. A short history of dicarboximide and phenylpyrrole fungicides

Dicarboximide fungicides were developed between the 1960s and the 1970s. They have strong antifungal properties with respect to many ascomycetes and related anamorphic fungi, including *Botrytis* spp., *Sclerotinia* spp., and *Bipolaris* spp. Owing to their prominent

fungicidal effect, dicarboximide fungicides have been widely used throughout the world, and the emergence of resistant fungi has been reported (q.v. Fungicide Resistance Action Committee, 2010). However, the mode of action and the resistance mechanisms of the dicarboximides have not been well understood. A biochemical study reported that dicarboximides had little effect on respiration or the biosynthesis of sterol, nucleic acids, proteins or chitin (Pappas & Fisher, 1979). However, the application of dicarboxyimides to fungal cells caused hyphal swelling and the bursting of tips (e.g. Eichhorn & Lorenz, 1978). The synthesis of cell walls was stimulated by dicarboximide treatment without any noticeable changes in their constituents (Hisada et al., 1978). The fungicides interfered with fungal membranes but had no effect on ion leakage or water permeability (Yoshida & Yukimoto, 1993). It was also reported that the dicarboximides induced membrane lipid peroxidation in some fungi by interfering with flavin-containing enzymes (Edlich et al., 1988). In addition, their fungicidal toxicity was reduced by piperonyl butoxide and α-tocopherol, which are a cytochrome P-450 inhibitor and an antioxidant, respectively (Leroux et al., 1992). Some dicarboximide resistant isolates of *Alternaria alternata* and *Botrytis cinerea* exhibited slightly enhanced catalase activity in the absence of the fungicides (Steel, 1996; Steel & Nair, 1995). These observations suggested that the mode of action of the fungicides might be related to reactive oxygen species, and the resistance to these fungicides might also be associated with a scavenging mechanism (Steel, 1996). On the other hand, *Neurospora crassa* mutants resistant to dicarboximides showed increased sensitivity to high osmolarity (Grindle, 1983; 1984). In *N. crassa*, at least six genes: *os-1*, *os-2*, *os-4*, *os-5*, *cut*, and *sor(T9)* were involved in the sensitivity to osmotic stress (Mays, 1969; Murayama & Ishikawa, 1973). Of the osmotic sensitive mutants, *os* mutants were resistant to dicarboximide and aromatic hydrocarbons, whereas *cut* and *sor(T9)* mutants were not (Grindle & Temple, 1982). That is, these studies have shown that fungicide resistance and hyper-osmosensitivity constitute the pleiotropic phenotypes of the same mutant gene. The explanations of the resistant mechanisms were incapable of proving these mutant traits.

Phenylpyrrole fungicides, another class of fungicides dealt with in this paper, were developed for agricultural use in the 1990s, with medical antibiotic pyrrolnitrin produced by *Pseudomonas* bacteria, as a lead compound. The compounds have as broad a fungicidal spectrum as the lead compound (Gehmann et al., 1990). The structure of phenylpyrrole fungicides was different from that of dicarboximide fungicides. In addition, there was a difference in the then proposed mode of action. However, in *B. cinerea*, most induced laboratory mutants that were resistant to dicarboximides also showed a cross resistance to phenylpyrroles. Moreover, the pleiotropy of fungicide resistance genes in *B. cinerea* and other plant pathogenic filamentous fungi including *Cochliobolus heterostrophus* was also recognized (Beever, 1983; Faretra & Pollastro, 1991; 1993; Gafur et al., 2001; Leroux et al., 1992; Matsuura & Tsuda, 1993; Yoshimi et al., 2003). Thus, these observations have led to the belief that both classes of the fungicides have the same mode of action, relating to the hyperosmotic adaptation of filamentous fungi. This was indispensable for elucidating the resistant mechanisms and thus understanding the osmotic signaling system in fungi based on a molecular approach.

3. Characteristics of the osmotic signaling system of filamentous fungi

Before discussing the osmotic signaling system of filamentous fungi, we briefly describe the modeled hyperosmotic adaptation mechanism in budding yeast. SLN1, a histidine kinase (HisK), is involved in sensing hyperosmotic stimulation. The transduced signal is

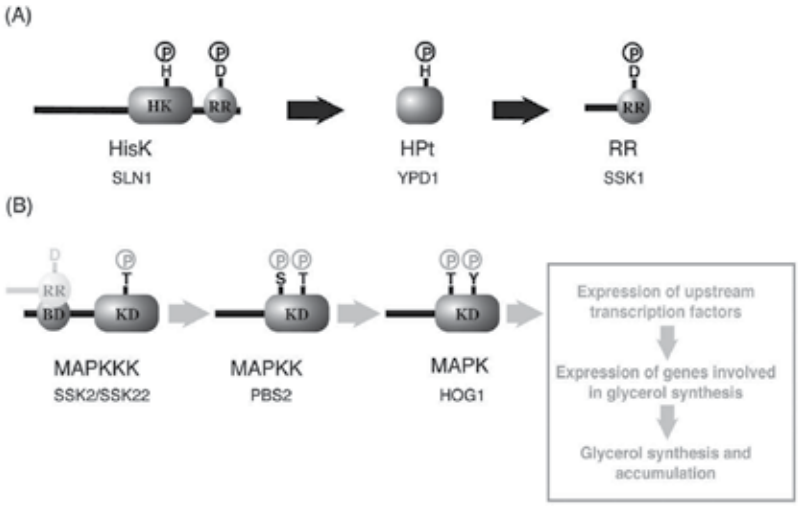

Fig. 1. Schematic of the osmotic signaling pathway of budding yeast
The osmotic signaling pathway consists of two types of signaling systems: (A) a two-component regulatory system and (B) a HOG1 MAPK signaling system.
The figure shows the steady state of the cell (under non-hyperosmotic conditions).
The light area in (B) shows the change in the HOG1 MAPK signaling system under hyperosmotic conditions.
HK: histidine kinase domain, RR: response regulator domain, BD: SSK1 binding domain, KD: kinase domain.

Genome	HisK	HPT	RR*
<Ascomycetes - Yeasts>			
Saccharomyces cerevisiae	1	1	2
Schizosaccharomyces pombe	3	1	2
<Ascomycetes - Filamentous Fungi>			
Neurospora crassa	11	1	2
Aspergillus nidulans	15	1	3
Cochliobolus heterostrophus	21	1	3
Botrytis cinerea	20	1	2
Magnaporthe grisea	10	1	2
Fusarium graminearum	16	1	2
<Basidiomycetes>			
Cryptococcus neoformans	7	1	2

Table1. Number of genes involved in the two-component regulatory system of fungi
* *Rim 15* ortholog is not included.

transmitted to the downstream HOG1 MAP kinase (MAPK) signal system (HOG pathway: Fig. 1B) through a histidine-containing phosphotransfer (HPt) protein YPD1 and a response regulator (RR) protein, SSK1, (a two-component regulatory system: Fig. 1A). Eventually, the activated HOG pathway induces intracellular glycerolgenesis and the cell adapts to the hyperosmotic environment. In this model, hyperosmotic stimulation is transmitted as follows.

When yeast cells are not exposed to hyperosmotic stress, histidine residues in the SLN1 histidine kinase domain present on the cell membrane are autophosphorylated and aspartyl residues in the SLN1 receiver domain are also phosphorylated. This phosphate is transferred to a YPD1 histidine residue and the SSK1 protein is phosphorylated by phosphorylated YPD1. When the cells are exposed to hyperosmotic stress, the phosphorylation of SLN1 is inhibited. Consequently, YPD1 and SSK1 are dephosphorylated. Dephosphorylated SSK1 combines with the SSK2/SSK22 proteins (MAPKK kinases in the HOG pathway), causing the autophosphorylation of these kinases. Phosphorylated SSK2/SSK22 phosphorylates the PBS2 protein (a downstream MAPK kinase), and the phosphorylated PBS2 protein phosphorylates the HOG1 MAPK. The phosphorylated HOG1 protein moves to the nucleus, inducing the gene expression of upstream transcription factors such as HOT1. Eventually, these regulatory factors activate the expression of genes involved in glycerol synthesis (q.v. Hohmann, 2002).

The hyperosmotic adaptation mechanism of filamentous fungi, including plant pathogenic filamentous fungi and human infectious fungi, is similar to but more complicated than that of budding yeast. While budding yeast has Sln1 as a sole HisK gene, filamentous fungi have several HisK genes per genome (Table 1). For example, fungal genome information elucidated eleven HisK genes in *N. crassa*, fifteen in *Aspergillus nidulans*; twenty-one in *C. heterostrophus*; twenty in *B. cinerea*; ten in *Magnaporthe grisea*; sixteen in *Fusarium graminearum*; and seven in *Cryptococcus neoformans.* The gene family in these organisms can be classified into at least ten groups according to the domain structure and the homology of the DNA sequences (Catlett et al., 2003). The ortholog of *Sln1*, which is essential for the hyperosmotic adaptation of budding yeast, is found in many ascomycetes, and is probably the ascomycete specific class of HisK. However, gene disruptants in *Cochliobolus heterostrophus* and *B. cinerea* do not show definite phenotypic changes, and their roles in these fungi are unknown (our unpublished data). In filamentous fungi, the HisK gene, which is classified as Group III, plays an important role in the osmotic stress response.

4. Group-III Hisk and its role in filamentous fungi

In 1997, the *os-1* gene of *N. crassa* was cloned, which bestows dicarboximide resistant and hyper-osmosensitive phenotypes. Sequence analysis revealed that this gene codes HisK, which is known as *nik-1* (Alex et al., 1996; Schumacher et al., 1997). Subsequently, the pleiotropic fungicide resistance genes in *B. cinerea* (*Daf1*; also called *BcOS1* or *Bos1*) and in *C. heterostrophus* (*Dic1*) were cloned (Cui et al., 2002; Oshima et al., 2002; Yoshimi et al., 2004). These two genes also code HisK and share a high homology with *os-1*. However, the structure of these HisKs is very different from that of SLN1 identified in budding yeast.

A genealogic study of the fungal Hisk genes, using fungal genome information, has led to the classification of *os-1*, *Daf1* and *Dic1* orthologs into a unique group (Group III) different from that of *Sln1* (Catlett et al., 2003), and has shown that the Group III HisK gene is generally present in Dikarya, regardless of the classification groups of fungi or their ecological status, e.g., human infectious *Candida albicans* (ascomycete), *C. neoformans*

(basidiomycete); phytopathogenic *M. grisea, Fusarium* spp. (ascomycetes), and *Ustilago maydis* (basidiomycete). The exceptions are budding yeast and fission yeast.

What are the structural characteristics of Group III HisK? SLN1 is believed to have two transmembrane domains at the *N* terminus and to be localized to the membrane. The localization and extracellular region of SLN1 is believed to be essential for sensing extracellular osmotic changes (Ostrander & Gorman, 1999; Reiser et al., 2003). However, a Group III HisK lacks transmembrane domains. A study using a GFP-fusion HisK protein has shown that a Group III HisK is cytoplasmic (Viaud et al., 2006). A domain consisting of around 90 amino acid residues is repeated five to seven times at the *N* terminus of a Group III HisK (Fig. 2). This domain is often found in proteins involved in the signaling system of prokaryotes, and the proteins are called HAMP (**H**istidine kinase, **A**denyl cyclase, **M**ethyl accepting chemotaxis protein, **P**hosphatase). Studies have shown that the HAMP domain is important in relation to the role of the sensor kinase, and is involved in intermolecular interactions (Pollard et al., 2009; Swain & Falke, 2007; Tao et al., 2002). In *N. crassa*, *B. cinerea* and *C. heterostrophus*, the deletions of the HAMP domain or amino acid-substituted mutations resulted in hyper-osmosensitivity and fungicide resistance, clearly indicating that this domain is essential for the function of Group III HisK (Cui et al., 2002; Ochiai et al., 2001; Yoshimi et al., 2004). Futhermore, intramolecular interactions of the HAMP domains of Group III HisK was demonstrated using two hybrid assays (Meena et al., 2010). However, the role and function of the HAMP domains of Group III HisK in molecular interactions is not fully elucidated, and further clarification at the molecular level is still required.

What are the functional differences between Group III HisKs and SLN1 in budding yeast? As mentioned at the beginning of the paper, when budding yeast cells are exposed to hyperosmotic stress, HOG1 MAPK is phosphorylated and glycerol accumulates in the cells. Glycerol accumulation as a result of hyperosmotic stimulus is also observed in filamentous fungal cells, e.g. *N. crassa* and *C. heterostrophus* (Fujimura et al., 2000; Tanaka et al., 2006). Moreover, fungicide treatment also induces glycerol accumulation in these filamentous fungal cells. In filamentous phytopathogenic fungi, i.e. *Colletotrichum lagenarium*, *C. heterostrophus* and *B. cinerea*, phenylpyrrole, fungicide treatments abnormally induce the phosphorylation of the HOG1 MAPKs of those fungi (Kojima et al., 2004). However, in mutants lacking the Group III HisKs of those filamentous fungi, the phosphorylation of HOG1 MAPK and glycerol accumulation due to exposure to hyperosmotic stress or the fungicides are not observed (Fujimura et al., 2000; Yoshimi et al., 2005). That is, in filamentous fungi, a Group III HisK functions as a responsible upstream factor in an osmotic signaling system, to sense and transduce osmotic signals, in the same way as SLN1 in budding yeast. In addition, budding yeast is innately insensitive to dicarboximide and phenylpyrrole fungicides. However, the introduction of *Hik1*, a Group III HisK gene of *M. grisea*, results in phenotypic alterations: transformants are no longer tolerant to the fungicide and the phosphorylation of HOG1 MAPK occurs due to the fungicide treatment (Motoyama et al., 2005b). These results indicate that Group III HisK is an essential factor that regulates the phosphorylation of HOG1 MAPK in response to both hyper-osmolarity and the fungicides. They also suggest that Group III HisK itself, or the phospho-relaying interactions of Group III HisK in intramolecules or intermolecules with other factors, e.g. HPt and RRs, are the targets of the fungicides. The fungicides mimic hyperosmotic stress that forces fungal cells to induce physiological adaptation via Group III HisK as if the cells were in a hyperosmotic condition, although they are not exposed to hyperosmotic stress.

Until now, the fungicide categorized as an aromatic hydrocarbon, the aforementioned antibiotic pyrrolnitrin, and bacteria-derived polyketide antibiotic ambruticin were considered to have similar modes of action to dicaroboximides and phenylpyrroles (Motoyama et al., 2005b; Okada et al., 2005; Vetcher et al., 2007).

Budding yeast possesses a sole HisK, SLN1 and its mutant exhibits lethality (Posas et al., 1996). The reason for this is believed to be that in mutants defective in *Sln1*, the HOG1 MAPK is constitutionally activated, and improper physiological changes are induced. However, in filamentous fungi, mutants defective in Group III HisKs do not exhibit lethality. This difference is probably not due to the type of HisK involved or the difference in the genes controlled by the downstream HOG pathway, but to the difference between a single and a multiple HisK-based two-component regulatory system. It is very interesting and important to know how several HisKs interact with and regulate HPt and RRs in the signaling systems, because this will enable us to understand the modes of action of dicarboximide and phenylpyrrole fungicides at the molecular level.

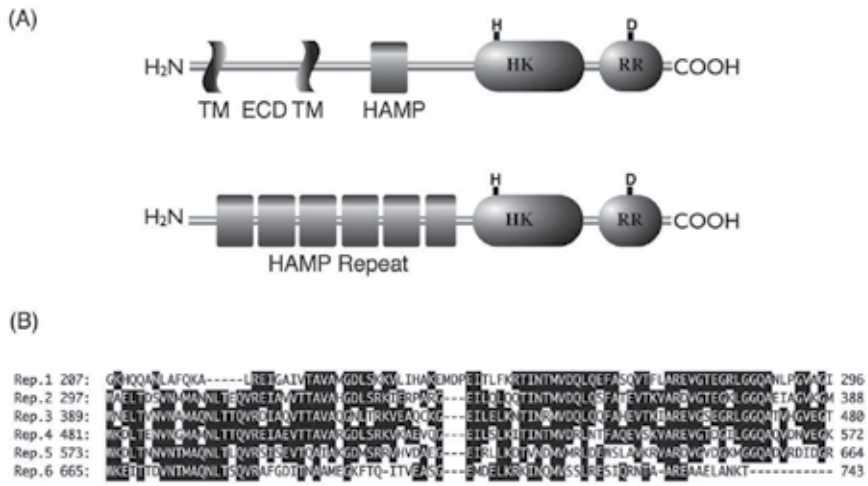

Fig. 2. (A) Schematic of the structure of a SLN1 histidine kinase and a Group III histidine kinase, (B) an example of the HAMP sequence (*Cochliobolus heterostrophus* Dic1). HAMP of Group III HisK is repeated five times in ascomycetous yeasts such as *Candida*, six times in filamentous Ascomycetes and seven times in Basidiomycetes.
TM: transmembrane domain, ECD: extracellular region of SLN1, HMAP: see the main text

5. Other HisKs involved in the osmotic signaling system

It is known that in some fungi, such as *C. neoformans*, HisKs other than Group III HisKs are involved in the hyperosmotic stress response. Mutants deficient in the *Tco1* (also called *CnNIK1*) gene coding a Group III HisK in this fungus show strong phenylpyrrole resistance but do not become hyper-osmosensitive. Tco2, a HisK belonging to a class specific to this fungus, is believed to control the hyperosmotic stress response of this fungus (Bahn et al., 2006). However, the *Tco2* gene disruptants show weak phenylpyrrole resistance, and therefore Tco1 and Tco2 are believed to have overlapping functions associated with the HOG pathway. In *C. heterostrophus* and *B. cinerea*, Group III HisKs control the hyperosmotic

stress response, regardless of the type of osmolytes (Yoshimi et al., 2003; Izumitsu et al., 2007). However, *Hik1* gene disruptants in *M. grisea* are highly sensitive to hyperosmotic stress with sorbitol, but not with certain solutes (KCl, NaCl and glycerol). In *M. grisea*, the involvement of HisK(s) other than Hik1 or an unknown hyperosmotic adaptation mechanism for these solutes has been suggested (Motoyama et al., 2005a).

6. RR involved in the osmotic signaling system and the mode of action of the fungicides

In *Neurospora crassa*, mutants of *os-1*, a Group III HisK gene, and those of *os-2*, a HOG1 MAPK gene, exhibit almost the same hyperosmotic sensitivity and fungicide resistance (Fujimura et al., 2000). However, HOG1 MAPK gene disruptants in *C. heterostrophus* and *B. cinerea* do not show as strong a hyperosmotic sensitivity and fungicide resistance as the Group III HisK gene (Fig. 3; Izumitsu et al., 2010). This suggests that in these plant pathogenic filamentous fungi, a signal stream from Group III HisK is divergent in down streams. There is the possibility of signal branching at each step of the two-component regulatory system and the HOG1 MAPK signaling pathway. In this work, signal divergence is shown to be the result of the involvement of the two RRs (Table 1) composing the two-component regulatory system in the hyperosmotic stress response.

The RR protein is a key element in the two-component signaling system. It governs output responses via its phosphorylation level, which is under the control of an upstream regulator HisK (West & Stock, 2001). Two conserved RR proteins homologous to Ssk1 and Skn7 in *S. cerevisiae* have been identified in several fungal species (Catlett et al., 2003). However, the specific responses that these proteins govern have only been characterized in certain yeast species, not in filamentous fungi (Bahn et al., 2006; Cottarel 1997; Krems et al., 1996; Nakamichi et al., 2003; Posas et al., 1996; Singh et al., 2004). The role of the response regulators in the filamentous fungus was first characterized in a study using *C. heterostrophus* RR disruptant (Izumitsu et al., 2007).

Ssk1 mutants of *C. heterostrophus* showed increased sensitivity to hyperosmotic stress and moderate dicarboximide and phenylpyrrole resistance, implying that *Ssk1* plays a role in osmotic adaptation and fungicide sensitivity. Although the role of Ssk1 in a two-component signaling system and a high-osmolarity glycerol pathway has been well characterized in yeast, only one functional analysis has been conducted on the Ssk1 homologue in filamentous fungi. In that report, an *Aspergillus nidulans ssk1* (*sskA*) mutant showed an osmosensitive phenotype and a deficiency of HOG1 MAPK phosphorylation (Furukawa et al., 2005). The data from the study using *C. heterostrophus* suggested that the Ssk1-type response regulator plays roles in high-osmolarity adaptation and in the mode of action of the fungicides. The results obtained with *C. heterostrophus* also indicate that the other response regulator, Skn7, plays a role in the osmotic adaptation and moderate resistance of Ssk1 and Skn7 RRs are involved in high-osmolarity adaptation and fungicide sensitivity. However, the two proteins show different mechanistic functions in the response pathway. The disruption of the *Ssk1* gene prevents the phosphorylation of HOG1 MAPK in both the high-osmolarity stress and the fungicide treatments, whereas the *Skn7* mutation does not affect the phosphorylation of HOG1 MAPK (Fig. 4). Various morphological observations of the *Ssk1* and *Skn7* mutants compared with wild-type cells after the application of the fungicides also indicated a difference in function between the two RRs (Fig. 5). The wild type develops heavily swollen hyphae with inflated cells, and hyphal growth is strongly

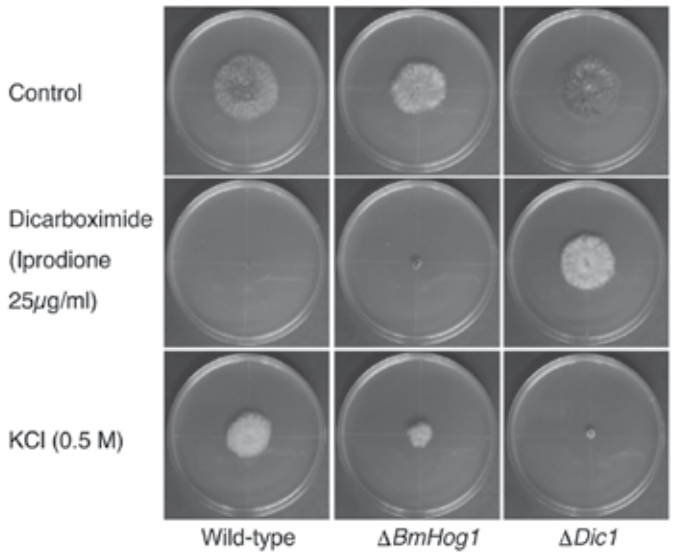

Fig. 3. Resistance to the dicarboximide fungicide, iprodione, and sensitivity to hyperosmotic condition with KCl in the wild-type, HOG1-MAPK (*BmHog1*) disruptant and Group III HisK (*Dic1*) disruptant of *Cochliobolus heterostrophus*

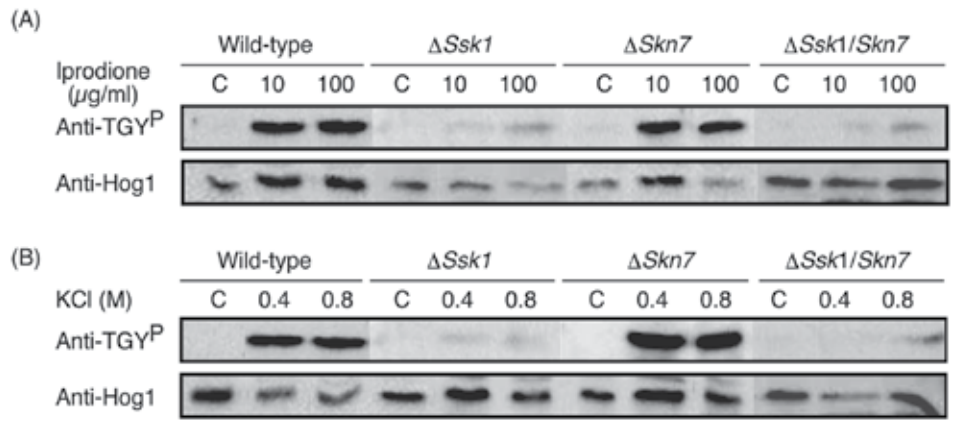

Fig. 4. HOG1-MAPK phosphorylation in the *Cochliobolus heterostrophus* wild-type strain, the *Ssk1* mutant strain, the *Skn7* mutant strain, and the *Ssk1*/*Skn7* double-mutant strain induced by the fungicide and osmotic stress. (A) Prepared mycelia of the strain tested were incubated in CM medium with or without 10 and 100 μg/ml iprodione for 10 min. Phosphorylated BmHog1 was detected using anti-dually phosphorylated p38 antibody (indicated by Anti-TGY^P). The total amount of BmHog1 was measured using anti-Hog1 C-terminus antibody (indicated by Anti-Hog1). (B) Prepared mycelia of the strain tested were incubated in CM medium with or without 0.4 and 0.8 M KCl for 10 min

inhibited by the fungicides, whereas both of the mutants showed partially restricted growth of hyphae, indicating incomplete fungicidal activity. In addition, the *Skn7* mutant develops swollen hyphae and inflated cells similar to those of the wild type, and the *Ssk1* mutant does not. Applications of dicarboximide and phenylpyrroles to *N. crassa* and *C. heterostrophus* mycelia cause abnormal accumulations of cellular glycerol, resulting in cell inflation and hyphal swelling (Tanaka et al., 2006; Zhang et al., 2002). These results suggest that only Ssk1 controls HOG1 MAPK-phosphorylation, which, under osmotic and fungicide stress conditions, seems to result in the accumulation of cellular glycerol. Moreover, Skn7 appears to play other roles in high-osmolarity adaptation and fungicide sensitivity that are independent of the activation of HOG1 MAPK.

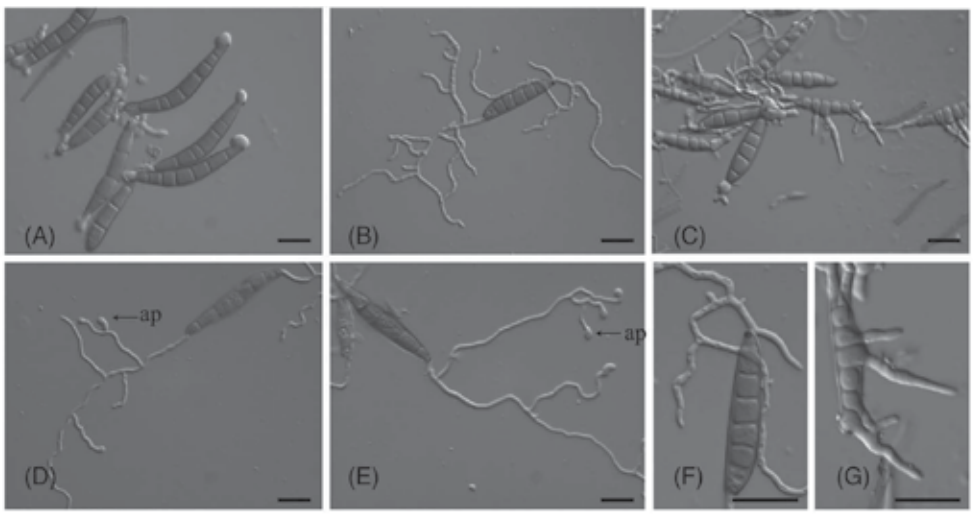

Fig. 5. Effect of dicarboximide treatment on the *Cochliobolus heterostrophus* wild-type strain, the *Ssk1* mutant strain, the *Skn7* mutant strain, and the *Ssk1/Skn7* double-mutant strain. (A) Wild-type strain incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (B), (E), (F) *Ssk1* mutant incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (C), (G) *Skn7* mutant incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (D) *Ssk1/Skn7* double-mutant strain incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (E) Untreated wild-type strain. No differences were observed between the untreated wild-type strain and the untreated mutant strains (data not shown). ap = appressorium. Scale bars = 50 µm

The phenotypes of the *Ssk1*, *Skn7*, and *Dic1* (Group III HisK) mutants are comparable but not identical. As mentioned above, Dic1 is the HisK responsible for osmotic adaptation and fungicide sensitivity in this fungus (Yoshimi et al., 2004; Yoshimi et al., 2005). All the phenotypic characteristics of the *Ssk1* and *Skn7* mutants are also observed in the Group III HisK mutants. In contrast to the *Ssk1* and *Skn7* single mutants, the *Ssk1/Skn7* double-mutant cells clearly show higher resistance to the fungicides than either single-mutant strain alone. Furthermore, the double-mutant strains are much more sensitive to the osmotic stress than the single-mutant strains. The dose–response of the *Ssk1/Skn7* double mutant to high osmolarity and fungicide exposure parallels that of the Group III HisK mutant. The above data clearly indicate that there are two osmotic signaling pathways in this fungus: a Group

III HisK => SSK1 => HOG pathway and a Group III HisK => SKN7 pathway. These two pathways are believed to contribute to hyperosmotic adaptation and the onset of fungicidal action equally and additively.

The discovery of the involvement of two osmotic signaling pathways in the down streams of Group III HisK provide a new insight into the mode of action of dicarboximide and phenylpyrrole fungicides. As mentioned in the above section, Group III HisK is probably a primary target of these fungicides or a core mediator of their fungicidal action, and the main mode of action of these fungicides is the abnormal phosphorylation of the HOG1 MAPK controlled by Group III HisK and consequent improper gene expression. Our results strongly indicate that improper signal mediation by the "Group III HisK => Skn7" pathway, which will cause the abnormal expression of the genes needed for hyper-osmotic adaptation, along with the "Group III HisK => SSK1 => HOG" pathway, is also required for the full activity of these fungicides. Moreover, with *B. cinerea*, the improper activation of "Group III HisK => SKN7" alone is sufficient to arrest colonial growth (Izumitsu et al., 2010). These facts implied that not only the primary target but also the genes abnormally expressed under the controls of each pathway are also important if we are to understand the mode of action of the fungicide. The identification of the genes that provide the greatest fungicidal activity will promote the development of new fungicides.

Today, the involvement of two osmotic signaling pathways in the down streams of Group III HisK is widely recognized in several filamentous fungi, e.g. *A. nidulans*, *M. grisea* (Aguirr et al., 2008; Hagiwara et al., 2007). However, in *M. grisea,* the Group III HisK => SSK1 => HOG pathway plays a major role in the hyperosmotic adaptation and the Group III HisK => SKN7 pathway plays a minor role, owing to the difference between the phenotypes of the two pathways (Motoyama et al., 2008). Fig. 6 shows a generalized osmotic signaling system in filamentous fungi.

7. Interesting characteristics regulated by the osmotic signaling system

The above discussion focused on cellular adaptation to osmotic stress and fungicidal action. However, some studies have shown that the osmotic signaling system of filamentous fungi is involved in various other important functions.

In *B. cinerea*, a Group III HisK gene disruptant forms very few conidia (Viaud et al., 2006). This characteristic can also be found in a downstream HOG1 MAPK gene disruptant (Segmüller et al., 2007). In these two disruptants, sclerotia are formed in very large quantities. In *B. cinerea,* a sclerotium is recognized as a survival organ for overwintering, and is prerequisite for sexual reproduction (Faretra et al., 1987). That is, it is suggested that in this fungus, the osmotic signaling system serves as a switch between asexual and sexual reproduction modes. In addition to this role in morphogenesis, the osmotic signaling system of *B. cinerea* plays a critical role in plant infection. Both the Group III HisK and HOG1 MAPK disruptants are largely non-pathogenic to host plants (Izumitsu et al., 2010; Segmüller et al., 2007; Viaud et al., 2006). The fact that in *B. cinerea*, the mutations of the fungicide resistance genes interferes with its pathogenicity, and morphogenesis may provide a scientific basis for understanding the well-known fact that there is a loss of fitness associated with dicarboximide resistance, resulting in a decrease in the frequency of resistant strains when dicarboximides are no longer applied (q.v. Fungicide Resistance Action Committee, 2010). In addition to this fungus, genes involved in the osmotic signaling system: *Tco1* and *Skn7* in *C. neoformans* (Bahn et al., 2006), *CaNik1* (also called as *Cos1*) and *Ssk1* in *C. albicans* (Calera et al., 2000; Yamada-Okabe et al.,

1999), *Ssk1* in *M. grisea* (Motoyama et al., 2008), and *AbNik1* in *Alternaria brassicicola* (Cho et al., 2009), are known to confer pathogenicity.

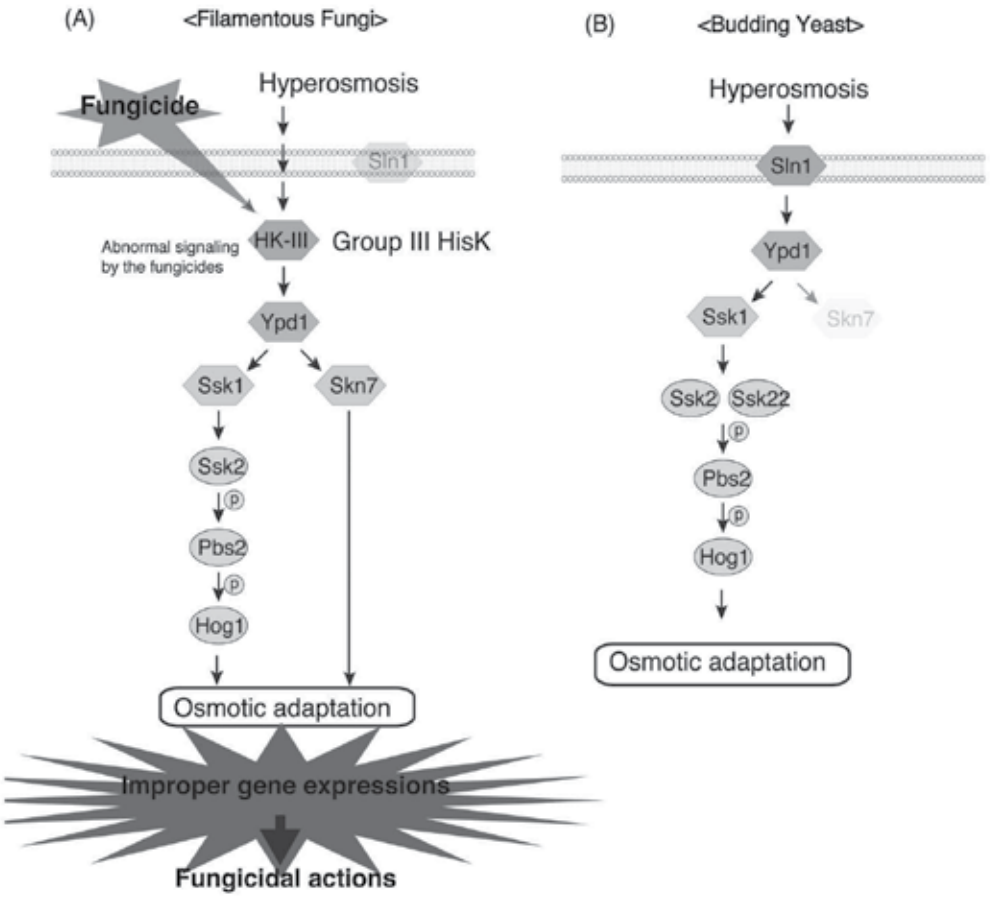

Fig. 6. Model illustration of the osmotic signaling systems of filamentous fungi (A) and budding yeast (B).

8. Concluding remarks

The osmotic signaling system of filamentous fungi consists of a two-component regulatory system and a HOG1 MAPK signaling system, as does the budding yeast used in the modeling of the osmotic signaling system. This system seems to be evolutionarily conserved among fungi, and yet has diverse functions and roles. This diversity is believed to be the result of the evolution of the environmental adaptability and survival strategies of each fungal species. The progress and accumulation of work on fungal genome science is promoting functional studies on the species without genomic sequence data. In addition, comparative studies using many species will lead to an understanding of the diverse roles of the signaling system. This knowledge is expected to be used to provide basic information that will aid the development of both new fungal control methods and new fungicides.

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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