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Herbicides

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Edited by Andrew J. Price and Jessica A. Kelton

HERBICIDES - ADVANCES IN RESEARCH

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Meet the editors

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Contents

Preface

Since the commercial production of herbicides began, herbicides have become an integral part of agriculture. Increased herbicide use has contributed to increased food and feed production, reduced farm labor requirements, improved control of invasive weed species, and a number of other positive benefits for the world's population. As herbicide use continues, research is required to improve upon existing herbicide formulations and application methods, to develop additional uses of herbicide compounds, and to further understand the mechanisms by which herbicides function in order to ensure the future benefits of herbicides.

However, with this increase in chemical inputs into production systems, many concerns have been raised in regards to the detrimental impact of these products to non-target species, including humans, and to the environment. In this respect, research has been necessary to reduce environmental degradation, identify potential contamination risks from herbicide use, and to ensure the protection of human health and wellbeing.

Needless to say, herbicide research is a critical component of both agricultural and environmental science. With continued research efforts in both fields, effective herbicides with reduced impact, rapid contamination detection methods, and unique applications of herbicidal compounds will, undoubtedly, continue to be developed.

In this book, contributing authors have provided a broad scope of topics related to recent herbicide research. Research detailed in these chapters is particularly focused on herbicides in agricultural settings or their impact on the surrounding environment. Topics range from herbicide mode of action investigation, herbicide degradation research, and bio-herbicide development to novel concepts for herbicide contamination detection and even the potential for application of herbicide compounds in the medical field.

The information provided in this book serves as a valuable tool for describing many areas of current herbicide research affecting both agricultural use and environmental impact. **Herbicides - Advances in Research** should be particularly useful to beginning and established scientists with interests in continuing to develop research projects focused on understanding herbicide functions, environmental behavior, and new applications of herbicidal compounds. It is hoped that this book will serve the scientific community as a source of current, vital research information to help shape future research and understanding of herbicides.

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Chapter 1

Agrochemicals and Bacterial Diversity in Cultivated Tropical Soils

Joseph Addo Ampofo

Additional information is available at the end of the chapter

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1. Introduction

1.1. General

Agrochemicals provide yield protection, prevent and cure crop disease, provide insulation to reduce energy use and provide countless other benefits that increase the standard of living for civilization as a whole. While the chemicals industry has made good progress reducing its overall environmental footprint, chemicals can also create a negative impact on human health and the environment when their production and use are not managed responsibly (Edwards, 1975; Tu, 1990; Zulalian, 1990).

1.2. Agriculture in Ghana

Urban food needs is increasing in developing countries, including Ghana (Figure 1), with growing populations. Increasingly vegetables are grown in urban and peri-urban areas to meet this demand. Agriculture has been Ghana's most important economic sector over the years, employing more than 60% of the national work force, mainly in the small landholders on formal and informal basis (Gerken *et al*, 2001) and accounting for about 40% of the total GDP and export earnings. Ghana has climatic zones that range from dry savanna to wet forest and run in east-west bands across the country.

Agriculture crops including yams, grains, cocoa, oil palms, kola nuts and timber, form the base of Ghana's economy. Compared to this, the contribution of the more traditional vegetables such as okra, pepper, tomato, onion and egg plants to the agricultural GDP is low. However, considering their contribution to agricultural GDP and the land area devoted to the cultivation of all crops, the traditional vegetables produce more value per area. For example, the share in total area cultivated and value for vegetables (tomato, okra, pepper and

eggplant) is 3.0 and 7.0%, respectively, while for cereals (maize, millet, sorghum and rice), these values are 35.0 and 8.0%, respectively (Table 1).

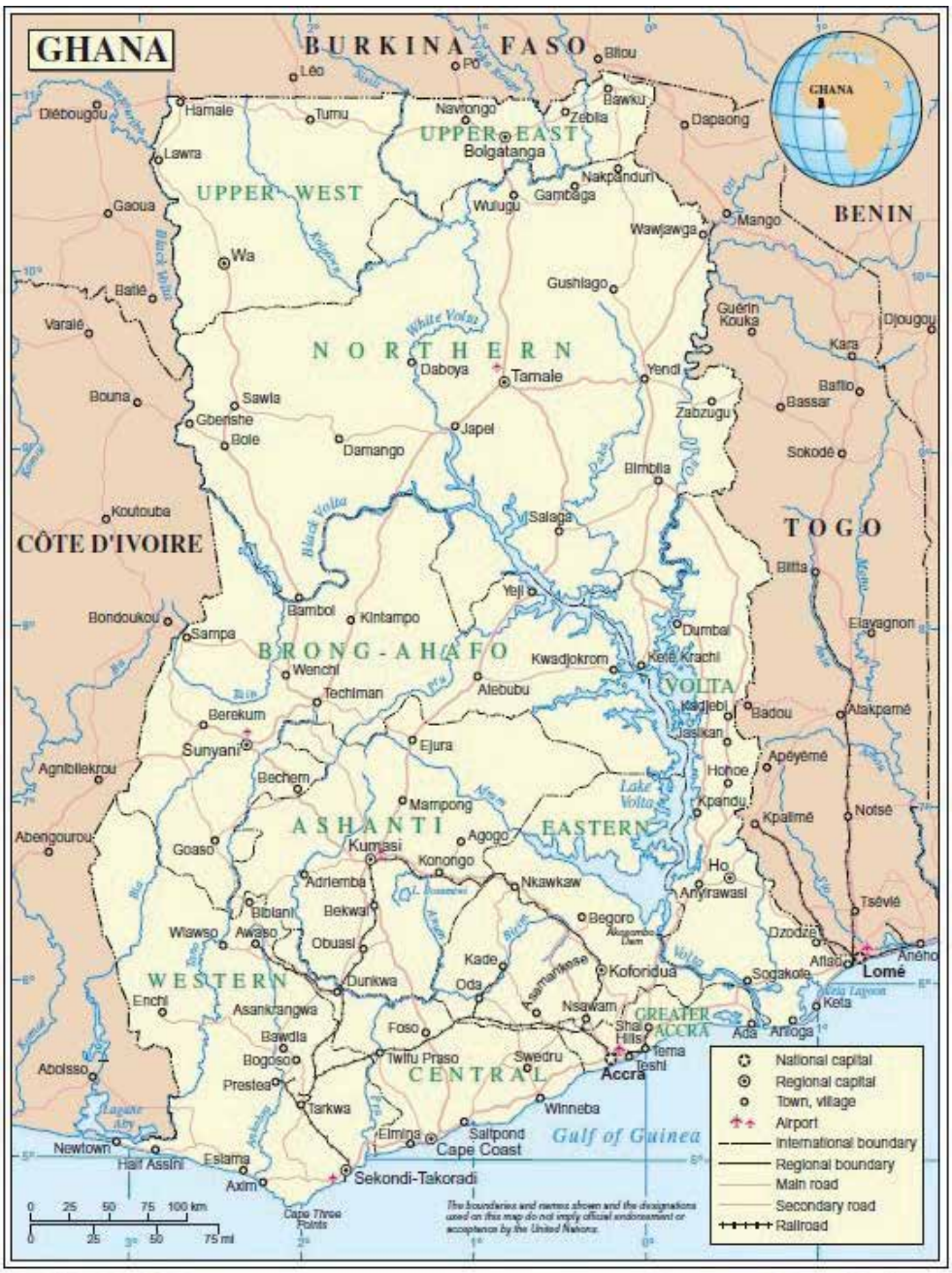

Figure 1. Map of Ghana, its boundaries and Lake Volta. Also shown are some major rivers.

Crops	Share in value (%)	Share in area (%)	Rate of growth (%)	
			Area	Yield
Maize (*Zea mays*)	4.80	17.0	2.00	3.00
Rice (*Oryza glaberrina*)	1.00	2.23	8.00	2.00
Millet (*Pennisetum glaucum*)	1.30	7.27	2.00	-
Sorghum (*Sorghum bicolor*)	1.24	8.41	2.00	1.50
Cassava (*Manihot esculenta*)	19.2	12.1	2.00	3.00
Cocoyam (*Xanthosoma roseum*)	10.2	6.08	2.00	-
Yam (*Dioscorea* spp.)	16.7	6.32	2.36	0.75
Plantain (*Musa* spp.)	13.0	5.32	2.00	-
Groundnut (*Arachis hypogaea*)	3.83	4.66	2.36	3.00
Tomato (*Solanum lycopersicum*)	2.79	0.61	1.00	2.00
Okra (*Hibiscus esculentus*)	3.16	0.85	3.00	2.00
Pepper (*Capsicum annuum*)	0.14	1.71	3.00	2.00
Eggplant (*Solanum melongena*)	0.56	0.07	3.00	2.00
Beans (*Phaseolus vulgaris*)	15.3	5.12	1.00	2.00
Cocoa (*Theobroma cacao*)	0.92	18.8	5.00	2.00
Oil Palm (*Elaeis guineensis*)	0.07	1.58	2.00	2.00
Rubber (*Ficus elastic*)	0.06	0.06	4.00	2.00
Coffee (*Coffea Arabica*)	0.16	0.02	4.00	2.00
Cotton (*Gossypium hirsutum*)	0.12	0.32	8.00	2.00
Tobacco (*Nicotiana tabacum*)	3.47	0.06	8.00	2.00
Orange (*Citrus sinensis*)	0.09	0.55	3.00	2.00
Pineapple (*Ananas comusus*)	0.09	0.06	3.00	2.00
Total crops/growth rates	98.2%	99.2%	3.31%	1.58%

Source: Nurah, 1999

Table 1. Growth rates and contributions of different crops to agricultural GDP

1.3. Application of agrochemicals in agriculture

Vegetable production in Ghana typically occurs in intensely managed, irrigated smallholder farms with relatively high pesticide inputs. Surveyed vegetable crops (tomato, pepper,

okra, eggplants (or garden eggs) and onion) cover approximately 0.4% of the cultured land of Ghana, equating to 58,270 ha in 1998 (Gerken *et al*., 2001). An estimated average pesticide rate of 0.08 liters ai/ha is applied to these vegetables. Relatively lower quantities are applied to cereals (0.03 liters ai/ha1) and higher quantities to cocoa (0.5 liters ai/ha). The compounds applied in vegetable production include organochlorine and organophosphate insecticides. In contrast to the traditional organochlorines, organophosphates are not highly persistent, but some can be highly toxic to some aquatic organisms (Castillo *et al*., 2006). Intensively managed vegetable farms are also characterized by an extensive network of drainage systems where surplus water may flow into local streams and rivers. Consequently, the aquatic ecosystems located downstream of vegetable farmlands might be vulnerable due to intensive pesticide use, drainage systems, and high precipitation rates typical for tropical areas where vegetable production occurs.

1.4. Non-point source agricultural pollution

Non-point source agricultural pollution is regarded as the greatest threat to the quality of surface waters in rural areas. One of the most important routes leading to non-point source agricultural pollution of surface waters in rural areas is runoff. Runoff from agricultural fields introduces pesticides, soil, organic matter, manure and fertilizer into small streams, increasing the volume of stream discharge and changing water quality (Neumann and Dudgeon, 2002). The impacts of such runoff are well documented (Cooper, 1993; Castillo *et al*., 1997; DeLorenzo *et al*., 2001).

Traditional vegetable farming systems (i.e. without any chemical input) are incapable of meeting demand. For instance, pests and diseases, which pose big problems in vegetable production, require intensive pest management to control them. Chemical pesticide use is a common practice to control pests and diseases in vegetable cultivation. However, besides their beneficial effects, pesticides are accepted as having potential environmental and public health impacts as well. If improperly used, pesticides can cause direct human poisoning, accumulate as residues in food and the environment or lead to the development of resistant strains of pests. These problems can arise from misuse of the pesticides or over-reliance on them, particularly if the users are not aware of these potential problems.

A total of 43 pesticides are in use in vegetable farming in Ghana (Table 2). This figure was obtained as a direct summation of pesticides applied on farms, but it could be lower than the actual number of pesticides in use. The pesticides comprised insecticides, fungicides and herbicides. Herbicides (44%) were the class of pesticides most used in vegetable farming in the areas surveyed, followed by insecticides (33%) and fungicides (23%).

In Table 2 the classification of these pesticides by the type of pests they control, active ingredient, chemical group and World Health Organization (WHO) Hazard Category is presented. The herbicides and fungicides used are mostly under WHO Hazard Category III, with a few under Hazard Category II. All the insecticides used are under Hazard Category II, which WHO classifies as moderately hazardous. This category includes organochlorines (OCs), organophosphates (OPs) and pyrethroids. Endosulfan was the only OC mentioned in use in the survey.

Pesticide type (% of total number in use)	Active Ingredient	Chemical Group	Hazard Category (WHO)	Registered for use on
Herbicide (44%)	Pendimethalin	Dinitroaniline	III	Tomatoes, onions
	2, 4-D	Aryloxyalkanoic acide	II	Rice, sugarcane
	Propanil	Anilide	III	Rice
	MCPA-Thioethy1	Aryloxyalkanoic acide	III	Not registered
	Oxadiazon	Oxadiazole	III	Not registered
	Oxyfluorfen	Diphenyl ether	III	Not registered
	Bensulfuron-methy1	Sulfonylurea	III	Rice
	Glyphosate	Glycine derivative	III	Various crops
	Paraquat dichloride	Bipyridylium	II	Various crops
	Acifluorfen	Diphenyl ether	III	Not registered
	Metolachlor	Chloroacetamide	III	Not registered
	Phenmedipham	Carbamate	III	Not registered
Fungicide (23%)	Mancozeb	Carbamate	III	Mangoes, vegetables
	Matalaxyl-M	Acylalanine	II	Not registered
	Thiophanate-methyl	Benzimidazole	III	Various crops
	Carbendazim	Benzimidazole	III	Not registered
	Benomyl	Benzimidazole	III	Not registered
Insecticide (33%)	Lambda-cyhalothrin	Pyrethroid	II	Vegetables
	Chlorpyrifos	Organophosphorus	II	Citrus, public health
	Endosulan	Organochlorine	II	Cotton
	Dimethoate	Organophosphorus	II	Not registered
	Cypermethrin	Pyrethroid	II	Not registered
	Deltamethrin	Pyrethroid	II	Various crops

Table 2. Types of Pesticides applied in vegetable production in Ghana

Technical endosulfan, a mixture of two stereoisomers, that is, α- and β-endosulfan in the approximate ratio of 7:3 (Shetty *et al.*, 2000; Kennedy *et al.*, 2001), is a chlorinated pesticide for control of a large spectrum of insect pests on a wide range of crops (Aguilera-del Real *et al.*, 1997). It is used in many countries throughout the world for the control of pests on fruits, vegetables, tea, tobacco, and cotton (Antonious and Byers, 1997; Sethunathan *et al.*, 2002). Because of such abundant usage, and the potential for accumulation in the environment (endosulfan is not readily detoxified by soil microorganisms), residues are detectable in soils, sediments, and crops at harvest time (Goebel *et al.*, 1982; U.S. Department of Health and Human Services, 1990). Although the metabolites of endosulfan, that is., sulfate, diol, ether,

hydroxy ether, and lactone, have been shown to occur (Maier-Bode, 1968; Schuphan *et al.*, 1968), only the sulfate metabolite is significant as a residue (Antonious and Byers, 1997).

Although the impacts are complex and often unknown or sometimes open to debate, some negative effects are well documented, such as chemicals found in the environment that are persistent, bioaccumulative and or toxic (e.g. PCBs, dioxins) (Waiwright, 1978; Moorman, 1989). Concern has been raised about chemicals which interfere with the normal function of hormonal systems of human and animals (i.e. endocrine disrupters), and substances which impact on children's health (De Reuck, *et al*, 1979).

1.5. Activity of selected agrochemicals

Glyphosate, the main component of Ceresate, is a non-selective, non-residual herbicide used against annual or biennial herbaceous monocotyledons, herbaceous dicotyledonous and perennial weeds. It is absorbed by foliage and transported through plant and is very effective on many deep-roots perennial species. It is metabolized or broken down by some plants, while other plants do not break it down. It is not usually absorbed from the soil by plants (Rueppel *et* al, 1977; McEwen & Stephenson, 1979; Eberbach & Douglas, 1983). Glyphosate remains unchanged in the soil for varying lengths of time, depending on soil texture and organic matter content. The half-life of glyphosate can range from 3 to 130 days.

Dimethoate (Cerox) is an organophosphorus insecticide with a contact and systemic action. Dimethoxon, an oxygen analogue metabolite of dimethoate, appears to play a dominant role in its toxicity for insects and mammals (Bohn, 1964; Koppel etal, 1986). Hydrolytic degradation is the main inactivating pathway of dimethoate in the environment. The half-life of dimethoate in different plants is between 2 and 5 days. Degradation in soil is dependent on the type of soil, temperature, moisture, and pH level.

Paraquat is a selective herbicide used to control most annual grasses and certain broadleaf weeds in field corn, potatoes, rice, cotton, soybeans, tobacco, peanuts and sunflowers. It is used in both pre-emergence and early post-emergence weed control (McErtenson, 1992). Paraquat is not subject to microbial degradation. Slight losses of paraquat can result from photodecomposition and volatilization. Its soil half-life is 90 days.

1.6. Microorganisms in soil

Microorganisms present in soil include Actinomycetes, Fungi, Algae, Bacteria and Protozoa. Most organisms are found in the top layers of soil, usually the top 2-3 centimeters, since this is typically where most of the organic matter is located (Alexander, 1979). The organisms are usually concentrated close to root surfaces in the rhizosphere, within living and dead roots, on soil particles, or among aggregates of soil particles. The rhizosphere is the region of the soil that is immediately adjacent to and affected by plant roots. It is a dynamic region where interaction takes place between plants, soil, microorganisms, nutrients and water.

Microorganisms play a major role in the breakdown of pesticides in the soil. Many microbes are capable of utilizing pesticides as sources of carbon and most pesticides studied are attacked

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at one or more sites by microorganism e.g. the bacteria *Hydrogenomones* can degrade DDT completely to carbon dioxide (McEwen & Stephenson, 1979).

Edwards (1975) lists possible effects on living organisms in soil contaminated with insecticides to include (i) direct toxic effect to microbial life in the soil, (ii) affecting organisms genetically to produce populations resistant to pesticides, (iii) sub-lethal effects resulting in alterations in behavior or changes in metabolic or reproductive activities, and, (iv) absorption into the bodies of soil fauna and passing on to other organisms.

The study determined the effects of some selected agrochemicals on bacterial population in the soil, and investigated the effect of agrochemicals on plant growth.

2. Materials and methods

2.1. The agrochemicals used

The agrochemicals used were obtained from the open market. Each agrochemical was new and sealed in bottles of one litre volume. The products included (i) Cerox, an insecticide containing dimethoate 400g / L; (ii) Ceresate, a herbicide containing glyphosate IPA 4% w/w SL; and, (iii) Paraquat, a herbicide with composition, paraquat DCL 24% w/w SL.

2.2. Selection of viable seeds for planting

Undamaged bambara groundnut (*Vigna subterranea*) seeds of similar sizes were surface-sterilized by immersing for 5 minutes in 0.1% mercuric chloride (HgCl) solution and washed in six changes of sterile distilled water. The seeds were next washed in 70% ethanol for 3 minutes, and rinsed twice with sterile distilled water. The sterile seeds were placed on water agar (0.1% agar) in large Petri dishes and incubated at room temperature for 5 days. The vigorously germinating seeds were selected for planting.

2.3. Planting of seeds in experimental soil

Five seeds were sowed in each pot, and the seedlings were thinned to one after they had survived. There were four replicates for each soil treated type. The plants received full sunlight up to mid-day each day and were protected from rains. They were watered daily with 20ml tap water per pot. Once a week, each pot received, in addition, 10ml Sachs' solution to augment the nutrient content in the soil.

2.4. Application of agrochemicals

On the second week of planting, the agrochemicals were applied to the soil with the seedlings. Pots labelled A served as control, no agrochemical was applied. Pots labelled B were sprayed with Cerox. Pots labelled C were sprayed with Ceresate, and Pots labeled D were sprayed with Paraquat. Spraying was done using a spray bottle. The agrochemicals were diluted with sterile distilled water according to the manufacturer's recommendation as follows:

Ceresate:	5 ml:300 ml of distilled water
Cerox:	1.8 ml:300 ml of distilled water
Paraquat:	1.2 ml :300 ml of distilled water

2.5. Assessment of extent of growth of experimental plants

The following records were made of the bambara groundnut plants: number of leaves, leaf length and leaf broadness. These measurements were taken once a week, for four weeks with the use of a ruler.

2.6. Assessment of nodulation

After six weeks, the plants were harvested and the roots thoroughly washed. The nodules were detached and counted.

2.7. Enumeration of total heterotrophic bacterial populations in soils

Soil samples were taken one day after planting of the seedlings and another set of samples on the third week after planting, from each of the pots to determine the bacterial population present in each soil treatment type. The soil samples were taken from between 2 - 8cm away from the stem of each seedling. One gram of each soil sample was dissolved in 9ml of sterile saline water and thoroughly mixed. Serial dilutions were made of each solution and 1ml plated on Nutrient Agar supplemented with yeast extract. All plates were incubated at 37^0C for a maximum of 48hrs.

2.8. Enumeration of *Rhizobium* sp. populations in each soil

Congo red Yeast-extract Mannitol Agar (YMA) (Hann, 1966) was inoculated with 1g of each soil treatment type. Incubation was at 30^0C for 5 days.

2.9. Determination of bacterial diversity in the soils

The Phene Plate (PhP) System which deals with Finger Printing of Bacteria in Microplates (Kuhn *et* al, 1991; Kuhn & Mollby, 1993) was used in the determination.

The bacteria to be tested were first pre-cultivated on appropriate agar media such as Blood agar, Brain Heart Infusion agar, Brilliant Green agar, Cereus selective agar, Deoxycholate Citrate agar, Eosin Methylene Blue agar, KF Streptococcus agar, MacConkey agar, Nutrient agar, Standard Plate Count agar, SS agar, Staphylococcus Medium, Triple Sugar Iron. The same pre-cultivation conditions were used for all strains in the test series.

A multichannel pipette with sterile tips was used to fill all wells in the PhP plate with suspending substrate. Aliquots of 0.320 - 0.375ml of the substrate were dispensed into all eight wells of 'Column 1' in the plate, and 0.150ml into all the other wells. All wells in 'Column 1' were inoculated with eight different types of bacteria colonies. The plates were left for at least one hour, after which the bacterial suspensions in the first column were homogenized with

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the aid of the multichannel pipette. Quantities of 25μl of the bacterial suspensions in the first column were then transferred to all the other wells in each row with the multichannel pipette. Colonies suspected to be anaerobic were covered with sterile paraffin oil. Each plate was covered by a sterile lid and put in a wet chamber to avoid drying. The plates were incubated at 37^0C. The colour of each well was assessed after 16, 40 and 64 hours of incubation. An optical microplate reader connected to a computer with the PhP software was used. Three readings were made, after 16, 40 and 64 hours, respectively. The absorbance was measured at 620nm.

2.10. Statistical analysis

The Statgraphics Plus for Windows version 4.0 was employed to test for significant differences between the various means of parameters of the differently treated soils and those of the untreated soil.

3. Results and discussion

3.1. Enumeration of population of rhizobia in the differently treated soil types

Total viable count studies using Congo red YMA produced the *Rhizobium* sp. population numbers indicated in Table 3. There were high population numbers per gram of soil in the cerox-treated soil and the non-treated soil, respectively. Ceresate- and paraquat-treated soils had very low population numbers per gram of soil. There was no statistically significant difference between the means of the population numbers in the ceresate-treated and paraquat-treated soils. There was, however, statistically significant difference in the means of the population numbers in the non-treated soil, cerox-treated soils and those of the ceresate-treated and paraquat-treated soils.

Treatment Type Soil	Mean number of *Rhizobium*spp. Population (g^{-1}soil) x 10^4
Cerox-treated soil	138 (± 12.11)
Ceresate-treated soil	20 (± 4.11)
Paraquat-treated soil	12 (± 3.55)
Non-treated soil	180 (± 9.99)

Table 3. Rhizobium spp. population numbers in soils with the different treatments.

3.2. Assessment of extent of growth of experimental plants

Plants were assessed four weeks after germination. On the basis of plant growth and the extent of nodulation recorded in Table 4, the plants could be described as follows:

- Non-treated soil:- plants grew well with deep green foliage, had highest mean leaf number and mean leaf length. They also formed the highest number of nodules and were the largest.

- Cerox-treated soil:- plants showed moderate growth and nodulation.
- Ceresate- and paraquat-treated soils:- plants showed stunted growth and yellowish-green foliage and formed the smallest number of mean number of nodules per plant, 3 – 10 nodules, as compared to 30 – 44 mean nodules per plant of the non-treated soil and the cerox-treated soil.

There was no statistically significant difference between the means of leaf length of plants cultured in cerox-treated soil and those of plants in paraquat-treated soil. There was also no significant difference between means of leaves of plants in cerox-treated soil and those in the non-treated soil.

There was no statistically significant difference between the mean leaf numbers of plants cultured in cerox- and ceresate-treated soils. There was no significant difference between the mean leaf sizes of plants cultured in cerox-treated soil, paraquat-treated soil and the non-treated soil. There was also no significant difference between the mean leaf sizes of plants cultured in the cerox-treated soil and the ceresate-treated soil.

There was no significant difference between the mean number of nodules of plants cultured in ceresate- and paraquat-treated soils.

Treatment Type	Mean leaf length (cm)	Mean leaf Number	Mean leaf size (cm)	Mean number of nodules
Cerox-treated	6.13 (±0.79)	11 (±2.94)	2.29 (±0.55)	30 (±5.16)
Ceresate-treated	3.36 (±0.86)	10 (±1.15)	1.57 (±0.28)	3 (±2.83)
Paraquat-treated	5.45 (±0.55)	6 (±2.16)	2.06 (±0.39)	8 (±5.45)
Non-treated soil	6.36 (0.40)	15 (±2.50)	2.37 (±0.24)	44 (±5.72)

Table 4. Growth and nodulation of the bambara groundnuts raised in the differently treated soils.

3.3. Total heterotrophic bacteria counts in soils after chemical application

The results in Table 5 show that all the differently treated soil types had total viable bacteria present. The mean number of viable heterotrophic bacteria recorded for the soil samples varied from 40 x 10^4 cfu g^{-1} to 61 x 10^5 cfu g^{-1}. Paraquat-treated soil recorded the least number of heterotrophic bacteria followed by Ceresate and the Cresox. The non-treated soil recorded the highest mean viable heterotrophic bacteria count. There was statistically significant difference between the means of the 4 variables at the 95.0% confidence level with the treatment types. There was, however, no significant difference between the various means before treatment, treatment after day 1, and treatment after 3 weeks at the 95.0% confidence level.

Treatment Type	Value Before Treatment	Value after Treatment 1 Day	Value After Treatment 3 weeks
Cerox	60×10^5 (±90.65)	18×10^5 (±258.20)	10×10^5 (±182.57)
Ceresate	40×10^5 (±75.28)	73×10^4 (±29.44)	70×10^4 (±52.28)
Paraquat	56×10^5 (±45.09)	41×10^4 (±54.77)	40×10^4 (±65.83)
Non-treated	52×10^5 (±19.90)	54×10^5 (±496.66)	61×10^5 (±258.20)

Table 5. Mean values of total heterotrophic bacteria count surviving after treatment with Agrochemicals.

3.4. Analysis of the diversity indices of the bacterial flora in the soils

The diversity indices of the bacterial flora were high (more than 0.90) for both the non-treated soil and the cerox-treated soil (Tables 6a and 6b). However, the diversity indices of the bacterial flora for the ceresate- and paraquat-treated soils had values of less than 0.90 (Table 6c and 6d). A high Di (maximum value is +1) means that the assayed isolates were evenly distributed into different types, whereas low Di (minimum value is 0) means that one or few types of bacteria dominated the studied population (Kuhn *et al*, 1991; Kuhn & Mollby, 1993; Ampofo & Clerk, 2003).

3.5. Similarities between the bacterial populations in the differently treated soil

The PhP software used (Kuhn & Mollby, 1993) also calculated the population similarity coefficients (Sp) between the different treatments. Sp coefficients were performed according to the unweighted-pair group method using the average linkages method. High Sp coefficients (<0.5) means that the two compared samples shared many identical genera. Low Sp coefficients (>0.5) means different bacterial populations (Sneat & Sokal, 1973). The mean similarities are presented in Table 7. Comparison between the differently treated soil types, i.e. cerox-treated soil, ceresate-treated soil, and paraquat-treated soil showed Sp values all below 0.50, an indication of related populations with high diversity indices of bacteria. Comparison between the populations of bacteria from the non-treated soil and the agrochemically treated soils, however, showed Sp values greater than 0.5, an indication that the related populations were of low diversity indices Kuhn *et* al, 1991; Kuhn & Mollby 1993; Ampofo & Clerk, 2003; Gabrielson *et* al, 2003).

This study has confirmed detrimental effect of insecticide on bacterial populations in the soil. Total heterotrophic counts, rhizobial counts as well as the number of nodules of all samples taken from the chemically treated soils were all low as compared to values obtained for the untreated soil. However the effect of the insecticide was minimal in all cases as compared to the effects of the herbicides on the soil fauna.

Sample Name and No.		No. of isolates	Di value
Non-treated soil	1	24	0.992
	2	24	0.908
	3	24	0.974
	4	24	0.962
Mean Diversity			**0.959**
		a)	
Sample Name and No.		**No. of isolates**	**Di value**
Non-treated soil	1	24	0.989
	2	24	0.978
	3	24	0.987
	4	24	0.962
Mean Diversity			**0.980**
		b)	
Sample Name and No.		**No. of isolates**	**Di value**
Non-treated soil	1	24	0.898
	2	24	0.862
	3	24	0.855
	4	24	0.915
Mean Diversity			**0.880**
		c)	
Sample Name and No.		**No. of isolates**	**Di value**
Non-treated soil	1	24	0.842
	2	24	0.814
	3	24	0.882
	4	24	0.880
Mean Diversity			**0.850**
		d)	

Table 6. a) Diversity among bacterial flora in the non-treated soil,b) Diversity among bacterial flora in the cerox-treated soil, c) Diversity among bacterial flora in the ceresate-treated soil, d) Diversity among bacterial flora in the paraquat-treated soil.

Parameter	Population of	Compared to	Sp value
Soil treatment type	Cerox	Ceresate	0.35
	Cerox	Paraquat	0.49
	Ceresate	Paraquat	0.13
No treatment	Non-treated	Cerox	0.51
	Non-treated	Ceresate	0.55
	Non-treated	Paraquat	0.52

Table 7. Similarities between the bacterial populations for the different soil treatment types.

Chemicals exert number of different toxic effects on a bacterial cell. It is difficult or even impossible to deduce the toxic mechanism of a specific chemical by just looking at its molecular structure, although chemicals with similar structures and/or physico-chemical properties are expected to have similar modes of action. Several studies have been done on the Quantitative Structure-Activity Relationships (QARs), but still knowledge is scarce. There are general rules though, such as lipophilic chemicals being more prone to disturb the bacterial membrane than hydrophilic chemicals, and electrophilic chemicals often forming irreversible covalent bonds to their target site at nucleophilc entities in biological molecules, such as proteins and DNA. A chemical may have multiple modes of toxic action and at low concentration it may even be used as a nutrient.

The effect of glyphosate on soil microbes has been studied by several authors because glyphosate, unlike most other herbicides, kills the plant by blocking a biochemical pathway which is also essential for most of the bacteria and fungi. It is known (Eberbach & Douglas, 1983) that glyphosate blocks certain biochemical pathways that are essential for growth of bacteria and the low number in bacteria population (10×10^5) as compared to the population in the non-treated soil (61×10^5) is evident enough to support this. In similar experiments conducted in Australia by the CSIRO Lands and Water the herbicides Ally®, Hoegrass® and Paraquat® were applied directly onto soil without any stubble cover at two and five times the recommended rate. In most situations this low level of functioning continued up to nine weeks. However, when the chemical was applied directly to the soil or to growing plants, the stress time for soil organisms was reduced. The research showed that it takes six weeks for the microbial activity to return to normal.

The Phene-Plate (PhP) system for biochemical finger printing of bacteria, which is based on measurements of the kinetics of biochemical tests, was suitable in using to type total of 384 isolates of bacteria in microplates. The system included mathematical models and had the

advantage of calculating the diversity index (Di) of the bacterial populations present in each of the treated soils, as well as calculating the similarity coefficient (Sp) (Kuhn *et* al, 1991; Kuhn & Mollby, 1993) between the populations of bacteria in the different treatments.

The current gaps in knowledge about the characteristics effects and exposure patterns of existing chemicals must be filled. Given the large knowledge gaps about chemicals on the market, it is important to generate and assess information regarding their potential risks by means of appropriate legal and regulatory instruments, voluntary agreements and economic incentives. A scientific, rules-based approach requires reliable information on effects and exposure as the basis for risk management decisions, where such information is not available, more and more countries may take precautionary approach. Workers and the public must take a more active role in monitoring and contributing to chemical safety management discussions. To facilitate this, good data from research institutions on health and environmental impacts must be more widely available.

Policies need to be established to ensure that this information is reliable, and presented in a way that is useful to all potential users for decision-making, including workers, the general public and the government. Further, governments and industry should work toward educating the public with respect to chemical safety and, where feasible, provide public interest groups with resources that allow them to play the equitable role in policy discussions.

The half-life for glyphosate is between 3 -130 days (Eberbach & Douglas, 1983), hence the effect of glyphosate on the soil bacteria was still evident. Dimethoate has a half-life of about 3 - 5 days (Bohn, 1964), but its effect on bacteria growth was still evident after three weeks. This means, dimethoate had still not been degraded or the recovery rate for the microorganisms was very slow. The half-life of paraquat is not known, but it is known that paraquat is not easily degraded and sticks to the surface soil for a longer period, because it is not leached easily. Pots treated with this agrochemical showed the effects after three weeks of application.

The following recommendations are made from this study

- Agricultural biotechnology may offer a possible alternative that may permit higher yield levels without intensive use of agrochemicals.
- If the herbs to be eliminated can easily be uprooted, then it is more advisable to do manual elimination, especially in small farming systems.

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References

[1] Aguilera-del realA.; Valverde-García, A.; Fernandez-Alba, A.R.; Camacho-Ferre, F. ((1997). Behaviour of endosulfan residues in peppers, cucumbers and cherry tomatoes grown in Greenhouse: Evaluation by decline curves. *Pestic. Sci.* , 51, 194-200.

[2] Alexander, M. (1979). Introduction to Soil Microbiology. Kreiger Publishing Company. , 3.

[3] Ampofo JA & Clerk GC ((2003). Diversity of bacteria in sewage treatment plant used as fish culture pond in southern Ghana*Aquaculture Research*, , 34, 1-9.

[4] Antonious, G. F, & Byers, M. E. (1997). Fate and movement of endosulfan under field conditions. *Environ. Toxicol. Chem.* , 16, 644-649.

[5] Bohn, W. R. (1964). The disappearance of dimethoate from soil. J. Econ. Entomol., 57(6), , 798-799.

[6] Castillo, L. E, De La Cruz, E, & Ruepert, C. (1997). Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America. *Environ. Toxicol. Chem.* , 16, 41-51.

[7] Castillo, L. E, Martínez, E, Ruepert, C, Savage, C, Gilek, M, Pinnock, M, & Solis, E. (2006). Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon., Costa Rica. *Science of the Total Environment* , 367, 418-432.

[8] Cooper, C. M. (1993). Biological effects of agriculturally derived surface-water pollutants on aquatic systems-a review. J. Environ. Qual. , 22, 402-408.

[9] De Reuck, J, Colardyn, F, & Willems, J. (1979). Fatal encephalopathy in acute poisoning with organophosphorus insecticides. A Clinicopathologic study of two cases. Clin. Neurol. Neurosurg., 81 (4), , 247-254.

[10] Delorenzo, M. E, Scott, G. I, & Ross, P. E. (2001). Toxicity of pesticides to aquatic microorganisms: A review. *Environ. Toxicol. Chem.* , 20, 84-98.

[11] Eberbach PL & Douglas LA ((1983). Persistence of glyphosate in a sandy loam *Soil Biology and Biochemistry* 15, , 485-487.

[12] Edwards, L. (1975). Effects of pesticides on soil organisms. *Soil Biology and Biochemistry*. 12, , 285.

[13] Gabrielson, J, Kuhn, I, Colque-navarro, P, Hart, M, & Iversen, A. McKenzie D & Mollby R ((2003). Microplate-based microbial assay for risk assessment and (eco)toxic fingerprinting of chemicals. *Analytica Chimica Acta*, 485, , 121-130.

[14] Gerken, A, Suglo, J. V, & Braun, M. (2001). Pesticide policy in Ghana. MoFA/PPRSD, ICP Project, Pesticide Policy Project/GTZ, Accra.

[15] Goebel, H, Gorbach, S. G, Knauf, W, Rimpau, R. H, & Huttenbach, H. (1982). Properties, effects, residues, and analytics of the insecticide endosulfan. *Residue Rev.*, , 83, 1-165.

[16] Hann, N. J. (1966). The Congo Red reaction in bacteria and its usefulness in the identification of rhizobia. *Canadian Journal of Microbiology*, 12, , 725-733.

[17] Kennedy, I. R, Sánchez-bayo, F, Kimber, S. W, Hugo, L, & Ahmad, N. (2001). Off-site movement of endosulfan from irrigated cotton in New South Wales. *J. Environ. Qual.* , 30, 683-696.

[18] Koppel, C, Forycki, Z, & Ibe, K. (1986). Hemoperfusion in severe Dimethoate poisoning. Intensive Care Med., 12 (2), , 110-112.

[19] Kuhn I & Mollby R ((1993). The PhP RS system- a simple microplate method for studying coliform bacterial populations *Journal of Microbiol. Methods* , 17, 255-259.

[20] Kuhn, I, & Allestam, G. Stenstrom TA & Mollby R. ((1991). Biochemical fingerprinting of water coliform bacteria- a new method for measuring the phenotypic diversity and for comparing different bacterial populations. Appl. Environ. Microbiol., 57 (11), , 3171-3177.

[21] Maier-bode, H. (1968). Properties, effect, residues and analytics of the insecticide endosulfan. *Residue Rev.* , 22, 2-44.

[22] Mcertensson, A. M. (1992). Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. *Soil Biology and Biochemistry*. 24, , 435-445.

[23] McEwen FL & Stephenson GR ((1979).) Uses and Significance of Pesticides in the EnvironmentA Wiley Interscience Publication, , 229-252.

[24] Moorman, J. B. (1989). A review of pesticide effects on microorganisms and microbial processes related to soil fertility. *J. Prod. Agric.*, 21, , 14-23.

[25] Neumann, M, & Dudgeon, D. (2002). The impact of agricultural runoff on stream benthos in Hong Kong, China. *Water Res.* , 36, 3103-3109.

[26] Nurah, G. K. (1999). A baseline study of vegetable production in Ghana. National Agricultural Research Project (NARP) Report, Accra, 151 pp.

[27] Rueppel, M. L, Brightwell, B. B, Schaefer, J, & Marvel, J. T. (1977). Metabolism and degradation of glyphosate in soil and water. *Agriculture and Food Chemistry* 25, , 517-528.

[28] Schuphan, I, Ballschmiter, K, & Tolg, G. (1968). Zum metabolismus des endosulfans in ratten and mausen. *Z. Naturforsch. B 23*, 701-706.

[29] Sethunathan, N, Megharaj, M, Chen, Z, Singh, N, Kookana, R. S, & Naidu, R. (2002). Persistence of endosulfan and endosulfan sulfate in soil as affected by moisture regime and organic matter addition. *Bull. Environ. Contam. Toxicol.*, , 68, 725-731.

[30] Shetty, P. K, Mitra, J, Murthy, N. B. K, Namitha, K. K, Savitha, K. N, & Raghu, K. (2000). Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermohyalospora* MTCC 1384. *Curr. Sci., 79*, 1381-1383.

[31] Sneath PHA & Sokal RR ((1973).) Numerical Taxonomy: The Principles and Practice of Numerical ClassificationW.H. Freeman, San Francisco.

[32] Tu, C. M. (1990). Effect of four experimental insecticides on enzyme activities and level of adenosine triphosphate in mineral and organic soils. *J. Health Environ. Sci.*, B25 (6), , 787-800.

[33] U.S.Department of Health and Human Services (1990). Toxicological profile for endosulfan. Agency for toxic substance and disease registry, Atlanta.

[34] Waiwright, M. (1978). A review of the effect of pesticides on microbial activity in soil. *J. Soil. Sci.*, 29, , 287-298.

[35] Zulalian, J. (1990). Study of the absorption, excretion, metabolism, and residues in tissues of rats treated with carbon-14-labeled pendimethalin, Prowl herbicide. *J. Agric. Food Chem.*, 38, , 1743-1754.

Chapter 2

Sorption of Terbuthylazine in Organic Matter Amended Soils: Effects on Eisenia Fetida and Lumbricus Terrestris

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Additional information is available at the end of the chapter

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1. Introduction

Pesticides are important tools in agriculture that help to minimize economic losses caused by weeds, insects, and pathogens. Although their use has helped to increase crop yields and value, they may also contribute to environmental degradation [1].

Pesticides are recognized as a source of potential adverse environmental impacts and their persistent in surface and ground waters has grown considerably [2]. Some soil applied herbicides reach surface and ground waters by the losses associated with runoff and leaching processes [3]. These losses are attenuated by the natural process of sorption, since degradation, transport, and biological activity of pesticides are greatly influenced by sorption on soil constituents [2].

In order to compensate for the losses caused by transport and degradation processes, some pesticides applicators are exceeding limits on labels which greatly exceed those required for control of the target organisms, and the excessive quantities added increase the environmental impact of these compounds [4, 5]. Because of this, public concern over the residues of pesticides in environment, food and related commodities has increased over the last decades. In Europe, pesticides are considered hazardous substances in accordance with current legislation regarding water [6, 7].

Traditional agricultural requires the use of herbicides, and prevention of ground water pollution is much cheaper than restoring polluted aquifers. Thus, it is of maximum interest that development of agricultural strategies continues to be directed to the decrease in pesticide movement [8].

Triazine herbicides have been largely used in agriculture worldwide for selective and non-selective control of broadleaf and small seeded grass weeds in diverse crops, such as cotton (*Gossypium hirsutum*, L.), maize (*Zea mays,* L.), soybean (*Glycine max*, L.), groundnut (*Arachis hypogea*, L.) and vineyards (*Vitis vinifera*, L.). However, due to their physicochemical properties (in particular, their relatively long persistence) there have been numerous reports of their presence in surface or ground waters [3, 9]. Several bioremediation strategies have been proposed to reduce the presence of pesticides in soil from which they can reach groundwater, such as remediation by enhancing the microbial population able to degrade specifically the target compounds. This strategy has been approached by addition of organic exogenous matter of different origin [9, 10, 11].

However, the influence of organic matter on soil properties and sorption process depends upon the type, amount and dominant components of the added organic materials [12, 13]. This aspect is of great interest, since it supposes an important advance in the behavior of the herbicides in the soil after the addition of different sources of organic matter.

Earthworms are one of the important components in decomposer communities and contribute significantly to the organic decomposition, nutrient cycling and soil formation [14]. Continuous application of pesticides may present risks to lead to soil pollution affect soil fauna [15]. For this reason, the use of earthworms for toxicity testing is highly recommended by the European Communities [16] and are considered as preferred bioindicators for assessing the environmental health status of chemical pollution [17, 18]. Earthworm species such as *Eisenia fetida* is considered as a suitable biomonitors to determine the ecological hazard of heavy metals and chemicals contaminated soil because of its low cost, easy culturing and the standardization of the acute and sub-chronic ecotoxicological tests [14, 19].

Of the potential biomarkers, earthworm glutathione-S-transferase and cellulalse enzymes are shown to respond to toxin exposure [14]. Glutathione-S-transferase is an important detoxification enzyme and its activity has been used as a potential bioindicator and biomarker of earthworms for heavy metals, pesticides and PAHs exposure [14, 15, 20]. Also, cellulase activity of earthworms indicates their role in the decomposition of plant litter and other cellulosic materials. It has been used as a biomarker of a pesticide contamination on earthworms [14, 20].

However, the biological relevance of *Eisenia fetida* is still open to debate [21] since they are often less susceptible to pollutants than other species [22] and rarely found in conventionally tilled agricultural soils. In this respect, Ma and Bodt [23] found and different levels of chlorpyrifos insecticide sensitivity to earthworms (*Eisenia* sp.< *Aporrectodea* sp.< *Lumbricus* sp.).

Few studies have been performed comparing different sources of organic matter types on the sorption and mobility of herbicides. For this reason, the objective of this study was to investigate the sorption and mobility of terbuthylazine herbicide in a soil amended with three organic amendment and their effects on acute toxicity and morphological alterations in two earthworm species (*Eisenia fetida* and *Lumbricus terrestris*).

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2. Material and methods

2.1. Soil, organic amendment and herbicide characteristics

The soil used in this experiment is a Plagic Antrosol [24]. The main soil characteristics are shown in Table 1.

	Soil	PM	MSW	CM
pH (H_2O)	8.6 ± 0.2	7.1 ± 0.3	6.2 ± 0.3	8.3 ± 0.2
CO_3^{2-} (g kg^{-1})	203 ± 12			
Fine sand (g kg^{-1})	142 ± 35			
Coarse sand (g kg^{-1})	387 ± 26			
Silt (g kg^{-1})	242 ± 19			
Clay (g kg^{-1})	229 ± 10			
Clay types	Smectite: 66% Kaolinite: 20% Illite: 14 %			
Organic matter (g kg^{-1})	1.1 ± 0.2	614 ± 26	469 ± 15	764 ± 29
Humic acid-C (mg kg^{-1})	18.5 ± 2.4	672 ± 1.4	1030 ± 17	461 ± 13
Fulvic acid-C (mg kg^{-1})	9.8 ± 1.1	715 ± 10	711 ± 10	631 ± 24
Total N (g kg^{-1})	0.4 ± 0.1	38.8 ± 2.9	17.3 ± 1.3	29.2 ± 2.1
Fe (mg kg^{-1})	35.8 ± 3.7	180 ± 22	815 ± 38	407 ± 28
Cu (mg kg^{-1})	9.7 ± 1.3	1.6 ± 0.3	82.6 ± 9.8	24.2 ± 1.8
Mn (mg kg^{-1})	11.3 ± 2.1	4.2 ± 0.9	75.6 ± 8.1	14.1 ± 1.2
Zn (mg kg^{-1})	8.1 ± 1.5	3.3 ± 0.8	134 ± 13	10.3 ± 1.6
Cd (mg kg^{-1})	6.5 ± 1.2	0.35 ± 0.07	1.1 ± 0.3	0.28 ± 0.09
Pb (mg kg^{-1})	0.36 ± 0.11	0.94 ± 0.12	82.4 ± 3.6	5.3 ± 0.8
Ni (mg kg^{-1})	2.9 ± 0.7	1.3 ± 0.2	13.6 ± 1.5	2.4 ± 0.6
Cr (mg kg^{-1})	5.3 ± 0.6	0.12± 0.02	19.4 ± 1.7	0.29 ± 0.04

Table 1. Characteristics of the experimental soil and organic amendment (mean ± standard error). Data are the means of four samples.

Soil pH was determined in distilled water with a glass electrode (soil:H_2O ratio 1:2.5). Soil texture was determined by the Robinson's pipette method [25] and quantification and dominant clay types were determined by X-ray diffraction. Total carbonates were measured by estimating the quantity of the CO_2 produced by HCl addition to the soil [26]. Soil organic matter was determined by the method of Yeomans and Bremner [27]. Humic and fulvic acids-

fractions were extracted with 0.1 M sodium pyrophosphate and 0.1 M sodium hydroxide at pH 13 [28]. The supernatant was acidified to pH 2 with HCl and allowed to stand for 24 h at room temperature. To separate humic acids-fraction from fulvic acids-fraction, the solution was centrifuged and the precipitate containing humic acids-fraction was dissolved with sodium hydroxide [27]. After the removal of humic acids-fraction, the acidic filtrate containing the dissolved fulvic acid-fraction was passed through a column of XAD-8 resin. The adsorbed fulvic was then recovered by elution with 0.1 M NaOH, desalted using Amberlyst 15-cation-exchange resin, and finally freeze-dried. The carbon content of humic and fulvic acids-fractions were determined by the method described. Total N was determined by the Kjeldhal method [26]. After nitric and perchloric acid digestion, total Ca, Mg, Fe, Cu, Mn, Zn, Cd, Pb, Ni and Cr concentrations were determined by atomic absorption spectrometer and K was determined by atomic emission spectrometer, according to MAPA methods [26].

The organic amendment applied were the organic fraction of a municipal solid waste (MSW), poultry manure (PM) and cow manure (CM). The general properties of the organic amendment are shown in Table 1. Organic matter was determined by dry combustion, according to the official methods of the Spanish Ministry of Agriculture [26]. Humic and fulvic acids-fraction were extracted, separated and determined by the methods previously described. Total N was determined by the Kjeldhal method [26]. After nitric and perchloric acid digestion, total Ca, Mg, Fe, Cu, Mn, Zn, Cd, Pb, Ni and Cr concentrations were determined by atomic absorption spectrometer and K was determined by atomic emission spectrometer, according to MAPA methods [26].

Table 2 shows the acidic functional group contents of humic acids isolated from both organic amendment. The carboxyl group content was estimated by direct potentiometric titration at pH 8, the phenolic hydroxyl group content was estimated as two times the change in charge between pH 8 and pH 10, and the total acidity was calculated by addition [29].

	Total acidity (mol kg^{-1})	**COOH**	**Phenolic OH**
PM	3.99 ± 0.13	2.99 ± 0.09	0.99 ± 0.05
MSV	4.29 ± 0.04	3.19 ± 0.03	1.10 ± 0.03
CM	2.81 ± 0.02	2.00 ± 0.03	0.80 ± 0.01

Table 2. Acidic functional group contents (mean ± standard errors) of humic acids isolated from PM, MSW and CM

The herbicide used in this experiment was the terbuthylazine. Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) is a selective herbicide for the control of broadleaf and grass weeds in forestry, lucerne (*Medicago sativa*, L.), maize (*Zea mays*, L.), sweetcorn (*Zea mays*, L. var. *rugosa*), peas (*Pisum sativum*, L.), orchard and non-cropland, with a water solubility of 8.5 mg l^{-1} at 20 ºC. It is absorbed by roots and inhibits Hill reaction and CO_2 sorption in the chlorophyllic function [30].

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2.2. Incubation procedure

Two kg of soil were pre-incubated at 25 ºC for 7 days at 30–40% of their water-holding capacity, according to Moreno et al. [31], prior to the treatments. After this pre-incubation period, soil samples were treated with three concentrations of terbuthylazine (1, 10 and 50 μg terbuthylazine g^{-1} soil) and treated with MSW at a rate of 10% or PM at a rate of 7.6% or CM at a rate of 5.8%, respectively, in order to applying the same amount of organic matter to the soil. A non-mended treated as well as a amended non-treated soil were used as controls.

The incubation treatments are detailed as follows:

1. C1, control soil, soil non-polluted and non-organic amended

2. C2, soil treated with 1 μg terbuthylazine g^{-1} soil and non-organic amended

3. C3, soil treated with 10 μg terbuthylazine g^{-1} soil and non-organic amended

4. C4, soil treated with 50 μg terbuthylazine g^{-1} soil and non-organic amended

5. MSW1, soil non- treated and amended with MSW

6. MSW2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with MSW

7. MSW3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with MSW

8. MSW4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with MSW

9. PM1, soil non- treated and amended with PM

10. PM2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with PM

11. PM3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with PM

12. PM4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with PM

13. CM1, soil non- treated and amended with CM

14. CM2, soil treated with 1 μg terbuthylazine g^{-1} soil and amended with CM

15. CM3, soil treated with 10 μg terbuthylazine g^{-1} soil and amended with CM

16. CM4, soil treated with 50 μg terbuthylazine g^{-1} soil and amended with CM

Triplicate treatments were kept in semi-closed microcosms at 25 ºC for 3, 15, 45 and 90 days, respectively.

Twenty two earthworms of the species *Eisenia fetida* (approximately 210 mg fresh weight) and *Lumbricus terrestris* (approximately 190 mg fresh weight) were included in each microcosm. Each microcosm was covered with fine nylon mesh to prevent the soil loss and to keep earthworms from escaping. *Lumbricus terrestris* were collected in the field, in an area that has not been treated with pesticides for 20 years, whereas *Eisenia fetida* were bred in laboratory cultures on organic amendment materials, vermicomposts principally.

2.3. Adsorption studies

For adsorption studies the treatments used were:

1. S, non-organic amended control soil (10 g of soil)
2. S+CM, soil amended with CM at rate of 10% (10 g of soil + 1 g of CM)
3. S+PM, soil amended with PM at a rate of 12.4% (10 g of soil + 1.24 g of PM)
4. S+MSW, soil amended with MSW at a rate of 16.3% (10 g of soil + 1.63 g of MSW)

Terbuthylazine sorption was determined according to Cabrera et al. [32] criteria. Triplicate samples (5 g) of the non-amended and organic amended soil (S, S+CM, S+PM, S+MSW) were treated with 10 ml of terbuthylazine (50%:50%, v/v) solution (initial concentrations, Ci, ranging from 5 to 50 μM in 0.01 $CaCl_2$). Previously, it was determined that equilibrium was reached in less than 24 h, and that no measurable degradation occurred during this period. Equilibrium concentrations (Ce) in the supernatants were determined by HPLC. Sorption isotherms were fitted to Freundlich equation ($Cs=Kf \times Ce^{1/nf}$) and sorption coefficients Kf and 1/nf were calculated.

2.4. Herbicide analysis

Herbicide was extracted twice with methanol (Merck, Darmstadt, Germany) at 1:2 soil/solution ratio for 15 min. Extracts were mixed and rotary-vacuum evaporated almost to dryness at 40 ºC. The residue was dissolved in 2 ml of methanol and analyzed by HPLC [30]. Terbuthylazine was analyzed using a Beckman, System Gold, Autosampler 508 HPLC chromatograph coupled to a Waters 2996 diode-array detector. The analytical conditions were: Nova-Pack C18 column (159 mm length X 3.9 mm internal diameter), eluent mixture, 50:50 acetonitrile/water at a flow rate of 1 ml min^{-1}, 25 μl injection volume, and UV detection at 220 nm [33]. External calibration curves with four standard solutions between 0.2 and 26 μM were used in the calculations.

2.5. Earthworm analysis

Earthworm cocoon production was determined after 30 days of exposure. Cocoons were collected by hand sorting and weighed, and then incubated for four additional weeks as described by Maboeta et al. [34]. Cocoons were cultured in Petri dishes at 25±1 ºC covered with three moist filter papers. According to Xiao et al. [14], the filter papers in these dishes were changed every three days to prevent bacterial growth. At the end of the experiment (30 days), the weight of per cocoon and number of juveniles per cocoon were determined.

After 3, 15, 45 and 90 incubation days for each treatment, three worms were selected and placed on wet filter paper in Petri dishes for 24 h to clear gut contents, and their weights were recorded after blotting them dry on paper towels. Earthworms were digested in the 1:1 nitric-perchloric extract after digestion at 450 ºC for 6 h. The terbuthylazine was measured by the method previously mentioned. Cellulase activity was measured as described by Mishra and Dash [35], and glutathione-S-transferase activity was measured according to the method described by Habig et al. [36] and Saint-Denis et al. [37].

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2.6. Statistical analysis

Two-ways analysis of variance (ANOVA) was performed for all parameters, considering two variables involved (incubation time and the terbuthylazine concentration applied to the soil) using the Statgraphics v. 5.0 software package [38]. The means were separated by the Tukey's test, considering a significance level of $P<0.05$ throughout the study. For the ANOVA, triplicate data were used for each treatment and every incubation day.

3. Results

3.1. Sorption studies

Sorption isotherms of terbuthylazine on soil, soil+CM, soil+PM and soil+MSW are shown in Figure 1. The results indicated that sorption of terbuthylazine on organic amended soils significantly increased compared to non-organic amended soil. For each organic amended soil, the herbicide sorption with MSW was higher than with PM and CM.

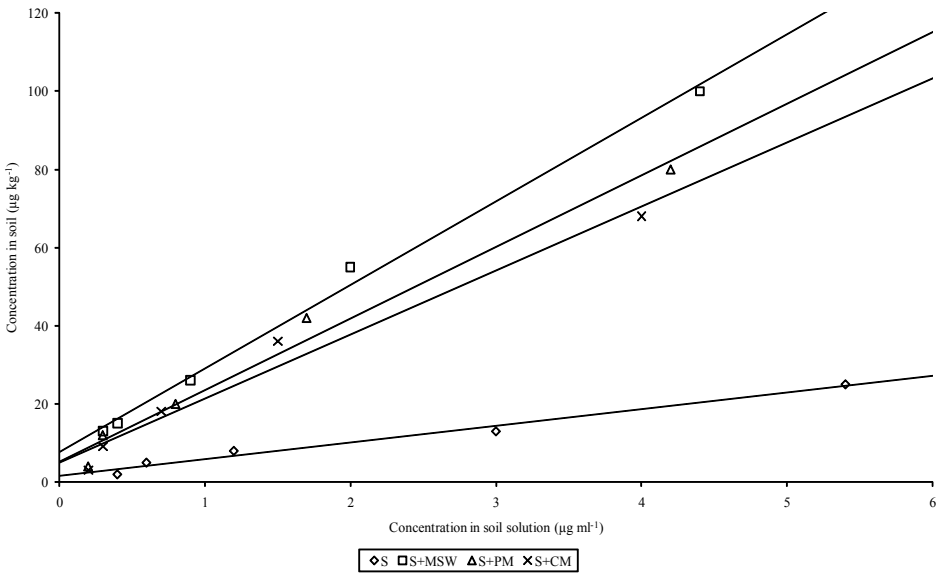

Figure 1. Terbuthylazine sorption isotherms in non-amended and organic amended soils. Symbols are experimental data points, whereas lines are the Freundlich-fit sorption isotherms.

Sorption isotherms were fit to the Freundlich equation and sorption coefficients Kf and 1/nf were calculated (Table 3). The results indicated that Kf values significantly increased in organic amended soils than for non-organic amended soils. However, terbuthylazine sorption increased by a factor of 4.5 upon amendment with MSW, whereas for PM and CM, the factor increased 4 and 3.4, respectively. Again, the results indicate significant differences between S

+MSW and S+CM treatments. Also, the 1/nf coefficients significantly decreased in organic amended soils than for non-organic amended soil. For organic amended soils, the 1/nf coefficient was higher in the soil amended with MSW, followed by PM and CM, respectively.

	Kf	1/nf	R^2
S	6.93a ± 0.95	0.92a ± 0.05	0.963
S+MSW	31.22c ± 2.46	0.80c ± 0.06	0.943
S+PM	28.03bc ± 2.03	0.83bc ± 0.05	0.958
S+CM	23.48b ± 1.99	0.86b ± 0.04	0.977

Table 3. Freundlich sorption coefficients Kf and 1/nf and standard error for terbuthylazine in non-amended and organic amended soils. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

3.2. Effect of terbuthylazine on weight on earthworms

In treated and non-organic amended soils, the *Eisenia fetida* weight decreased during the experimental period and when increased the terbuthylazine concentration in soil (Table 4). At the end of the experiment, the worm weight decreased 23.6%, 31.4% and 39.1% in soil treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively. For *Lumbricus terrestris*, the weight decreased 25.6%, 33.6% and 41.8% in soil treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively.

The application of organic matter to non-treated soil increased the worm weight. However, this increased depended of the organic matter type. At the end of the experiment, *Eisenia fetida* weight increased 20.3%, 15.1% and 11.3% in MSW, PM and CM-amended soils, compared to control soil, whereas *Lumbricus. terrestris* weight increased 18.6%, 13.9% and 10.1% in MSW, PM and CM-amended soils, compared to control soil. However, the ANOVA analysis indicated no significant differences between these treatments.

In treated and organic amended soils, the both worms weight increased respect to the treated and non-organic amended soils. This increase was higher for MSW, followed by PM and CM-amended soils.

The non-treated and organic amended soils have the highest mean cocoon numbers (Table 5). For both worms, the cocoon numbers were highest in MSW followed by PM and CM-amended soils. Terbuthylazine treatments in organic amended soils decreased the cocoon numbers. However, this decrease was lowest in MSW followed by PM and CM-amended soils.

The average weight per cocoon was also higher in non-polluted and organic amended soils, compared to control soil. Again, the average weight per cocoon in terbuthylazine treated and organic amended soils were highest in MSW followed by PM and CM-amended soils.

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	Eisenia fetida				*Lumbricus terrestris*			
	Incubation days				**Incubation days**			
	3	**15**	**45**	**90**	**3**	**15**	**45**	**90**
C1	208ab ± 12	211b ± 14	211b ± 9	212b ± 10	192ab ± 5	190ab ± 8	195ab ± 7	199ab ± 10
C2	206ab ± 15	190a ± 20	173a ± 15	162a ± 13	188ab ± 10	172a ± 10	160a ± 8	148a ± 14
C3	201ab ± 17	180a ± 11	164a ± 16	145a ± 18	180a ± 11	163a ± 15	150a ± 10	132a ± 13
C4	197ab ± 19	164a ± 23	140a ± 12	129a ± 16	170a ± 13	156a ± 11	134a ± 8	116a ± 12
MSW1	216b ± 13	233b ± 16	244b ± 19	255b ± 14	203ab ± 11	215b ± 15	222b ± 12	236b ± 17
MSW2	215b ± 12	217b ± 18	213b ± 18	220b ± 17	198ab ± 13	195ab ± 12	197ab ± 10	201ab ± 13
MSW3	214b ± 10	215b ± 13	209ab ± 10	205ab ± 11	194ab ± 10	190ab ± 11	186ab ± 13	182a ± 14
MSW4	213b ± 13	211b ± 10	204ab ± 13	197ab ± 13	190ab ± 14	188ab ± 10	182a ± 11	179a ± 12
PM1	215b ± 14	229b ± 17	233b ± 12	244b ± 11	198ab ± 12	206b ± 15	219b ± 12	227b ± 14
PM2	214b ± 12	212b ± 13	209ab ± 18	207ab ± 18	195ab ± 10	194ab ± 12	194ab ± 8	192a ± 11
PM3	213b ± 11	210ab ± 15	199ab ± 13	189a ± 15	193ab ± 11	188ab ± 15	180a ± 16	172a ± 15
PM4	212b ± 15	205ab ± 12	190a ± 11	180a ± 11	190ab ± 12	181a ± 10	175ab ± 12	168a ± 13
CM1	213b ± 17	220b ± 15	225b ± 14	236b ± 18	196ab ± 11	202ab ± 13	210b ± 10	219b ± 12
CM2	221b ± 15	201ab ± 10	195ab ± 15	193ab ± 14	193ab ± 10	193ab ± 9	180a ± 12	186ab ± 11
CM3	210ab ± 11	195ab ± 12	180a ± 10	174a ± 11	190ab ± 8	185ab ± 10	170a ± 13	162a ± 13
CM4	209ab ± 13	190a ± 17	173a ± 12	165a ± 13	188ab ± 6	179a ± 11	164a ± 11	157a ± 14

Table 4. Changes in weight (mean ± standard error) (mg) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

	Eisenia fetida			*Lumbricus terrestris*		
	Cocoon numbers	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**	**Cocoon numbers**	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**
C1	2.93ab ± 0.35	8.76b ± 0.47	3.09b ± 0.36	2.63b ± 0.22	8.15b ± 0.31	2.81ab ± 0.17
C2	2.05a ± 0.22	6.64ab ± 0.24	2.49ab ± 0.17	1.78a ± 0.18	6.06ab ± 0.23	2.21a ± 0.14
C3	1.74a ± 0.15	5.68a ± 0.31	2.34a ± 0.21	1.54a ± 0.24	5.20a ± 0.16	2.13a ± 0.10
C4	1.18a ± 0.17	4.88a ± 0.25	1.98a ± 0.13	1.03a ± 0.10	4.33a ± 0.13	1.77a ± 0.15
MSW1	3.42b ± 0.22	8.95b ± 0.39	3.37b ± 0.28	3.03b ± 0.34	8.31b ± 0.24	3.03b ± 0.17
MSW2	2.90ab ± 0.17	7.50b ± 0.25	2.94b ± 0.17	2.51b ± 0.19	6.76ab ± 0.17	2.60ab ± 0.13
MSW3	2.69ab ± 0.13	7.12ab ± 0.20	2.87b ± 0.13	2.33b ± 0.15	6.43ab ± 0.22	2.54ab ± 0.19
MSW4	2.37ab ± 0.13	6.97ab ± 0.22	2.55ab ± 0.11	2.06ab ± 0.17	6.24ab ± 0.25	2.22a ± 0.16

	Eisenia fetida			*Lumbricus terrestris*		
	Cocoon numbers	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**	**Cocoon numbers**	**Average weigh of per cocoon (mg)**	**Number of juveniles per cocoon**
PM1	3.29b ± 0.25	8.91b ± 0.45	3.29b ± 0.22	2.92b ± 0.19	8.27b ± 0.35	2.96b ± 0.19
PM2	2.68ab ± 0.20	7.39b ± 0.18	2.82b ± 0.16	2.34b ± 0.22	6.67ab ± 0.24	2.46ab ± 0.23
PM3	2.41ab ± 0.18	6.82ab ± 0.24	2.73ab ± 0.19	2.19ab ± 0.15	6.28ab ± 0.26	2.41ab ± 0.18
PM4	2.10a ± 0.15	6.38ab ± 0.21	2.39a ± 0.23	1.91ab ± 0.11	6.10ab ± 0.28	2.12a ± 0.15
CM1	3.17b ± 0.19	8.83b ± 0.53	3.21b ± 0.17	2.83b ± 0.14	8.21b ± 0.39	2.89a ± 0.29
CM2	2.47ab ± 0.10	7.09ab ± 0.21	2.68ab ± 0.19	2.16ab ± 0.12	6.35ab ± 0.22	2.38ab ± 0.21
CM3	2.19a ± 0.11	6.42ab ± 0.15	2.58ab ± 0.13	1.88ab ± 0.15	5.78a ± 0.21	2.28ab ± 0.12
CM4	1.90a ± 0.13	5.95a ± 0.13	2.23a ± 0.15	1.66a ± 0.17	5.35a ± 0.18	1.98a ± 0.16

Table 5. Cocoon production, average weight of cocoons (mg) and number of juveniles per coccon (mean ± standard error) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

The number of juveniles per cocoon decreased when terbuthylazine concentration increased. This decrease was higher for *Lumbricus terrestris* than for *Eisenia fetida*. The application of organic matter in terbuthylazine treated soils increased this parameter. Again, this increase was higher MSW followed by PM and CM-amended soils.

3.3. Biochemical assay

At the end of the experiment and for 50 μg terbuthylazine g^{-1} soil treatment, the cellulase activity of *Eisenia fetida* and *Lumbricus terrestris* worms was significantly recudec (29.4% and 31.1%) compared to the control soil (Table 6). The application of organic matter in herbicide treated soil increased the cellulase activity. At the end of the incubation day and for the higher concentration of terbuthylazine, the *Eisenia fetida* cellulase activity decreased 25%, 21.4% and 19% in soils amended with CM, PM and MSW, respectively, compared to organic amended and non-treated soils. For *Lumbricus terrestris*, cellulase activity decreased 26.8%, 23.9% and 21.5% in soils amended with CM, PM and MSW, respectively, compared to organic amended and non-treated soils.

Compared to the control soil, the glutathione-S-transferase activity of *Eisenia fetida* decreased 12.3%, 19.6% and 30.9% in soils treated with 1, 10 and 50 μg terbuthylazine g^{-1} soil, respectively, whereas for *Lumbricus terrestris* the glutathione-S-transferase activity decreased 14%, 21% and 32.4%, respectively (Table 7). At the end of the experiment, the glutathione-S-transferase activity of both worms had higher increase in MSW, PM and CM-amended soils, respectively, compared to control soil. The application of organic matter in herbicide treated soils increased the glutathione-S-transferase activity. Again, this increase was higher in MSW followed by PM and CM-amended soils.

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4. Discussion

Our results indicated that terbuthylazine induced negative effects on weight, reproductive and enzymatic activities on the both earthworms. These negative effects increased with increasing herbicide concentration and/or exposure time. These results are in accordance with Brunninger et al. [39] who studied the toxicity of terbuthylazine on the growth and reproduction of *Eisenia andrei* over a period of three generations.

The weight loss may indicate a feeding inhibition situation, with the earthworms regulating the intake of the terbuthylazine by reducing consumption rate and thus affecting their subsequent growth rate. This strategy is commonly used by earthworms to avoid poisoning with herbicides and heavy metals [40].

The decrease of earthworm cellulase and gluthatione-S-transferase activities possibly is due to a physiological adaptability to compensate for pesticide stress. To overcome the stress situation, animals require high energy, and this energy demand may have led to protein catabolism [41]. Furthermore, this decrease in protein content might be a result of mechanical lipoprotein formation, which is used to repair damaged cells, tissues, and organs [40].

	Eisenia fetida				***Lumbricus terrestris***			
	Incubation days				**Incubation days**			
	3	**15**	**45**	**90**	**3**	**15**	**45**	**90**
C1	556b ± 22	624bc ± 28	610bc ± 19	590b ± 20	527b ± 19	542b ± 15	575bc ± 18	558bc ± 13
C2	550b ± 19	543b ± 18	538b ± 17	529ab ± 22	520b ± 15	508b ± 12	496b ± 17	489b ± 17
C3	544b ± 20	529ab ± 20	510ab ± 19	491ab ± 17	511b ± 17	492b ± 18	470ab ± 14	450ab ± 15
C4	530ab ± 17	498ab ± 15	456a ± 21	416a ± 13	510b ± 11	479b ± 13	425a ± 18	384a ± 12
MSW1	563b ± 26	679c ± 25	710c ± 19	748c ± 22	539b ± 10	568bc ± 18	602c ± 20	687c ± 18
MSW2	558b ± 19	652c ± 21	684c ± 17	703c ± 15	520b ± 15	547b ± 14	589bc ± 13	595bc ± 21
MSW3	549b ± 14	624bc ± 18	643bc ± 22	663c ± 19	511b ± 13	530b ± 11	559b ± 13	539b ± 19
MSW4	536b ± 17	560b ± 24	582b ± 26	606bc ± 20	505b ± 12	520b ± 15	528b ± 10	676c ± 17
PM1	570b ± 24	668b ± 26	700c ± 15	738c ± 21	528b ± 20	560b ± 17	608c ± 15	616c ± 18
PM2	552b ± 23	601bc ± 17	643bc ± 19	683c ± 18	515b ± 18	547b ± 19	590bc ± 22	568bc ± 13
PM3	540b ± 19	590b ± 15	628bc ± 22	642bc ± 16	504b ± 14	530b ± 12	546b ± 19	568bc ± 13
PM4	529ab ± 22	541b ± 20	561b ± 20	580b ± 13	493b ± 17	519b ± 17	522b ± 15	514b ± 16
CM1	575b ± 19	647c ± 24	680c ± 17	700c ± 22	524b ± 13	555b ± 15	586bc ± 15	650c ± 19
CM2	539b ± 21	565b ± 17	596b ± 23	642bc ± 17	510b ± 21	532b ± 12	558b ± 19	588bc ± 15
CM3	520ab ± 18	540b ± 18	573b ± 22	525ab ± 13	498b ± 19	517b ± 11	524b ± 18	483b ± 13
CM4	500ab ± 19	509ab ± 21	516ab ± 20	525ab ± 13	480b ± 18	496b ± 15	482b ± 19	476ab ± 11

Table 6. Cellulase activity (mean± standard error) (mg glucose mg protein hour^{-1}) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

	Eisenia fetida				*Lumbricus terrestris*			
	Incubation days				Incubation days			
	3	15	45	90	3	15	45	90
C1	118ab ± 10	116ab ± 11	121ab ± 10	120ab ± 12	110ab ± 9	113ab ± 11	117ab ± 10	115ab ± 11
C2	116ab ± 9	111ab ± 13	108a ± 9	105a ± 10	110ab ± 7	106a ± 10	104a ± 11	98.9a ± 6.2
C3	113ab ± 8	108a ± 12	103a ± 11	96a ± 8	108a ± 10	104a ± 11	95.3a ± 8.6	90.9a ± 5.8
C4	110a ± 11	102a ± 9	90a ± 10	82.9a ± 6.9a	104a ± 6	99.6a ± 7.8	82.4a ± 7.2	77.7a ± 4.9
MSW1	130ab ± 12	140ab ± 14	152b ± 15	161b ± 13	123ab ± 13	131b ± 12	142b ± 10	154b ± 10
MSW2	127ab ± 10	134b ± 8	140b ± 10	148b ± 16	118ab ± 12	127b ± 11	135b ± 9	140b ± 12
MSW3	122ab ± 11	130ab ± 10	135b ± 13	141b ± 15	115ab ± 11	120ab ± 13	126b ± 11	132b ± 11
MSW4	118ab ± 12	121ab ± 11	123ab ± 11	126ab ± 11	109a ± 7	112ab ± 10	115ab ± 12	117ab ± 10
PM1	128ab ± 13	136b ± 17	147b ± 10	156b ± 11	120ab ± 10	129ab ± 11	140b ± 11	148b ± 11
PM2	124ab ± 10	130ab ± 12	135b ± 12	142b ± 13	117ab ± 10	124ab ± 12	130b ± 10	134b ± 12
PM3	120ab ± 8	125ab ± 9	129ab ± 11	134b ± 15	112ab ± 9	118ab ± 11	120ab ± 11	124ab ± 10
PM4	115ab ± 9	119ab ± 10	121ab ± 9	120ab ± 11	107a ± 9	111ab ± 9	107ab ± 9	109ab ± 9
CM1	124ab ± 12	130ab ± 15	140b ± 12	150b ± 10	117ab ± 10	125b ± 10	136b ± 12	142b ± 13
CM2	123ab ± 10	128ab ± 11	133b ± 10	135b ± 12	113ab ± 11	116ab ± 8	123ab ± 9	127b ± 11
CM3	118ab ± 11	122ab ± 10	123ab ± 12	125ab ± 13	106a ± 8	113ab ± 7	115ab ± 9	118ab ± 10
CM4	111ab ± 9	112ab ± 10	113ab ± 11	113ab ± 10	100a ± 9	104a ± 9	108a ± 8	106a ± 9

Table 7. Glutathione-S-transferase activity (mean ± standard error) (nmol mg protein min^{-1}) of *Eisenia fetida* and *Lumbricus terrestris* exposed to different concentrations of terbuthylazine herbicide. Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($p<0.05$)

However, these negative effects were higher in *Lumbricus terrestris* than for *Eisenia fetida*. Therefore, the sensitivity of each worm is different to the terbuthylazine herbicide. According to Ma and Bodt [23] this can be due to some physiological property of the worms or to factors governing the exposure to herbicide. Studies on the effect of benomyl and carbofuran on earthworms have similarly shown that the toxicity is much greater to *Lumbricus terrestris* than for *Eisenia fetida* when tested under standardized conditions in soil substrates [42].

The addition of organic matter to the herbicide treated soil increased the earthworm weight, reproductive and enzymatic activities probably due to the sorption of terbuthylazine with the organic matter. These results are in agreement with Dolaptsoglou et al. [43] and Cabrera et al. [30, 32], who found a decrease of terbuthylazine in the soil solution after the addition of organic matter to soil due to the herbicide sorption.

The terbuthylazine sorption isotherms and Freundlich sorption coefficients obtained in this study, suggested that organic matter play a fundamental role in the sorption of the herbicide

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in agricultural soils, probably as a result of the humic substances containing several major functional groups, such as carboxyl, phenolic, alcohol and carbonyl [44, 45]. However, our results also suggested that the chemical composition of the organic matter influenced in the terbuthylazine sorption.

Several studies of metal complexation with organic matter indicated that the sorption of heavy metals increased when the humic acid-fraction content increased in the organic matter, compared to the fulvic acid-fraction content, probably due to the humic acid-fraction possess a higher number of carboxylic groups than fulvic acid-fraction [12, 13].

The terbuthylazine sorption isotherms and Freundlich sorption coefficients indicated higher herbicide sorption in MSW-amended soils, followed by PM and CM. Therefore, and similar to the heavy metals complexation, the sorption of herbicide increased with the humic acid-fraction content in the organic amendment applied to the soil. The higher sorption probably caused a larger decrease of herbicide in the soil solution, and therefore, lowest availability of terbuthylazine availability for earthworms. This fact probably is the responsible of the increase in earthworm weight, reproductive and enzymatic activities.

5. Conclusions

It can be concluded that the sensitivity of earthworm to pesticides differ depending on the taxonomic species, *Lumbricus terrestris* being more sensitive than *Eisenia fetida* to terbuthylazine herbicide. The application of organic matter have a positive effect on reducing the toxic effect of terbuthylazine on both *Eisenia fetida* and *Lumbricus terrestris*, which is attributable to their capability of absorbing the pesticide decreasing its concentration in soil solution. This positive effect will depend on the organic amendment characteristics, those with higher amount or reactive humic acid being the most effective.

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References

[1] Farenhorst, A. Importance of soil organic matter fractions in soil-landscape and regional assessments of pesticide sorption and leaching in soil. Soil Science Society America Journal 2006; 70(3) 1005-1012.

[2] Cox, L., Cecchi, A., Celis, R., Hermosín, M. C., Koskinen, W.C., Cornejo, J. Effect of exogenous carbon on movement of simazine and 2,4-D in soils. Soil Science Society of America Journal 2001; 65(6) 1688-1695.

[3] Blanchoud, H, Moreau-Guigon, E, Farrugia, F, Chevreuil, M, Mouchel, JM. Contribution by urban and agricultural pesticide uses to water contamination at the scale of the Marne watershed. Science of Total Environment 2007; 375(1-3) 168-79.

[4] Gerstl, Z., Nasser, A., Mingelgrin, U. Controlled release of pesticides into soils from clay-polymer formulations. Journal of Agriculture and Food Chemistry 1998a; 46(9) 3797-3802.

[5] Gerstl, Z., Nasser, A., Mingelgrin, U. Controlled release of pesticides into water from clay-polymer formulations. Journal of Agriculture and Food Chemistry 1998b; 46(9) 3803-3809.

[6] Directive 2000/60/EC of 23 of October of 2000, related to Communitary Marc to Actuation about Water Politic. Diary Official of European Communities (DOCE), L 321, 19 of December of 2000.

[7] Directive 2006/11/EC of the European Parliament and Council of 15 of February of 2006, related to Pollution Caused by Dangerous Substances tipped in the Aquatic Environment of the Community. Diary Official of European Communities (DOCE), L 64, 4 of March of 2006.

[8] Cox, L., Celis, R., Hermosin, M.C., Becker, A., Cornejo, J. 1997. Porosity and herbicide leaching in soils amended with olive-mill wastewater. Agriculture, Ecosystems and Environment 65(2) 151-161.

[9] Delgado-Moreno, L., Peña, A. Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. Science of Total Environment 2009; 407(5) 1489-1495.

[10] Delgado-Moreno, L., Peña, A. Organic amendments from olive-mill wastes as a strategy to modify degradation of sulfonylurea herbicides in soil. Journal of Agriculture and Food Chemistry 2007; 55(15) 6213-8.

[11] Abdelhafid, R., Houot, S., Barriuso, E. Dependence of atrazine degradation on C and N availability in adapted and non-adapted soils. Soil Biology and Biochemistry 2000; 32(3) 389-401.

http://dx.doi.org/10.5772/54948

[12] Tejada, M., Hernández, M.T., García, C. Application of two organic wastes in a soil polluted by lead: Effects on the soil enzymatic activities. Journal of Environmental Quality 2007; 36(1) 216-225.

[13] Tejada, M., Moreno, J.L., Hernández, M.T., García, C. Soil amendments with organic wastes reduce the toxicity of nickel to soil enzyme activities. European Journal of Soil Biology 2008; 44(1) 129-140.

[14] Xiao, N., Jing, B., Ge, F., Liu, X. The fate of herbicide acetochlor and its toxicity to *Eisenia fetida* under laboratory conditions. 2006; Chemosphere 62(8) 1366-1373.

[15] Booth, L.H., Hodge, S., O'Halloran, K. Use of cholinesterase in *Aporrectodea caliginosa* (Oligochaeta, Lumbricidae) to detect organophosphate contamination: comparison of laboratory test, mesocosms, and foeld studies. Environmental Toxicology Chemistry 2000; 19(2) 417-422.

[16] EC. 2004. Biological test method: tests for toxicity of contaminated soil to earthworms (*Eisenia foetida*, *Eisenia andrei*, or *Lumbricus terrestris*). Report EPS 1/RM/43, Environment Canada, Environmental Technology Centre, Ottawa, ON, Canada.

[17] Zhou, S.P., Duan, C.Q., Hui, F.U., Chen, Y.H., Wang, X.H., Yu, Z.F. Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods. Journal of Environmental Science 2007; 19(7) 854-858.

[18] Chakra Reddy, N., Venkateswara Rao, J. Biological response of earthworm, *Eisenia foetida* (Savigny) to an organophosphorus pesticide, profenofos. Ecotoxicology and Environmental Safety 2008; 71(2) 574-582.

[19] Castellanos, L.R., Hernández, T.C.S. Earthworm biomarkers of pesticide contamination: current status and perspectives. Journal of Pesticide Science 2007; 32(4) 360-371.

[20] Tejada, M., Masciandaro, G. Aplication of organic wastes on a benzo(a)pyrene polluted soil. Response of soil biochemical and role of *Eisenia fetida.* Ecotoxicology and Environmental Safety 2011; 74(4)668-674.

[21] Capowiez, Y., Raulth, H., Castagliola, G., Mazzia, C. Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). Biology and Fertility of Soils 2005; 41(3) 135-143.

[22] Spurgeon, D.J., Weeks, J.M. Evaluation of factors influencing results from laboratory toxicity tests with earthworms. In: Sheppard, S.C., Brembridge, J.D., Holmstrup, M., Posthuma, L. (eds.) Advances in earthworm ecotoxicology. SETAC, Pensacola, pp 15-25; 1998.

[23] Ma, W., Bodt, J. Differences in toxicity in the insecticide chlorpyrifos to six species of earthworms (Oligachaeta, Lumbricidae) in standardiezed soil tests. Bulletin of Environmental Contamination and Toxicology 1993; 50(6) 864-870.

[24] FAO. Carte mondiale des sols. Légende révisée. pp. 125; 1989.

[25] Soil Survey of England and Wales. Soil Survey Laboratory methods, Tech. Monogr. vol. 6, Harpenden, UK; 1982.

[26] MAPA. Métodos oficiales de análisis. Ministerio de Agricultura, Pesca y Alimentación; 1986.

[27] Yeomans, J.C., Bremner, J.M. A rapid and precise method for routine determination of organic carbon in soil. Communications in Soil Science and Plant Analysis 1988; 19(13) 1467-1476.

[28] Kononova, M.M. Soil organic matter, (second ed.), Pergamon Press, Oxford; 1966.

[29] Ritchie, J.D., Perdue, E.M. Proton-binding study of standard and reference fulvic acids, humic acids, and natural organic matter. Geochimica et Cosmochimica Acta 2003; 67(1) 85-96.

[30] Cabrera, A., Cox, L., Velarde, P., Cornejo, J. Terbuthylazine persistence in an organic amended soil. Journal of Environmental Science and Health, Part B 2008; 43(8) 713-716.

[31] Moreno, J.L., García, C., Hernández, T. Toxic effect of cadmium and nickel on soil enzymes and the influence of adding sewage sludge. European Journal of Soil Science 2003; 54(2) 377-386.

[32] Cabrera, A., Cox, L., Fernández-Hernández, A., García-Ortiz Civantos, C., Cornejo, J. Field appraisement of olive mills solid waste application in olive crops: Effect on herbicide retention. Agriculture, Ecosystems and Environment 2009; 132(3-4) 260-266.

[33] Bruna, F., Pavlovic, I., Celis, R., Barriga, C., Cornejo, J., Ulibarri, M.A. Organohydrotalcites as novel supports for the slow release of the herbicide terbuthylazine. Applied Clay Science 2008; 42(1-2), 194-200.

[34] Maboeta, M.S., Reinecke, A.J., Reinecke, S.A. 1999. Effects of low levels of lead on growth and reproduction of the Asian earthworm *Perionyx excavates* (Oligochaeta). Ecotoxicology and Environmental Safety 1999; 44(3) 236-240.

[35] Mishra, P.C., Dash, M.C. Digestive enzymes of some earthworms. Experientia 1980; 36 1156-1157.

[36] Habig, W.H., Pabst, M.J., Jakoby, W.B. Glutathione-s-transferases. Journal of Biological Chemistry 1974; 249 7130-7139.

[37] Saint-Denis, M., Labrot, F., Narbonne, J.F., Ribera, D. Glutathione, glutathione-related enzymes, and catalase activities in the earthworm *Eisenia fetida andrei*. Archives of Environmental Contamination and Toxicology 1998; 35(4) 602-614.

[38] Statistical Graphics Corporation. Statgraphics 5.0. Statistical Graphics System, Educational Institution Edition; Manugistics, Inc., Rockville, MD, USA p. 105; 1991.

http://dx.doi.org/10.5772/54948

[39] Brunninger, B., Viswanathan, R., Beese, F. Terbuthylazine and carbofuran effects on growth and reproduction within three generations of *Eisenia andrei* (Oligochaeta). Biology and Fertility of Soils 1994 ; 18(2) 83-88.

[40] Ribeiro, S., Sousa, J.P., Nogueira, A.J.A., Soares, A.M.V.M. Effect of endosulfan and parathion on energy reserves and physiological parameters of the terrestrial isopod *Porcellio dilalatus*. Ecotoxicology and Environmental Safety 2001; 49 131-138.

[41] Mosleh, Y.Y., Paris-Palacios, S., Couderchet, M., Vernet, G. Acute and sublethal effects of two insecticides on earthworms (*Lumbricus terrestris* L.) under laboratory conditions. Environmental Toxicology 2003; 18(1) 1-8.

[42] Haque, A., Ebing, W. Toxicity determination of pesticides to earthworms in the soil substrates. Zeitschrift fuer Pflanzenkrankheiten und Pflanzenschutz 1983; 90(4) 395-408.

[43] Dolaptsoglou, Ch., Karpouzas, D.G., Menkissogh-Spirondi, U., Eleftherohorinos, I., Voudrias, E.A. Influence of different organic amendments on the degradation, metabolism, and adsorption of terbuthylazine. Journal of Environmental Quality 2007; 36(6) 1793-1802.

[44] Sluzny, C., Graber, E.R., Gerstl, Z. Sorption of s-triazine herbicides in organic matter amended soils: Fresh and incubated systems. Water, Air and Soil Pollution 1999; 115(1-4) 395-410.

[45] Datta, A., Sanyal, S.K., Saha, S. 2001. A study on natural and synthetic humic acids and their complexing ability towards cadmium. Plant Soil 2001; 235(1) 115-125.

Chapter 3

Phytotoxicity of Phenolic Acids From Cereals

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Additional information is available at the end of the chapter

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1. Introduction

Allelopathic interactions between individuals of different plant species or those of the some species are caused by plant-produced allelochemicals. Once released into the environment, passively or actively they can influence germination, growth and development of neighboring plant either negatively or positively [1, 2]. Most allelochemicals are characterized by multi-functional phytotoxicity and are often also important for general defense. Generally stressed donor plants increased allelopathic activity due to increased production of allelochemical. Factors such as placement of residues, nutrient status, environmental conditions and microbial activity also affect allelopathy [3-5].

In recent years there has been an increasing focus on the prospects of exploiting allelopathy for controlling weeds but also insects and diseases. Allelopathy plays an important role in the agro ecosystem leading to a wide array of interactions between crop-crop, crop-weed and tree-crop. Generally, these interactions are harmful to the receiver plants but provide a selective benefit to the donor. Several members of crops exhibit allelopathic interactions that play a significant role in the complex environment of the agro ecosystem.

The allelochemicals are released largely by plant residues that are left in the fields after the harvest of a crop or through use of cover crops. Research on allelopathic interactions has been focused in agricultural crops as on option in the development of integrated weed management strategies, reducing environmental effects and the cost of crop protection [6-10]. Secondary metabolites with allelopathic properties are thought to protect plants against competing plants. For instance, allelopathic activity of decomposing wheat (*Titicum aestivun* L.), and oat (*Avena sativa* L.) straw on some crop species has been reported (11). Allelopathic potential of rye [12-14] and rice [6, 15, 16] has been extensively studied. In cereals such as, maize (*Zea may* L.), wheat (*Titicum aestivun* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.), rice (*Oryza*

sativa L.) and sorghum (*Sorghum bicolor*) a variety of allelochemicals have been identified including hydroxamic acids, cumarines, alkaloids, flavonoids and phenolic acids.. The allelopathic activity of these cereals may arise from one or the combined action of a group of allelochemicals. For example, hydroxamic acids appear to be responsible for the allelopathic effect of wheat, maize and rye [13, 17-19], indole alkaloids in allelopathic effect of barley [20, 21] and phenolic acid in the allelopathic effect of rice and sorghum [15, 22-25]. Rye is an example of a plant which provides excellent weed suppression through allelopathic mechanism. Rye and its residues which strongly inhibit germination and seedling growth of several dicot and monocotyledonous plants species. Several studies have demonstrated the allelopathic characteristic of rye residues are in agreement with the contents of hydroxamic acids in the plants and their degradation products [17, 26-29]. The allelopathy of an allelochemicals depends on the target species, dose, structure and their physicochemical properties. Both biotic and abiotic factor can trigger the allelopathic potential of a plant [3, 30-33]. The effectiveness of allelochemicals is therefore considered to be highly dynamic.

A basic step to understanding the allelophatic properties of a compound is to evaluate its phytotoxic properties. Phytotoxicity in plants may have some of the following effects: toxicity to the radicle growth of monocots and dicots; inhibitory effect on the energy metabolism of chloroplasts and mitochondria and modification of the binding affinity of the receptor sites of membranes. Parameters such as chemical stability, lipophilia, and acid-base or electrophilic-nucleophile interactions can be involved in the molecular mechanism of action or the dynamic in the environment.

Lipophilia is an essential parameter to establish the quantitative structure-activity relationship of phytotoxicity.

The n-octanol/water partition coefficient (kow) provides direct information on lipophilicity that describes the tendency of distribution of a solute from the aqueous phase into organic constituents of environmental compartments and even into biological membranes [34,35]. This has made it one of the most commonly reported physico-chemical properties of drugs, pesticides and other chemicals [36-38].

Numerous researchers have realized that there is a close parallel between the retention of compounds in reverse phase high performance liquid chromatographic columns (RP-HPLC) and octanol-water partition coefficients (kow). This technique is rapid and has the advantages that small samples suffice, the substances need not be pure and the exact volume of the phases need not be known.

Studies have tried to link this correlation to biological activity [39-45]. However, lack of success may be due to insufficient homogeneity or chemical diversity in the data set.

To gain a deeper understanding of the potential allelopathic properties of phenolic acids, we compared the phytotoxic activity of a series of phenolic acids, some of which are present in cereal cultivars (Figure 1).

The structural effect and role of molecular lipophyilia determined from RP-HPLC method were analyzed from the standpoint of a structure-phytotoxicity relationship using lettuce seeds

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(*Lactuca sativa*) and the alga *Chlorella vulgaris*, one of the most commonly used species in microalgae toxicity bioassay testing [46].

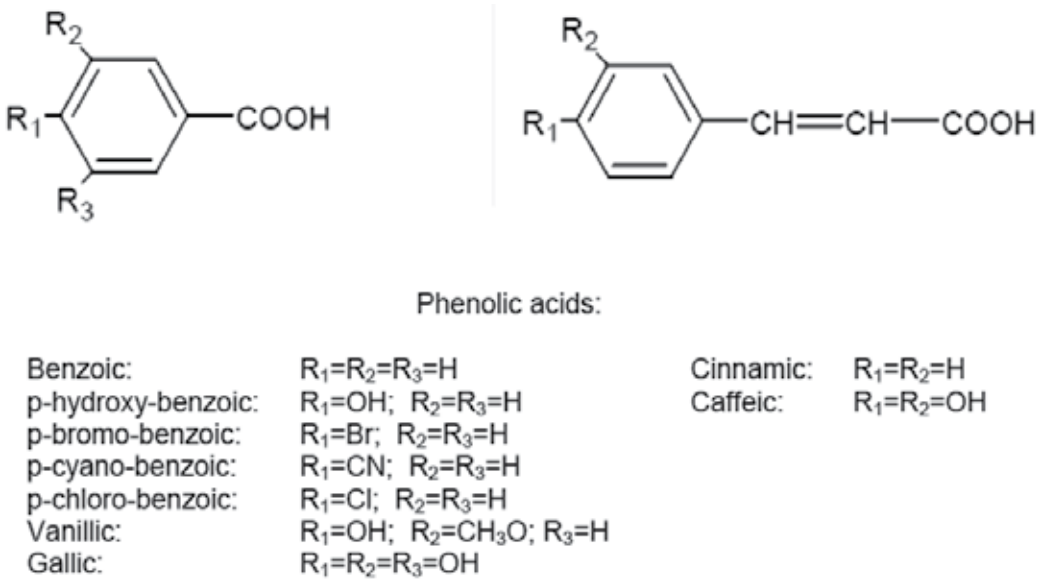

Figure 1. Structure of phenolic acids.

2. Experimental

Chemicals: Carboxylic acids were obtained from a commercial source (Aldrich Chemical Co.)

LogP_{HPLC} values: The capacity factor (k′) for the compounds used in this study were determined from $k' = (t_R - t_M)/ t_M$, where t_R is the retention time of the compound and t_M is the retention time of the non-retained compound (thiourea). RP-HPLC was carried out in a C_{18} column with mobile phase water (pH:3 phosphoric acid)/ acetonitrile 60:40 v/v [47]. The relation between k′ and n-octanol-water partition coefficients were established by linear regression of logk′ and logkow values obtained from the literature. LogP_{HPLC} values were derived from the relationship:

$$\log P_{HPLC} = 1.08 \log k' + 1.72$$

Germination assays: 45 lettuce seeds were uniformly placed on Petri dishes covered with cotton film. In order to maintain individual compound concentrations, each plate was watered with 8 mL of an aqueous solution of 100 or 250 μg/mL of each compound. The plates were sealed and incubated at 25 ± 2^0 C in an 8:16 h light: dark cycle for 6 d. Controls were incubated only with water. Each assay was replicated three times Germination inhibition was expressed as percentage of the control.

Antialgal test: Test compounds were dissolved in nutrient growth medium (Gibco) with the aid of either ultrasound or gentle heating. *Chlorella vulgaris* (Laboratory of Microbiology, Faculty of Science, University of Chile) was grown in nutrient growth medium. Samples were incubated at 25^0 C for 10 d in test tubes containing 4.0 x10^4 colony forming units (CFU) under continuous cold white fluorescent light with an intensity of 200 ft. c. The growth of *C. vulgaris* was assessed by turbidity measured by the spectrophotometric method at 600 nm.

Percentage inhibition was obtained as 100 (Ts – Tc)/(100 – Tc), where Ts is the sample transmittance and Tc the control transmittance.

3. Results and discussion

Table 1 shows the germination inhibition activity of phenolic acids (Figure 1). In the concentration range studied (100 – 250 μgmL^{-1}), cinnamic, p-bromo, p-chloro and benzoic acids showed the highest levels of germination inhibition of lettuce seeds. The effect of water-soluble inhibitor compounds associated with allelopathic plants is often more pronounced on the growth of an indicator than on its germination, and depends on the dose and the receptor plant. In fact, previous studies demonstrated that when phenolic acids are exuded from wheat, barley, wild oat and cucumber plants [24, 25], they did not inhibit germination but did inhibit the growth of *Brassica kaber* at a lower concentration than those used in our assays.

Phenolic acid	Germination inhibition (%)		antialgal activity (%)		logk´	log P_{HPLC}
	100 (μgmL^{-1})	250 (μgmL^{-1})	100 (μgmL^{-1})	250 (μgmL^{-1})		
Cinnamic	77.0	98.7	5.1	11.3	0.30	2.08
Benzoic	2.6	86.3	0.0	0.0	0.23	1.97
p-hydroxy- benzoic	0.0	5.3	0.0	0.0	- 0.41	1.28
Vanillic	2.1	4.2	0.0	0.0	- 0.37	1.32
Caffeic	0.0	0.0	100	100	- 0.51	1.17
Gallic	0.0	0.0	57.6	81.1	- 1.15	0.48
p-bromo benzoic	16.3	83.1	47.7	48.3	0.98	2.78
p-cyano benzoic	35.6	53.4	11.3	17.7	- 0.1	1.61
p-chloro benzoic	100	100	ND	ND	0.83	2.62

Table 1. Inhibitiory effect (%) on germination of lettuce seeds (*L. sativa*), antialgal activity (*C. vulgaris*) and values of logk´ and log P_{HPLC} of phenolic acids.

Each value corresponds to the mean of three samples; replicate values showed errors below 5% in all cases. ND: not determined. The data were analyzed by one-way ANOVA.

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In addition, we observed that caffeic acid did not inhibit germination but it stimulated seedling growth, particularly the root elongation of lettuce, in the concentration range 50-500 μgmL^{-1}.

Since measuring seedling length is often complicated due to curling and other morphological alterations, seedling fresh weight may be a better bioindicator to evaluate stimulation activity. Figure 2 shows the relation of the percentage of fresh biomass increase with respect to caffeic acid concentration; the greatest increase was observed at 250 μgmL^{-1}.An increase in plant growth produced by a chemical structure stimulator may be related to the stimulation of auxin-induced growth or to an improvement of the growth substrate because of organic matter enrichment. Further studies are necessary to establish the cause of the stimulation observed.

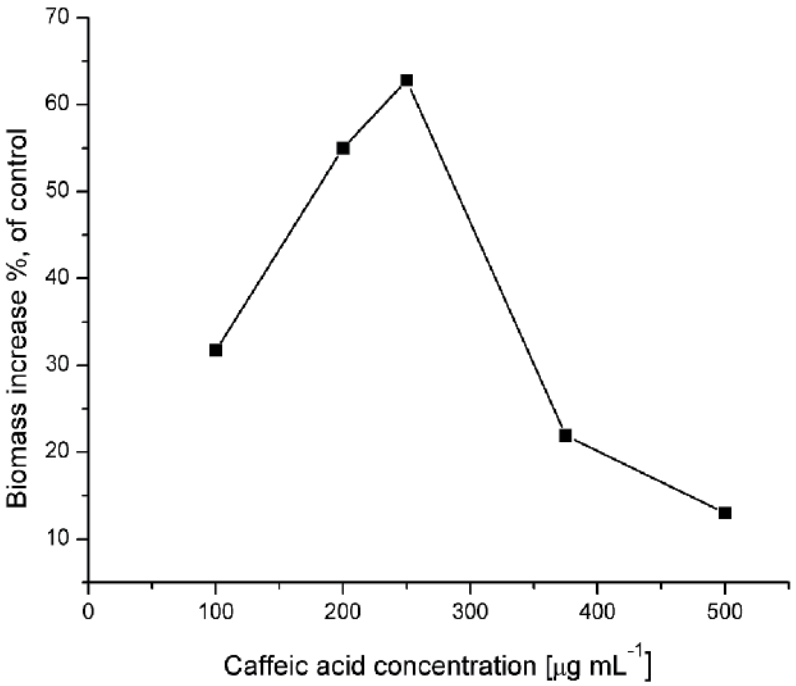

Figure 2. Effect of caffeic acid on fresh biomass increase of lettuce seedlings with respect to control (values correspond to the mean of three samples, replicate values had errors below 5% in all cases).

The phytotoxicity of the compounds involved in allelopathic effects depends upon the target species. Microalgae responded rapidly to environmental changes owing to their short germination time. Green microalgae such as *Chlorella* are taxonomically classified as plants bearing some similarity to higher plants. For this reason, a microalgae test may be used to evaluate herbicidal activity against higher plants [46]. The phytotoxicity of carboxylic acids was also

examined against the fresh water green alga *Chlorella vulgaris*. The percentages of *in vitro* growth inhibition are given in Table 1: gallic, caffeic and p-bromo benzoic acids showed the highest antialgal activity.

The chemical mechanism of the phytotoxicity of phenolic acids is not well understood. However, carboxylic acids and phenols are known to induce uncoupling of oxidative phosphorylation and photophosphorylation in mitochondria and chloroplasts. The uncoupling activity depends upon carboxylic function ionization. Pka values of the series studied were in the small range 4.0-4.5; therefore the substantial differences between the observed activities may not depend upon the ionization of the O-H bond of carboxylic function.

Hansch [48], proposed a theory to rationalize the relationship between the chemical structure and biological activity of auxins. The hypothesis assumes that auxins with an aromatic ring and side chain react with a plant substrate via two points, one on the side chain and another on the aromatic ring. A critical step should be the movement of the compounds from solution to the action sites. The lipophilia parameter is essential in the penetration rate and dynamics in the physiological system. This hypothesis must be considered to understand the mechanism of action the phenolic acids.

Although cinnamic and caffeic acids have identical side chains, they showed opposite phytotoxicities. At the concentration of 250 μgL^{-1}, cinnamic acid had the highest germination inhibition activity and low antialgal activity, while caffeic acid stimulated the growth of lettuce seedlings and showed the highest antialgal activity. This suggests that the difference of activity may arise from the structure of the aromatic ring. Compounds with carboxylic function bonding at the aromatic ring and with hydroxyl group substituents such as p-hydroxy benzoic acid, vanillic, caffeic and gallic acids did not show germination inhibition effects but the p-bromo, p-chloro and p-cyano derivatives showed significant activity.

These results suggest that part of the phytotoxicity may be related at the step of interaction of the aromatic ring with the substrate plant and the lipophilic-hydrophilic balance may have a fundamental role.

As mentioned above, the kow determined from the traditional shake-flash method is generally accepted as being a useful parameter in structure-activity relationship studies of correlation with biological activity of compounds and the dynamic processes of a chemical in the environment. It is now almost equally accepted that the correlation between the capacity factor (k′) obtained from RP-HPLC method and kow to be used for simple and rapid estimation of partition coefficients.

With this in mind, we established a RP-HPLC method to evaluate the lipophilia of phenolic acids, as shown in figure 3.

A linear regression between the capacity factor (k′) and logkow from literature sources was obtained ($R^2 = 0.99$). From this regresion log P_{HPLC} values were derived by a single equation (see experimental). Table 1 gives the logk′and logP_{HPLC} values

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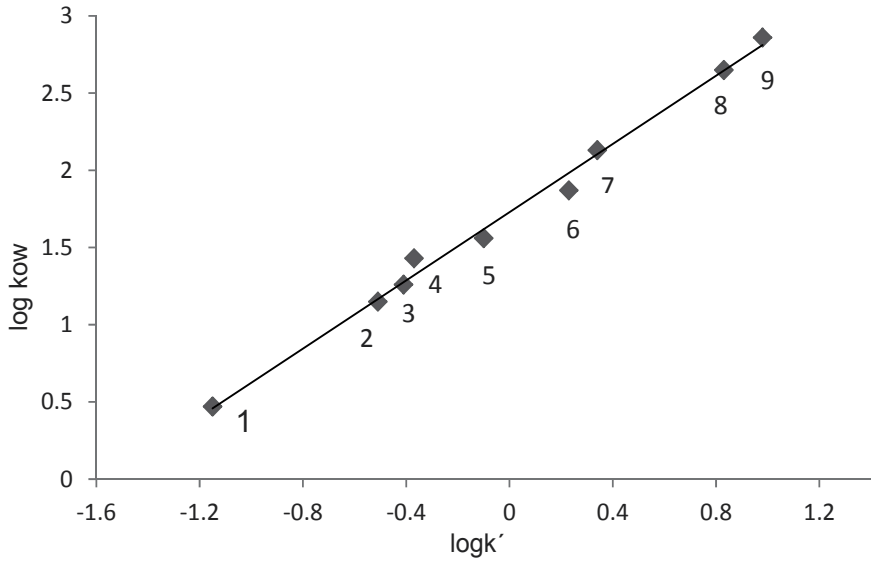

Figure 3. Relationships between the logkow (literature data) and determined logk´ values. (Phenolic acids: 1 = gallic; 2 = caffeic; 3 = p-hydroxy benzoic; 4 = vanillic; 5 = p-cyanobenzoic; 6 = benzoic; 7 = cinnamic; 8 = p-chlorobenzoic; 9 = p-bromobenzoic).

Even on the basis of the limited number of compounds studied, the correlation obtained shows a promise as a simple; direct and rapid method for the estimation of lipophilicity parameters of new phenolic acids and or those for which this data does not exist.

Positive and negative logk´ values were obtained, which may arise from the lipophilic character of the aromatic substituents. Lipophilia values can be obtained from the parameter pi (π) as a measure of the lipophilic properties of the aromatic substituents [49]. Compounds with negative logk´ have substituents with negative π values ($\pi_{CH3)} = - 0.04$; $\pi_{(OH)} = - 0.62$; $\pi_{(CN)} = - 0.31$) and compounds with positive logk´ have zero or positive π values ($\pi_{H} = 0$; $\pi_{Br^-} = 1.02$; $\pi_{Cl^-} = 0.7$).

More clarity about the lipophilic effect of the substituents on the capacity factor is obtained from the relation between logk´ values for p-substituent compounds and the π values. Figure 4 illustrates that logk´ increased linearly with the lipophilic character of the substituents.

These results suggest strongly that part of the lipophilic properties of phenolic acids arise from the different lipophilic characters of the aromatic ring substituents.

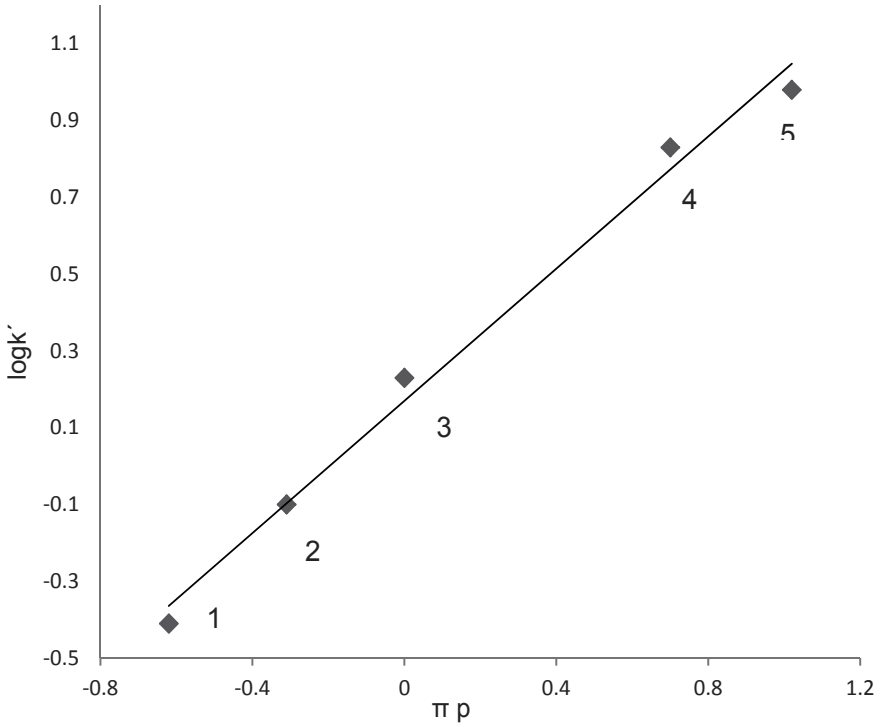

Figure 4. Relationship between logk´ values and πp values for p-substituteed phenolic acids (1 = OH; 2 = CN; 3 = H; 4 = Cl; 5 = Br).

The role of lipophilia on the phytotoxicity of the series studied can be analyzed from the logk´or log P_{HPLC} values

In general, the compounds with positives logk´or $logP_{HPLC}$ values ≥ 2.0 showed more germination inhibition activity and the compounds with negative values did not show significant activity. Specifically, logk´ values of caffeic and cinnamic acids suggest that the different activity observed may arise from the different lipophilic character of these compounds. A clearer relationship was obtained from a regression between percentage of germination inhibition activity at the 250 μgmL^{-1} concentration and the $logP_{HPLC}$ values. Figure 5 shows that germination inhibition activity increased with the lipophilic character of the molecules until the range of 2.0-2.6 values where the higher activity was observed. With the limited data at hand, it is not possible to generalize this observation to other series of compounds at this time, but there are reasons to believe that the parameters logk´ or $logP_{HPLC}$ may be appropriate indicators to infer the lipophilic requirement for the germination inhibition activity of phenolic acids.

Antialgal activity cannot be rationalized in terms of the logk´ or $logP_{HPLC}$ parameters because only the caffeic, gallic and p-bromobenzoic acids displayed significant activity, and they showed positive and negative logk´ values. It is important to emphasize that caffeic acid

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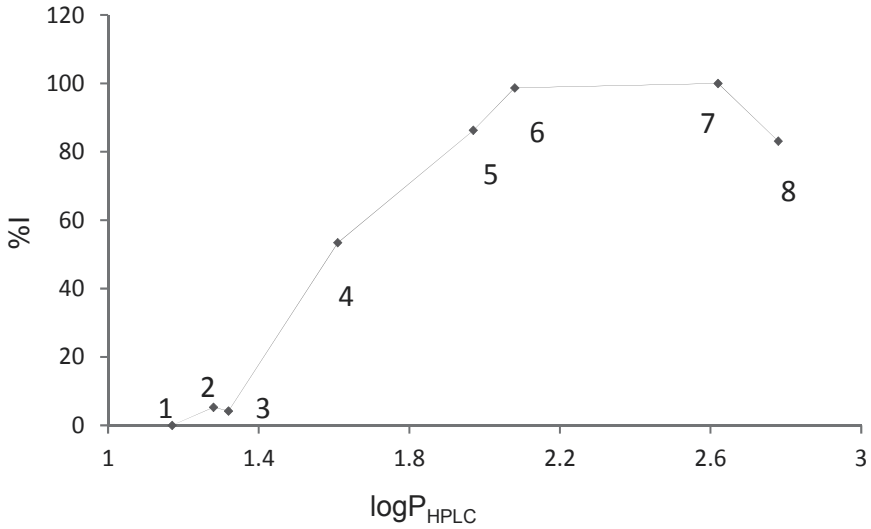

Figure 5. Relationships between $logP_{HPLC}$ values and percentage of inhibition germination activity (I%) for phenolic acids (1 = caffeic; 2 = p-hydroxybenzoic; 3 = vanillic; 4 = p-cyanobenzoic; 5 = benzoic; 6 = cinnamic; 7 = p-chlorobenzoic; 8 = p-bromobenzoic)

(logk′= - 0.51) displayed the highest antialgal activity and the opposite effect on the germination and growth of the lettuce plants.

These results show that the phytotoxic effect depends of receptor species, it is necessary to understand other molecular aspects of phytotoxic interaction to clarify these results.

4. Conclusions

- The series of phenolic acids studied shown varied phytotoxic activity against the germination of lettuce seeds and growth of microalga *C. vulgaris.*
- Caffeic acids showed a particular behavior against the target species stimulated the growth of lettuce plants and inhibited the growth of *C. vulgaris*.
- Part of phytotoxic activity against seed germination is related to the lipophilic character of phenolic acids and it can be inferred from logk′ or $logP_{HPLC}$ parameters.
- Although the concentrations used are probably greater than in the field, these results contribute to the knowledge of the relative role of the allelopathy of the naturally occurring phenolic acids of plants with agricultural importance.

The necessity of understanding other aspects such as the chemical stability and persistence in the soil are essential prerequisites if the application of allelochemicals is to become an alternative in the control of weeds.

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References

[1] [1] Harborne J B. Introduction to Ecochemical Biochemistry. Academic Press, London 1977.

[2] [2] Rice E C. Allelopathy 2nd ed. Academic Press Orlando 1984.

[3] [3] Einhellig F A. Interactive effects of allelochemicals and environmental stress. Chou C H- Waller G R. (eds) Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones. Institute of Botany. Academia Sinica Science Monograph Series N0 9 Taipei ROC 1989; pp 101-118.

[4] [4] Einhellig F A. Mechanism and mode of action of allelochemicals. In "The Science of Allelopathy" (Putman A R, Tang C S. Eds.) John Willey & Sons Inc New York 1986; pp 171-175.

[5] [5] Einhellig F A. Interaction involving allelopathy in cropping systems. Agronomy Journal 1996; 88 886-893.

[6] [6] Bhowmik P C. Inderfit. Challenges and opportunities in implementing allelopathy for natural weed management. Crop. Protection 2003; 22 661-671.

[7] [7] Weston L A. Utilization of allelopathy for weed management in agro ecosystems. Agronomy Journal 1996; 88 860 – 866.

[8] [8] Swranton C J. Marphy S D. The role of integrated weed management (IWM) in agro ecosystem health. Weed Science 1996; 44 437 – 445.

[9] [9] Batish D R, Singh H P, Kohli R K, Kaur S. Crop allelopathy and its role in ecology agriculture, Journal Crop Production 2001; 4 121 -161.

[10] [10] Fomsgaard I S. Chemical ecology in wheat plant - pest interaction. How the use of modern techniques and multidisciplinary approach can throw new light on a well-known phenomenon: Allelopathy. Journal Agricultural and Food Chemistry 2006; 54 987 – 990.

[11] [11] Dias L S. Allelopathic activity of decomposing straw of wheat and oat and associated soil on some crop species. Soil & Tillage Research 1991; 21 113-120.

[12] [12] Putnam A R., De Frank J. Use of phytotoxic plant residues for selective weed control. Journal Crop Protection 1983; 2 173-181.

[13] [13] Sicker D, Frey M, Schulz M, Gierl A. Role of Natural Benzoxazolinones in survival strategy of plants. In: Jeong K W. (ed). International Review of Cytology. A Survey of Cell Biology. Academic press, San Diego 2000; pp 319-346.

[14] [14] Burgos N R, Talbert R E. Differential activity of allelochemical from *Secale cereale* in seedling bioassay. Weed Science 2000; 48 302-310.

[15] [15] Ahn J K., Chung I M. Allelophatic potential of rice hulls on germination and seedling growth of Barnyard grass. Agronomy Journal 2000; 92 1162 – 1167.

[16] [16] Chung I M., Kim K H., Ahn J K., Ju H J. Allelophathic potential evaluation of rice cultivars on *Echinochloa crus-galli* Korean. Journal Weed Science 1997; 17 52 – 58.

[17] [17] Copaja S V, Villarroel E, Bravo H R, Pizarro L, Argandoña V H. Hydroxamic acids in *Secale cereale* L. and the relationship with their antifeedant and allelopathic properties. Zeitschrift fur Naturforschung 2006; 61c 670-676.

[18] [18] Wu H., Haig T, Pratley J, Lemerle D, An M. Allelochemicals in wheat (*Triticum aestivum* L.) production and exudation of 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one. Journal of Chemical Ecology 2001; 27 1691-1700.

[19] [19] Macias F A, Marin D, Oliveros-Bastidas A, Castellano D, Simonet A M, Molinillo J M G. Structure-activity relationships (SAR) studies of Benzoxazolinones, their degradation products and analogues. Phytotoxicity on standard target species (STS). Journal Agricultural and Food Chemistry 2005; 53 538-548.

[20] [20] Bravo H R, Iglesias M J, Copaja S V, Argandoña V H. Phytotoxicity of indole alkaloids from cereals. Revista Latinoamericana de Química 2010; 38 123-129.

[21] [21] Overland L. The role of allelopathic substances in the "Smother crop" Barley. American Journal of Botany. 1966; 53 423-432.

[22] [22] Guenzi W D, Mac Calla T M. Phenolic acids in oat, wheat, sorghum and corn residues and their phytotoxicity. Agronomy Journal 1996; 58 303 – 304.

[23] [23] Solomonsson A., Theander O. Anian P. Quantitative determination by GLC of phenolic acids as ethyl derivatives in cereal straws. Journal Agricultural and Food Chemistry 1978; 26, 830 – 835.

[24] [24] Baghestani A, Limieux C, Baziramakenga R, Simarda R R determination of allelochemicals in spring cultivars cereal of different competitiveness. Weed Science 1999; 47 498-504.

[25] [25] Yu J Q, Matsui Y. Phytotoxic substances in root exudates of cucumber (*Cucumis sativ*es L.) Journal Chemical Ecology 1994; 20 21 – 31.

[26] [26] Barnes I P, Putman A R. Rye residues contribute weed suppression in no-tillage cropping system. Journal Chemical Ecology 1983; 9 1045-1057.

[27] [27] Barnes I P, Putman A R. Evidence for allelopathy by residues and aqueous extracts of rye (*Secale cereale* L.). Weed Science 1986; 34 384-390.

[28] [28] Barnes I P, Putman A R. Role of Benzoxazinones in allelopathy by rye (*Secale cereale* L.). Journal Chemical Ecology 1987; 13 889-906.

[29] [29] Pérez F J, Ormeño-Nuñez J. Weed growth interference from temperate cereals: effect of a hydroxamic acids-exuding rye (*Secale cereale* L.) cultivar. Weed Research 1993; 33 115-119.

[30] [30] Gianoli E. Niemeyer H M. Environmental effects on the accumulation of hydroxamic acids in wheat seedling: The importance of plant growth rate. Journal Chemical Ecology 1997; 23 543-551.

[31] [31] An M. Johnson I R. Lovett J V. Mathematical modeling of residues allelopathy: The effects of intrinsic and extrinsic factors. Plant and Soil 2002; 246 11-22.

[32] [32] Matsuo H.Taniguchi K. Hiramoto K. Yamada, Ichinose Y. Toyada k. Tareda K. Shiraishi T. Gramine Increase associated with rapid and transient systemic resistance in barley seedling induced by mechanical and biology stresses. Plant Cell Physiology 2001; 42 1103-1111.

[33] [33] Kato-Noguchi H. Effect of light-irradiation on allelopathic potential of germinating maize. Phytochemistry 1999; 52 1023-1027.

[34] [34] Karickhoff S W. Semi-empirical estimation of sorption of hydrophobic pollutants in natural sediments. Chemosphere 1981; 10 833-846.

[35] [35] Kenaga E E. Correlation of bioconcentration factors of chemicals in aquatic and terrestrials organism with their physical and chemical properties. Environmental Science Technology 1980; 14 553-536.

[36] [36] Leo A C, Elkins D. Partition coefficients and their uses. Chemical Review 1971; 71 525 616.

[37] [37] Finizzio A, Vighi M, Sandroni D. Determination of octanol/water partition coefficient (Kow) of pesticides: critical review and comparison of methods. Chemosphere 1977; 34 131 – 161.

[38] [38] Laak A M. Tsai R S. Donné-Opderkelder G M. Carrup P A. Testa B. Timmerman H. Lipophilicity and hydrogen-bonding capacity of H_1-antihistamine agents in relation to their central sedative side-effects. European Journal of Pharmaceutical Sciences 1994; 2 373-384.

[39] [39] Baker J K, Rauls D O, Borne R F. Correlation of biological activity and high-pre-assure liquid chromatography retention Index for a series of Propranolol Barbiturate and Anthralinic acid analogues. Journal of Medicinal Chemistry 1979; 22 1301 – 1306.

[40] [40] Nahum A, Horvath C. Evaluation of octanol-water partition coefficients by using high performance liquid chromatography. Journal of Chromatography 1980; 192 315 – 322.

[41] [41] Unger S H, Chiang G H. Octanol physiological buffer distribution coefficients of lipophilic amines by reversed phase high-performance liquid chromatography and their correlation with biological activity. Journal of Medicinal Chemistry 1981; 24 262 – 270.

[42] [42] Han S-Y., Qiao J-Q, Zhang Y-Y, Yang L-L, Lian H-Z, Ge X, Chen H-Y. Determination of n-octanol/water partition coefficient for DDT-related compounds by RP-HPLC with novel dual-point retention time correction. Chemosphere 2011; 83 131 – 136.

[43] [43] Valko K, Bavan C, Reynolds D. Chromatographic hydrophobicity index by fast gradient RP-HPLC: High-throughput alternative to log P/log D. Analytical Chemistry 1997; 69 2022 – 2029.

[44] [44] Guo R, Iang X, Chen J, Zhang Q, Kettrup A. Prediction of soil adsorption coefficients from retention parameters on three reversed-phase liquid chromatographic columns. Analytical Chemistry 2002; 74 655 – 660.

[45] [45] Hollosy F, Lorand T, Orfi L, Eros D, Keri G, Idei M. Relations hip between lipophilicity and antitumor activity of molecule library of Mannich ketones determined by high-performance liquid chromatography, C log calculation and cytotoxicity test. Journal of Chromatography 2002; 768 361 – 368.

[46] [46] Rioboo C, González D, Herrero C, Cid A. Physilogical response of fresh water microalga (*Chlorella vulgaris*) to triazinne and phenyl-urea herbicides. Aquatic toxicology 2002; 59 225 – 235.

[47] [47] Mimick D J, Frenz J H, Patrick M A, Brend D A. A compressive method for determining hydrophobicity constants by reversed-phase high-performance liquid chromatography. Journal of Medicinal Chemistry 1988; 31 1923 – 1933.

[48] [48] Hansch C, Muir R M, Fujita T, Maloney P. P, Geiger F, Streich M. The Correlation of biological activity of plant growth regulators and *Chloromycetin* derivatives with Hammett constants and partition coefficients. Journal Chemical Society 1963; 85 2817 – 2824.

[49] [49] Hansch C, Leo A. Substituent constants for correlation analysis in Chemistry and Biology. J. Wiley: New York. 1979.

Chapter 4

Weed Seed Rain Dynamics and Ecological Control Ability in Agrophytocenosis

Vytautas Pilipavičius

Additional information is available at the end of the chapter

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1. Introduction

Evolving together with agricultural plants, weeds have adapted their growth and biological cycle of development. The dispersal of weed seeds in agriculture fields is increased by current grain harvesting technology after seed set [1, 2]. Herbicides are used to prevent new weed seed bank additions. Although herbicides cannot control all weeds, they may partially control them, thus weeds ripen fewer seed numbers. Therefore, infest soil, straws and awns by seeds [3]. Intensive use of herbicides following the traditional crop growing technologies, however, does not entirely solve the problem of weediness [4]. Surviving weeds after herbicide applications are able to produce new seeds [6], depending on species, significantly decreasing total seed production [5]. Even a few weed plants left undamaged by herbicides can produce considerable weed seed amounts [7]. Previous research of Leguizamon and Roberts (1982) revealed that after cultivation in early April of a sandy loam soil with 9500 apparently viable seeds m^2 in 0–10 cm, 295 seedlings m^2 emerged, of which about half survived to maturity in July. Seeds were dispersed from mid-June to November and 136,460 m^2 were returned to the soil, representing a 14-fold increase in the seed bank. Application of soil-active herbicides reduced the numbers of weeds and the total seed output, but that of tolerant species was increased. Maximum numbers of seeds were 59,980 m^2 for *Chenopodium album*, 39,430 m^2 for *Stellaria media* and 37,580 m^2 for *Veronica persica* [8]. Today more attention is given to ecological problems which arise through use of herbicides [9]. Pesticides are leaching through the soil and into groundwater far more commonly than the projected ones a decade ago. Point sources may be widespread but are not the sole cause; it is also clear that many pesticides are leaching to groundwater from routine, nonpoint source labeled use. Controlled plot studies show the intermittent, often rapid delivery of many pesticides to shallow groundwater. Generally, the concentrations of pesticides in groundwater are low, in the 0.1–5.0 $\mu g\ L^{-1}$ range. Even at these concentrations there are concerns for long-term, chronic exposure to a large segment of the public through drinking-water supplies [10].

According to Seralini et al. (2012) agricultural edible GMOs and formulated pesticides must be evaluated very carefully by long term studies to measure their potential toxic effects. First long-term research study "Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize" showed worrying results in rat pathology (tumor development, liver congestions and necrosis, etc.). The experimental object in this study was Roundup-tolerant genetically modified maize (from 11% in the diet), cultivated with or without Round-up, and Roundup alone (from 0.1 ppb in water) [79]. In recent years ecological and economic factors provided a need and a necessity to decrease the use of herbicides or even to refuse them entirely [11]. Research and policies to resolve the problems of agricultural impacts on the environment will require a new focus on integrated farm-management systems that enhance efficiency and reduce off-site impacts [10]. The quality of weed control in today's agriculture depends on the ability to eliminate seeds, which are still in the soil and to limit the amount of additions [1, 12], as well integrating non-chemical – ecologically acceptable – weed control means.

The research hypothesis: most weed seeds ripened in the crop would be removed from the field together with spring barley harvested in the late milk-early dough growth stage of maturity. Accordingly in the late milk-early dough growth stage spring barley maturity, the highest dry matter yield and energy is accumulated. *The aim* of this work was to evaluate weed seed rain dynamics, and implications on ecological and economic management.

2. Evaluation of spring barley agrophytocenosis

Field experiment site and soil type. A field experiment was conducted at 54°52'N and 23°49'E. The soil of the experimental site is *Calcari-Epihypogleyic Luvisols – LVg-p-w-cc* drained clay loam on sandy light loam. The soil agrochemical characteristics: pH_{KCl} 7.08-7.25, humus 2.22-2.45%, mobile P_2O_5 – 245.0-251.3 mg kg^{-1} and mobile K_2O – 93.6-110.5 mg kg^{-1}. Agrochemical soil properties were established using the infrared ray system PSCCO/ISI IBM – PC 4250. Soil samples for agrochemical analysis were taken from 0–20 cm soil layer from 10 sites of all treatments and their replications, making combined samples.

Experimental design. The experiment was arranged as a randomized complete block design with treatments including the following harvest timing, which was made on the basis of spring barley maturity stages by Zadoks [13] and Meier [14].

Spring barley was harvested at the stages of maturity:

1. Stem elongation 39-41*, 37-39, 31
2. Heading 57-59, 55, 57-59
3. Early milk 71-73, 69-71, 69-71
4. Milk (medium milk) 75, 73-75, 73
5. Late milk-early dough 77-83, 77-83, 77-83
6. Dough 87, 85, 87
7. Hard 92, 91-92, 92

* - decimal code for spring barley development during experimental years: 1997, 1998 and 1999.

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Experimental treatments were replicated four times. Total size of each experimental plot was 96 m^2 (4x24m) and results recording plot size – 66 m^2 (3x22m).

Spring barley growing conditions. The preceding crop for spring barley was winter wheat *Triticum aestivum* (1997), spring barley *Hordeum vulgare* (1998) and cultural amaranth *Amaranthus spp.* (1999) [1]. In every year of the experiment, double-row barley cv '*Roland'* was grown on different fields. Herbicides were not used in the experimental field for evaluation of alternative weed control. Complex phosphorus, potassium and nitrogen fertilizers (60 kg ha^{-1} of active compounds) "*Azofoska*" (N:P:K ratio 1:1:1 by 16%) were applied on spring barley in spring after sowing before sprouting. Soil tillage in every year of the trial was the same. Each year, mouldboard ploughing at approx. 24 cm depth was accomplished in September. Autumn and spring loosening at approx. 8 cm depth was performed in October and April respectively, while spring loosening with harrow at approx. 4 cm depth was accomplished in early May, just prior to crop sowing.

Spring barley productivity. At stem elongation, heading, early milk, milk (medium milk) and late milk-early dough growth stages of maturity spring barley was harvested by frontal reaper for biomass and at dough and hard stages by combine harvester for grain.

Whole-plant silage was prepared from spring barley biomass harvested at early milk, milk (medium milk), late milk-early dough and dough stages of maturity. Spring barley green biomass was chopped up with the grinding-mill and ensiled in 3 L volume glass jar taking into account method used by Wilson and Wilkins [15].

Laboratorial analyses of spring barley whole-plant biomass, grain and silage: dry matter, crude protein, crude fat, crude fibre, crude ash [16] and metabolizable energy for ruminants (cows) in MJ kg^{-1} of dry matter [17] were determined at each harvesting growth stage in prepared samples for analyses. Drying plant samples at 103°C for 4 hours, there was established the amount of dry matter and burning at 550°C for 3.5 hours in muffle-furnace, there was established the amount of crude ash. Crude protein was established by the *Kjeldahl* method and crude fat by direct extraction with petrol-ether for 6 hours in *Sokslet* device. The concentration of crude fibre was established by plant samples boiling with adequate concentration of sulphuric acid and potassium alkali, filtered, separated, washed, dried, weighed and burned at 500°C for 3 hours in muffle-furnace [16]. Metabolizable energy [MJ kg^{-1}] in dry matter of fodder for ruminants (cows) was established depending on gas production (CO_2 and CH_4) *in vitro* and fodder chemical composition, by the Hohenheim fodder value test. 200 mg of fodder sample with cow rumen fluid, micro- and macro-elements, buffer- and reduction-solutions is placed in the special test-tube and incubated in a rotary thermostat by 39 °C for 24 hours [17]. Silage fermentation analysis was made according to standard methods used in Agrochemical centre of Lithuanian institute of agriculture. It evaluated silage pH, concentrations of lactic, acetic and butyric acids.

Crop weediness. Weed samples were taken at the early milk stage of spring barley maturity. There were 10 samples taken from every experimental plot by wire frame of 20x30cm. Air-dried weeds were divided into species, counted and weighed.

Weed seed rain. Dynamics of weed seed rain in spring barley agrophytocenosis was established according to Rabotnov [18] method and other weed seed rain experiments [8, 19, 20]. Fifty troughs were laid out in each of four replications, in chess-order, in tens (Figure 1 and 2). In total, two hundred troughs were used. Weed seeds from the troughs were collected every 2-4 days. The collected seeds were divided into species and counted.

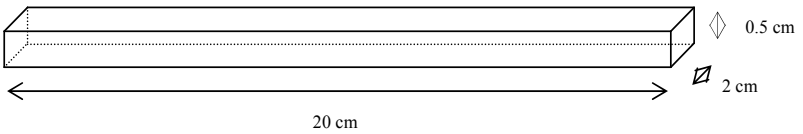

Figure 1. Schema of trough used for the weed seed rain establishment [1]

Figure 2.Troughs for collecting weed seeds in spring barley crop, photographs by Vytautas Pilipavičius

Meteorological conditions. The Lithuanian territory occupies intermediate geographical position between west Europe oceanic climate and Eurasian continental climate. The climate of the Lithuanian territory forms in different radiation and circulation conditions. Differences in these conditions hardly cross the boundaries of microclimatic differences; therefore, Lithuania belongs to the western region of the Atlantic Ocean continental climatic area [21]. Meteorological conditions during experimental years were established utilizing data of Kaunas (Noreikiškės) meteorological station situated in vicinity of the experimental fields. Meteorological factors taken included: average air temperatures, sum of active air temperatures (> 10^{o}C), rainfall (mm) and sunlight duration in hours.

Economic treatment evaluation. In order to evaluate the economics of alternative and conventional harvest technologies the valuations of agricultural machinery were used [22-24]. The price of fodder spring barley is evaluated in 320 Lt t^{-1}, and the normative price of straws in 27

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Lt t^{-1} [the ratio of national currency Litas (Lt) and Euro (€) is 1 € = 3.4528 Lt]. The value of spring barley biomass at the late milk-early dough growth stage of maturity was determined according to the energetic value of its grains and straws. Calculating the costs of spring barley harvested at the late milk-early dough growth stage of maturity includes autumn soil ploughing, autumn and spring continuous cultivation and cultivation with harrowing, sowing, green biomass pressing into rolls and their rolling with pellicle as well as transporting were evaluated. Calculating the costs of spring barley harvested at the hard stage of maturity includes autumn soil ploughing, autumn and spring continuous cultivation and cultivation with harrowing, sowing, as well as the cereal harvesting, grain and straw managing were evaluated. The tractor *MTZ-80/82*, the cereal harvester SK-5 „*Niva*" as well as various agricultural machinery were used for these proceedings.

Statistical data assessment. The research data were statistically evaluated by dispersion analysis ANOVA method applying *Selekcija* [25, 26] and *SigmaStat* [27] software packages. Degrees of phenomena interdependence and their directions were established by correlation-regression analysis applying *SigmaPlot* software package [28]. Reliability of dependencies was evaluated by the *p* criterion.

3. Weediness of spring barley agrophytocenosis

The field experiments were carried out in separate fields with different weed infestations (Table 1). The experiment initiated on a very weedy field. The second year of the experiment trial was moved to the field where weed density was established more than three times and weed air-dry biomass was 2.6 times less comparing with the spring barley agrophytocenosis of the first year experiment. During the experiment in 1999 weed density was 135 weeds m^{-2}, i.e. analogically as in 1998 but their air-dry biomass was more than 6 times less and weighed only 18.9 g m^{-2}. During the three year experiment in spring barley agrophytocenosis, annual weeds dominated accounting for 68-98% of crop air-dry weed biomass and 84-98% of the total weed number. Perennial weeds comprised 2-32% of total weed air-dry biomass and 2-16% of the total weed number in the crop. Our results are similar to previous research indicating in Lithuania prevailing weeds as short-lived annual dicotyledons that comprise 70-90% of total spread weeds [4, 29]. Consequently, in the experimental spring barley agrophytocenosis, annual weeds prevailed that are commonly spread by seeds while perennials commonly propagate by vegetative parts and spreading by seeds is less important except for infesting new soils. However, Zwerger [30] pays high attention to the perennial weed spreading by seeds analyzing potential danger of *Cirsium arvense* spreading. From annual weeds in the crop prevailed *Chenopodium album*, *Stellaria media* and *Sonchus asper* while from perennial ones - *Sonchus arvensis*. During all three years of experiment, 40 weed species, 32 annual and 8 perennial, were found. Twenty-six weed species were established in spring barley agrophytocenosis during the first year, 19 during the second and 21 during the third year of the experiment (Table 1).

Weeds	Weed density and air-dry biomass					
	1997		1998		1999	
	weeds m^{-2}	g m^{-2}	weeds m^{-2}	g m^{-2}	weeds m^{-2}	g m^{-2}
Amaranthus spp. L.	0	0.0	0	0.0	10.83	0.14
Anthemis arvensis L.	0	0.0	0	0.0	0	0.0
Anthemis tinctoria L.	0	0.0	0	0.0	0	0.0
Apera spica-venti (L.) P.Beauv.	0	0.0	0	0.0	0	0.0
Atriplex patula L.	0	0.0	0	0.0	0	0.0
Avena fatua L.	0	0.0	0	0.0	0	0.0
Capsella bursa-pastoris (L.) Medik.	17.1	1.37	2.50	0.44	13.33	1.40
Chaenorrhinum minus (L.) Lange	0.4	0.01	1.25	0.04	2.50	1.57
Chenopodium album L.	29.5	131.3	70.0	53.96	66.25	5.67
Cirsium arvense (L.) Scop.	2.9	5.58	2.08	3.43	0.83	0.25
Crepis tectorum L.	3.3	0.88	0	0.0	0	0.0
Elytrigia repens (L.) Nevski	0	0.0	0	0.0	2.5	2.3
Erysimum cheiranthoides L.	62.1	6.39	1.67	0.19	1.25	0.08
Euphorbia helioscopia L.	3.8	0.18	0.83	0.20	0.83	0.05
Fallopia convolvulus (L.) A. Löve	2.1	0.14	5.42	1.45	0	0.0
Galeopsis tetrahit L.	0	0.0	1.67	0.29	0	0.0
Galinsoga parviflora Cav.	0	0.0	0.83	0.17	0	0.0
Galium aparine L.	2.5	0.30	2.08	1.08	0	0.0
Lamium purpureum L.	1.2	0.05	0	0.0	0.83	0.18
Medicago lupulina L.	1.2	0.18	0	0.0	0	0.0
Mentha arvensis L.	0	0.0	0	0.0	1.67	0.28
Myosotis arvensis (L.) Hill.	1.7	0.13	0	0.0	0	0.0
Plantago major L.	2.5	0.13	0.42	0.81	2.92	0.06
Poa annua L.	7.5	0.50	0	0.0	5.0	0.10
Polygonum aviculare L.	0.4	0.07	0	0.0	0	0.0
Polygonum laphatifolium L.	8.3	0.91	3.75	0.56	0.42	0.01
Raphanus raphanistrum L.	0.4	0.17	0	0.0	0	0.0
Rumex crispus L.	0	0.0	0	0.0	0	0.0
Sinapis arvensis L.	147.9	69.23	1.67	1.05	0	0.0
Sonchus asper (L.) Hill.	16.4	8.98	3.33	5.21	0.87	0.44
Sonchus arvensis L.	0.3	0.17	15.84	24.77	6.21	3.14

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Weeds	Weed density and air-dry biomass					
	1997		1998		1999	
	weeds m^{-2}	g m^{-2}	weeds m^{-2}	g m^{-2}	weeds m^{-2}	g m^{-2}
Spergula arvensis L.	0	0.0	0.42	0.25	0	0.0
Stellaria graminea L.	0	0.0	0	0.0	0.42	0.01
Stellaria media (L.) Vill.	37.9	17.13	7.08	3.79	9.17	2.73
Thlaspi arvense L.	4.6	0.49	0	0.0	0.42	0.08
Tripleurospermum inodorum (L.) Sch. Bip.	34.2	10.92	0	0.0	2.92	0.22
Trifolium pratense L.	1.2	0.02	0	0.0	0	0.0
Tussilago farfara L.	0	0.0	1.25	0.12	0	0.0
Veronica arvensis L.	2.5	0.08	0	0.0	4.17	0.09
Viola arvensis Murray	3.3	0.18	0.42	0.04	1.67	0.05
Annual	388.3	249.54	102.92	68.72	120.46	12.81
Perennial	7.0	5.90	19.59	29.13	14.55	6.13
All weeds	395.3	255.4	122.5	97.8	135.0	18.9

Table 1. Composition, density and air-dry biomass of weed species in agrophytocenosis of spring barley on separate fields [1, 31]

Weed density linearly depended on weed air-dry biomass. With increase of air-dry weed biomass by 1 gram per square meter weed density enlarges by 1.21 weed plants. There was established opposite dependence of weed air-dry biomass on weed density. It showed change of weed air-dry biomass by 0.7 g m^{-2} with change of weed density by 1 plant (Figure 3).

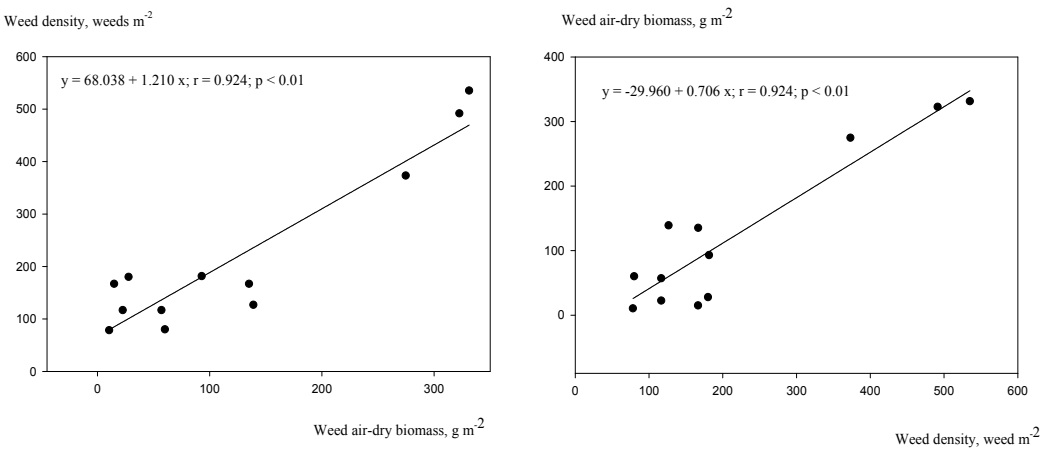

Figure 3. Relationship between weed density and weed air-dry biomass [1, 31]

4. Weed seed rain

4.1. Weed seed rain initiation

Dispersed weed seeds in spring barley agrophytocenosis during three years of the experiment belonged to 29 weed species from 12 families (Table 2). Weed seed rain in spring barley begins when spring barley is at stem elongation stage and increases to the hard stage of maturity. Ephemeral weeds of short vegetation *Stellaria media* and *Poa annua* as well the early summer weed *Chenopodium album* matured and began to disperse their seeds at the stem elongation of spring barley.

Winter annual weeds such as *Capsella bursa-pastoris* ripened and dispersed seeds at heading growth stage of spring barley, usually in the third ten-day period of June. Spring barley changing into early milk stage of maturity, *Lamium purpureum*, *Apera spica-venti*, *Atriplex patula*, *Veronica arvensis*, *Sonchus asper* and *Myosotis arvensis* ripened and began to pour seeds. At milk (medium milk) stage of spring barley maturity, *Thlaspi arvensis*, *Raphanus raphanistrum*, *Spergula arvensis*, *Galium aparine*, *Fallopia convolvulus* and *Polygonum laphatifolium* ripened and began to pour seeds. Spring barley changing from milk into dough stage of maturity, *Sinapis arvensis*, *Sonchus arvensis*, *Erysimum cheiranthoides* and *Cirsium arvense* ripened and began to disperse seeds. At dough stage of spring barley maturity, *Avena fatua*, *Crepis tectorum*, *Anthemis arvensis* and *Anthemis tinctoria* ripened their seeds. At dough stage of spring barley maturity, all weed species of agrophytocenosis which seeds were ripened except in 1998 when *Crepis tectorum* seeds matured and began to disperse only at hard stage of spring barley maturity [2].

The experimental data showed that *Crepis tectorum*, *Cirsium arvense* and *Sonchus arvensis* ripened and began to disperse seeds the latest. However, during separate experimental years the beginning of seed ripeness and start of seed rain for some weed species lasted more than presented the first growth stages of spring barley through the uneven meteorological conditions during separate years of field experiment. As example can serve, *Chenopodium album* seeds began to disperse at stem elongation stage of spring barley in 1997, at heading growth stage in 1999 and at milk (medium milk) maturity in 1998. Mainly it depended on the year climatic conditions (see subchapter 4.3) and on general crop stand weediness (Figure 4).

4.2. Weed seed rain dynamics

Weed seed rain is more intensive in weedier cereal crop, considering weed density and especially weed air-dry weight. It was confirmed by the correlation-regression analysis. Weed seed rain linearly and positively depended on weed dry weight $r = 0.842^{**}$ and on weed density $r = 0.686^{*}$. Weed air-dry biomass increase of 1 g m^{-2} induced increase of weed seed rain by 11.7 seeds m^{-2} while increase in weed density by one plant enhanced weed seed rain by 7.3 seeds m^{-2}. Hence, total weed seed rain was more dependent on the weed air-dry biomass than on weed density (Figure 4).

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Family	Species	The beginning of seed rain		
		97	98	99
Boraginaceae Juss.	*Myosotis arvensis* (L.) Hill.	M.	N.	M.e.
Chenopodiaceae Less.	*Atriplex patula* L.	M.	M.	M.e.
	Chenopodium album L.	S.e.	M.e.	He.
Cruciferae B. Juss.	*Capsella bursa-pastoris* (L.) Medik.	M.	M.	He.
	Erysimum cheiranthoides L.	M.l.-D.e.	D.	N.
	Raphanus raphanistrum L.	M.	M.l.-D.e.	N.
	Sinapis arvensis L.	M.l.-D.e.	M.l.-D.e.	M.l.-D.e.
	Thlaspi arvense L.	M.	N.	M.
Compositae Giseke	*Anthemis arvensis* L.	D.	N.	N.
	Anthemis tinctoria L.	D.	N.	N.
	Cirsium arvense (L.) Scop.	D.	M.l.-D.e.	D.
	Crepis tectorum L.	D.	H.	N.
	Sonchus asper (L.) Hill.	M.l.-D.e.	M.	M.e.
	Sonchus arvensis L.	D.	M.l.-D.e.	D.
	Tripleurospermum inodorum (L.) Sch. Bip.	D.	N.	D.
Caryophyllaceae Juss.	*Stellaria media* (L.) Hill.	S.e.	M.e.	He.
	Spergula arvensis L.	N.	M.l.-D.e.	N.
Euphorbiaceae J. St. Hill.	*Euphorbia helioscopia* L.	N.	N.	M.
Labiatae Juss.	*Lamium purpureum* L.	M.e.	M.	N.
Poaceae Bernhart	*Apera spica-venti* (L.) P. Beauv.	N.	M.e.	N.
	Avena fatua L.	N.	M.	N.
	Poa annua L.	S.e.	N.	N.
Polygonaceae Lindl.	*Fallopia convolvulus* L.	D.	M.	M.
	Polygonum lapathifolium L.	M.	M.l.-D.e.	M.
	Polygonum aviculare L.	M.	N.	N.
	Rumex crispus L.	D.	N.	N.
Rubiaceae Juss.	*Galium aparine* L.	M.l.-D.e.	M.l.-D.e.	N.
Scrophulariaceae Juss.	*Veronica arvensis* L.	M.	M.e.	M.
Violaceae Juss.	*Viola arvensis* Murr.	N.	M.l.-D.e.	N.

Note. Spring barley growth stages of maturity: S.e. – stem elongation, He. – heading, M.e. – early milk, M. – milk, M.l.-D.e. – late milk-early dough, D. – dough, H. – hard, N. – weed seed rain was not established.

Table 2. Weed seed rain initiation in spring barley agrophytocenosis [1, 2]

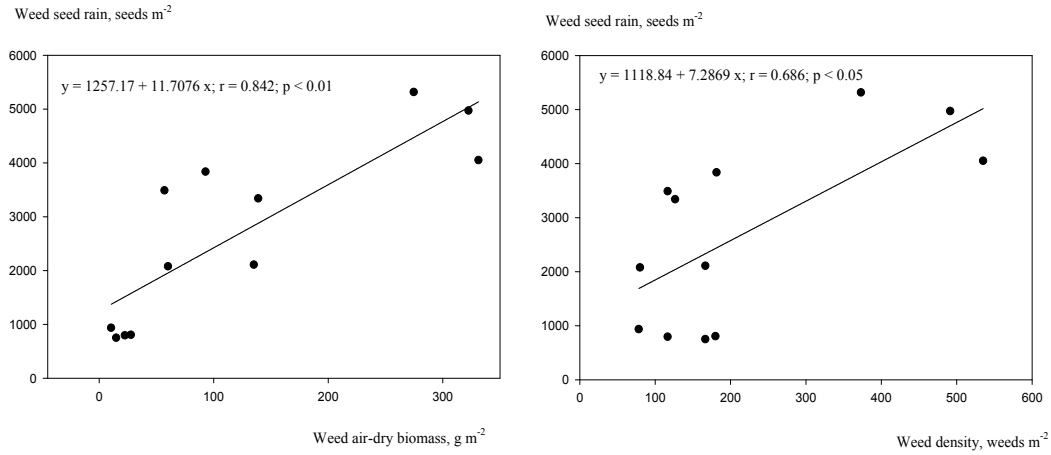

Figure 4. Weed seed rain dependence on weed air-dry biomass and density [1, 31]

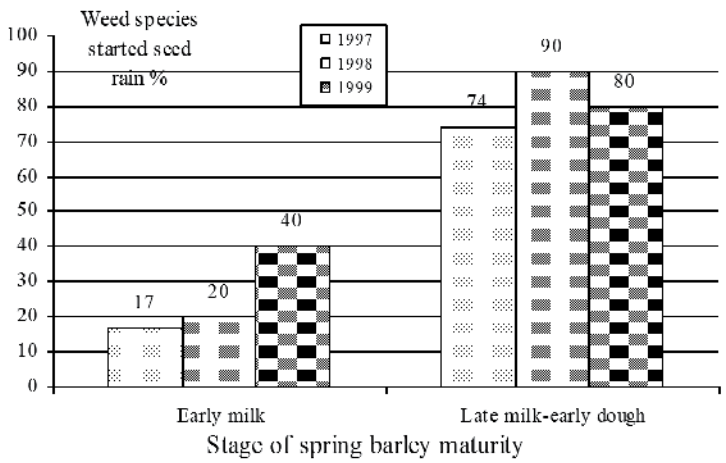

Figure 5. Weed species [%] started seed rain depending on spring barley crop maturity

It was established that seed rain depended directly on plant density of *Stellaria media* r = 0.711*, *Sonchus asper* r = 0.918*** and *Capsella bursa-pastoris* r = 0.474. Accumulated *Stellaria media, Sonchus asper* and *Capsella bursa-pastoris* air-dry biomass in the crop had adequate influence on their seed rain, respectively r = 0.833**, r = 0.786* and r = 0.766* [32]. When spring barley was ripening, weed seed rain was more intensive (Figure 5 and 6). It is in conformity with data of other researchers indicating that, until cereal harvesting, some weed species are able to pour out all their ripened seeds [33].

Weed seed rain during separate years of the experiment varied in accordance with the spring barley crop weediness. However, seeds matured and dispersed 29 (Figure 7) of 40 weed species (Table 1) grown in spring barley agrophytocenosis. Presumptively it was influenced by the low density of some weed species and limiting solar light to others by successful smothering

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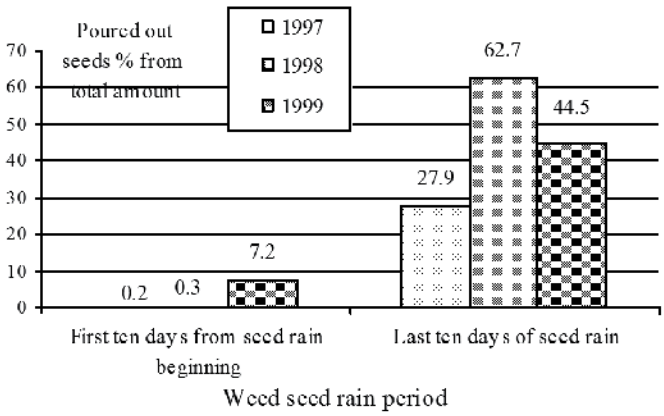
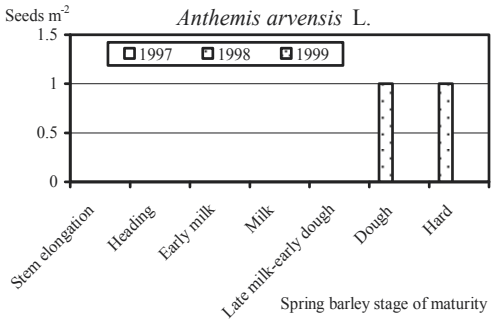


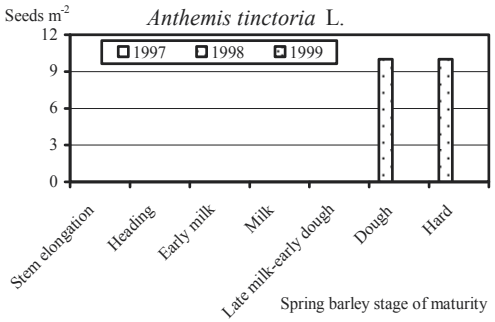
Figure 6. Weed seed rain intensity during vegetation in agrophytocenosis of spring barley

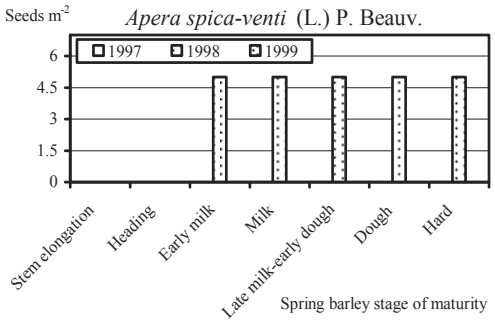
by spring barley. The most important weed species in weed seed rain dynamics biologically belonged to annual weeds. Dispersed seeds of *Stellaria media* in spring barley agrophytocenosis composed 19-29% from total seed rain during experimental years. Seed rain of *Chenopodium album* covered from 6% to 63% of total weed seed rain while *Capsella bursa-pastoris* 6%-10%. Seed rain of *Sonchus asper* and *Sinapis arvensis* was essential only during the first year of experiment with 26% and 11% of seeds from total number of dispersed ones, accordingly. From perennial weeds only *Sonchus arvensis* showed significant seed rain covering 4.8% of total dispersed weed seed number during the second year of experiment. Seed rain of all other weeds in spring barley agrophytocenosis jointly consisted from 11% to 19% from total number of dispersed weed seeds (Figure 7).

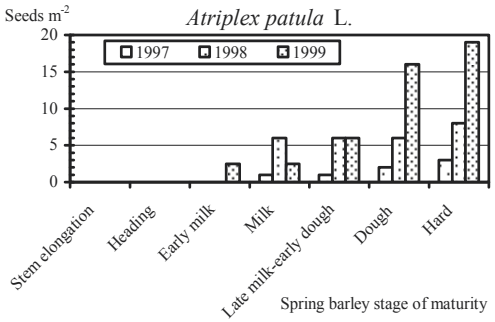
The data of the field trial proved that weeds ripened regularly. Analyzing seed rain of all weed species of spring barley agrophytocenosis were established 4543 seeds m^{-2} in 1997, 2753 seeds m^{-2} in 1998 and 821 seeds m^{-2} in 1999 (Table 3).

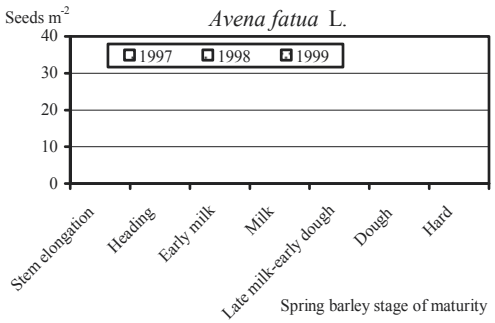
Different number of dispersed weed seeds depended on crop and meteorological conditions. Initially, weed seed rain every year of the experiment was slow with low numbers of weed species and low numbers of dispersed seeds. At medium milk stage of spring barley maturity, dispersed seed covered just 6%-23% of total seeds. At late milk-early dough stage of spring barley maturity, it already covered 27%-42% of total dispersed weed seed number. Usually, weed seeds which were left in the crop could be taken from the field together with harvest (biomass of spring barley for silage) and would not infest the soil. Harvesting spring barley for biomass or silage at medium milk stage of maturity, 77%-94% of weed seeds would be removed from the field while harvesting at late milk-early dough stage of maturity, 58%-73% of weed seeds could be removed from the field. When harvesting cereal at hard stage of maturity, most of the weed seeds already are dispersed on the soil and naturally increase weed infestation in the following crop of the crop rotation.

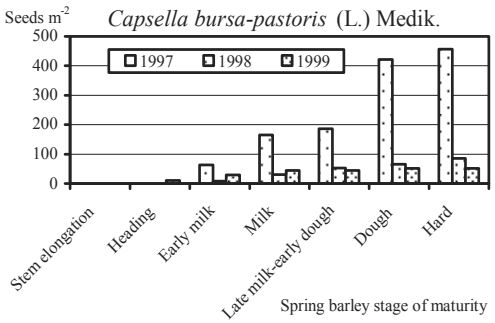

Seeds m-2
Anthemis arvensis L.
1997 1998 1999
1.5
1
0.5
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

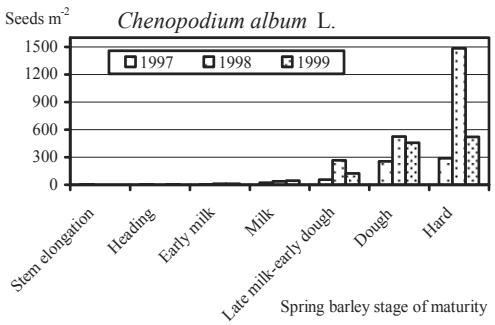

Seeds m-2
Anthemis tinctoria L.
1997 1998 1999
12
9
6
3
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

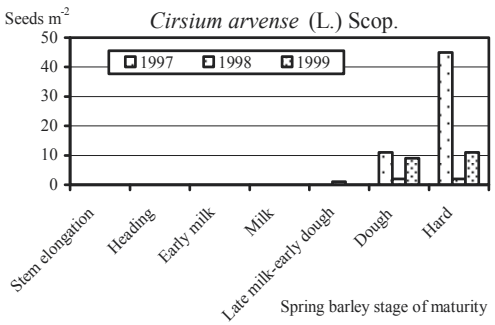

Seeds m-2
Apera spica-venti (L.) P. Beauv.
1997 1998 1999
6
4.5
3
1.5
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Atriplex patula L.
1997 1998 1999
20
15
10
5
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

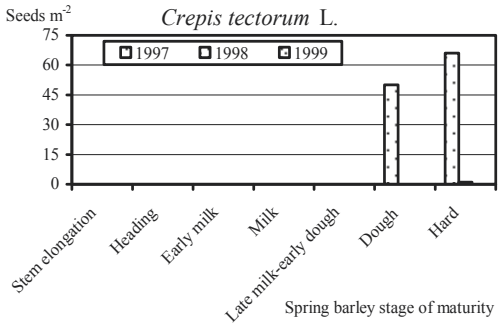

Seeds m-2
Avena fatua L.
1997 1998 1999
40
30
20
10
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

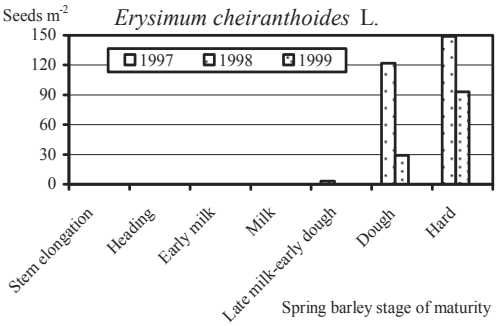

Seeds m-2
Capsella bursa-pastoris (L.) Medik.
1997 1998 1999
500
400
300
200
100
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

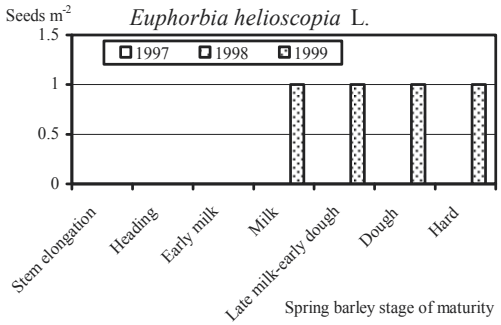

Seeds m-2
Chenopodium album L.
1997 1998 1999
1500
1200
900
600
300
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

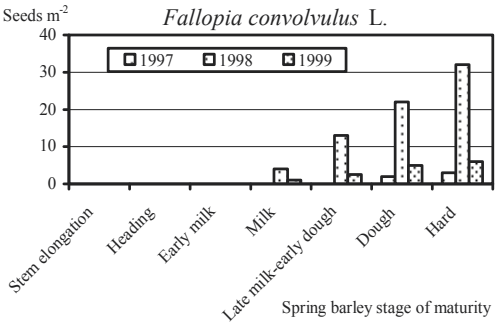

Seeds m-2
Cirsium arvense (L.) Scop.
1997 1998 1999
50
40
30
20
10
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

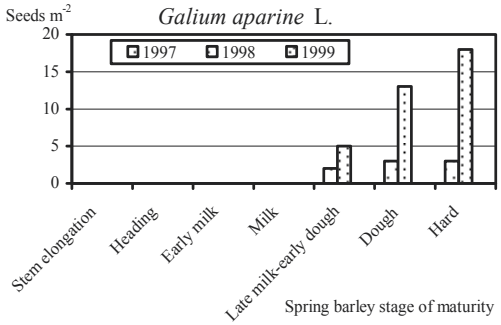
http://dx.doi.org/10.5772/55972

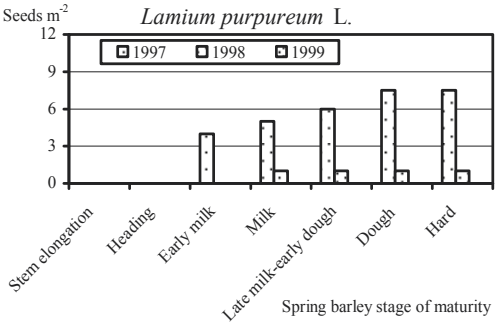


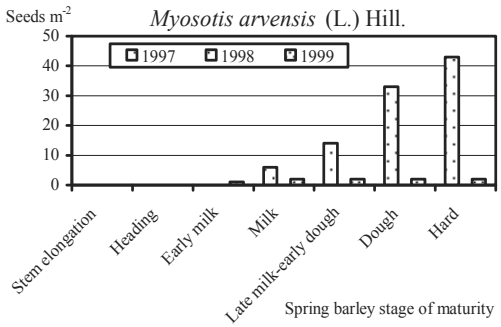


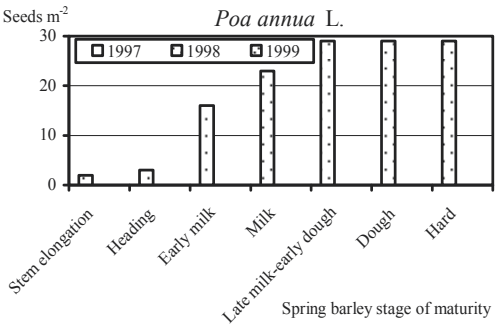


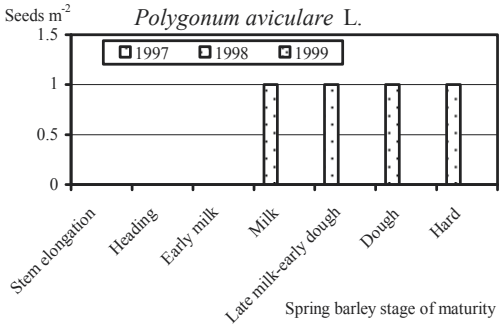


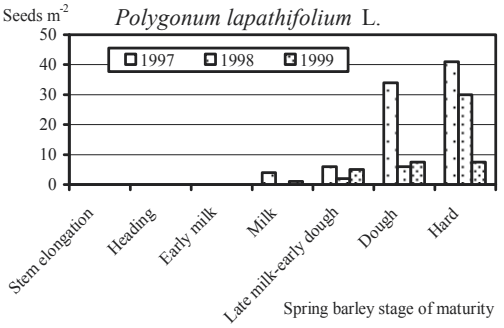


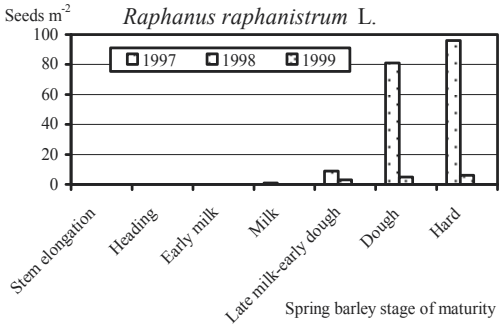


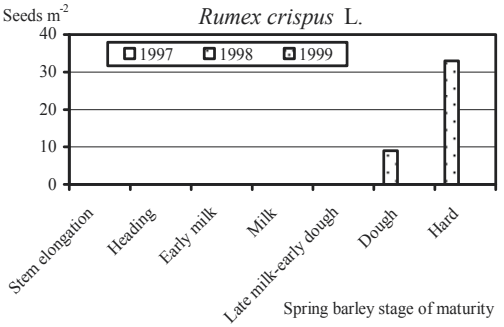


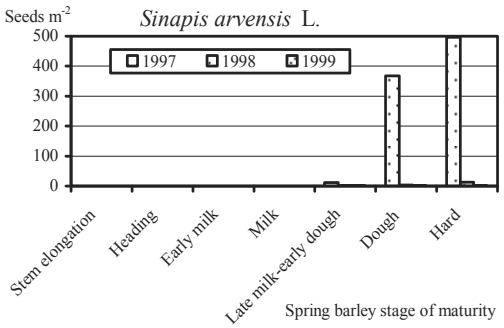


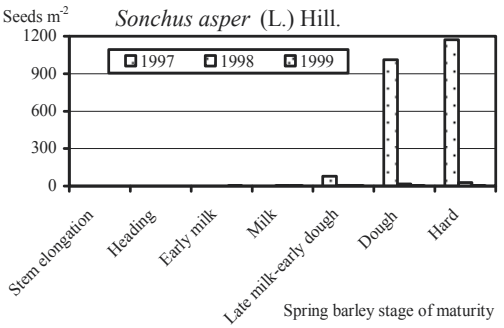

Seeds m-2
Polygonum aviculare L.
1997 1998 1999
1.5
1
0.5
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

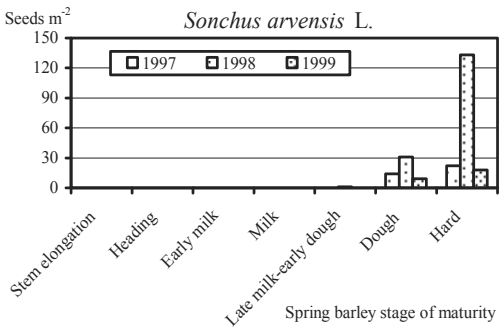

Seeds m-2
Polygonum lapathifolium L.
1997 1998 1999
50
40
30
20
10
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

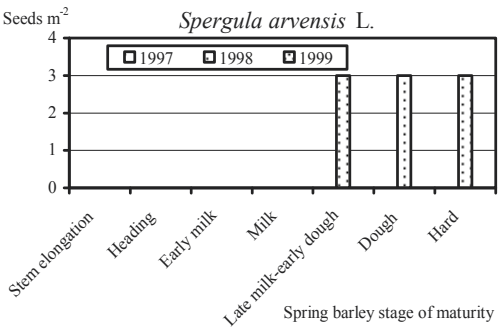

Seeds m-2
Raphanus raphanistrum L.
1997 1998 1999
100
80
60
40
20
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Rumex crispus L.
1997 1998 1999
40
30
20
10
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Sinapis arvensis L.
1997 1998 1999
500
400
300
200
100
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Sonchus asper (L.) Hill.
1997 1998 1999
1200
900
600
300
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Sonchus arvensis L.
1997 1998 1999
150
120
90
60
30
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity


Seeds m-2
Spergula arvensis L.
1997 1998 1999
4
3
2
1
0
Stem elongation
Heading
Early milk
Milk
Late milk-early dough
Dough
Hard
Spring barley stage of maturity

http://dx.doi.org/10.5772/55972

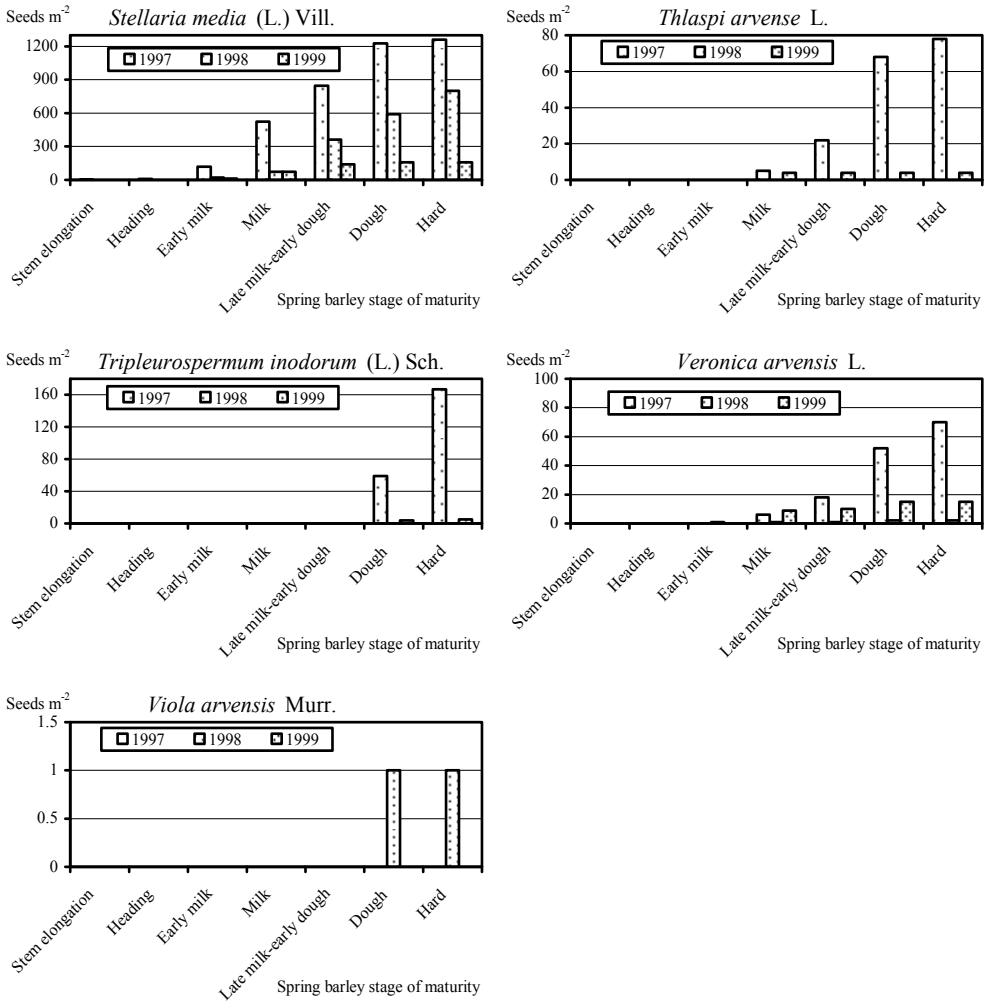


Figure 7. Weed species seed rain dynamics in spring barley agrophytocenosis [1, 2]

Moreover, most weed seeds which, together with crop biomass, get in silage [35-38], in manure [36, 37, 39], in sewage [40] in compost [41], or going through alimentary canal of cattle [35, 42], lost their germinating power and would not infest the crop in the future.

4.3. Weed seed rain and meteorological conditions

Weed seed rain increases during the time of cereal ripening (Figure 4, 6, Table 3) but it decreases in separate vegetation periods depending on change of meteorological conditions. Growth and development of all plants are influenced by environmental factors from which meteorological ones are highly important [43].

Stages of spring barley maturity	Weed seed rain					
	1997		1998		1999	
	seed m^{-2}	%	seed m^{-2}	%	seed m^{-2}	%
Stem elongation	8**	0.17	0**	0.0	0**	0.0
Heading	16**	0.35	0**	0.0	12**	1.5
Early milk	207**	4.6	47**	1.7	60**	7.3
Milk (medium milk)	764**	16.8	161**	5.8	189**	23.0
Late milk-early dough	1289**	28.4	731**	26.6	343**	41.8
Dough	3871	85.2	1331**	48.3	744*	90.6
Hard	4543	100	2753	100	821	100
LSD_{05}	707.0	-	417.7	-	71.5	-
LSD_{01}	968.4	-	572.1	-	97.9	-

Table 3. Total weed seed rain in the crop of spring barley [1, 9, 34]; * $p < 0.05$; ** $p < 0.01$

Meteorological conditions such as temperature, rainfall, and sunlight at sprouting and germination stage influenced vegetation and can determine plant density in the crop. For example, germination of *Solanum elaeagnifolium* [44] and *Matricaria perforata* [45] depends on temperature, germination of *Rumex obtusifolius* depends on temperature and light [46], germination of *Ranunculus repens* depends on soil humidity and temperature [47]. Growth and biomass accumulation of *Chenopodium album* [48], *Bromus tectotum* and *Taeniatherum asperum* [49] also depends on meteorological conditions. In our experiments there was determined linear relationship between weed biomass and weed seed rain (Figure 4). Logically, weed seed rain could be influenced by the meteorological factors such as air temperature, rainfall and sunlight duration. According to the sum of active air temperature and precipitation, the vegetation period during the first and the second experimental years was wet and during the third experimental year – not humid enough (Figure 8).

Weed seed rain changed dynamically, increasing and decreasing during vegetation regardless of total seed number dispersed during separate years of experiment (Figure 7). In our experiment, established weed seed rain fluctuations significantly depended on active air temperature (> 10°C), rainfall and sunlight duration (Figures 9-11). Weed seed rain regularly intensified with increase of sum of active air temperature (Figure 9) as well as with increase of sunlight duration (Figure 11). This phenomenon is based on plant physiological processes such as development and water circulation in plant tissues that are significantly dependent on sunlight and environmental temperature. In contrast to the sum of active air temperature and sunlight duration, rainfall inhibited weed seed rain (Figure 10). Jointly, during rainy periods, active air temperatures decreased and shortened sunlight duration which leads to an increase of humidity accumulation in plants. Excess humidity amounts reaching weed seeds managed to slow physiological maturation and as well inhibited seed rain. Statistically reliable non-linear dependencies of total weed seed rain on active air-temperatures $r^2=0.528^{**}$, $r^2=0.538^{**}$, $r^2=0.119^{*}$,

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rainfall r^2=0.567**, r^2=0.608**, r^2=0.155* and sunlight duration r^2=0.512**, r^2=0.418**, r^2=0.136* are presented in figures 9-11.

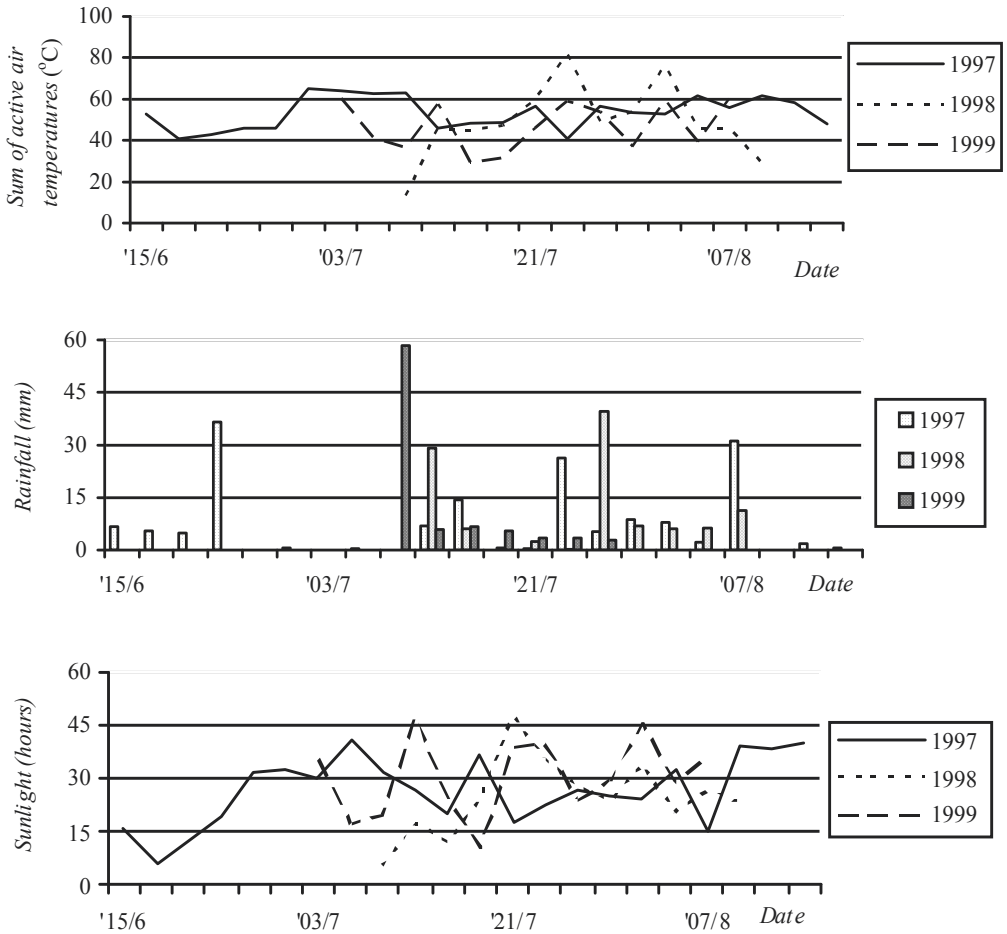

Figure 8. Meteorological conditions: active air temperatures, rainfall and sunlight dynamics during experimental years, Kaunas (Noreikiškės) Meteorological station [43]

4.4. Weed seeds in grains

The later the cereal harvest, the fewer amounts of weed seeds get into grain, but the more of them infested the soil [12]. In cereal grain yield of hard maturity (in the sample of 100 g), on average, are found less weed seeds by 820 when comparing with grain yield of dough maturity. Such decrease makes up to 21 million (12–39 million) fewer weed seeds in crop yield from 1 ha with a biomass of approximately 38 kg (13–53 kg). This regularity motivates the necessity of earlier spring barley harvesting not only because of frequently experienced grain losses but also because of weed seed spreading limitation [50].

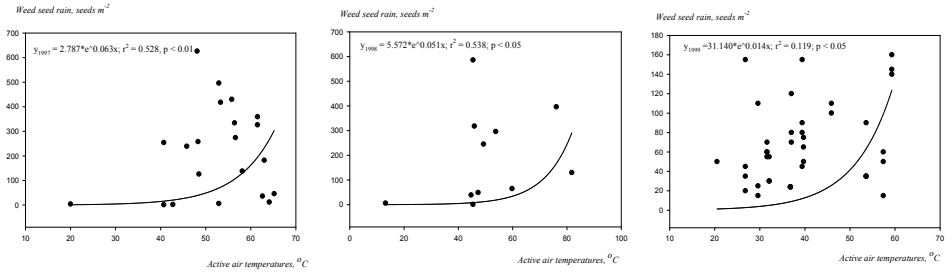

Figure 9. Weed seed rain dependence on active air temperatures [43]

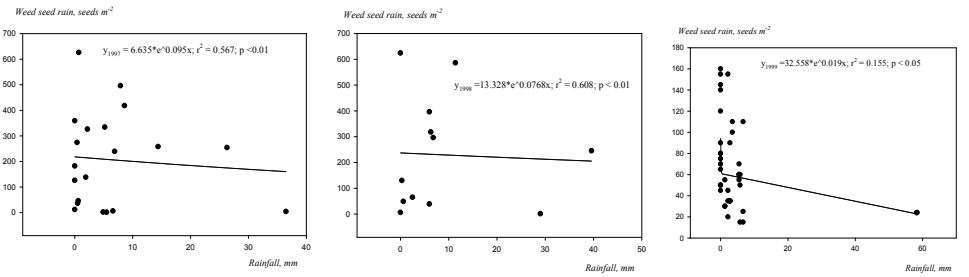

Figure 10. Weed seed rain dependence on rainfall [43]

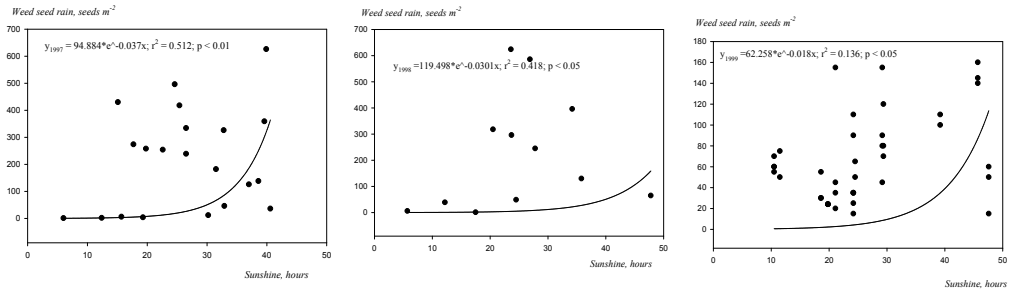

Figure 11. Weed seed rain dependence on sunshine duration [43]

4.5. Spring barley crop productivity

Spring barley dry matter yield increased significantly while cereal matured from stem elongation to late milk-early dough growth stages. In the further growth stages of spring barley - dough and hard – total above-ground dry matter yield decreased significantly (Figure 12). The yield of dry matter begins to decrease at anthesis complete growth stage of spring barley [51]. The optimal period for gathering cereal is considered 4 weeks after heading [52] or 2-3 weeks before hard growth stage, when dry matter yield reaches maximum and begins to decrease [53]. The maximum increase of dry matter in cereal is characteristic from heading till

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milk stage but the biggest yield accumulates in milk-dough and dough stages of maturity [54], thereafter it decreased slightly for the two-row cultivars [55]. Dynamics of dry matter in cereal can be influenced by meteorological conditions, soil, fertilization and other factors [56]. However, dynamics of dry matter accumulation in cereals depends on decrease of assimilation surface when leaves decline and on allocation and transformation of assimilation products [51, 53, 57]. The general decrease of dry matter yield is influenced by decrease of vegetative biomass [58]. Growth stages of spring barley and other cereals can be theoretically divided into three groups according accumulation dynamics of harvest: increase, reach of maximum, and decrease. The logical solution is to limit yield losses, i.e. to refuse the third group. By cutting cereal at milk-dough stages of maturity, it would be possible to achieve that. Of course, then it would be necessary to refuse conventional harvesting of cereal for grain applying an alternative use of all above-ground biomass for forage at such stage of maturity when maximum yield of dry matter and metabolizable energy is reached [1, 59].

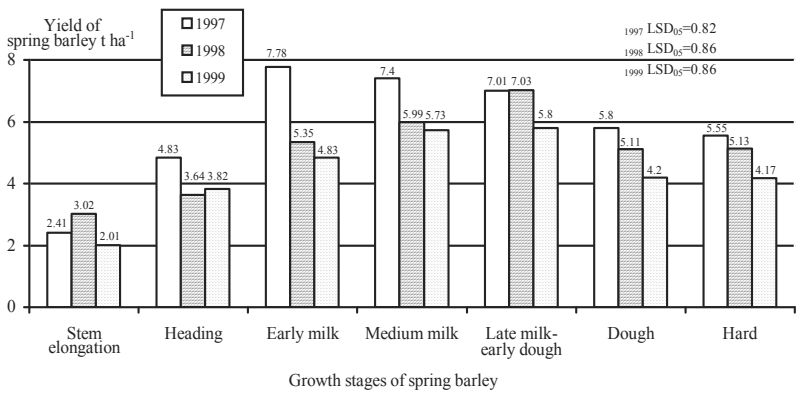

Figure 12. Spring barley dry matter yield at seven stages of growth and maturity [1, 59, 60]

The concentration of crude protein, crude fibre, crude fat and crude ash variation of each year of the experiment preserved analogical tendency (Table 4). The concentration of crude protein and crude ash was the greatest at stem elongation growth stage and as the spring barley matured the concentration of crude protein and crude ash decreased. However, in the grain of dough and hard growth stages, concentration of these components increased but in the straw it decreased. Therefore, total yield of crude protein and crude ash decreased significantly at dough and hard growth stages compared with milk and late milk-early dough stages of spring barley maturity. The concentration of crude fibre and crude fat tended to increase or decrease as the spring barley matured. However, the yield of crude fibre and crude fat at dough and hard growth stages decreased significantly (Table 4).

Likewise, as in our experiment, the greatest concentration of nutrition at stem elongation growth stage of spring barley and other cereals was determined. The concentration of nutrition essentially decreased to minimum at the end of vegetation [61] and remained constant near

maturity [55, 62]. At the end of cereal vegetation, growth of DM is zero and biological yield does not increase but even begins to decrease [63]. Losses of DM in spring barley yield can be decreased additionally using nitrogen fertilizers. However, spring barley loses a part of whole-plant DM yield before reaching hard stage in variables of the trials fertilized and non-fertilized by nitrogen [64]. That is because the index of green plant surface area decreases to zero when respiration occurs in plant ears, which requires energy. So, if photosynthesis does not occur, spring barley matures about 3 weeks before harvesting using non-replenished energetic resources. Moreover, development of DM in plant organs fully influences not only the product (grain) but also the growth of a plant and biological yield [59]. Usually the differences between agricultural plants and their varieties are seen in differences of speed usage of DM of assimilation tissues. In some cases, when general biomass of cereal increases, grain yield does not increase because of the development of some assimilation products in vegetative organs [53]. The metabolizable energy (ME) in spring barley yield for ruminants (cows) is given in Table 5. Metabolizable energy is energy directly intaken and used in an animal's organs. Total forage energetic losses are rejected beforehand, which are experienced in an animal's organs for various reasons (energetic losses with feces, urine and intestine gas and energy necessary for digestion processes) [16, 65].

The ME (MJ kg^{-1} DM) was similar to the chemical composition dynamics. In contrast to the ME content (MJ kg^{-1} DM), the amount of ME per hectare increased significantly as the spring barley matured to the late milk-early dough growth stage, and likewise, DM, digestible organic matter in the dry matter, crude protein and crude ash yield decreased significantly at dough and hard growth stages.

Growth stage	In dry matter							
	Crude protein		Crude fibre		Crude fat		Crude ash	
	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}
				1997				
Stem elongation	12.98	0.31	27.32	0.66	2.42	0.06	10.76	0.26
Heading	9.60	0.46	32.13	1.55	1.69	0.08	7.99	0.39
Early milk	6.61	0.51	31.61	2.45	2.30	0.18	9.46	0.74
Milk (medium milk)	7.16	0.53	28.44	2.10	2.20	0.16	7.40	0.55
Late milk-early dough	7.71	0.54	25.27	1.77	2.11	0.15	5.34	0.37
Dough	-	0.32#	-	1.64#	-	0.12#	-	0.25#
Grain	7.91	0.186	6.42	0.15	2.37	0.06	2.63	0.06
Straw	3.94	0.136	43.31	1.49	1.81	0.06	5.86	0.19
Hard	-	0.31#	-	1.62#	-	0.10#	-	0.21#
Grain	8.55	0.213	6.69	0.17	2.63	0.06	2.46	0.06
Straw	3.07	0.094	47.48	1.45	1.23	0.04	4.91	0.15

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Growth stage	In dry matter							
	Crude protein		Crude fibre		Crude fat		Crude ash	
	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}
LSD_{05}		0.05		0.28		0.02		0.06
			1998					
Stem elongation	16.60	0.50	28.57	0.86	1.74	0.05	11.30	0.34
Heading	9.66	0.35	28.90	1.05	2.73	0.10	8.49	0.31
Early milk	8.70	0.47	26.68	1.43	2.33	0.12	6.64	0.36
Milk (medium milk)	8.23	0.49	25.84	1.55	2.04	0.12	6.01	0.36
Late milk-early dough	6.88	0.48	22.10	1.55	2.39	0.17	5.21	0.37
Dough		0.32#	-	1.37#	-	0.09#	-	0.28#
Grain	-	0.20	5.32	0.116	2.65	0.058	2.89	0.06
Straw	9.20	0.12	42.85	1.251	1.25	0.036	7.54	0.22
Hard	4.04	0.39#	-	1.17#	-	0.10#	-	0.18#
Grain	-	0.30	5.08	0.146	2.75	0.08	2.47	0.07
Straw	10.54	0.09	45.73	1.028	1.03	0.02	5.05	0.11
LSD_{05}		0.05		0.24		0.02		0.05
			1999					
Stem elongation	14.26	0.29	23.93	0.48	2.31	0.05	10.97	0.22
Heading	9.95	0.38	28.44	1.09	2.18	0.08	6.04	0.23
Early milk	7.60	0.37	21.70	1.05	2.16	0.10	6.75	0.33
Milk (medium milk)	7.98	0.46	25.05	1.43	2.64	0.15	5.87	0.34
Late milk-early dough	7.13	0.41	23.17	1.34	2.18	0.13	4.38	0.25
Dough	-	0.33#	-	1.00#	-	0.10#	-	0.19#
Grain	11.59	0.25	5.56	0.12	3.15	0.07	2.70	0.06
Straw	3.60	0.07	43.42	0.88	1.51	0.03	6.70	0.13
Hard	-	0.34#	-	0.93#	-	0.09#	-	0.19#
Grain	11.93	0.27	5.79	0.13	2.89	0.07	2.89	0.06
Straw	3.78	0.07	42.48	0.79	1.45	0.03	6.84	0.13
LSD_{05}		0.07		0.21		0.02		0.06

LSD_{05}, the least significant difference; # - total yield (grain + straw)

Table 4. Effect of spring barley growth stage at harvesting on yield chemical composition [1, 59, 60]

When spring barley grain matures at hard growth stage compared with dough stage, the yield of DM, crude protein, crude fibre and ME increases. The yield of crude fat and crude ash almost does not differ. However, when the quality of straw becomes worse, the general value of yield remains fewer than at milk-dough stage. Martin and Seibold [66] determined comparable results: ME of 9.56 MJ kg^{-1} DM at heading stage of maturity and ME of grain 12.93 MJ kg^{-1} DM and 6.80 MJ kg^{-1} DM of straw at hard stage of spring barley maturity.

Growth stage	ME, MJ kg^{-1} DM			ME, GJ ha^{-1}		
	1997	**1998**	**1999**	**1997**	**1998**	**1999**
Stem elongation	10.80	9.24	9.97	26.03	27.91	20.04
Heading	10.00	9.02	8.61	48.30	32.83	32.89
Early milk	8.38	9.54	8.01	65.20	51.04	38.69
Milk (medium milk)	8.49	9.45	8.56	62.74	56.61	49.05
Late milk-early dough	8.60	9.67	8.64	60.29	67.98	50.11
Dough	-	-	-	51.76#	42.18#	38.94#
Grain	11.97	11.30	11.84	28.13	24.75	25.93
Straw	6.85	5.97	6.47	23.63	17.43	13.01
Hard	-	-	-	50.69#	46.80#	36.02#
Grain	12.44	12.50	11.01	30.98	36.00	25.32
Straw	6.44	4.80	5.72	19.71	10.80	10.70
LSD_{05}	-	-	-	**6.78**	**7.66**	**7.70**

ME, metabolizable energy; DM, dry matter; # - total yield (grain + straw)

Table 5. Energetic value of spring barley over-ground biomass (whole-plant) at seven stages of growth and maturity [1, 59, 60]

Positive, statistically reliable, linear dependence of spring barley crude protein [t ha^{-1}] r_{1997}=0.736***, r_{1998}=0.317, r_{1999}=0.858***, crude fibre [t ha^{-1}] r_{1997}=0.964***, r_{1998}=0,937***, r_{1999}=0.961***, crude fat [t ha^{-1}] r_{1997}=0.960***, r_{1998}=0.911***, r_{1999}=0.957*** and crude ash [t ha^{-1}] r_{1997}=0.689***, r_{1998}=0.335, r_{1999}=0.646*** on dry matter yield [t ha^{-1}] and linear dependence of metabolizable energy [Gj ha^{-1}] on spring barley dry mass [t ha^{-1}], r_{1997}=0.992***, r_{1998}=0.985***, r_{1999}=0.983***, crude protein [t ha^{-1}] r_{1997}=0.750***, r_{1998}=0.420*, r_{1999}=0.844***, crude fibre [t ha^{-1}] r_{1997}=0.967***, r_{1998}=0.900***, r_{1999}=0.948***, crude fat [t ha^{-1}] r_{1997}=0.926***, r_{1998}=0.931***, r_{1999}=0.953*** and crude ash yields [t ha^{-1}] r_{1997}=0.671***, r_{1998}=0.385*, r_{1999}=0.576** were established [59].

Digestibility *in vitro* of spring barley organic matter in the dry matter depended on spring barley stage of maturity. The highest digestibility *in vitro* was established at growth stage of stem elongation 73–78% (except 1998) and at later growth stages it decreased. Digestibility of

spring barley whole-plant biomass at stem elongation was less compared with barley grain digestibility at dough and hard stages of maturity (digestibility *in vitro* to 89%). Spring barley metabolizable energy directly depended on barley growth stages and fodder digestible organic matter in the dry matter digestibility *in vitro*, $r = 0.995\text{-}0.998$ at $P < 0.0001$ [67]. Ensiling spring barley biomass harvested at early milk, milk, late milk-early dough and dough stages of maturity, silage chemical composition directly depended on cereal stage of maturity. Whole plant silage produced from cereals of later stages of maturity, late milk-early dough and dough stages of maturity, has less crude protein and crude ash concentration, lower digestibility *in vitro* by ruminants and fewer accumulated metabolizable energy MJ kg^{-1} of silage dry biomass [68]. Nykänen et al. [69] reported the highest organic matter digestibility in peas (710–800 g kg^{-1}), vetches and spring barley had an organic matter digestibility of 670 g kg^{-1}, while the other spring cereals had the lowest values (550–610 g kg^{-1}). The highest organic matter digestibility of spring barley silage was found processing silage from biomass of earlier stage (milk) of spring barley maturity [68].

4.6. Economic evaluation of technology

Cereals in Lithuania are some of the most important agricultural crops. In 2011, cereal crop area comprised 51.7% of all crops [70] while conventionally they cover 60-64 % of crop area [71]. The biggest part of grain (approx. 70 %) is used for forage [72]. With the increasing intensity of agricultural production, spring barley is becoming one of the most important cereals in Lithuania [73, 74]. Spring barley covers more than 23% of total cereal crop area in the country [70]. Edwards et al. [75] proposed that it would be more purposeful to use the whole plant for forage than to feed animals with separate processed grain and straw. Silage significantly decreases cereal processing costs; expensive combining, straw processing, grain transport, grain cleaning and grain drying can be omitted. Moreover, inevitable grain losses, especially due to unfavourable meteorological conditions during the harvest can be avoided. When preparing whole plant silage from late milk-early dough and dough stages of spring barley maturity, higher nutritive value was achieved when evaluating total metabolizable energy received from plot area compared with earlier harvested for biomass or harvested at hard maturity for grains spring barley whole plant above-ground plant part energetic value as fodder for ruminants [68]. Of special interest is, whether in technology can be reduced unnecessary input use [77]. Therefore, the aim of this research was to determine economical efficiency of different spring barley growing and yield harvesting [at late milk-early dough suitable for silage and hay making and hard (grains and straws are obtained) stages of maturity] technologies as well as the economical background.

Due to the maturing process of spring barley, dry matter yield is gradually accumulated by reaching maximum at the late milk-early dough growth stages. The dry matter yield decreased significantly as the spring barley matured from late milk-early dough to hard growth stage (see subchapter 4.5). When harvesting spring barley at two different growth stages, the costs during separate years varied from 604.7 to 869.1 Lt ha^{-1} and depended on the different yields and proceedings. The costs associated with harvesting spring barley at the late milk-early

Growth stage of harvesting and operation	Costs [Lt ha^{-1}]*		
	Year		
	1997	1998	1999
Late milk-early dough	**659.24**	**674.40**	**604.73**
Autumn plough	76.09	76.09	76.09
Autumn loosening	26.03	26.03	26.03
Spring loosening	26.03	26.03	26.03
Spring loosening with harrow	24.82	24.82	24.82
Sow	106.75	106.75	106.75
Harvest	16.50	16.50	16.50
Press to rolls	199.50	214.13	176.67
Rolls involve in film	126.04	126.40	104.28
Transport	57.48	57.65	47.56
Hard	**840.52**	**869.13**	**749.94**
Autumn plough	76.09	76.09	76.09
Autumn loosening	26.03	26.03	26.03
Spring loosening	26.03	26.03	26.03
Spring loosening with harrow	24.82	24.82	24.82
Sow	106.75	106.75	106.75
Harvest	350.52	405.42	323.77
Press straw to rolls	125.74	92.45	76.84
Grain transport	25.02	28.94	23.12
Straw transport	22.25	16.36	13.59
Grain clean	7.47	8.64	6.90
Grain dry	49.80	57.60	46.00

Table 6. Cost structure of spring barley harvested at two growth stages [76]; * 1 € = 3.4528 Lt

dough stage decreased by 19-22% (Table 6), when compared with the control treatment, i.e. hard stage maturity.

When harvesting at the hard stage of maturity, the value of spring barley yield mainly depended on the grain value (91-94% of the spring barley yield value). The grain value at the late milk-early dough stage of maturity was much lower and made 71-77% of the spring barley biomass value. Comparing spring barley biomass yield value at the late milk-early dough stage of maturity with grain and straw yield value at the hard stage of maturity, it was determined that it was by 12-19% lower (Table 7).

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Growth stage	Production	Yield value [Lt ha^{-1}]*		
		Year		
		1997	1998	1999
Late milk-early dough	Biomass for silage	707.00	857.65	663.91
Hard	Grain + straw	879.42	982.35	786.49
	Grain	796.80	921.60	736.00
	Straw	82.62	60.75	50.49

Table 7. Value of spring barley yield at two growth stages [76]; * 1 € = 3.4528 Lt

Analyzing the economical effect of different technologies, it was determined that the profit increased when harvesting spring barley at the late milk-early dough stage of maturity compared to the hard stage of maturity. In 1997 the profit increased by 22.7 %, in 1998 by 61.8 % and in 1999 by 61.9 %, respectively (Table 8). The larger profit and smaller costs influenced the larger productive profitability, which increased 1.6 times in 1997, 2.1 times in 1998 and 2.0 times in 1999 while harvesting spring barley at the late milk-early dough stage of maturity [76].

Growth stage	Profit [Lt ha^{-1}]*			Profitability [%]		
	Year					
	1997	1998	1999	1997	1998	1999
Late milk-early dough	47.75	183.25	59.18	7.2	27.2	9.8
Hard	38.91	113.22	36.55	4.6	13.0	4.9

Table 8. Profit and profitability of spring barley harvested at two growth stages [76]; * 1 € = 3.4528 Lt

Economical calculations show that costs on the average were 819.9 Lt ha^{-1} and production value was 882.8 Lt ha^{-1}, when spring barley were grown according to conventional farming technology. Therefore, the average profit was 61.9 Lt ha^{-1}, and profitability 7.7 %. When spring barley was grown according to alternative technology, the costs were 646.1 Lt ha^{-1}, while yield value, profit and profitability were 742.9 Lt ha^{-1}, 96.7 Lt ha^{-1} and 15.0 % respectively. Other authors [78] determined analogous value of spring barley yield 771-846 Lt ha^{-1} according to economical evaluation of crop technologies. Economical evaluation of technologies for spring barley growth and harvest determined that the alternative farming technology – harvesting spring barley at the late milk-early dough stage of maturity –is more effective. Compared with the conventional farming technology, costs decreased by 21.2 %, profit and profitability increased 1.5 and 1.9 times, respectively. The economical efficiency of the spring barley growth technologies directly depended on the dry matter yield. Linear relationships between spring barley yield and costs and between the yield and received profit were recognized (Figure 13). With the increase of the dry biomass yield of spring barley by 1 t ha^{-1}, growing costs decreased on the average by 50 Lt ha^{-1} and the received profit increased by 24 Lt ha^{-1} [76]. Additionally, the

alternative technology of spring barley growth and harvest reduces weed seed spreading and weediness of the future crop.

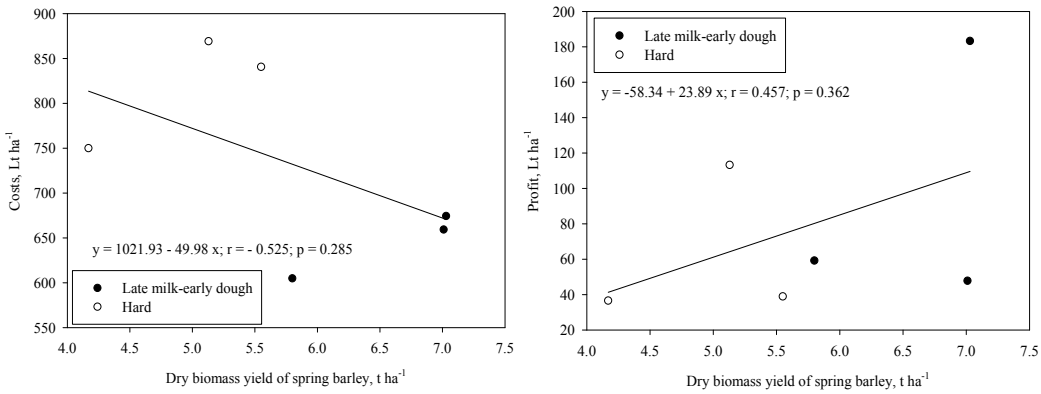

Figure 13. Costs and profit of spring barley technology depending on dry biomass yield (1 € = 3.4528 Lt) [76]

5. Conclusion

Spring barley agrophytocenosis on separate experimental plots was distinguished for different weed infestation: 395 weeds m^{-2} and 255 g m^{-2} air-dry biomass of weeds in 1997, 122 weeds m^{-2} and 98 g m^{-2} in 1998 and 135 weeds m^{-2} and 19 g m^{-2} in 1999.

Analyzing seed rain of all weed species in spring barley agrophytocenosis, there were established 4543 seeds m^{-2} in 1997, 2753 seeds m^{-2} in 1998 and 821 seeds m^{-2} in 1999. Weed seed rain was dependent on weed dry weight r=0.842** and on weed density r=0.686*. Consequently, it is very important to minimize accumulated weed biomass in the crop by weed control means before ripening and dispersal; new weed seeds build the soil weed seedbank and further field weediness.

Weed seed rain during vegetation non-linearly depended on active air temperature sum r^2= 0.528**, 0.538*, 0.119*; on rainfall r^2= 0.512**, 0.418*, 0.136* and on sunlight duration r^2= 0.567**, 0.608**, 0.155*. Increasing sum of active air-temperatures and sunlight duration increased weed seed rain by 12-54% and 14-51%, respectively. In contrast to the air temperatures and sunlight, rainfall inhibited weed seed rain by 16-57%.

Weed seed rain in spring barley agrophytocenosis began at the stem elongation stage and gradually increased until hard stage of maturity. At medium milk stage of maturity, 6-23% weed seeds were dispersed out and at late milk-early dough stage of maturity, 27-42% of weed seeds were dispersed. When harvesting cereal at milk or late milk-early dough stage of maturity, non-mature weed seeds are taken from the field together with crop yield and did not infest the soil. When harvesting cereals at medium milk stage of maturity and at late milk-early dough stage of maturity, 77-94% and 58-73%, of new weed seeds are removed from the

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field, respectively. Accordingly, it helped to control weed seed dispersal and potential weediness of future crops.

Growing and developing spring barley gradually accumulated dry biomass and metabolizable energy that reached the largest amount at milk and late milk-early dough stages. At later stages of spring barley maturity, yield and amount of metabolizable energy in spring barley decreased. Spring barley whole-plant dry matter yield at late milk-early dough maturity stage reached 7.03 t ha^{-1} and 5.80 t ha^{-1} accumulating 68.0 Gj ha^{-1} and 50.1 Gj ha^{-1} of metabolizable energy, respectively.

Alternative cereal harvesting (late milk-early dough stage of maturity, when grain humidity is 38-45 %) is promising. Harvesting of the largest crop yield could make it be possible to reduce the price of concentrated forage as well as to decrease weediness. By making whole-plant silage or haylage from cereals at late milk-early dough stage of maturity, more than 20% greater dry matter yield could be harvested.

Harvesting spring barley at the late milk-early dough growth stage helps to avoid expensive combining, grain and straw managing. Comparing these alternative and conventional technologies economically, it was established that using alternative technology, costs decreased by 21%, profit increased 1.5 times and profitability increased 1.9 times.

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References

[1] Pilipavičius V. Vasarinių miežių (*Hordeum vulgare* L.) derliaus kaupimosi ir piktžolių sėklų byrėjimo dinamika / Dynamics of spring barley (*Hordeum vulgare* L.) yield accumulation and of weed seed rain. Daktaro disertacija / dr. disertation. Kaunas-Akademija: LZUU, 2000. 102.

[2] Pilipavičius V. Preventive weed control in lower input farming system. In: 5th EWRS Workshop on Physical Weed Control, 2002, Pisa, Italy. 46-56.

[3] Ciuberkis S. The spreading of weed seeds in the fields of crop rotation. In: Challenges for Weed Science in a Changing Europe: 9th EWRS Symposium, 1995, Budapest, Hungary. 161-165.

[4] Pilipavičius V. Herbicides in winter wheat of early growth stages enhance crop productivity. In: Herbicides- properties, synthesis and control of weeds. Edited by Mohammed Naguib Abd El-Ghany Hasaneen. Rijeka: InTech; 2012. p471-492. ISBN 978-953-307-803-8. Available from: http://www.intechopen.com/articles/show/title/herbicides-in-winter-wheat-of-early-growth-stages-enhance-crop-productivity

[5] Fawcett RS, Slife FW. Effects of 2,4-D and Dalapon on weed seed production and dormancy. Weed Science 1978; 26(6) 543-547.

[6] Meinlschmidt E., Karch K. Einfluß reduzierter Herbizidendosen auf Unkrautunterdrückung und Samenproduktion. Journal of Plant Diseases and Protection 1994; Sp.iss.XIV 373-380.

[7] Pedersen JO., Rasmussen IA. The influence of herbicides on weed seed production. In: Side effect of Pesticides – Weeds: 7th Danish Plant Protection Conference 1993; 73-83.

[8] Leguizamon ES., Roberts HA. Seed production by an arable weed community. Weed Research 1982; 22(1) 35-39.

[9] Pilipavičius V. Three- year assessment of weed dynamics in herbicide-free barley crop: a field study. Žemdirbystė-Agriculture 2006; 93(3) 89-98.

[10] Hallberg GR. Pesticides pollution of groundwater in the humid United States. Agriculture, Ecosystems & Environment 1989; 26(3-4) 299-367.

[11] Wacker P. Bekämpfung von Unkräutern bei der Getreideernte. Landtechnik 1989; 6 215-219.

[12] Pilipavičius V. Weed spreading regularity and adaptivity to abiotical factors. Summary of the review of scientific works presented for dr. habil. procedure: Biomedical sciences, agronomy. Kaunas-Akademija: Lithuanian university of agriculture, 2007. 30.

[13] Zadoks JC., Chang TT., Konzak CF. A decimal code for the growth stages of cereals. Weed Research 1974; 14 415-421.

[14] Meier U. Growth Stages of Mono-and Dicotyledonous Plants. BBCH-Monograph, 1997. 572.

[15] Wilson RF., Wilkins RJ. An evaluation of laboratory ensiling techniques. Journal of the Science of Food and Agriculture 1972; 23(3) 377-385.

[16] Nauman C., Bassler R. Die chemische Untersuchungvon Futtermitteln. Methodenbuch Band III, VDLUFA. Verl. Darmstadt. 1993, 1, 2, 3, 4, 5, 6, 8, Anhang, 1-7.

[17] Naumann C., Bassler R. Bestimmung der Netto-Energie-Laktation (Schatzmethode) - Gasbildung nach Hohenheimer Futterwerttest. Die chemische Untersuchung von Futtermitteln. Metodenbuch III, 2 Erganzungslieferung. VDLUFA. Verl. Darmstadt. 1988, 25.1, 1-8.

[18] Rabotnov SA. Методы изучения семеного размножения травянистых растений в сообществах. Полевая геоботаника. Москва-Ленинград; 1960. p20-39.

[19] Moss SR. The production and shedding of *Alopecurus myosuroides* Huds. seeds in winter cereals crops. Weed Research 1983; 23 45-51.

[20] Stancevičius A., Girkutė A. Piktžolių sėklų byrėjimo dinamika javų pasėliuose. LŽŪA mokslo darbai: Žemės ūkio intensyvinimas 1972; 28 25-34.

[21] Basalykas A., Bieliukas K., Chomskis V. (1958). Lietuvos TSR fizinė geografija / Physical geography of Lithuania. Vilnius: Mokslas; 1958.

[22] Naginė V., Skrebutenienė I., Malunavičienė V. Mechanizuotų agroserviso darbų įkainiai. Pagrindinio žemės dirbimo darbai. Vilnius: Darbo ekonomikos ir mokymo metodikos tarnyba; vol.31. 2004.

[23] Naginė V., Skrebutenienė I., Malunavičienė V. Mechanizuotų agroserviso darbų įkainiai. Pasėlių priežiūra ir šienapjūtės darbai. Vilnius: Darbo ekonomikos ir mokymo metodikos tarnyba; vol.33. 2004.

[24] Naginė V., Skrebutenienė I., Malunavičienė V. Mechanizuotų agroserviso darbų įkainiai. Derliaus nuemimo darbai. Vilnius: Darbo ekonomikos ir mokymo metodikos tarnyba; vol.35. 2004.

[25] Tarakanovas P. Nauja kompiuterinės programos versija bandymo duomenų apdorojimo dispersinės analizės metodu / A new version of the computer programme for trial data processing by the method of analysis of variance. Žemdirbystė-Agriculture 1997; 60 197-213.

[26] Tarakanovas P. Statistinių duomenų apdorojimo programų paketas *Selekcija*. Akademija: LŽI; 1999. 57.

[27] Spss Science: SigmaStat® Statistical Software Version 2.0. User's Manual. USA, 1997.

[28] Spss Science: SigmaPlot® 2000 User's Guide. Exact Graphs for Exact Science. USA, 2000. 435.

[29] Pilipavičius V. Competition of weeds and spring barley in organic and conventional agriculture. Vagos 2005; 68(21) 30-43.

[30] Zwerger P. Zur Samenproduktion der Acker-Kratzdistel (*Cirsium arvense* (L.) Scop.). Journal of Plant Diseases and Protection 1996; Sp.iss.XV 91-98.

[31] Pilipavičius V. Ausfall von Unkrautsamen in Abhängigkeit von Unkrautbiomasse und Unkrautdichte. Journal of Plant Diseases and Protection 2002; Sp.iss.XVIII 239-245.

[32] Pilipavičius V. The Influence of *Stellaria media* (L.) Vill., *Sonchus asper* (L.) Hill., *Capsella bursa-pastoris* (L.) Medik. plants air-dry weight and density on their seed rain in the spring barley crop. Žemdirbystė-Agriculture 2001; 76 38-46.

[33] Kohout V. Herbologie / Weed science. Plevele a jejich regulace / Weeds and their control. Praha: Czech university of agriculture; 1996, 116.

[34] Pilipavičius V., Lazauskas P. Regulierung der Unkrautsamenverbreitung im Getreide. Journal of Plant Diseases and Protection 2000; Sp.iss.XVII 469-472

[35] Blackshaw RE., Rode LM. Effect of ensiling and rumen digestion by cattle on weed seed viability. Weed Science 1991; 39 104-108.

[36] Grigas A. Daugiamečių žolių sėklinių pasėlių ir sėklų piktžolėtumas bei piktžolių sėklų gyvybingumo išsilaikymas. LŽMTI mokslo darbai 1980; 25 105-117.

[37] Grigas A. Issledovanie sochranenija ziznessposobnosti semian sornych rastenii. In: Proceedings Zashchita rastenii v respublikach pribaltiki i Belorusii, Chiast I. 1981, Dotnuva - Akademija, Lithuania, 113-114.

[38] Grigas A., Smulkienė B. Piktžolių sėklų gyvybingumas, skirtingą laiką joms išbuvus silose. LŽMTI mokslinių straipsnių rinkinys: Agronomija 1989; 63 93-101.

[39] Sarapatka B., Holub M., Lhotska M. The effect of farmyard manure anaerobic treatment on weed seed viability. Biological Agriculture and Horticulture 1993; 10 1-8.

[40] Grigas A., Smulkienė B. Piktžolių sėklų gyvybingumas, skirtingą laiką joms išbuvus skystame mėšle. LŽMTI mokslinių straipsnių rinkinys: Agronomija 1989; 63 83-92.

[41] Tereshchuk V. Sources of weed infestation of agricultural land and the problems of weed control. In: Challenges for Weed Science in a Changing Europe: 9th EWRS Symposium, 1995, Budapest, Hungary. 135-141.

[42] Grigas A. Sėklų, perėjusių galvijų virškinamąjį traktą, gyvybingumas. LŽMTI mokslo darbai: Agronomija 1987; 35 165-175.

[43] Pilipavičius V. Piktžolių sėklų byrėjimo priklausomumas nuo meteorologinių faktorių / Dependence of weed seed falling on meteorological factors, precipitation and sunlight duration. Vagos 2002; 53(6) 17-21.

[44] Gmira N., Douira A., Bouhache M. Ecological grouping of *Solanum elaeagnifolium*: a principal weed in the irrigated Tadla plain (central Moroko). Weed Research 1998; 38 87-94.

[45] Thomas AG., Lefkovitch SL., Woo SL., Bowes GG., Peschken DP. Effect of temperature on germination within and between diploid and tetraploid populations of *Matricaria perforata* Merat. Weed Research 1994; 34 187-198.

[46] Benvenuti S., Macchia M., Miele S. Light, temperature and burial depth effects on *Rumex obtusifolius* seed germination and emergence. Weed Research 2001; 41 177-186.

[47] Harris SM., Doohan DJ., Gordon RJ., Jensen KIN. The effect of thermal time and soil water on emergence of *Ranunculus repens*. Weed Research 1998; 38 405-412.

[48] Röhring M., Stützel H. Canopy development of *Chenopodium album* in pure and mixed stands. Weed Research 2001; 41 111-128.

[49] Dakheel AJ., Radosevich SR., Barbour G. Effects of temperature and moisture on growth, interference and photosyntesis of *Bromus tectorum* and *Taeniatherum asperum*. Weed Research 1994; 34 11-22.

[50] Pilipavičius V. Piktžolių sėklų kiekis grūduose priklausomai nuo vasarinių miežių brandos tarpsnio / Weed seed getting into grain according to spring barley maturity stage. Vagos 2006; 71(24) 51-57.

[51] Svihra J., Talapka S. Rastovo-produkcny a akumulacny proces vybranych odrod jarneho jacmena. Rostlina vyroha 1995; 41 249-253.

[52] Striegl M. Faktory ovlivnujici tvorbu biomasy u jarnich obilnin. Rostlina vyroba 1982; 28 363-370.

[53] Petr J., Cierny V., Gruska L. Formirovanije urozaja osnovnych selskochozeistvennych kultur. Moskva. 1984.

[54] Korneva EJ., Davidov BN., Koskin EI. Optimizacija srokov uborki jacmenia dlia briketirovanija. In: Biologiceskie osnovy povyshenija prodnktivnosti selskochozeistvennych kultur: proceedings. 1984, Moskva. 96-99.

[55] Majdoub A., Mehouachi M., Yahyaoui A., Rahmani L. Chemical composition and nutritive value of three barley cultivars grown under the semi-arid conditions of northwestern Tunisia. Barley and Wheat Newsletter, ICARDA 1994; 13 15-19.

[56] Repka J., Rimar J., Lorencik L. Vplyv podneho typu na produkcne procesy a produkciu porastov polnych plodin. Rostlina vyroba 1978; 24 1235-1245.

[57] Petr J. Weather and yield. Praha: Developments in crop science 20, 1991.

[58] Mašauskienė A., Leistrumas K., Dovydaitis V. Miežių ir avižų bei vikių ir avižų mišinio derliaus bei cheminės sudėties dinamika pieninės, pieninės-vaškinės, vaškinės ir kietosios brandos fazėse. LŽMTI mokslo darbai: Agronomija 1982; 28 126-132.

[59] Pilipavičius V. The effect of harvest growth stage on the yield, chemical composition and metabolizable energy of spring barley. Scientia Agriculturae Bohemica 2004; 35 41-51.

[60] Pilipavičius V. Vasarinių miežių sausųjų medžiagų ir apykaitos energijos derliaus kaupimosi dinamika / Dynamics of spring barley dry matter and metabolic energy yield accumulation. Žemdirbystė - Agriculture 2000; 72 91-102.

[61] Baier J. Obsah zivin v susine ozime psenice v probehu ontogeneze. Rostlinna vyroba 1986; 32 459-471.

[62] Ayub M., Dewi IAP., Tanveer A. Forage yield and quality of barley as influenced by nitrogen application and harvest dates. Pakistan Journal of Biological Sciences. 1999; 2(4) 1278-1282.

[63] Kupka J., Novak V., Lipavsky J. Dynamika obsahu fosfoni a tvorba susiny v ontogenezi jarniho jecmene. Rostlina vyroba 1977; 23 741-744.

[64] Pettersson R. Above-ground growth dynamics and net production of spring barley in relation to nitrogen fertilization. Swedish Journal of Agricultural Research 1989; 19 135-145.

[65] Mikulionienė S., Stankevičius, R. Zootechninė pašarų analizė ir jų energetinė vertė, 2nd edition. Akademija: LZUU; 2003.

[66] Martin J., Seibold R. DLG-Futterwerttabellen. Wiederkäuer. 7., erweiterte und überarbeitete Auflage. Universität Hohenheim. Frankfurt am Main: DLG-Verlag; 1997.

[67] Pilipavičius V. Spring barley over-ground biomass digestibility *in vitro*. Veterinarija ir Zootechnika 2012; 58(80) 65-69.

[68] Pilipavičius V. Whole plant silage nutritive value from spring barley of different maturity. Veterinarija ir Zootechnika 2007; 37(59) 61-66.

[69] Nykänen A., Jauhiainen L., Rinne M. Biomass production and feeding value of whole-crop cereal legume silages. Agronomy Research 2009; 7(Special issue II) 684–690.

[70] Statistics Lithuania (2011). Statistikos departamentas prie LRV (2011), M5010302: Agricultural crops in country / Žemės ūkio augalai šalyje. http://db1.stat.gov.lt/statbank/default.asp?w=1229/ (accessed 30 August 2012).

[71] Šapolienė A. Agriculture in Lithuania 2003. Vilnius: Statistikos departamentas prie Lietuvos Respublikos Vyriausybes; 2004. 105.

[72] Rudys E. Lietuvos žemės ūkio ekonominė apžvalga 1993-1997 / Economical review of Lithuanian agriculture 1993-1997. Vilnius: LRZUM, LAEI. 1999. 136.

[73] Nedzinskienė T., Vegys R., Dabkevičiūtė H., Dabkevičienė D. Vasarinių miežių auginimas pagal intensyvią technologiją priesmėlio dirvose. LŽI mokslinių straipsnių rinkinys: Agronomija 1993; 72 89-98.

[74] Surkus J., Semaska J., Semaskiene R. Kompleksiškas pesticidų ir karbamido naudojimas miežiuose. Zemdirbyste-Agriculture 1999; 68 90-107.

[75] Edwards RA., Donalson E., MacGregor AW. Ensilage of whole-crop barley. I-Effects of variety and stage of growth. Journal of the Science of Food and Agriculture 1968; 19 656-660.

[76] Pilipavičius V., Makutėnienė D., Makutėnas V. Economical evaluation of spring barley yield at different stages of maturity. Journal of Plant Diseases and Protection 2006; Sp.iss.20 867-873.

[77] Swinton SM. Economics of site-specific weed management. Weed Science 2005; 53 259-263.

[78] Kinderiene I. Energy and economic comparison of measures designed for erosion reduction on hilly soils. Zemdirbyste – Agriculture 2005; 89 39-52.

[79] Séralini G.-E., Clair E., Mesnage R., Gress S., Defarge N., Malatesta M., Hennequin D., Spiroux de Vendōmois J. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food and Chemical Toxicology 2012; http://dx.doi.org/10.1016/j.fct.2012.08.005

Chapter 5

Determination of Pesticides in Water and Vegetable Matter by Manual Shaking-Enhanced, Ultrasound-Assisted Emulsification Microextraction Combined with Gas Chromatography-Mass Spectrometry

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Additional information is available at the end of the chapter

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1. Introduction

A chromatographic method combined with mass spectrometry is the key technique used in analysis of trace-level compounds present in complex matrices. However, success depends on the enrichment and extraction of target analytes from the matrix. This study investigated the development of a microextraction technique for use in the analysis of pesticides present at trace levels in field water samples and vegetable matter.

The most common extraction techniques used in environmental analysis are liquid–liquid extraction (LLE) [1, 2] and solid-phase extraction (SPE) [3, 4], both of which are time-consuming and use large volumes of samples. As a result, much attention is being paid to the development of more efficient and environmentally friendly extraction techniques, such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME). SPME, a solvent- free technique, was developed by Arthur and Pawlizyn [5]; however, the fiber is expensive and fragile, and the problem of sample carry-over cannot always be eliminated [4, 6-8]. LPME, which was introduced by Cantwell and co-workers [9], minimizes solvent usage and solvent variation; this technique is of interest to many analysts. Single-droplet microextraction (SDME) [10-13], solvent-bar microextraction (SBME) [14, 15] and hollow fiber-LPME (HF-LPME) [16-19] have been developed in the past few years but longer extraction times are required to obtain good extraction efficiencies. Efforts to overcome these limitations led to the development of dispersive liquid–liquid microextraction (DLLME) with the advantages of short extraction times, ease of operation, and small amounts of solvents used [20-22]. In DLLME, a water-immiscible extraction solvent, which is dissolved in a water-miscible dispersive solvent,

is introduced rapidly by syringe into an aqueous sample in a conical centrifuge tube. A cloudy mixture containing fine droplets of the extraction solvent dispersed entirely in the aqueous phase is formed. The organic phase drop, which precipitates in the bottom of the tube after centrifugation, is injected into an injection port of gas chromatography (GC) or high-performance liquid chromatography (HPLC) for further analysis.

Lately, a novel microextraction technique called ultrasound-assisted emulsification microextraction (USAEME) was developed by Garcia-Jares and co-workers [23]. In USAEME, a very small volume of water-immiscible extraction solvent is mixed with an aqueous sample solution by ultrasound-assisted emulsification to form fine droplets for extracting analytes, obviating the need of a dispersive solvent in DLLME [24]. The ultrasound-assisted emulsification is carried out at 25ºC for 10 min [23, 25] or for 9 min [26]. Recently, a few reports indicated that the use of manual shaking before ultrasound-assisted emulsification enhanced extraction efficiency. Fuh and co-workers used ultrasound with occasional manual shaking to generate a cloudy suspension [26]. The approach reported by Fontana and co-workers mixed honey samples with extraction solvent, Triton X-114, and used manual shaking to generate a homogeneous solution [27]. The work performed by Huang *et al.* showed that manual shaking for 10 s before ultrasound-assisted emulsification enhances the extraction efficiency of organochlorine pesticides (OCPs) by >100% in aqueous samples [28].

In this study, a new technique, manual shaking-enhanced, ultrasound-assisted emulsification microextraction (MS-USAEME) has been developed. Carbamate pesticides were chosen as the target analytes to evaluate the performance of the proposed method. Carbamate pesticides have been used for decades in many countries to increase agricultural production, are acetylcholinesterase inhibitors that allow acetylcholine to accumulate in the human body, resulting in health problems. Their residues can appear in fruit and vegetables, and are usually distributed in aqueous environments by leaching and runoff from soil into ground and surface water because of their high solubilities in water [29-31]. In order to detect trace amounts of the pesticides, the effects of changes of various experimental parameters, such as the nature and volume of the extraction solvent, duration of ultrasound emulsification, the effect of manual shaking and the addition of salt, were investigated and optimized. The MS-USAEME technique is simple and efficient. The objective of this study was to investigate the use of MS-USAEME for the extraction of carbamate pesticides from field water and vegetable matter.

2. Experimental

2.1. Reagents and materials

The carbamates, propoxur (99.8% purity) and pirimicarb (99% purity), were purchased from Fluka (Steinheim, Germany). Carbaryl, (99.8% purity) and methiocarb (99.5% purity) were purchased from Chem Service (West Chester, PA, USA). Carbofuran (98% purity) was purchased from Aldrich (Saint Louis, MO, USA). Stock 1 g L^{-1} solutions of each pesticide were prepared in methanol (HPLC-grade) and stored at 4 ºC. Mixed working standard solutions

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were prepared daily with deionized (DI) water purified with a Milli-Q system (Millipore, Bedford, MA, USA).

1-Octanol (99% purity), 1-nonanol (98% purity), 1-decanol (98% purity) and sodium chloride (NaCl, 99.5% purity) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 1-Undecanol was purchased from TCI (Tokyo, Japan). HPLC-grade methanol was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2. Sample preparation

Field water samples (from Cyonglin, Hsinchu, Taiwan) were passed through a 0.45 μm pore size membrane filter (Millipore, Bedford, MA, USA) and stored at 4 ºC.

A sample of lettuce (*Lactuca sativa*) (from Siluo Township, Yunlin County, Taiwan) was chopped in a food chopper, then 2 L of deionized (DI) water was added per gram of chopped lettuce and the mixture homogenized in a food homogenizer. A 15 mL sample of the homogenized lettuce was centrifuged at 1398 × g for 10 min in a benchtop centrifuge; the supernatant liquid was then passed through a 0.45 μm pore size membrane (Millipore, Bedford, MA, USA) and stored at 4 ºC.

2.3. Instrumentation

Analysis was performed on an Agilent gas chromatograph (6850 series, Wilmington, DE, USA) equipped with a split/splitless injector and coupled with an Agilent mass spectrometer (5978B series). A DB-5 MS UI fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) was used for separation of the analytes. Initially the column temperature was held at 140 ºC for 1 min, then ramped to 270 ºC at 20 ºC min^{-1} and kept at that temperature for 3.5 min. The carrier gas was helium (purity 99.9995%) that had been further purified by passage through an Agilent helium gas purifier (model RMSH-2) and the flow rate was 1.0 mL min^{-1}. The inlet was operated at 300 ºC and was used in the pulsed splitless mode. Ionization was operated in the electron impact (EI) mode at 70 eV. The temperature of the ion source was 230 ºC and the temperature of the quadrupole mass filter was 150 ºC. The MS was operated in full scan mode and a mass range of *m*/*z* 50–250 was scanned to confirm the retention times of the analytes. The selected ion monitoring (SIM) mode was used for the determination of the target compounds. Two selection ions were used for quantitation, and scan start times of the compounds were studied by gas chromatography-mass spectrometry (GC-MS): *m*/*z* 110, 152 (Propoxur); *m*/*z* 103, 164 (Carbofuran); *m*/*z* 115, 144 (Carbaryl); *m*/*z* 109, 153 (Methiocarb); *m*/*z* 166, 238 (Pirimicarb). The mass spectrometer was turned ON at 3.5 min and OFF after 11 min, to avoid filament breaking.

The ultrasonic water bath was obtained from Branson Ultrasonics (Danbury, CT, USA). The ultrasound frequency and power were 42 Hz and 100 W, respectively.

2.4. Analytical procedure

A 10 mL sample of DI water was placed in a glass centrifuge tube and spiked with five carbamate analytes at the required concentrations; 2.5 g of NaCl was added and dissolved

completely. A 10 μL portion of 1-octanol (the extraction solvent) was added and the tube was manually shaken gently for 10 s, and then immersed in an ultrasonic water bath. The levels of water bath and solution in the tube were both the same. During ultrasonication for 3 min at room temperature, the bath and the tube contents became cloudy due to the dispersion of fine droplets of 1-octanol within the aqueous bulk. After centrifugation at 1398 × g for 3 min in a benchtop centrifuge, the extraction solvent floated on the aqueous phase; the floating extraction phase (3 μL) was collected with a 25 μL microsyringe and transferred to a microtube. One μL of this extractant was injected into the GC for analysis (Figure 1). The above procedure was applied to field water and to the supernatant liquid from the lettuce samples mentioned in section 2.2.

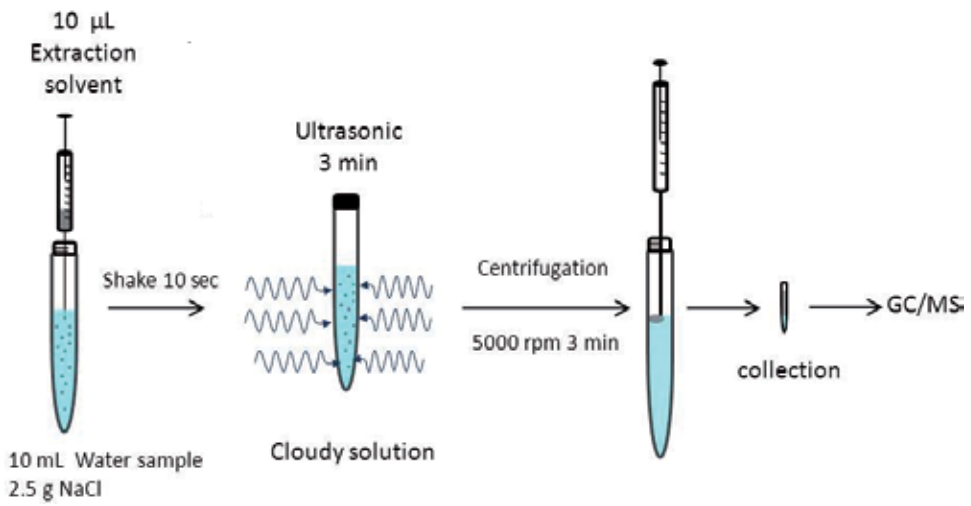

Figure 1. The analytical procedure.

3. Results and discussion

3.1. Optimization of the experimental conditions

In order to obtain the most effective extraction of carbamate pesticides, it is important to determine the optimum conditions for the analysis. The variable parameters include the nature and volume of the extraction solvent, the ultrasonication time, the ionic strength and the effect of manual shaking. The behavior of five carbamate pesticides was studied under various extraction conditions. The calculation of enrichment factor (EF) for this method was (concentration of analyte in the floating phase, C_{org}) divided by (initial concentration of analyte in the aqueous sample, C_0).

$$EF = C_{org} / C_0$$

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3.1.1. Selection of extraction solvent

The selection of an extraction solvent is the most important experimental parameter in this method. An appropriate extraction solvent must have: (1) low toxicity, (2) immiscibility with water and (3) high extraction ability for the target analytes. On the basis of these criteria, to achieve good extraction of carbamates from aqueous samples, alcohols 1-octanol, 1-nonanol, 1-decanol and 1-undecanol were chosen as potential extraction solvents. The final selection of solvent was decided on the basis of extraction efficiency. Comparison of EF obtained with each of the four extraction solvents showed that 1-octanol was the most effective; see Figure 2. It seems that carbamates have a better affinity for a slightly polar solvent, 1-octanol.

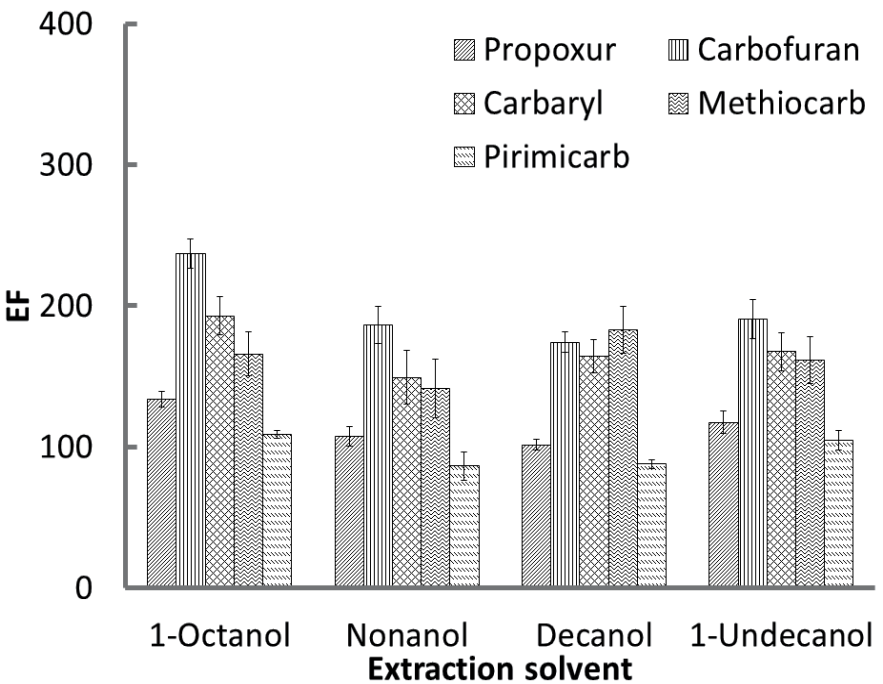

Figure 2. Selection of extraction solvent ($n = 3$). Samples were spiked with 50 μg L^{-1} of each analyte. Extraction conditions: aqueous sample volume 10.0 mL; extraction solvent volume 15 μL; ultrasonication time: 5 min; salt addition: 1 g NaCl.

3.1.2. Effect of the volume of extraction solvent

The volume of the floating phase is increased with increasing volume of the extraction solvent; however, the analytes are diluted as a result of the increased volume of the floating phase. To estimate the influence of volume of extraction solvent on the procedure, the volume of extraction solvent (1-octanol) was varied in the range 8 to 15 μL. The results showed that the EF of the analytes decreased when the volume of the extraction solvent was added above 10 μL. This may be due to the dilution of extracts (Figure 3). Therefore, 10 μL of extraction solvent was chosen as the optimal volume for further studies.

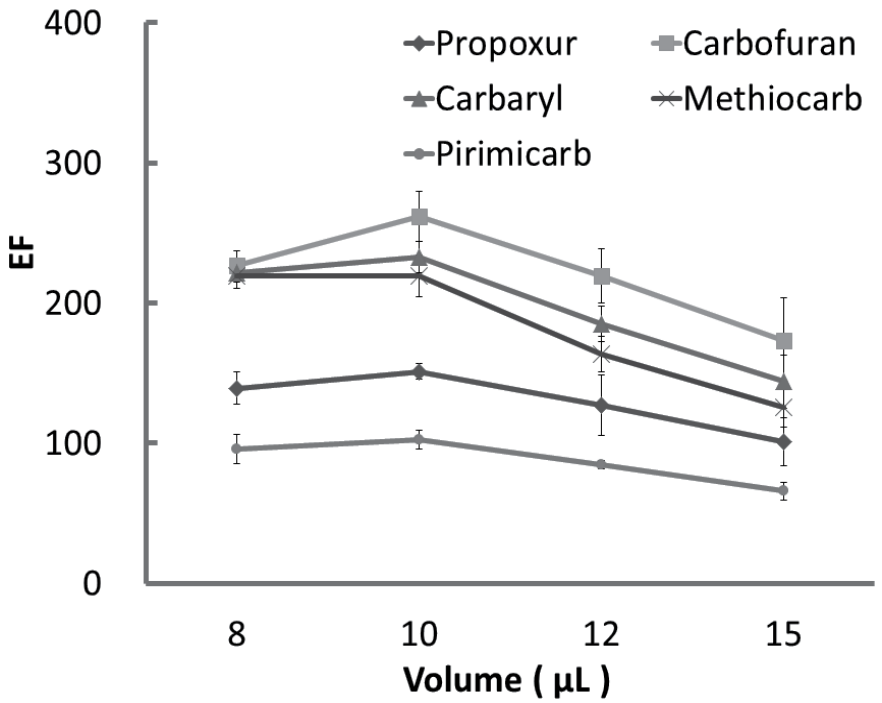

Figure 3. Effect of the extraction solvent volume (n = 3). Samples were spiked with 50 µg L^{-1} of each analyte. Extraction conditions: aqueous sample volume 10.0 mL; extraction solvent 1-octanol; ultrasonic time: 5 min; salt addition: 1 g NaCl.

3.1.3. Effect of ultrasonication time

The ultrasonication time might affect extraction efficiency because it affects both emulsification and the mass transfer process. To examine this effect, the EF was monitored with extraction times varying from 0 to 7 min (Figure 4). The maximum EF was achieved after ultrasonication for 3 min and no significant variation was observed with extraction times longer than 3 min. This is probably due to the fact that the ultrasonic water bath generates the emulsion quickly, rapidly making a very large contact surface area between the extraction solvent and the aqueous phase. Therefore 3 min was taken to be the optimum extraction time.

3.1.4. Effect of manual shaking

Manual shaking was essential to ensure that the extraction solvent and aqueous samples were adequately mixed before the ultrasound treatment when the ultrasonic extraction time was as brief as 3 min. A study done by Huang and co-workers [28] found that maximum peak area was achieved with 10 s of manual shaking and there was no significant increase for longer times. Here, with manual shaking, the EF was significantly higher than the EF value obtained from no manual shaking. The shaking assured that the aqueous sample was mixed well with the extraction solvent, thus generating the emulsion quickly (Figure 5).

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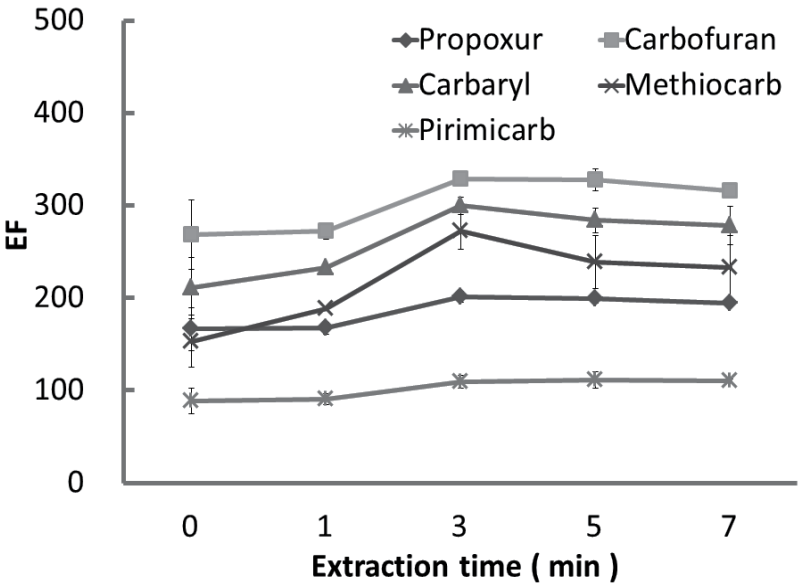

Figure 4. Effect of ultrasonic extraction time (n = 3). Samples were spiked to 50 μg L^{-1} of each analyte. Extraction conditions: aqueous sample volume 10.0 mL; extraction solvent 1-octanol; extraction solvent volume 10 μL; salt addition: 1 g NaCl.

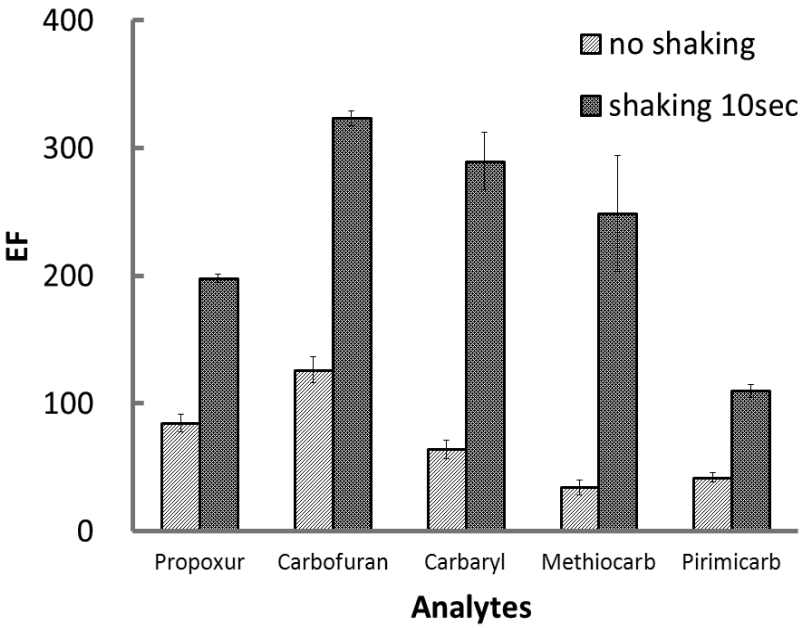

Figure 5. Effect of hand-shaking (n = 3). Samples were spiked to 50 μg L^{-1} of each analyte. Extraction conditions: aqueous sample volume 10.0 mL; extraction solvent 1-octanol; extraction solvent volume 10 μL; ultrasonication time: 3 min; salt addition: 1 g NaCl.

3.1.5. Effect of the amount of salt added

Ionic strength is an important determinant of extraction efficiency. During the extraction procedure, adding salt increases ionic strength, which leads to the "salting out" phenomenon, commonly discussed in liquid-liquid extraction. The presence of salt decreases the solubility of target analytes in aqueous phases, and improves their partition from aqueous to organic layer. This was observed on the increasing EFs when NaCl was added from 0 to 2.5 g (Figure 6). However, adding salt also eliminates the emulsion between aqueous and organic layers which results in the larger volume of floating phase left after extraction. When more salt was added, more floating phase was obtained. The larger volume of floating phase offsets the enhanced partition of the pesticides in organic layer. There was no significant change of the EF as the amount of salt added was increased from 2.5 to 3.0 g. On the basis of this result, 2.5 g of NaCl was added to the aqueous sample solution for further studies.

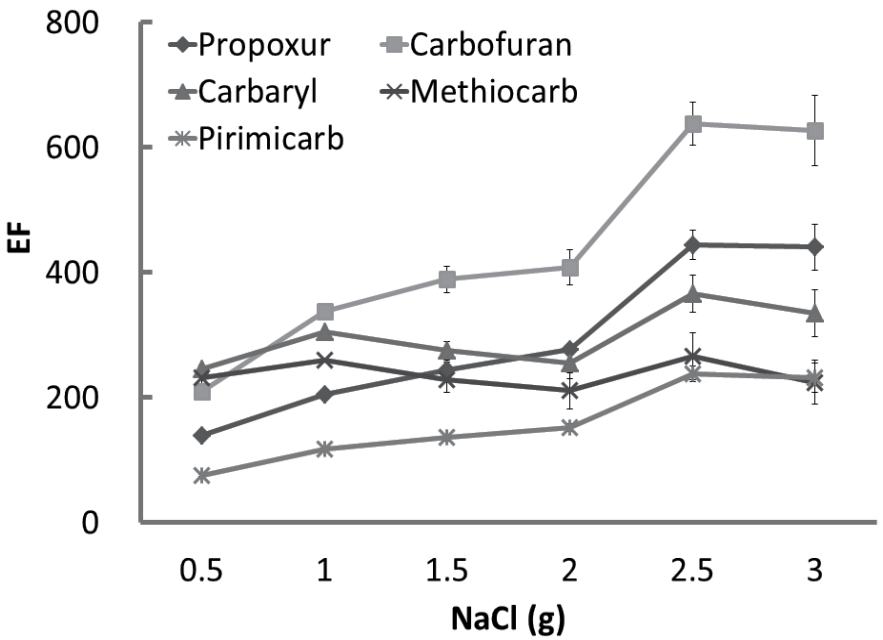

Figure 6. Effect of salt addition (n = 3). Samples were spiked to 50 μg L^{-1} of each analyte. Extraction conditions: aqueous sample volume 10.0 mL; extraction solvent 1-octanol; extraction solvent volume 10 μL; ultrasonication time: 3 min.

3.2. Comparison of methods

The proposed method requires simple equipment: a 25 μL syringe, 10 μL of low toxicity extraction solvent, and an ultrasonic water bath, used to emulsify the extraction solvent and sample to form fine droplets for extraction. Compared to DLLME, solvent terminated dispersive liquid-liquid microextraction (ST-DLLME) and dispersive liquid-liquid microextraction

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combined with sweeping micellar electrokinetic chromatography (DLLME-sweeping-MEKC) method, this method avoids the use of a high toxicity extraction solvent and a large amount of dispersive solvent, which increases the solubility of the analytes in water during extraction. Unlike other extraction methods such as SPE, SPME, LPME, single drop microextraction (SDME) and quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based extraction, there is no need for this method to use a packed solid phase cartridge, a fragile fiber coated with a polymeric phase, a section of hollow fiber, a metal stand or clean-up sorbent. The apparatus needed for MS-USAEME is simpler than that required by the above-mentioned methods. According to the results in Table 1, the proposed method, which uses less organic solvent, maintains the advantage of short extraction times and makes the extraction more efficient.

Methods	Linearity (ng mL^{-1})	MDL (ng mL^{-1})	RSD (%)	Extraction time (min)	EF	Ref.
HF-LPME[a]-HPLC-UV	1–1,000	0.024–5.5	1.90–9.53	30	294-873	[32]
HF-LPME[a]–GC–MS	1–400	0.2–0.8	4.86–7.81	20	37-144	[30]
SPME[b]–GC–MS	–	1.2–4.6	13–17	120	-	[33]
SPME[b]–HPLC–MS	50–5,000	1–10	1–6	90	-	[34]
DLLME[c]–HPLC-UV	5–500	0.4–1.0	4.7–6.5	1	101-145	[35]
ST-DLLME[d]-GC-MS	0.005-20	0.001-0.5	2.3-6.8	10	-	[36]
UASEME-HPLC-DAD	0.3-200	0.1-0.3	3.4-4.8	3	170-246	[37]
DLLME-sweeping-MEKC[e]	10-500	2.0-3.0	4-7-6.5	1	491-1834	[38]
SDME[f]-GC-MS	0.05-200	0.02	0.6-13.1	15	-	[39]
QuEChERS[g]-LC-MSMS	1-20	1	<20	21	-	[40]
This work	0.05-100	0.013-0.026	6.8-16.9	3	237-638	This work

a) HF-LPME: hollow fiber liquid phase microextraction.

b) SPME: solid phase microextraction.

c) DLLME: dispersive liquid liquid microextraction.

d) ST-DLLME: solvent terminated dispersive liquid-liquid microextraction

e) DLLME-sweeping-MEKC: dispersive liquid-liquid microextraction combined with sweeping micellar electrokinetic chromatography

f) SDME: single drop microextraction.

g) QuEChERS: quick, easy, cheap, effective, rigged and safe procedure.

Table 1. Comparison of methods.

3.3. Analytical performance

Linearity (LR), regression coefficient (R^2) and EF were investigated under optimized experimental conditions. The LR of the method was evaluated using water samples spiked with the selected compounds at various concentrations. The performance of the proposed method is summarized in Table 2. The linear calibration of the targeted carbamate pesticides was examined in the range 0.05 to 100 μg L^{-1}. Linear plots yielded R^2 ≥0.9972. The EF for all of the carbamate pesticides tested was in the range from 237 to 638. The results indicate that MS-USAEME combined with GC-MS was sensitive enough for the detection of these five carbamate pesticides.

Carbamate	LR (μg L^{-1})	R^2	EF
Propoxur	0.05~100	0.9987	444
Carbofuran	0.05~100	0.9988	638
Carbaryl	0.05~100	0.9993	365
Methiocarb	0.05~100	0.9972	266
Pirimicarb	0.05~100	0.9980	237

Propoxur : 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20, 100 μg L^{-1}

Carbofuran : 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20, 100 μg L^{-1}

Carbaryl : 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20, 100 μg L^{-1}

Methiocarb: 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20, 100 μg L^{-1}

Pirimicarb : 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20, 100 μg L^{-1}

Table 2. Analytical performance.

3.4. Analysis of real samples

In order to investigate the influence of the sample matrix in real samples, the determination of carbamate pesticides in water and vegetable matter was done using the method described here (Figure 7). All of the real samples were spiked with 5 μg L^{-1} methiocarb and 0.1 μg L^{-1} of the other four analytes to calculate the recovery of the targeted compounds. The reproducibility of the method was satisfactory; the RSD ranged from 6.8 to 16.9%. The relative recoveries were calculated by the ratios of the concentration of the carbamate pesticides in real samples and the concentrations of the analytes extracted in ultrapure water samples. Both samples were spiked with the same amount of pesticides. For all target compounds, the relative recoveries of field water and vegetables were within 77 to 114%. The definition of absolute recovery was determined by the ratio of extracted concentration in real samples and concentration spiked in the real sample. The absolute recoveries of target analytes were between 5.3 to 30.6%. The method detection limit (MDL) was calculated as three times the standard deviation of seven replicate runs of water and vegetable samples spiked with low concentrations of the analytes. MDL ranged from 0.013 to 0.022 μg L^{-1} for field water and 0.017 to 0.026 μg L^{-1} for the vegetable sample (Table 3).

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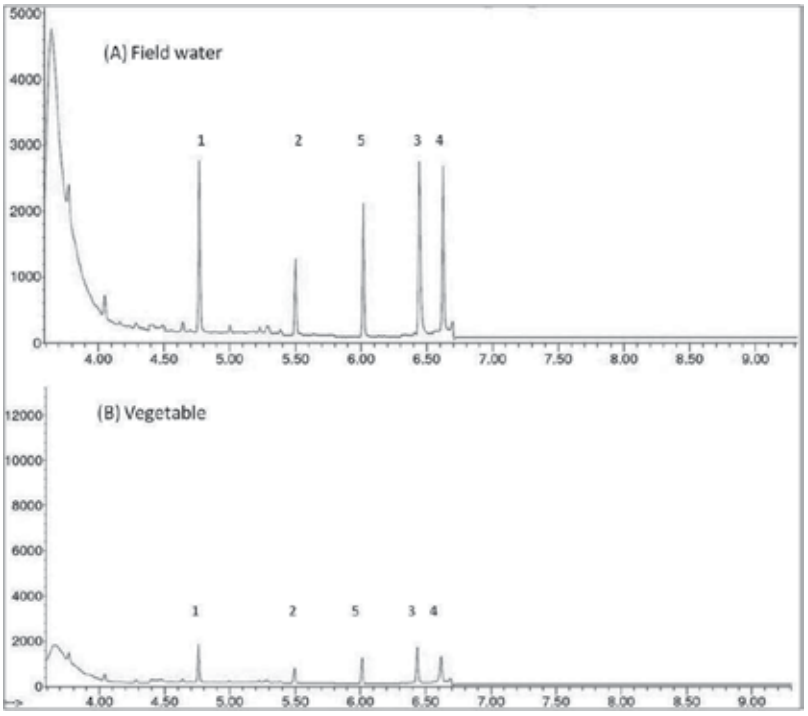

Figure 7. GC-MS chromatogram of A) field water sample and B) lettuce spiked with 5 carbamate pesticides at 0.5 μg L^{-1}; Extraction conditions: samples volume 10.0 mL; extraction solvent 1-octanol; extraction solvent volume 10 μL; ultrasonication time: 3 min; salt addition : 2.5 g NaCl. 1. Propoxur. 2. Carbofuran. 3. Carbaryl. 4. Methiocarb. 5. Pirimicarb.

Compound	Field water				Vegetable sample			
	MDL (μg L^{-1})	RSD% (n=6)	Absolute recovery (%)[a]	Relative recovery (%)[b]	MDL (μg L^{-1})	RSD% (n=6)	Absolute recovery (%)[a]	Relative recovery (%)[b]
Propoxur	0.017	8.2	17.3	82	0.022	7.9	14.5	98
Carbofuran	0.022	10.9	30.6	114	0.026	7.2	20.3	110
Carbaryl	0.019	6.8	13.1	77	0.023	11.3	11.3	96
Methiocarb	0.017	16.9	7.9	110	0.017	8.0	5.3	105
Pirimicarb	0.013	8.2	10.3	85	0.023	11.5	8.4	100

[a] Absolute recoveries were determined by the ratio of the extracted concentration to the spiked concentration in the real sample.

[b] Relative recoveries were determined by the ratio of the concentration found in the real sample to the concentration in deionized water samples. Both samples were spiked with the same amount of analytes. The extraction yields obtained from deionized water were considered as 100%.

Concentrations used for testing absolute recovery and relative recovery were: propoxur: 0.1 μg L^{-1}; carbofuran: 0.1 μg L^{-1}; carbaryl: 0.1 μg L^{-1}; methiocarb: 5 μg L^{-1}; pirimicarb: 0.1 μg L^{-1}. MDL: spiked with 0.05 μg L^{-1} each compound.

Table 3. Analysis of real samples.

4. Concluding remarks

This paper describes a simple, rapid extraction method using GC-MS to analyze carbamate pesticides in field aqueous and lettuce samples. Manual shaking for 10 s before ultrasonication is essential for effective extraction when the ultrasonication time is as brief as 3 min. The method performed well; repeatability, EF and recovery were satisfactory and the analysis can be done in a short time. Compared to other microextraction methods, the method described here is environmentally friendly and has the advantages of speed, simplicity, frugal use of organic solvent (10 μL/sample) and low cost.

Acknowledgements

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References

[1] Yasuhara, A, Shiraishi, H, Nishikawa, M, Yamamoto, T, Uehiro, T, Nakasugi, O, et al. Determination of organic components in leachates from hazardous waste disposal sites in Japan by gas chromatography-mass spectrometry. J Chromatogr A. (1997).

[2] Puig, D, & Barcelo, D. Determination of phenolic compounds in water and waste water. TRAC-TREND ANAL CHEM. (1996). , 15(8), 362-75.

[3] Kelly, M. T, & Larroque, M. Trace determination of diethylphthalate in aqueous media by solid-phase microextraction-liquid chromatography. J Chromatogr A. (1999). , 841(2), 177-85.

[4] Magdic, S, & Pawliszyn, J. B. Analysis of organochlorine pesticides using solid-phase microextraction. J Chromatogr A. (1996). , 723(1), 111-22.

[5] Arthur, C. L, & Pawliszyn, J. Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal Chem. (1990). , 62(19), 2145-8.

[6] Hiroyuki, K. Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. J Chromatogr A. (1996).

[7] Acedo-valenzuela, M-I, Galeano-díaz, T, Mora-díez, N, & Silva-rodríguez, A. Determination of neutral and cationic herbicides in water by micellar electrokinetic capillary chromatography. Anal Chim Acta. (2004). , 519(1), 65-71.

[8] Fei, T, Li, H, Ding, M, Ito, M, & Lin, J-M. Determination of parabens in cosmetic products by solid-phase microextraction of poly(ethylene glycol) diacrylate thin film on fibers and ultra high-speed liquid chromatography with diode array detector. J Separ Sci. (2011). , 34(13), 1599-606.

[9] Jeannot, M. A, & Cantwell, F. F. Solvent microextraction into a single drop. Anal Chem. (1996). , 68(13), 2236-40.

[10] Zhao, L, & Lee, H. K. Application of static liquid-phase microextraction to the analysis of organochlorine pesticides in water. J Chromatogr A. (2001). , 919(2), 381-8.

[11] Saraji, M, & Farajmand, B. Application of single-drop microextraction combined with in-microvial derivatization for determination of acidic herbicides in water samples by gas chromatography-mass spectrometry. J Chromatogr A. (2008).

[12] Ahmadi, F, & Assadi, Y. Hosseini SMRM, Rezaee M. Determination of organophosphorus pesticides in water samples by single drop microextraction and gas chromatography-flame photometric detector. J Chromatogr A. (2006).

[13] Jeannot, M. A, & Cantwell, F. F. Solvent microextraction as a speciation tool: determination of free progesterone in a protein solution. Anal Chem. (1997). , 69(15), 2935-40.

[14] Jiang, X, & Lee, H. K. Solvent Bar Microextraction. Anal Chem. (2004). , 76(18), 5591-6.

[15] Melwanki, M. B, Huang, S-D, & Fuh, M-R. Three-phase solvent bar microextraction and determination of trace amounts of clenbuterol in human urine by liquid chromatography and electrospray tandem mass spectrometry. Talanta. (2007). , 72(2), 373-7.

[16] Shen, G, & Lee, H. K. Hollow fiber-protected liquid-phase microextraction of triazine herbicides. Anal Chem. (2001). , 74(3), 648-54.

[17] Chen, P-S, Huang, S-P, Fuh, M-R, & Huang, S-D. Determination of organochlorine pesticides in water using dynamic hook-type liquid-phase microextraction. Anal Chim Acta. (2009). , 647(2), 177-81.

[18] Psillakis, E, & Kalogerakis, N. Hollow-fibre liquid-phase microextraction of phthalate esters from water. J Chromatogr A. (2003).

[19] Huang, S-P, Chen, P-S, & Huang, S-D. Dynamic headspace time-extended helix liquid-phase microextraction. J Chromatogr A. (2009). , 1216(20), 4347-53.

[20] Rezaee, M, & Assadi, Y. Milani Hosseini M-R, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid-liquid microextraction. J Chromatogr A. (2006).

[21] Farajzadeh, M. A, Bahram, M, & Jonsson, J. A. Dispersive liquid-liquid microextraction followed by high-performance liquid chromatography-diode array detection as an efficient and sensitive technique for determination of antioxidants. Anal Chim Acta. (2007). , 591(1), 69-79.

[22] Nagaraju, D, & Huang, S-D. Determination of triazine herbicides in aqueous samples by dispersive liquid-liquid microextraction with gas chromatography-ion trap mass spectrometry. J Chromatogr A. (2007).

[23] Regueiro, J, Llompart, M, Garcia-jares, C, Garcia-monteagudo, J. C, & Cela, R. Ultrasound-assisted emulsification-Microextraction of emergent contaminants and pesticides in environmental waters. J Chromatogr A. (2008).

[24] Wang, W-X, Yang, T-J, Li, Z-G, Jong, T-T, & Lee, M-R. A novel method of ultrasound-assisted dispersive liquid-liquid microextraction coupled to liquid chromatography-mass spectrometry for the determination of trace organoarsenic compounds in edible oil. Anal Chim Acta. (2011). , 690(2), 221-7.

[25] Ozcan, S, Tor, A, & Aydin, M. E. Determination of selected polychlorinated biphenyls in water samples by ultrasound-assisted emulsification-microextraction and gas chromatography-mass-selective detection. Anal Chim Acta. (2009). , 647(2), 182-8.

[26] Lin, S-L, & Fuh, M-R. Orthogonal array optimization of ultrasound-assisted emulsification microextraction for the determination of chlorinated phenoxyacetic acids in river water. J Chromatogr A. (2010). , 1217(21), 3467-72.

[27] Fontana, A. R, Camargo, A. B, & Altamirano, J. C. Coacervative microextraction ultrasound-assisted back-extraction technique for determination of organophosphates pesticides in honey samples by gas chromatography-mass spectrometry. J Chromatogr A. (2010). , 1217(41), 6334-41.

[28] Wei, S-Y, Leong, M-I, Li, Y, & Huang, S-D. Development of liquid phase microextraction based on manual shaking and ultrasound-assisted emulsification method for analysis of organochlorine pesticides in aqueous samples. J Chromatogr A. (2011). , 1218(51), 9142-8.

[29] Wu, Y. S, & Lee, H. K. Li SFY. Separation and determination of pesticides by capillary electrophoresis. I. Rapid separation of fifteen *N*-methylcarbamate insecticides via micellar electrokinetic chromatography. J Microcolumn Sep. (1998). , 10(3), 239-47.

[30] Zhang, J, & Lee, H. K. Application of liquid-phase microextraction and on-column derivatization combined with gas chromatography-mass spectrometry to the determination of carbamate pesticides. J Chromatogr A. (2006). , 1117(1), 31-7.

[31] Molina, M, Pérez-bendito, D, & Silva, M. Multi-residue analysis of N-methylcarbamate pesticides and their hydrolytic metabolites in environmental waters by use of solid-phase extraction and micellar electrokinetic chromatography. Electrophoresis. (1999). , 20(17), 3439-49.

[32] Hylton, K, & Mitra, S. Barrier film protected, and mixed solvent optimized microscale membrane extraction of methyl carbamate pesticides. J Chromatogr A. (2007).

[33] Carabias-martínez, R, García-hermida, C, Rodríguez-gonzalo, E, & Ruano-miguel, L. Behaviour of carbamate pesticides in gas chromatography and their determination with solid-phase extraction and solid-phase microextraction as preconcentration steps. J Separ Sci. (2005). , 28(16), 2130-8.

[34] Sagratini, G, Manes, J, Dario, G, Damiani, P, & Pico, Y. Analysis of carbamate and phenylurea pesticide residues in fruit juices by solid-phase microextraction and liquid chromatography-mass spectrometry. J Chromatogr A. (2007). , 1147(2), 135-43.

[35] Wu, Q, Zhou, X, Li, Y, Zang, X, Wang, C, & Wang, Z. Application of dispersive liquid-liquid microextraction combined with high-performance liquid chromatography to the determination of carbamate pesticides in water samples. Anal Bioanal Chem. (2009). , 393(6), 1755-61.

[36] Chen, H, Chen, R, & Li, S. Low-density extraction solvent-based solvent terminated dispersive liquid-liquid microextraction combined with gas chromatography-tandem mass spectrometry for the determination of carbamate pesticides in water samples. J Chromatogr A. (2009). , 1217(8), 1244-8.

[37] Wu, Q, Chang, Q, Wu, C, Rao, H, Zeng, X, Wang, C, et al. Ultrasound-assisted surfactant-enhanced emulsification microextraction for the determination of carbamate pesticides in water samples by high performance liquid chromatography. J Chromatogr A. (2010). , 1217(11), 1773-8.

[38] Zhang, S, Li, C, Song, S, Feng, T, Wang, C, & Wang, Z. Application of dispersive liquid-liquid microextraction combined with sweeping micellar electrokinetic chromatography for trace analysis of six carbamate pesticides in apples. Anal Methods. (2010). , 2(1), 54-62.

[39] Chen, H, Chen, R, Feng, R, & Li, S. Simultaneous Analysis of Carbamate and Organophosphorus Pesticides in Water by Single-Drop Microextraction Coupled with GC-MS. Chromatographia. (2009). , 70(1), 165-72.

[40] Chung, W. C, & Chan, T. P. Validation and use of a fast sample preparation method and liquid chromatography-tandem mass spectrometry in analysis of ultra-trace levels of 98 organophosphorus pesticide and carbamate residues in a total diet study involving diversified food types. J Chromatogr A. (2010). , 1217(29), 4815-24.

Chapter 6

Degradation of Cyclohexanedione Oxime Herbicides

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José Luis Alonso-Prados and Pilar Sandín-España

Additional information is available at the end of the chapter

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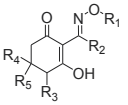
1. Introduction

The use of herbicides is the most common practice for weed control, not only in agricultural fields, but also in urban and industrial areas and communication routes. However, the growing number of detections of herbicide residues in different environmental matrices [1-5] has increased public concern about the widespread use of these compounds. In this sense, the European Union has developed a Community legal framework concerning the commercialization of plant protection products on the market (Regulation EC Nº 1107/2009) and the sustainable use of pesticides (Directive 2009/128/EC) in order to protect human and animal health and the environment from possible risks associated with the use of pesticides. Both documents emphasize that pesticides and their residues shall have no unacceptable effects on the environment, having particular regard to contamination of water bodies as well as the impact on non-target organisms.

In this sense, to meet more stringent regulations, the agrochemical industry tends to develop herbicides with low environmental persistence, effectiveness at a low application rate and minimal non-target organism toxicity. Consequently, new families of herbicides such as imidazolines, sulfonylureas or cyclohexanediones have appeared in the market in the last years.

It is important to note that Regulation EC Nº 1107/2009 describes "residues" as the substances resulting from the use of pesticides, including their metabolites, breakdown or reaction products (e.g. substances resulting from water treatment). This is particularly important for new families of pesticides, like cyclohexanedione herbicides, because the scientific literature about their environmental behaviour and persistence in the environment is quite limited. Cyclohexanedione oxime herbicides (CHD) have been developed during the last 30 years. While alloxydim-sodium was the first herbicide of this family discovered and introduced into the market in 1978 [6], profoxydim was the last CHD herbicide registered in 1998 [7]. This class

of herbicides also includes butroxydim, clethodim, cycloxydim, sethoxydim, tepraloxydim and tralkoxydim (Table 1) [8].



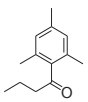
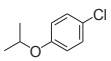
Herbicide	R_1	R_2	R_3	R_4	R_5
Alloxydim	$-CH_2-CH=CH_2$	$-CH_2-CH_2CH_3$	$-COOCH_3$	$-CH_3$	$-CH_3$
Butroxydim	$-CH_2CH_3$	$-CH_2CH_3$	H		H
Clethodim	$-CH_2CH=CH-Cl$	$-CH_2CH_3$	H		H
Cycloxydim	$-CH_2CH_3$	$-CH_2-CH_2CH_3$	H		H
Profoxydim		$-CH_2-CH_2CH_3$	H		H
Sethoxydim	$-CH_2CH_3$	$-CH_2-CH_2CH_3$	H		H
Tepraloxydim	$-CH_2CH=CH-Cl$	$-CH_2CH_3$	H		H
Tralkoxydim	$-CH_2CH_3$	$-CH_2CH_3$	H		H

Table 1. Structures of cyclohexanedione herbicides.

In general, this family of herbicides is used for post-emergence control of annual and perennial grass weeds in broad-leaved crops (including sugar beet, soybean, oilseed rape), except profoxydim which is used for the control of grass weeds in rice [9] and tralkoxydim which is used for the control of annual winter grass weeds in wheat and barley fields [7].

The site of CHD herbicides action is on acetyl-Coenzyme A carboxylase (ACCase), a key early enzyme in the lipid biosynthesis pathway. The inhibition of this enzyme prevents fatty acid formation and the lack of lipids results in loss of cell integrity of membranes and no new growth.

The chemical structure of the CHD herbicides is shown in Table 1. These compounds show a keto-enol tautomerism due to the presence of two ketone groups, as well as two isomers *E*/*Z* relating to the alkyl side chain bound to oxime ether group. The herbicidal activity of these compounds are mainly due to the cyclohexane-1,3-dione ring and the oxyimino group, although its activity can be increased depending on the different functionalization of the substituents R_1-R_5 [6,8].

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As was mentioned before, CHD herbicides have been developed to reduce the adverse effects of some herbicides and to fulfil environmental requirements set by the international legislation. However, some of their physico-chemical properties (Table 2), such as the polar and non-volatile character, and the adsorption and partition coefficients make them highly mobile. These features increase the possibility for these herbicides to reach aquatic bodies, becoming potential contaminants of this compartment.

Regarding the persistence of CHD herbicides in the environment, these xenobiotics are susceptible to rapid degradation due to the action of biotic and abiotic processes. For example, these herbicides are decomposed at a pH below 5 and above 10, and they are also photochemically and thermally unstable. Furthermore, these compounds are readily degraded through microbial and plant metabolism. In some cases the degradation is so fast that it is questioned if herbicidal activity is maybe due to some degradation products [10-12].

In this sense, to estimate the persistence of these compounds in the environment, it is of utmost importance to investigate the factors affecting the behaviour of CHD herbicides and the routes involved in their degradation, as well as to identify the degradation products formed.

Herbicide	Trade Name	Water Solubility ($mg\ L^{-1}$)	V.p. (20-30 ºC)* (mPa)	Henry's Constant ($Pa\ m^3\ mol^{-1}$)	Log K_{ow} (pH 7)	pK_a	DT_{50} in soil (days)	K_{oc}	Soil Mobility (pH 7)
Alloxydim	Clout, Kusagard, Fervin [13]	$> 2{\cdot}10^6$ (sodium salt)	< 0.133	—	0.20	3.7 [14]	2-10	60 [12]	—
Butroxydim	Falcon	6.9	$1{\cdot}10^{-3}$	$5.79{\cdot}10^{-5}$	1.90	4.36	9	6-1270	large variable
Clethodim	Select [13]	5.45 [15]	$1{\cdot}10^{-2}$	$1.4{\cdot}10^{-7}$ [15]	4.14 [15]	4.47 [15]	1-3	900 [12]	very high [15]
Cycloxydim	Focus, Laser, Stratos [16]	53	0.01	$6.1{\cdot}10^{-5}$	1.36	4.17	< 1	<10-183	high to very high [17]
Profoxydim	Aura, Tetris	5.31	$1.7{\cdot}10^{-1}$	$1.76{\cdot}10^{-2}$	3.9	5.91	3-13	81-5983 [18]	large variable [18]
Sethoxydim	Poast, Nabu	> 4700	< 0.013	$1.39{\cdot}10^{-6}$ [19]	1.65	4.1 [12]	1	100 [12]	high [20]
Tepraloxydim	Aramo	430	$2.7{\cdot}10^{-2}$	$8.74{\cdot}10^{-6}$	0.2	4.58	5.2-14	3.7 [21]	high [14]
Tralkoxydim	Achieve, Grasp, Splendor	6.7	$3.7{\cdot}10^{-4}$	$2{\cdot}10^{-5}$	2.1	4.3	2-5	30-300	very high [22]

* V.p.: Vapour pressure.

Table 2. Physico-chemical properties of CHD herbicides. Unless otherwise noted, data were compiled from Pesticide Manual [7].

2. Transformation processes affecting persistence of cyclohexanedione oxime herbicides

Following their application, the environmental fate of herbicides depends to a great extent on biotic and abiotic degradation processes. As a result of these degradation processes, different by-products, often with unknown properties, may be formed before they achieve the complete mineralization. The extent of degradation as well as the nature of by-products formed depend on the chemical structure and physico-chemical properties of the parent compound (Table 2), characteristics of the compartment in which the herbicide is present and also the environmental conditions [23].

A large number of kinetic models to describe the transformation of pesticides are available. However, the simplest model that can provide a sensible and adequate description of the decline curves is preferred [24]. At relatively low concentrations of pesticides (approximately $1 \cdot 10^{-3}$ M), the model that best describes the rate of degradation of many xenobiotics is one that follows a first order kinetics

$$C = C_0 \cdot e^{-k \cdot t} \quad (1)$$

where C is the herbicide concentration at time t, C_0 is the initial concentration of herbicide and k is the rate constant of the transformation process.

Herbicide transformation is often expressed in terms of half-life ($t_{1/2}$) because it is a more intuitive parameter than the rate constant, k. This parameter is defined as the time taken for herbicide concentration to fall to half its initial value and it is related to the rate constant, k, by means of the Equation 2.

$$t_{1/2} = \ln 2 / k \quad (2)$$

Under field conditions, transformation processes occur simultaneously with other processes leading to herbicide dissipation. In these cases, the term DT_{50} value is more appropriate than $t_{1/2}$ and reflects the time for the dissipation of 50% of the initial concentration.

It should be noted that half-lives vary in a wide range depending on the nature of herbicides, the compartment characteristics as well as the environmental conditions, so caution should be taken in making comparisons between herbicides.

2.1. Abiotic processes

The main abiotic transformation processes affecting the efficiency, persistence and fate of herbicides include reactions initiated by light, temperature, reactions in aqueous media (as a reaction medium and pH variations) and reactive substances present in the compart-

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ments. Below we review the most important studies about abiotic degradation of CHD herbicides to date.

2.1.1. Hydrolysis

As mentioned previously, CHD herbicides can be potential contaminants of the water compartment due to their physico-chemical properties. In this compartment, hydrolysis reactions are one of the main abiotic transformation process affecting herbicides [25]. This process can be particularly important in groundwater where other abiotic transformation processes such as thermal degradation or photolysis are not relevant. Moreover, it is well-known that the moisture content in soil also affects the persistence of herbicides [26].

Hydrolysis is a pH-dependent process and the rates of transformation can significantly vary among herbicides. For instance, some herbicides may undergo hydrolysis at pH extremes, while a slight variance of pH could give rise to a fast degradation of those herbicides that are pH-sensitive [27,28].

The penetration of herbicides in plants is also affected by the pH of the water used in the pesticide mixture and, hence, the effectiveness of the herbicide could be affected by this parameter too. Usually, the absorption by plants is higher when herbicides are in their non-ionized form. As CHD herbicides are weak acids (pKa ≈ 3.7-5.9), an increase in pH of the aqueous solution leads to an increase of ionized herbicide molecules (anionic form) and therefore, they are absorbed more slowly across the plant cuticle and its phytotoxicity would be lower.

As a first approach, Iwataki and Hirono (1979) [29] observed that alloxydim CHD herbicide was hydrolyzed in aqueous solution under acidic and basic conditions.

The influence of pH on the abiotic transformation of clethodim in aqueous solution was studied by Falb *et al.* [30]. These authors stated that clethodim is an acid labile herbicide and its degradation increased as acidity increased (Figure 1). At neutral pH, no degradation of clethodim was observed and a total recovery was obtained, while at a pH 6 and 5 the herbicide recoveries decreased by 8% and by 37%, respectively, after 20 hours.

Others CHD herbicides such as cycloxydim, profoxydim or tralkoxydim have also been reported to undergo hydrolysis. Profoxydim hydrolysis depends on the pH value with degradation rates relatively low [9]. Cycloxydim is also unstable in acidic aqueous media with half-lives of 1, 7, 104 and 102 days at pH 3, 5, 7 and 9, respectively [12].

Aqueous solutions of sethoxydim are found to be unstable at room temperature or when kept at -20° C; only 6 and 24% of the parent sethoxydim remained after 72 hours, respectively [11]. The disappearance of sethoxydim was attributed to hydrolysis reactions.

Regarding butroxydim and tralkoxydim, both herbicides are hydrolyzed in water. The hydrolysis of both CHD herbicides was faster in acidic media than under neutral or basic conditions [7]. At a pH of 5 the herbicide butroxydim is degraded by an acid hydrolysis reaction with a DT_{50} of 10.5 days, while at a neutral pH (pH = 7) the half-life exceeded 8 months and at a pH of 9 the hydrolytic degradation was negligible [7]. In the same way, the stability of

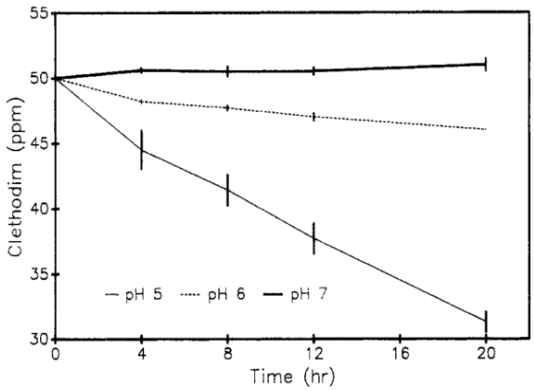

Figure 1. Degradation kinetics of technical clethodim at different pH values [30].

tralkoxydim increases with increasing pH. The value of DT_{50} for this herbicide was only 6 days at a pH of 5, whereas at a pH of 9 87% of the compound remained unchanged ($DT_{50} \approx 139$ d) [7].

2.1.2. Chlorination

Reactions with chemicals of anthropogenic origin are other important routes of abiotic degradation of pesticides in water. Residual chlorine is one of these substances commonly present in water bodies as a consequence of its use in the water and wastewater treatment plants. A residual concentration of chlorine species is maintained after disinfection processes in order to guarantee disinfected water through the distribution system or during storage. It means that residual chlorine could also react with other xenobiotic compounds present in waters such as herbicides.

Degradation of pesticides by action of residual chlorine is of great relevance if we consider that nowadays the reuse of treated wastewater for irrigation of crops, urban landscapes and other recreational areas is a common practice. Furthermore, it should be noted that some farmers also use drinking water or treated water for their pesticide preparation.

It is known that numerous pesticides are degraded during the processes of disinfection by chlorine or other forms of chlorine [31-33]. However, there is little information about the fate of CHD herbicides in the presence of residual chlorine. In this sense, our research group has carried out different studies on the chemical behaviour of CHD herbicides in the presence of hypochlorite and chloramines, two of the most common agents employed for water disinfection.

In preliminary studies to establish a method for the determination of tepraloxydim residues in drinking water, Sandín *et al.* [34] demonstrated that the presence of residual chlorine in laboratory distilled water rapidly degraded the herbicide tepraloxydim. Therefore, these authors have performed a thorough study of tepraloxydim degradation in chlorinated waters. They showed that the reaction between the herbicide and hypochlorite was very fast with a half-life below 5 seconds. Degradation of tepraloxydim was also observed when chloramines

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were added to the herbicide solution (Figure 2a), although it was slower than in the presence of hypochlorite ($t_{1/2}$ = 4.5 h) due to the lower oxidation potential of chloramines. Similar results were obtained when tepraloxydim was dissolved in tap water with a molar ratio of 1:10 (herbicide:total chlorine) and half-life of 0.86 h was calculated (Figure 2b) [34].

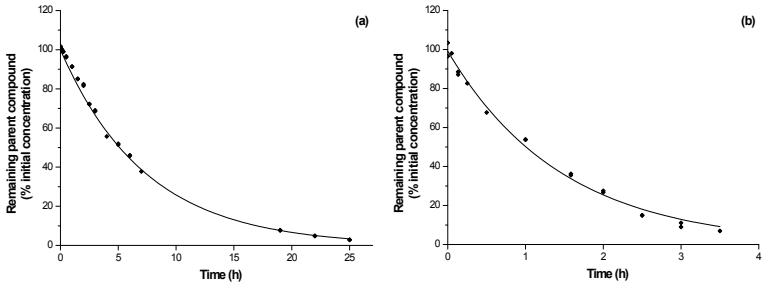

Figure 2. Degradation rate of tepraloxydim in chloramine solution (a) and in tap water (b) [34].

Our research group has also carried out other detailed studies about degradation of alloxydim and clethodim in the presence of hypochlorite and chloramines [35,36]. In the same manner as tepraloxydim, the degradation rates of both herbicides were very fast in the presence of hypochlorite with half-lives less than 1 second for alloxydim [35] and 20 seconds for clethodim [36]. The concentration of these two herbicides diminished more slowly in the presence of chloramines than in the presence of hypochlorite, showing half-lives equal to 8 min and 15.4 h for alloxydim and clethodim, respectively [35,36].

2.1.3. Photolysis

Photolysis by sunlight is one of the primary degradation routes for pesticides in different environmental compartments. Photochemical processes in the environment include all those reactions initiated by solar radiation. It should be noted that, in many cases, the thermal degradation of pesticides is associated with the absorption of the solar radiation energy.

Photodegradation of pesticides depends on various factors such as: the chemical structure and electronic absorption spectrum of the pesticides, the radiation source and its intensity, the time of exposure or the presence of other substances in environmental media. Moreover, there are two types of photochemical processes that can lead to the transformation of a pesticide: direct and indirect photolysis [37]. In the first one, the transformation of the pesticide is the result of direct absorption of solar radiation. In the second process, other compounds present in the compartment absorb firstly solar radiation to form reactive species that can subsequently react with pesticides resulting in their transformation.

Although the experimental design is not described, Iwataki and Hirono [29] observed that alloxydim was unstable and quantitatively decomposed when it was exposed to UV or sunlight. These authors also noted the thermal degradation of this herbicide at 120º C, but they did not clarify whether such degradation is related to radiation exposure [29]. Regarding the

thermal degradation of CHD herbicides, Soeda *et al.* [38] also evaluated the transformation of alloxydim when it was heated to 30, 40 and 50 ºC in a dark incubator. The thermal transformation of alloxydim was observed and 6.2% of alloxydim was degraded after 20 days of incubation at the highest temperature tested.

Falb *et al.* [30] investigated the photolytic behaviour of aqueous solutions of clethodim and the effect of adjuvants on the photolysis rates. These authors stated that photolysis reactions contributed to the degradation of clethodim to a greater extent than hydrolysis reactions. Also, the photodegradation rates of clethodim were strongly affected by the addition of adjuvants. So, the rates of degradation under UV light and sunlight were increased with addition of adjuvants up to 7 fold and up to 27 fold over the control, respectively [30]. Similar findings were also obtained by Bridges *et al.* [39]. McMullan [40] noted that clethodim efficacy was enhanced as a consequence of the presence of adjuvants in the spray solution. This effect was attributed to an increase of the adsorption rate of the herbicide and thus, a reduction of its photodegradation. These findings are in agreement with data published for sethoxydim by McInnes *et al.* [41] and Hazen and Krebs [42]. These researches suggested that spraying late at day may improve CHD herbicide efficacy due to a reduction in the amount of UV light.

The lability of cycloxydim to temperature and radiation has been also investigated. This herbicide was stable at room temperature but it becomes unstable above 30º C, decomposing at 200º C [7].

Besides the hydrolysis reactions previously mentioned, Campbell and Penner [11] identified the direct photolysis as an efficient pathway of sethoxydim degradation in aqueous solutions. These authors exposed aqueous solutions of sethoxydim to artificial light and observed that only 2% remained after 3 h. In the same way, they also observed a rapid photodegradation on glass disks of sethoxydim dissolved in n-hexane (81% of the herbicide was transformed after 1 h). In agreement with these results, Shoaf and Carlson [10] showed that sethoxydim was completely degraded within seconds in aqueous media either in incandescent or UV light at pH 3.3 and 6.0 and methanolic solutions of the herbicide were transformed by more than 50% after 10 min of exposition to UV light.

In our research group, different experiments have been carried out to study the photodegradation of sethoxydim in natural waters (mineral, well and river) and under natural and simulated sunlight in order to obtain results close to field conditions [43]. The degradation rates in natural waters were lower than in ultrapure water. For example, photodegradation of sethoxydim-lithium in natural water was approximately 5 times slower than in ultrapure water showing a half-live of 436.9 ± 0.8 min for river water and 82.1 ± 0.7 min for ultrapure water. Results indicated that the degradation of sethoxydim-lithium has a strong dependence on the composition of the water sample. The retardant effect observed in natural waters was attributed to the presence of increasing concentrations of TOC (Total Organic Carbon) where river water has the highest concentration of TOC (2.865 mg L^{-1}) and ultrapure water has the lowest (0.005 mg L^{-1}) [44].

An extensive research was conducted to study the effect of different natural substances commonly present in aqueous systems on the degradation rates of alloxydim [45] and

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clethodim [46]. Previous studies carried out in ultrapure water have proved that direct photolysis contributed appreciably to the degradation of both herbicides, so alloxydim and clethodim dissolved in ultrapure water were completely degraded in 4 h and 2.5 h under simulated sunlight, respectively [46]. To evaluate the effect of matrix composition, different substances that can be found in natural waters such as HA (humic acids), nitrate and ferric ions were added to aqueous solutions of alloxydim and clethodim. Figure 3 shows the photodegradation curves of clethodim in the presence of various concentrations of HA, nitrate ions and Fe(III) ions in ultrapure water under simulated solar radiation. In the case of clethodim, the presence of increasing concentrations of HA retarded the photodegradation compared to ultrapure water (Figure 3a) [46]. Analogous findings were observed for alloxydim herbicide in the presence of HA [45]. The retarding effect observed suggests that HA could be acting as an "optical filter" absorbing most of the photons emitted from the radiation source and thereby slowing the direct photochemical reaction of clethodim. In the presence of nitrate ions, the degradation rates of both herbicides, clethodim (Figure 3b) and alloxydim, were not affected at all [45,46]. On the contrary, the addition of ferric ions to ultrapure water resulted in a notable increase of the photolysis rate of clethodim (Figure 3c) and alloxydim compared to the direct photolysis. Several authors described this enhanced effect of Fe(III) ions as a result of the formation of hydroxyl radicals [47,48]. Furthermore, it has also been reported in the literature that the organic molecules can form a complex with the Fe(III) ions and later undergoes a direct photolysis [49].

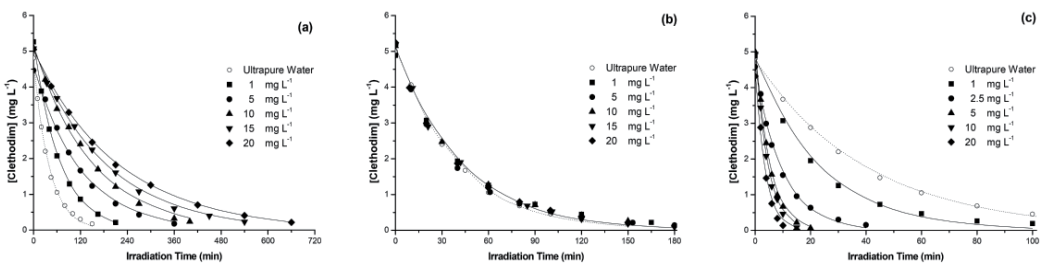

Figure 3. Photodegradation of clethodim in the presence of various concentrations of HA (a), nitrate ions (b), and Fe(III) ions (c) in ultrapure water under simulated Fe(III) ions [46].

It is noteworthy to mention that degradation of CHD herbicides occurs on soil surfaces and in plant leaves, although in many cases it is not clear if degradation is due to photolytic or biotic processes. In this sense, Hashimoto *et al.* [50] studied the fate of ^{14}C-alloxydim-sodium in soybean plants. It was observed that alloxydim was easily degraded on the leaf surface with half-life over 1-2 days [50] whereas the herbicide was detected even after 28 days in the plant. Therefore, Hashimoto *et al.* [50], like other authors [38,51], considered that the easy dissipation of the radiolabeled alloxydim from leaf surfaces was probably the result of photochemical reactions.

In order to simulate photolysis on soil surfaces, Soeda *et al.* [38] irradiated with UV light a methanol solution of ^{14}C-alloxydim spotted on TLC plates. Under these conditions, alloxydim

was photolyzed 33% (254 nm) and 9% (365 nm) after 20 minutes of irradiation. Ono *et al.* [52] also concluded that photolysis of alloxydim occurred when a soil spiked with an aqueous solution of this herbicide was exposed to sunlight.

Several authors studied the photodegradation of sethoxydim on glass slides and TLC plates as a model for soil and plant surfaces. UV irradiation (282 nm) of sethoxydim on TLC plates resulted in a large decomposition of this herbicide due to a direct photolysis process, since the absorbance spectrum of sethoxydim and the UV emission spectrum overlap at 282 nm [10]. Campbell and Penner [11] also evaluated the fate of sethoxydim on glass exposed to light and they observed a rapid photodegradation on this surface. Analogous conclusions were obtained when photodegradation experiments were carried out in the presence of different adjuvants [42]. Regarding the fate of sethoxydim on plant surfaces, it was observed that sethoxydim photodegradation on leaf surface occurred simultaneously to the uptake by corn plant leaf [42] and sugar beet [53]. In soil and under field conditions, the persistence of sethoxydim was highly affected by moisture and light [10,54].

2.2. Biotic processes

Besides abiotic pathways, biotic processes strongly affect the fate and persistence of pesticides in the environment. Biotic processes of pesticides refer to those transformations mediated by living organisms. Due to their ubiquitous nature, transformations involving microorganisms are the primary routes of biotic degradation of pesticides, although biological reactions in plants and animals can contribute significantly to their breakdown. There are a variety of factors affecting the biotic processes, including environmental conditions (temperature, moisture, pH, oxygen content), biological diversity or pesticides properties.

Biological transformations include many reactions (oxidation/reduction, hydrolysis, or conjugation/condensation) regularly catalysed by enzymes as a consequence of three major strategies [55]: (i) cometabolism where the degradation of the pesticide is coincidental to the general metabolic activity of an organism and provides no source of energy; (ii) catabolism where an organism uses the pesticide as an energy source; and (iii) processes in which extracellular enzymes secreted by an organism degrade pesticides.

Moreover, as biotic and abiotic processes usually occur simultaneously, sometimes it is complicated to determine the degree to which each contributes to degradation.

There are few scientific studies regarding the biotic processes affecting CHD herbicides; some of them refer to the difficulty of distinguishing between biotic and abiotic processes since both may occur simultaneously and may have common transformation products as a consequence of common reactions.

As mentioned before (Section 2.1.3.), photolysis was considered the main dissipation route of alloxydim from soybean plants [50]. However, some radioactivity was found as conjugates of aglycone components, which indicated that biotic transformation also occurs in the plant, reaching 5% of the total degradation of alloxydim. Similar results were obtained for alloxydim when it was sprayed on several crops such as sugar beet or wild oat [38,51]. In unsterilized soil, the half-life of alloxydim was 5 to 6 days under dark incubation [52]. In this research, the

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evolution of $^{14}CO_2$ was investigated as a measure of microorganism activity in the soil. Cumulative amounts of $^{14}CO_2$ were detected over 28 days of incubation, suggesting that alloxydim can be utilized by soil microorganisms during metabolism.

After the application of sethoxydim on sugar beet leaves, the herbicide was rapidly degraded (half-life < 1 day). Moreover, up to 20% of sethoxydim was translocated to untreated leaves and to the roots in three days, where it decreased gradually as a consequence of metabolism by the plant [53]. Considering the transformation product identified, the main metabolic mechanism proposed for sethoxydim may indicate that oxidation was partially catalysed by enzymes in addition to direct oxygenation. Ishihara *et al.* [53] suggested that metabolites of sethoxydim should be regarded as target compounds when a crop residue is analyzed because these compounds remain in sugar beet as conjugates and non-conjugates 60 days after treatment. Similarly, other researches have shown a rapid degradation of sethoxydim in both grasses and dicotyledonous crops; although it is uncertain to what extent the biotic or abiotic processes are involved [56,57]. Sethoxydim is degraded in soil by microorganisms with a half-live of 25 days [58]. Despite its short half-life, the parent herbicide and/or its transformation products caused considerable root inhibition over a period of 150-280 days depending on initial concentration of sethoxydim. Therefore, Roslycky [58] pointed out that it could be expected to be a potential danger as a result of the cumulative effects of sethoxydim and/or its transformation products in soil.

The dissipation of tralkoxydim in several crops and water-soil systems has also been documented [59-62]. Under these conditions, both biotic and abiotic processes took place concurrently and a rapid degradation was observed. In maize and wheat crops, part of tralkoxydim entering the foliar tissue was probably degraded by means of non-catalysed reactions or by metabolism in plant cells [59,60]. Although abiotic processes are the major routes affecting tralkoxydim in the water-soil system, biotic processes are responsible for faster dissipation of this herbicide in the water layer as compared to plain water and soil sediment, probably due to higher microbial activity in water [62].

Several studies concerning the metabolism of CHD herbicides in animals, primarily rats, have been conducted. In general, CHD herbicides were rapidly absorbed and excreted *via* the urine and faeces [63]. The fate of ^{14}C-alloxydim orally administered to rats for 7 days has been reported [64]. After daily dosing for 7 days, radioactivity was almost quantitatively eliminated in the urine and faeces within 2 days of the last dose. Unchanged alloxydim was excreted, mainly in the urine, whereas the remaining herbicide was degraded to different transformation products. The major transformation pathway of alloxydim in rats was oxidation and subsequent hydrolysis reactions involving enzymatic catalysis, although non-enzymatic reactions such as thermal degradation also occur to a lesser extent. Analogous results were reported for various CHD herbicides such as butroxydim, sethoxydim, and cycloxydim, which were rapidly excreted within 7, 2 and 5 days, respectively [7,63].

3. Transformation products

Taking into account human safety and environmental protection, the ideal herbicide would be the one that, after acting against the target weed, could be completely mineralized to inorganic compounds as final products such as H_2O, CO_2, NH_4^+, NO_3^-. However, the complete mineralization often occurs slowly in the environment and different intermediate compounds can be formed prior to complete mineralization. Several terms have been used for these intermediate compounds including "degradates", "breakdown products", etc. In general, "transformation products" is used as a general term for those compounds formed during biotic and abiotic processes; compounds resulting from abiotic degradations are referred to as "by-products", "degradation products" or "photoproducts" (if they are formed by photodegradation processes). "Metabolites" refer to compounds formed as a result of biological transformations and the term "residue" includes both parent compound and transformation products [55].

The understanding of overall consequences for herbicide use is limited, due to the fact that most studies have focused on the parent compound regardless of their transformation products. However, transformation products can behave very differently from the parent compound as a consequence of its different chemical structure [23]. In this sense, diverse studies have confirmed that many transformation products of pesticides are more persistent, and present a higher toxicity and/or a higher mobility compared to their parent compounds [65-67]. Furthermore, different authors have suggested that the phytotoxicity of some CHD herbicides is due not only to the parent compounds but also to their transformation products [10-12,68]. Therefore, researches involving these transformation products have become essential in order to better understand the behaviour of pesticides and to avoid underestimating the risk derived from their use.

Literature about transformation products of CHD herbicides is very limited and many studies have only reported the detection of transformation products without performing a detailed identification of them [10,11,30,69,70]. In this sense, QTof mass analyzer coupled to HPLC has been applied as a valuable tool for the identification and structural elucidation of transformation products of CHD herbicides in aqueous matrices [45,46]. Thus, QTof mass analyzer provides accurate masses for both parent and product ions in combination with the possibility of performing MS/MS acquisitions obtaining more structural information. For instance, on the basis of the exact mass measurements and fragmentation patterns provided by QTof, it was possible to elucidate the structures of nine clethodim photoproducts previously separated by a HPLC system [46].

Table 3 compiles the main biotic and abiotic transformation products of CHD herbicides in different matrices from the information available from open literature.

As mentioned before, the herbicides of the CHD family present two isomers, *E* and *Z*, due to the presence of the alkyl side chain bound to oxime ether group. These herbicides are marketed as the *E*-isomers, but the isomerization around the N-O bond seems to occur easily, making the corresponding *Z*-isomer a plausible transformation product of both biotic and abiotic

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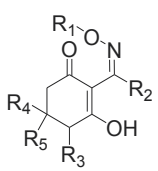
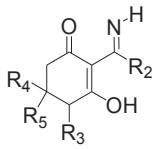
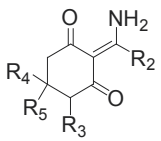
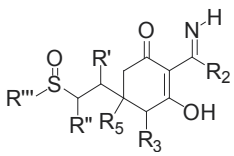
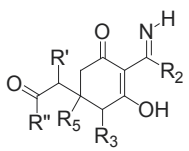
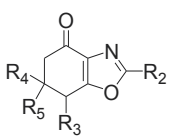
processes. Several authors have stated that some *E*-isomers of CHD herbicides may equilibrate with the *Z*-isomer in polar solvents [30,46,71] or in chlorinated water [34,36]. Moreover, it has been reported that isomerization can be induced by light [45,46] and temperature [72].

For example, equilibrium between both tepraloxydim isomers took about 7 days, with a final ratio between isomers of 2:1 (*Z:E*) [34]. Rapid isomerization has been observed for clethodim in ultrapure water reaching 4% in a freshly prepared solution and 40% after two months [36]. Clethodim *Z*-isomer was also identified as a transformation product formed resulting from the exposition to simulated solar light [46]. In the same way, the corresponding *Z*-isomer of alloxydim was detected as a photoproduct after the exposure to UV radiation ($\lambda > 290$ nm) [45].

An important feature of the oxime ether bond is its relatively low dissociation energy (ca. 53 kcal mol^{-1}). Therefore, it is expected that one of the most important reactions of CHD herbicides is the cleavage of the N-O bond to yield two possible dealkoxylated compounds, imine and/or amine (Table 3). Thus, photodegradation of alloxydim in aqueous solution was investigated under simulated and natural solar light and the main photoproduct obtained was the imine [45,73]. Both studies revealed that the amount of alloxydim imine formed can be influenced by the composition of aqueous solution and the intensity of radiation source [45]. Other authors have stated the formation of alloxydim amine in the soil and plants, although it is not clear if a biotic or abiotic mechanism is involved. In this sense, Ono *et al.* [52] identified the amine of alloxydim by thin-layer chromatography and mass spectrometry when the photodegradation of this herbicide was studied in sterilized soil. Alloxydim was also readily degraded in sugar beet [38] and soybean [50] and the main transformation product was identified as the amine compound. After 10 days of treatment with alloxydim, Soeda et al. [38] detected the corresponding amine as the main transformation product in sugar beet extracts. This transformation product persisted for 42 days and its formation seemed to occur by photoreduction on plant leaves [38]. Hashimoto et al. [50] and Veerasekaran and Catchpole [51] obtained similar results in some susceptible and resistant plants, suggesting that a large part of alloxydim transformation to amine occurred directly from the surface of the leaves as a result of abiotic degradation. The metabolism of alloxydim sodium in rats has been reported by Takano *et al.* [64]. Alloxydim amine was identified as a major metabolite of alloxydim in the rat liver, while low yields were observed in urine and faeces [64].

Transformation Product	Matrix (transformation process) *	Herbicide	Reference
Z- isomer	Water (p,c)	Alloxydim	[45]
		Clethodim	[36,46]
		Tepraloxydim	[34]
Imine	Water (c,h,p)	Alloxydim	[45]
		Clethodim	[46]

Transformation Product	Matrix (transformation process) *	Herbicide	Reference
	Soil (p, b)	Cycloxydim	[12]
		Sethoxydim	[11]
		Tepraloxydim	[34]
		Butroxydim	[7]
		Cycloxydim	[12]
		Tralkoxydim	[59]
	Plant (p, b)	Cycloxydim	[74]
		Profoxydim	[7]
	Animal (b)	Alloxydim	[64]
		Profoxydim	[7]
Amine	Water (p)	Alloxydim	[29]
	Soil (p)	Alloxydim	[52]
	Plant (p, b)	Alloxydim	[38,50,51]
		Sethoxydim	[53]
Imine sulfoxide	Water (p)	Clethodim	[46]
	Soil (b)	Cycloxydim	[12]
		Sethoxydim	[53]
	Plant (b)	Cycloxydim	[12]
		Cycloxydim	[12]
		Sethoxydim	[53]
	Animal (b)	Cycloxydim	[12]
Imine ketone	Water (p)	Clethodim	[46]
			
Oxazole	Water (h,p)	Alloxydim	[29]
		Cycloxydim	[12]
	Soil (b, p, h)	Tepraloxydim	[34]
		Alloxydim	[29,52]
		Butroxydim	[7]
		Cycloxydim	[12]
		Tralkoxydim	[12,59]

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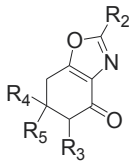
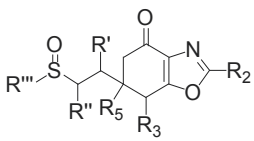
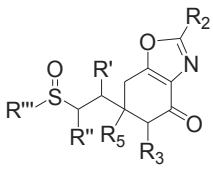
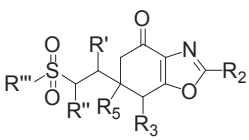
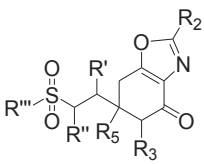
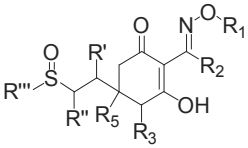
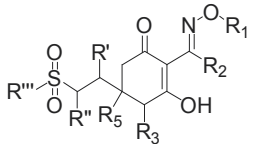
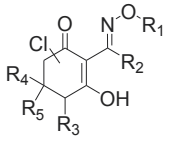
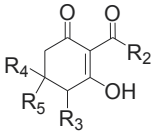
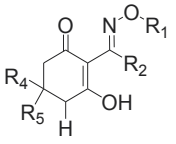
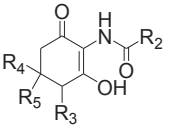
Transformation Product	Matrix (transformation process) *	Herbicide	Reference
	Plant (p, t, b)	Alloxydim	[38,50,51]
		Cycloxydim	[74]
		Tralkoxydim	[61]
	Animal (b)	Alloxydim	[64]
Oxazole sulfoxide	Water (h,p)	Cycloxydim	[12]
	Soil (p,b)	Cycloxydim	[12]
	Plant (t, b)	Cycloxydim	[12]
		Sethoxydim	[53]
	Animal (b)	Cycloxydim	[12]
Oxazole sulfone	Soil (n.d.)	Cycloxydim	[12]
	Plant (t)	Sethoxydim	[53]
			
Sulfoxide	Water (p,c)	Clethodim	[36,46]
		Cycloxydim	[12]
		Profoxydim	[75]
	Soil (p)	Cycloxydim	[12]
	Plant (b)	Clethodim	[7]
		Cycloxydim	[12]
		Profoxydim	[7]
		Sethoxydim	[53]
	Animal (b)	Clethodim	[7]
		Cycloxydim	[12]

Transformation Product	Matrix (transformation process) *	Herbicide	Reference
		Profoxydim	[7]
Sulfone 	Water (c, h, p)	Clethodim	[36]
		Sethoxydim	[70]
	Soil (n.d.)	Cycloxydim	[12]
	Plant (b)	Clethodim	[7]
		Cycloxydim	[12]
		Sethoxydim	[53]
	Animal (b)	Clethodim	[7]
		Cycloxydim	[12]
		Profoxydim	[7]
Chlorinated 	Water (c)	Alloxydim	[35]
Ketone 	Soil (b)	Alloxydim	[52]
		Butroxydim	[7]
	Animal (b)	Alloxydim	[64]
Demethoxycarbonylated 	Water (h)	Alloxydim	[29]
	Soil (b)	Alloxydim	[52]
	Plant (b)	Alloxydim	[50]
	Animal (b)	Alloxydim	[64]
Amide 	Water (h)	Alloxydim	[29]
	Animal (b)	Alloxydim	[64]
Glutaric acid derivative	Soil (b)	Alloxydim	[52]
		Butroxydim	[7]
		Tralkoxydim	[59]

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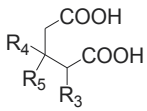
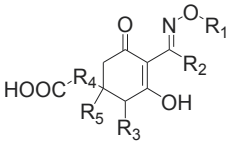
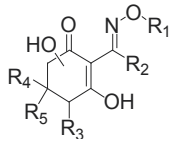
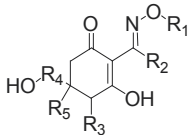
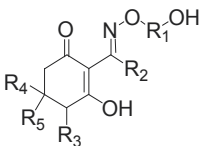
Transformation Product	Matrix (transformation process) *	Herbicide	Reference
	Plant (b)	Tralkoxydim	[61]
Carboxylic acid derivative 	Soil (n.d.)	Tralkoxydim	[59]
Hydroxylated derivative 	Water (c)	Clethodim	[36]
	Soil (b)	Cycloxydim	[12]
		Cycloxydim	[12]
		Alloxydim	[64]
		Profoxydim	[7]
	Animal (b)	Tralkoxydim	[61]
			

Table 3. Biotic and abiotic transformation products of CHD herbicides in different matrices. *(h: hydrolysis, c: chlorination, p: photodegradation, t: thermal, b: biotic, n.d.: no defined).

Sevilla *et al.* [46] performed the identification of transformation products formed during the photolysis of aqueous solutions of clethodim. By means of HPLC coupled to Qtof, the structures for nine photoproducts detected were proposed, being clethodim imine the major photoproduct identified [46]. In the same way, up to six products were detected and isolated by Campbell and Penner [11] when aqueous solutions of sethoxydim were exposed to artificial light. Five of these products were transitory and only one appeared to be the single end product, which was identified as sethoxydim imine. Imine was also detected as a by-product of cycloxydim when the herbicide was subjected to solar radiation on the soil surface and under acidic conditions [12].

As for other transformation products, the formation of oxazoles and isoxazoles was frequently observed as a consequence of both biotic and abiotic transformations and their presence has been detected in several matrices (water, soil, plant, animal) (Table 3). In this sense, studies of thermal degradation of alloxydim gave two oxazoles as transformation products at 120 ºC with a ratio between them of 3:2 [29]. The mechanism of the formation of these compounds involved the loss of alkoxy group, -OR_1 (Table 1), the Beckmann rearrangement and subsequent intramolecular cyclization. Conversion of alloxydim to oxazoles and isoxazoles also has been reported in plants treated with this herbicide [38,50,51]. In these studies, the authors suggested that the transformation occurs mainly on leaf surfaces and it was probably due to abiotic processes such as photolysis or/and thermal degradation.

Hydrolysis reactions of CHD herbicides in aqueous matrices can also lead to the formation of oxazoles and isoxazoles. The fate of tepraloxydim in aqueous solutions free of chlorine has been evaluated by Sandín *et al.* [34]. Concomitantly with the disappearance of tepraloxydim, a transformation product (identified as the corresponding oxazole) was monitored. The molar ratio of tepraloxydim oxazole detected was 7% after 7 days in solution and it increased to 12.5% after 21 days [34]. In the same way, cycloxydim is labile in aqueous media under hydrolytic conditions. After 32 days at a pH of 7, 7% of oxazole was detected, while at a pH of 3 higher yields of this transformation product were observed [12].

The biotic contribution to the formation of oxazoles in plants is not well-known. The metabolic pathway of some CHD herbicides has been reported mainly in rats. Oxazoles of alloxydim and tralkoxydim were identified as minor metabolites in both urine and faeces [12,64].

Oxidative reactions are often involved in the transformation process of many herbicides. The mechanism of these reactions occur through (i) physico-chemical oxidations involving molecular oxygen or reactive species present in the media (acids, radicals or singlet oxygen) or (ii) biological oxidations which are mediated by enzymes. In general, the main transformation products observed as a result of these reactions include hydroxylated compounds and, in the case of S-containing herbicides like some CHD herbicides, sulfoxides, and sulfones. Sulfoxidation of herbicides may be of great importance since, in some cases, sulfoxides and sulfones are suspected to show biological/toxicological activity to target and/or non-target organisms [55,76]. Moreover, these oxidized compounds are reported to present a higher water solubility and minor soil sorption than parent pesticides, thus a higher possibility to reach and contaminate ground and surface water is expected [55].

Degradation of clethodim in chlorinated water either with sodium hypochlorite or chloramines led to a single transformation product that was identified as clethodim sulfoxide [36]. Subsequent degradation of the transformation product clethodim sulfoxide was followed and it degraded mainly to clethodim sulfone, although other minor products were detected. As a result of photolysis reactions in aqueous solutions, Sevilla *et al.* [46] have also identified oxidative transformation products such as clethodim sulfoxides, sulfoxides of clethodim imine and the ketone imine of the herbicide [46]. Roberts [12] has reported that the photolysis of cycloxydim on the soil surface led mainly to cycloxydim S-oxides whereas imine and oxazole were minor products. Shoaf and Carlson [70] observed that photolysis of sethoxydim solutions led to the formation of the corresponding sulfone, as well as the formation of five other

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unidentified products. Moreover, sethoxydim sulfone was also detected during both acid and alkaline hydrolysis, although a higher conversion to this product was observed under basic conditions.

Sulfoxides and sulfones are also common transformation products during the biotic degradation of CHD herbicides in plants and animals. For example, sethoxydim was rapidly converted to its sulfoxides and sulfone in sugar beet [53]. The oxidation of the sulphur atom was partially catalysed by enzymes in addition to direct oxygenation. These authors also noted that the abundance of sulfoxide metabolites was generally higher than that of the sulfone type metabolite at the early stages after application, whereas the situation was opposite on day sixty [53]. Other studies conducted with cycloxydim on soybean have shown that its major metabolite on green foliage, stems and beans was the sulfoxide [12] while other oxidation products, such as sulfone and hydroxylated compounds, were found as minor metabolites. Roberts [12] reported the metabolism of cycloxydim in rats. This herbicide was almost completely eliminated within 5 days, with cycloxydim sulfoxide being the major metabolite in urine. Cycloxydim sulfoxide was administered to goats in order to emulate consumption of this major plant metabolite [12]. Under these conditions, cycloxydim sulfoxide remained almost unchanged in the urine, although low residues of sulfone and secondary sulfoxides were present in milk and the liver. This finding points out the higher stability of some CHD transformation products compared to the parent compound.

As mentioned previously, degradation products of some herbicides can also present an undesirable herbicidal activity against non-target plants. However, these data are still scarce for CHD herbicides. In this sense, our research group is currently studying the phytotoxicity of CHD degradation products by means of bioassays. This technique has shown to be a useful tool to screen the phytotoxicity of CHD herbicides, showing good sensitivity, low cost and reproducibility [77]. Thus, we have investigated the phytotoxicity of alloxydim and its main chlorinated transformation product on wheat with bioassays in a hydroponic culture [35,68]. Results showed that the degradation product of alloxydim caused a 32% reduction in root growth of wheat plants although this phytotoxic effect occurred at a higher dose than for the parent compound.

In a photodegration study of sethoxydim in aqueous media six degradation products were detected, where five of them were transitory [11]. A phytotoxicity experiment revealed that two of these transitory products had herbicidal activity when they were applied to *Echinochloa crus-galli*, whereas imine showed no significant injury [11]. Although quantitative analysis of these transformation products was not made and their relative herbicidal potencies could not be determined, Campbell and Penner [11] suggested the possibility that some of these transformation products actually induce the phytotoxic effects on grasses.

Table 3 shows the main transformation products of CHD herbicides, the matrix, and the processes where they are generated. Many of them are formed as a result of the combination of two or more of the reactions discussed above.

4. Analytical determination

In order to fulfil the environmental requirements of new international regulations, it is of great importance to develop reliable and sensitive methods for the determination of pesticides and their residues. In this sense, the analysis of CHD herbicides and their transformation products entails some difficulties. CHD herbicides are effective at low doses, thus trace concentrations are expected to be found in the environment. Therefore, analytical methods must provide high sensitivities. It is also important to bear in mind that CHD herbicides are polar and chemically unstable, which makes the analysis more difficult. The situation becomes more complex when transformation products are present due to the lack of analytical standards and scarce information available.

Depending on the physico-chemical properties of pesticides, the type of matrix, and the level of concentration required, analytical methods often involve preliminary steps of sample preparation. These pretreatments consist in interference removal from the matrix and concentration of the analytes of interest. Nowadays, together with classical techniques such as liquid extraction with organic solvents, more recent techniques such as solid-phase extraction (SPE), solid-phase micro-extraction (SPME), stir-bar-sorptive extraction (SBSE), or QuEChERS are commonly used prior to analytical determination [78]. Once the sample preparation is completed, the qualitative and quantitative analysis of pesticide residue are traditionally carried out using chromatography techniques since these allow separating complex mixtures. Gas chromatography (GC) and liquid chromatography (LC) are the most common techniques used for the determination of CHD residues. Although GC has been applied to the analysis of CHD herbicides, methods based on LC are more suitable for the analysis of CHD residues due to their low volatility, thermolability and the polar character. This technique is applicable not only for the parent compound but also for their transformation products.

Ono *et al.* [79] compared three different analytical methods (HPLC, GC and ultraviolet spectrophotometry) for the determination of alloxydim herbicide and the three transformation products in different crops and soils. An extraction and clean-up step was necessary before their determination. The HPLC method was found to be most suitable and the lowest limit of detection for alloxydim and all its transformation products was 0.01 ppm. Recoveries of alloxydim and three transformation products were 75-93% in various crops and 85-92% in soils. A method for the determination of the total content of alloxydim-sodium and five of its degradation products in ground water was described, using derivatization with hydrogen peroxide followed by GC-MS [80]. Derivatization of these compounds was carried out in order to obtain more stable and volatile products. An additional clean-up process was necessary to remove interferences caused by the presence of reactives used during the derivatization procedure. The detectable limit achieved was 0.1 μg L^{-1}, expressed as alloxydim-sodium equivalent, and the recoveries ranged from 53 to 85% [80].

Falb *et al.* [69] developed an LC method for the separation of clethodim and several transformation products formed during photolysis and hydrolysis of the herbicide in solution. Up to 31 and 19 photolytic and hydrolytic products were separated, although further identification was not achieved. Multi-residue methods for the analysis of clethodim and some of its

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transformation products in fruits and vegetables have also been described [81,82]. For example, Klein and Alder [82] achieved the simultaneous determination of clethodim and five of its metabolites (two sulfoxides and three sulfones) by LC-MS after an extraction procedure with organic solvents and a clean-up step using SPE. These authors stated the influence of the matrix during the extraction and clean-up of clethodim, since decomposition of clethodim to the corresponding sulfoxides occurred in avocado, hindering to calculate recoveries of clethodim in this matrix. However, good recoveries (80-120%) were obtained for clethodim and its transformations products in most matrices at a concentration level 0.01 mg kg^{-1} [82].

An analytical method for sethoxydim in several crops was established using HPLC with UV detection by Gomyo and Ono [83]. The herbicide was extracted from crops with organic solvents prior to the analysis by HPLC. The lowest detection limit was 0.02 ppm and the recoveries ranged from 79 to 87%. In a later work, Gomyo *et al.* [84] reported a study showing a comparison between HPLC and GC methods for the determination of sethoxydim and ten of its transformation products in different crops. The experimental data showed that the HPLC method was more suitable for these compounds. After the extraction with methanol from crops, sethoxydim and all its transformation products were converted to sulphone derivatives by reaction with hydrogen peroxide and afterwards a clean-up step was performed using a Woelm column. This method provided a detection limit of 1.0 to 0.05 ppm for sethoxydim and its metabolites [84]. Hu *et al.* [85] established a multi-residue method based on LC-APCI/MS for the determination of sethoxydim and different pesticides in several aqueous matrices. The extraction recoveries of this herbicide in distilled water were 64%, whereas it was impossible to recover it from treated and raw water, probably due to degradation processes during the extraction step. Analogous findings were achieved by Shoaf and Carlson [70] during the optimization of a HPLC method for the determination of sethoxydim in aqueous solutions. These authors stated that recoveries of the parent compound were improved considerably at acidic pH values.

A SPME-HPLC-UV method has been reported for the determination of profoxydim herbicide in rice fields [86]. The technique of SPME was applied on-site at a flooded rice field in real time. This technique allowed extracting the target analyte under field conditions, decreasing time-consuming sample shipment and later sample preparation in the laboratory. Moreover, the stability of profoxydim during the storage of SPME fiber was increased during the storage compared to aqueous samples. The detection limit of the SPME-HPLC-UV method for the detection of profoxydim was 5 μg L^{-1} [86]. Tsochatzis *et al.* [87] developed and validated a multi-residue HPLC-DAD method for the separation and determination of nine commonly applied rice pesticides, including profoxydim, in paddy water samples. Preliminary clean-up of water samples and isolation of pesticides was performed on SPE cartridges. The limit of detection (LOD) and quantification (LOQ) for profoxydim herbicide were 0.4 μg L^{-1} and 2 μg L^{-1}, respectively. The method was subsequently employed for the determination of pesticides in paddy fields and surface water systems located in the Axios river basin (Greece). Profoxydim was detected at a relatively high concentration (6.3 μg L^{-1}) close to its dose of application [87].

In 2005, Lehotay *et al.* [1] applied QuEChERS for the determination of 229 pesticides, including cycloxydim and sethoxydim in two representative commodities (lettuce and orange). Recov-

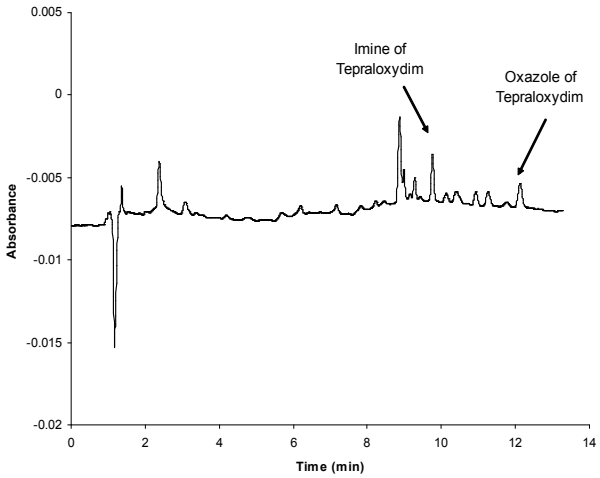

Figure 4. Chromatogram of commercial mineral water fortified with 0.1 µg. L^{-1} metabolites tepraloxydim oxazole and tepraloxydim imine (Modified from [71]).

eries of cycloxydim and sethoxydim vary from 80-89% and 50-69%, respectively. The low recoveries could be due to degradation processes, incomplete extraction and pH of the sample. Therefore, further investigations and modifications of the QuEChERS method are needed for the determination of these herbicides.

Liska *et al.* [88] developed a multi-residue method for the detection and quantification of 50 pesticides, including alloxydim-sodium and sethoxydim herbicides, in ultrapure and river Rhine water. Trace enrichment of water samples was necessary to obtain enough sensitivity for further analysis by LC-DAD. The detection limits achieved were 0.5 mg L^{-1} for sethoxydim and 1 mg L^{-1} for alloxydim-sodium in both types of waters. Sandín *et al.* [71] have established a method for direct analysis of tepraloxydim and its main metabolites in water. It has been demonstrated that chlorine content added to disinfect tap water is a critical parameter for the determination of tepraloxydim. In this work an analytical method for determination of two metabolites of tepraloxydim, oxazole and imine, has been validated to an LOQ of 0.1 µg L^{-1} in tap and commercial mineral water (Figure 4).

Different studies have shown that the methods based on the technique LC coupled with electrospray ionization (ESI) tandem mass spectrometry (MS), using previous SPE as the extraction procedure, are a powerful tool for the analysis of CHD herbicides. For example, Shen *et al.* [89] proposed a multi-residue method for the simultaneous analysis of six CHD herbicides employing LC-MS/MS. The method was successfully used to determine these herbicides in rice and corn, obtaining recoveries within 70.0-97.9% at the spiked levels of 5-20 ng g^{-1}. Marek *et al.* [90] described a multi-residue method for the determination of alloxydim, clethodim and sethoxydim in river water and distilled water after extraction/clean-up with C18/SAX. The recoveries of the herbicides from distilled water were 117% (alloxydim), 96% (clethodim) and 89% (sethoxydim).

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5. Conclusion

New herbicides, like cyclohexanedione oximes which are effective at low doses and easily decomposable, have been developed in recent years in order to reduce herbicide impact in the environment. However, degradation of these herbicides does not guarantee their detoxification and, in many cases, transformation products are more toxic, mobile and/or persistent than their active substances. Therefore, it is of utmost importance to improve our knowledge about these herbicides in order to minimize the possible adverse effects of their residues on human health, non-target organisms and the environment.

In this sense, this review provides an overview about the environmental behaviour of cyclohexanedione oxime herbicides under biotic and abiotic conditions. The most relevant studies in literature have been compiled and significant aspects such as factors affecting the behaviour of this herbicide family, as well as degradation routes and transformation products formed have been discussed. Moreover, illustrative examples about sample preparation, methods of determination and analytical techniques used for the analysis of cyclohexanedione herbicides and their transformation products have been described.

Although the availability of new scientific information on cyclohexanedione herbicides and their environmental fate and behaviour is increasing in recent years, more data are still needed. Thus, a better understanding of the degradation mechanism of cyclohexanedione herbicides is important for studying the fate and the effects of herbicides in the environment. In this sense, since most studies are conducted under laboratory conditions, more field research should also be desirable.

Moreover, to assess the overall impact of these herbicides, a major emphasis must be done on investigating their transformation products. It would also be interesting to perform monitoring programs of the parent compounds together with their transformation products in aqueous media because they could be potential contaminants of this compartment.

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References

[1] Lehotay SJ, de Kok A, Hiemstra M, van Bodegraven P. Validation of a Fast and Easy Method for the Determination of Residues from 229 Pesticides in Fruits and Vegetables Using Gas and Liquid Chromatography and Mass Spectrometric Detection. Journal of AOAC International 2005;88(2) 595-614.

[2] Guzzella L, Pozzoni F, Giuliano G. Herbicide Contamination of Surficial Groundwater in Northern Italy. Environmental Pollution 2006;142(2) 344-353.

[3] Buttiglieri G, Peschka M, Fromel T, Muller J, Malpei F, Seel P, Knepper TP. Environmental Occurrence and Degradation of the Herbicide N-Chloridazon. Water Research 2009;43(11) 2865-2873.

[4] Laabs V, Amelung W, Pinto AA, Wantzen M, da Silva CJ, Zech W. Pesticides in Surface Water, Sediment, and Rainfall of the Northeastern Pantanal Basin, Brazil. Journal of Environmental Quality 2002;31(5) 1636-1648.

[5] Loos R, Wollgast J, Huber T, Hanke G. Polar Herbicides, Pharmaceutical Products, Perfluorooctanesulfonate (Pfos), Perfluorooctanoate (Pfoa), and Nonylphenol and Its Carboxylates and Ethoxylates in Surface and Tap Waters around Lake Maggiore in Northern Italy. Analytical and Bioanalytical Chemistry 2007;387(4) 1469-1478.

[6] Iwataki I. Cyclohexanedione Herbicides: Their Activities and Properties. Boca Raton: CRC Press; 1992.

[7] Tomlin CDS., editor. The Pesticide Manual: A World Compendium. 14 ed., Hampshire: BCPC Publications; 2006.

[8] Watson KG. Cyclohexane-1,3-Dione Oxime Ether Grass-Specific Herbicides and the Discovery of Butroxydim. Australian Journal of Chemistry 2011;64(4) 367-372.

[9] Walter H. Profoxydim: Development of a Herbicide from Laboratory to Field. In: Prado RD, Jorrín JV. (eds.) Uso De Herbicidas En La Agricultura Del Siglo XXI. Córdoba: Servicio Publicaciones Universidad de Córdoba; 2001. p19-30.

[10] Shoaf AR, Carlson WC. Stability of Sethoxydim and Its Degradation Products in Solution, in Soil, and on Surfaces. Weed Science 1992;40(3) 384-389.

[11] Campbell JR, Penner D. Abiotic Transformations of Sethoxydim. Weed Science 1985;33(4) 435-439.

[12] Roberts TR., editor. Metabolic Pathways of Agrochemicals. Cambridge: Royal Society of Chemistry; 1998.

[13] Wiley-VCH., editor. Ullmann's Agrochemicals. Weinheim: Wiley VCH Verlag; 2007.

[14] Kibler E, Landes M, Von der Heyde J, Jahn D, Munger P. Bas 620 H - a New Selective Herbicide for Post-Emergence Control of Grass Weeds in Broadleaf Crops. In: The

1999 Brighton Conference - Weeds, 15-18 November 1999, Brighton, UK. BCPC Publications; 1999.

[15] European Food Safety Authority. Conclusion on the Peer Review of the Pesticide Risk Assessment of the Active Substance Clethodim. EFSA Journal 2011;9(10) 2417-2512.

[16] FAO/WHO., editor. Pesticide Residues in Food 1992. Evaluations Part 1 - Residues. Plant Production and Protection Paper Nº 118. Rome: FAO/WHO; 1993.

[17] European Food Safety Authority. Conclusion on the Peer Review of the Pesticide Risk Assessment of the Active Substance Cycloxydim. EFSA Journal 2010;8(7) 1669-1742.

[18] Sanco/11198/2011 Final. Review Report for the Active Substance Profoxydim. European Commission 2011.

[19] Edwards D. Reregistration Eligibility Decision (Red) for Sethoxydim. United States Environmental Proction Agency 2005.

[20] Rubien L, Durkin PR. Sethoxydim [Poast]-Human Health and Ecological Risk Assessment. Final Report. USDA, Forest Service 2001.

[21] Sanco/10388/2002 - Rev. 4. Review Report for the Active Substance Tepraloxydim. European Commission 2004.

[22] European Food Safety Authority. Conclusion Regarding the Peer Review of the Pesticide Risk Assessment of the Active Substance Tralkoxydim. EFSA Scientific Report 2008;1391-78.

[23] Rathore HS, Nollet LML., editors. Pesticides: Evaluation of Environmental Pollution. Boca Raton: CRC Press; 2012.

[24] Sanco/10058/2005. Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. European Commission 2006.

[25] Barceló D, Hennion M-C. Trace Determination of Pesticides and Their Degradation Products in Water. Amsterdam: Elsevier Science; 1997.

[26] Hultgren RP, Hudson RJM, Sims GK. Effects of Soil Ph and Soil Water Content of Prosulfuron Disipation. Journal of Agricultural and Food Chemistry 2002;50(11) 3236-3243.

[27] Dannenberg A, Pehkonen SO. Investigation of the Heterogeneously Calalyzed Hydrolysis of Organophosphorus Pesticides. Journal of Agricultural and Food Chemistry 1998;46(1) 325-334.

[28] Santos TCR, Rocha JC, Alonso RM, Martínez E, Ibáñez C, Barceló D. Rapid Degradation of Propanil in Rice Crop Fields. Environmental Science and Technology 1998;32(22) 3479-3484.

[29] Iwataki I, Hirono Y. The Chemical Structure and Herbicidal Activity of Alloxydim-Sodium and Related Compounds. In: Geissbühler H, Brooks GT, Kearney PC. (eds.) Advances in Pesticide Science. Oxford: Pergamon Press; 1979. p235-243.

[30] Falb LN, Bridges DC, Smith AE. Effects of Ph and Adjuvants on Clethodim Photodegradation. Journal of Agricultural and Food Chemistry 1990;38(3) 875-878.

[31] Losito I, Zambonin CG, Palmisano F. Degradation of Chlortoluron in Water Disinfection Processes: A Kinetic Study. Journal of Environmental Monitoring 2000;2(6) 582-586.

[32] Rahman MM, Kim T-H, Kwon G-S, Yang JE, Park M, Kim J-E. Removal Efficiency of the Herbicide Oxadiazon in Treatment Processes for Drinking Water. Journal of the Korean Society for Applied Biological Chemistry 2009;52(3) 252-257.

[33] Mascolo G, Lopez A, James H, Fielding M. By-Products Formation During Degradation of Isoproturon in Aqueous Solution. Ii: Chlorination. Water Research 2001;35(7) 1705-1713.

[34] Sandín-España P, Llanos S, Magrans JO, Alonso-Prados JL, García-Baudín JM. Optimization of Hydroponic Bioassay for Herbicide Tepraloxydim by Using Water Free from Chlorine. Weed Research 2003;43(6) 451-457.

[35] Sandín-España P, Santín I, Magrans JO, Alonso-Prados JL, García-Baudín JM. Degradation of Alloxydim in Chlorinated Water. Agronomy for Sustainable Development 2005;25(2) 331-334.

[36] Sandín-España P, Magrans JO, García-Baudín JM. Study of Clethodim Degradation and by-Product Formation in Chlorinated Water by Hplc. Chromatographia 2005;62(3-4) 133-137.

[37] Larson RA, Weber EJ. Reaction Mechanisms in Environmental Organic Chemistry. Boca Raton: Lewis Publishers; 2000.

[38] Soeda Y, Ishihara K, Iwataki I, Kamimura H. Fate of a Herbicide ^{14}c-Alloxydim-Sodium in Sugar Beets. Journal of Pesticide Science 1979;4(2) 121-128.

[39] Bridges DC, Smith AE, Falb. LN. Effect of Adjuvant on Foliar Absorption and Activity of Clethodim and Polar Degradation Products of Clethodim. Weed Science 1991;39(4) 543-547.

[40] McMullan PM. Grass Herbicide Efficacy as Influenced by Adjuvant, Spray Solution Ph, and Ultraviolet Light. Weed Technology 1996;10(1) 72-77.

[41] McInnes D, Harker KN, Blackshaw RE, Born WHV. The Influence of Ultraviolet Light on the Phytotoxicity of Sethoxydim Tank Mixtures with Various Adjuvants. In: Foy CL. (ed.) Adjuvants for Agrochemicals. Boca Raton: CRC Press 1992. p205-213.

[42] Hazen JL, Krebs PJ. Photodegradation and Absorption of Sethoxydim as Adjuvant-Influenced Surface Effects. In: Foy CL. (ed.) Adjuvants Agrochemicals. Boca Raton (USA): CRC Press; 1992. p195-203.

[43] Sevilla-Morán B, Mateo-Miranda MM, Alonso-Prados JL, García-Baudín JM, Sandín-España P. Sunlight Transformation of Sethoxydim-Lithium in Natural Waters and Effect of Humic Acids. International Journal of Environmental Analytical Chemistry 2010;90(3-6) 487-496.

[44] Merck Millipore. http://www.millipore.com/ (accessed 1 October 2012).

[45] Sevilla-Morán B, Sandín-España P, Vicente-Arana MJ, Alonso-Prados JL, García-Baudín JM. Study of Alloxydim Photodegradation in the Presence of Natural Substances: Elucidation of Transformation Products. Journal of Photochemistry and Photobiology A 2008;198(2-3) 162-168.

[46] Sevilla-Morán B, Alonso-Prados JL, García-Baudín JM, Sandín-España P. Indirect Photodegradation of Clethodim in Aqueous Media. By-Product Identification by Quadrupole Time-of-Flight Mass Spectrometry. Journal of Agricultural and Food Chemistry 2010;58(5) 3068-3076.

[47] Mazellier P, Jirkovsky J, Bolte M. Degradation of Diuron Photoinduced by Iron(Iii) in Aqueous Solution. Pesticide Science 1997;49(3) 259-267.

[48] Galichet F, Mailhot G, Bonnemoy F, Bohatier J, Bolte M. Iron(III) Photo-Induced Degradation of Isoproturon: Correlation between Degradation and Toxicity. Pest Management Science 2002;58(7) 707-712.

[49] Boule P., editor. The Handbook of Environmental Chemistry. Vol. II, Part L: Environmental Photochemistry. Berlin: Springer-Verlag; 1999.

[50] Hashimoto Y, Ishihara K, Soeda Y. Fate of Alloxydim-Sodium on or in Soybean Plants. Journal of Pesticide Science 1979;4(3) 299-304.

[51] Veerasekaran P, Catchpole AH. Studies on the Selectivity of Alloxydim-Sodium in Plants. Pesticide Science 1982;13(4) 452-462.

[52] Ono S, Shiotani H, Ishihara K, Tokieda M, Soeda Y. Degradation of the Herbicide Alloxydim-Sodium in Soil. Journal of Pesticide Science 1984;9(3) 471-480.

[53] Ishihara K, Shiotani H, Soeda Y, Ono S. Fate of the Herbicide Sethoxydim in Sugar Beet. Journal of Pesticide Science 1988;13(2) 231-237.

[54] Koskinen WC, Reynolds KM, Buhler DD, Wyse DL, Barber BL, Jarvis LJ. Persistence and Movement of Sethoxydim Residues in Three Minnesota Soils. Weed Science 1993;41(4) 634-640.

[55] Somasundaram L, Coats JR., editors. Pesticide Transformation Products. Fate and Significance in the Environment. Washington D.C.: ACS Symposium Series; 1991.

[56] Smith AE, Hsiao AI. Persistence Studies with the Herbicide Sethoxydim in Prairie Soils. Weed Research 1983;23(5) 253-257.

[57] Campbell JR, Penner D. Sethoxydim Metabolism in Monocotyledonous and Dicotyledonous Plants. Weed Science 1985;33(6) 771-773.

[58] Roslycky EB. Microbial Response to Sethoxydim and Its Degradation in Soil. Canadian Journal of Soil Science 1986;66(3) 411-419.

[59] Mills MS, Simmons ND. Assessing the Groundwater Contamination Potential of Agricultural Chemical: A Flexible Approach to Mobility and Degradation Studies. Pesticide Science 1998;54(4) 418-434.

[60] Srivastava A, Gupta KC, Singh G. Dissipation of Tralkoxydim Herbicide from Wheat Crop and Soil under Sub-Tropical Conditions. Pesticide Science 1995;43(1) 53-55.

[61] Hadfield ST, Wilson A, Kuet SF, Mason R. The Metabolism of Tralkoxydim in Field-Grown Spring Wheat and Maize Plant Cell Suspension Culture. Pesticide Science 1994;40(3) 193-200.

[62] Srivastava A, Gupta KC. Dissipation of Tralkoxydim in Water-Soil System. Journal of Pesticide Science 1994;19(3) 145-149.

[63] FAO/WHO., editor. Pesticide Residues in Food 1999. Evaluations Part 1 - Residues. Plant Production and Protection Paper Nº 157. Rome: FAO/WHO; 1999.

[64] Takano R, Hashimoto Y, Ono S, Soeda Y. Metabolism of Alloxydim-Sodium in Rats. Journal of Pesticide Science 1984;9(2) 275-283.

[65] Tixier C, Meunier L, Bonnemoy F, Boule P. Phototransformation of Three Herbicides: Chlorbufam, Isoproturon, and Chlorotoluron. Influence of Irradiation on Toxicity. International Journal of Photoenergy 2000;2(1) 1-8.

[66] Durand G, Barceló D, Albaigés J, Mansour M. Utilisation of Liquid Chromatography in Aquatic Photodegradation Studies of Pesticides: A Comparison between Distilled Water and Seawater. Chromatographia 1990;29(3-4) 120-124.

[67] Osano O, Admiraal W, Klamer HJC, Pastor D, Bleeker EAJ. Comparative Toxic and Genotoxic Effects of Chloroacetanilides, Formamidines and Their Degradation Products on Vibrio Fischeri and Chironomus Riparius. Environmental Pollution 2002;119195-202.

[68] Sandĭn-España P, Loureiro I, Escorial C, Chueca C, Santĭn-Montanya I. The Bioassay Technique in the Study of the Herbicide Effects. In: Soloneski S, Larramendy ML. (eds.) Herbicides, Theory and Applications. Rijeka: InTech; 2011. p431-454.

[69] Falb LN, Bridges DC, Smith AE. Separation of Clethodim Herbicide from Acid and Photodegradation Products by Liquid Chromatography. J. AOAC International 1991;74(6) 999-1002.

[70] Shoaf AR, Carlson WC. Analytical Techniques to Measure Sethoxydim and Breakdown Products. Weed Science 1986;34(5) 745-751.

[71] Sandín-España P, González-Blázquez JJ, Magrans JO, García-Baudín JM. Determination of Herbicide Tepraloxydim and Main Metabolites in Drinking Water by Solid-Phase Extraction and Liquid Chromatography with Uv Detection. Chromatographia 2002;55(11/12) 681-686.

[72] Curtin DY, Grubbs EJ, McCarty CG. Uncatalysed Syn-Anti Isomerization of Imines, Oxime Ethers, and Haloimines. Journal of American Chemical Society 1966;88(12) 2775-2786.

[73] Sandín-España P, Sevilla-Morán B, Calvo L, Mateo-Miranda M, Alonso-Prados JL. Photochemical Behavior of Alloxydim Herbicide in Environmental Waters. Structural Elucidation and Toxicity of Degradation Products. Microchemical Journal 2012;Article in Press.

[74] Huber R, Hamm R, Ohnsorge U, Tuerk W. The Metabolism of Cycloxydim in Soybeans. In: Brighton Crop Protection Conference-Pests and Diseases, 21-24 November 1988, Brighton, UK. BCPC Publications; 1988.

[75] Sánchez P, Kubitza J, Dohmen GP, Tarazona JV. Aquatic Risk Assessment of the New Rice Herbicide Profoxydim. Environmental Pollution 2006;142(1) 181-189.

[76] Monaco TJ, Weller SC, Ashton FM. Weed Science: Principles and Practices. 4th ed., New York: John Wiley & Sons; 2002.

[77] Rea G, Polticelli F, Antonacci A, Lambreva M, Pastorelli S, Scognamiglio V, Zobnina V, Giardi MT. Computational Biology, Protein Engineering, and Biosensor Technology: A Close Cooperation for Herbicides Monitoring. In: Soloneski S, Larramendy ML. (eds.) Herbicides, Theory and Applications. Rijeka: InTech, Published; 2011. p93-120.

[78] Picó Y. Analysis of Pesticides (New Generation) and Realted Compounds. In: Meyers RA. (ed.) Encyclopedia of Analytical Chemistry. Supplementary Volumes S1 - S3. Chichester: John Wiley & Sons; 2009. p1-38.

[79] Ono S, Toyama N, Sugioka K, Tokieda M, Nomura O, Tsukada S. Residue Analysis of Alloxydim-Sodium and Its Degradation Products. Journal of Pesticide Science 1980;5(4) 575-584.

[80] Gomyo T, Kobayashi S, Soeda Y. Analytical Method for the Total Content of Alloxydim-Sodium Herbicide and Its Degradation Products in Groundwater by Gas Chromatography Mass Spectrometry. Analytical Sciences 1991;7(1) 23-27.

[81] Ishimitsu S, Kaihara A, Yoshii K, Tsumura Y, Nakamura Y, Tonogai Y. Determination of Clethodim and Its Oxidation Metabolites in Crops by Liquid Chromatography with Confirmation by Lc/Ms. Journal of AOAC International 2001;84(4) 1172-1178.

[82] Klein J, Alder L. Applicability of Gradient Liquid Chromatography with Tandem Mass Spectrometry to the Simultaneous Screening for About 100 Pesticides in Crops. Journal of AOAC International 2003;861015–1037.

[83] Gomyo T, Ono S. Residue Analysis of Herbicide Sethoxydim. Journal of Pesticide Science 1987;12(4) 741-743.

[84] Gomyo T, Kawakami H, Tokieda M, Sugioka K, Kobayashi S, Ono S. Residue Analysis of Herbicide Sethoxydim and Its Metabolites in Crops. Journal of Pesticide Science 1987;12(4) 729-737.

[85] Hu J-Y, Aizawa T, Magara Y. Analysis of Pesticides in Water with Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometry. Water Research 1999;33(2) 417-425.

[86] Eisert R, Jackson S, Krotzky A. Application of on-Site Solid-Phase Microextraction in Aquatic Dissipation Studies of Profoxydim in Rice. Journal of Chromatography A 2001;909(1) 29-36.

[87] Tsochatzis ED, Tzimou-Tsitouridou R, Menkissoglu-Spiroudi U, Karpouzas DG, Maria Papageorgiou G. Development and Validation of an Hplc-Dad Method for the Simultaneous Determination of Most Common Rice Pesticides in Paddy Water Systems. International Journal of Environmental Analytical Chemistry 2012;92(5) 548-560.

[88] Liska I, Brouwer ER, Ostheimer AGL, Lingeman H, Brinkman UAT, Geerdink RB, Mulder WH. Rapid Screening of a Large Group of Polar Pesticides in River Water by on-Line Trace Enrichment and Column Liquid Chromatography. International Journal of Environmental Analytical Chemistry 1992;47(4) 267-291.

[89] Shen J-c, Ye W-x, Xie L-q, Zhao Q-h, Xiao L-l. Analysis of Cyclohexanedione Herbicides in Rice and Corn by Lc-Ms/Ms. Journal of Chinese Mass Spectrometry Society 2009;30(4) 201-207.

[90] Marek LJ, Koskinen WC, Bresnahan GA. Lc/Ms Analysis of Cyclohexanedione Oxime Herbicides in Water. Journal of Agricultural and Food Chemistry 2000;48(7) 2797-2801.

Chapter 7

Kinetics and Mechanism of Inhibition of Oxidation Enzymes by Herbicides

E. A. Saratovskikh

Additional information is available at the end of the chapter

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1. Introduction

"A lack of knowledge in the area of biology of grown plants and specific features of the medium of their dwelling in each specific field cannot be compensated by an excess of pesticides, fertilizers, or melioration."

Academician D.N. Pryanishnikov, 1934

Pesticides as a whole and herbicides in particular are substances with high biological activity. They can exert a toxic effect on many components of cells: enzymes, structural and functional proteins, lipoproteids, polysaccharides, nucleic acids, and others. The elucidation of the mechanism of toxic effect is an important challenge, the solution of which would allow one to establish the real and potential danger of application of these or other compounds for human and non-targent organisms. Despite the enormous scale of production and use of chemical means for cultivated plant protection, there is still much unknown on the mechanism of their action. It is considered that, probably, each pesticide acts through a unique mechanism. For example, the acting components of pesticides, namely, zenkor, lontrel, roundup, kusagard, setoxidim, basagran, tilt, and tachigaren, belong to different classes of chemical compounds. According to available literature data (Table 1), they interact with various enzymatic systems, have their own specific binding sites, and are characterized by different mechanisms of action.

Much data concerning the influence of herbicides and fungicides on various components of the living cell (Fedtke, 1982; Kadyshev, 1970; Fudel-Osipova, 1981), in particular, on some enzymes (Mathew et al., 1998; Forthoffer et al., 2001; Banas et al., 2000; Knecht & Löffler, 1998; Du, 2000a; Gruys et al., 1993; Nosanchuk et al., 2001; Kiyomiya et al., 2000) have been reported. For instance, anticholinesterase compounds, organophosphorus pesticides, carbamates and triazines (Grin & Goldberger 1968), are structurally similar to substrates and

competitively inhibit their activity. The effect was evaluated for fries of Mediterranean fishes *Dicentrarchus labrax* (Varo et al., 2003) and for rats (*Maple amber, M. arrow*) fed with soybean after treatment with zenkor and atrazine (Mathew et al., 1998). It was shown that herbicide basagran suppresses the antiphosphatecholinesterase activity and results in an increase in the hydroxylase activity (Al-Mendofi & Ashton, 1984; Forthoffer et al., 2001).

The growth of fungi *Cryptococcus neoformans* was suppressed by glyphosate due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (Nosanchuk et al., 2001). A non-productive four-membered complex is formed between the enzyme, pesticide, and phosphate (Du, 2000b). Octahedral coordination is performed by the metal ion: Co glyphosate enzyme as in 3-deoxy-D-arabiheptulosonate-7-phosphate synthase localized in cytosols (Ganson & Jensen, 1988).

Oxidative phosphorylation is performed by Zn-containing enzymes. Dinoseb, pentachlorophenol, dichlorodiphenyltrichloroethane, and Sevin separate oxidative phosphorylation in mitochondria of Palma Christi (Kuz`minskaya, 1975) and decrease the ATP content in glycols of soybean (Gruenhagen & Moreland, 1971). Chloro-containing organic pesticide endosulfan reacts with glutathione (cofactor of glutathione peroxidase), considerably decreasing the activity of the enzyme. The loss of secretory reactions in thylakoids of adrenocortical steroidogenic cells and changes in the enzyme activity indicate that the pesticide was involved in the oxidative reactions (Dorval et al., 2003).

The formation of complexes of vegetable peroxidase with various substrate-inhibitors was established (Ugarova & Lebedeva, 1978). Both the direct participation of the metal in the substrate addition to the protein part of the molecule and providing of a relationship between the flavine group and apoenzyme under the action of the metal are assumed. The neighborhood of the pyridine nitrogen atom to the carboxyl group in picolinic acid (picloram) is manifested in the ability to complexation and metal removal from enzymes (Shcheglov et al., 1967).

The tests on human and rat tissues showed that tachigaren and its metabolites (four enzymes synthesizing pyrimidine) inhibit mitochondrial [EC 1.3.99.11]. This results in the changes in the pyridine–nucleotide pool that provides the work of immune cells. The reaction is reversible and its mechanism is uncompetitive with respect to the substrate and cofactor ubiquinone (Knecht & Löffler, 1998).

On the other hand, diverse xenobiotics, both pesticides and metals, are abundant in considerable amounts in the nature, namely, in air, soil, and water (Banas et al., 2000; Knecht & Löffler, 1998; Du, 2000a). If these xenobiotics get into the human organism, they may cause various diseases (Gruys et al., 1993; Nosanchuk et al., 2001). In the presence of pesticides with ligand properties, their combined effect on living organisms can be enhanced or weakened.

The ability of the environment to self-purification, *i.e.*, decomposition of contaminants, is determined, to a great extent, by the occurrence of enzymatic redox processes in cells of plants and microorganisms. One of the enzymes performing the redox processes in biological systems is NADH-oxidoreductase (NADH-OR) (Tukhvatullin et al., 2001; Sommerhalter et al., 2004;

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Common name	Range of application	Mechanism action	Reference
Zenkor, Metribuzin	selective to dicotyledons and solanaceous	complexes with membrane lipids	Ziegler et al., 1982
Lontrel, Clopyralid	a wide spectrum of action	similar to auxin	Hall et al., 1985
Kusagard	selective to dicotyledons, beet, cotton	lesion meristem tissues	Iwataki & Hirono, 1978
Roundup, Glyphosate	for struggle against perennial weeds	inhibitor of enolpyruvateshekemate-phosphate synthase	Amrhein et al., 1980
Setoxidim	selective to dicotyledons, beet, solanaceous		
Basagran, Bentazon	selective to grains	inhibitor of photo- ; protein- ; lipids- ; RNA - synthesis	Trebst & Wietoska, 1975 Osama & Ashton, 1984
Tachigaren, Hymexazol	selective to the grass, sugar beet	inhibitor of dehydrogenase (mitochondrial)	Knecht & Löffler, 1998
Titl, Propiconazole		7-etoxyrezofurine O-diethylase; inductor glutathione S-transferase	Levine & Oris, 1999; Egaas et al., 1999
Lontrel metal complexes	a wide spectrum of action	inhibitor of NADH-oxidoreductase	Saratovskikh, 2005; Saratovskikh, 2007

Table 1. Pesticides mechanism action from literary

Bagirov et al., 1989; Lycholat & Bilchuk, 1998) possessing a broad substrate specificity. This enzyme is in the composition of the monooxygenase system that utilizes substrates and transforms xenobiotics into the lowly toxic state. NADH-OR, [EC 1.6.99.25] from the methyl-otroph *Methylococcus capsulatus* (strain M) (Burbaev et al., 1990) transfers electrons for the mixed reduction of oxygen to water, methane transformation to give methanol in the active center of methane hydroxylase, and the reduction of dioxygen to water in the active center of cytochrome oxidase. The enzyme studied consists of four subunits, each including FAD and the iron-sulfur cluster, 2Fe-2S (Tsuprun et al., 1987; Bagirov et al., 1989). NADH-OR functions according to the following scheme:

$$NADH + A_{ox} \xrightarrow{\text{NADH-}OR} \text{NAD}^+ + A_{\text{red}}$$

where A is acceptor.

The most part of biological oxidation processes is performed by an array of carriers, which are grouped in the electron transfer chain and the respiratory chain, one end of which contains the active metabolite and the $1/2O_2$–H_2O system is localized on another end. Among the main components of the chain electron transfer are nicotinamide (pyridine) coenzymes NADH and NADFH.

The sequence of electron transfer from NADH to an electron acceptor is still unknown. However, by analogy with other reductases, one can suggest that the electrons are transferred

from NADH to FAD and then to the iron sulfur 2Fe-2S cluster and to the electron acceptor. Neotetrazolium chloride (NT) was used in this work as the artificial electron acceptor.

Enzymes of this type are present in the cells of almost all organisms. Therefore, the general features of the interaction of this enzyme with pesticides can also be applied to NADH-OR from other organisms.

It is well known that nucleotides play an important role in organisms: energy and regulatory processes and biosynthesis. Nicotinamide adenine dinucleotide functions together with several vitally important enzymes. Therefore, it was of interest to perform a kinetic study of some widely used pesticides on the activity of the enzyme acting together with NADH of one of the enzymes performing oxidation and on nucleotide NADH.

Here we present the data showing the formation of complexes of a series of pesticides with dinucleotide NADH and data on the kinetics of NADH-OR inhibition by commercial herbicides and fungicides of various structures and several complexes of the herbicide lontrel with doubly charged metal ions.

2. Materials and methods

2.1. Compounds, concentrations and replicates

2.1.1. Pesticides

The active substances of the herbicides and fungicides (their formulas are shown below) were isolated from commercial preparations by extraction (Saratovskikh et al., 1988). After isolation the purification was as follows: glyphosate (roundup), tachigaren, and basagran were purified by double recrystallization from water (Mel`nikov, 1987); kusagard and setoxidim were subjected to chromatography on a column with SiO_2; tilt was obtained as nitrate followed by the isolation of the base; lontrel was recrystallized from benzene and then twice recrystallized from hexane; zenkor was recrystallized from hexane and then from a hexane–benzene mixture. The chemical and structural formulas of the used pesticides and lontrel metal complexes are presented in Table 2.

2.1.2. Metal salts

Co, Mn, Ni, and Cu acetates and Fe lactate for the synthesis of metal complexes (pure grade, Reakhim, USSR) were purified by double recrystallization from water.

2.1.3. Reagents

Commercial NADH (nicotinamide adenine dinucleotide) and NT (Sigma no. 2251, Reanal, Hungary) as an artificial electron acceptor were used.

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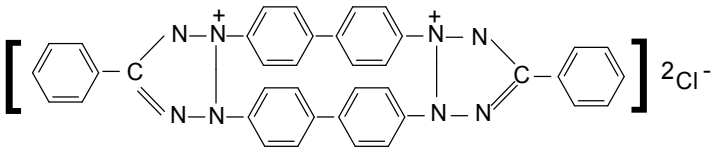


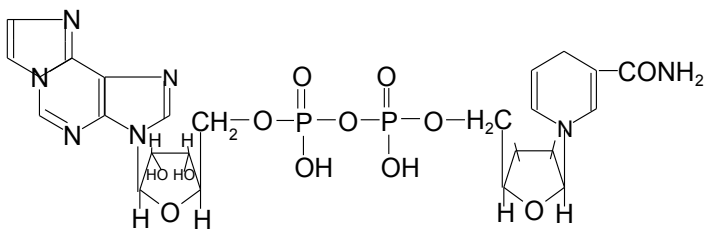
2.2. Compound synthesis

2.2.1. The metal complexes of lontrel (ML_2)

ML_2 where L is lontrel, were synthesized by refluxing ethanolic solutions of lontrel with the corresponding divalent metal salts (Aliev et al., 1988; Saratovskikh, 1989).

2.2.2. The synthesis of ε-NADH

The synthesis of ε-NADH for the study of complexation with pesticides was carried out according to (Lichina et al., 1978; Lichina et al., 1979). Ethenonicotinamide adenine dinucleotide (ε-NADH) has the structure



The degree of modification of adenine ε-NADH is 100%.

2.3. Enzyme inhibition

2.3.1. Bacterial culture preparation

Methane-oxidizing bacteria *M. capsulatus* (strain M) were grown in a 20-L flow-type fermenter in a 10-L salt medium at 42°C. The rate of supply of air mixed with the gas-main natural gas was 300+100 L min^{-1} (Burbaev et al., 1990). The flow rate was 0.24 m^3 h^{-1}.

The cell suspension was collected, concentrated by separation, and washed twice with a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. The cells were destroyed in a DKM-5 semiatomated disintegrator (produced at the Institute of Problems of Chemical Physics of the RAS, Chernogolovka). The cell-free preparation was centrifuged for 30 min at 3000g, the supernatant was centrifuged for 1 h at 65000g, and the precipitated membrane structures and the supernatant fraction ($SF_{65\text{-}1}$) were collected separately, frozen, and stored in liquid nitrogen until used.

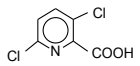
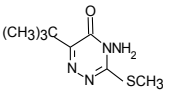
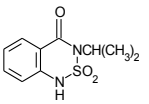
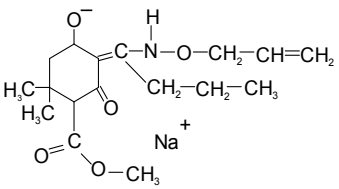
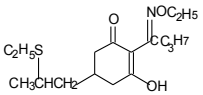
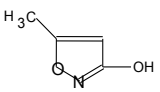
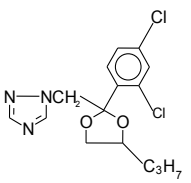
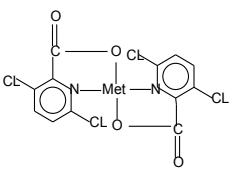
№	Common names of pesticides	Chemical formula	*K*, 10^{-3} M with ε-NADH
1.	Lontrel, Clopyralid 3,6-Dichloropicolinic acid		11.7 ± 0.4
2.	Zenkor, Metribuzin 4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one		22.0 ± 2.0
3.	Basagran, Bentazon 3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide		not determined
4.	Roundup, Glyphosate N-(phosphonomethyl) glycine	$(OH)_2POCH_2NHCH_2COOH$	2.2 ± 0.4
5.	Kusagard Sodium salt of 2-(1-allyl-oxyamino butylidene)-5,5-dimethyl-4-methoxycarbonylcyclohexane-1,3-dione		2.5 ± 0.1
6.	Setoxydim 2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one		2.8 ± 0.7
7.	Tachigaren, Hymexazol 5-methyl-1,2-oxazol-3-ol		1.8 ± 0.4
8.	Titl, Propiconazole 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole		0.46 ± 0.06
9.	Lontrel metal complexes $M(L)_2$:		
	$Cu(L)_2$		4.6 ± 0.2
	$Co(L)_2$		3.1 ± 0.1
	$Ni(L)_2$		4.7 ± 0.3
	$Fe(L)_2$		0.55 ± 0.06
	$Mo(L)_2$		2.2 ± 0.1

Table 2. Industrial and nomenclature names and chemical formula of the investigated substances and the complexation constants of pesticides with ε-NADH

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2.3.2. NADH-OR isolation and purification

The fraction $SF_{65\text{-}1}$ (500 mL, 60 mg of protein mL^{-1}) was passed through a column (30x7 cm) with DEAE-cellulose 52 (Whatman, UK), and the column was washed with 1 L of $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. NADH-OR was eluted using a linear gradient of 0-0.35 *M* NaCl in the same phosphate buffer. The protein fraction with the maximum NADH-OR-activity was eluted with 0.2 *M* NaCl. The eluate was collected, concentrated under argon by ultrafiltration through the Vladipor porous membranes under a 5 atm pressure to 60 mg mL^{-1} of the protein, and fractionated successively on a column with Sephadex G-75 (4x70 cm) and a column with Sepharose 2B (4x80 cm) (Pharmacia, Sweden) in a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. The enzyme preparation with a specific activity (with respect to NT) of 1.3 μmol L^{-1} min^{-1} (mg protein)$^{-1}$ (20 °C) was collected, concentrated by ultrafiltration to 21 mg mL^{-1} of the protein, frozen, and stored in liquid nitrogen until used.

2.3.3. Determining Enzyme activity and inhibition constant (K_i)

The activity of NADH-OR was determined from the rate of reduction of NT to formazan in a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 8.0. The rate of formazan formation was estimated (Burbaev et al., 1990) from the change in the absorbance at 550 nm using a Specord M-40 (GDR) spectrophotometer. The reaction was carried out in 3-mL cells (10x10 mm). The reaction mixture contained 0.1 mL of NADH-OR (1 mg of the protein), 0.3 mL of the test compound, 0.1 mL of NADH ($1.0 \cdot 10^{-3}$ mol L^{-1}), and $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 8.0, added up to 3 mL. The reaction was initiated by adding 0.2 mL of a solution of NT ($1.5 \cdot 10^{-3}$ mol L^{-1}).

The study was carried out by the traditional Michaelis–Menten procedure. The first task was to elucidate the dependence of the rate constant for the enzymatic formation of formazan on the pesticide concentration and to determine I_{50}, *i.e.*, the concentration of the pesticide inhibitor, resulting in a twofold decrease in the maximum rate of the enzymatic reaction. The second stage included two series of experiments: (1) at a constant NT concentration and variable NADH concentrations, and (2) at a constant NADH concentration and variable NT concentrations.

The K_i values were calculated from the equation (Dixon & Webb, 1979; Emanuel & Knorre, 1969)

$$K_i = (I_{50} \cdot K_m) / (SV / v - K_m),$$

where K_i is the inhibition constant; I_{50} is the concentration of the pesticide inhibitor; K_m is the determined Michaelis constant for NT or NADH; v is the rate; S is the concentration of NT or NADH; V is the maximum rate determined from the Lineweaver–Burk plot.

The Michaelis constant in the presence of the inhibitor (K_m^1) is the following:

$$K_m^1 = [(1/v)/(1/S)] \cdot V$$

The K_m^1 and V values are dictated by the inhibition type.

The Hill coefficients were determined by the Hill formula (Dixon & Webb, 1979; Cornish–Bowden, 1976)

$$Y = \left(K_h I^h\right) / \left(1 + K_h I^h\right),$$

where Y is the degree of protein saturation with the ligand and is equal to the ratio of the number of occupied binding sites to the total number of binding sites; K_h is the association constant in the case where the concentration of the complex is as follows:

$$[E_h \cdot I_h] = K_h [E_h] \cdot [I]^h$$

where h is the Hill coefficient describing the degree of allostericity and equal to the number of molecules of the ligand, in this case, the pesticide inhibitor; I is the concentration of the pesticide inhibitor.

2.3.4. Studies on bacteria Beneckea harveyi

Lyophilized preparation of marin "luminescent" bacteria *Benechea harveyi* (strain B 1 7 – 667F) and *Photobacterium phosphoreum* (Zhmur & Orlova, 2007; Kuz`mich et al., 2002) was stored in a freezing box and used prior to use. The lyophilic preparation of bacteria was suspended in a 0.85% solution of NaCl.

To determine toxicity, 0.3–0.5 mL of suspended bacteria was added to 0.5 mL of the studied water. A 0.85% solution of NaCl or water from an aquarium was used as a control. The measurements were carried out by the instrumental method with a BLM-8801 luminometer (SKTB "Nauka," USSR) with detection on a voltmeter by a decrease in the bioluminescence intensity in the presence of a sample of analyzed water compared to the control.

The 50% (and more) decrease in the luminescence intensity indicates that the aqueous medium is toxic. The bioluminescence intensity of bacteria is determined by the activity of intracellular metabolic processes involving the luciferase enzyme. The decrease in luminescence can be due to the inhibition of the enzyme itself and to the influence of toxicants to other units of the metabolic chain.

The toxicity coefficient was calculated by the formula

$$T = \left[\left(I_c - I_t\right) / I_t\right] \times 100\%,$$

where I_c is the bioluminescence intensity in the control, and I_t is the luminescence intensity in the tested sample.

At $T \leq 19\%$ the tested sample is not toxic. At $19 < T \leq 50\%$ the tested sample is considered toxic, whereas at $T > 50\%$ the sample is strongly toxic.

Each toxicological experiment was carried out at least three times and then the results obtained were statistically processed.

2.4. Instrumental analysis

2.4.1. Fluorimetric measurements

The fluorescence spectra of the etheno-modified compounds were recorded on an Aminco–Bowman spectrofluorimenter (US) in 3.5-mL quartz cells. The fluorescence excitation wave-

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length for ε-NADH is 312 nm, and the fluorescence emission maximum is 420 nm. The fluorescence intensity of ε- NADH was measured in a 0.025 M tris-HCI buffer (pH = 6.8) at 20^0C. The concentration of ε-NADH equal to $1 \cdot 10^{-4}$ M was used in experiments. The fluorescence spectrum of ε-NADH was accepted to be 1, and then a solution of nucleotide was titrated in the cell with an aqueous solution of the studied pesticide in the concentration from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-2}$ M.

The complexation constants of pesticides with nucleotides were calculated from the experimental titration curve for each point. The obtained values were averaged. Theoretical titration curves were calculated from the values of complexation constants obtained by the experimental data (Saratovskikh et al., 1988).

2.4.2. Electron spin resonance analyses

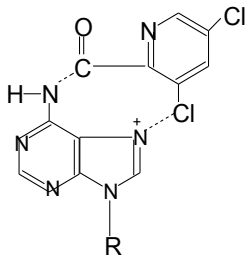
ESR spectra were recorded at 77 K on an SE/X2544 Radiopan radiospectrometer (Poland) at a 10 mW microwave radiation and a magnetic field modulation of 0.4 mT. The samples were prepared in a $2.0 \cdot 10^{-2}$ *M* tris-HCl buffer, pH 7.0. ESR spectra were recorded in 50% glycerol.

3. Results and discussion

The study of the inhibition of NADH-OR by pesticides and metal complexes of herbicide lontrel (see Table 2) was started from the consideration of their interaction with coenzyme NADH.

Figure 1. Excitation and fluorescence spectra of ε-NADH.

It is known (Blagoyi et al., 1991) that polynucleotides, particularly, pyridinenucleotides, form complexes of various types, including charge-transfer complexes, and are highly reactive towards a series of metals. However, the introduction of the etheno group does not almost change the electronic structure of the nucleotide fragment of a NADH molecule. Therefore, the complexation of pesticides with NADH was judged about on the basis of the value of fluorescence quenching of its chemical analog, modified dinucleotide ε-NADH in which the adenine fragment is subjected to etheno-modification. Figure 1 illustrates the excitation and fluorescence spectra of ε-NADH.



Figures 2 and 3 represent the obtained dependences of the change in the fluorescence intensity of ε-NADH on the concentration of various quenchers. When the concentration of pesticide (or metal complex) increases, the fluorescence quenching of compound ε-NADH is observed, which is not accompanied by a shift of the position of the excitation maximum and fluorescence emission. The absence of spectral changes in all cases considered indicates the absence of changes in the ground and excited levels of the modified based upon the interaction with pesticides. Fluorescence quenching was observed at the pesticide and lontrel metal complexes concentrations ranging from 10^{-6} to 10^{-3} M. Such low concentrations of the quencher exclude the assumption that the quenching proceeds via the Stern–Volmer mechanism due to random collisions. Therefore, the result of quenching is the formation of a covalent bond with the adenine fragment, as it is shown in the scheme of the [NADH–lontrel] complex

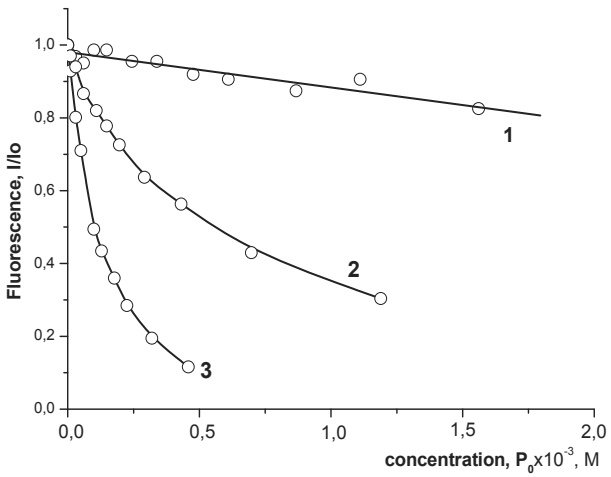


Figure 2. Dependences of the fluorescence intensity of ε-NADH on the pesticide concentration: (1) tilt; (2) kusagard; (3) zenkor. The concentration of ε-NADH is $1 \cdot 10^{-5}$ M. Solid lines are theoretical curves, and points are experimental data.

The mathematical model of the process was considered to refine the mechanism of formation of complexes [ε-NADH–pesticide] and to estimate their stability constants. It was assumed that the pesticides interact with ε-NADH according to the scheme

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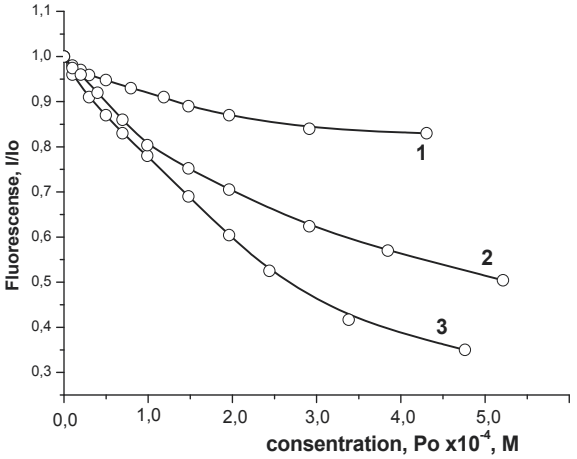

Figure 3. Dependences of the fluorescence intensity of ε-NADH on the concentration of the lontrel metal complexes: (1) Fe(L)2; (2) Ni(L)2; (3) Cu(L)2. The concentration of ε-NADH is $1 \cdot 10^{-5}$ M. Solid lines are theoretical curves, and points are experimental data.

$$[A] + n[P] \leftarrow \frac{\kappa^{+1}}{\kappa^{-1}} \rightarrow [\Pi] \tag{1}$$

where A is the concentration of etheno-modified units of the nucleotide (adenine) in ε-NADH, P is the pesticide concentration, Π is the concentration of the reaction product = complex, $K = \kappa^{+1} / \kappa^{-1}$ is the complexation constant, and n is the stoichiometric coefficient equal to the number of equivalent binding sites.

At equilibrium the process is described by the following system of equations:

$$K[A] \times [P]^n = [\Pi] \tag{2}$$

$$[A] + [\Pi] = [A_0] \tag{3}$$

$$[P] + n[\Pi] = [P_0] \tag{4}$$

Equations 2–4 make it possible to determine the values of the complexation constant from the experimentally determined concentration

$$[A] = \frac{I - I_\kappa}{I_0 - I_\kappa} A_0,$$

where I_o is the fluorescence intensity of free ε-NADH, I_κ is the limiting value of fluorescence intensity of ε-NADH at the maximum concentration of the quencher, and I is the fluorescence intensity of ε-NADH at the given concentration of the quencher.

$$K = \frac{[A_0] - [A]}{[A]\{[P_0] - n([A_0] - [A])\}^n} \tag{5}$$

The value of stoichiometric coefficient n should preliminarily be determined from the data obtained at a rather high pesticide concentration at which the following equation is fulfilled:

$$[P_o] >> n([A_o] - [A]). \tag{6}$$

After condition (6) is fulfilled, equation (5) can be rewritten in the form

$$\ln\left(\frac{[A_0] - [A]}{[A]}\right) = \ln K + n\ln[P_0], \tag{7}$$

from which it follows that the dependence of $\ln \ln \frac{[A_0] - [A]}{[A]}$ on $\ln[P_o]$ is a straight line with the angular coefficient equal to n.

As can be seen from Fig. 4, stoichiometric coefficient n is equal to 1±0.2 for all pesticides and metal complexes evaluated. Therefore, we may conclude that only one pesticide molecule interact with one molecule of ε-NADH. Having determined the value of n at high P_o, one can find the value of K at other pesticide concentrations. The use values of P_o should not be too low, since at very low $[P_o]$ relative errors of the values of $([A_o] - [A])$ and $([P_o] - n[A_o] - [A])$ can be too high and the errors of complexation constant determination will be unacceptably high.

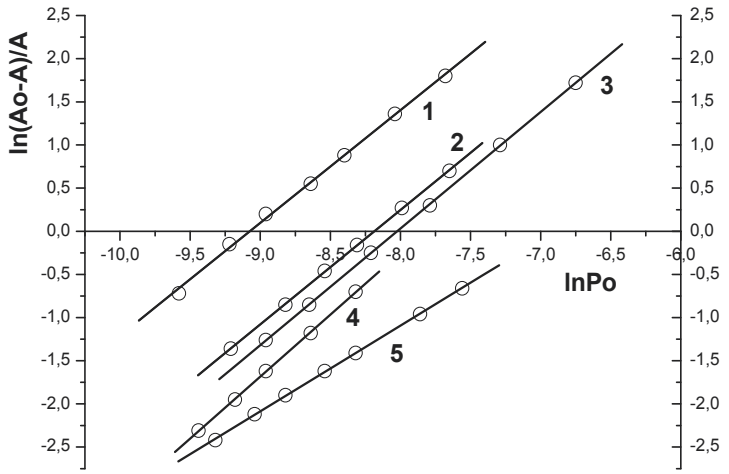

Figure 4. Dependences of the $\ln \frac{Ao - A}{A}$ on the lnPo. (1) zenkor; (2) $Cu(L)_2$; (3) setoxidim; (4) $Mo(L)_2$; (5) $Co(L)_2$.

Figures 2 and 3 show the experimental dependences of I/I_o on $[P_o]$ and the corresponding theoretical curves for the pesticides and $Cu(lontrel)_2$ complexes ($Cu(L)_2$) calculated by the

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values found for n and K. Satisfactory coincidence of the experimental and theoretical data indicates that the developed model is valid.

As can be seen from Table 2, of the synthesized pesticides, zenkor has the lowest complexation constant (K) with ε-NADH ($K = 2.1 \cdot 10^4$ M^{-1}) and tilt has the highest one ($K = 4.6 \cdot 10^2$ M^{-1}). It is noteworthy that the complexation constant of the lontrel metal complexes with ε-NADH is substantially lower than the corresponding constant for lontrel. It is known (Luisi et al., 1975) than in solution NADH exists predominantly in a folded conformation in which the adenine part of the molecule is localized near the nicotine amide part of the nucleotide. About 90% dinucleotide exists in this conformation in solution. The rest 10% exist in solution in the "open" conformation when the nicotine amide part is remote from the adenine structure. Therefore, it can be assumed that the decrease in the complexation constants with ε-NADH for the metal complexes compared to lontrel indicates steric hindrances appeared upon the formation of the [NADH–$M(L)_2$] complex.

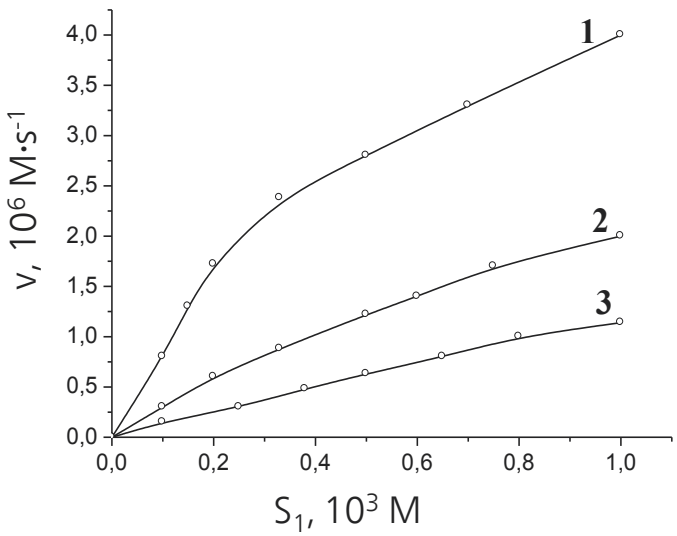

Figure 5. Kinetic curves for the oxidation rate of NADH-OR *vs.* the concentration of NADH at a constant concentration of NT in the absence of an inhibitor (1) and in the presence of $0.33 \cdot 10^{-4}$ (2) and $1.00 \cdot 10^{-4}$ mol L^{-1} (3) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 1.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

The effect of pesticides on the activity of NADH-OR is illustrated by Figs 5–8. The experimental kinetic curves for the rate of NADH-OR oxidation *vs.* concentration of the NADH (S_1) substrate at an invariable NT concentration are presented in Fig. 5. The plots converted to the Lineweaver–Burk coordinates are shown in Figs 6–8. Figure 6 shows the pattern of OR inhibition by lontrel as a function of the concentration of NADH (at a constant NT concentration). The intersection of these straight lines in one point on the ordinate (see Fig. 6) indicates that the herbicide lontrel inhibits NADH-OR and competes with NADH for the region of binding with the enzyme. The $1/S_1$ intercept on the abscissa was used to calculate the inhibition constant (S_1 is the NADH concentration, S_2 is the NT concentration).

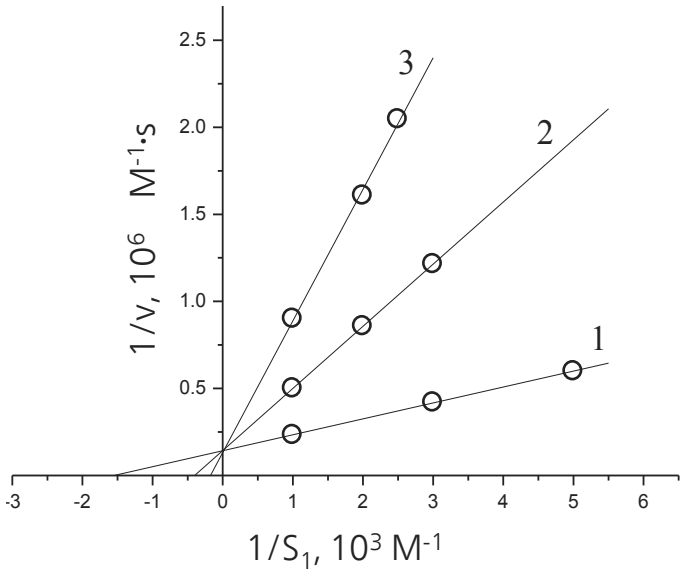

Figure 6. Inhibition of the NADH-oxidoreductase by lontrel (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $0.33 \cdot 10^{-4}$ (2) and $1.00 \cdot 10^{-4}$ mol L^{-1} (3) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 1.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

It can be seen from Tables 3 and 5 that the lontrel complex with the copper ion, although follows a competitive mechanism of inhibition with respect to NADH, still inhibits the oxidation of NADH almost 30 times stronger than the parent lontrel. The I_{50} values are equal to $1.1 \cdot 10^{-3}$ and $3.3 \cdot 10^{-4}$ mol L^{-1} (K_i are $1.0 \cdot 10^{-4}$ and $6 \cdot 10^{-6}$ mol L^{-1}).

Pesticide	*I*50 mol L^{-1}	*h*	for NADH				for NT			
			V_{max} mol/ L s	S_1 mol L^{-1}	$K_i \cdot 10^4$	Type**	V_{max} mol/ L s	S_2 mol L^{-1}	$K_i \cdot 10^4$	Type**
Zencor	$5.00 \bullet 10^{-4}$	1.952	-	$4.93 \bullet 10^{-3}$	0.25	A	$0.23 \bullet 10^{-6}$	$3.39 \bullet 10^{-4}$	8.94	B
Lontrel (L)	$1.10 \bullet 10^{-3}$	1.726	-	$1.23 \bullet 10^{-3}$	1.00	A	$1.88 \bullet 10^{-6}$	$6.98 \bullet 10^{-4}$	7.42	B
Bazagran	$6.00 \bullet 10^{-4}$	2.086	$1.82 \bullet 10^{-6}$	$1.83 \bullet 10^{-4}$	12.80	B	$0.26 \bullet 10^{-6}$	$2.55 \bullet 10^{-4}$	8.40	B
Kuzagard	$27.0 \bullet 10^{-2}$	1.575	-	$9.86 \bullet 10^{-3}$	14.00	A	-	$5.72 \bullet 10^{-3}$	158.9	A
Tachigaren	$2.70 \bullet 10^{-3}$	1.920	-	$2.47 \bullet 10^{-3}$	21.00	A	-	$5.30 \bullet 10^{-3}$	4.55	A
Roundup	$1.70 \bullet 10^{-3}$	1.328	$3.33 \bullet 10^{-6}$	$6.17 \bullet 10^{-4}$	22.0	C	$0.21 \bullet 10^{-6}$	$2.00 \bullet 10^{-4}$	42.90	B
Tilt	$2.20 \bullet 10^{-3}$	2.483	$1.25 \bullet 10^{-4}$	$5.98 \bullet 10^{-4}$	23.00	C	-	$13.00 \bullet 10^{-3}$	1.52	A
Setoxidim	$17.0 \bullet 10^{-2}$	1.832	$2.00 \bullet 10^{-6}$	$7.59 \bullet 10^{-4}$	397.50	C	-	$11.00 \bullet 10^{-3}$	8.04	A

* In the absence of an inhibitor *V*max = $7.40 \cdot 10^{-6}$ mol L^{-1} s^{-1}; $S_1 = 6.58 \cdot 10^{-3}$ mol L^{-1}; $S_2 = 2.65 \cdot 10^{-3}$ mol L^{-1}.

** The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 3. Effect of inhibitors on NADH-oxidoreductase*

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It can be seen from Fig. 7 that the herbicide roundup does not compete with NADH for the enzyme binding site.

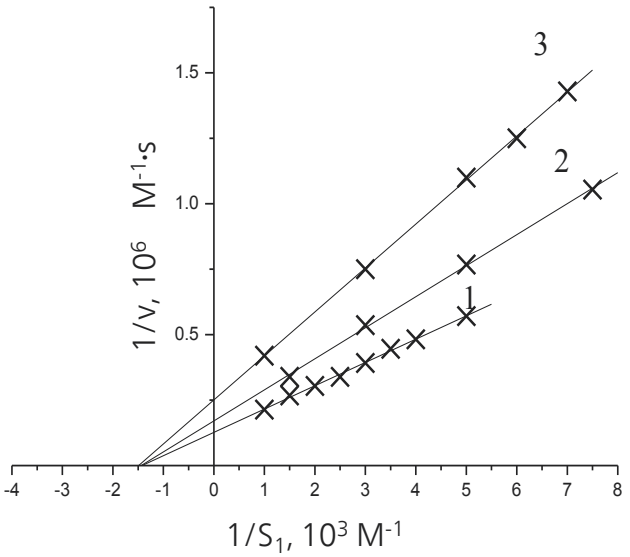

Figure 7. Inhibition of the NADH-oxidoreductase with roundup (in the Lineweaver-Burk coordinates) in the absence of the inhibitor (1) and in the presence of $1.17 \cdot 10^{-3}$ (2) and $2.50 \cdot 10^{-3}$ mol L^{-1} (*3*) roundup; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 2.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

The dependences of the reciprocal reaction rate on the reciprocal concentration of the NT electron acceptor (with the NADH concentration remaining constant) are shown in Figs 8 and 9. The herbicides lontrel and zenkor (see Fig. 8, Table 3) also inhibit the rate of electron transfer from the NADH-OR active center to NT. The inhibition pattern is uncompetitive (K_i are equal to $7.42 \cdot 10^{-4}$ and $8.94 \cdot 10^{-4}$ mol L^{-1}, respectively). The lontrel complex with the copper ion exhibits noncompetitive inhibition, while the complex with cobalt exerts mixed inhibition (see Fig. 9, Table 4).

The Michaelis constants (K_m) calculated without inhibitors are $6.6 \cdot 10^{-4}$ and $2.47 \cdot 10^{-3}$ mol L^{-1} for NADH and NT, respectively. It was determined in the preliminary experiment that all compounds under study reversibly inhibit NADH-OR.

The data on the effect of other herbicides, fungicides, and lontrel metal complexes on the rate of NADH oxidation and the rate of NT reduction with NADH-oxidoreductase are presented in Table 3.

Of all the compounds studied, the highest inhibitory activities were found for zenkor and basagran (I_{50} are $5.0 \cdot 10^{-4}$ and $6.0 \cdot 10^{-4}$ mol L^{-1}, respectively). Lontrel, roundup, tachigaren, and tilt inhibit NADH-OR somewhat less efficient (I_{50} are $1.1 \cdot 10^{-3}$, $1.7 \cdot 10^{-3}$, $2.7 \cdot 10^{-3}$, and $2.2 \cdot 10^{-3}$ mol L^{-1}, respectively, see Table 3). Kusagard and setoxidim exhibit weak antireductase activities; they depress the enzyme activity when are present in higher concentrations: $2.7 \cdot 10^{-2}$ and $1.7 \cdot$

10^{-2} mol L^{-1}, respectively. In terms of the K_i values with respect to NADH, the herbicides and fungicides can be arranged in the following activity sequence: zenkor > lontrel > basagran > > kusagard > tachigaren > roundup > tilt > setoxidim. This sequence is similar to the sequence of complexation constants of these compounds with NADH given in Table 2.

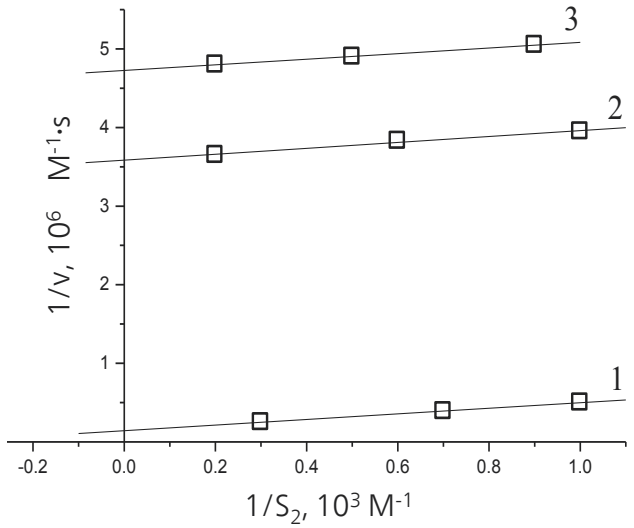

Figure 8. Inhibition of the NADH-oxidoreductase with zenkor (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $3.33 \cdot 10^{-4}$ (2) and $5.00 \cdot 10^{-4}$ mol L^{-1} (3) of zenkor; $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NT} = 7.2 \cdot 10^{-5}$ - $6.4 \cdot 10^{-3}$ mol L^{-1}.

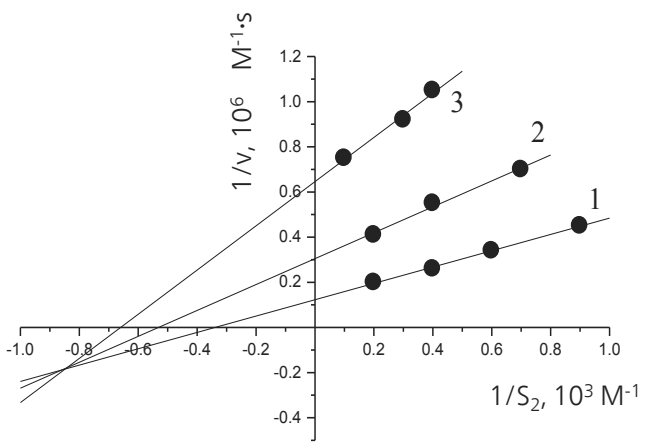

Figure 9. Inhibition of the NADH-oxidoreductase with zenkor (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $3.33 \cdot 10^{-4}$ (2) and $5.00 \cdot 10^{-4}$ mol L^{-1} (3) of zenkor; $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NT} = 7.2 \cdot 10^{-5}$ - $6.4 \cdot 10^{-3}$ mol L^{-1}.

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Lontrel, zenkor, basagran, and roundup inhibit the reduction of NT in a uncompetitive manner, apparently, due to nonspecific interaction with the protein matrix outside the enzyme active center. This interaction could induce conformational changes around the electron transfer site, which result in the inhibition of enzymatic activity. Meanwhile, kusagard, setoxidim, tilt, and tachigaren compete with NT for the binding region on the enzyme. These differences can be due to different structures of the pesticides examined.

Inhibitor – solt	S_2, 10^{-4} M	K_i, 10^{-4} M^{-1}	Type *	h	Inhibitor – $M(L)_2$	
					K_i, 10^{-4} M^{-1}	Type *
$Cu(CH_3COO)_2$	13.2	0.7	A	1.0	4.0	C
$(NH_4)_6Mo_7{\cdot}O_{24}$	6.6	4.4	A	1.0	0.4	A
$Co(CH_3COO)_2$	8.8	0.02	A	1.0	13.1	D
$Fe(C_3H_9COO)_2$	4.4	4.1	A	1.0	11.7	C
$Ni(CH_3COO)_2$	14.7	0.7	A	1.0	11.7	C
$Mg(CH_3COO)_2$		no inhibition			3.6	A
$MgSO_4$		no inhibition				
$Zn(CH_3COO)_2$		no inhibition			2.5	D
$ZnSO_4$		no inhibition				
$Mn(CH_3COO)_2$		no inhibition			22.3	D
$MnSO_4$		no inhibition				

In the absence of an inhibitor $V_{max} = 2.8{\cdot}10^{-6}$ mol L^{-1} s^{-1}; $S_2 = 3.3{\cdot}10^{-4}$ mol L^{-1}.

* The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 4. Kinetic parameters of inhibition of NADH-oxidoreductase at the artifical electron acceptor NT.

The metal complexes of lontrel are known (Saratovskikh et al., 1988; Saratovskikh et al., 1990) to exhibit herbicide activities *in vivo*. In addition, as noted above, the complex formed by the herbicide lontrel with the copper ion exhibits a much higher inhibitory activity than the starting lontrel. Therefore, we carried out an additional study of a series of complexes of these pesticides with different doubly charged metal ions, $M(L)_2$, and the salts of these metals.

As can be seen from the experimental kinetic curves of the dependence of the oxidation rate of NADH-OR on the NADH concentration at a constant NT concentration presented in Fig. 10, the addition of $Ni(ac)_2$ in the concentrations from $3.3{\cdot}10^{-6}$ to $5{\cdot}10^{-4}$ M inhibits oxidoreductase functioning and decreases the rate of formation of the reaction product formazan, and at a considerable inhibitor concentration the reaction rate can decrease to zero. Similar studies were carried out with other salts. The dependences of the rate of the enzymatic reaction on the concentration of salt of the metal-inhibitor can be determined from the obtained curves (Fig. 11). It is seen that Mg(II) does not inhibit NADH-OR even at the highest of the concentrations studied, namely, 10^{-2} M. The Zn(II) and Mn(II) salts also exerted no inhibitory effect, and the replacement of the SO_4^{2-} anion by $(CH_3COO)_2^{2-}$ does not change the character of the process.

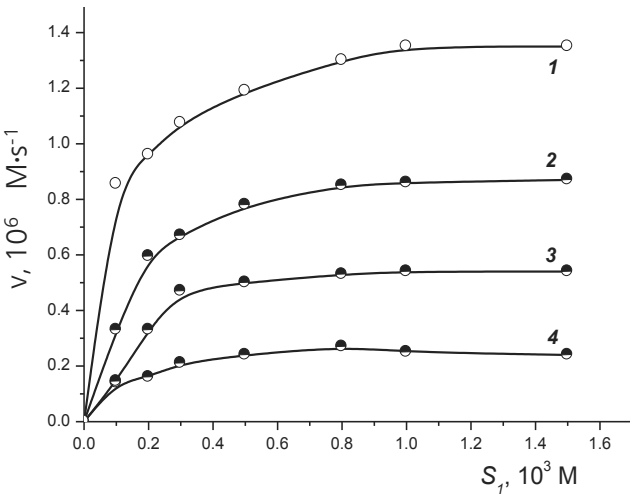

Figure 10. Kinetic curves of the dependence of the oxidation rate of NADH-oxidoreductase on the NADH concentration at a constant concentration of NT. C_{NT} = 6.68·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.0·10^{-4} – 1.5·10^{-3} M; (1) entry without inhibitors; in the presence of Ni(ac)$_2$ in the concentration (2) 3.3·10^{-6} M; (3) 3.0·10^{-5} M, and (4) 1.7·10^{-4} M.

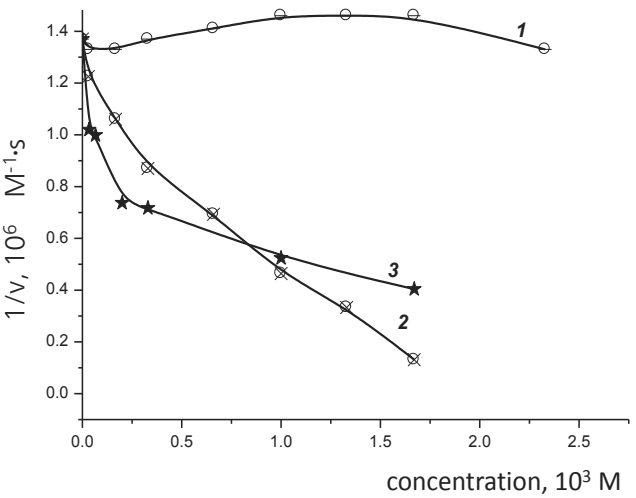

Figure 11. Dependences of the enzymatic reaction rate on the concentration of the metal salts: (1) Mg(ac)$_2$; (2) Mo(am)$_6$; (3) Fe(acac)$_2$. C_{NT} = 6.68·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.0·10^{-4}–1.5·10^{-3} M.

The dependences of the reciprocal rate of the reductase reaction on the inverse concentration of the artificial electron accetor (NT) at a fixed NADH concentration for Fe(acac)$_2$ are presented in Fig. 12. The considered metal salts inhibit the reduction of NT, competing with the artificial electron acceptor for the binding region with NADH-OR. As follows from Table 4, the corresponding complexes, except for Mo(L)$_2$, do not compete with the electron acceptor.

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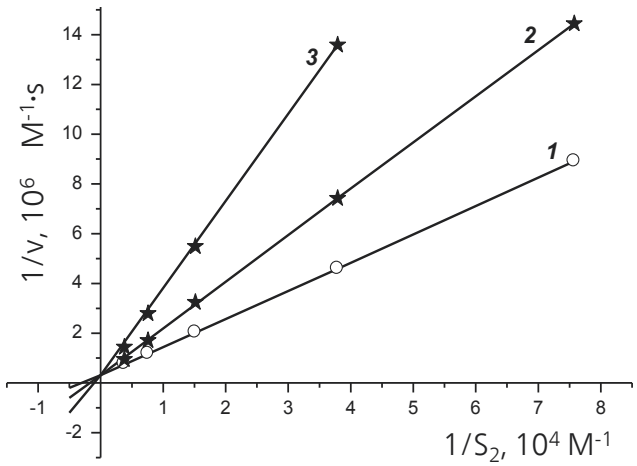

Figure 12. Dependences of the reciprocal inhibition reaction rate of NADH-oxidoreductase by Fe(acac)$_2$ on the inverse NT concentration at a constant concentration of NADH (in the Lineweaver–Burk coordinates); C_{NT} = 1.0·10^{-5}–7.0·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.43·10^{-3} M; (1) without an inhibitor; in the presence of Fe(acac)$_2$ in the concentration (2) 3.0·10^{-4} M and (3) 5.0·10^{-4} M.

When determining the influence of the metal salts on the reduction of NT, the maximum values for the inhibition constants were found for Mo(VI) and Fe(II): K_i = 4.4·10^{-4} M^{-1} and 4.1·10^{-4} M^{-1}, respectively. The minimum value K_i = 2.0·10^{-6} M^{-1} was calculated for Co(II). The inhibition constants of Cu(L)$_2$, Co(L)$_2$, Fe(L)$_2$, and Ni(L)$_2$ are one to three orders of magnitude higher than those for the corresponding salts. On the contrary, Mo(L)$_2$ showed K_i an order of magnitude lower than that of Mo(II). The phenomena of the appearance of or increase in the inhibitory effect were also observed for the metal complexes with other organic ligands (Tatjanenko et al., 1985).

The following series can be arranged for an increase in the values of K_i with the change in the NT concentration for the metal salts: Co(II) < Cu(II) ~ Ni(II) < Fe(II) ~ Mo(VI). A similar regularity is observed for the lontrel complexes in the activity sequence by the values of K_i: Mo(L)$_2$ < Zn(L)$_2$ < Mg(L)$_2$ < Cu(L)$_2$ < Ni(L)$_2$ = Fe(L)$_2$ < Co(L)$_2$ < Mn(L)$_2$; the electron-donor properties of the metal exert a strong effect on the strength of the bond between the inhibitor and enzyme.

The results of the influence of (ML$_2$) on the enzymatic activity of NADH-OR are presented in Table 4. On going from the salt to complexes, the type of inhibition changes completely: from the same competitive type for all metal salts to several different variants, namely, mixed for Co(L)$_2$ and uncompetitive for Ni(L)$_2$, Fe(L)$_2$, and Cu(L)$_2$. Only Mo(VI) and Mo(L)$_2$ retain one, competitive, type of inhibition. The Mg(II), Zn(II), and Mn(II) ions do not inhibit NADH-OR, whereas Mg(L)$_2$ compete with an electron acceptor for the binding site on the enzyme and Zn(L)$_2$ and Mn(L)$_2$ have the mixed type of inhibition with respect to both NT and NADH. Interestingly, as shown above, Mg(L)$_2$, Zn(L)$_2$, and Mn(L)$_2$, in turn, do not interact with NADH. The formation constants of the M(L)$_2$–NADH complexes ($K_{c/f}$: Fe < Mo < (Co) < Cu < Ni) show a direct correlation with the inhibition constants of NADH-OR by an electron donor for both the complexes and metal salts K_{iM+}: Co < Ni < Cu < Mo < Fe (Table 5).

Inhibitor – solt	I_{50}, M	V_{max}, Mc^{-1}	S_1, 10^{-4} M	K_i, 10^4 M^{-1}	Type*	Inhibitor – $M(L)_2$		
						I_{50}, M	K_i, 10^{-4} M^{-1}	Type*
$Cu(CH_3COO)_2$	$3.3 \cdot 10^{-5}$	$1.1 \cdot 10^{-6}$	3.9	1.2	D	$3.3 \cdot 10^{-4}$	0.06	A
$(NH_4)_6Mo_7 \cdot O_{24}$	$3.3 \cdot 10^{-4}$	$1.4 \cdot 10^{-6}$	3.0	8.8	D	$8.5 \cdot 10^{-4}$	0.1	A
$Co(CH_3COO)_2$	$1.3 \cdot 10^{-6}$	$9.7 \cdot 10^{-7}$	1.2	0.01	B	$1.5 \cdot 10^{-3}$	13.7	C
$Fe(C_3H_9COO)_2$	$3.3 \cdot 10^{-4}$		4.6	14.2	A	$1.1 \cdot 10^{-3}$	1.1	A
$Ni(CH_3COO)_2$	$3.3 \cdot 10^{-5}$	$7.7 \cdot 10^{-7}$	3.2	0.9	D	$2.0 \cdot 10^{-3}$	12.4	C
$Mg(CH_3COO)_2$	no inhibition					$2.0 \cdot 10^{-3}$	12.7	C
$MgSO_4$	no inhibition							
$Zn(CH_3COO)_2$	no inhibition					$1.0 \cdot 10^{-3}$	10.2	C
$ZnSO_4$	no inhibition							
$Mn(CH_3COO)_2$	no inhibition					$3.0 \cdot 10^{-3}$	3.8	A
$MnSO_4$	no inhibition							

In the absence of an inhibitor $V_{max} = 2.8 \cdot 10^{-6}$ mol L^{-1} s^{-1}; $S_2 = 3.3 \cdot 10^{-4}$ mol L^{-1}.

* The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 5. Kinetic parameters of inhibition of NADH-oxidoreductase at the substrate NADH.

Among the lontrel complexes with metal ions, only the complexes with Mg and Mo proved to be competitive reductase inhibitors with respect to NT. The lontrel complexes with Cu, Ni, and Fe ions inhibit the enzyme noncompetitively, while the lontrel complexes with Mn, Zn, and Co display a mixed type of inhibition.

Table 5 demonstrates the kinetic parameters of inhibition of NADH-oxidoreductase with respect to the substrate NADH. The highest inhibition ability is shown by Co(II): $I_{50} = 1.33 \cdot 10^{-6}$ M. The copper and nickel salts exhibits the equal values: $I_{50} = 3.3 \cdot 10^{-5}$ M. The weak antireductase activity was demonstrated by Fe(II) and Mo(VI), namely, $I_{50} = 3.3 \cdot 10^{-4}$ M. The metal salts can be arranged in the following series by the calculated values of I_{50}: $Co(ac)_2 < Ni(ac)_2 = Cu(ac)_2 < Fe(acac)_2 = Mo(am)_6$.

The inhibitory effect of the complexes ZnL_2, CoL_2, FeL_2, and MoL_2 is similar to that of the parent lontrel (I_{50} are $1.0 \cdot 10^{-3}$, $1.5 \cdot 10^{-3}$, $1.1 \cdot 10^{-3}$, and $0.85 \cdot 10^{-3}$ mol L^{-1}, respectively), while the inhibitory effects of MgL_2, MnL_2, and NiL_2 are much lower.

The Mg, Zn, Co, and Ni complexes with lontrel exhibit noncompetitive inhibition with respect to NADH, whereas Mn, Cu, Fe, and Mo complexes inhibit the enzyme competitively. The difference between the inhibition patterns may be related to the difference between the acceptor abilities of the metal ions.

A comparison of the data presented in Tables 4 and 5 shows that the metal salts inhibit the enzyme in much lower concentrations. The value of I_{50} of a salt is one to three orders of magnitude lower than that of the complexes of the corresponding metal. The maximum difference in the values of I_{50} is observed for Co(II) and $Co(L)_2$: $1.33 \cdot 10^{-6}$ and $1.5 \cdot 10^{-3}$ M, respectively. The minimum difference is characteristic of molybdenum: Mo(VI) $I_{50} = 3.3 \cdot 10^{-4}$

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M, $Mo(L)_2$ I_{50} = $8.5 \cdot 10^{-4}$ M; *i.e.*, the antireductase activity of the metal ion is threefold higher than that of the corresponding metal complex with lontrel. The order of increasing I_{50} (*i.e.*, decreasing the antireductase activity) in the case of the complexes $Cu(L)_2 < Mo(L)_2 < Zn(L)_2 < Fe(L)_2 = L < Co(L)_2 < Ni(L)_2 = Mg(L)_2 < Mn(L)_2$ is reciprocal to that presented above for the salts of the same metals.

The highest of the calculated inhibition constants with respect to the electron donor (NADH) belongs to Fe(II) and Mo(VI), and the values of K_i are$14.2 \cdot 10^{-4}$ M^{-1} and $8.8 \cdot 10^{-4}$ M^{-1}, respectively. The lowest value ($K_i = 1.4 \cdot 10^{-6}$ M^{-1}) was determined for Co(II).

The character of inhibition of NADH-OR with copper acetate at different substrate (NADH) concentrations at a fixed concentration of NT is shown in Fig. 13. The intersection of the straight lines in one point but not in the axis indicates that the inhibition follows the so-called mixed type. The same character of inhibition was demonstrated by $Mo(am)_6$ and $Ni(ac)_2$. The $Cu(L)_2$, $Mo(L)_2$, and $Fe(L)_2$ complexes compete with NADH for the binding site on NADH-OR (Table 5). The strength of the bond between $Cu(L)_2$ and NADH-OR is nearly 20 times higher than that of Cu(II); $K_i = 6.0 \cdot 10^{-6}$ M^{-1} and $1.2 \cdot 10^{-4}$ M^{-1}, respectively. A similar situation was observed for molybdenum and iron and their complexes.

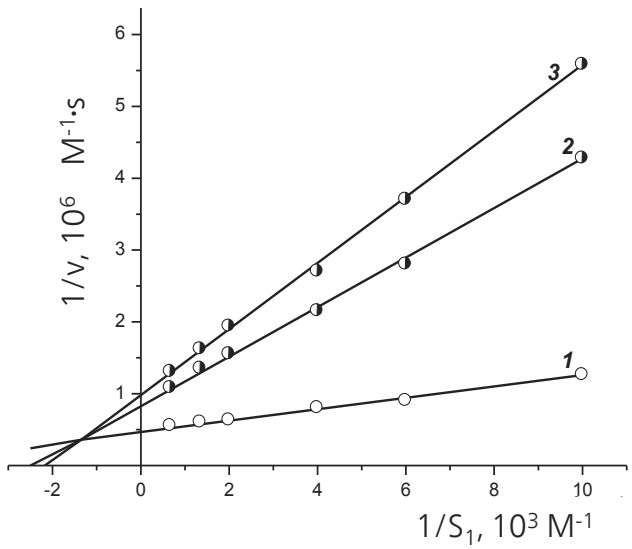

Figure 13. Dependences of the reciprocal inhibition reaction rate of NADH-oxidoreductase by $Cu(ac)_2$ on the inverse NT concentration at a constant concentration of NT (in the Lineweaver–Burk coordinates). $C_{NT} = 6.68$ 10^{-3} M; $C_{enzyme} = 2.83 \cdot 10^{-7}$ M; $C_{NADH} = 1.0 \cdot 10^{-4}$–$1.5 \cdot 10^{-3}$ M; (1) without an inhibitor; in the presence of $Cu(ac)_2$ in the concentration (2) $3.0 \cdot 10^{-5}$ M and (3) $1.7 \cdot 10^{-4}$M.

On the contrary, the strength of the bond between $Ni(L)_2$ and NADH-OR decreases: $K_i = 0.9 \cdot 10^{-4}$ M^{-1} for Ni(II) and $K_i = 12.4 \cdot 10^{-4}$ M^{-1} for $Ni(L)_2$. The inhibition constant increases by two orders of magnitude on going from the salt to the cobalt complex: $0.014 \cdot 10^{-4}$ and $3.8 \cdot 10^{-4}$ M^{-1}, respectively. However, $Co(ac)_2$ inhibits oxidoreductase manifesting the noncompetitive

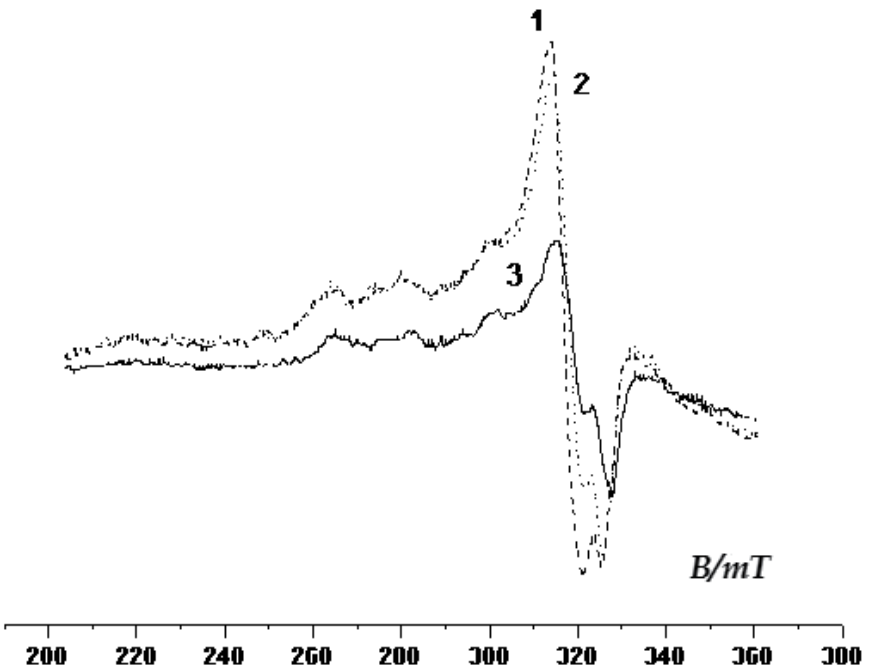

Figure 14. EPR of the metal complex (1) CuL_2 = $5.0 \cdot 10^{-4}$ mol L^{-1}; (2) CuL_2 =$5.0 \cdot 10^{-4}$ mol L^{-1} in the presence of NADH = $6.0 \cdot 10^{-4}$ mol L^{-1}; (3) CuL_2 = $5.0 \cdot 10^{-4}$ mol L^{-1} in the presence of NADH = $6.0 \cdot 10^{-4}$ mol L^{-1} and enzyme. L is lontrel. The conditions of measurements: 77 K, microwave 10 mW, magnetic field modulation 0.4 mT.

character of inhibition for NADH. At the same time, the $Co(L)_2$ complex does not compete with NADH for the binding site on the enzyme.

Of the compounds considered, only $Fe(acac)_2$ competes with an electron acceptor for the binding site in the active center of the enzyme. The same character of inhibition is retained in the iron complex with respect to NADH, but K_i for $Fe(L)_2$ is 14 times lower than that for Fe(II): $1.1 \cdot 10^{-4}$ and $14.2 \cdot 10^{-4}$ M^{-1}, respectively.

The change in the pattern of inhibition by the lontrel complexes with doubly charged metal ions may be due to the fact that the interaction of these complexes with the protein involves other protein ligands (thiol groups, the imidazole part of histidine, and other amino acid residues of the peptide chain). In addition, the metal ions in these complexes can be reduced by the enzyme, as it was shown by ESR for the lontrel complexes with copper ions (Fig. 14).

Our results indicate that the herbicides, fungicides, and lontrel metal complexes can react with NADH-OR in the cavity of the protein matrix in which either NADH binding or electron transfer to a natural or artificial electron acceptor takes place. An additional interaction of these compounds beyond the enzyme active center cannot also be dismissed. The structure, size, and spatial configuration of the pesticide molecule are also significant. Apparently, the large size of kusagard and sedoxidim molecules prevents them from entering the cavity of the protein globule, which may account for the weak inhibition of NADH-OR by these com-

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pounds. Apparently, the combination of these factors is responsible for the different mechanisms of NADH-OR inhibition by the considered compounds.

The Hill factor (*h*) is nearly equal or close to 2 for all of the compounds, which indicates that two inhibitor molecules can be attached simultaneously to the enzyme both inside and probably outside the active center (Table 3). This parameter substantially distinguishes pesticides and metal complexes from metal salts. The Hill factor for the considered metal salts is close to unit (Table 5), indicating the possibility of the addition of only one metal cation to the enzyme.

Evidently, the metal cation tends to negatively charged groups of amino acids, namely, carboxyl, sulfide, and amide groups. In addition, Mn, Mg, and Zn for the least stable complexes with carbonyl, and Ni and Fe form the strongest complexes with this group (Holtzclaw & Collman, 1957; Isatt et al., 1954), which agrees with our data. Thus, the interaction of this kind near the active center of the enzyme can result in conformational changes in the region of electron transfer, which is manifested as noncompetitive and mixed types of inhibition. It is known (Tsuprun et al., 1987; Fitzpatrick et al., 2005) that the 2Fe-2S cluster is in the composition of the active center of NADH-OR. The change in the character of inhibition on going from divalent metal salts to their lontrel complexes can additionally be due to the interaction of the complexes not only with the protein matrix but also with the metal of the 2Fe-2S cluster.

Figure 15 represents the scheme of direction of the inhibitor attack. The intramolecular electron transfer in the active center of NADH-OR proceeds from flavine adenine dinucleotide (FAD) to the iron-sulfur cluster 2Fe-2S and further to an artificial electron acceptor (Bayer et al., 1996; Du et al., 2000b; Ganson & Jensen, 1988). For competitive inhibition, the cation occupies the site of NT and this breaks the chain of electron transfer. The fact that the metal salts have much lower I_{50} compared to the complexes indicates easiness of this interaction. The determining factor in the behavior of the metal salts is the structure of the electronic shells of the cation.

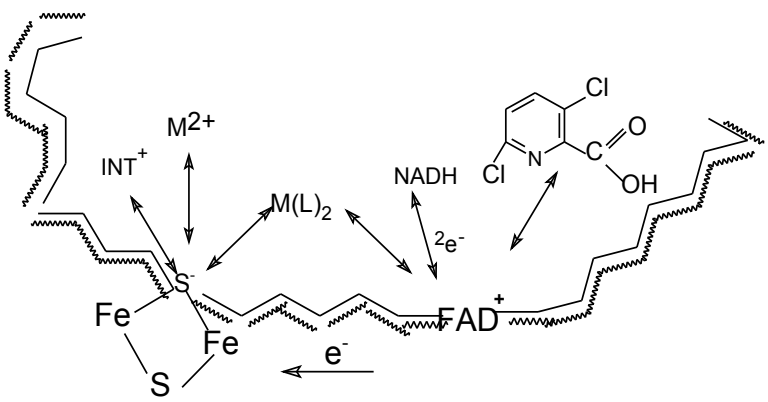
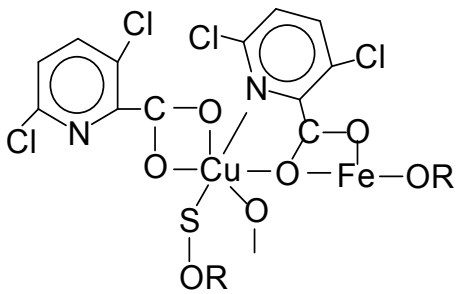


Figure 15. Scheme of direction of the attack from different inhibitors.

Present in the composition of the complex, the metal cannot act as a free cation, since it is significantly affected by the ligand environment. The considered ligand (lontrel) is capable of occupying the site of NADH, donor of two electrons, in the active center of the enzyme. Probably, the interaction with iron of the cluster occurs through the carboxyl of the ligand and due to a high electron density of chloropyridine. As a result, the ligand or complex inhibits the NADH-binding region of the electron-transfer chain.



We have previously reported (Aliev et al., 1988) that the lontrel complexes can exist in solution in both the dimeric and polymeric forms. The structures of the complexes allow them to play the role of both the electron-donor and electron-acceptor. It can be assumed that the complexes form polymer chains in which the ligands acts as a "bridge" and the metal of the complex pulls electrons of the 2Fe-2S cluster of the active center of the enzyme. The existence of two binding sites in the active center likely explains the fact that K_i of the complex (in the case of Mo, with respect to the electron acceptor; in the case of Cu, Mo, and Fe, with respect to the electron donor) is one to two orders of magnitude lower than that of the salt.

Obviously, the evaluated compounds, herbicides, fungicides, and metal complexes of herbicide lontrel, can retard oxidation processes in plants and living organisms. However, the effect of these compounds on various components of the ecosystem is manifested in a diverse character of reactions of the organisms, in the multiphase character of these reactions, and in the possible transition of one effect to another. To analyze the influence of the studied compounds on the living organisms, we chose hydrobionts – marine "luminescent" bacteria *Benechea harveyi* (strain B 1 7 – 667F), (Zhmur & Orlova, 2007; Kuz`mich et al., 2002). It is validly considered (Tsvetkov & Konichev, 2006) that hydrobionts are the most appropriate objects for the study of biochemical adaptations to the toxic action and, as a consequence, they are the most popular laboratory test-objects and object-indicators for calibrations of contaminations under natural conditions. On the other hand, when the effect of chemical toxicants on biological systems is studied, the time of the toxicological analysis itself compared to the rate of formation of metabolites in the living organism is significant. Bacteria *Beneckea harveyi* allow researchers to obtain a fast response and to compare their effect on the change in the enzymatic (luciferase) activity. It is important that the inactivation of only one enzyme is controlled *in vitro* on a model system.

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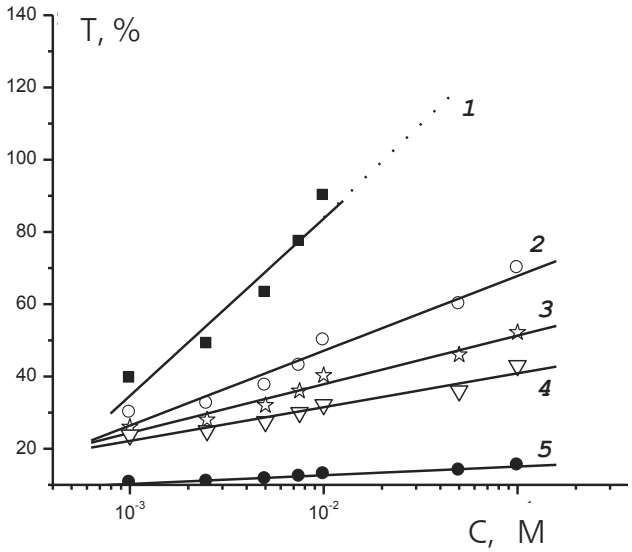

Figure 16. Change in the toxicity of the pesticides towards "luminescent" bacteria *Beneckea harveyi vs.* concentration of the pesticide in the concentration range from 10^{-1} to 10^{-3} M. (1) zenkor; (2) lontrel; (3) roundup; (4) basagran; (5) tachigaren.

The results of measurements of toxicity of herbicides and fungicide tachigaren with respect to luminescent bacteria *Beneckea harveyi* are presented in Fig. 16. The plots show that the toxicity of solutions increases proportionally to the concentration with an increase in the pesticide concentration. However, the rates of toxicity increase differ for different substances: the rate of zenkor is considerably higher than those of other compounds, whereas for tachigaren the toxicity coefficient (T, %) increases with the lowest rate. Zenkor has the highest toxicity of all the compounds evaluated. Lontrel, roundup, and basagran differ from each other to a lower extent. The lowest toxicity was determined to be tachigaren. In fact, even at the highest of the studied concentrations, 10^{-1} M, tachigaren remains to be a nontoxic compound with T < 19%. In the concentration range from $3 \cdot 10^{-3}$ to 10^{-1} M basagran exhibits a weak toxicity: 24 < T ≤ 40%. In the concentration range from 10^{-3} to $3 \cdot 10^{-3}$ M, all compounds (except zenkor) are lowly toxic with T ≤ 50%. Beginning from the concentrations 10^{-3} M (zenkor), 10^{-2} M (lontrel), and 10^{-1} M (roundup), they become highly toxic compounds.

The results of measurements of pesticide toxicity are presented in Table 6. Parameter EC_{50} corresponds to the toxicant concentration resulting in the 50% decrease in the luminescence of bacteria. The values of EC_{50} increase in the order zenkor < lontrel < roundup < basagran < tachigaren. An analysis of the data in Table 6 shows that the pesticides are arranged in the decrease in toxicity in the same sequence, which is retained at all concentrations studied.

It was discussed previously that the metal complexes of herbicide lontrel are characterized by a considerable antireductase activity. However, there are no literature data on the quantitative estimation of their toxicity towards hydrobionts. The results of the study of the influence on *Beneckea harveyi* are given in Fig. 17. It is seen that all the metal complexes are toxic even at a

concentration of 10^{-7} M. The toxicity increases linearly with an increase in the concentration for all complexes. Curves 1–4 corresponding to the complexes of different metals are parallel, indicating the same rate of toxicity increasing on the concentration of ML_2. At all concentrations the toxicity coefficients of the complexes decrease in the series $CuL_2 > CoL_2 > MnL_2 > MgL_2$. As follows from Table 6, EC_{50} measured by the "probit analysis" method (Loshadkin et al., 2002) change in the same order: $CuL_2 > CoL_2 > NiL_2 > MoL_2 > MnL_2 > ZnL_2 > L > MgL_2$. It should especially be mentioned that the toxicity of almost all lontrel complexes with respect to *Beneckea harveyi* turned out higher than that of the starting herbicide. The value of EC_{50} for CuL_2 is more than two orders of magnitude lower and that for ZnL_2 is four orders of magnitude lower than that for lontrel. The exclusion is the MgL_2 complex, whose toxicity is insignificantly lower than that of the starting lontrel.

№	Pesticide	EC_{50}		ML_2	EC_{50}	
		M	g/l		M	g/l
1.	Zenkor	$(4.4\pm0.1)\cdot10^{-3}$	0.94	CuL_2	$(1.3\pm0.1)\cdot10^{-5}$	0.0058
2.	Lontrel	$(8.0\pm0.3)\cdot10^{-3}$	1.54	CoL_2	$(3.0\pm0.2)\cdot10^{-4}$	0.13
3.	Roundup	$(2.0\pm0.1)\cdot10^{-2}$	3.38	NiL_2	$(5.0\pm0.2)\cdot10^{-4}$	0.22
4.	Basagran	$(2.9\pm0.1)\cdot10^{-2}$	7.01	MoL_2	$(7.0\pm0.3)\cdot10^{-4}$	0.33
5.	Tachigaren	$(1.0\pm0.2)\cdot10^{-1}$	9.91	MnL_2	$(1.6\pm0.1)\cdot10^{-3}$	0.69
6.				ZnL_2	$(2.0\pm0.1)\cdot10^{-3}$	0.89
7.				MgL_2	$(1.0\pm0.1)\cdot10^{-2}$	4.06

Table 6. Toxicity values (EC_{50}) for the pesticides and lontrel metal complexes (ML_2) measured by the *Beneckea harveyi* biotest.

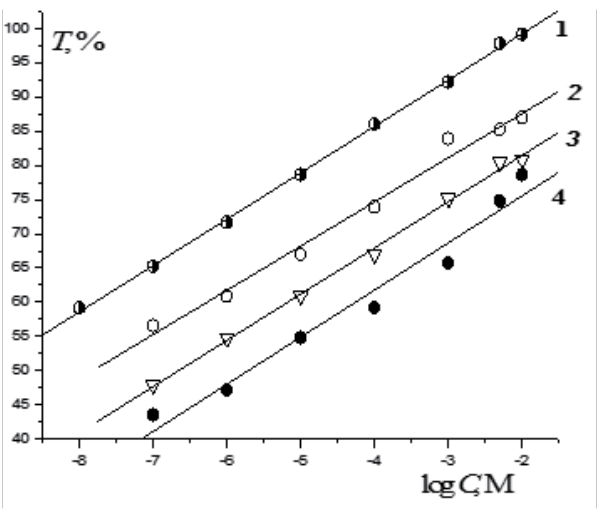

Figure 17. Change in the toxicity index determined on "luminescent" bacteria *Beneckea harveyi vs.* logarithm of the concentration of the metal complexes: (1) CuL_2; (2) CoL_2; (3) MnL_2; (4) MgL_2.

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A comparison of the data in Table 6 shows that metal complexe toxicity is manifested at concentrations two orders of magnitude lower than that of any pesticide considered. Similarly, the EC_{50} parameters of the pesticides and metal complexes indicate thatthe values of toxicity of the latter are two orders of magnitude higher than that of all pesticides and, particularly, herbicide lontrel. In the range of the concentrations studied, both the pesticides and the complexes of seven metals are arranged in the same sequence by toxicity decreasing.

The study performed of the toxicity of herbicides, fungicides, and metal complexes of lontrel towards bacteria *Beneckea harveyi* showed high toxicity. This fact indicates the antiluciferase activity of these compounds. The determined values of EC_{50} correlate with both the complexation constants (K) of these compounds with NADH and inhibition constants (K_i) of enzyme NADH-OR by these compounds.

4. Conclusion

Thus, despite the substantial differences in the chemical structures, all of the herbicides, fungicides and lontrel metal complexes studied inhibit NADH-OR at both the electron-donor and the electron-acceptor sites. These compounds inhibit the NADH-binding region and, perhaps, the intramolecular electron transfer from FAD to the 2Fe-2S iron-sulfur cluster and further to an artificial electron acceptor. This conclusion is consistent with the published data on the interruption of the electron transfer chain (Tissut et al., 1984; Macherel et al., 1982; Higgins et al., 1981) by pesticides and the involvement of metals in this process (Bayer et al., 1996; Du et al., 2000b; Ganson & Jensen 1988). The character of inhibition changes on going from the metal salts to their complexes: all the metal salts compete with an electron acceptor for the binding site, and the complexes compete with an electron donor. It is very important that in some cases, of Mo, Cu, and Fe, the strength of the bond with the enzyme increases on going from the metal ion to the ligand and to the complex ($M^{+2} < L < ML_2$), which should result in an increase in the toxic properties of the complexes compared to the metals and pesticides.

The enzyme NADH-OR is abundant in nature and is found in both unicellular and multicellular organisms; therefore, broad-scale practical use of herbicides and fungicides may entail their accumulation in living organisms and severe environmental consequences.

Evidently, one of the mechanisms of formation of toxicity of herbicides seems to be inhibition of redox processes in organisms of different trophic levels. In birds and mammals (including people), the inhibition of the oxidative enzymes decreases the protective functions of the organism and results in various maladies. Moreover, almost all well understood diseases of modern man are caused by a poor ecological state of the environment (Gichev, 2003; Klyushnikov, 2005; Mogush, 1984; Isaev, 1997; Lisichkin & Chernov, 2003).

Several thousands of pesticides are produced in the world. About 180 pesticides are widely used (Mel'nikov, 1987). Maximum allowable concentrations are substantiated only for 30 of them (Fomin, 2000). The assertion about an exclusive importance of their use for the enhancement of agricultural productivity is not substantiated (Yablokov, 1990; Fisher et al., 2002;

Yudanova, 1989; Khan, 1980; Moses, 1988; Paasivitra, 1988). The use of pesticides is an example for gaining a short-term profit of chemical companies owing to the long-term detriment for all society (Skurlatov et al., 1994; Yablokov, 1990; Suley & Uilcoks, 1983; Kurdyukov, 1982).

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References

[1] Aliev, Z.G; Atovmyan, L.O.; Saratovskikh, E.A.; Krinichnyi, V.I. & Kartsev, V.G. (1988). Synthesis, structure, and spectral characteristics of copper-complexes with picolinic-acid derivatives. *Izv. Acad. Nauk SSSR, Ser.: Khim.,* No. 11, pp. 2495-2502.

[2] Al-Mendofi, O. & Ashton, F.M. (1984). Bentazon influence on selected metabolic processes of isolated bean leaf cells. *J. Plant. Growth. Regul.* Vol. 3, No. 2, pp. 121–126.

[3] Amrhein, N.; Deus, B.; Gehrke, P. & Steinrücken, H.C. (1980). The site of the inhibition of the shikimate pathway by glyphosate. II. Interference of glyphosate with chorismate formation *in vivo* and *in vitro. Plant Physiol*. Vol. 66, No. 5, pp. 830-834.

[4] Bagirov, R.M.; Stukan, R.A.; Gvozdev, R.I.; Korshunova, L.A.; Knizhnik, A.Zh.; & Akentreva, N.R. (1989). The study of the active centre of the reductase component membrane-related methane monoxigenase of *Methylococcus-capsulatus* (strain M) gamma-resonance spectroscopy. *Biofizika*, Vol. 34, pp. 949-952.

[5] Banas, A.; Banas, W.; Stenlid,G. & Stymne, S. (2000). Selective increase in acyl hydrolase activity by graminicides in whea. *Biochem. Soc. Trans*., Vol. 28, No. 6, pp. 777-779.

[6] Bayer, M.; Walter, K. & Simon, H. (1996). Purification and partial characterisation of a reversible artificial mediator accepting NADH oxidoreductase from *Clostridium thermoaceticum. Eur. J. Biochem*., Vol. 239, No. 3, pp. 686-691.

[7] Blagoyi, Yu.P.; Galkin, V.L. & Gladchenko, G.O. (1991). Metal complexes of nucleic acids in solutions, Naukova dumka, Kiev. 272 p.

[8] Burbaev, D.Sh.; Moroz, I.A.; Gvozdev, R.I. & Korshunova, L.A. (1990). Binuclear iron-sulphur centers membrane-related methane monoxigenase of *Methylococus capsulatus. Biofizika*, Vol. 35, pp. 779-782.

[9] Cornish-Bowden, A. (1976). *Principles of Enzyme Kinetics*, Butterworths, L.–Boston. 280 p.

[10] Dixon, M. & Webb, E.C. (1979). *Enzymes*. Longman Group Ltd., L.–N. Y.–Toronto. 810 p.

[11] Dorval, J.; Leblond, V.S. & Hontela, A. (2003). Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochlorine pesticide. *Aquat. toxicol*. Vol. 63. No. 3, pp. 229-241.

[12] Du, W.; Wallis, N.G.; Mazzulla, M.J.; Chalker, A.F.; Zhang, L.; Liu, W.S.; Kallender, H. & Payne, D.J. (2000). Characterization of Streptococcus pneumoniae 5-enolpyruvyl-shikimate 3-phosphate synthase and its activation by univalent cations. *Eur. J. Biochem*., Vol. 267, No. 1, pp. 222-227.

[13] Du, W.; Wallis, N.G. & Payne, D.J. (2000). The kinetic mechanism of 5-enolpyruvylshikimate-3-phosphate synthase from a gram-positive pathogen *Streptococcus pneumonia. J. Enzyme Inhib*., Vol. 15, No. 6, pp. 571-581.

[14] Egaas, E.; Sandvik, M.; Fjeld, E.; Kallqvist, T.; Goksoyr, A. & Svensen, A. (1999). Some effects of the fungicide propiconazole on cytochrome P450 and glutathione S-transferase in brown trout (Salmo trutta). *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol*., Vol. 122, No. 2, No. 3, pp. 337-344.

[15] Emanuel, N.M. & Knorre, D.G. (1969). *The rate of chemical kinetics*. Vysshaya shkola, Moscow. p. 260.

[16] Fedtke, C. (1982). *Biochemistry and Physiology of Herbicide Action*, Springer-Verlag, Berlin-Heidelberg-New York. 22 p.

[17] Fisher, G.; Velthuizen, H.,; Shah, M. & Nachtergaele, F.O. (2002). *Global Agro-ecological Assessment for Agriculture in the 21st Century: Methodology and Results*. Elsevier, Berlin. 120 p.

[18] Fitzpatrick, P.F.; Orville, A.M.,; Nagpal, A. & Valley, M.P. (2005). Nitroalkane oxidase, a carbanion-forming flavoprotein homologous to acyl-CoA dehydrogenase. *Arch. Biochem. Biophys*., Vol. 433, pp. 157-165.

[19] Fomin, G.S. (Ed.) (2000). *Water. Control of chemical, bacterial and radiation safety according to international standards: An encyclopedic reference book.* Protector, Moscow. 848 p.

[20] Forthoffer, N.; Helvig, C.; Dillon, N.; Benveniste, I.; Zimmerlin, A.; Tardif, F. & Salaun, J.P. (2001). Induction and inactivation of a cytochrome P450 confering herbicide resistance in wheat seedlings. *Europ. J. Drug. Metab. Pharmacokinet*., Vol. 26, No. 1-2, pp. 9-16.

[21] Fudel-Osipova, V.I. (Ed.) (1981). *Fiziologo-biologicheskyi mekhanizm deistviya pestitsidov* [*Physi-ological and Biological Mechanism of Pesticide Action*]. Naukova dumka, Kiev, 256 p.

[22] Ganson, R.J. & Jensen, R.A. (1988). The essential role of cobalt in the inhibition of the cytosolic isozyme of 3-deoxy-*arabino*-heptulosonate-7-phosphate synthase from *Nicotiana silvestris* by glyphosate. Arh. Biochem. Biophys. Vol. 260, No. 1, pp. 85-93.

[23] Gichev, Yu.P. (2003). *Environmental Contamination and Ecological Stipulations of Human Pathology:* Analytical Review, (Ser. Ecology, Issue 68), GPNTB SO RAS, Novosibirsk.

[24] Grin, D. & Goldberger, R. (1968). *Molecular aspects of life*. Mir, Moscow. 400 p.

[25] Gruenhagen, R.D. & Moreland, D.E. (1971). Effect of herbicides on ATP levels in excised soybean hypocotyls. *Weeds,* Vol. 19, No. 4, pp. 319–323.

[26] Gruys, K.J.; Marzabadi, M.R.; Pansegrau, P.D. & Sikorski, J.A. (1993). Steady-state kinetic evaluation of the reverse reaction for *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase. *Arch. Biochem. Biophys*., Vol. 304, No. 2, pp. 345-351.

[27] Hall, J.C.; Bassi, P.K.; Spencer, M.S. & Vanden Born, W.H. (1985). An evaluation of the role of ethylene in herbicidal injury induced by picloram and clopyralid in rapeseed and sunflower plants. *Plant Physiol*., Vol. 79, pp. 18–23.

[28] Higgins, J.J.; Best, D.J.; Hammond, R.S. & Scott, D. (1981). Methaneoxidizing microorganisms. *Microbiol. Res.,* Vol. 45, pp. 556-590.

[29] Holtzclaw, H.F. & Co1lman, J.P. (1957). Infrared absorption of metal chelate compounds of 1,3-diketones. *J. Amer. Chem. Soc.,* Vol. 79, No. 13, pp. 3318-3322.

[30] Isaev L.K. (Ed.) (1997). *Impact on the human body hazardous and harmful environmental factors. Metrological aspects.* Book 1, Tom 1-2. PAIMS, Moscow.

[31] Isatt, R.M.; Haas, C.G.; Block, B.P. & Ferne1ius, W.C. (1954). Studies on coordinat1on compounds. XII. Calculation of thermodynamic formation constants at varying ionic strengths. *J. Phys. Chem*., Vol. 58, No. 12, pp. 1133-1136.

[32] Iwataki, I. & Hirono, Y. (1978). The chemical structure and herbicidal activity of alloxidim-Na and related compounds. In: Abstr. Vol. Fifth Int. Union for Pure and Appl. Chem. (IUPAC) Congr. No. 11-12, Zürich.

[33] Kadyshev, Ch. Sh. (1970). *Gerbicidy i fungicidy kak antimetabolity ingibitory fermentnykh sistem* [*Gerbicides and Fungicides asAntimetabolites and Inhibitors of Enzyme Systems*], Fan, Tashkent, 83 p.

[34] Khan, S. (1980). *Pesticides in the environmental*. Elsevier, Amsterdam, Oxford. 240 p.

[35] Kiyomiya, K.; Matsushita, N.; Matsuo, S. & Kurebe, M. (2000). Cephaloridine-induced inhibition of cytochrome C oxidase activity in the mitochondria of cultured renal epithelial cells (LLC-PK(1)) as a possible mechanism of its nephrotoxicity. *Toxicol. Appl. Pharmacol*., Vol. 167, No. 2, pp. 151-156.

[36] Klyushnikov, V.Yu. (2005). Human Organism as an Indicator for Environmental Contamination. *Sensor*, No. 3, pp. 26-33.

[37] Knecht, W. & Löffler, M. (1998). Species-related inhibition of human and rat dihydroorotate dehydrogenase by immunosuppressive isoxazol and cinchoninic acide derivatives. *Biochemical pharmacology,* Vol. 56. Issue 9, pp. 1259-1264.

[38] Kurdyukov, V.V. (1982). *Aftereffect of pesticides in plant and animal organisms*. Kolos, Moscow. 128 p.

[39] Kuz`mich, V.N.; Sokolova, S.A. & Krainiukova, A.N. (Eds.) (2002). *Guidelines on the definition of the methods of biotesting of the toxicity of water, bottom sediments, pollutants and drilling solutions*. REFIA, NIA-Priroda, Moscow. 118 p.

[40] Kuz`minskaya, U.A. (1975). *Biochemical characterization of subcellular structures of the liver under the influence of pesticides. (The mechanism of action of organochlorine and urea pesticide.)*: Aftoref. ... d. med. sciense. Kiev. 42 p.

[41] Levine, S.L. & Oris, J.T. (1999). Enhancement of Acute Parathion Toxicity to Fathead Minnows Following Pre-exposure to Propiconazole. *Pesticide Biochemistry and Physiology,* Vol. 65, Issue 2, pp. 102-109.

[42] Lichina, M.V.; Shugalyi, A.V.; Gvozdev, R.I. & Todorov, I.N. (1979). Modification of DNA dibromètilacetatom. I. Interaction with the free grounds and with the grounds available for reaction in the structure of the DNA molecule. *Bioorganic chemistry*, Vol. 5, No. 5, pp. 664-670.

[43] Lichina, M.V.; Shugalyi, A.V.; Chernyi, D.I. & Todorov, I.N. (1978). Modification of DNA dibromethile-acetate: about possibilities of use in the analysis of the peculiarities of the structures. Dokl. AN SSSR. Vol. 243, No. 2, pp. 516-519.

[44] Lisichkin G.V. & Chernov N.N. (Eds.) (2003). *Man and the environment of his dwelling.* Mir, Moscow. 460 p.

[45] Loshadkikn, N.A.; Gladkikh, V.D.; Goldenkov, V.A.; Siniczin, A.N.; Darina, L.V. & Bulanova, L.P. (2002). Probit method in the assessment of the effects of physiologically active substances at low levels of impacts. *Ras. Chem. J*., Vol. 46, No. 6, pp.63-67.

[46] Lycholat, Y. & Bilchuk, V. (1998). Lawn grass enzyme activity regulation by metals under air pollution. *Toxicol. Lett*., Vol. 95, No. 1001, pp. 234.

[47] Luisi, P.L.; Baici, A.; Bonner, J.F. & Aboderin, A.A. (1975). Relationship between fluorescence and conformation of NAD^+ bound to dehydrogenases. *Biochem*., Vol. 14, No. 2, pp. 362–370.

[48] Macherel, D.; Ravanel, P. & Tissut, M. (1982). Affes of bicetal carbamates on mitochondrie and chloroplast. *Pestic. Biochem. and Physiol.,* Vol. 18, No. 3, pp. 280-288.

[49] Mathew, R.; Kacew, S. & Khan, S.U. (1998). Bioavailability in rats of bound pesticide residues from tolerant or susceptible varieties of soybean and canola treated with metribuzin or atrazine. *Chemosphere*, Vol. 36, No. 3, pp. 588–596.

[50] Mel`nikov, N.N. (1987). *Pesticides. Chemistry, Technology, and Use*. Khimiya, Moscow. 712 p.

[51] Mogush G. (1984). *Acute poisoning, diagnosis, treatment*. Bucharest. 579 p.

[52] Moses, M.M.D. (1988). *Monitoring the international code of conduct of the distribution and use of pesticides on North America. The Pesticide Education and Action Project, USA*. PAN International, New York. 324 p.

[53] Nosanchuk, J.D.; Ovalle, R. & Casadevall, A. (2001). Glyphosate inhibits melanization of *Cryptococcus neoformans* and prolongs survival of mice after systemic infection. *J. Infect. Dis*., Vol. 183, No. 7, pp. 1093-1099.

[54] Osama, Al-M. & Ashton, F. M. (1984). Bentazon influence on selected metabolic processes of isolated bean leaf cells. *J. Plant Growth Regul*., Vol. 3, No. 2, pp. 121–126.

[55] Paasivitra, J. (1988). Organochlorine compounds in the environment. *Water Sci. Technol*., Vol. 20, No. 2, pp. 119-129.

[56] Pryanishnikov, D.N. (1934). Agricultural chemistry. Selkhozisdat. Moscow-Leningrad. 399 p.

[57] Saratovskikh, E.A. (1989). Synthesis of bidentate complexes of 3,6-dichloropicolinic acid. *Russian Chemical Bulletin*, Vol. 38, No. 10, pp. 2140-2141.

[58] Saratovskikh, E.A.; Kondrat'eva, T.A.; Psikha, B.L.; Gvozdev, R.I. & Kartsev, V.G. (1988). Complex-formation of some pesticides with adenosine triphosphoric acid. *Izv. Akad. Nauk SSSR, Ser.: Khim.*, No. 11, pp. 2501–2506.

[59] Saratovskikh, E.A.; Korshunova, L.A.; Gvozdev, R.I. & Kulikov, A.V. (2005). Inhibition of the nicotinamide adenine dinucleotide-oxidoreductase reaction by herbicides and fungicides of various structures. *Rus. Chem. Bull*., Int. Ed Vol. 54, No. 5, pp. 1322-1326.

[60] Saratovskikh, E.A.; Korshunova, L.A.; Roshchupkina, O.S. & Skurlatov, Yu.I. (2007). Inhibition of NADH-oxidoreductases by metal compounds. *Rus. J. physical chem. B*. Vol. 26, No. 8, pp. 46-53.

[61] Saratovskikh, E.A.; Papina, R.I. & Kartsev, V.G. (1990). Binding of ATP by pesticides as a possible primary mechanism of inhibition of seed germination and plants growth. *Sel'skokhoz. Biol*., No. 5, pp.152-159.

[62] Shcheglov, Yu.V.; Sokolov, M.C. & Kasihin, A.N. (1967). Herbicidal activity and the synthesis of piclorame and some other derivatives of 2-picoline. *Agrochemistry,* No. 5, pp. 105–111.

[63] Skurlatov, Yu.I.; Duka, G.G. & Miziti, A. (1994). *Vvedenie vekologicheskuyu khimiyu* (Introduction in Ecological Chemistry), Vysshaya shkola, Moscow. 400 p.

[64] Sommerhalter, M.; Lieberman, R.L. & Rosenzweig, C. (2004). X-ray crystallography and biological metal centers: is seeing believing. *Inorg. Chem*., Vol. 20, No. 20, pp. A-I.

[65] Suley, M. & Uilcoks, B. (Eds). (1983). *Biology of nature protection*. Mir, Moscow. 430 p.

[66] Tatjanenko, L.V.; Moshkovsky, Yu.Sh.; Zakharova, I.A.; Ponticelli, G.; Massacesi, M. & Devoto, G. (1985). Inhibitorial effect of the Cu(II) and Ni(II) complexes with polyamines and imidazole derivatives on the Ca, Mg- dependent ATP-ase substrate. *Polyhedron*. Vol. 4, No. 1, pp. 159-162.

[67] Tissut, M.; Ravanel, P. & Macherel, D. (1984). Study of some natural and synthetic compounds inhibiting the electron transfer in plant mitochondria [amides, carbamates, flavonoides, phenols; herbicides]. *Physoil. Veg*., Vol. 22, No. 5, pp. 607-614.

[68] Trebst, A. & Wietoska, H. (1975). Hemmung des photosynthetischen elektronentransports von chloroplasten durch metribuzin. *Z. Naturforsch. C*., Vol. 30, No. 7/8, pp. 499-504.

[69] Tukhvatullin, I.A.; Gvozdev, R.I. & Andersson, K.K. (2001). Chemistry of natural compounds, bioorganic and biomolecular chemistry. Structural and functional model of methane hydroxylase of membrane-bound methane monooxygenase from *Methylococcus capsulatus* (Bath). *Rus. Chem. Bull*., Vol. 50, No. 10, pp. 1867-1876.

[70] Tsuprun, V.L.; Akentreva, N.P.; Tagunova, I.V.; Orlova, E.V.; Grigoryan, A.N.; Gvozdev R.I. & Kiselev, N.A. (1987). Electron microscopy methane-monoxygenase methane-oxidizing bacteria *Methylococcus capsulatus*. *Dokl. Akad. Nauk SSSR*, Vol. 292, No. 2, pp. 490-493.

[71] Tsvetkov, I.L. & Konichev, A.S. (2006). *Ecological biochemistry of hydrobionts*. MGOU, Moscow. 104 p.

[72] Ugarova, N.N. & Lebedeva, O.V. (1978). The structure and functions of horseradish peroxidase. *Biocemistry,* Vol. 43, No. 10, pp. 1731-1742.

[73] Varo, I.; Navarro, J.C.; Amat, F. & Guilhermino, L. (2003). Effect of dichlorvos on cholinesterase activity of the European sea bass (*Dicentrarchus labrax*). *Pest. Biochem. Physiol*., Vol. 75, No. 3, pp. 61-72.

[74] Yablokov, A.V. (1990). *Yadovitaya priprava* (Poisonous Relish), Mysl', Moscow. 125 p.

[75] Yudanova, L.A. (1989). *Pestitsidy v okruzhayushchei srede* (Pesticides in the Environment), GPNTB SO AN SSSR, Novosibirsk. 140 p.

[76] Ziegler, W. (1982). Ionenkanäle in planaren bimolekularen lipidmembranen, erzeugt durch das herbizid sencor 7OWP. *Biológia* (ČSSR), Vol. 37, No. 11, pp. 1071-1077.

[77] Zhmur, N.S. & Orlova, T.L. (2007). *Methodology to determine the toxicity of the water, aqueous extracts of soil, sewage sludge and wastes on the changing levels of chlorophyll fluorescence and the number of algal cells.* Biological methods of control. The Federal register FR.1.39.2007.03223. AKVAROS, Moscow. 48 p.

Chapter 8

Involvement of Lignin-Modifying Enzymes in the Degradation of Herbicides

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Additional information is available at the end of the chapter

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1. Introduction

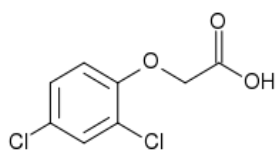
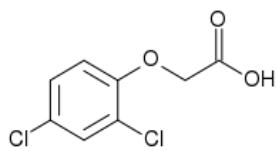
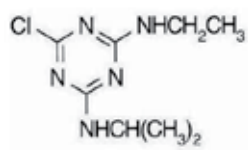
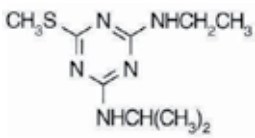
The high demand for food due to the increase in the world population has led to an increasing use of plant protection products, also known as pesticides, in order to improve productivity. However, along with the success in food production, the accumulation of these persistent chemicals in soil and water is harmful to the environmental and human health [1]. In recent years, agricultural pesticide application has increased all over the world. A total of 5,197 mil lbs of pesticides were used worldwide in 2007 [2]. There are many different types of pesticides; each is meant to be effective against specific pests. The term "-cide" comes from the Latin word "to kill." Among them, herbicides account for the largest market (around 40%) share, followed by insecticides (17%) and fungicides (10%). Herbicides are chemicals used to kill undesired plants, such as weeds, and they are used extensively in home gardens and in agriculture. Due to the relative predominance of herbicides, the present review will focus on this class of compounds.

Herbicides have well defined pros and cons associated with their use. Their use tends to increase yields, and thus makes a significant difference in food production, particularly in countries that struggle periodically against famines. On the other hand, they can cause water pollution when erosion and/or rainwater carry the chemicals off the farms together with the eroded soils after each rainfall. Herbicides vary in their potential to persist in the soil. They are chemically heterogeneous and their structure is one of the main features that determine persistence. For example, some substitutions on aromatic rings (-F, -Cl, -NO_2, -NH_2, -CF_3, -

SO_3H) [1] in picloram, atrazine, flazasulfuron and other herbicides (Table 1), have a marked influence on persistence. Furthermore, water insoluble molecules are less susceptible to microbial attack because they may sorb tightly to particles and thus stay less available [3].

The herbicide persistence in soil is also affected by the properties of soil such as organic matter content, moisture, pH, microbial activity, among other particularities. Generally, soils with high contents in organic matter have low loss of chemicals through volatilization as a consequence of a stimulated adsorption to soil particles [3]. On the other hand, herbicides that sorb tightly to soil particles have low mobility which decreases the possibility of groundwater contamination. The organic matter also serves as an energy source for microorganisms that degrade pesticides. Furthermore, the soil pH may affect the adsorption processes, changing the mobility and bioavailability of the herbicide [1].

The presence of native microorganisms (fungi, bacteria, protozoans, etc) can greatly influence the toxicity and persistence of herbicides. The degradation process by native microorganisms produces less toxic products and it is probably the most important pathway for the breakdown of herbicides. However, microorganisms require special environmental conditions such as adequate temperature, oxygen and nutrient supply for growth and herbicide degradation [1].

Chemical group	Common name	Water solubility and persistence	Chemical structure
Phenoxy acetic	2,4 D	900 ppm 1 month	
	2,4,5 T	278 ppm 1 month	
Triazines	Atrazine	33 ppm (22°C) 5-7 months	
	Ametryne	185 ppm (20°C) 3-6 months	

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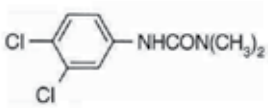
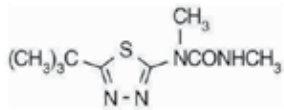
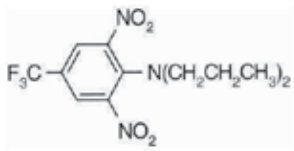
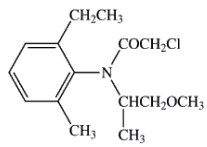
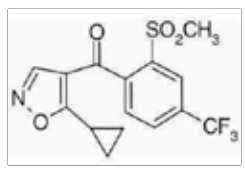
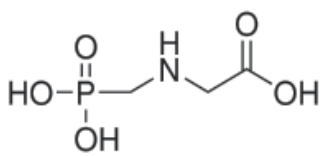
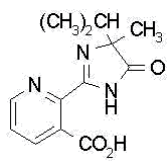
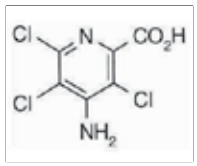
Chemical group	Common name	Water solubility and persistence	Chemical structure
Ureas	Diuron	42 ppm 4-8 months	
	tebuthiuron	2,500 ppm 12-15 months	
dinitroanilines	Trifluralin	0.3 ppm 6 months	
acetanilides	metolachlor	488 ppm (20°C) 50 days	
isoxalone	isoxaflutole	6 ppm (20°C) 38 days	
Glycine derivative	Glyphosate	15,700 ppm 30-90 days	
imidazolinones	Imazapyr	11,272 ppm 3-6 months	

Chemical group	Common name	Water solubility and persistence	Chemical structure
Picolinic acid	Picloram	Soluble 3 months	

* Water solubility at 25°C. ** Soil average persistence at recommended rates [4]

Table 1. Chemical properties of the most common herbicides

2. General bioremediation processes

The impact of hazardous herbicide residues on the environment has led to the necessity of finding feasible technologies to remediate these sites. The conventional remediation methods use physical and chemical processes, such as incineration, adsorption on resins and UV irradiation. These methods generally generate excellent contaminant removal; however, from an ecological viewpoint, they are not friendly since they produce unwanted by-products and hazardous residues, besides the danger of human exposure to contaminants.

An innovative technology for complementing or substituting the conventional methods and which presents the same or even improved efficiency is bioremediation. By definition, bioremediation is the use of biological processes to clean up polluted sites. Such biological methods have the potential of being less expensive and more eco-friendly than the physical and chemical treatments [5]. Bioremediation is based on biological systems such as living organisms (bacteria, fungi and plant) and enzymes. They are effective systems to treat a polluted site because they are able to modify the chemical structure of the contaminant into less hazardous end-products [5]. Among these possibilities, microorganisms have become the most attractive option in bioremediation strategies [1].

The naturally occurring bacteria, fungi or plants in a contaminated site are commonly used in bioremediation but microorganisms may be isolated elsewhere and inoculated into the site with the goal of accelerating the remediation. This process is called bioaugmentation [6]. Other terms are commonly used in bioremediation studies. When a chemical compound is broken down into water and CO_2, the biodegradation is completed and the process is called mineralization. On the other hand, the term biotransformation usually refers to the process whereby a molecule is converted into a different molecule which is not always less toxic than the precursor. Another term usually used is bioaccumulation or biosorption. This process does not involve any transformation of the molecule and refers to the concentrative transfer of substances into living or dead biomass. The microorganisms can still transform the molecule but are unable to grow on it, in other words, the compound is not used as an energy source [7].

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Bioremediation can be developed by two main methods: in situ and ex situ. In situ methods treat the materials in place, while ex situ methods are based on excavation or removal of the polluted materials for processing in another place, such as in bioreactors and biopiles, where optimal environmental conditions for rapid biodegradation may be provided. The in situ bioremediation is more advantageous because it avoids the transport of hazardous materials and is more cost-effective. On the other hand, this process can be limited in some cases since the remediation is not effective in low-permeability soils [1].

3. Herbicide bioremediation studies

Bacteria and fungi are the main microorganisms capable of degrading herbicides. Most bioremediation studies have been focused on bacteria. Among those, several species of *Pseudomonas* have been found to degrade a wide range of herbicide groups [8-10]. Generally, the bacteria strains utilize the herbicide as the sole carbon and/or nitrogen source. In order to metabolize pollutants, bacteria must take the molecule inside the cell where the enzymes are located [11].

Although bacteria are the main agents used in most commercial bioremediation processes, filamentous fungi have received much attention in recent years, especially white rot fungi (WRF) or ligninolytic fungi [12]. Most WRF belong to the Basidiomycete group and comprise the organisms that are capable of degrading lignin, a polymer found in wood [13]. These fungi have been extensively investigated since the mid-1980s as bioremediation agents because of some particular characteristics. First, as filamentous fungi, WRF present extensive branching and mycelial growth, hence they are able to spread out in the environment reaching the pollutants more efficiently than bacteria. Second, they possess non-specific and radical-based enzymatic systems for degrading lignin. These oxidative enzymes allow them to degrade a broad range of pollutants, including low-solubility compounds, without the necessity of incorporating them into the cells. Third, WRF prefers to grow on substrates such as agricultural crop wastes (straw, corn cobs, sawdust, peanut shells) which are inexpensive and may be used as additional nutrient sources [5,14]. In this context, the primary focus of this review is on the main white-rot species which have been studied for herbicide transformation.

4. White-rot fungi and their lignin-modifying enzymes

White-rot fungi are known as the most efficient lignin degraders. Lignin is a polymer found in wood and vascular tissues [15]. In the cell wall, lignin is covalently associated with carbohydrate components (cellulose and hemicelluloses) forming a matrix that protects the cell wall against microbial attack [16]. Unlike most natural polymers, lignin is irregular and non-repeating. Its biosynthesis is the result of oxidative polymerization of several phenylpropanoid precursors, such as coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol. The polymerization occurs at random by various carbon-carbon and ether bonds resulting in an irregular structure (Fig. 1)

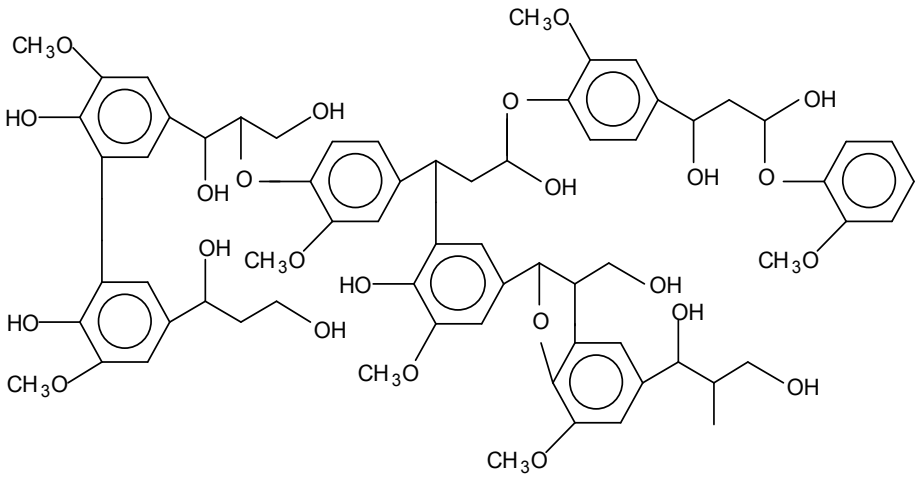

Figure 1. General structure of lignin

Lignin resists attacks by most microorganisms; anaerobic processes tend not to attack the aromatic rings at all and the aerobic breakdown of lignin is slow. In nature, only basidiomyceteous white-rot fungi (WRF) are able to degrade lignin efficiently. Wood decayed by white-rot fungi is pale in color because of oxidative bleaching and loss of lignin and often retains a fibrous texture.

In order to become the most effective wood degraders in nature, WRF developed non-specific and radical-based mechanisms for degrading lignin. WRF variously secrete one or more of four extracellular enzymes that are involved not only in the lignin degradation but also in the degradation of several pollutants. The four major lignin-modifying enzymes (LMEs) are: lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) and laccase (Lac, EC 1.10.3.2) [17]. All these enzymes act via generation of free radicals which represent an efficient way to reach their substrates.

The LMEs are highly non-specific and, thus, capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. White-rot fungi usually secrete one or more of the LMEs in different combinations. Distribution of white-rot fungi into groups according to their enzymatic systems have been undertaken [18-20]. General classification is based on the capacity of different fungi to produce one or a combination of three ligninolytic enzymes: LiP, laccase and MnP. However, the absence or non-detection of these enzymes in some white-rot fungi, the sequencing of white-rot fungal genomes and the discovery of new enzymes has led to the distinction of different groups [21]. Generally, white-rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP and VP):

a. Laccase, MnP and LiP (*Trametes versicolor, Bjerkandera adusta*)

b. laccase and at least one of the peroxidases (*Lentinus edodes, Pleurotus eryngii, Ceriporiopsis subvermispora*)

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c. only laccase (*Schizophyllum commune*)

d. only peroxidases (*Phanerochaete chrysosporium*)

The most frequently observed LMEs among the white-rot fungi species are laccases and MnP and the least are LiP and VP.

A considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published in past years [22-24]. A general description of the mechanisms and functions of the main ligninolytic enzymes of the white-rot fungi is given in Panel 1.

Enzyme: LACCASE (EC 1.10.3.2)

Active site/prosthetic group: four copper atoms.

Catalytic mechanism: catalysis of the one electron oxidation of phenolics, aromatic amines and other aromatic hydrogen donors with simultaneous reduction of O_2 to H_2O.

General reaction: 4 benzenediol + O_2 → 4 benzosemiquinone + 2 H_2O

Mediators: 3-HAA, ABTS, HBT, RBB

Function: Removal of polyphenols, degradation of pesticides and textile dyes, bleaching, removal of lignin, synthesis of melanin, formation of basidiomes, generation of hydroxyl radicals, stress defense

Main laccase producer fungi: *Ganoderma lucidum, Pleurotus eryngii, Pleurotus ostreatus, Rigidoporus lignosus, Trametes versicolor*

Enzyme: LIGNIN PEROXIDASE (LiP, EC 1.11.1.14)

Active site/prosthetic group: Heme (Iron protoporphyrin IX)

Catalytic mechanism: oxidation of lignin side chains by one electron abstraction in the presence of H_2O_2, generating reactive radicals. Cleavage of C-C and ether bonds in lignin. Cleavage of aromatic rings.

General reaction: 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 → 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O

Mediators: VA, 2Cl-14DMB

Function: Degrades lignin (non-phenolic lignin units), mineralizes recalcitrant aromatic compounds.

Main LiP producer fungi: *Bjerkandera adusta, Phanerochaete chrysosporium, Trametes cervina, Trametes versicolor*

Enzyme: MANGANESE PEROXIDASE (MnP, EC 1.11.1.13)

Active site/prosthetic group: Heme (Iron protoporphyrin IX)

Catalytic mechanism: Oxidize Mn^{2+} into Mn^{3+}. Mn^{3+} acts as a diffusible oxidizer on phenolic or non-phenolic lignin units and amino-aromatic compounds

General reaction: 2 Mn^{2+} + 2 H^+ + H_2O_2 → 2 Mn^{3+} + 2 H_2O

Mediators: Chelated Mn^{3+}

Co-oxidants: unsaturated fatty acids and thiols

Function: Oxidation and depolymerization of lignin, degradation of dyes, phenols and other recalcitrant compounds

Main MnP producer fungi: *Ceriporiopsis subvermispora, Irpex lacteus, Lentinus edodes, Pleurotus ostreatus, Trametes versicolor*

Enzyme: VERSATILE PEROXIDASE (VP, EC 1.11.1.16)

Active site/prosthetic group: Heme (Iron protoporphyrin IX). Hybrid molecular architecture with multiple binding sites

Catalytic mechanism: Combination of the catalytic properties of LiP and MnP; Oxidation of Mn^{2+} to Mn^{3+} and also phenolic and nonphenolic aromatic compounds.

General reaction: donor + H_2O_2 → oxidized donor + 2 H_2O

Mediator: Chelated Mn^{3+}

Function: Oxidizes phenols, hydroquinones, dyes, amines, aromatic alcohols and xenobiotics.

Main VP producer fungi: *Bjerkandera adusta, Pleurotus eryngii*

3-HAA (3-Hydroxyanthranilic acid), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)), HBT (1-hydroxybenzotriazole), RBB (Remazol brilliant blue), VA (veratryl alcohol), 2Cl-14DMB (2-Chloro-1,4-dimethoxybenzene).

Panel 1. Lignin-modifying enzymes of the white-rot fungi [17]

The LMEs may function cooperatively or separately from each other, however, auxiliary enzymes (unable to degrade lignin on their own) are necessary to complete the process of lignin and/or xenobiotics degradation: aryl alcohol oxidase (AAO, EC 1.1.3.7), aryl alcohol dehydrogenase (AAD, EC 1.1.1.90), glyoxal oxidase (GLOX, EC 1.2.3.5), quinone reductase (QR, EC 1.1.5.1), cellobiose dehydrogenase (CDH, EC 1.1.99.18), superoxide dismutase (SOD, EC 1.15.1.1), glucose 1-oxidase (GOX, EC 1.1.3.4), pyranose 2-oxidase (P2Ox, EC 1.1.3.4) and methanol oxidase (EC 1.1.3.13). These are mostly oxidases generating H_2O_2 and dehydrogenases. Cytochrome P450 monooxygenases are also significant components involved in the degradation of lignin and many xenobiotics.

Recent additions to the enzymatic system of white-rot fungi include dye-decolorizing peroxidases or DyP involved in the oxidation of synthetic high redox-potential dyes and nonphenolic lignin model compounds [30] and aromatic peroxygenases (APOs) that catalyze diverse oxygen transfer reactions which can result in the cleavage of ethers [31-32].

Other important components of the lignin degradative system of white-rot fungi are low molecular mass oxidants such as hydroxyl radicals (•OH) and chelated Mn^{3+} (Mn^{3+} mainly chelated by oxalic acid), produced through the action of the ligninolytic enzymes. These are particularly important during the early stages of wood decay and can also act on the degradation of xenobiotics [24,33-35].

5. Environmental applications of ligninolytic enzymes

Ligninolytic enzymes are highly non-specific and, thus, capable of degrading a wide range of recalcitrant compounds structurally similar to lignin. In recent years, the capability of white-rot fungi and their enzymes to biodegrade several xenobiotics and recalcitrant pollutants has generated considerable research interest in the area of industrial/environmental microbiology. As a consequence, a considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published [22-27,36-40]. Among the industrial applications of the ligninolytic enzymes found in WRF are: a). paper whitening, b). degradation of industrial dyes from the textile industry and c). degradation of xenobiotic

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compounds, including polycyclic aromatic hydrocarbons, phenolics, herbicides and other pesticides. In this review, we focused on the degradation of herbicides by white-rot fungi with emphasis on work done in the last decade.

6. Isolation and laboratory maintenance of white-rot fungi

There are about 10,000 species of white-rot fungi in the world. However, not more than 3 dozen have been more properly studied. Among these, the majority of studies have focused on *Phanerochaete chrysosporium*, followed by *Trametes versicolor, Bjerkandera adusta* and *Pleurotus sp* [40]. White-rot fungi have great potential for biotechnological applications. For this reason, there has been a growing interest in screening for new white-rot fungi species and strains. A classical strategy is to collect basidioma or mycelia of white-rot fungi in forests, dead trees, and lignocellulosic crop residues showing signs of attack by fungi. Samples of basidioma or mycelia are aseptically transferred onto potato dextrose agar or malt extract agar and sub-cultured until the obtainment of pure mycelia. Identification is based on morphological, physiological, biochemical and genetic characteristics of the basidioma, hyphae and spores. More recently, molecular biology techniques are being used in the identification of new isolates. Advancements in molecular methods have permitted a more rational study of the phylogenetic relationships within the various organisms. Non-coding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA seem to be one of the most frequently employed analytical tools [41].

There are several options for the maintenance of white-rot fungi in the laboratory. Continuous growth methods of preservation in which white-rot fungi are grown on agar (e.g., malt extract agar, potato dextrose agar and yeast extract agar) are typically used for short-term storage. For long-term storage, preservation in distilled water (method of Castellani [42]), mineral oil and anhydrous silica gel are some of the indicated methods. These are low cost methods but none is considered permanent. Lyophilization and liquid nitrogen refrigeration (cryo-preservation) are expensive methods but are considered permanent.

7. Herbicide biotransformation and detoxification by white-rot fungi

Phanerochaete chrysosporium is the main white-rot fungus used in herbicide biodegradation studies. The lignin-degrading system of *P. chrysosporium* comprises the peroxidases LiP and MnP. Studies have demonstrated that these enzymes are responsible, at least in part, for the transformation of several persistent pollutant molecules including toxic herbicides [43]. The biotransformation of triazine by *P. chrysosporium* was studied in liquid cultures [44]. The herbicide atrazine was partly transformed into polar dechlorinated and/or N-dealkylated metabolites. The removal of the ethyl side chain was the preferred reaction and produced metabolites which were not further transformed by the fungus. They found that the N-dealkylation was supported by the mycelium and not by purified peroxidases. Thus, the authors suggested that cytochrome-P450 could have been involved in the degradation.

Using non-sterile substrates, *P. chrysosporium* demonstrated a satisfactory growth and significant effect on the degradation of several herbicides such as simazine and trifluralin [45], bentazon and 2-methyl-4-chlorophenoxyacetic acid (MCPA) [46-47]. Thus, the systems based on non-sterile substrates offer good perspectives of practical application since the preparation of large amounts of sterile substrates is expensive.

The herbicide diuron has also been degraded by *P. chrysosporium* in several culture conditions. Higher degradation efficiency (95%) was found in ashwood culture as compared to the liquid culture (75%) [48]. The authors suggested that MnP could be responsible for the degradation since it was the dominant enzyme detected in the cultures. In liquid medium, the degradation performance of *P. chrysosporium* is not very good when compared with other white-rot species in the same experiments. Within 7 days the depletion of diuron, linuron and propanil was not higher than 34% [49]. In another work, no *Phanerochaete* strain was able to degrade more than 42% of the herbicides chlortoluron, isoproturon and diuron during 5 days [50]. However, *P. chrysosporium* was more efficient than *Trametes versicolor* in cleaving diketonitrile, which is derived from the herbicide isoxaflotole [51]. Enzyme preparations derived from these organisms, namely LiP and MnP, were unable to catalyze the transformation even in the presence of mediators.

Solid state cultures (SSC) seem to be great systems for cultivation of ligninolytic fungi as well as for herbicide degradation. Isoproturon [52] and bentazon [46-47] were extensively degraded by *P. chrysosporium* in wheat straw cultures. In vitro tests suggest the action of LiP and MnP in the degradation of both herbicides although the cytochrome P450 activity can not be ruled out. For isoproturon, non-identified hydroxylated metabolites were formed in SSC as well as in pure enzyme tests. Furthermore, a second degradation reaction seems to occur with isoproturon since N-demethylated metabolites were also formed in both systems.

Bjerkandera adusta is one of the most promising strains of white-rot fungi for bioremediation. This fungus possesses high ligninolytic activity and produces several extracellular lignin-degrading enzymes, including LiP, MnP and laccase [53]. Also, it is one of the few species to produce VP [54]. *B. adusta* has demonstrated a great ability to degrade pollutant molecules, especially polycyclic aromatic hydrocarbons [55] and synthetic dyes [56-57]. The removal of the herbicides chlortoluron, isoproturon and diuron by *B. adusta* and other fungi was reported in shaking liquid cultures [50], *B. adusta* was the most efficient WRF able to degrade the three herbicides. Removal of chlortoluron, diuron and isoproturon reached 98%, 92% and 88%, respectively, after two weeks. On the other hand, it was reported that *B. adusta* was able to transform efficiently the herbicide linuron but to lower extents than propanil and diuron [49]. The authors suggested that the low depletion of some molecules may be due to the absence of phenolic or aromatic amine groups. Unfortunately, no efforts were made to evaluate which enzymatic system from *B. adusta* could be involved in these herbicide transformations. Nevertheless, in vitro experiments using purified versatile peroxidase from *B. adusta* proved that this enzyme is able to transform halogenated phenolic pesticides including the herbicide bromoxynil [54]. This enzyme polymerized bromoxynil molecules producing a dimer and a trimer as the main products with the loss of bromide atoms and hydroxyl groups. Nevertheless, VP from *B. adusta* was able to oxidize only three of 13 halogenated pesticides tested. It was

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suggested that the peroxidase-mediated reactions are limited by the presence of phenoxyl groups and by the electronic properties of the substituents on the aromatic ring.

The genus *Pleurotus* includes edible and medicinal species which are found both in temperate and tropical climates. They are one of the most important cultivated mushrooms in the world and are commonly referred to as "oyster mushrooms". *Pleurotus ostreatus* and *P. pulmonarius* are the main species applied in bioremediation studies. These species are able to degrade PAH, chlorinated biphenyls and industrial dyes [36, 58-59]. *Pleurotus* species are known to be good producers of ligninolytic enzymes, mainly laccases [59], versatile peroxidase (VP) and manganese peroxidase (MnP) [24]. *P. pulmonarius*, cultured in pasteurized straw solid medium in association with bacteria, was able to degrade the highly persistent herbicide atrazine [60]. This study demonstrated that *P. pulmonarius* acts on the atrazine molecule producing chloro-metabolites which were further dechlorinated by bacteria, producing non-toxic metabolites. In liquid cultures, *P. ostreatus* was able to reduce the concentration of several herbicides. During five days, the herbicides chlortolurun, isoproturon and diuron were depleted 35, 42 and 33%, respectively [50]. Another study demonstrated maximum depletions of 42, 81 and 94% for diuron, linuron and propanil, respectively, during seven days [49]. In the latter studies no investigation about the enzymatic system involved in herbicide transformations was carried out.

Trametes versicolor seems to be highly promising for herbicide bioremediation. It is a good lignin degrader and produces Lac, MnP and LiP under certain culture conditions [61], being one of the most-studied laccase-producing fungus. Direct involvement of its laccase in herbicide transformation has been demonstrated. Recently, in vitro degradation experiments of glyphosate using several ligninolytic purified enzymes in combination with different mediators was carried out [62]. Laccase from *T. versicolor* was able to rapidly degrade the herbicide, but only in the presence of mediators such as ABTS, $MnSO_4$ and Tween 80. The best glyphosate degradation by laccase (90.1%) was obtained in the presence of all these mediators in the same reaction. The laccase-mediator reactions produced and accumulated the metabolite AMPA, a compound frequently found in soil exposed to glyphosate. Another work demonstrated that the compound diketonitrile, the active principle of the herbicide isoxaflutole, was transformed by laccase, purified from *T. versicolor* cultures, in the presence of the mediator ABTS [51]. The main metabolite produced in the reaction was benzoic acid, an inactive compound also produced by tolerant plants. Laccase from this white-rot species also has been reported to degrade metabolites from phenylurea herbicides commonly found in the fields. These are hydroxylated metabolites produced by fungi and plants as part of their phenylurea herbicide metabolization. The hydroxylation of their aromatic rings makes phenylureas substrates for laccases and thus ready for further degradation [63]. Purified laccases were able to transform the metabolites 2-hydroxyphenyl-urea (2-HF) and 4-hydroxyphenyl-urea (4-HF) into several products without mediator addition. The results suggested that these enzymes from *T. versicolor* allow the formation of polymers between the hydroxyphenylurea products, as deduced from the formation of insoluble compounds in the reaction medium [64].

In vivo studies have also been carried out. *T. versicolor* strains were able to deplete diuron, linuron and propanil from the culture medium after seven days, reaching 37%, 47% and 94%

pesticide transformation, respectively [49]. It was demonstrated that the herbicide diuron was the more extensively removed phenylurea in cultures of the *Trametes* species, with 72% transformation by *Trametes sp* and 33% by *T. versicolor* [50]. *T. versicolor* was able to degrade diuron and atrazine [65], chlornitrofen (CNP) and nitrofen (NIP) [66]. In the last work, the authors suggest that extracellular ligninolytic enzymes are not involved in the initial step of CNP and NIP degradation. However, the possibility of an effective participation of cytochrome P450 in the degradation of CNP and NIP has been emphasized.

Trametes versicolor has also been monitored for soil bioremediation in microcosms of non-sterile soils under water stress. Under this condition, this species was able to decompose atrazine without correlation with the production of ligninolytic enzymes [67] and to act on a pesticide mixture containing herbicides (simazine and trifluralin) and an insecticide (dieldrin) [45]. This type of study is very important because the capability of a given fungus to act in a soil microcosm can be strongly affected by competition with the native soil microflora and by the adverse conditions such as water stress.

Coriolopsis fulvocenerea, *Cerrena maxima* and *Coriolus hirsutus* were investigated as atrazine decomposers in liquid cultures [68]. Among the several fungi strains, the authors found that the best degraders were those that produce laccases. The results showed that these fungi degrade at least 80-92% of atrazine after 40 days. Further, cultures with addition of laccase inducers such as guaiacol and syringaldazine, were more effective in decreasing the atrazine concentration, suggesting an important role of these enzymes in atrazine degradation [69]. Recently, it was studied the effect of laccase from *C. hirsutus* on atrazine binding to soil [70]. Laccase introduction into the soil caused a rapid increase in irreversibly bound atrazine. This mechanism probably decreases the herbicide toxicity and thus contributes to atrazine detoxification in soil.

Recently, the decrease of the concentration of the herbicides bentazon and diuron in *Ganoderma lucidum* cultures was reported [71-72]. The action of the ligninolytic enzymes on bentazon was confirmed by in vitro experiments with the crude enzyme filtrates. After 24 hours, bentazon was completely degraded by the liquid culture filtrate. Also, solid state cultures using corn cobs as substrates were a good medium for the production of laccase and MnP as well as for the transformation of bentazon.

The white-rot fungus *Phlebia brevispora* metabolizes the herbicide CNP (chlornitrofen) by nearly 80% within 28 days of incubation with the production of five metabolites [73]. *P. vispora* was also able to degrade a highly toxic compound, a dibenzo-ϱ-dioxin, present as a contaminant of commercially produced CNP herbicide [73]. The transformation of phenylurea herbicides by *Phlebia radiate* after five days has also been reported [50].

Hypholoma fasciculare is another white-rot fungus capable of degrading different herbicide classes [65]. This fungus possesses a great ability to degrade the triazine herbicides terbuthylazine and atrazine and the phenylurea herbicide diuron in liquid cultures and also in biobed bioremediation system. No relationship between the ligninolytic activity and the fungi capacity to degrade pesticides was found, although similar mechanisms could be involved in degradation of all the compounds.

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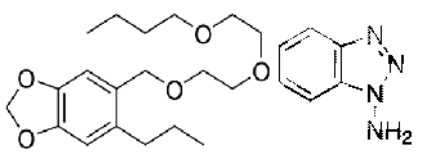
Lentinus subnudus was highly efficient in removing atrazine (78%) and metalachlor (94%) from contaminated soils after 25 days [74]. No attempts were done to identify the participation of the ligninoloytic enzymes in the process.

8. Are ligninolytic enzymes actually involved in herbicide degradation?

WRF have been demonstrated to be capable of transforming and/or degrading a wide range of herbicide classes. Two mechanisms or systems have been proposed. The first is transformation in the extracellular space and it involves lignin-degrading enzymes. These powerful capabilities of WRF reside in the fact that many pollutants have structural similarities to lignin and because ligninolytic enzymes are non-specific they can also act on the pollutant molecules. Furthermore, the transformation of some compounds can be enhanced with the use of small molecules, called mediators, which can extend the enzymatic reactivity of enzymes towards the substrates; this process is often referred to as the enzyme-mediator system. The participation of extracellular enzymes in the transformation of several herbicides by WRF was conclusively demonstrated by studies performed with purified enzymes [46,47,49,51,54,62,63].

The second system of WRF involved in herbicide transformation is an intracellular enzymatic mechanism, represented mainly by cytochrome P450. Purification of fungal cytochrome P450, in order to obtain conclusive data, has been accomplished in only a few studies, due to the difficulties in keeping the activation of the enzymes during microsome preparation. Hence, most conclusions were drawn from the results of indirect experiments consisting in the addition of specific cytochrome P450 inhibitors to the culture medium, such as piperonyl butoxide and 1-aminobenzotriazole [66,75-76] (Fig. 2). Direct evidence is also available. Some experiments were carried out with the microsomal fraction isolated from *Pleurotus ostreatus* [77]. The authors found that the microsomes transformed the pesticides in vitro in a NADPH-dependent reaction. In Table 2 and 3, representative studies were listed in which ligninolytic enzymes of different WRF were used for removing herbicides. These tables show only those studies in which direct or strong indirect evidence is presented about the participation of enzymes (laccases, peroxidases and cytochrome P450 monooxygenases) for the removal of herbicides by WRF.



piperonil butoxide 1-aminobenzotriazole

Figure 2. Specific cytochrome P450 inhibitors

Pesticide/metabolite(s)	Treatment conditions	Fungal source	Ref.
Glyphosate/AMPA	In vitro assay. The presence of ABTS, Mn^{2+} and Tween 80 resulted in the removal of 90.1% of glyphosate.	*Trametes versicolor*	[62]
2,4-dichlorophenol (DCP): metabolite of 2,4-D	In vitro assay. Laccase/mediator system. Laccase was immobilized on chitosan.	*Coriolus versicolor* (syn. *Trametes versicolor*)	[78]
Dymron	In vitro assay. Laccase/mediator system. Over 90% of the herbicide was degraded by the addition of ABTS as mediator.	*Trametes* sp.	[79]
Bromofenoxim; Bromoxynil; Dichlorophen; Niclosamide	In vitro assay. Laccase/mediator system. Addition of syringaldehyde or acetosyringone resulted in higher rates of enzymatic degradation.	*Coriolopsis gallica*	[49]
Bentazon	In vitro assay. Crude enzymes from liquid and solid state cultures were applied. A synergistic effect of ABTS, Mn^{2+}, H_2O_2, and Tween 80 was observed.	*Ganoderma lucidum*	[72]
Isoxaflutole. In plants and soils, isoxaflutole is rapidly converted to a diketonitrile derivative (DKN)	The WRF are able to convert the DKN to inactive benzoic acid analogue when cultured in liquid media. The DKN derivative was incubated with fungal extracellular oxidases, without or with their specific redox m ediator. Both LiPs and MnPs presenting high specific activities were unable to catalyze the enzymatic transformation of DKN, with or without mediators, during 12-h incubations. Laccase transformed the herbicide when incubated with 1 mM ABTS.	*Phanerochaete chrysosporium* and *Trametes versicolor*	[51]
Pentachlorophenol (PCP)	Submerged cultures in the presence and absence of laccase inducers. Cultures actively producing laccase removed 70% of initial PCP. The removal of PCP was less than 20 percent in the cultures with low laccase activity.	*Pleurotus pulmonarius*	[80]

ABTS = 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate), AMPA ((aminomethyl)phosphonic acid).

Table 2. Studies where strong evidences of participation of laccase in the biodegradation of herbicides were found

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Enzymes	Pesticide/ metabolite(s)	Treatment conditions	Fungal source	Ref.
MnP Manganese peroxidase	Glyphosate/AMPA	In vitro assay. Addition of Mn^{2+} was essential for the oxidative activity of the enzyme. A reaction mixture containing Mn^{2+} and Tween 80 with or without H_2O_2 resulted in total degradation of the herbicide.	*Nematoloma frowardii*	[62]
	Pentachlorophenol (PCP)	Fungal inoculation in soil microcosms contaminated with PCP. Addition of wheat straw increased enzyme production and PCP degradation.	*Anthracophyllum discolor*	[82]
	Bentazon	In vitro assay. Crude enzymes from liquid and solid state cultures were applied. A synergistic effect of ABTS, Mn^{2+}, H_2O_2, and Tween 80 was observed.	*Ganoderma lucidum*	[72]
VP Versatile peroxidase	Bromoxynil; Dichlorophen; PCP	In vitro assay. Purified versatile peroxidase (VP) able to transform several pesticides. Analysis of reaction products by GC–MS showed the presence of 2,3,5,6-tetrachloroquinone among the products from pentachlorophenol oxidation, while the main product from dichlorophen was 4-chlorophenol-2,2-methylenequinone. Several polymers were obtained from the peroxidase oxidation of bromoxynil. In all cases, VP mediated dehalogenation reactions.	*Bjerkandera adusta*	[54]
CYP450 enzymes / Cytochrome P450 monooxygenase	Azinphos-methyl; Phosmet; Terbufos	In vitro assay. Pesticide transformation by microsomal fraction. Addition of NADPH in the reaction mixture was required.	*Pleurotus ostreatus*	[77]
	trans-Chlordane	Static liquid cultures. Addition of cytochrome P450 inhibitors reduced degradation activity and demonstrated involvement of CYP450 enzymes in the pesticide metabolism.	*Phlebia lindtneri, Phlebia brevispora* Phlebia aurea	[76]
	Cloronitrofen (CNP) and nitrofen (NIP)	Extracellular LiP, MnP and Laccase did not catalyze the oxidation of either CNP or NIP. Piperonyl butoxide, an inhibitor of cytochrome P450, suppressed fungal oxidation of CNP and NIP to their hydroxylated products. The inhibition resulted in increasing the amount of reductively dechlorinated and nitro-reduced products.	*Trametes* (*Coriolus*) *versicolor*	[66]

Table 3. Studies where evidence of participation of peroxidases and CYP450 enzymes in the biodegradation of herbicides

9. Concluding remarks

The ability of WRF to transform a wide variety of herbicide classes makes them suitable candidates to detoxify contaminated water and soils. Nevertheless, numerous WRF species and herbicide classes are still not explored. Ligninolytic enzymes, such as laccases and peroxidases, have shown to be responsible for many herbicide transformations, although their action remains to be confirmed in most studies. In this context, also the cytochrome P450 system has demonstrated to have a very important role in bioremediation of numerous types of pollutants including herbicides, although further studies must be done in order to understand the action of this system in transformation of herbicides by WRF.

The employment of ligninolytic enzyme preparations could be advantageous over the direct use of white-rot fungi, considering that these preparations can be more easily standardized, facilitating accurate dosage. Enzyme application is simpler than the use of microorganisms and can be rapidly modified according to the characteristics of the herbicide to be removed. In addition to this, analysis of metabolite compounds produced by enzyme preparations is easier than analysis of the metabolites produced by the whole white-rot fungi.

There is a need for introducing new methodologies for the elucidation of the chemical structures of the metabolites produced by the action of ligninolytic enzymes. This should also facilitate the elucidation of the metabolic routes used by the white-rot fungi in herbicide degradation. Furthermore, the proof that the process of degradation corresponds to a real detoxification is a crucial step that must be overcome before its practical use.

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References

[1] Gavrilescu M. Fate of pesticides in the environment and its bioremediation. Engineering in Life Sicence 2005;5(4),497-526.

[2] United States Environmental Protection Agency. EPA. http;//epa.gov (acessed 5 october 2012)

[3] Chowdhury A, Pradhan S, Saha M, Sanyal N. Impact of pesticides on soil microbiological parameters and possible bioremediation strategies. Industrial Journal of Microbiology 2008;48,114-127.

[4] Rodrigues BN, Almeida FS. Herbicides Guide. Iapar, Brazil. 1998. p648.

[5] Gianfreda L, Rao MA. Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzyme and Microbial Technology 2004;35,339-354.

[6] Vidali M. Bioremediation. An overview. Pure and Applied Chemistry 2001;73(7), 1163-1172.

[7] Bennet JW, Wunch KG, Faison BD. Use of fungi in Biodegradation. In: Hurst CJ (eds) Manual of Environmental Microbiology. ASM Press Washington, 2002; p 960-971.

[8] Bellinaso MDL, Greer CW, Peralba MC, Henriques JAP, Gaylarde CC. Biodegradation of the herbicide trifluralin by bacteria isolated from soil. FEMS Microbiology and Ecolology 2003;43,91-194.

[9] Dwivedi S, Singh BR, Al-Khedhairy AA, Musarrat J. Biodegradation of isoproturon using a novel *Pseudomonas aeruginosa* strain JS-11 as a multi-functional bioinoculant of environmental significance. Journal of. Hazardous Materials 2011;185 (2-3), 3938-944.

[10] Mandelbaum RT, Allan DL, Wackett LP. Isolation and characterization of a *Pseudomonas* sp that mineralizes the s-triazine herbicide atrazine. Applied and Environmental Microbiology 1995;61,1451-1457.

[11] Barr DP, Aust SD. Mechanisms white rot fungi use to degrade pollutants. Environmental Science and Technology 1994;28 (2)79-87.

[12] Asgher M, Bhatti HN, Ashraf M, Legge RL. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 2008;19,771-783.

[13] Pointing SB. Feasibility of bioremediation by white-rot fungi. Applied Microbiogy and Technology 2001;57,509-526.

[14] Reddy CA. The potential for white-rot fungi in the treatment of pollutants. Current Opinion in Biotechnology 1995;6:,320-328.

[15] Have RT, Teunissen PJM. Oxidative mechanisms involved in lignin degradation by white-rot fungi. Chemical Reviews 2001;101,3397-3413.

[16] Leonowicz AL, Matuszewska A, Luterek J, Ziegenhagen D, Wajtas-Wasilewska M, Nam-Seok C, Hofrichter M, Rogalski J. Biodegradation of Lignin by white rot fungi. Fungal Genetics and Biology 1999;27,175-185.

[17] Maciel GM, Bracht A, Souza CGM, Costa AM, Peralta RM. Fundamentals, diversity and application of white-rot fungi. In: Silva AP, Sol M (Eds) Fungi: types, environmental impact and role in disease, Nova Science Publishers, Inc. p409-458

[18] Hatakka A. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiology Reviews 1994;13,125-135

[19] Nerud F, Misurcova Z. Distribution of ligninolytic enzymes in selected white-rot fungi. Folia Microbiologica 1996;41,264-266

[20] Tuor U, Winterhalter K, Fiechter A. Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. Journal of Biotechnology 1995;41,1-17

[21] Kuhar S, Kapoor M, Kapoor R, Sharma KK, Singh A, Kuhad RC. Biodiversity of ligninolytic fungi. In Kuhad RC, Singh A (Eds) Lignocellulose biotechnology: future prospects. New Delhi: I K International p37-53

[22] Ruiz-Duenas FJ, Martinez AT. Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microbial Technology 2009;2,164-177

[23] Kersten P, Cullen D. Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Fungal Genetics and Biology 2007;44,77-87.

[24] Hofrichter M Review: lignin conversion by manganese peroxidase (MnP). Enzyme and Microbial Technology 2002;30,454–466.

[25] Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60,551-565.

[26] Wesenberg D, Kyriakides I, Agathos SN. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnology Advances 2003;22,161-187

[27] Baldrian P. Fungal laccases – occurrence and properties. FEMS Microbiolology Review 2006;30,215-242.

[28] Lundell TK, Makela MR, Hilden K. Lignin-modifying enzymes in filamentous basidiomycetes: ecological, functional and phylogenetic review. Journal of Basic Microbiology 2010;50,5-20

[29] Conesa A, Punt PJ. van den Hondel C. Fungal peroxidases: molecular aspects and applications. Journal of Biotechnology 2002;93,143-158

[30] Liers C, Bobeth C, Pecyna M, Ullrich R, Hofrichter M. DyP-like peroxidases of the jelly fungus *Auricularia auricula*-judae oxidize nonphenolic lignin model compounds and high-redox potential dyes. Applied Microbiology and Biotechnology 2010;85,1869-1879.

[31] Liers C, Arnstadt T, Ullrich R, Hofrichter M. Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. FEMS Microbiology Ecology 2011;78(1),91-102

[32] Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T. New and classic families of secreted fungal heme peroxidases. Applied Microbiology and Biotechnology 2010;87,871-897

[33] Hammel KE, Kapich AN, Jensen KA, Ryan ZC. Reactive oxygen species as agents of wood decay by fungi. Enzyme and Microbial Technology 2002;30,445-453

[34] Watanabe T, Teranishi H, Honda Y, Kuwahara M. A selective lignin-degrading fungus, *Ceriporiopsis subvermispora*, produces alkylitaconates that inhibit the production of a cellulolytic active oxygen species, hydroxyl radical in the presence of iron and H_2O_2. Biochemical and Biophysical Research Communications 2002;297,918-923

[35] ten Have R, Teunissen PJM. Oxidative mechanisms involved in lignin degradation by white-rot fungi Chemical Reviews 2001;101,3397-3413.

[36] Pozdnyakova NN, Rodakiewicz-Nowak J, Turkovskaya OV, Haber J. Oxidative degradation of polyaromatic hydrocarbons catalyzed by blue laccase from *Pleurotus ostreatus* D1 in the presence of synthetic mediators. Enzyme and Microbial Technology 2006;39,1242–1249.

[37] Aust SD, Swaner PR, Stahl JD Detoxification and metabolism of chemicals by white-rot fungi. In: Gan JJ, Zhu PC. Aust SD, Lemley AT (eds) Pesticide decontamination and detoxification, American Chemical Society, Washington, DC. Oxford University Press, 2003, p3-14.

[38] Couto SR, Herrera JLT. Industrial and biotechnological applications of laccases: A review. Biotechnology Advance 2006;24,500-513.

[39] Riva S. Laccases: Blue enzymes for green chemistry. Trends in Biotechnology 2006;24(5),219-226.

[40] Dashtban M, Schraft H, Syed T, Qin W. Fungal biodegradation and enzyme modification of lignin. International Journal of Biochemistry and Molecular Biology 2010;1,36-50

[41] Prewitt ML, Diehl S, McElroy TC, Diehl WJ. Comparison of general fungal and basidiomycete-specific ITS primers for identification of wood decay fungi. Forest Products Journal 2008;58,68-71

[42] Castellani A. Viability of some pathogenic fungi in distilled water. Journal of Tropical Medicine and Hygiene 1939;42,225-226

[43] Marcos-Urrea E, Reddy CA. Degradation of chloro-organic pollutantsw by white rot fungi. In: Sing SN (Ed) Microbial degradation ox xenobiotics. Springer Berlin Heidelberg 2012. p31-66

[44] Mougin C, Laugero C, Asther M, Dubroca J, Frasse P, Asther M. Biotransformation of the herbicide atrazine by white rot fungus *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 1994;60(2),705-708.

[45] Fragoeiro S, Magan N. Impact of *Trametes versicolo*r and *Phanerochaete chrysosporium* on differential breakdown of pesticide mixtures in soil microcosms at two water potentials and associated respiration and enzyme activity. International Biodeterioration and Biodegradation 2008;62,376-383.

[46] Castillo MDP, Ander P, Stenstrom J, Torstensson L. Degradation of the herbicide bentazon as related to enzyme production by *Phanerochaete chrysosporium* in two solid substrate fermentation systems. World Journal of Microbiology and Biotechnology 2000;16,289-295.

[47] Castillo MDP, Andersson A, Ander P, Stenstrom J, Torstensson L. Establishment of the white rot fungus *Phanerochaete chrysosporium* on unsterile straw in solid substrate fermentation systems intended for degradation of pesticides. World Journal of Microbiology and Biotechnology 2001;17,627-633.

[48] Fratila-apachitei LE, Hirst JA, Siebel MA, Gijzen HJ. Diuron degradation by *Phanerochaete chrysosporium* BKM-F-1767 in synthetic and natural media. Biotechnology Letters 1999;21,147-154.

[49] Torres-Duarte C, Roman R, Tinoco R, Vazquez-Duhalt R. Halogenated pesticide transformation by a laccase-mediator system. Chemosphere 2009;77,687-692.

[50] Khadrani A, Seigle-Murandi F, Steiman R, Vroumsia T. Degradation of three phenylurea herbicides (chlortoluron, isoproturon and diuron) by micromycetes isolated from soil. Chemosphere 1999;38(13),3041-3050.

[51] Mougin C, Boyer F-D, Caminade E, Rama R. Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. Journal of Agricultural and Food Chemistry 2000;48:4529-4534.

[52] Castillo MDP, Wirén-Lehr SV, Scheunert I, Torstensson L. Degradation of isoproturon by the white rot fungus *Phanerochaete chrysosporium*. Biology and Fertility Soils 2001;33,521-528.

[53] Nakamura Y, Sungusia MG, Sawada T, Kuwahara M. Lignin-Degrading Enzyme Production by *Bjerkandera adusta* Immobilized on Polyurethane Foam. Journal of Bioscience and Bioengineering 1999;88(1),41-47.

[54] Davila-Vazquez G, Tinoco R, Pickard MA, Vazquez-Duhalt R. Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta.* Enzyme and Microbial Technology 2005;36,223-231.

[55] Field JA, Jong ED, Costa GF, De Bont JAM. Biodegradation of Polycyclic Aromatic Hydrocarbons by New Isolates of White Rot Fungi. Applied Environmental and Microbiology 1992;58,2219-2226.

[56] Heinfling A, Bergbauer M, Szewzyk U. Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. Applied Microbiology and Biotechnology 1997;48,261-266.

[57] Eichlerová I, Homolka L, Nerud F. Decolorization of high concentrations of synthetic dyes by the white rot fungus *Bjerkandera adusta* strain CCBAS 232. Dyes and Pigments 2007;75,38-44.

[58] Zeddel A, Majcherczyk A, Hu°ttermann A. Degradation of polychlorinated biphenyls by white-rot fungi *Pleurotus ostreatus* and *Trametes* in a solid state system. Toxicological and Environmental Chemistry 1993;40,255–266.

[59] Tychanowicz GK, Zilly A, Souza CGM, Peralta RM. Decolorization of industrial dyes by solid-state cultures of *Pleurotus pulmonarius.* Process Biochemistry 2004;39,855–859.

[60] Masaphy S, Levanon D, Henis Y.Degradation of atrazine by the lignocellulolytic fungus *Pleurotus pulmonarius* during solid-state fermentation. Bioresource Technology 1996;56,207-214.

[61] Rogalski J, Lundell T, Leonowicz A, Hatakka A. Production of laccase, lignin peroxidase and manganese-dependent peroxidase by various strains of *Trametes versicolor* depending on culture conditions. Acta Microbiologica Polonica 1991;40,221-234.

[62] Pizzul L, Castillo MDP, Stenstrom J. Degradation of glyphosate and other pesticides by ligninolytic enzymes. Biodegradation 2009;20,751-759.

[63] Jolivalt C, Neuville L, Boyer FD, Kerhoas L, Mougin C. Identification and formation pathway of laccase-mediated oxidation products formed from hydroxyphenylureas. Journal of Agricultural and Food Chemistry 2006;54,5046-5054.

[64] Jolivalt C, Raynal A, Caminade E, Kokel B, Le Goffic F, Mougin C. Transformation of N′, N′-dimethyl-N-(hydroxyphenyl)ureas by laccase from the white rot fungus *Trametes versicolor.* Applied Microbiology and Biotechnology 1999;51,676-681.

[65] Bending GD, Friloux M, Walker A. Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiology Letters 2002;212,59-63.

[66] Hiratsuka N, Wariishi H, Tanaka H. Degradation of diphenyl ether herbicides by the lignin-degrading basidiomycete *Coriolus versicolor*. Applied Microbiology and Biotechnology 2001;57,563-571.

[67] Bastos AC, Magan N. *Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions. International Biodeterioration and Biodegradation 2009;63,389-394.

[68] Koroleva OV, Stepanova EV, Landesman EO, Vasilchenko LG, Khromonygina VV, Zherdev AV, Rabinovich ML. Enzyme immunoassay of herbicide decomposition by soil and wood decay fungi. Appl. Biochem. Microbiol., 2002;38, 355-360.

[69] Gorbatova ON, Koroleva OV, Landesman EO, Stepanova EV, Zherdev AV. Increase of the detoxification potential of Basidiomycetes by induction of laccase biosynthesis. Applied Biochemistry and Microbiology 2006;42(4),414-419.

[70] Davidchik VN, Kulikova NA, Golubeva LI, Stepanova EV, Koroleva OV. *Coriolus hirsutus* laccase effect on atrazine adsorption and desorpton by different types of soil. Applied Biochemistry and Microbiology 2008;45(4),405-410.

[71] Coelho JD, Oliveira AL, Souza CGM, Bracht A, Peralta RM. Effect of the herbicides bentazon and diuron on the production of ligninolytic enzymes by *Ganoderma lucidum*. International Biodeterioration and Biodegradation 2010;64(2),156-161.

[72] Coelho JD, Souza CGM, Oliveira AL, Bracht A, Costa MAF, Peralta RM. Comparative removal of bentazon by *Ganoderma lucidum* in liquid and solid state cultures. Current Microbiology 2010;60(5),350-355.

[73] Kamei I, Kondo R. Simultaneous degradation of commercially produced CNP herbicide and of contaminated dioxin by treatment using the white rot fungus *Phlebia brevispora.* Chemosphere 2006;65,1221-1227.

[74] Nwachukwu EO, Osuji JO. Bioremedial degradation of some herbicides by indigenous white rot fungus, *Lentinus subnudus*. Journal of Plant Sciences 2007;2(6),619-624.

[75] Mougin C, Laugero C, Asther M, Chaplain V. Biotransformation of s-triazine herbicides and related degradation products in liquid culture by the white rot fungus *Phanerochaete chrysosporium.* Pesticide Science 1997;49,169-177.

[76] Xiao P, Mori T, Kondo R. Biotransformation of the organochlorine pesticide transchlordane by wood-rot fungi. New Biotechnology, 2011;29(1),107-115.

[77] Jauregui J, Valderrama B, Albores A, Vazquez-Duhalt R. Microsomal transformation of organophosphorus pesticides by white rot fungi. Biodegradation, 2003;14(6), 397-406.

[78] Zhang J, Xu Z, Chen H, Zong Y. Removal of 2,4-dichlorophenol by chitosan-immobilized laccase from Coriolus versicolor. Biochemical Engineering Journal, 2009;45(1), 54-59.

[79] Maruyama T, Komatsu C, Michizoe J, Ichinose H, Goto M. Laccase-Mediated Oxidative Degradation of the Herbicide Dymron. Biotechnology Progress 2006;22(2), 426-430.

[80] Souza DF, Costa SC, Dacome AS, Souza CGM, Bracht A, Peralta RM. Pentachlorophenol removal by *Pleurotus pulmonarius* in submerged cultures. Brazilian Archives of Biology and Technology 2011;54(2),357-362

[81] Cea M, Jorquera M, Rubilar O, Langer H, Tortella G, Diez MC. Bioremediation of soil contaminated with pentachlorophenol by *Anthracophyllum discolor* and its effect on soil microbial community. Journal of Hazardous Materials 2010;181(1–3),315-323.

Chapter 9

Herbicides as Potential Chemotherapeutic Agents Against Parasitic Protozoa

Wanderley de Souza

Additional information is available at the end of the chapter

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1. Introduction

Herbicides refer to a large number of compounds widely used to kill plants that interfere with the growth of desired crops, thereby improving the productivity of the crop system. One group of herbicides that includes compounds generally designated as dinitroanilines has been shown to interfere with plant cells by interrupting mitosis and the formation of multinucleated cells. Research has shown that these effects are due to interference with microtubules, i.e., a cytoskeleton structure that is ubiquitous in eukaryotic cells and plays a fundamental role in several biological processes, including the determination and maintenance of cell shape, the motility of several cell types that use flagella and cilia for locomotion, the intracellular transport of organelles, and the movement of chromosomes during cell division. Other processes involving microtubules are not as well characterized. Previous research has shown that dinitroanilines interfere with microtubules by binding to sites on the surface of the longitudinal contacts established between the tubulin subunits that contain lysine and arginine residues, which in turn bind to the nitrile group of dinitroaniline [1,2].

Microtubules are made of α-and β-tubulin heterodimers that form long (i.e., several μmeters in length), filamentous, tubular structures when polymerized. The number of tubulin isotypes varies according to the organism species (e.g.,six types of α-tubulin and seven types of β-tubulin are found in human cells). They can be very dynamic structures that undergo constant assembly and disassembly in cells. Tubulin molecules may be post-translationally modified by polyglutamylation, polyglycylation, phosphorylation, acetylation, detyrosination/tyrosination, and removal of the penultimate glutamic acid residue found in α tubulins. In addition, an increased number of proteins can interact with microtubules; these proteins are known as microtubule-associated proteins (MAPs)and include dynein, kinesin, etc., all of which interfere

with the stability of the microtubules and their function. Further data on microtubule composition and dynamics can be found in an excellent review by Gardner et al. [3].

2. Dinitroanilines

Dinitroanilines correspond to a family of herbicides that were originally discovered through studies evaluating dyes and chemical synthesis intermediates. The most important member of the group is trifluralin, which is widely used in soybean production. The family is divided into the following two subfamilies: the methylanilines, which includes trifluralin, pendimethalin, benefin, dinitramine, fluchloralin, and profluralin, and the sulfonylanilines, which includes oryzalin and nitralin [1,4,5].Initial studies showed that these compounds inhibit cell division by interfering with the assembly of microtubules, thereby interfering with the formation of the plant cell walls and chromosome movement during the mitotic process, which ultimately leads to the appearance of multinucleated cells [6].

One characteristic feature of several pathogenic protozoa is the presence of a large number of structures in which microtubules are a major component. In the case of the Trypanosomatidae family, which includes such important pathogenic species as *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania*, subpellicular microtubules are located immediately below the plasma membrane, establishing connections between them, the plasma membrane, and the profiles of the endoplasmic reticulum. They are seen throughout the protozoan body with exception to the region of the flagellar pocket [7]. This large group of organisms also contains the flagellar microtubules and intranuclear spindle microtubules involved in the process of nuclear division. In the case of Apicomplexa, which includes such pathogens as *Toxoplasma gondii*, *Plasmodium*, *Eimeria*, *Babesia,* etc., researchers have found subpellicular microtubules, i.e., a special type of microtubule that forms the conoid, spindle microtubules, and flagellar microtubules in microgametes [8]. In *Giardia lamblia,* the microtubules are associated with the adhesive disc (i.e., a structure involved in the attachment of the trophozoite to the intestinal epithelial cells) and form the spindle microtubules and flagella. In the case of trichomonads (e.g., *Trichomonas vaginalis* and *Tritrichomonas foetus*) microtubules form the flagella and such structures as the pelta-axostylar system and the spindle microtubules [9, 10].

In the following text, I will review the literature focused on the effects of herbicides on each group of pathogenic protozoa.

3. Trypanosomatids

The microtubules that are found in trypanosomatids, especially those that are subpellicular, are considered resistant to several compounds that usually depolymerize microtubules found in eukaryotic cells, including colchicine, vinblastine, and vincristine [11]. However, these organisms show some sensitivity to taxol [12]. Research has shown that trifluralin inhibits cell division in several members of the Trypanosomatidae family, including *Leishmania amazonen-*

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sis, *Leishmania mexicana*, *Leishmania infantum*, *Leishmania major*, *Leishmania panamensis*, *T.brucei*, and *T.cruzi* [13-15]. The half maximal inhibitory concentration (IC_{50}) for these protozoa ranges from 0.9 to 670 μM. In general, the *Leishmania* species were more sensitive to the herbicides than the *Trypanosoma* species [16]. In general, the amastigotes, which are the predominant and proliferative intracellular form, are more sensitive than the forms that grow in axenic media (i.e., promastigotes and epimastigotes). A microscopic analysis showed that trifluralin induced changes in the shape of *T. cruzi* epimastigotes (i.e., they became more rounded), affected the mitochondrion, interfered with the ingestion of macromolecules through the cytostome, decreased the number of horseradish peroxidase containing reservosomes, induced the appearance of multi-flagellated cells (i.e., probably due to interference with the cell division process), and blocked the process of metacyclogenesis; yet,trifluralin does not disrupt the subpellicular microtubules [17].

Some papers have described attempts to use dinitroanilines in vivo. For instance, promising results were observed when using topical applications of dinitroanilines to treat lesions induced by *L. major* and *L. mexicana* [14] and oral applications of dinitroanilines to treat the chronic phase of Chagas disease in mice. These results are similar to those obtained with benznidazole [18].

4. Apicomplexa

More information on the effect of herbicides is available for this group of eukaryotic microorganisms, especially *T.gondii*. Most of the studies on organisms within Apicomplexa were performed by Morrisette and her colleagues. The first paper in 1996 [19] showed that dinitroaniline herbicides inhibited intracellular division in the tachyzoites of *T. gondii*. This classical paper also demonstrated that oryzalin and ethalfluralin inhibited 50% of the protozoan growth at concentrations of 100 nM. In the case of trifluralin, the IC_{50} was 300 nM. These concentrations are very low; most importantly, even at concentrations that were 100 times higher, the drugs did not interfere with the human fibroblasts used to cultivate the protozoa. These compounds blocked the process of endodyogeny, i.e.,a special characteristic of cell division in *T. gondii* trophozoites where two daughter cells are formed inside a mother cell. Oryzalin, not ethalfluralin, disrupted the subpellicular microtubules. None of the compounds interfered with the structure of the conoid, i.e., a structure made of microtubules of a special type [20]. The authors also obtained mutant parasites that were resistant to the herbicides under investigation through chemical mutagenesis. Subsequently, the research showed that in the presence of oryzalin at a concentration of 2.5 μM, the tachyzoites retained the capacity to assemble the spindles and undergo nuclear division. However, due to disintegration of the subpellicular microtubules, the parasites were no longer able to invade new cells. At 2.5 μM, the compound interfered with the spindle microtubules, and the protozoa increased in size [21]. Morrissette and her co-workers further analyzed the obtained mutants and showed that they were localized in or near the M and N loops, i.e., domains that coordinate the lateral interactions between protofilaments [1, 22, 23]. Subsequently, several other oryzalin analogs were synthe-

sized, thereby leading to the acceptance of an antimitotic structure-activity relationship for dinitroanilines.

N^1,N^1-dipropyl-2,6-dinitro-4-(trifluoromethyl)-1,3benzenediamine is the most potent agent against *T. gondii* [5]. These studies were extended to *Plasmodium falciparum,* and the results indicated that trifluralin and oryzalin inhibited the progression of the protozoa inside erythrocytes by blocking the mitotic division with the accumulation of abnormal microtubular structures [24]. This research also demonstrated that trifluralin is active against the gametocytes of *P. falciparum*, thereby inducing disassembly of the subpellicular microtubules due to the formation of tubular structures containing disassembled microtubules. The researchers used labeled trifluralin and electron microscopy autoradiography to show that the compound binds to the tubular structures [25]. Oryzalin and trifluralin derivatives also showed activity against *Cryptosporidium parvum*. Several derivatives of these compounds were synthesized and, despite their reduced toxicity, showed similar activity [26].

5. Anaerobic protozoa

Oryzalin was tested against *Giardia lamblia* trophozoites. The obtained results showed that oryzalin inhibited parasite proliferation in an axenic culture. At 50 and 100 μM, most of the protozoa were killed. Morphological studies showed curling of the flagella in about 60% of the cells, elongation of the median body (i.e., a structure made of microtubules), changes in the shape of the cell, and blockage of cell division (Figures 1-3) [27].

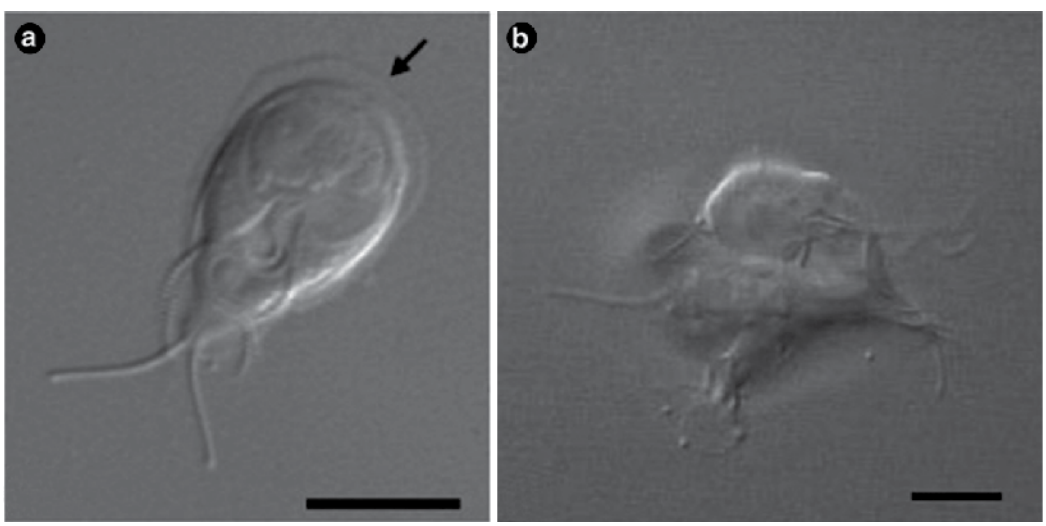

Figure 1. Light microscopy of the control (A) and oryzalin-treated (B) trophozoites of *Giardia lamblia*. The control cell displays a pyriform shape with four pairs of clearly identifiable flagella. In the treated cell, the loss of its normal shape is observed. Bar, 3 μm [27].

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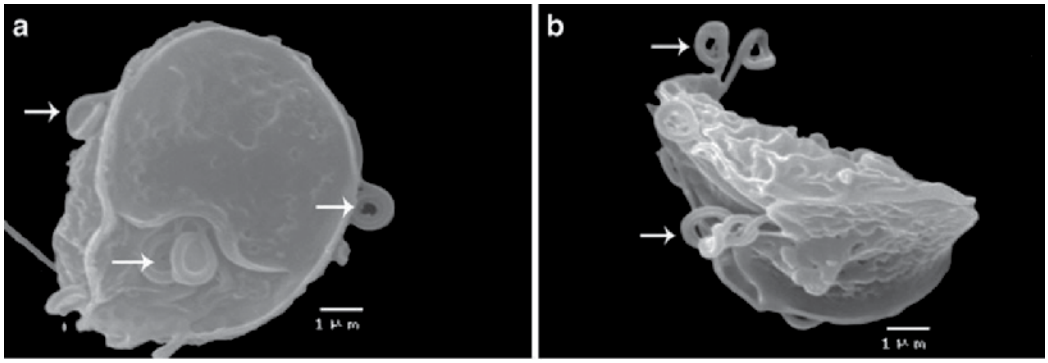

Figure 2. Scanning electron microscopy showing several alterations in the organization of the trophozoite form of *G. lamblia,* including shortening and curling of the flagella (arrows in a and b). Bar, 1µm [27].

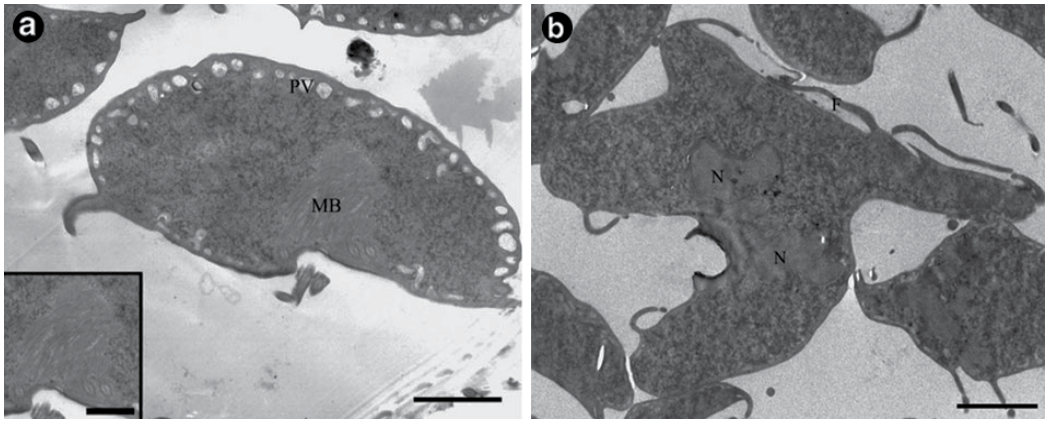

Figure 3. Transmission electron microscopy of thin sections of the control (A) and oryzalin-treated (B) trophozoites of *G. lamblia* where inhibition of protozoan division is clearly seen. Bar in A and B, 0.5 and 2 µm, respectively [27].

6. Trifluralin associated with phospholipid analogues

Phospholipid analogues, such as miltefosine, have been shown to be very effective against parasitic protozoa, especially *Leishmania donovani,* and are now considered the favorite pharmaceutical treatment for visceral leishmaniasis in India [28]. The association via molecular hybridization combines the pharmacophoric moieties of miltefosine and trifluralin, thereby leading to some compounds that are very active against *T.cruzi* and *L.amazonensis* (submitted for publication). The effects observed on the structural organization of the protozoa seem to also affect the membranes and cytoskeleton structures, thereby offering new possibilities in the treatment of parasitic diseases. Based on the preliminary results obtained with these compounds it seems to me that very soon some of them will be in the phase of clinical trials.

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References

[1] Morrissete, N. S, Mitra, A, Sept, D, & Sibley, L. D. Dinitroanilines bind α-tubulin to disrupt microtubules. Molecular Biology of the Cell.(2004). , 15, 1960-1968.

[2] Perez, E. A. Microtubule inhibitors: differentiating tubulin-inhibiting agents based on mechanism of action, clinical activity, and resistance. Molecular Cancer Therapy (2009). , 8, 2086-2095.

[3] Gardner, M. K, Zanic, M, & Howard, J. Microtubule catastrophe and rescue. Current Opinion in Cell Biology (2012). in press

[4] Mitra, A, & Sept, D. Binding and interaction of dinitroanilines with Apicomplexa and Kinetoplastid α-tubulin. Journal of Medicinal Chemistry. (2006). , 49, 5226-5231.

[5] Endeshaw, M. M, Li, C, De Leon, J, Yao, N, Latibeaudiere, K, Premalatha, K, Morrissette, N, & Werbovetz, K. A. Synthesis and evaluation of oryzalinanalogs against *Toxoplasma gondii*.Bionorganic& Medicinal Chemistry Letters.(2010). , 20, 5179-5183.

[6] Vaughn, K. C. LehnenLPJr. Mitotic disrupter herbicydes. Weed Sciences (1991). , 39, 450-457.

[7] De Souza, W. The sub-pellicular microtubules of trypanosomatids. Trends in Cell and Molecular Biology (2009). , 4, 5-13.

[8] Attias, M, & De Souza, W. A review of the Apicomplexa cytoskeleton. Trends in Cell and Molecular Biology (2009). , 4, 67-79.

[9] Campanati, L, & De Souza, W. The cytoskeleton of *Giardia lamblia*. Trends in Cell and Molecular Biology (2009). , 4, 49-61.

[10] Benchimol, M. The cytoskeleton of trichomonads.Trends in Cell and Molecular Biology. (2009). , 4, 25-39.

[11] Jordan, M. A, & Wilson, L. Microtubules as a target for anticancer drugs. Nature Reviews in Cancer (2004). , 4, 253-265.

[12] Baum, S. G, Wittner, M, Nadler, J. P, Horwirz, S. B, Dennis, J. E, Schiff, P. B, & Tanowitz, H. B. Taxol, a microtubule stabilizing agent, blocks the replication of *Trypanosoma cruzi*. Proceedings of the National Academy of Sciences USA.(1981). , 78, 4571-4575.

[13] Bienen, E. J, & Fong, D. Herbicides to curb human parasite infections: in vitro and in vivo effects of trifluralin on the trypanosomatid protozoans. Proceedings of the National Academy of Sciences USA.; (1993). , 90, 5657-5661.

[14] Chan, M. M, Tzeng, J, Emge, T. J, Ho, C. T, & Fong, D. Structure-function analysis of antimicrotubule dinitroanilines against promastigotes of the parasitic protozoan *Leishmania mexicana*. Antimicrobial Agents and Chemotherapy.(1993). , 37, 1909-1913.

[15] Fong, D. Plant microtubule inhibitors against trypanosomatids. Parasitology Today (1994). , 448-451.

[16] Traub-cseko, Y. M, Ramalho-ortigão, J. M, Dantas, A. P, De Castro, S. L, Barbosa, H. S, & Downing, K. H. Dinitroaniline herbicides against protozoan parasites: the case of *Trypanosoma cruzi*. Trends in Parasitology. (2001). , 17, 136-1341.

[17] Bogitsh, B. J, Middleton, R. L, & Ribeiro-rodrigues, R. Effects of the antitubulin drug trifluralin on the proliferation and metacyclogenesis of *Trypanosoma cruzi* epimastigotes. Parasitology Research.(1999). , 85, 475-480.

[18] Zaidenberg, A, Luong, T, Limessi, D, & Bleiz, J. Del Buenono MB, Quijano G, Drut R, Kozubsky L, Marron A, Buschiazzo H. Treatment of experimental chronic Chagas disease with trifluralin. Basic Clinical Pharmacology and Toxicology.(2006). , 98, 351-356.

[19] Stokkerman, T. J, Schawrtzman, J. D, Keenan, K, Morrissete, N. S, Tilney, L. G, & Roos, D. S. Inhibition of *Toxoplasma gondii* replication by dinitroaniline herbicides. Experimental Parasitology (1996). , 84, 355-360.

[20] Hu, K, Roos, D. S, & Murray, J. M. A novel plymer of tubulin forms the conoid of*Toxoplasma gondii*. Journal of Cell Biology (2002). , 156, 1039-1050.

[21] Morrissette, N. S, & Sibley, L. D. Disruption of microtubules uncouples budding and nuclear division in *Toxoplasma gondii*. Journal of Cell Science.(2002). , 115, 1017-1025.

[22] Ma, C, Tran, J, Li, C, Ganesan, L, Wood, D, & Morrissette, N. Secondary mutations correct fitness defects in *Toxoplasma gondii* with dinitroaniline resistance mutations. Genetics (2008). , 180, 845-856.

[23] Lyons-abbott, S, Sacket, D. L, Wloga, D, Gaertig, J, Morgan, R. E, & Werbovetz, K. A. Morrissette NS.α-tubulin mutations alter oryzalin affinity and microtubule assembly properties to confer dinitroaniline resistance. Eukaryotic Cell.(2010). , 9, 1825-1834.

[24] Fennel, B. J, Naughton, J. A, Dempsey, E, & Bell, A. Cellular and molecular actions of dinitroaniline and phosphorothioamidate herbicides on *Plasmodium falciparum*: tubulin as a specific antimalarial agent. Molecular and Biochemical Parasitology.(2006). , 145, 226-238.

[25] Kaidoh, T, Nath, J, Fujioka, H, Okoye, V, & Aikawa, M. Effectand localization of truflurlin in Plasmodium gametocytes: an electron microscopic study. Journal of Eukaryotic Microbiology, (1995). , 42, 61-64.

[26] Benbow, J. W, Bernberg, E. L, Korda, A, & Mead, J. R. Synthesis and evaluation of dinitroanilines for treatment of cryptosporidiosis. Antimicrobial Agents and Chemotherapy.(1998). , 42, 339-343.

[27] Terra, L. T, Campanati, L, & De Souza, W. Heterogeneity in the sensitivity of microtubules of *Giardia lamblia* to the herbicydeoryzalin. Parasitology Research. (2010). , 107, 47-54.

[28] Pearson, R. D. Development status of miltefosine as first oral drug in visceral and cutaneous leishmaniasis. Current Infectious Diseases Report. (2003). , 5, 41-42.

Chapter 10

Immunosensors: Concepts and Structures for Fast and Accurate Sensing

A. Rodríguez and E. Valera

Additional information is available at the end of the chapter

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1. Introduction

Biosensors based in interdigitated transducers have demonstrated the capability to fulfil the requirements of the market in a broad range of applications: their combination with biomolecules such as antibodies allows the development of high selectivity sensors. Their electrical excitation and readout makes easy to develop electronic equipment that perform automatically the operations required for the measurement and the fluidics manipulation involved in it. The sensitivity levels required for many applications can be reached using low cost interdigitated electrodes with finger sizes of several microns, thus eliminating the need for expensive submicron technologies. In another hand the use of plastic substrates helps further reducing the costs of the sensors. All this together makes these sensors excellent candidates to satisfy the demands of potentials users in terms of quality, cost, suitability of apparatus that can be developed, automatization, etc.

One of the important issues that arise when potential users are consulted is the time required to perform a test. The total time since the sample is introduced in the sensor until the measurement is ready, thus involving fluidic manipulation, functionalization, and the measurement itself often is desirable to be in the order of several (few) minutes or lower. While different schemes can be applied to optimize the duration of the fluidic manipulation to adapt it to the time per test required in each case, the time in which the electrical measurement is done may be important: A very common detection method is the impedance spectroscopy sweeping a wide frequency range. This is a powerful, but cumbersome tool to study the sensor performance providing trustworthy results. Performing impedance measurements in a broad range of frequencies is time consuming, especially if a high number of tests are to be done and when these tests involve low frequencies.

Markets demands faster alternatives capable of providing measurements in shorter times without compromising the requirements of sensitivity of the measurements.

In this chapter three strategies for the fast quantification are explained:

- Single Frequency measurements
- DC measurements
- Multi-sine burst signal

These techniques are analyzed and their advantages in comparison with the classical wide range impedance spectroscopy method are commented.

The technique has been applied to the detection of atrazine in foodstuff products. Though in this contribution atrazine has been chosen as the analytical target, this immunosensor technology can be used for a wide variety of compounds using the appropriate selective antibodies.

Many types of sensors have been developed for the detection and quantification of pesticides and traces of them. The MRL of a given pesticide, which often lies in the order of few tens of ppbs, determines the minimum sensitivity of these sensors. Also the quantification, or the detection, of one substance in the complex chemical matrix of some foods as wine, milk or juices also poses important requirements to their selectivity.

Currently, in the literature, good examples of impedimetric immunosensors devoted to food safety can be found [1]. Although these devices have demonstrated to be efficient, typically the impedance analysis in a wide frequency range is not fast, requires expensive equipments and qualified personnel. These features imply a great difficulty to obtain a commercial product. Therefore, if commercial devices are desired from this technology, some variations should be including in order accomplishing the market requirements.

In this chapter two cases of immunosensors are analysed. Firstly, an impedimetric immunosensor based on the analysis of only one frequency, instead of the broad frequency spectrum is studied. By means of this technique, the data treatment related to the analysis of the broad frequency spectrum is avoided. Alternatively, another measurement technique based on the application of a multi-sine burst signal is also explored, as a method to hardly reduce the cost related to the expensive equipments. Then, the advantages of a second device, the conductimetric immunosensor are also exanimate. In this device a secondary antibody labelled with gold nanoparticles is included in order to produce a conductive label on the electrodes that opens the possibility of applied simple and inexpensive DC measurements. In addition, but within the same device, an interesting change of substrate is studied.

2. Description of the immunosensors

In this section, structure, functionalization, working mechanisms, as well as the contribution of the immunosensors treated to obtain a commercial product are described.

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2.1. Interdigitated μ-electrodes (IDμE's)

Interdigitated μ-electrodes (IDμE's) were used as transducers for the immunosensors presented in this chapter. Interdigitated μ-electrodes are two coplanar electrodes (that works as counter and working electrodes) which have equal surface areas and each is presumed to contribute equally to the measured network impedance. The standard procedure of the electrodes fabrication, which can be made in large quantities and at low cost, is as follows:

Thin Au/Cr (~ 150 nm thickness) interdigitated μ-electrodes (IDμE's, ~ 5 μm thick and electrode gap) were patterned on a Pyrex 7740 glass substrate. The objective of the chromium layer is just to improve the adhesion of the gold to the Pyrex substrate, thus, the Cr layer is much thinner than the Au layer (aprox. 1:10 ratio).

Before metal deposition, a washing step of the substrate, using absolute ethanol, is required. The metal deposition was performed by means of sputtering deposition and the interdigitated μ-electrodes were then patterned on the Pyrex substrate by a photolithographic metal etch process. For the immunosensor measurements, arrays consisting on six IDμE's organized on a 1 cm^2 area were constructed.

Before functionalization, the samples were first cleaned in a solution of ethanol absolute 70% and Milli-Q water 30%. Then, the samples were plunged for 12 h in a solution of NaOH 2.5% in Milli-Q water. Afterwards, the 12 h the samples were rinsed in 100mL of Milli-Q water in order to neutralize the action of the NaOH. Finally, the arrays of IDμE's were dried with ethanol and N_2.

2.2. Impedimetric immunosensor based on singly frequency impedance measurements

The impedimetric immunosensor presented, whose detection method is based on single frequency impedance measurements, is a robust and *label-free device*. This device is based on the use IDμE's arrays, bioreagents specifically developed against the desired compound and on the *impedimetric change* that occurs when the immunoassay is performed on the electrodes surface.

An immunochemical competitive reaction (Figure 1), between the pesticide residues and the immobilized antigen on IDμE's for a small amount of the specific antibody, is performed on the electrodes. This reaction involves the competition between the free pesticide and a fixed amount of coated antigen for a limited amount of antibody (Ab). The amount of Ab captured on the IDμE surface, and hence the free antigen, is determined by means of impedimetric analysis.

As the immunosensor does not measure an amount of pesticide, because it measures an amount of antibody, the change in the impedance is due to the addition of antibody in the sensor surface, and not to the addition of molecules of pesticides. This approach has an important effect on the sensitivity of this immunosensor, because the molecules of antibody are much bigger than the molecules of pesticide and their effect on the impedance of the device is much higher. This feature represents an important advantage in comparison with other impedimetric immunosensors reported previously [2-4]. As a consequence, authors of these

works must reduce the electrode size to nanometer scale [2], or otherwise their limits of detection can only achieve tens of ppbs [3, 4].

Immunosensor functionalization consists on two main steps: i) the coating antigen (CA) immobilization; and ii) the specific antibody capture.

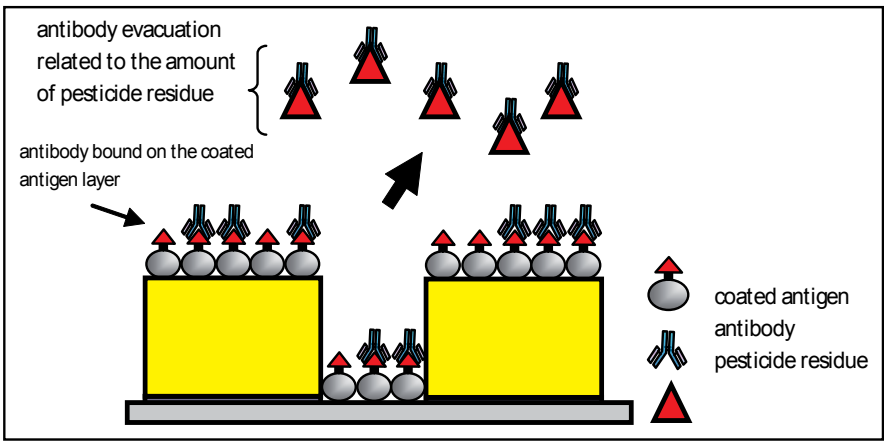

Figure 1. Immunosensor reaction. An amount of the specific antibody is bound on the coated antigen layer. Other quantity is evacuated of the IDμE's; this amount is related to the pesticide concentration.

As it has been demonstrated in the literature [1], the detection method based on impedance measurement in a wide frequency range, works correctly and accurate results can be obtained from it. Nevertheless, this method can be tedious and time consuming because computer processing is required to extract the values of all parameters involved. For this reason, an alternative method for the detection of pesticide based on measurements at single frequencies was raised.

By this method, single frequencies are applied, instead of the wide frequency range, for the atrazine detection. In the case the computer processing related to the fitting of the Nyquist plots of impedance spectra to the equivalent circuit is completely avoided because this detection method is based on the measurement of just the module (|Z|), the real part (Zre) or the imaginary part (Zim) of the impedance at a single frequency.

As it is well known, the inclusion of antibody on the immunosensor (antibody capture step) implies a variation in the immunosensor impedance (real and imaginary part of the impedance). This variation can be represented for the values of the equivalent circuit elements. Therefore, the changes of the values of the elements of the equivalent circuit are due to variations of immunosensor impedance. Then, the changes of the elements of the equivalent circuit are strongly related to the variation of |Z|, Zre and Zim.

This detection method consists on the measurements of the module, the real part or the imaginary part of the impedance of the immunosensor at a single frequency for the analysis. For this reason, both |Z|, Zre and Zim have been studied in order to analyze which of them

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have better performance for the different range of frequencies. Their performance is determined by comparing the limit of detection (LOD) for their response curves. The variation of the |Z|, Zre or Zim of the IDμE's, between the antigen incubation and the competition step is plotted against atrazine concentration to obtain the response curve. In order to find the best immunosensor response and improvements in the LOD, two kind of variation of the impedance have been taking into account:

i. Absolute difference:

$$\Delta Zabs = |Z(Ab) - Z(AT)|, \tag{1}$$

and

ii. Relative difference:

$$\Delta Zrel = \left|\frac{Z(Ab) - Z(AT)}{Z(AT)}\right| \tag{2}$$

where Z(Ab) is the impedance (module, real part or imaginary part) of the antibody captured and Z(AT) is the impedance (module, real part or imaginary part) of the antigen previously immobilized.

The impedance measurements were executed at room temperature, applying an AC signal of 25 mV of amplitude in the frequency range from 40 Hz to 1 MHz and using 0 Vdc bias potential, in diluted PBS solution (1.6 μS cm^{-1}). Likewise, all impedance measurements were done in a Faraday cage.

This method has been demonstrated using several, representing the ranges of the middle (30 kHz to 150 kHz) and high (150 kHz to 1 MHz) frequencies. The bandwidth was chosen in order to taking into account all regions of the impedance spectrum between the middle and high frequencies.

2.3. Conductimetric immunosensor based on DC measurements

Although this device is also based on the use IDμE's arrays, and bioreagents specifically developed against atrazine, this immunosensor includes a *secondary antibodies labelled with gold nanoparticles*. The deposition of this secondary antibody on the electrodes surface produces a detectable *conductimetric change*.

Again, an immunochemical competitive reaction is used for reach the pesticide detection. However in this case, a secondary antibody (Ab_2) is included (Figure 2). These secondary antibodies, linked to the gold particles, constitute a conductive film between the electrodes. Thus, the conductance of this film will depend on the concentration of gold labelled antibodies.

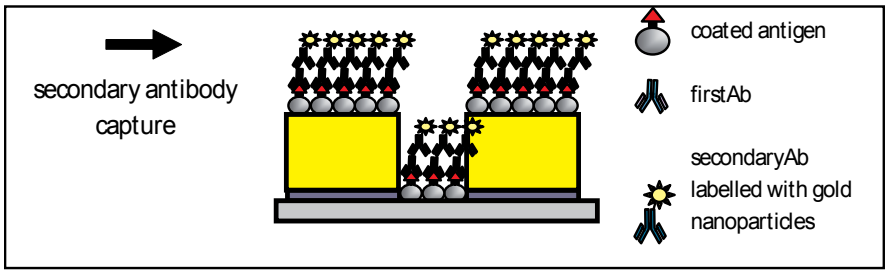

Figure 2. Schematic diagram of the complete assay system performed on the IDμE's for the conductimetric immunosensor.

The functionalization of this immunosensor consists in three main steps: i) the coating antigen (CA) immobilization; ii) the specific antibody capture (Ab_1); and iii) the capture of a non-specific antibody (Ab_2) labelled with gold nanoparticles.

The competition reaction is performed during step i and ii. During step iii, Ab_2 is linked to Ab_1, and then the Ab_2 concentration is related to the Ab_1 concentration included. Therefore, the concentration of the free pesticide tested is related to the amount of gold nanoparticles, which is measured by DC measurements. In this new structure, gold particles act as new *small fingers*, reducing the gap of the interdigitated μ-electrodes [5].

Using this conductimetric immunosensor has been demonstrated that pesticides residues can be detected by means of simple and inexpensive DC measurements, when gold nanoparticles are included as labels in the immunosensor [6, 7]. When a DC voltage is applied to an interdigitated μ-electrode with gold particles attached to it, the DC current which passed through the electrodes grows clearly in relation to the concentration of pesticide included.

As well as the impedimetric case, the conductive measurements were also carried out at room temperature, in a Faraday cage and without the use of any redox mediator. The two interdigitated electrodes were covered by a diluted PBS solution with a conductivity of 1.6 μS cm^{-1} and connected to the input of an Agilent 4156C Semiconductor Parameter Analyzer by means of standard probe tips.

In comparison with the impedimetric measurements performed for the typical impedimetric immunosensors, the conductive measurements are easier to execute, because impedance spectroscopy is not required and the fitting procedure to the equivalent circuit model is also completely avoided.

3. Functionalization of the immunosensors

For the immunosensors development, the biofunctionalization (immobilization) of the biological element onto the transducer surface is required

Over the coated layer of antigen, the free specific antibody is captured by affinity. In the case of the conductimetric immunosensor, a secondary antibody labelled with a gold nanoparticle

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is attached to the primary specific antibody in order to amplify the affinity event and obtain a good conductive response.

As the dielectric properties of the biological systems are very remarkable, the developed devices can exhibit a good impedimetric response.

3.1. Functionalization of the impedimetric immunosensor

In the case of the impedimetric immunosensor, the passive adsorption technique was used. By means of this method the chemical changes on the impedimetric immunosensor surface follow basically two steps:

i. Step I, antigen immobilization on the IDμE;

ii. Step II, specific antibody capture in the competition step.

The chemical recognition layer was deposited on top of the interdigitated μ-electrodes area (fingers and inter-digits space). These chemical procedures are schematically shown in Figure 3.

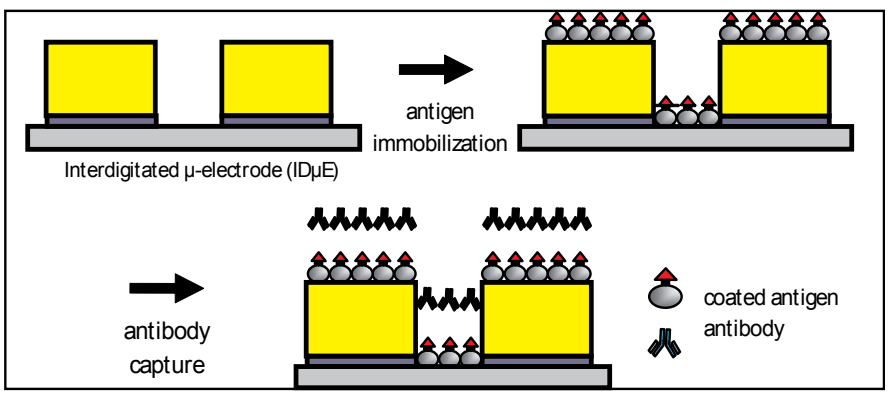

Figure 3. Schematic diagram of the complete assay system performed on the IDμE's for the impedimetric immunosensor.

3.2. Functionalization of the conductimetric immunosensor

In this case, a covalent immobilization technique was applied. Then, the chemical changes on the conductimetric immunosensor surface follow five steps, two previous steps for the immunosensor surface functionalization and other three steps for the immunosensor reaction:

i. Step I, protection of interdigitated μ-electrodes with N-acetylcysteamine;

ii. Step II, immunosensor surface functionalization with GPTS;

iii. Step III, covalent immobilization of the antigen on the IDμE;

iv. Step IV, specific primary antibody (Ab_1) capture in the competition step;

v. Step V, secondary labelled with gold antibody (Ab_2) capture.

Due to the covalent immobilization, the chemical recognition layer was deposited only on the gap of the interdigitated μ-electrodes. The complete functionalization procedures of the conductimetric immunosensor are schematically shown in Figure 4.

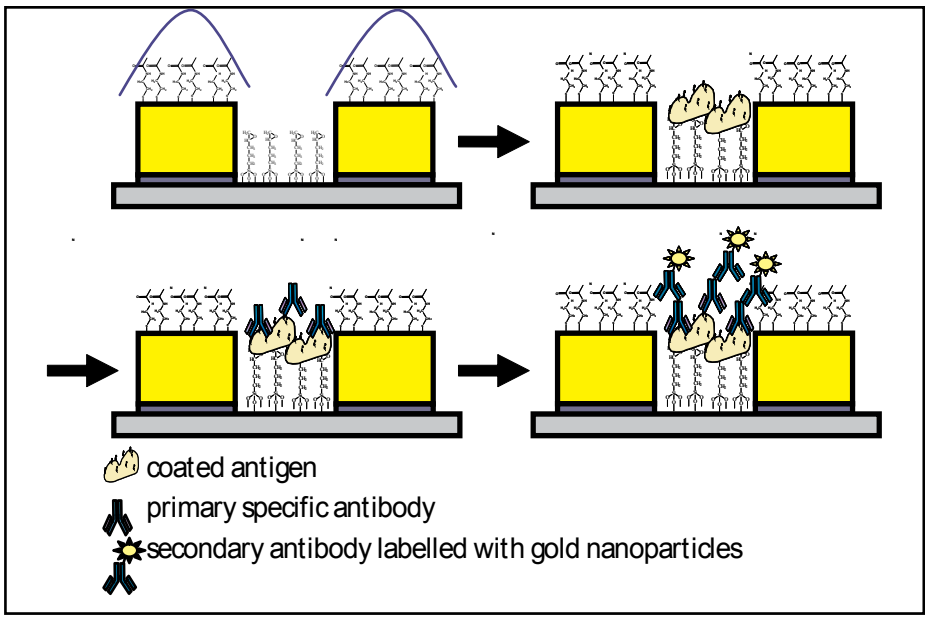

Figure 4. Schematic diagram of the complete assay system performed on the IDμE's for the conductimetric immunosensor.

4. Pesticide residues detection

Both types of immunosensors described in previous sections (impedimetric and conductimetric) have been tested for the detection of free pesticide samples. For that, *atrazine*, a widely used pesticide in the wine industry, as well as for the test of novel biosensors [8-12], has been used as pesticide of test.

Atrazine, a widely used selective herbicide for the control of annual grasses and broadleaved weeds, has often been found in drinking water, and therefore, they are a potential threat for the public health [13-16]. The European Community has established maximum residue level for residues of this herbicide in wine grapes in 50 $\mu g\ L^{-1}$.

The competitive reaction carried out on the interdigitated μ-electrodes has been performed in buffer solution (impedimetric and conductimetric immunosensor) and in wine samples (conductimetric immunosensor). The performance details of both types of devices as atrazine detectors are detailed below.

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4.1. Impedimetric immunosensor based on singly frequency impedance measurements

In this case, experiments were carried out with different atrazine concentrations (1.6 – 1000 μg L^{-1}) during the competition step in order to qualitatively show how the impedimetric immunosensor is sensitive to the atrazine concentration using the detection method of single frequencies. These experiments were performed using different ID's samples for every concentration.

Several frequencies of work between 35 kHz to 1 MHz were chosen in order to take into account all regions of the impedance spectrum between the middle and high frequencies. Frequencies below 30 kHz were excluded from the sensor characterization because their unstable response. As it is well known, these frequencies are related to the Warburg impedance and to the double-layer capacitance. Typically, in the impedimetric immunosensors, some scattering was found in the impedance variations at these frequencies due to the influence of these parameters. Therefore, these frequencies were found inadequate to obtain confident results.

Measurements were also differential in order to suppress the non-ideal effects related to the geometry or technology of the IDμE's. Due to that, the change in the impedance (ΔZ_{abs} and ΔZ_{rel}) was calculated between the Step I and the Step II of the impedimetric immunosensor functionalization. The impedance variations, in relation with different atrazine concentrations and with the frequency of work, were analyzed and the results are shown below. Several detection curves at different frequencies of work have been executed using the variation of the module of impedance (|Z|), the variation of the real part of the impedance (Zre) and the variation of the imaginary part of the impedance (Zim). These changes have been obtained taking into account the absolute and relative differences described above. In order to analyze the impedimetric immunosensor response, detection curves obtained at these frequencies were compared and studied separately for each frequency range, as it can be seen in Figures 5, 6 and 7.

These figures only show some examples of the detection curves obtained by means of the relative difference of impedance. Detection curves using the absolute difference of impedance are not included in this manuscript because their shape is very similar to the detection curves using the relative difference of impedance and because, as it will be noticed, important improvements in the features of the atrazine assay (LOD) have been found, at middle frequencies (35.37 kHz to 131.95 kHz), when the relative difference is used, in comparison to the absolute difference in all cases.

Figure 5 shows the response of a typical impedimetric immunosensor using the relative difference of |Z| at three different frequency ranges: from 35.37 kHz to 131.95 kHz (curves 5a); from 401.96 kHz to 602.70 kHz (curves 5b); from 738.01 kHz to 1 MHz (curves 5c). As additional examples, Figure 6 and 7 show the response of a typical impedimetric immunosensor using the relative difference of Zre and Zim respectively, for the frequency range from 738.01 kHz to 1 MHz. As it can be seen in the figures, the inclusion of the antibody in the Step II implies a higher increase in the absolute value of the impedance when low atrazine concentrations are included in the competition step at all the frequency ranges under study.

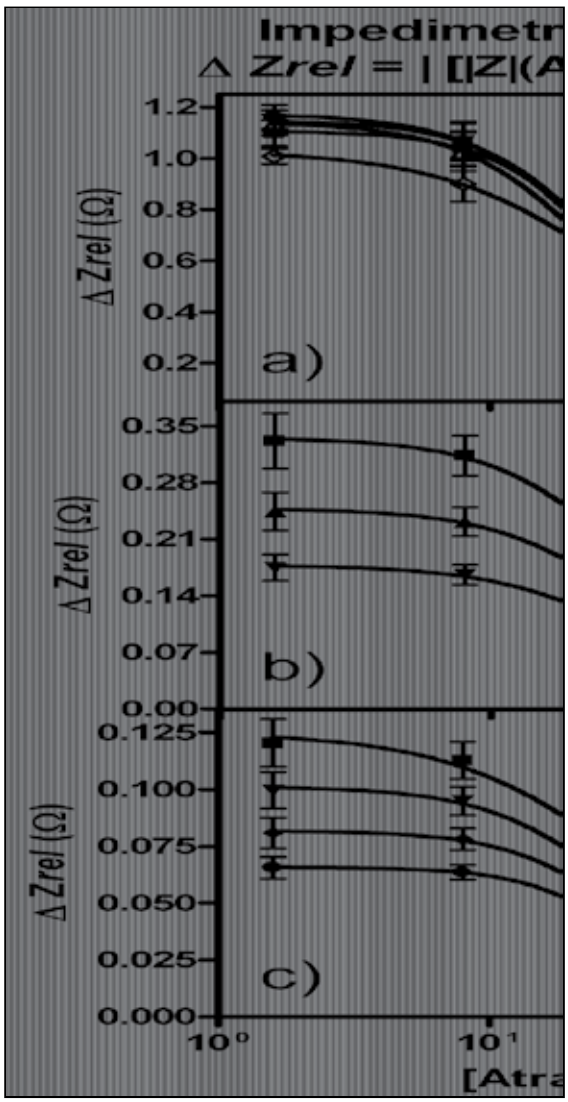

Figure 5. Impedimetric immunosensor responses using the relative difference of Zre at high frequencies from 738.01 kHz to 1 MHz.Impedimetric immunosensor responses using the relative difference of |Z| at: a) middle frequencies from 35.37 kHz to 131.95 kHz; b) high frequencies from 401.96 kHz to 602.70 kHz; c) high frequencies from 738.01 kHz to 1 MHz. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

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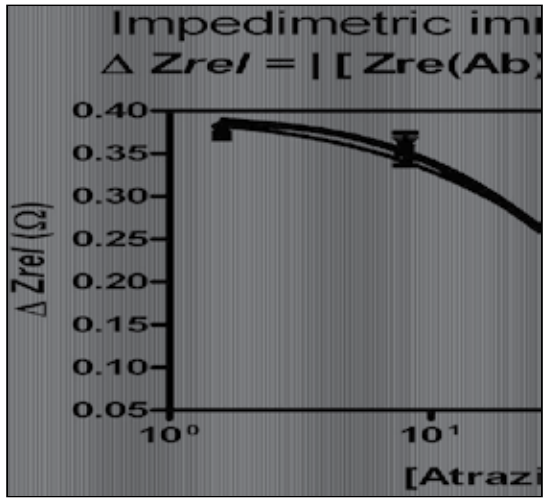

Figure 6. Impedimetric immunosensor responses using the relative difference of Zre at high frequencies from 738.01 kHz to 1 MHz.Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

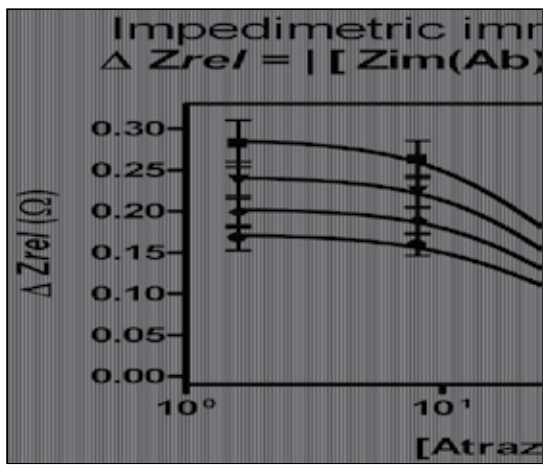

Figure 7. Impedimetric immunosensor responses using the relative difference of Zim at high frequencies from 738.01 kHz to 1 MHz. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

A characteristic of the behaviour of the impedimetric immunosensor and the detection method to single frequencies is that the difference between the response curves is very small, almost imperceptible. This can be appreciated in Figures 5a and 6. This could be used for the auto-tuning of the sensor and to reduce the effect of the dispersion of the initial capacitance of the

devices in the measurement, by using two or more frequencies in the measurement instead of only one.

The main influence of choosing the absolute or the relative difference is observed in the values of the features assays (LOD) obtained for the different ranges of frequency of work. This table summarizes the values of the LOD in μg L^{-1} for some frequencies of work used to represent each part of the spectrum and for the three kinds of impedance considered.

In Table 1 an important improvement in the LOD at middle frequencies (35.37 kHz to 131.95 kHz) is found when the relative difference is used, in comparison to the absolute difference in all cases. On the other hand, at high frequencies, from 401.96 kHz, the differences in LOD between the absolute difference and relative difference are small in comparison with the differences obtained at middle frequencies. In some cases the LOD of the absolute difference becomes the minimum.

Features of the atrazine assays	ΔZ	frequency of work (kHz)					
		35.37	**64.95**	**131.95**	**401.86**	**738.01**	**1000**
LOD, μg L^{-1}	$\lvert\lvert Z\rvert(Ab)-\lvert Z\rvert(AT)\rvert$	13.77±0.16	13.34±0.16	11.95±0.18	11.93±0.17	9.79±0.19	9.57±0.17
	$\left\lvert\frac{\lvert Z\rvert(Ab)-\lvert Z\rvert(AT)}{\lvert Z\rvert(AT)}\right\rvert$	10.02±0.21	8.32±0.22	6.87±0.23	9.87±0.20	8.56±0.23	12.06±0.17
	$\lvert Zre(Ab)-Zre(AT)\rvert$	14.30±0.13	13.63±0.16	15.96±0.17	11.03±0.39	9.83±0.23	8.41±0.22
	$\left\lvert\frac{Zre(Ab)-Zre(AT)}{Zre(AT)}\right\rvert$	8.95±0.22	7.62±0.23	5.76±0.25	19.16±0.37	8.25±0.30	8.01±0.25
	$\lvert Zim(Ab)-Zim(AT)\rvert$	28.61±0.29	19.60±0.25	9.55±0.36	7.43±0.28	7.19±0.23	7.81±0.23
	$\left\lvert\frac{Zim(Ab)-Zim(AT)}{Zim(AT)}\right\rvert$	12.52±0.21	11.95±0.17	7.51±0.21	9.10±0.23	8.61±0.22	9.46±0.21

[a] Parameters extracted from the four-parameter equation used to fit the standard curve.

Table 1. Features of the atrazine assays [a]. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

Comparing directly the three cases, it is clear that the best response is obtained using the Zre variation by means of relative difference at middle frequencies (*5.76 – 8.95 μg L^{-1}*), and that the worst response is obtained using the Zim variation by means of absolute difference also at middle frequencies (*19.60 - 28.61 μg L^{-1}*), although in all cases the limits of detection obtained are still lower than the MRL (50 μg L^{-1}) established by European Union.

These results can be seen more clearly in Figure 8. This figure shows the evolution of the LOD in the frequency ranges analyzed, for the variation of |Z| (curve 8a), Zre (curve 8b) and Zim (curve 8c). In Figure 8b is clear the improvement in the limit of detection at middle frequencies, when the relative difference of the Zre is used instead of the other impedance variation, as it was observed above.

Although the best responses were obtained using the real part of the impedance, when the module of the impedance is used to find the features of the assay, measurements show lower

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dispersion between different devices, what means a better compensation of the effects of geometry or technology of the IDμE's. As consequence the immunosensor resolution (LOD) is almost constant in the whole range of frequencies under study, both in the case of the absolute difference or relative difference, as it can be comprehended in Figure 8a. This is important because, the frequency of work can be chosen independently of the immunosensor resolution, therefore providing a degree of freedom to the design of the circuitry of control.

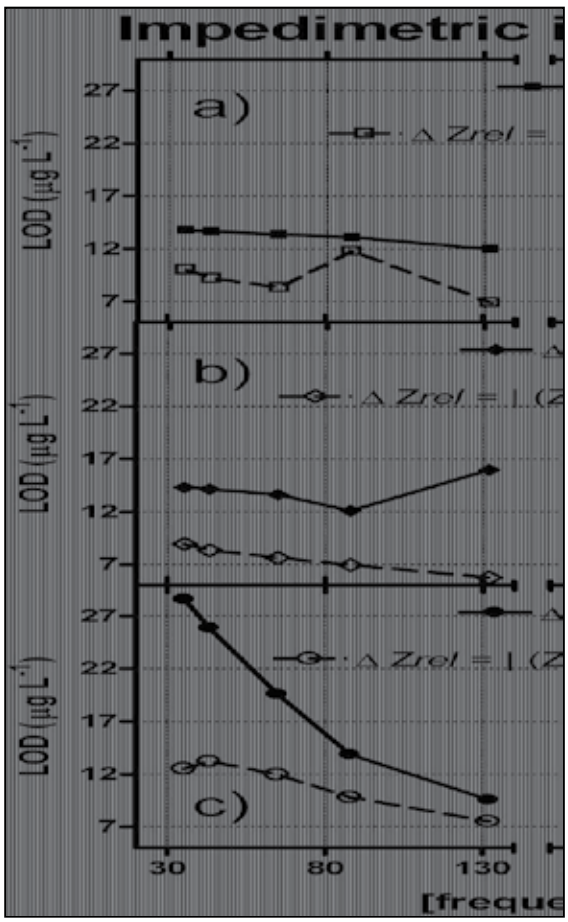

Figure 8. Evolution of the limit of detection by means of absolute and relative variation of: a) module of impedance; b) real part of impedance; c) imaginary part of impedance Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928,

4.1.1. Multi-sine burst signal

The characterization technique previously shown is based on the monitoring of the electrical response of the device under test after application of only one AC signal in a single frequency.

By the burst technique, a multi-sine signal is used in order to perform simultaneous measurements at different frequencies at the same time. These signals are useful for fast measuring biological objects which electrical properties changes quickly with time, in order to obtain the frequency transfer function free of modulation.

Mathematically, a multi-sine signal is formed by the sum of N tones, each one with its own phase. To be precise, in bio-impedance measurement scenario is just necessary a signal burst and not a continuous time signal. An example of a multi-sine burst signal can be seen in Figure 9.

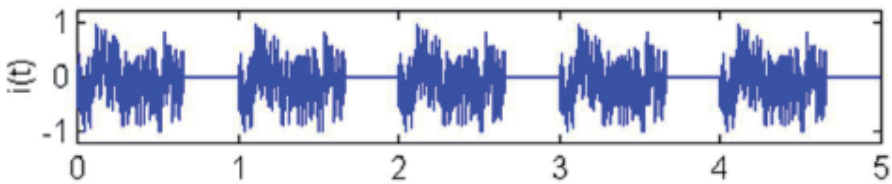

Figure 9. Five multi-sine burst signal with 1ms of period.

Signal generation and acquisition is a crucial part in the hardware implementation since they are the connector between the analog world and the discrete world of digital signal processing algorithms. Multi-sine signal is based on Bilateral Quasi-Logarithmic (BQL) [17] frequency distribution and is implemented on a custom arbitrary signal waveform generator based on a Field Programmable Gate Array (FPGA). Acquisition is done with a specific front-end connected to a fast A/D board which transfers the data to the Digital Signal Processor (DSP) silicon for real time processing. Thus, it is possible to make simultaneous measurements in a wide spectrum in a short time. The main idea is to use broadband signals because the energy is spread over the desired frequency range. A photo of the waveform generator can be seen in Figure 10, whereas Figure 11 shows a schematic of the signal generation and acquisition configuration.

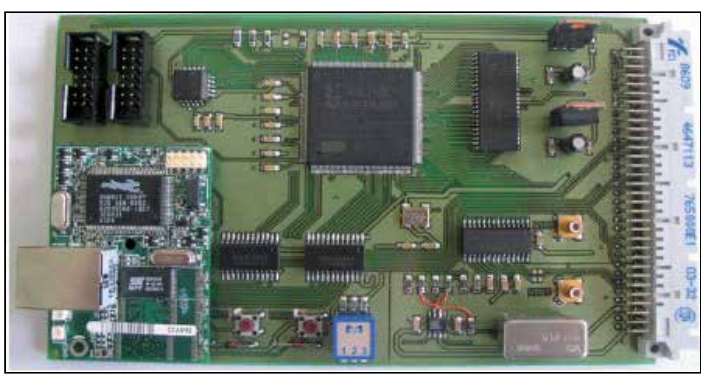

Figure 10. Multi-sine generator board.

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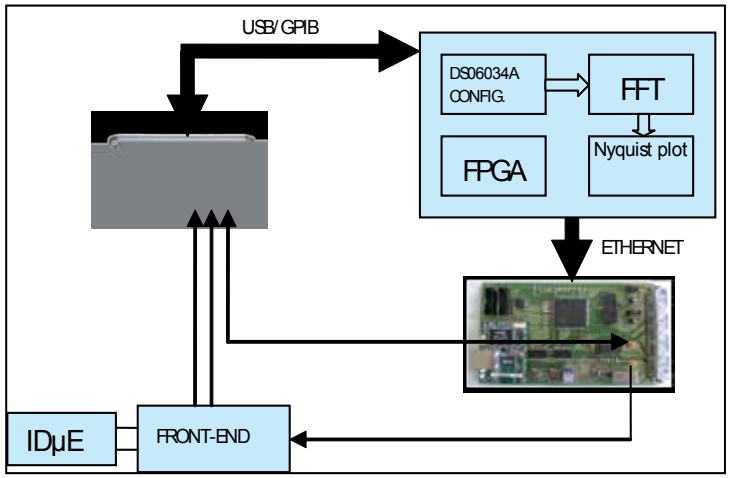

Figure 11. Signal generation and acquisition configuration.

In this case the data acquisition system was designed to only interpret the region semicircle and not the area of diffusion (Nyquist plot). Likewise, the number of points (fundamental frequencies) was chosen in 21 because some hardware limitations related to the memory, such as capacity and velocity (reading and writing) and to avoid difficulties in the design and benefits worsen. An example of the plots obtained by this method is shown in Figure 12.

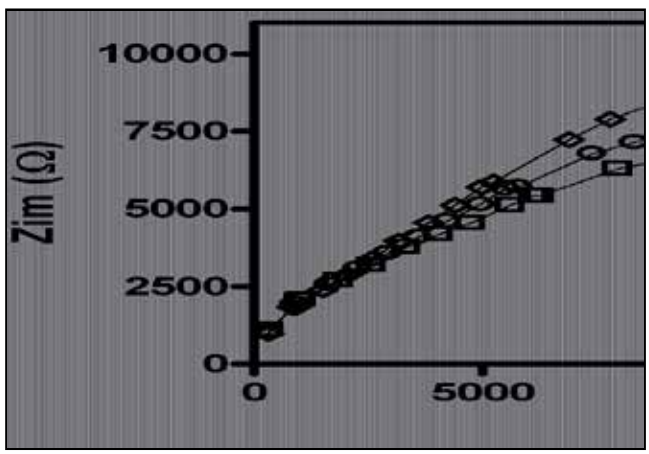

Figure 12. Immunosensor response obtained from a multi-sine burst signal.

In this figure we can see how the system is able to represent different curves that are related to the antibody concentration. Although in these curves the bandwidth is smaller in comparison with the previous case, the curves obtained are enough to appreciate differences in the impedance contribution. Then, these differences could be also related to differences in parameters of the equivalent circuit, and thus, this system could be used as detection system.

The work related to the multi-sine burst signal was performed in collaboration with Dr. R. Bragós and co-workers, members of the *Divisió Intrumentació i Bioenginyeria (UPC)*.

4.2. Conductimetric immunosensor

In the case of the conductimetric immunosensor, impedimetric measurements were discarded as detection method, because the inclusion of the gold particles (conductive elements) *subtracts* the contribution of the secondary antibody (resistive element). Therefore, only DC measurements were applied in this case.

4.2.1. Atrazine detection in buffer samples

Atrazine concentrations between 0.32 to 2000 μg L^{-1} during the competition step (Step IV) were included. The electrodes were covered by a diluted PBS solution and the measurements were executed to +25 and +100 mVdc bias. The results obtained by this method are represented in Figure 13.

The limits of detection obtained for the atrazine residues detection using the conductimetric immunosensors, when the competitive assay was performed in buffer solution, were *0.446 μg L^{-1}* (100 mVdc bias) and *1.217 μg L^{-1}* (25 mVdc bias), both far below the MRL.

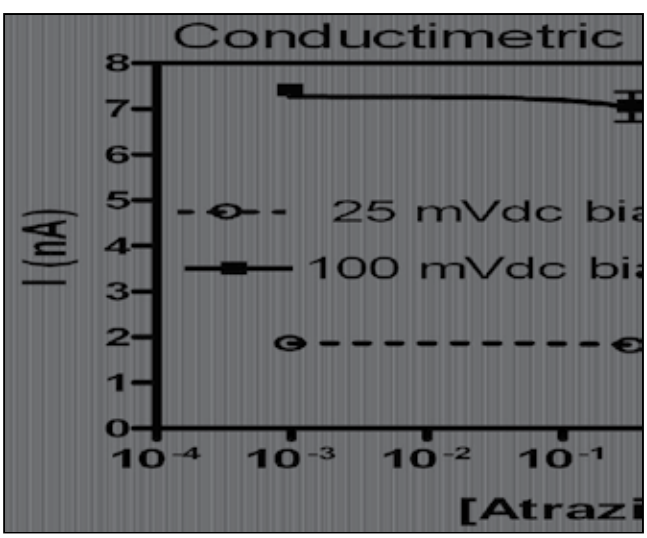

Figure 13. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for the atrazine detection in relation with the presence of gold particles (40 nm). Buffer solution was used for the competitive reaction. Measures were taken in diluted PBS solution. See Table 2 for the features of the atrazine assay.

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Features of the atrazine assays [a]	Conductimetric immunosensor (buffer)	
	25 mV	100 mV
IC_{50}, μg L^{-1}	8.47±0.19	5.29±0.14
LOD, μg L^{-1}	**1.217**	**0.466**
R^2	0.89	0.91

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 2. Features of the atrazine assays [a]

4.2.2. Atrazine detection in red wine samples

After the demonstration of both types of immunosensors for the detection of free pesticide in buffer samples, the conductimetric immunosensor was also studied using a complex matrix such as red wine samples. Red wine was chosen instead of other matrixes such as white wine, water or grape juice, because its strong matrix effect. Therefore, if the red wine matrix effect can be measured, the other matrix effects will be easier. Again, atrazine was used as pesticide of test.

Red wine samples were obtained from a local retail store and used, on a first instance, to evaluate the extension of the potential non-specific interferences. Prior measurements with the immunosensors, the wine samples were purified by solid-phase extraction (SPE) using LiChrolut RP-18 (500 mg, 6 mL) sorbent (Merck, Darmstadt, Germany) pre-conditioned with MeOH (3 mL), and MeOH:Mili-Q water (15:85, v/v, 3 mL) at a flow rate of 3 mL min^{-1}. The wine samples (3 mL) were loaded at 5mL min^{-1}, and the SPE cartridges washed with of MeOH:Mili-Q water (70:30, v/v, 1 mL), dried, and finally eluted with of MeOH:Mili-Q water (80:20, v/v, 1 mL). The fractions collected were diluted 1:50 in PBST and used for the impedimetric measurements [1].

Again, the experiments carried out in this section included atrazine concentrations (0.32 – 2000 μg L^{-1}) during the competition step (Step IV), the electrodes were covered by a diluted PBS solution, and the measurements were performed to +25 and +100 mVdc bias.

The results obtained by this way are shown in Figure 14. The limits of detection obtained for the detection of residues of atrazine, when the competitive assay was performed in red wine samples, were *0.489 μg L^{-1}* (100 mVdc bias) and *0.034 μg L^{-1}* (25 mVdc bias). As in the previous cases, the MRL required by EC was largely reduced.

4.2.3. New approach: A flexible device

As it was proven in the previous sections, both types of immunosensors described in this chapter have been able to detect residues of atrazine when buffer samples were used for the competitive assay. In both cases, the transducer was supported by a PYREX substrate. In this section a new approach, flexible plastic substrates, is introduced, in order to hardly reduce the cost of the device.

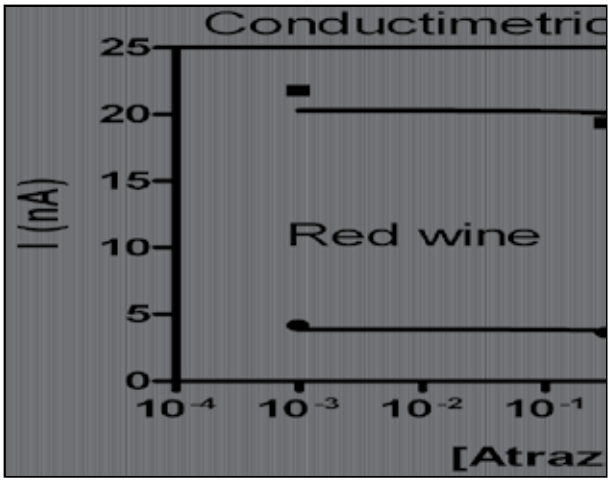

Figure 14. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for atrazine detection in relation with the presence of gold particles (40 nm). Red wine samples were used for the competitive assay. Measures were taken in diluted PBS solution. See Table 3 for the features of the atrazine assay.

Features of the atrazine assays [a]	Conductimetric immunosensor (red wine)	
	25 mV	100 mV
IC_{50}, µg L^{-1}	19.05±0.10	20.54±0.07
LOD, µg L^{-1}	**0.034**	**0.489**
R^2	0.96	0.98

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 3. Features of the atrazine assays [a]

4.2.3.1. Flexible interdigitated µ-electrode (FIDµE)

Although Pyrex properly complies with the conditions of isolation and compatibility necessary, the possibility of a flexible sensor was also explored. Therefore, flexible interdigitated µ-electrodes (FIDµE) for biosensor applications were fabricated. A sample of the FIDµE's developed can be seen in Figure 15.

The flexibility of the FIDµE's was reached using a plastic substrate. The plastic chosen as new substrate was polyethylene naphthalate (PEN), 0.075 mm, purchased from Goodfellow Cambridge Limited.

The fabrication procedure of the FIDµE's is as follows: Thin Au (150 - 200 nm thickness) interdigitated µ-electrodes (IDµE's) with, 30 µm thick with electrode gap of 30 µm were patterned on a PEN substrate. As a good adhesion between gold and the PEN substrate exist, the chromium layer was avoided. Before metal deposition (performed by sputtering), the PEN substrate was cleaned using absolute ethanol. Then, the interdigitated µ-electrodes were then patterned by a photolithographic metal etch process.

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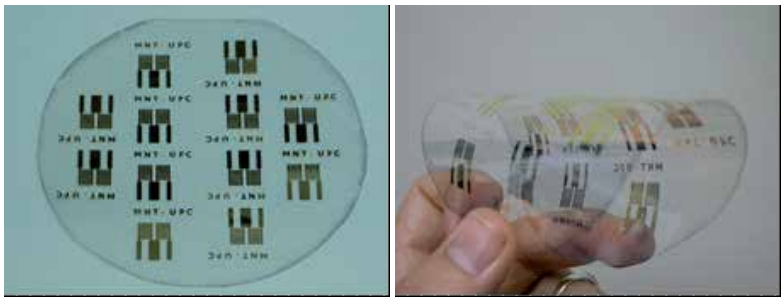

Figure 15. Flexible interdigitated μ-electrodes fabricated: a) top view; b) demonstration of flexibility. Reprinted from Microelectronic Engineering, 87/2, Enrique Valera, David Muñiz, Ángel Rodríguez,, Fabrication of flexible interdigitated μ-electrodes (FIDμEs) for the development of a conductimetric immunosensor for atrazine detection based on antibodies labelled with gold nanoparticles, 167–173, Copyright (2010), with permission from Elsevier

In order to apply FIDμE's as biosensor, the electrodes surface must be activated. For this reason, SiO_2 was deposited by sputtering on the electrodes surface, because silicon oxide surface contains reactive SiOH groups, which can be used for covalent attachment of organic molecules and polymers [18].

4.2.3.2. Conductimetric immunosensor using FIDμE's

The functionalization performed in this case followed the covalent immobilization technique previously explained. Atrazine concentrations between 0.32 – 2000 μg L^{-1} were included. The electrodes were covered by a diluted PBS solution and the measurements were performed with a bias of +100 mVdc. The response curve obtained can be seen in Figure 16. In this case, the curve is based on the current through the electrodes, related to amount of gold particles.

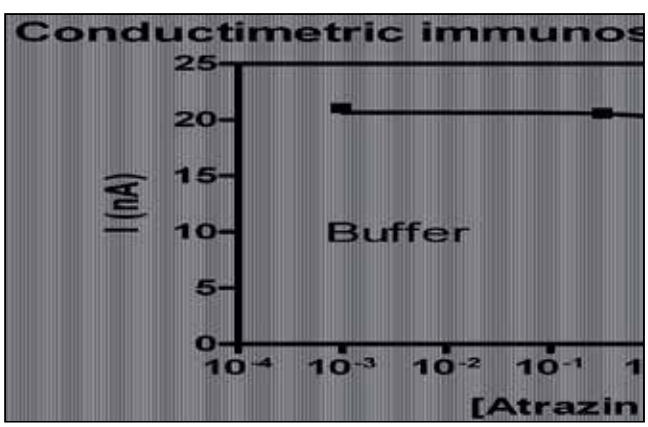

Figure 16. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for the atrazine detection in relation with the presence of gold particles (40 nm). Buffer solution was used for the competitive reaction. Measures were taken in diluted PBS solution. See Table 4 for the features of the atrazine assay.

In this case, the LOD is *2.975 μg L^{-1}* and the R^2 was 0.9922. Nevertheless, another important conclusion is that in this case, a very small gap is not needed to achieve the atrazine detection in low concentrations.

The most relevant analytical features of the conductimetric atrazine assay are summarized in Table 4.

Features of the atrazine assays [a]	Conductimetric immunosensor (buffer)
IC_{50}, μg L^{-1}	24.17±0.03
LOD, μg L^{-1}	**2.975**
R^2	0.99

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 4. Features of the atrazine assays [a]

5. Conclusions

In this chapter, two immunosensors based on interdigitated μ-electrodes together with three strategies for the excitation and readout of their response are described. The goal of the readout methods was to reduce the time of measurement. They have been implemented and characterized in depth.

It can be concluded that each of the methods represent an important reduction of the time of measurement and a reduction of the complexity, and cost of the electronics required for its implementation compared to broadband spectroscopy. Each of the proposed methods has particularities: Using the method of single frequency measurements, the use of non labelled antibodies is allowed, and the complexity of the electronics required is low. The maximum simplicity in the electronics circuitry is probably achieved with DC measurements but this makes necessary the use of antibodies labelled with conductive particles. In these two cases the measurements can be done in seconds. The expense in terms of sensitivity is low as it can be seen from the LOD obtained with each of them: in both cases the LOD is well below the MRL established for the detection of atrazine by the EC.

The third method, based in burst signals, allows for the fast measurement of the impedance of the devices, also in a few seconds, in the broad band from low to high frequencies used in the conventional impedance spectroscopy. Its advantage comes from the fact that it uses a broad band measurement and therefore it is expected to provide LOD similar to the conventional broadband spectroscopy. This method requires more complexity in the electronics to be used for the excitation of the sensors and also a higher complexity in the analysis of the readout signals of the devices. Nevertheless can been readily programmed in common portable devices with little extra electronics.

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References

[1] Ramón-azcón, J, et al. An impedimetric immunosensor based on interdigitated microelectrodes (IDμE) for the determination of atrazine residues in food samples. Biosensors & Bioelectronics, (2008). , 1367-1373.

[2] Laureyn, W, et al. Nanoscaled interdigitated titanium electrodes for impedimetric biosensing. Sensors and Actuators B, (2000). , 360-370.

[3] Hleli, S, et al. Atrazine analysis using an impedimetric immunosensor based on mixed biotinylated self-assembled monolayer. Sensors and Actuators B, (2006). , 711-717.

[4] Helali, S, et al. A disposable immunomagnetic electrochemical sensor based on functionalised magnetic beads on gold surface for the detection of atrazine. Electrochimica Acta, (2006). , 5182-5186.

[5] Valera, E, Rodríguez, Á, & Castañer, L. M. Steady-State and Transient Conductivity of Colloidal Solutions of Gold Nanobeads. Nanotechnology, IEEE Transactions on, (2007). , 504-508.

[6] Valera, E, et al. Conductimetric immunosensor for atrazine detection based on antibodies labelled with gold nanoparticles. Sensors and Actuators B, (2008). , 95-103.

[7] Valera, E, et al. Determination of atrazine residues in red wine samples. A conductimetric solution. Food Chemistry, (2010). , 888-894.

[8] Fredj, H. B, et al. Labeled magnetic nanoparticles assembly on polypyrrole film for biosensor applications. Talanta, (2008). , 740-747.

[9] Salmain, M, Fischer-durand, N, & Pradier, C. -M. Infrared optical immunosensor: Application to the measurement of the herbicide atrazine. Analytical Biochemistry, (2008). , 61-70.

[10] Helali, S, et al. Surface plasmon resonance and impedance spectroscopy on gold electrode for biosensor application. Materials Science and Engineering C, (2007). , 588-593.

[11] Zacco, E, et al. Electrochemical biosensing of pesticide residues based on affinity biocomposite platforms. Biosensors and Bioelectronics, (2007). , 1707-1715.

[12] Anh, T. M, et al. Detection of toxic compounds in real water samples using a conductometric tyrosinase biosensor. Materials Science and Engineering C, (2006). , 453-456.

[13] Steinheimer, T. R. HPLC Determination of Atrazine and Principal Degradates in Agricultural Soils and Associated Surface and Ground Water. J. Agric. Food Chem., (1993). , 588-595.

[14] Kaune, A, et al. Soil Adsorption Coefficients of s-Triazines Estimated with a New Gradient HPLC Method. J. Agric. Food Chem., (1998). , 335-343.

[15] Moore, A, & Waring, C. P. Mechanistic Effects of a Triazine Pesticide on Reproductive Endocrine Function in Mature Male Atlantic Salmon (Salmo salar L.) Parr. Pesticide Biochemistry and Physiology, (1998). , 41-50.

[16] Papiernik, S. K, & Spalding, R. F. Atrazine, Deethylatrazine, and Deisopropylatrazine Persistence Measured in Groundwater in Situ under Low-Oxygen Conditions. J. Agric. Food Chem., (1998). , 749-754.

[17] Ionescu, R. E, et al. Impedimetric immunosensor for the specific label free detection of ciprofloxacin antibiotic. Biosensors and Bioelectronics, (2007). , 549-555.

[18] Yuqing, M, Jianguo, G, & Jianrong, C. Ion sensitive field effect transducer-based biosensors. Biotechnology Advances, (2003). , 527-534.

Chapter 11

Biophoton Emissions in Sulfonylurea-Herbicide-Resistant Weeds

Hideki Nukui, Hidehiro Inagaki,
Hiroyuki Iyozumi and Kimihiko Kato

Additional information is available at the end of the chapter

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1. Introduction

In the 1950s, a new weed biotype was first reported to be resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) [1]. Since then, the number of weed biotypes resistant to various herbicides has increased dramatically and reported worldwide. There are 393 biotypes of 211 weed species that have evolved resistance to compounds from all the major groups of herbicides [2]. Biotypes of 127 weed species were found to be resistant to herbicides in the acetolactate synthase (ALS) inhibitor group, one of the most successful of the herbicide groups [2].

To control weeds efficiently, it is necessary to identify their herbicide resistance before applying herbicides in the field; however, some herbicides, including ALS inhibitors, act slowly. Therefore, it is difficult to quickly identify whether the weeds in the field are resistant. Recently, some rapid methods for identifying resistance to ALS inhibitors have been developed. For example, an identification method based on an *in vivo* ALS activity assay [3-7] and one based on the regrowth of roots or shoots from weed samples treated with herbicide [8-10] have been developed for identification of sulfonylurea (SU), the major compound group in ALS inhibitors. Here, we propose an alternative method of biophoton measurement for identifying herbicide resistance more simply and rapidly. In this chapter, we introduce the method by providing examples, particularly for detecting SU herbicide-resistant weeds.

2. What are biophotons?

All living organisms spontaneously generate ultraweak photon emissions, commonly referred to as "biophotons," which originate from biochemical reactions in their cells. Biophotons

can be detected easily, nondestructively, and in real time by using a photon counter equipped with highly sensitive photomultiplier tubes. They have accordingly gained considerable attention as a new tool by which to identify the condition of living organisms. The emission intensity of biophotons is extremely weak at 10^0–10^3 photons per second per square centimeter of surface area, and in a nearly continuous spectrum within the optical range of at least 200–800 nm [11, 12]. Biophotons are considered to be associated with the oxidative metabolic reactions essential for life activities, unlike the bioluminescence observed in fireflies, which employs a luciferin–luciferase system. Biophotons were first observed by Coli (1955) in seedlings of plants such as wheat and lentils [13]. Thereafter, this ultraweak biophoton emission was reported by many researchers at the organism, tissue, and cellular levels [11, 14]. It has been suggested that reactive oxygen species (ROS) are the driving force behind biophoton emission because biophotons disappear if the oxygen supply is cut off [15]. The majority of biophotons, if not all, are believed to be emitted in the process of oxidation of substances such as unsaturated fatty acids, amino acids, and polyphenols. These substances are peroxidized and excited by ROS or enzymes such as NADPH oxidase, peroxidase, lipoxygenase, and cytochrome P450. Excited carbonyl compounds generated through this process are considered to be important emitting molecules [15]. In some cases, the excited carbonyl compound is produced as a result of free radical recombination reactions [16]; however, because the quantum efficiency of excited carbonyl is relatively low, the biophotons detected from the living body, to some extent, appear to originate in fluorescent substances in cells, to which the excess energy of exited carbonyl is transferred [17]. In plants, chlorophyll is a good example of this energy transfer. In green seedlings containing large amounts of chlorophyll, biophoton emission was observed only in the range of red light, whereas its original spectrum of biophoton emission was between yellow and orange [18]. In addition, among ROS, only singlet oxygen species emit light. However, except in special cases, the concentration of singlet oxygen in the cell is not sufficiently high to produce biophotons that can be detected with a photon counter [17]. Therefore, the detailed mechanism of biophoton emission in the living body has not been fully clarified. Biophoton research is particularly advanced in the medical field, where photon counters have been experimentally applied to tumor detection [19], brain monitoring [20], and diagnosis of renal failure [21].

3. Biophoton emissions from plants in response to stress

In plants, the most important role of biophotons is to respond to biological (e.g., pathogens, insects, and wounds) and non-biological (e.g., temperature, drought, salt concentration, and chemicals) stimuli to which biophoton increments are primarily observed in plants that acclimatize to these stimuli. Biophoton emission occurs in response to the destruction of cells and as a result of controlled biochemical reactions in cells. Many studies have attempted to detect stress responses in plants using biophotons.

3.1. Response to biological stress

It is well known that pathogenic infections induce a significant increase in biophoton emissions in plants. On the basis of the disease-resistance reactions of plants, biophotons are classified

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into two types: the relatively weak emissions observed during the early stages of the resistance reaction [22], and strong emissions from cells exhibiting programmed cell death (PCD) during the middle stages of the resistance reaction to localize the pathogen from healthy cells [23]. In sweet potatoes undergoing PCD as a result of inoculation with *Fusarium oxysporum*, it was reported that the wavelength composition of photon emission considerably shifted toward a shorter wavelength as compared with that of untreated samples, indicating that this was a luminous phenomenon quantitatively different from the one observed under normal conditions [24]. In addition, in the early stage of the resistance reaction, biophoton emission has been proven to occur through the signaling cascade that occurs in resistance reactions [25]. Taking into consideration the first report on the direct link between resistance reaction and biophotons, the subsequent report presents a very important finding. Because of this direct linkage, biophotons were enhanced during the early resistance reaction in plants pretreated with plant activators that enhance the resistance reaction to pathogens [22]. Thus, the two types of biophoton emissions found in disease-resistance reactions are clearly based on controlled biochemical oxidative reactions in cells.

Other than pathogenic infection, exogenously applied plant hormones (gibberellins, auxins, salicylic acid, jasmonic acid) and herbivore damage are reported to induce biophoton emissions [24, 26, 27].

3.2. Response to non-biological stress

Biophoton emission from plants has been reported to dramatically increase under high temperatures that are fatal to plants [28]. This is a good example of biophoton emission in response to cell destruction, in which uncontrolled oxidative reactions occur. In addition, an increase in biophoton emission was observed during acclimation of the plants to high temperature (but not fatal levels) [24]. In contrast, because a transient increase in biophotons is also caused by a sudden decrease in temperature, it has been proposed that this phenomenon can be applied for testing the resistance of plants to frost damage [29].

In addition to the response to temperature, plants also exhibit biophoton emission responding to salt and drought stresses. When azuki bean sprouts were treated with NaCl solution, biophoton emission weakened up to 1 M, but increased at 4.5 M [30]. A mild increase in biophoton emission also occurred under dry conditions, and an extremely strong increase in biophoton intensity was observed when the sprouts that had been dried over many hours were permitted to absorb water again [31]. Thus, under conditions of severe stress that are rarely conducive for the survival of plants, the increment in biophoton emission is a result of the destructive oxidative process of cells.

3.3. Response to herbicide treatment

Chemicals such as herbicides can also act as a non-biological stress for plants. Paraquat is a typical photosystem I inhibitor that acts on the photosynthetic membrane system in photosystem I. Paraquat causes tissue damage by generating ROS through the reduction of molecular oxygen. Intense biophoton emission was observed from the leaves of tobacco plants

treated with paraquat (Figure. 1) [32]. Furthermore, it was confirmed that biophoton emission from leaves treated with paraquat decreased when antioxidants such as catechin were additionally applied [32]. Therefore, it was suggested that the biophoton emission from paraquat treatment was strongly related to the ROS generated in cells.

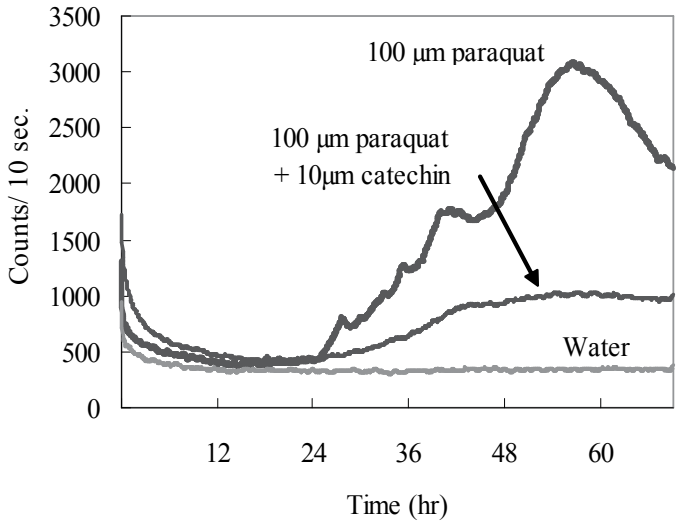

Figure 1. Effectiveness of catechin on biophoton emissions from leaves of tobacco treated with paraquat [32].

The effects of 18 herbicides on biophoton emission from cultured rice cells have been examined (Nukui *et al*., not published). Among the herbicides investigated, 10 increased the biophoton emissions from rice cells compared with solvent treatment, whereas six decreased emissions, and two had little effect when treated at 100 ppm (Table 1). Some typical results are shown in Figure 2.

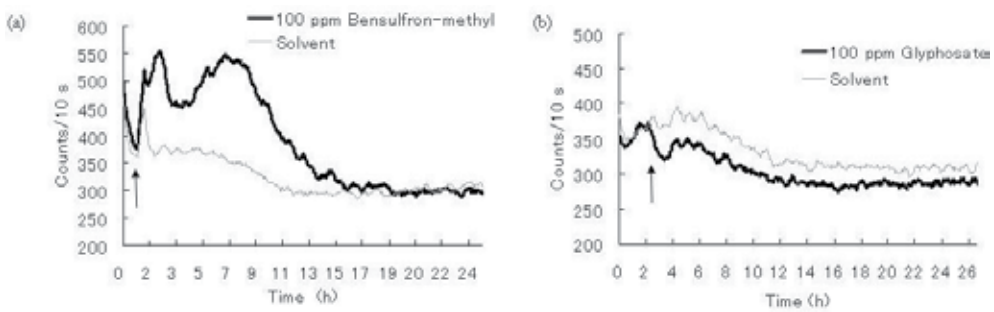

Figure 2. Biophoton emission from cultured rice cells treated with herbicides. (a), bensulfron-methyl; (b), glyphosate. Values represent the average of duplicates. Arrows indicate the time when cells were treated with the herbicides.

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Herbicide	Biophoton[z)]	Mode of action
Bensulfuron-methyl	◎	Inhibition of acetolactate synthase (ALS)
Simetryn	○	Inhibition of photosynthesis at photosystem II
Atrazine	◎	Inhibition of photosynthesis at photosystem II
Linuron	◎	Inhibition of photosynthesis at photosystem II
Oxadiazon	○	Inhibition of protoporphyrinogen oxidase(PPO)
Oxadiargyl	◎	Inhibition of protoporphyrinogen oxidase(PPO)
Diflufenican	×	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase
Amitrole	▲	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)
Glyphosate	▲	Inhibition of EPSP synthase
Glufosinate-ammonium	△	Inhibition of glutamine synthetase
Asulam	×	Inhibition of DHP (dihydropteroate) synthase
Dithiopyr	△	Microtubule assembly inhibition
Trifluralin	△	Microtubule assembly inhibition
Propham	◎	Inhibition of mitosis/microtubule polymerization inhibitor
Isoxaben	◎	Inhibition of cell wall (cellulose) synthesis
Dinoterb	◎	Uncoupling (Membrane disruption)
Dichlorprop	◎	Synthetic auxins (action like indoleacetic acid)
Pyributicarb	△	Unknown

z) ◎, increased remarkably; ○, increased; △, no effect; ▲, decreased; ×, decreased remarkably

Table 1. Effects of herbicides on biophoton emissions from cultured rice cells

Herbicides such as bensulfuron-methyl (BSM) induced an increase in biophoton emission from rice cells and then a decrease to control levels within 12–24 h after herbicide application (Figure 2). Although the precise mechanisms of biophoton emission in these cases (except for BSM treatment) were not investigated, these changes must reflect the biochemical reactions of rice cells to these chemicals. As described in detail below, in BSM-treated plants, the oxidative detoxification of BSM by P450 is responsible for biophoton emission.

4. How to measure biophotons from plant segments

4.1. Apparatus for biophoton measurement

Biophotons can be detected using a photon counter equipped with a highly sensitive photomultiplier tube. In our laboratory, we mainly use multi-sample photon counters, namely,

PCX-100 and CCSPC-01 (Hamamatsu Photonics, Hamamatsu, Japan) (Figure 3). The PCX-100 counter was equipped with a photomultiplier tube (R329; Hamamatsu Photonics, Hamamatsu, Japan) that provided a spectral response from 240 to 630 nm. It has a sample holder for 16 Petri dishes in the dark box and as the photomultiplier moved onto the samples, the biophotons from the samples were measured in rotation. The CCPPS-01 counter was equipped with a photomultiplier tube (R331P; Hamamatsu Photonics, Hamamatsu, Japan) that provided a spectral response from 300 to 650 nm. In the CCSPC-01 system, the photomultiplier was fixed, and a disc with 24 samples rotated under the photomultiplier. Because temperature affects biophoton emissions through e.g. changes in enzymatic activities, the photon counter was placed in an air-conditioned room.

Figure 3. Appearance of photon counter CCSPC-01

4.2. Sample preparation

Samples such as cut plant segments and cultured cells were used with the PCX-100 and CCSPC-01 counters for biophoton measurements. The procedure using cut plant segments treated with herbicides with the CCSPC-01 counter is shown in Figure 4. Plants were cut into 5-mm-long segments, and 0.5 g of these segments were set in the Petri dishes (60 mm in diameter), to which 2 mL of appropriate concentrations of herbicide solution or solvent (e.g., distilled water) were added. Dishes were then set in a sample holder in the dark box of the photon counter. Biophotons from each sample were continuously measured every 10 s. Because biophotons are luminescent from biological reactions, no special reagent was needed for their detection.

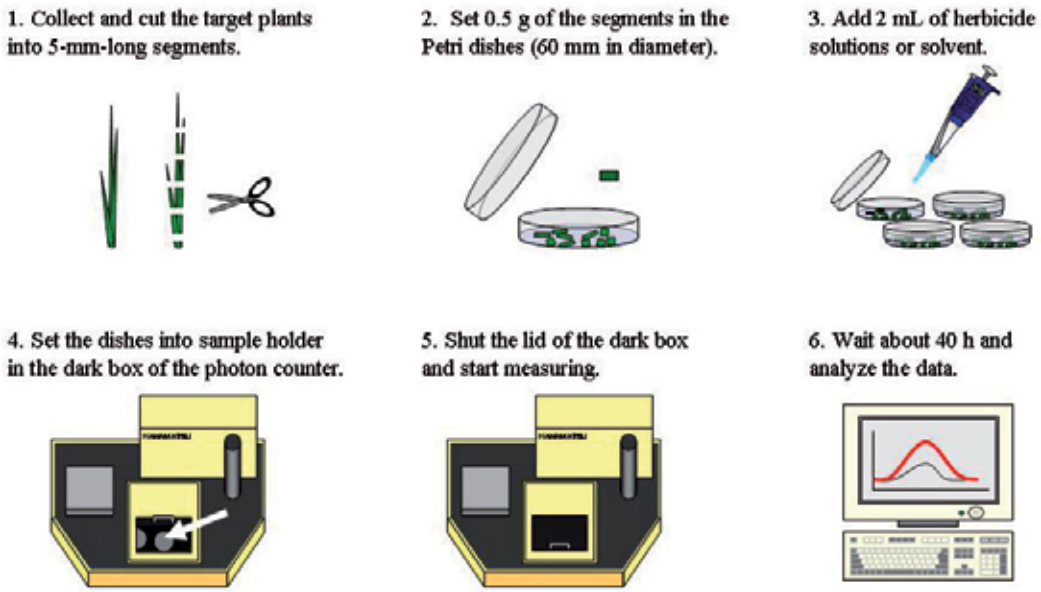

Figure 4. The procedure of measuring biophotons with the CCSPC-01counter using plant segments treated with herbicide.

It is preferred that all steps after sample collection are performed in a dark room because fluorescence from plant segments, or the Petri dishes due to excitation with room light during the sample preparation, contribute to measurement "noise".

5. Biophoton emissions in sulfonylurea herbicide-resistant weeds

5.1. Sulfonylurea herbicide resistance

Weed biotypes resistant to ALS-inhibiting herbicides have been reported worldwide [33], and have increased to 127 species [2]. Sulfonylurea (SU) herbicides are among the most potent ALS-inhibiting herbicides used worldwide, and biotypes resistant to SU herbicides have been found in many weed species. For example, *Scirpus juncoides* Roxb. var. *ohwianus* T. Koyama has evolved resistance to herbicides used in the paddy fields of Japan [34, 35], and the resistant biotypes cause serious problems for weed control in Japanese rice production [36, 37]. Mutations in the ALS genes, the target site of SU herbicides, have been reported as the molecular basis of SU resistance in weeds [36]. *S. juncoides* is reported to have at least two ALS genes, and an amino acid substitution at Pro197 or Trp574 in either of the two ALS proteins encoded by the two genes in all the examined resistant biotypes. This type of resistance is referred to as "target-site resistance."

Another type of herbicide resistance is referred to as "non-target-site resistance." Mutations in a non-target site, such as activation of herbicide metabolism or reduction of herbicide

absorption and translocation are the molecular bases underlying this type of herbicide resistance. For example, it has been reported that there is SU tolerance in cut leaves in rice and barnyardgrass (i.e., the metabolic half-time of SU was 2.6–4.8 h in rice and 12–50 h in barnyardgrass compared to 50 h or more in susceptible weeds) [38]. Herbicide-resistant weeds with non-target-site resistance have become a more serious problem than those with target-site resistance because non-target-site resistance tends to cause multiple herbicide resistance [39, 40].

5.2. Biophoton emissions in SU-resistant plants through target-site resistance

Biophoton emissions in *S. juncoides* have been investigated by employing 12 biotypes (seven SU-resistant and five SU-susceptible plants) collected from paddy fields in various regions of Japan [41]. Typical results of biophoton emissions from the culms of *S. juncoides* treated with BSM, one of the SU herbicides most commonly used in paddy fields in Japan, is shown in Figure 5 [41].

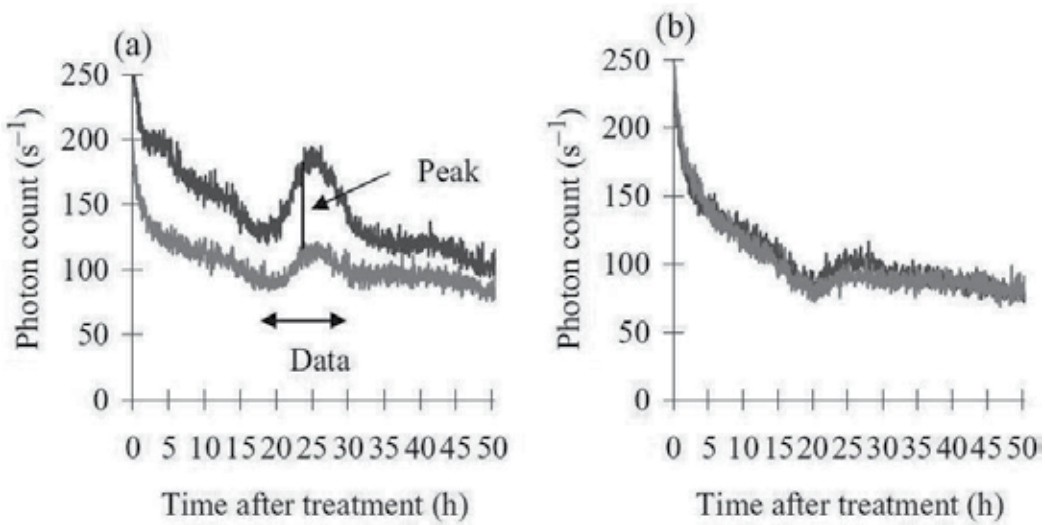

Figure 5. Examples of a typical time course of biophoton emissions from the culms of *Scirpus juncoides* treated with bensulfron-methyl (BSM) and water. (a) Typical resistant biotype and (b) typical susceptible biotype. The black line indicates the 100 ppm BSM treatment and the gray line indicates the water control [41].

It was observed that the resistant biotypes exhibited an increase in biophoton emissions with a peak at 25 h after 100 ppm BSM treatment. The emission intensity of the biophoton emissions varied depending on the concentrations of BSM. The difference in biophoton emission between resistant and susceptible biotypes is obvious with 10 and 100 ppm BSM treatment, whereas there were few differences with 1 ppm BSM treatment [41]. The emission intensity was different between plant organs (Figure 6) [41]. The increment in the biophoton emission was greater for the culms than for the roots. In the resistant biotype, a relatively higher increment in biophoton emission was observed in both the culms and roots, while the increment was

lower in the susceptible biotypes. This increment of biophoton emission in a resistant biotype was independent of the seed source or mutations in the ALS genes [41]. All seven resistant biotypes showed a higher increment in biophoton emissions than the five susceptible biotypes. The biophoton emissions in the resistant biotype were higher than those in the susceptible biotypes from the vegetative growth to the flowering stage. In particular, the difference in biophoton emission between the resistant and susceptible biotypes widened during the vegetative growth stage, and reached the maximum level at the flowering stage. However, at the seed-setting stage, the biophoton emissions were markedly enhanced in the susceptible biotypes. Therefore, at this stage, there were no distinct differences in biophoton emissions between the resistant and susceptible biotypes [41].

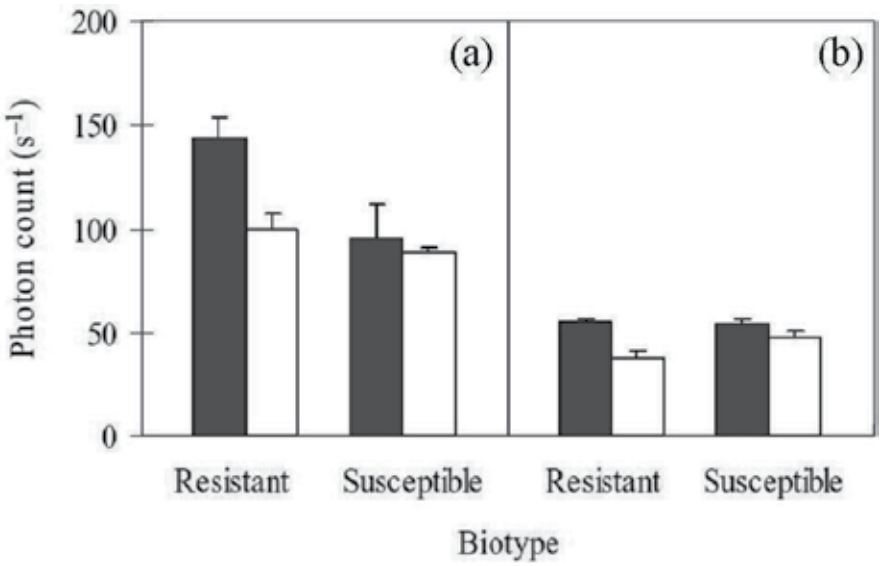

Figure 6. Biophoton generation in the (a) culm and (b) roots of resistant and susceptible biotypes of *Scirpus juncoides*. The bars indicate standard deviations (±SD). Bensulfron-methyl treatment (solid column); control (open) [41].

In addition to the case of *S. juncoides*, this increment in biophoton emission in SU-resistant biotypes has been confirmed in *Monochoria vaginalis* [42]. Figure 7 shows the result of biophoton emissions after BSM treatment from leaf segments of four SU-resistant and four SU-susceptible biotypes of *M. vaginalis* [42]. In the four resistant biotypes, distinct increments were independent of differences in the mutation sites of the ALS genes. In contrast, increments in the four SU-susceptible biotypes were less than that in the four SU-resistant biotypes. Therefore, it is suggested that biophoton emission could be a new indicator of SU-resistant biotypes in various weeds.

5.3. Biophoton emission in SU-resistant plants through non-target site resistance

For biophoton emission in SU-resistant plants through non-target site resistance, biophoton emissions from leaf segments of rice and 11 paddy weeds treated with BSM were measured [43]. There was a definite difference in biophoton emission among plant species, and rice and

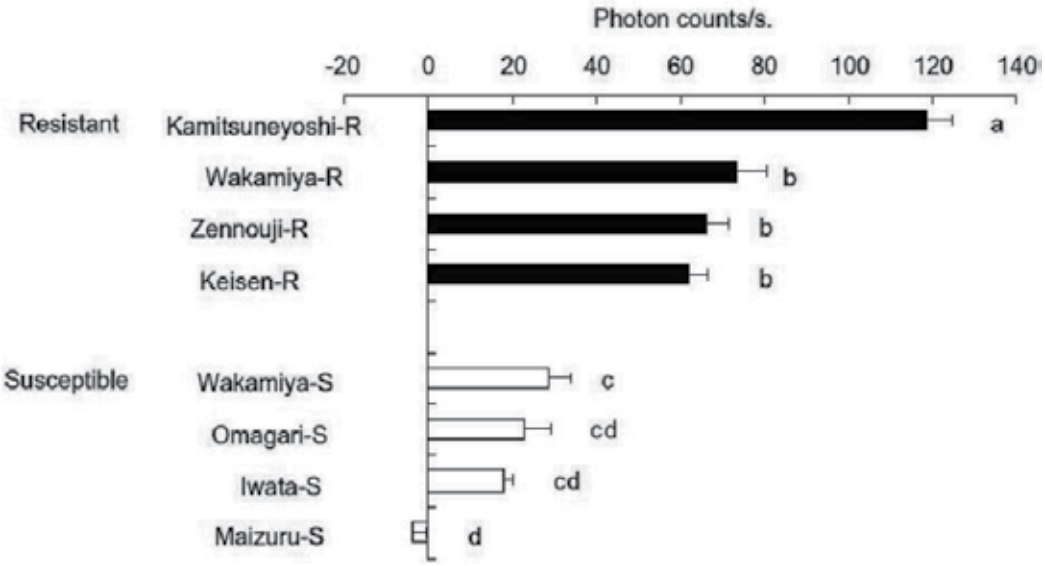

Figure 7. Biophoton generation from leaf segments of resistant and susceptible biotypes of *Monochoria vaginalis*. Values are the differences in averages from 24 to 40 h after treatment between the bensulfron-methyl application and water control. The bars indicate standard deviations (±SD). Different letters indicate a significant difference at the 5% level according to Tukey's Studentized Range Test [42].

barnyardgrass, which are reported to be tolerant of SU herbicide as described above, showed considerably higher biophoton emission than those of other weed species. The enhancement of biophoton emission in BSM-treated leaf segments compared to the water-treated control was 3.8 times higher in rice and 3.0 times higher in barnyardgrass [33]. It seems that the distinct differences in biophoton emissions among rice, barnyardgrass, and other weed species are consistent with their selectivity against SU herbicides.

6. Mechanism for biophoton emission in plants treated with SU herbicides

Chemical reactions such as oxidation are considered to be the source of energy for biophoton emissions. We previously studied biophoton emissions during plant disease response, particularly with regard to the involvement of ROS. During the disease response of cultured rice cells to *N*-acetylchitooligosaccharide elicitor, ROS seemed to be directly involved in biophoton emission [25, 44].

As shown in Figure 1, ROS is also involved in biophoton emission in paraquat-treated tobacco. Hideg and Inaba (1991) demonstrated that the leaves of paraquat-resistant tobacco treated with paraquat emitted weaker biophotons compared with tobacco plants sensitive to paraquat [45]. The superoxide dismutase (SOD) activity of the resistant plant was 3 to 6 times greater than that of sensitive plants. Apparently, higher SOD activity in

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resistant tobacco contributes to the elimination of the ROS induced by paraquat, resulting in weaker biophoton emission.

Recent studies suggested that oxidative metabolism by cytochrome P450 monooxygenase (P450) might be involved in biophoton emissions from herbicide-treated plants [42, 43]. P450s are a class of heme-containing enzymes that catalyze the biosynthesis of lignin, terpenoid, alkaloids, and many secondary compounds in plants. P450s are also known to play important roles in metabolizing herbicides, including SU [46,47], and are involved in non-target site herbicide resistance [48].

As described above, leaf segments of non-target site resistant plants such as rice and barnyardgrass, emit strong biophotons when treated with SU herbicide, and this biophoton emission was inhibited by the P450 inhibitor (malathion and piperonyl butoxide) treatment [42].

The involvement of P450s in biophoton emission from SU herbicide-treated plants was also confirmed in another experiment employing P450 gene-silenced cultured rice cells. P450s are known to make up one of the largest superfamilies of enzymes and catalyze diverse reactions in both animals and plants. In rice, *CYP81A6*, a P450 gene responsible for resistance to BSM, has been identified by map-based cloning [49]. We silenced the *CYP81A6* gene in rice cells and analyzed their response to BSM (Nukui *et al.*, unpublished). P450-silenced cells showed increased BSM sensitivity as expected. As shown in Figure 2, cultured rice cells, as well as leaf segments, emit biophotons when treated with BSM. Treatment with 100 ppm BSM induced an obvious biphasic biophoton emission in the control cell line. In contrast, P450-silenced cells did not show biophoton emission after BSM treatment. The enhancement of biophoton emission in BSM-treated cells compared to the water-treated control was 1.6 times greater in the control line and 1.0 times in the P450-silenced cell line (Figure 8). Moreover, the effects ALS inhibition on BSM-responsive biophoton emissions have been investigated. BSM inhibits the reaction of ALS, the first step in the biosynthesis of branched-chain amino acids (BCAA: Valine, Leucine, Isoleucin) [50], and the herbicidal activities of BSM is canceled by the application of BCAA [51]. Although the externally applied 1 mM BCAA recovered the cell growth of BSM-treated P450-silenced rice cells, it failed to recover BSM-responsive biophoton emissions. These results suggest that it was not decreased cell activity but the suppressed detoxifying reaction of P450 that reduced biophoton emissions in P450-silenced cells.

On the other hand, the fate of SU compounds is not well known for the target site-resistant weeds such as *S. juncoides* and *M. vaginalis* described above. There are only suggestive results. Figure 9 shows the effect of the P450 inhibitor malathion on biophoton emissions from leaf segments of *M. vaginalis*, which have been confirmed to be target site resistant to SU [42]. In resistant biotypes, malathion decreased biophoton emission from leaf segments treated with BSM, whereas in susceptible biotypes, malathion had no definite inhibitory effect on biophoton emission. This indicates that P450s are also involved in biophoton emissions from target site SU-resistant weeds. Therefore, we hypothesize that SU, which cannot bind to ALS because of ALS mutations, would also be detoxified with P450s, resulting in biophoton emission.

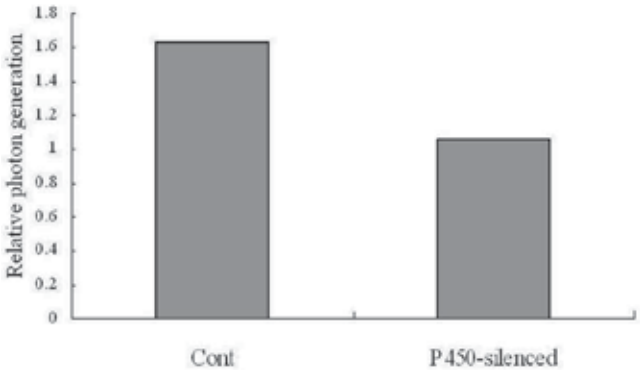

Figure 8. Biophoton emission from P450-silenced rice cells (P450-silenced) and control cells (Cont). Values represent the ratio of total biophoton emission from BSM-treated cells to that from solvent (control)-treated cells during 0-12 h after BSM or solvent application.

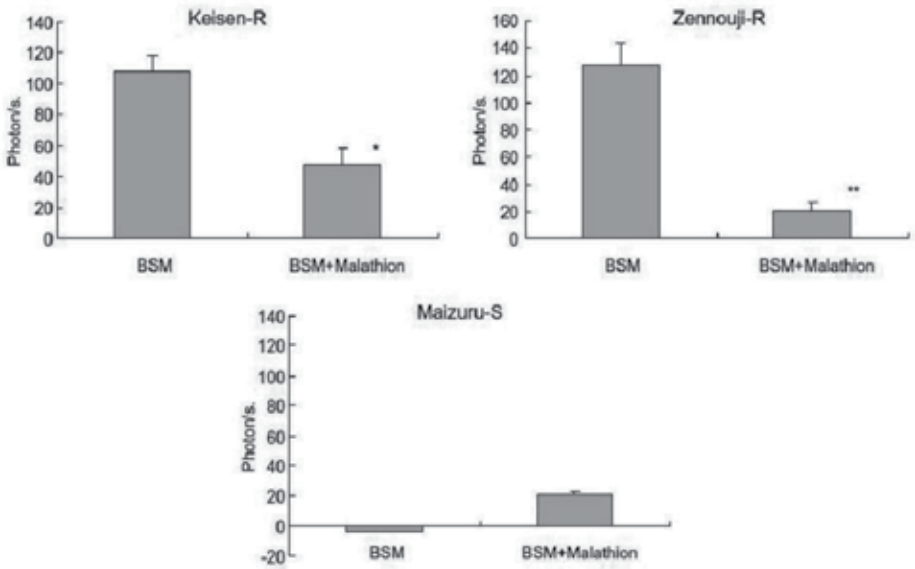

Figure 9. Effect of P450 inhibitor on biophoton emissions from leaf segments of *Monochoria vaginalis*. Values are the differences in averages 24–40 h after treatment between the bensulfron-methyl (BSM) or BSM + P450 inhibitor (malathion) and water control, respectively. The bars indicate standard deviations (±SD). * and ** indicate the significant differences at $P < 0.05$ and 0.01, respectively [42].

7. Conclusion

As described above for SU resistance, it is confirmed that both target site-resistant and non-target site-resistant plants can be distinguished from susceptible plants. In addition, from the analysis of the mechanisms of biophoton emissions, it is considered that weed biotypes with target site resistance to herbicides other than SU might also be distinguished from susceptible

biotypes by biophoton measurement, as long as the herbicides are subjected to detoxification by P450 enzymes. It is well known that P450s play major roles in the detoxification of many types of herbicides, and many resistant weed biotypes with enhanced P450 activities have been reported [48]. For example, rigid ryegrass has been reported to be resistant to diclofop-methyl, diuron, atrazine, simazine, and chlorsulfron for resistance mediated by P450s [48]. Biophoton measurement might be able to distinguish all these resistant biotypes from susceptible biotypes.

Although it is necessary to clarify the applicable range of herbicide resistance by using weed biotypes resistant to herbicides other than SU, our study suggests that biophoton measurement can be used in the identification of a broad spectrum of herbicide resistances and will become a useful tool for efficient weed control.

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References

[1] Hilton HW. Herbicide tolerant strain of weeds. Hawaiian Sugar Planters Association Annual Reports. 1957; p 69

[2] Heap I. The international Survey of Herbicide Resistant Weeds. www.weedscience.com (accessed 1 October 2012)

[3] Gerwick BC., Mireles LC., Eilers RJ. Rapid diagnosis of ALS/AHAS-resistant weed. Weed Technol. 1993; 7: 519-524

[4] Simpson DM., Stoller EW., Wax LM. An *in vivo* acetolactate synthase assay. Weed Technol. 1995; 9: 17-22

[5] Lovell ST., Wax LM., Simpson DM., McGlamery M. Using the *in vivo* acetolactate synthase (ALS) assay for identifying herbicide-resistant weeds. Weed Technol. 1996; 10: 936-942

[6] Uchino A., Watanabe H., Wang GX, Itoh K. Light requirement in rapid diagnosis of sulfonylurea-resistant weeds of *Lindernia* spp. Weed Technol. 1999; 13: 680-684

[7] Uchino A., Watanabe H. Effects of pyruvate and sucrose on acetolactate synthase activity in *Lindernia* species and *Schoenoplectus juncoides* in an *in vivo* assay. Weed Biol. Manag. 2007; 7: 184-187

[8] Boutsalis P. Syngenta quick-test: a rapid whole-plant test for herbicide resistance. Weed Technol. 2001; 15: 257-263

[9] Hamamura K., Muraoka T., Hashimoto J, Tsuruya A., Takahashi H., Takeshita T., Noritake K. Identification of sulfonylurea-resistant biotypes of paddy field weeds using a novel method based on their rooting responses. Weed Biol. Manag. 2003; 3: 242-246

[10] Ohno S., Yanagisawa K., Hanai R., Muraoka T. [Shoot regeneration method: a simplified method of evaluating sulfonylurea herbicide resistance.] J. Weed Sci. Technol. 2004; 49: 277-283 (in Japanese)

[11] Popp FA., Some essential questions of biophoton research and probable answers, in: Popp FA., Li KH., Gu Q. (eds.) Resent advances in Biophoton Research and it's Application, World Scientific, Singapore, 1992, p 1-42

[12] Inaba H. Ultraweak biophoton imaging and information characterization, in: Sueta T., Okoshi T. (eds.), Ultrafast and Ultra-Parallel Optoelectronics, Ohmsha/John Wiley and Sons, Tokyo, 1995, p570-580

[13] Colli L., Facchini U., Guidotti G., Lonati RD., Orsenigo M., Sommariva O. Further measurements on the bioluminescence of seedlings. Experientia 1955; 11: 479-81

[14] Beloussov L., Popp FA., Voeikov V., Wijk RV., editors. Biophotonics and Coherent Systems: Moscow University Press, Moscow; 2000

[15] Abeles, FB. Plant chemiluminescence. Annu. Rev. Plant Physiol. 1986; 37: 49-72

[16] Radoc K., Radenovic C., Jeremic M. Spontaneous ultraweak bioluminescence in plants: origin, mechanisms and properties. Gen. Physiol. Biophys. 1998; 17: 289-308

[17] Suzuki S., Usa M., Nagoshi T., Kobayashi M., Watanabe N., Watanabe H., Inaba H. Two-dimensional imaging and counting of ultraweak emission patterns from injured plant seedlings. J. Photobiol. B Biol. 1991; 9: 211-217

[18] Slawinski J., Grabikowski E., Ciesla L. Spectral distribution of ultraweak luminescence from germinating plants. J. Luminescence 1981; 24/25: 791-794.

[19] Musmeci F. Recent advances in biophoton research and its applications. World Scientific, Singapore, 1992, p 302-324

[20] Kobayashi M., Takeda M., Ito K., Kato H., Inaba H. Two-dimensional photon counting imaging and spatiotemporal characterization of ultraweak photon emission from a rat's brain in vivo. J. Neurosci. Methods 1999; 93: 163-168

[21] Agatsuma S., Nagoshi T., Kobatashi M., Usa M., Watanabe H., Sekino H., Inaba H. Hydroxyl radical-induced characteristics chemiluminescent spectra from plasma of hemodialysis patients. Clin. Chem. 1992; 38: 48-55

[22] Iyozumi, H., Kato, K., Kageyama, C., Inagaki, H., Yamaguchi, A., Furuse, K., Baba, K., Tsuchiya, H. Plant defense activators potentiate the generation of elicitor-responsive photon emission in rice. Physiol. Mol. Plant Pathol. 2005; 66: 68-74

[23] Bennett, M., Mehta, M., Grant, M. Biophoton imaging: a nondestructive method for assaying R gene responses. Mol. Plant-Microbe Interact. 2005; 18: 95-102

[24] Iyozumi, H., Kato, K., Makino, T. Spectral shift of ultraweak photon emission from sweet potato during a defense response. Photochem. Photobiol. 2002; 75: 322-325

[25] Kageyama C., Kato K., Iyozumi H., Inagaki H., Yamaguchi A., Furuse K., Baba K. Photon emissions from rice cells elicited by *N*-acetylchitooligosaccharide are generated through phospholipid signaling in close association with the production of reactive oxygen species. Plant Physiol. Biochem. 2006; 44: 901-909

[26] Kai, S, Ohya, T, Moriya, K, Fujimoto, T. 1995. Growth control and biophoton radiation by plant hormones in red bean. Jpn. J. Appl. Phys. 34: 6530-6538

[27] Yoshinaga N., Kato K., Kageyama C., Fujisaki K., Nishida R., Mori N. Ultraweak photon emission from herbivory-injured maize plants. Naturwissenschaften 2006; 93: 38-41

[28] Hauvaux M. Spontaneous and thermoinduced photon emission: new methods to detect and quantify oxidative stress in plants. Trends Plant Sci. 2003; 8: 409-413

[29] Agaverdiyev AS., Doskoch YE., Tarusov BN. Effect of low temperatures on the ultraweak luminescence of plants. *Biofizika* 1965; 10: 832-836

[30] Ohya, T., Kurashige, H., Okabe, H., Kai, S. Early detection of salt stress damage by biophotons in red bean seedling. Jpn. J. Appl. Phys. 2000; 39: 3696-3700

[31] Ohya T., Yoshida S., Kawabata R., Okabe H., Kai S. Biophoton emission due to drought injury in red beans: possibility of early detection of drought injury. Jpn. J. Appl. Phys. 2002; 41: 4766-477.

[32] Kato K., Makino T., Matsuura H. Japanese Patent 2004-233162. 2003

[33] Saari LL., Cotterman JC., Thill DC. Resistance to acetolactate synthase inhibiting herbicides. In: Powles SB., Holtum JAM. (eds.) Herbicide Resistance in Plants. Biology and Biochemistry, CRC Press, Boca Raton, FL, 1994, p 83-138

[34] Kohara H., Konno K., Takekawa M. [Occurrence of sulfonylurea- resistant biotypes of *Scirpus juncoides* Roxb. var. *ohwianus*. T. Koyama in paddy fields of Hokkaido prefecture, Japan.] J. Weed Sci. Technol. 1999; 44: 228-235 (in Japanese)

[35] Uchino A., Ogata S., Kohara H., Yoshida S., Yoshioka T., Watanabe H. Molecular basis of diverse responses to acetolactate synthase-inhibiting herbicides in sulfonylurea-resistant biotypes of *Schoenoplectus juncoides*. Weed Biol. Manag. 2007; 7: 89-96

[36] Itoh K., Uchino A. Sulfonylurea-resistant weeds in paddy rice fields of Japan. In: Clark JM., Yamaguchi I. (eds.) Agrochemical resistance: Extent, Mechanism, and Detection. The American Chemical Society, Washington, DC, 2002; p168-180

[37] Uchino A., Watanabe H., Kikuchi H., Miura Y., Ogata S., Usui T. et al. [Sulfonylurea-resistant paddy weeds in the six prefectures of the Tohoku district : the number of instances judged as infection by resistant biotypes from 1996-2003.] Tohoku Weed J. 2005; 5: 24-28 (in Japanese)

[38] Takeda S. Yuyama T., Ackerson RC. Herbicidal action and selectivity mechanism of bensulfuron methyl. Weed Research, Japan 1989; 34: 8-93 (in Japanese)

[39] Preston C., Tardif FJ., Christopher JT., Powles SB. Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. Pestic. Biochem. Physiol. 1996; 54: 123-134

[40] Preston C. Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci. 2004; 52: 448-453

[41] Inagaki H., Ishida Y., Uchino A., Kato K., Kageyama C, Iyozumi H., Nukui H. Difference in ultraweak photon emissions between sulfonylurea-resistant and sulfonylurea-susceptible biotypes of *Scirpus juncoides* following the application of a sulfonylurea herbicide. Weed Biol. Manag. 2008; 8: 78-84

[42] Inagaki H., Imaizumi T., Wang GX., Tominaga T., Kato K., Iyozumi H., Nukui H. Sulfonylurea-resistant biotypes of *Monochoria vaginalis* generate higher ultraweak photon emissions than the susceptible ones. Pestic. Biochem. Physiol. 2009; 95: 117-120

[43] Inagaki H., Imaizumi T., Wang GX., Tominaga T., Kato K., Iyozumi H., Nukui H. Spontaneous ultraweak photon emission from rice (*Oryza sativa* L.) and paddy weeds treated with a sulfonylurea herbicide. Pestic. Biochem. Physiol. 2007; 89: 158-162

[44] Kato K., Honzawa Y., Iyozumi H. Nukui H. Quantitative correlation between elicitor-responsive photon emission and hydrogen peroxide, induced with N-acetylchitohexaose. Jpn. J. Phytopathol. 2010; 76: 142-148 (in Japanese)

[45] Hidge E., Inaba H. Dark adapted leaves of paraquat-resistant tobacco plants emit less ultraweak light than susceptible ones. Biochem. Biophys. Res. Commun. 1991; 178: 438-443

[46] Shirakura S., Ito K., Barefoot AC., Aizawa H., Ishizuka K. Differences in absorption, translocation and metabolism of azimsulfron between rice (*Oryza sativa*) and flat sedge (*Cyperus serotinus*). Weed Res. Japan 1995; 40:299-307

[47] Yun MS., Deng F., Usui K. *O*-dealkylation of counarin ethers and effects of inducers, inhibitors, and sulfonylureas on rice cytochrome P-450. J. Weed Sci. Tech. 1999; 44: 341-348

[48] Yuan JS., Tranel PJ., Stewart Jr, CN. Non-target-site herbicide resistance: a family business. Trends Plant Sci. 2006; 12: 6-13

[49] Pan G., Zhang X., Liu K., Zhang J., Wu X., Zhu J., Tu J. Map-based cloning of a novel rice cytochrome P450 gene CYP81A6 that confers resistance to two different classes of herbicides. Plant Mol. Biol. 2006; 61: 933-943

[50] Takeda S., Erbes DL., Sweetser PB., Hay JV., Yuyama T. Mode of herbicidal and selective action of DPX-F5384 between rice and weeds. Weed Res. Japan 1986; 31: 157-163

[51] Sengnil K., Usui K., Ishizuka K. Recovery from growth inhibition and acetolactate synthase activity in rice suspension-cultured cells treated with bensulfron methyl. Weed Res. Japan 1992; 37: 28-34

Chapter 12

Risk Assessment of Herbicide Resistant Crops with Special Reference to Pollen Mediated Gene Flow

Amit J. Jhala and Linda M. Hall

Additional information is available at the end of the chapter

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1. Introduction

The world population is projected to become a staggering 8.3 billion by 2030 from about 6 billion today, which will aggravate food insecurity especially in developing countries [31]. By 2050, developing countries will account for 93% of cereal and 85% of meat demand growth [73]. In agricultural crop production systems, insects, diseases and weeds continue to threaten sustainability and account for ~40% loss in crop production. Availability of farm land and productivity is decreasing because of soil erosion, degradation and annexation of farm land for alternative uses. The availability of water for agricultural crops is also decreasing. Drought, storm, flood and heat waves are predicted to occur more frequently and would have a large impact on crop productivity [19]. Since atmospheric concentrations of greenhouse gases continue to rise at rates that are both unprecedented and alarming, efforts have been made to understand their implications on crop production [2]. Higher growing season temperatures can have dramatic impacts on agricultural productivity, farm incomes and food security [8]. Salinity and other soil toxicities are likely to be much more problematic in some areas. In semi-arid regions, reduction in production of primary crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) are predicted in the next two decades [58].

Ability of the agriculture sector to support a growing population has been a concern and continues to be on high priority on the global policy agenda [5]. Simultaneous demands for replacement of oil-based fuels and plant-based bio-products, the desire for reduction in pesticide usage and crops to tolerate abiotic stress will place pressure on agricultural production systems. New agricultural technologies are required to ensure global food security and support conservation of water and land. Crop cultivars with higher yields and resistivity are required to meet food requirements in a sustainable manner without causing disruption to the environment. In her book "Silent Spring", Carson R. suggested finding a biological solution

of pest control in agriculture as an alternate of using chemical pesticides [18]. One approach is to develop genetically engineered crops. Technological responses to the need for bio-products, new crops and stress-tolerant crops have been predicated on the use of genetic engineering.

In addition to traditional uses of crops for food, feed and fiber, agricultural crops are being utilized for biodiesel, bio-products, industrial chemicals, bio-degradable plastics and plant-based pharmaceuticals [14]. Genetic engineering is increasingly used to accomplish this goal and viewed by researchers as a key technology to provide innovations. Long-term sustainability of technical applications has become quite important during the last two decades resulting from ecological concern, environmental awareness and new rules and regulations.

Introduction of herbicide-resistant crops at a commercial scale was controversial; however, this new technology has provided many benefits to growers, consumers, and environment [3]. In many parts of the world, agricultural pesticides have been used in excess of requirements, leaving residue in food and soil resulting in environmental pollution. Agro-chemicals are ineffective against viruses and only partly effective against many plant pathogens. The most cost effective and environmentally friendly method would be to deploy cultivars that have been developed for resistance to various agents causing biotic stress. For example, transgenic cotton in India, which is genetically engineered to contain the *Bt* toxin, has led to decrease use of insecticides and improved yields [12]. China is the major producer of cotton and Chinese growers are amongst the largest users of pesticides in cotton. A survey suggests that following introduction of *Bt* cotton, reduction in pesticide use was from 55 to 16 kg formulated product per hectare and the reduction in number of pesticide application was from 20 to 7 [46]. The other benefits of herbicide-resistant crops have been described in [9, 25].

Besides the benefits of herbicide-resistant crops, their commercialization has created great controversy among government agencies, business consortia, researchers and certain non-profit organizations about the consequences of herbicide-resistant crops and their negative impact on food, feed and environment [3, 27, 80]. The relevance of assessing weedy characteristics when considering the invasiveness of herbicide-resistant crops has been the subject of much debate [90]. Gene flow from transgenic plants to wild relatives may cause wild plants to acquire traits that improve their fitness [29]. Mere presence of wild relatives in a given area does not necessarily imply interspecific gene flow would happen; however, a long term co-existence in a given habitat may signal the need to assess the likelihood of spontaneous gene transfer from GE crops to their wild relatives [68]. For example, jointed goatgrass (*Aegilops cylindrica*), a wild relative of wheat can acquire the herbicide tolerant trait of wheat, and can therefore, thrive in crop fields unless applications of other herbicides are made [41, 42]. Similarly, the survey work in Africa suggested that wild and weedy sorghum occurred intermixed with and adjacent to cultivated sorghum and may pose risk of inter-specific transgene movement [64]. Weedy rice is an important weed in rice growing regions in more than 50 countries [57]. The concern for the introgression of the transgene into the weedy rice may result in more difficult to manage hybrid is a serious consideration for commercial production of novel rice [21, 93].

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Plant-derived pharmaceuticals and industrial compounds may have an impact on human and animal health or public perception if they are found in the food or feed systems [38]. For example, the "Starlink" maize incident illustrates how genetically-engineered crop cultivars intended for special purposes may mix with commodity crops. The maize was grown exclusively for animal consumption before determination of whether it was suitable for human consumption. Within a single year, it entered the commodity corn grain supply of the USA [43]. Lack of a channelized production system left growers to decide whether to sell the grains for human or animal consumption. Management of externalities and of the possible unintended economic effects that arise in this context is critical and poses different concerns. The regulatory agencies worldwide are struggling to develop a risk assessment procedure prior to commercial release of engineered crops intended for plant molecular farming and for specialty chemicals [4, 83].

Adventitious presence (AP) is the low level presence of genetically engineered seeds in conventional and organic seeds, in addition to other unwanted materials [53]. Commingling has long been acknowledged and thresholds for AP have been established in conventionally grown crops. However, more recently AP became an issue after commercialization of transgenic crops. With respect to approved crops, the issue is not agronomic performance, food safety, environmental protection, or animal or human health; however, AP is more related with economic concerns, market access, contract specifications, and consumer preferences [53]. Pollen-mediated gene flow from herbicide-resistant to conventional crops and their crop volunteers are the major source of AP in subsequent crops and may create the problems in trade, especially with the European Union (EU) where strict regulations are prevailing for growing, importing and regulating herbicide-resistant crops [11]. These issues related with market and trade will become more complicated as the number of herbicide-resistant crops and traits increase in the future and there is little harmony in regulation of novel crops or their threshold levels internationally.

The aim of the research described in this chapter was to test the following objectives:

1. To discuss risk assessment of herbicide-resistant crops with flax as a model species
2. To determine the occurrence and distribution of weedy and wild relatives of flax and their potential hybridization with commodity flax to predict the risk of inter-specific transgene movement
3. To determine intra-specific pollen-mediated gene flow in flax under natural field conditions and to evaluate the potential for co-existence of herbicide-resistant and organic flax

2. Risk assessment of herbicide-resistant crops with flax as a model species

The cultivation of flax dates back to more than 6,000 years mainly for seed oil and fiber [1]. In addition to the traditional industrial and non-food uses of flax, with the increasing information on molecular biology derived from identification and expression of genes, the potential for the production of novel flax for quality traits has been developed [58, 62]. With the introduction

of high α-linolenic acid (ALA) flax cultivars [73], the world market is increasing dramatically for the flax based products [61].

Flax is a poor competitor and thus, flax fields should be kept free from weeds [89]. Herbicide resistant flax was registered and withdrawn due to market considerations. Few effective herbicides have been registered for controlling weeds in flax [15]. There is an opportunity to develop flax cultivars with enhanced agronomic traits including reduced maturity period and better nitrogen use efficiency. Considering the utility of flax or flax based products for various purposes, genetically engineered flax is under development in Canada. Before genetically engineered flax is commercialized; however, environmental biosafety assessment must be quantified. The following are five considerations of environmental risk assessment of genetically engineered crops adopted by the Canadian Food Inspection Agency (CFIA):

- Potential of the plant with novel trait (PNT) to become a weed of agriculture or be invasive of natural habitats
- Gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive
- Potential for the PNT to become a plant pest
- Impact of the PNT or its gene products on non-target species, including humans
- Impact on biodiversity

3. Inter-specific pollen-mediated gene flow in flax

If the transgene(s) move by pollen-mediated gene flow (*transfer of genetic information between sexually compatible plant populations via cross-pollination*) from herbicide-resistant crops to wild relatives and if it has effect on fitness, weediness, diversity or population size, it is considered as one of the consequences of genetically engineered crops [65]. Gene flow is a natural, biological process which occurs to some degree in all flowering plant species [28]. There is a possibility that generating novel crops for food quality, better weed control or insect control may have effects on other plant populations, especially to closely related species of crops [26, 27]. Some traits may provide a possible benefit to wild or weedy relatives [68, 69]. Therefore, to evaluate the potential introgression of herbicide-resistant flax with its closely related species, a meta-analysis to study the occurrence of wild relatives of flax, their hybridization with cultivated flax and the possibility of transgene movement was quantified.

The evolution of many crop plants has been the result of recurrent cycles of hybridization from wild and weedy relatives [69]. In plant breeding and cultivar development programs, crop wild relatives have been used as a source of gene pool which can be transferred into crop cultivars to expand genetic basis by transferring desirable traits to increase resistivity to biotic and abiotic stresses. However, after commercial production of herbicide-resistant crops, concerns have been raised about the possibility of the sexual transfer of crops' genes to wild relatives through recurrent back crossing [27]. Natural hybridization is known to occur in

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many cultivated crops and the potential for transgene movement from herbicide-resistant crops to closely related species has been documented [17].

Flax belongs to family Linaceae which is composed of 22 genera [88] and approximately 300 species [45]. Linaceae is placed in the order Malpighiales [6]. It has been estimated that about 63 wild and weedy species of flax occur in North America [47]. The North American species of *Linum* were grouped into two sections, *Linum* (Eulinum) and *Cathartolinum*. Based on petal color, North American *Linum* includes two groups: the blue flowered and yellow-flowered [87, 71]. Twenty five yellow-flowered species of *Linum* are found in western North America and Central America. The greatest density of this segment of the genus is in east-central Mexico and point to that region as the probable area of origin and establishment of the yellow flowered *Linum* species in North America [71]. *Linum rigidum* Pursh occurs in extreme southern Florida and also has a vast range in the Great Plains extending from the northern Mexico to western Canada [63].

Interspecific hybridization has been attempted in many crops to improve germplasm, however, in *Linum*, such achievements have been very limited because of the difficulties in the successful hybridization between different species of the genus [47]. Attempts have been made to transfer the rust resistance from a wild relative to cultivated flax [23]. We hypothesize that cultivated flax is more likely to hybridize with closely related species having a similar ploidy level based on reported work [7, 36, 37, 75, 92]. Our objective was to predict the potential risk of inter-specific transgene movement from herbicide-resistant flax prior to commercial release.

Interspecific hybridization in *Linum* was first reported between flax and *L. narbonense* [101]. Later, successful hybridization of *L. usitatissimum* with *L. africanum, L. angustifolium, L. corymbiferum, L. floccosum, L. pallescens* and *L. tenue* were reported [7, 36, 54, 66, 85]. All crosses produced fertile F_1 hybrids in at least one direction, presumably due to their similarity in ploidy levels and size of chromosomes (Fig. 1). Crosses among five taxa, *L. africanum, L. angustifolium, L. corymbiferum, L. decumbens* and *L. usitatissimum* were highly successful in at least one direction with hybrid progeny exhibiting 80 to 90% germination [44]. Inter specific hybridization between species other than cultivated flax are also successful. When *L. strictum* was used as a male parent, it successfully hybridized with *L. africanum*, *L. angustifolium*, and *L. floccosum* [75].When *Linum crepetans* and *L. humile* crossed with *L. hirsutum* and *L. hispanicum*, respectively, they produced fertile plants [36, 37, 75] (Fig. 2).

In summary, interspecific hybridization studies indicate that cultivated flax has the potential to hybridize with at least nine wild relatives with a haploid chromosome number of 15 (Fig. 1). *Linum africanum, L. angustifolium,* and *L. pallescens* were crossed with *L. usitatissimum* and all reciprocal crosses were successful (Fig. 1). Therefore, further studies should be conducted to determine if hybrids between these three species occur and retain transgenes from herbicide-resistant flax in the natural ecosystem through hybridization and introgression with other wild relatives (Fig. 1).

There have been studies of successful hybridization among taxa other than n=15 (Fig. 2). The species with a haploid chromosome number of 9, constitute the largest group in the genus *Linum* [34]. Some crosses between species of taxa n=9, *L. alpinum, L. altaicum, L. austriacum, L.*

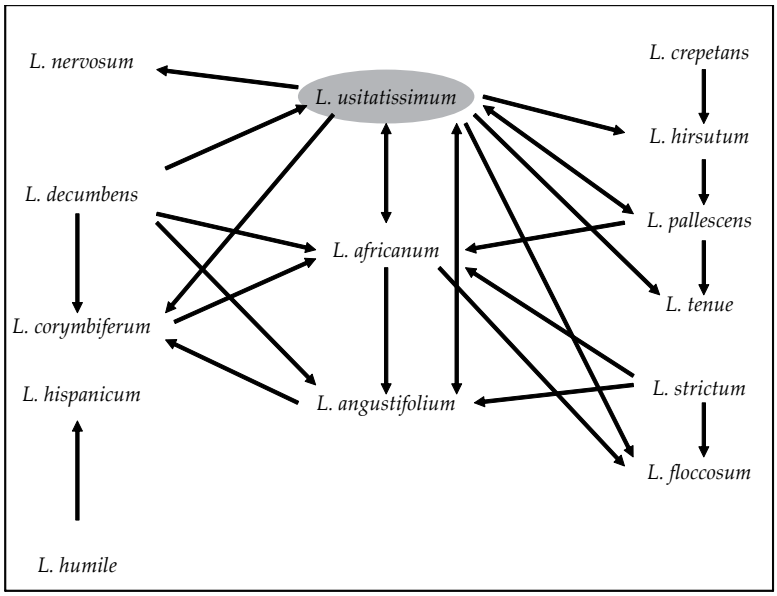

Figure 1. Inter-specific crosses among *Linum* species (n=15) that resulted in fertile progeny. Arrows indicate the direction of the cross (male to female). These are the closely related species with the greatest potential to hybridize with flax [47].

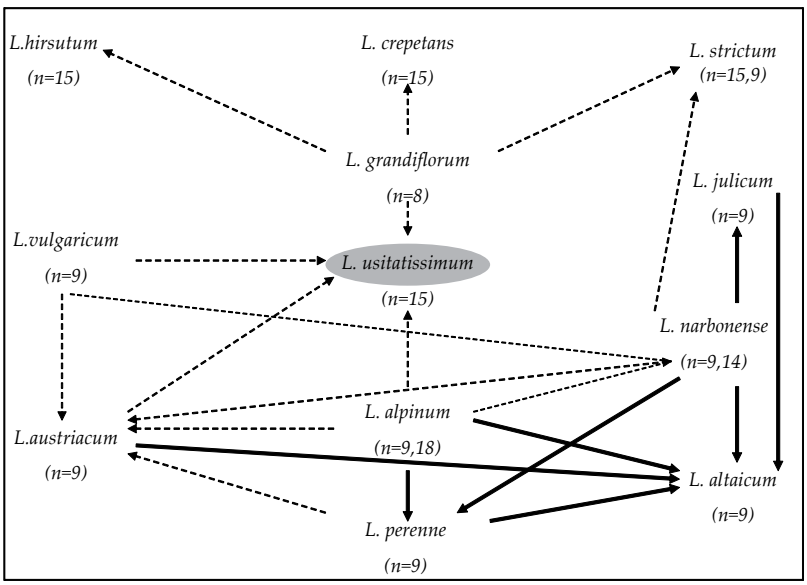

Figure 2. Interspecific hybridization in *Linum* (species with different chromosome numbers). Arrows indicate the direction of the cross (male to female). Solid lines indicate fertile F_1 hybrids were obtained with viable seed production. Dotted lines indicate hybridization occurred, but F_1 hybrids were not obtained with embryo rescue and/ or treatments with colchicine [47].

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julicum, *L. narbonense* and *L. perenne* produced hybrid plants [36, 37]. The pairing of chromosomes of this species revealed that *L. altaicum* differs by one reciprocal translocation from *L. alpinum, L. austriacum, L. julicum, L. narbonense,* and *L. perenne* [37].

Interspecific hybridization between *Linum* species with different chromosome numbers was also studied. Crosses between *L. alpinum* (n=9, 18), *L. austriacum* (n=9), *L. vulgaricum* (n=9) and *L. usitatissimum*; as well as crosses between other species with n=15 (i.e., *L. crepetans*, *L. hirsutum, L. strictum, L. usitatissimum*) with *L. grandiflorum* (n=8), either did not produce any seeds, or failed to produce fertile plants [7, 54, 66, 75, 76] (Fig. 2). These results suggest that similarity in chromosome numbers play an important role in successful inter-specific hybridization in *Linum* species. Therefore, species with different chromosome numbers have no or minimal risk of transgene movement.

Three closely related species of flax are reported to occur in western Canada. Two of them, *L. rigidum* and *L. sulcatum*, have the same chromosome numbers as cultivated flax (n=15). While inter-specific hybridization has not been documented for these species, hybridization of flax with other n=15 species suggests outcrossing may occur. Further research on selected *Linum* species, including a greenhouse study to quantify distribution, flowering time, preferred habit or population and hybridization potential with cultivated flax is warranted to determine whether introgression of the transgene occurs.

4. Intra-specific pollen-mediated gene flow in flax

Intra-specific gene flow is important as it is one of the main factors, along with seed impurity, that could contribute to adventitious presence by cross pollination with conventional cultivars and may cause problems during trade if the herbicide-resistant trait is not approved in the other country. Herbicide-resistant crops may cross pollinate with conventional crops and may introduce transgenes in conventional or organic crops [22]. In Canada and the USA, herbicide-resistant crops are not segregated from conventional or organic crops once the introduced trait has been unconfined released by government agencies [16]. Pollen-mediated gene flow or other sources of adventitious presence, however, may pose problems for the export of herbicide-resistant crop seeds to the countries where the novel trait has not been approved or deregulated [59]. In anticipation of the commercialization of herbicide-resistant crops, field trials have been conducted in many crops to determine distribution of gene flow and the appropriate isolation distances required between herbicide-resistant and conventional crop cultivars to meet AP thresholds.

There were two crop production systems (conventional and organic) prior to introduction of herbicide-resistant crops. However, after the commercial production of herbicide-resistant crops in 1996, the area, crop species, and number of countries growing herbicide-resistant crops have been increasing rapidly [32]. In addition to increase in the herbicide-resistant crop acreages, the concerns are also increasing about the co-existence of three production systems, adventitious presence, and transgene movement from herbicide-resistant crops to conventional and organic crops [40, 67]. Some studies reported that crop-to-crop gene flow can cause

more ecological and economical concerns than crop-to-wild gene flow because in any crop, when an herbicide-resistant cultivar would be grown commercially, gene flow to a conventional cultivar of the same crop may occur more frequently than to wild relatives [27, 28].

A major concern with the commercial cultivation of herbicide-resistant crops is the possibility of introduction of unwanted traits into conventional crop production systems. Adventitious presence of genetically engineered crop seeds in conventional crops may occur by means of pollen-mediated gene flow, admixture in seed lots and also by crop volunteers. Canadian flaxseed is being exported (> 80%) mainly to the EU, the USA, Japan and South Korea Flax Council of Canada, 2007). Growing of agricultural crops represents an open system, and thus a complete separation of plant material or crop production systems is not possible. Considering this fact and to protect the consumer's choice, the EU has defined a 0.9% labeling threshold for the adventitious presence of genetically engineered seeds in conventional products. In 1996, the herbicide (sulfonylurea) resistant flax was registered in Canada [60], but very soon it was de-registered at the request of the Flax Council of Canada, primarily to avoid the trade issue with the EU. This decision has halted further genetic modification in flax.

Currently, flax has been re-discovered as a source of functional foods [33]. In 2005, approximately 200 new flax based food and personal care products were introduced in the US market. This suggests that flax has the highest growth potential in functional food industry because of high level of omega-3 fatty acids [61]. Few studies have suggested that consuming flaxseeds in daily diet can reduce the risk of cardiovascular diseases [14, 61, 94], and certain cancers for example, breast cancer [61, 86]. Flax fibers are also becoming an integral part of new composite materials utilized in automobile and construction industry [58]. Therefore, considering the market demand for various applications, flax is required to be genetically modified. Considering this fact, Canada has decided to move forward for re-visiting genetic engineering of flax, but after risk assessment prior to commercialization by conducting research on environmental biosafety for science based decision making.

The future situation of co-existence of conventional, organic and novel flax cultivars and concern over potential movement of herbicide-resistant traits into commodity crops have illustrated the shortage of information on the gene flow among flax cultivars at intermediate distances. Therefore, the objective of this study was to determine the crop-to-crop gene flow in flax under the field conditions from high α-linolenic acid cultivar (high ALA 18:3 *Cis* $\Delta^{9, 12, 15}$) to low α-linolenic acid cultivar (low ALA 18:3 *Cis* $\Delta^{9, 12, 15}$) under the western Canadian climatic conditions.

Field experiments were conducted at two locations, Edmonton Research Station (EdRS) and Ellerslie Research Station (ElRS), University of Alberta in 2006 and 2007 to determine pollen-mediated gene flow in flax at an inter-mediate distance up to 50 m in western Canada. Flax cultivar "AC McDuff" was used as a pollen source (20x20 m in the center, Fig.3) and "Solin" cultivar "SP 2047" was used as a pollen receptor (120x120 m, Fig.3). Both the flax cultivars flowered synchronously. Prior to harvesting, the entire field was divided in eight arms or replicates (Fig. 3). A binder was used to cut all flax plants in the first 3.0 m receptor area to avoid contamination. After 3.0 m, samples were harvested by combine in 1.5 m block distance up to 12.5 m and then in 5.0 m blocks up to 50 m in all 8 replicates. Thiobarbituric acid (TBA)

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test determines relative linolenic fatty acid content in oil extruded from flax seed [13]. This method was used for screening flax seeds from receptor populations collected from the field experiments from different distances.

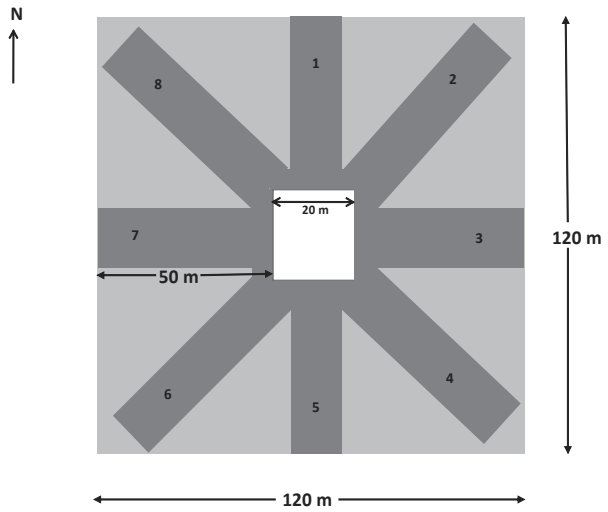

Figure 3. Design of pollen-mediated gene flow experiment in flax at all the locations and years. The pollen source flax cultivar (AC MCDuff) was seeded in the area of 20x20 m in the center of the field. The pollen receptor solin flax cultivar (SP 2090) was grown in surrounding 120x120 m area. After flowering but before harvesting, the pollen receptor area was divided in eight blocks and flax seed samples were collected at specific distances.

Gene flow was tested at various distances (up to 50 m) at each location. At EdRS research site, maximum frequency of gene flow (0.0166) was observed at the minimum mean distance of 0.1 m from the pollen source (Fig. 3). At ElRS site in 2006, 748,800 seeds were screened and the frequency of gene flow was 0.0145 and 0.00011, respectively at 0.1 and 35 m distance from the pollen source. In 2007, maximum frequency of gene flow (0.0242) was recorded at the mean distance of 0.1 m from the pollen source at EdRS site in 2007. Gene flow was quantified up to 25 m distance at EdRS site in 2007 with 13 seeds with high ALA. Some rare gene flow was observed (0.000028) after screening 476,800 seeds at 35 m distance from the pollen source at ElRS site in 2007.

The frequency of gene flow was highest in the recipient plants closest to the source, 0.0166 at EdRS, 2006; 0.0144 at ElRS, 2006; 0.0242 at EdRS, 2007 and 0.0186 at ElRS, 2007 (Fig. 4). At all four locations, gene flow ranged from 0.0001 to 0.001 at 7 m and from 0 to 0.0001 at 25 m from the mean distance from the pollen source. Some rare gene flow events were observed at 35 m mean distance from the pollen source, however at low frequency (0.00109). Several thousand seeds were analyzed at 45 m distance but no gene flow was detected at any location, however, unless all available seeds did not tested, zero gene flow cannot be interpreted but the number of seeds sampled suggest the maximum power value of test which increases the validity of the results obtained in this study.

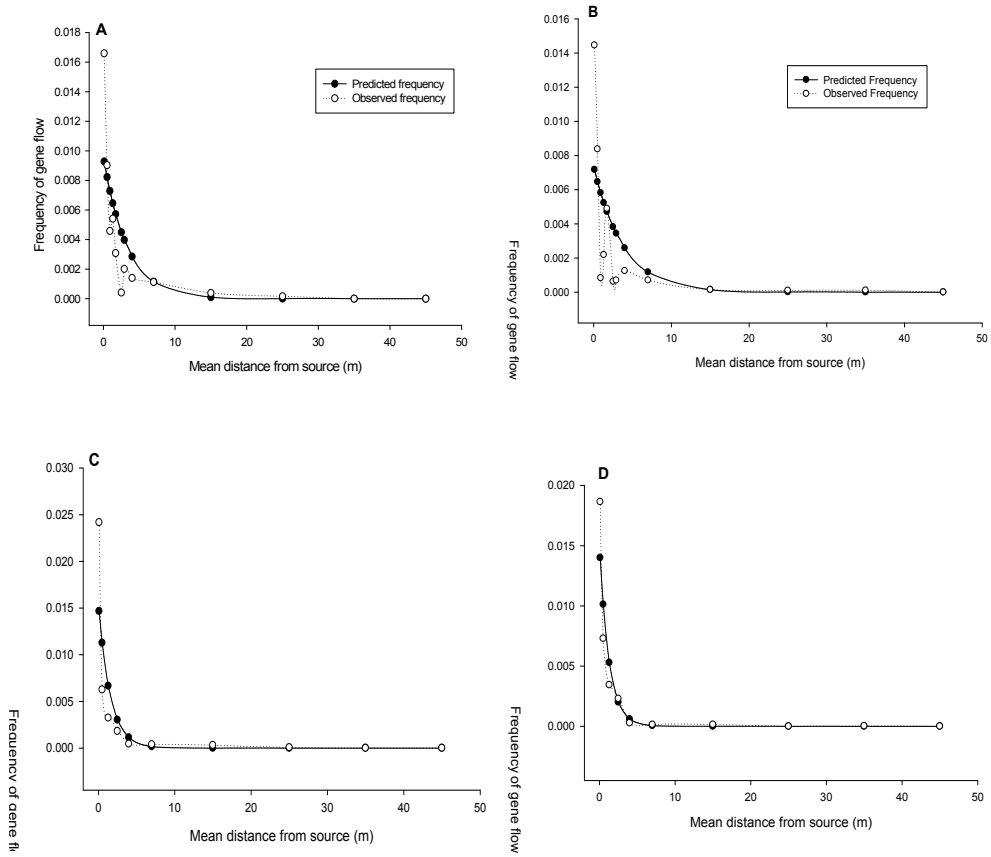

Figure 4. Pollen-mediated gene flow in flax at various locations and years (A) Edmonton Research Station (EdRS), 2006; (B) Ellerslie Research Station (ElRS), 2006; (C) Edmonton Research Station (EdRS), 2007; and (D) Ellerslie Research Station (ElRS), 2007. The triangle indicates the distance, where 50% (O_{50}) reduction in the frequency of gene flow and the arrow indicates the distance where 90% (O_{90}) reduction in gene flow from the pollen source (m) [48].

The frequency of gene flow in this study was almost equal to the other reported work, especially for samples, adjacent to the pollen source [24, 44, 52]. At 0.5 m, average gene flow was reduced to 0.0077; and at 1.0 m, 0.0027. Frequency of gene flow was reduced from 0.0126 to 0.0033, when flax cultivars were grown 1.25 to 0.25 m apart, respectively. In a similar experiment, [70] reported that frequency of gene flow in flax varied from 0 to 0.003, depending on the spacing between plants and climatic conditions.

Flax cultivars AC McDuff and SP 2047 were used in this experiment because they have similar flowering periods, which provides better chances of gene flow to occur. But for the flax cultivars with different flowering periods, the gene flow may be more or less than observed in this study. Environmental conditions and wind direction or speed did not appear to influence the amount of pollen-mediated gene flow in this study over two growing seasons. Dillman reported that sticky flax pollen was primarily disseminated by honey bees and thrips [24]. Subsequent experiments suggest that honey bee was the most frequent visitor of flax

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flowers followed by bumble bees and thrips [79]. In this study, pollinators may have played a major role in gene flow between two flax cultivars.

5. Conclusion

Flax is the second most important oilseed crop in western Canada. Because flax has unique oil and fiber attributes, it is being evaluated as a model plant species for bio-industrial and nutraceutical products. The development of flax with novel traits has raised concerns regarding the pollen-mediated gene flow to conventional flax and closely related species. As a part of pre-commercialization risk assessment of herbicide-resistant flax, intra and inter-specific pollen-mediated gene flow were quantified. Results from the average value of all location-year suggest that the frequency of gene flow at 0.1 m distance was in the range from 0.0185 to 0.01 and declined rapidly with distance from the pollen source. The minimum frequency of gene flow was recorded at 35 m distance from the pollen source. Thus, pollen-mediated gene-flow from herbicide-resistant flax to commodity flax may occur at short distances but would be rare beyond 35 m.

The occurrence of flax wild relatives and reported inter-specific hybridization was reviewed to initiate the evaluation of environmental risk of inter-specific transgene movement from herbicide-resistant flax. Inter-specific hybridization and cytogenetic studies between flax and congeneric species demonstrated that cultivated flax has ability to hybridize and form viable F_1 plants with at least nine species of *Linum,* which are reported to occur in Asia and Europe indicates that inter-specific gene flow will be negligible in North America. Hybridization of flax with many other wild relatives reported to occur in North America has either not been studied or reported. More research is required to study species distribution, sympatry, concurrent flowering, ploidy level and sexual compatibility of flax wild relatives.

In summary, the results suggest that pollen-mediated gene flow in flax will be a minor contributor to adventitious presence of herbicide-resistant flax. Minimum threshold levels will have to be established based on the impurities arising from various transgene contributors such as gene flow from other flax cultivars, occurrence of gene introgression from closely related species, seed-and pollen-mediated gene flow from herbicide-resistant flax volunteers, and admixture during production, transportation or trade.

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References

[1] Allaby, R. G, Peterson, G. W, Merriwether, D. A, & Fu, Y. B. Evidence of the domestication history of flax (*Linum usitatissimum* L.) from genetic diversity of the *sad2* locus. Theor.Appl.Genet. (2005). , 112, 58-65.

[2] Anderson, K, & Bows, A. Reframing the climate change challenge in light of post-2000 emmission trends. Philosophical Transactions of the Royal Society of London Series A: Physics and Engineering Sciences (2008). , 366, 3863-3882.

[3] Andow, D. A, & Zwahlen, C. Assessing environmental risks of transgenic plants. Ecological Letters (2006). , 9, 196-214.

[4] Andow, D. A. A growing concern: Protecting the food supply in an era of pharmaceutical and industrial crops: Technical report, Union of Concerned Scientists, (2004). Washington, D.C.

[5] AnonymousUnited nations millennium development goals. United Nations,Washington DC 2000; Accessed:07/13, (2009).

[6] Apg, I. I. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. Bot. J. Linnean Soc. (2003). , 141, 399-436.

[7] Bari, G. MBE Godward. Interspecific crosses in *Linum*. Euphytica, (1970). , 19, 443-446.

[8] Battisti, D. S, & Nylor, R. Historical warnings of future food insecurity with unprecedented seasonal heat. Science, (2009). , 323, 240-244.

[9] Beckie, H. J, Harker, K. N, Hall, L. M, Warwick, S. I, Legere, A, & Sikkema, P. H. A decade of herbicide-resistant crops in Canada. Can. J. Plant Sci. (2006). , 86, 1243-1264.

[10] Beckie, H. J, Harker, K. N, Hall, L. M, Warwick, S. I, Legere, A, & Sikkema, P. H. A decade of herbicide-resistant crops in Canada. Can. J. Plant Sci., (2006). , 86-1243.

[11] Beckie, H. J. MDK Owen. Herbicide-resistant crops as weeds in North America. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources (2007). , 044, 1-22.

[12] Bennett, R. M, Ismael, Y, Khambhampati, U, & Morse, S. Economic impact of genetically modified cotton in india. AgBioForum (2004). , 7, 96-100.

[13] Bhatty, R. S, & Rowland, G. G. Measurement of alpha-linolenic acid in the development of edible oil flax. J. Am. Oil Chem. Soc., (1990). , 67(6), 364-367.

[14] Bloedon, L. T, & Szapary, P. O. Flaxseed and cardiovascular risk. Nutr. Rev. (2004). , 62, 18-27.

[15] Brook, H. ed). Crop Protection 2008 Alberta Agriculture and Food Agdex 606-1, Edmonton, Canada, (2008). , 539.

[16] Brookes, G, & Barfoot, P. Co-existence in North American agriculture: Can GM crops be grown with conventional and organic crops? (2004). PG Economics Ltd., Dorchester, UK.

[17] Carrie, L. Morjan LHR. How species evolve collectively: Implications of gene flow and selection for the spread of advantageous alleles. Molecular Ecology (2004). , 13, 1341-1356.

[18] Carson, R. Silent spring. Houghton Miffin Company (1962). New York, USA.

[19] Challinor, A, Ewert, F, Arnold, S, Simelton, E, & Fraser, E. Crops and climate change: Progress, trends, and challenges in simulating impacts and informing climate change. J. Exp. Bot. (2009). , 60, 2775-2789.

[20] Chapotin, S. M, & Wolt, J. D. Genetically modified crops for the bioeconomy: Meeting public and regulatory expectations. Transgenic Res., (2007). , 16, 675-688.

[21] Chen, L. J, Lee, D. S, Song, Z. P, Suh, H. S, & Lu, B. R. Gene flow from cultivated rice (*Oryza sativa* L.) to its weedy and wild relatives. Annals of Botany (2004). , 93, 67-73.

[22] Colbach, N, Devaux, C, & Angevin, F. Comparative study of the efficiency of buffer zones and harvest discarding on gene flow containment in oilseed rape. A modelling approach. Eur.J.Agron. (2009). , 30(3), 187-198.

[23] Deshpande, R. B. Wild safflower (*Carthamus oxyacantha* bieb.)- a possible oilseed crop for the desert and arid regions. Indian J. Genet. Plant Breeding (1952). , 12, 10-14.

[24] Dillman, A. C. Natural crossing in flax. Agronomy Journal (1962). , 30, 279-286.

[25] Duke, S. O. Taking stock of herbicide-resistant crops ten years after introduction. Pest Management Science (2005).

[26] Ellstrand, N. C. Scientists evaluate potential environmental risks of transgenic crops. California Agriculture (2006). , 60, 119-120.

[27] Ellstrand, N. C. When crop transgenes wander in California, should we worry? California Agriculture, , 60-116.

[28] Ellstrand, N. C. Current knowledge of gene flow in plants: Implications for transgene flow. Philos.Trans.R.Soc.Lond.Ser.B-Biol.Sci., (2003). , 358(1434), 1163-1170.

[29] Ellstrand, N. C, & Schierenbeck, K. A. Hybridization as a stimulus for the evolution of invasiveness in plants? Euphytica, (2006). , 148, 35-46.

[30] Faircloth, W. H, Patterson, M. G, Monks, C. D, & Goodman, W. R. Weed management programs for glyphosate-tolerant cotton (*Gossypium hirsutum* L.). Weed Technol. (2001). , 15, 544-551.

[31] FAOWorld agriculture 2030: Main findings.Food and Agriculture Organization, Rome, Italy. 2009. Accessed: 07/03, (2009).

[32] Firbank, L. G, Rothery, P, May, M. J, Clark, S. J, Scott, R. J, & Stuart, R. C. Effects of genetically modified herbicide-tolerant cropping systems on weed seedbanks in two years of following crops. Biol. Letters (2006). , 2(1), 140-143.

[33] Fitzpatrick, K. Innovation in western Canadian functional food. Cereal Foods World (2007). , 52, 289-290.

[34] Gill, K. S. Linseed. Indian Council of Agricultural Research, New Delhi, India (1987). , 386.

[35] Gill, K. S. Evolutionary relationship among *Linum* species. University of California, Riverside, CA, USA (1966). , 1-243.

[36] Gill, K. S, & Yermanos, D. M. Cytogenetic studies on genus *Linum*.I. hybrids among taxa with 15 as haploid chromosome number. Crop Science (1967). , 7(6), 623-626.

[37] Gill, K. S, & Yermanos, D. M. Cytogenetic studies on genus *Linum*. II. hybrids among taxa with 9 as haploid chromosome number. Crop Science (1967). , 7(6), 627-631.

[38] Graff GD Regulation of transgenic crops intended for pharmaceutical and industrial usesIn: Just, Richard E., Julian M. Alston and David Zilberman (eds.), Regulating Agricultural Biotechnology: Economics and Policy. Springer, New York (2006). , 647-661.

[39] Gulden, R. H, Francois, T, Clarence, S. J, Sikkema, P. H, & Hamill, A. S. Conventional vs. glyphosate-resistant cropping systems in ontario: Weed control, diversity and yield. Weed Science, (2009). , 57, 665-672.

[40] Hall, L. M. AKC Topinka, RL Nielson. Intraspecific gene flow: Influencing factors. In: Gulden,R. H. and C. J. Swanton (eds.), The First Decade of Herbicide Resistant Crops

in Canada. Canadian Weed Science Society-Societe canadienne de malherbologie, Sainte-Anne-de-Bellevue, Quebec, (2006). , 87-100.

[41] Hanson, B. D. CA Mallory Smith, WJ Price. Interspecific hybridization: Potential for movement of herbicide resistance from wheat to jointed goatgrass (*Aegilops cylindrica*) Weed Technology (2005). , 19, 674-682.

[42] Hanson, D. E, Ball, D. A, & Mallory-smith, C. A. Herbicide resistance in jointed goatgrass (*Aegilops cylindrica*): Simulated responses to agronomic practices Weed Technology (2002). , 16, 153-163.

[43] Haslberger, A. GMO contamination of seeds. Nature Biotechnology (2001).

[44] Henry, A. W, & Tu, C. Natural crossing in flax. J. Am. Soc. Agron., (1928). , 20, 1183-1192.

[45] Heywood, V. H. Flowering plants of the world. Oxford University Press, Oxford, UK (1993). , 207-208.

[46] Huang, J. R, Hu, C, & Pray, F. Quiao, S Rozelle. Biotechnology as an alternative to chemical pesticides: A study of bt cotton in china. In: 5th international cosortium on agricultural biotechnology research, International Cosortium on Agricultural Biotechnology Research, Ravello, Italy (2001).

[47] Jhala, A. J, Hall, L. M, & Hall, J. C. Potential hybridization of flax (*Linum usitatissimum* L.) with weedy and wild relatives: An avenue for movement of engineered genes? Crop Science (2008). , 48, 825-840.

[48] Jhala, A. J, Bhatt, H, Keith, A, & Hall, L. M. Pollen mediated gene flow in flax: Can genetically engineered and organic flax coexist? *Heredity* (2011). , 106, 557-566.

[49] Jhala, A. J, Weselake, R, & Hall, L. M. Genetically engineered flax: Potential benefits, risks and mitigation of transgene movement. Crop Sci. (2009). , 49, 1943-1954.

[50] Johnson, W. G, Bradley, P. R, Hart, S. E, Buesinger, M. L, & Massey, R. E. Efficacy and economics of weed management in glyphosate resistant corn (*Zea mays* L.). Weed Technology (2000). , 14, 57-65.

[51] Johnson, K. L, Raybould, A. F, Hudson, M. D, & Poppy, G. M. (2007). How does scientific risk assessment of GM crops fit within the wider risk analysis? Trends Plant Sci., 12:(1) 1-5.

[52] Kadam, B. S, Kulkarni, R. K, & Patel, S. M. Natural cross pollination in linseed. The Madras Agricultural Journal (1938). , 26, 4-9.

[53] Kershen, D. L, & Mchughen, A. Adventitious presence: Inadvertent commingling and coexistence among farming methods. CAST Commentary, QTA (2005). , 2005, 1-1.

[54] Kikuchi, M. Cytological studies of the genus *Linum.* Japanese J. Genetics (1929). , 4, 201-210.

[55] Koul, O, & Dhaliwal, G. S. eds). Transgenic Crop Protection: Concepts and Strategies Science Publishers Inc., Enfield, NH, USA, (2004).

[56] Kruger, G. R, Johnson, W. G, Weller, S. C, Owen, M, Shaw, D. R, & Wilcut, J. W. US grower views on problematic weeds and changes in weed pressure in glyphosate-resistant corn, cotton, and soybean cropping systems. Weed Technology (2009). , 23, 162-166.

[57] Kumar, V, Bellinder, R. R, Brainard, D. C, Malik, R. K, & Gupta, R. K. Risks of herbicide-resistant rice in india: A review. Crop Protection (2008). , 27, 320-329.

[58] Kymäläinen, H, & Sjöberg, A. Flax and hemp fibres as raw materials for thermal insulations. Building and Environment, (2008).

[59] Mallory-smith, C, & Zapiola, M. Gene flow from glyphosate-resistant crops. Pest Manag.Sci. (2008). , 64, 428-440.

[60] Mchughen, A, Rowland, G. G, Holm, F. A, Bhatty, R. S, & Kenaschuk, E. O. CDC triffid transgenic flax. Can. J. Plant Sci. (1997). , 77, 641-643.

[61] Morris, H. M. Flax: A health and nutrition primer. Flax Council of Canada, Winnipeg, Canada (2007).

[62] Moryganov, A. P, Galashina, V. N, Dymnikova, N. S, Stokozenko, V. G, & Danilov, A. R. Modification of flax fibers. Fibre Chemistry (2008). , 40, 234-240.

[63] Mosquin, T, & Hayley, D. E. Evolutionary relationships of the southern florida populations of *Linum rigidum* (Linaceae). Can.J. Bot. (1967). , 45, 1277-1283.

[64] Phipps, R. H. PJR. Environmental benefits of genetically modified crops: Global and european perspective on their ability to reduce pesticide use. Journal of Animal and Feed Sciences (2002). , 11, 1-18.

[65] Poppy, G, & Crops, G. M. Environmental risks and non-target effects. Trends Plant Sci. (2000). , 5, 4-6.

[66] Ray, C. J. Cytological studies in the flax genus *Linum*. American J. Bot. (1944). , 31, 441-448.

[67] Rieger, M. A, Lamond, M, Preston, C, Powles, S. B, & Roush, R. T. Pollen-mediated movement of herbicide resistance between commercial canola fields. Science (Wash.) (2002). , 296(5577), 2386-2388.

[68] Rieseberg, L. H, Kim, M. J, & Seiler, G. J. Introgression between the cultivated sunflower and a sympatric wild relative, *Helianthus petiolaris* (asteraceae). Int J Plant Sci. (1999).

[69] Rieseberg, L. H, & Wendel, J. F. Introgression and its consequences in plants. In: Harrison,R. (ed.), Hybrid Zones and the Evolutionary Process. Oxford University Press, Oxford, UK, (1993). , 70-109.

[70] Robinson, B. B. Natural cross pollination studies in fiber flax. Agronomy Journal (1937). , 29(8), 644-649.

[71] Rogers, C. M. Yellow flowered speceis of *Linum* in central America and western North America. Brittonia (1968). , 20, 107-135.

[72] Rosegrant, M. W, & Cline, S. A. Global food security: Challenges and policies. Science (2003). , 302, 1917-1919.

[73] Rowland, G. G. An EMS-induced low-linolenic acid mutant in McGregor flax (*Linum usitatissimum* L.). Can J Plant Sci (1991). , 71, 393-396.

[74] Runge, C. F, & Ryan, B. (2004). The global diffusion of plant biotechnology: International adoption and research in 2004. Accessed:02/10, 2009. http:// www.apec.umn.edu/faculty/frunge/globalbiotech04.pdf.

[75] Seetaram, A. Interspecific hybridization in *Linum*. Euphytica (1972). , 21, 489-495.

[76] Sharma, R. R, & Khanna, A. N. Development of fruit in some interspecific crosses of the genus *Linum*. Indian Oilseeds J. (1964). , 8, 34-80.

[77] Shaw, D. R, Givens, W. A, Farno, L. A, Gerard, P. D, Jordan, D, & Weller, S. C. Using a grower survey to assess the benefits and challenges of glyphosate-resistant cropping systems for weed management in US corn, cotton and soybean. Weed Technology (2009). , 23, 134-149.

[78] Sikkema, P. H, & Soltani, N. Herbicide resistant crops in eastern Canada. In: Gulden,R. H. and C. J. Swanton (eds.), The First Decade of Herbicide Resistant Crops in Canada. Canadian Weed Science Society, Sainte Anne de Bellevue, (2007). Quebec, , 3-13.

[79] Smirnov, V. M. (1956). Cross pollination in flax by bees. Pchlevodstvo, , 9, 53-55.

[80] Snow, A, Andow, D. A, Gepts, P, Hallerman, E. M, Power, A, & Tiedje, J. M. (2005). Genetically engineered organisms and the environment: Current status and recommendations. Ecol. App., , 15, 377-404.

[81] Snow, A. A, Andersen, B, & Jorgensen, R. B. Costs of transgenic herbicide resistance introgressed from *brassica napus* into weedy *B. rapa*. Molecular Ecology, , 8, 605-615.

[82] Snow, A. A, Andow, D. A, Gepts, P, Hallerman, E. M, Power, A, & Tiedje, J. M. Genetically engineered organisms and the environment: Current status and recommendations. Ecological Applications : A Publication of the Ecological Society of America (2005). , 15, 377-404.

[83] Stewart, P. A, & Knight, A. J. Trends affecting the next generation of U.S. agricultural biotechnology: Politics, policy, and plant-made pharmaceuticals. Technol. Forecast. Soc. Change, (2005). , 72, 521-534.

[84] Surman, W. GM crops in argentina.New Agriculturist,WREN media, Suffolk, UK 2007;. Accessed:12/ 15, (2009).

[85] Tammes, T. The genetics of the genus *Linum*. bibl. genet. Bibl. Genet. (1928). , 4, 1-36.

[86] Thompson, L. U, Chen, J. M, Li, T, Straaser, W. K, & Goss, P. E. Dietary flaxseed alters tumor biological markers in post menopausal breast cancer. Clin Cancer Res (2005). , 11, 3828-3835.

[87] Vaisey-genser, M, & Morris, D. H. History of the cultivation and uses of flaxseed. In: Muir,A. D. and N. D. Wescott (eds.), The Genus *Linum*. Taylor and Francis Ltd, New York New York, USA, (2003).

[88] Vromans, J. Molecular genetic studies in flax (*Linum usitatissimum* L.). Wageningen University, Wageningen, The Netherlands, (2006). , 22-39.

[89] Wall, D. A. Fluazifop-P tank-mixtures with clethodim for annual grass control in flax (*Linum usitatissimum* L.). Weed Technololy (1994). , 8, 673-678.

[90] Warwick, S. I, Beckie, H. J, & Hall, L. M. Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of New York Academy of Science (2009). , 1168, 72-99.

[91] Winkler, H. Linaceae, trib I. linoideae- eulineae. In: Engler,A. (ed.), Die Nature-Lichen Pfanzenfamilien Nebst Ihren Gattungen Und Wichtigeren Arten, Insbesondere Den Nutzpflanzen. W. Engelmann, Leipzig, (1931). , 111-120.

[92] Yermanos, D. M, & Gill, K. S. Induction of polyploidy in *Linum* species. Crop Science (1967).

[93] Zhang, W. Q, Linscombe, S. D, Webster, E, Tan, S. Y, & Oard, J. Risk assessment of the transfer of imazethapyr herbicide tolerance from clearfield rice to red rice (*Oryza sativa* L.). Euphytica (2006). , 152, 75-86.

[94] Zhao, G, Etherton, T. D, Martin, K. R, West, S. G, Gillies, P. J, & Etherton, K. M. Dietary α-linolenic acid reduces inflammatory and lipid cardiovascular risk factors in hypercholesterolemic men and women. Journal of Nutrition (2004). , 134, 2991-2997.

Chapter 13

Recent Advances in the Extraction of Triazines from Water Samples

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Additional information is available at the end of the chapter

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1. Introduction

The use of herbicides in agriculture has helped to improve crop quality and yield. However, the presence of such substances has also caused serious environmental pollution problems. Triazine herbicides are a group of herbicides applied in agriculture for pre- and post-emergence weed control. The first report of the use of triazine derivatives was in 1952 by J.R. Geigy from Switzerland but it was not until 1954 that chlorazine was used as a herbicide, followed by simazine in 1955 [1]. During subsequent years the amount of commercially available triazines increased. The main triazine herbicides are derived from s-triazine, a six member heterocycle with symmetrically located atoms in which positions 2, 4 and 6 are substituted. The stereochemical stability of s-triazines is large enough to persist in environmental samples from several months to many years [2].

A list of common *s*-triazines and some of their properties are given in Table 1. The two most common *s*-triazines analyzed in waters are atrazine and simazine. The chemical common name depends on the substituent in position 2 (or R1 in Table 1), when a –Cl group is contained the names end with –azine, while $–SCH_3$ and $–OCH_3$ end with –tryn and –ton, respectively. The thermodynamical properties also depend on the substitutes, the acidity decreases according the following order $-OCH_3$<$-SCH_3$<-Cl and the solubility in water are higher in acidic conditions. The *s*-triazines which contain $–SCH_3$ group are more polar than the –Cl and $–OCH_3$ compounds according to the partition coefficient between n-octanol and water K_{ow} (log P) [3]. The toxicity of these substances has promoted the development of new analytical methodologies to evaluate their impact to the environment and human health.

Maximum residue limits for *s*-triazines in water samples are in the μg l^{-1} order. This fact demands better quality and accurate analytical methodologies. Moreover, these concentration levels require performing an initial stage of concentration and purification of the analytes prior to their analysis. The analytical procedure usually is comprised of five steps: sampling, sample preparation, separation, detection and data analysis, but sampling and sample preparation are the critical steps of the analytical process. Over 80% of the analysis time is spent on these two steps. If one of these steps is not followed adequately, the performance of the procedure will be affected and the results will be inconsistent [4].

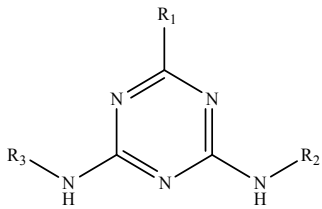
Compound	R_1	R_2	R_3	pK_a	K_{ow} (log P)
					
Atrazine	Cl	C_2H_5	$CH(CH_3)_2$	1.68	2.7
Propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	1.85	2.9
Simazine	Cl	C_2H_5	C_2H_5	1.65	2.3
Terbutylazine	Cl	C_2H_5	$C(CH_3)_3$	1.88	3.1
Terbutemon	OCH_3	C_2H_5	$C(CH_3)_3$	4.20	3.6
Ametryn	SCH_3	C_2H_5	$CH(CH_3)_2$	4.00	3.1
Prometryn	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	4.05	3.3
Simetryn	SCH_3	C_2H_5	C_2H_5	4.00	2.8
Terbutryn	SCH_3	C_2H_5	$C(CH_3)_3$	4.40	3.7

Table 1. pKa and K_{ow} (log P) of common *s*-triazines herbicides

Adequate sample preparation is a requisite for analytical techniques. Analysts have responded to this challenge, so in this work recent sample extraction techniques for analysis of *s*-triazines in water samples are overviewed.

2. Sample preparation

The sample preparation concept is based on converting a real matrix into a sample suitable for analysis. This process involves a change in the chemical environment of the sample. An initial step in the design of an extraction method is the knowledge of the physical and chemical properties such as lipophilicity and the predominance of acid-basic species neutral or ionic.

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Matrix effects are the main problem in extracting analytes. A matrix effect can be defined as the influence of a property of the sample, independent of the presence of the analyte, on the recovery efficiency. In water samples a pre-concentration step is required prior to measurement of triazines. A pre-concentration factor of several orders of magnitude (200-1000 fold) is mandatory to reach the low detection limits necessary for identification and analysis of these herbicides, especially in highly organic matter content samples such as wastewaters. The common extraction methods used for isolation of polar compounds from water matrices are the liquid-liquid extraction (LLE) and the solid phase extraction (SPE).

2.1. Liquid-liquid extraction

LLE has been used in the past for the extraction of triazines from environmental water samples [5]. LLE is based on the partition coefficient of the analytes between two liquid phases of low solubility. In the case of triazines the common sample volume used is 1 l (pH adjusted to 7) and it is mixed with an organic solvent, such as methylene chloride (at least 2x50 ml). The aqueous layer is then discarded, the organic phase evaporated and concentrated to a volume of 5 ml and the solvent is exchanged. The obtained extract is analyzed by a separation technique (gas or liquid chromatography). The main disadvantages of this procedure are: the use of large volumes of organic solvents, limited pre-concentration factors and tedious procedures.

In recent years, the scientific community has shown an increased interest in the development of environmentally friendly laboratory activities. Green analytical chemistry pursues the aim of replacing toxic reagents by clean ones. Also the development and improvement of new sample preparation techniques is a fast growing trend in analytical chemistry. In this context, liquid phase microextraction techniques have evolved from the use of tens of ml of solvent to the use of drop-based (μl) systems. The different approaches employed for the liquid microextraction of triazine herbicides from environmental matrices are mainly: hollow fiber liquid phase microextraction (HFLME) and dispersive liquid-liquid microextraction (DLLME).

2.1.1. Hollow fiber liquid phase microextraction

HFLME is a membrane based separation technique. It can be sub-classified into two-phase and three-phase systems. The two-phase system is the most used system in the extraction of trazines from aqueous samples. The two-phase systems are also referred as microporous membrane extraction. It is comprised of an aqueous phase and a hydrophobic porous membrane impregnated with a suitable organic solvent (Figure 1). The aqueous phase usually contains the analyte and it is called the donor phase while the organic solvent is the receiving/acceptor phase [6].

The extraction process involves partitioning of the analyte from the aqueous sample into the organic solvent which impregnates the hollow fiber (HF) and the diffusion through the membrane into the bulk receptor/acceptor phase. These systems are suitable for extraction of compounds with large partitioning coefficients in the organic phase. Polypropylene is the material commonly used for triazines using two-phase HFLME systems.

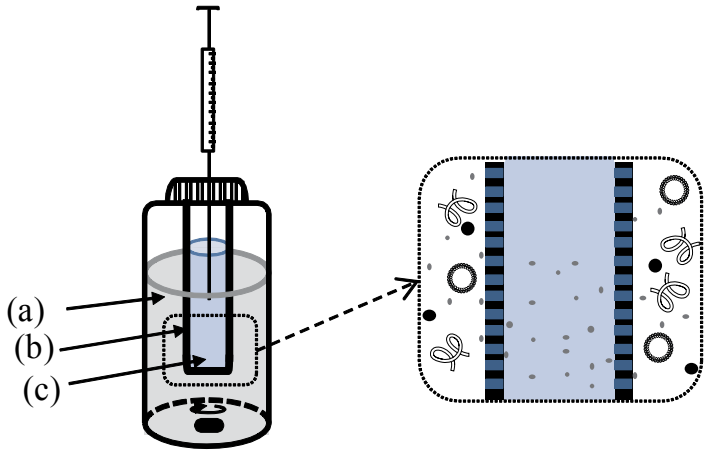

Figure 1. Representation of a hollow fiber liquid phase microextraction system. (a) donor phase, (b) hollow fiber with organic phase, (c) acceptor phase.

The common steps included on the HFLME are: a) cleaning of the HF, b) conditioning of the HF impregnating it with the extraction solvent, c) adding a specific volume of the solvent into the HF, d) immersing the HF into the sample for a definite time, e) aspiring the preconcentrated sample for its analysis.

A system based on the use a polypropylene HF (1.5 cm X 0.6 mm i.d.) containing 3 μl of toluene as organic solvent was used for the extraction of simazine, atrazine, propazine, simetryn and prometryn from 3.0 ml of water samples. The organic phase was analyzed by gas chromatography-mass spectrometry (GC-MS). The effect of salt addition, agitation, pH and exposure time were evaluated. The most critical variable was the pH value required (>4.0) which determines the formation of the suitable extractable analyte form. The method described provides good enrichment factors (<150), good precision (<3.5%, expressed as relative standard deviation, RSD) with limits of detection (LODs) in the range of 0.007-0.063 μg l^{-1}[7].

The use of phosphorus-oxygen compounds as co-extraction solvents has been proposed for isolation of pesticides from water samples including triazine herbicides. Atriazine, simazine and propazine were extracted using a polypropylene HF (3.3 cm X 0.3 mm i.d.) filled with a *n*-dihexylether solution containing 10% of tri-*n*-octylphosphine oxide ($(C_8H_{17})_3PO$) and 10% of tri-n-butylphosphonate ($(C_4H_9O)_3PO$). The dipolar moment from the P-O bond increases the polarity of the extraction solvent, allowing the isolation of the triazines and the other pesticides evaluated. The extraction was optimal when the donor pH was fixed to 8.0, using the organic phase, above mentioned and a contact time of 4 h in a 250 ml of a water sample. The system was coupled to high performance liquid chromatography- mass spectrometry (HPLC-MS) as separation and detection technique. Under these conditions LODs from 0.061 to 0.26 were obtained for triazine compounds [8].

Over the past decades, carbon nanotubes have elicited interest due to their chemical and physical properties. At the nanoscale, an increase of the contact surface area is observed.

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Following the new tendencies, a polypropylene HF (3.0 cm X 0.6 mm i.d.) was impregnated with a suspension composed by n-octanol and multiwalled carbon nanotubes, intended for simazine, simetryn, propazine, and prometryn isolation. The HF was immersed into the sample (15.0 ml) containing 30 μl of chlorobenzene and 2.25 g of NaCl for 20 minutes. Then, the HF was washed with water and immersed in 50 μl of methanol for elution of the analytes. The analysis of the methanolic solution by HPLC with ultraviolet detection (HPLC-UV) gives a LOD of in the range of 0.08-0.15 μg l^{-1} [9].

2.1.2. Dispersive liquid-liquid microextraction

DLLME is a miniaturized LLE technique based on a ternary component solvent system composed of a certain amount of the sample, a disperser solvent and an extraction solvent. The extraction steps involved on the DLLME are (Figure 2): a) a volume of the sample is placed in a tube with conic bottom, b) the disperser and extraction samples are injected into the sample, c) the mixture is then mixed and a cloudy solution is formed in the test tube. A higher contact area between the organic-aqueous phases is obtained due to the formation and dispersion of micro-drops of organic phase. Subsequently, equilibrium state is achieved quickly, resulting in a reduction in the extraction time. The final step is the centrifugation and depending on the density of the extraction solvent, d) it sediments at the bottom of the test tube or e) floats at the top of the solution. Finally, a definite volume of the pre-concentrated sample is recovered and analyzed.

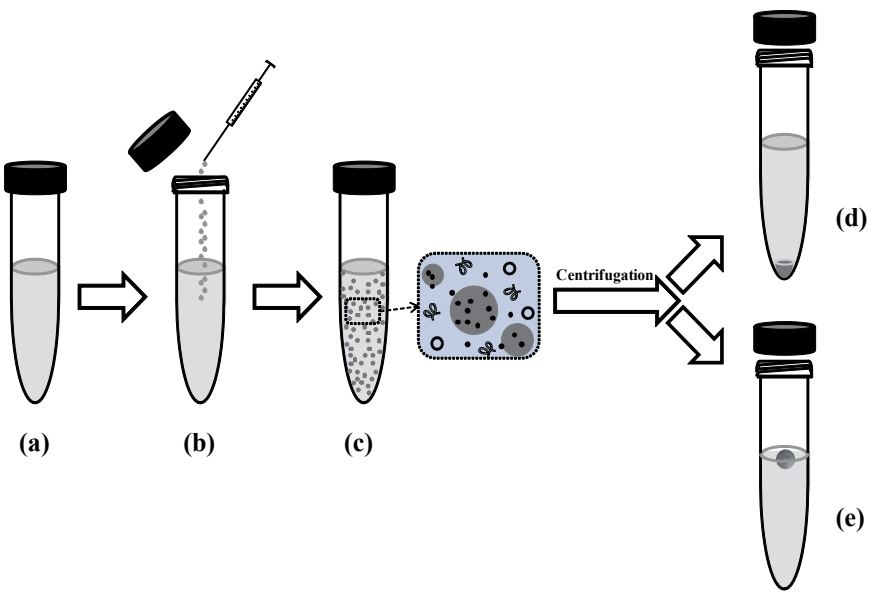

Figure 2. Representation of the dispersive liquid-liquid microextraction methodology

Chlorobenzene is a suitable extraction solvent for triazine isolation, and it was applied for DLLME of atrazine, simazine, prometryn, propazine and simetryn from water samples. The

methodology uses 5.0 ml of water samples containing 4% (w/v) of sodium chloride mixed with 12.0 μl of chlorobenzene and 1.0 ml of acetone (disperser solvent). The mixture was centrifuged and a 2 μl sample was analyzed by GC-MS. The method proposed has a LODs between 0.021 and 0.12 μg l^{-1} with a precision <5%, expressed as RSD [10].

The search for new extraction solvents is a key trend in the solvent extraction evolution. In this sense, ionic liquid, which is an ionic media resulting from the combination of organic cations with various anions has attracted attention for its special features such as: low-vapour pressure, high viscosity, dual polarity and a wide range of miscibility with water and other organic solvents [11].

The ionic liquid 1-hexyl-3-methylimidazolium hexafluorophosphate has been evaluated as extraction solvent of atrazine, prometryn, and simazine. The proposed methodology prepared a solution mixing water sample (10 ml) and the ionic liquid (60 μl) in a conical test tube. The test tube was heated in water bath at 70 °C for 5 min and was thereafter cooled with ice for 30 min until the solution became turbid. The dispersion was centrifuged for 10 min at 3800 rpm, the upper aqueous phase was removed and the ionic liquid phase was dissolved in 100 μl of methanol for HPLC-UV analysis. Under optimal condition the LODs of the reported method are in the range from 0.46 to 0.89 μg l^{-1}, with precisions below 10% (RSD) [12].

Following the same tendency, a DLLME method coupled to microwave assisted extraction (MAE) was design for extracting ametryne, prometryne, and terbutryn. A 10 ml microwave tube was filled with 5.0 ml of water sample. Then, 40 μl of 1-butyl-3- methylimidazolium tetrafluoroborate and 500 μl of a 0.2 g ml^{-1} disperser solution of lithium bis[(tri-fluoromethane)sulfonyl]imide were added. The suspension was irradiated under microwave power of 30 W during 90 seconds. After cooling, the suspension was centrifuged at 5000 rpm for 6 minutes. The aqueous phase was removed and the ionic liquid phase was stored for HPLC-UV analysis. The LODs were between 0.52 and 1.30 μg l^{-1} with precisions <10%, as RSD [13].

In the case of DLLME using extraction solvents with lower density than water, there has been reported the use of DLLME based on the solidification of a floating organic droplet for analysis of atrazine and simazine in water samples. The conditions proposed for the sample treatment are: 10 μl of 1-undecanol (ϱ=0.83 g ml^{-1}) as extraction solvent, 100 μl of acetonitrile as disperser solvent, NaCl 5% (w/v) and 5ml of water sample. The mixture was then centrifuged for 3 min at 400 rpm and then transferred into an ice bath. After 5 minutes the extraction solvent solidified and was transferred into a clean conical tube, where it melts quickly at room temperature. The extract was then analyzed by GC-MS. The LODs reported were in the range of 0.52-1.30 μg l^{-1} with precisions <5.0%, as RSD [14].

Simultaneous DLLME and microwave assisted extraction was also applied in the analysis of cereal samples. The method involved the use of 1-dodecanol, methanol and water in order to extract the solid sample. Although the technique reported does not involve the analysis of water samples, it is an interesting example of coupled techniques which generates a dynamic and simple methodology for extraction of triazines from complex samples [15].

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2.2. Solid phase extraction

Solid phase extraction (SPE) is the main separation technique used for trace enrichment of triazines from aqueous samples. The use of cartridges or disk forms, allows a high degree of flexibility. In the last years, there has been a considerable interest in designing new selective and sensitive stationary phases for extracting triazine compounds. Selectivity is related with the extraction mechanism used during isolation of the analytes. The most important interactions between the solid phase and the analytes are represented in Figure 3.

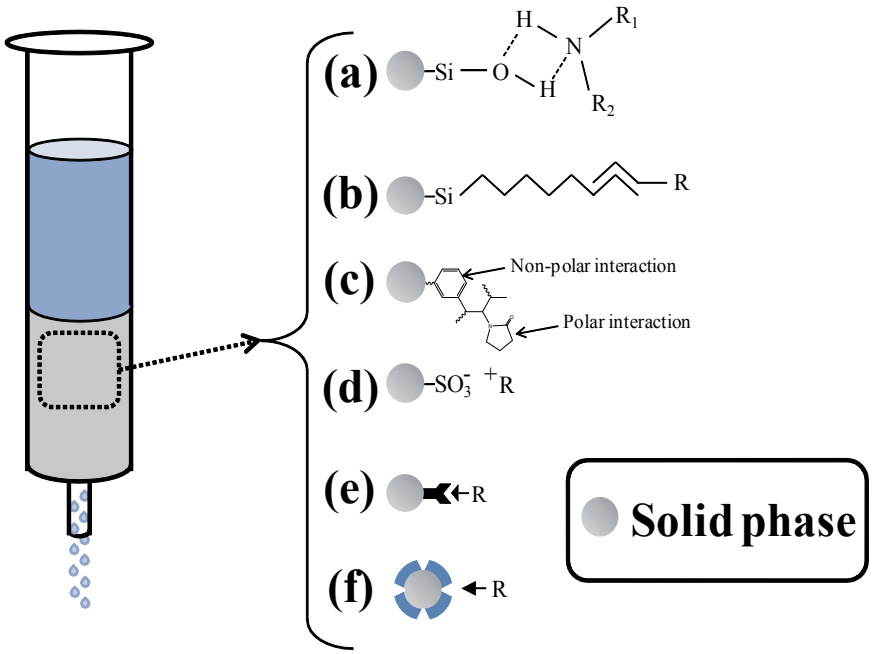

Figure 3. Representative interactions mode between the solid phase and the analytes during SPE

SPE using polar solid phases (Fig 3.a) has been applied for isolation of atrazine, ametryn, prometryn, terbuthylazine, terbutryn and simazine. Initially, a solution of 10 ml of water sample, 50 ml of acetonitrile and 10 g of NaCl was prepared. Then a 25 ml portion of the mixture was evaporated to dryness in a vacumm evaporator at 50 ºC. The residue was dissolved in hexane and it was then subjected to SPE clean-up with Florisil cartridges using hexane and acetone/hexane (80:20) as condition and elution solvents. The sample extract was analyzed by HPLC-MS, achieving limits of quantification (LOQs) in the range of 0.02-0.05 mg l^{-1} with precisions < 10% [16].

SPE, based on C18 and polymeric phases (Fig 3.b and c), has been widely used for determination of triazine in water samples [17-23]. The common amount of water sample passing through the cartridge is 100 ml, followed by a drying step. Triazine compounds are eluted with a few ml of a solvent such as acetonitrile, ethyl acetate or methanol. The LODs achieved depend

on the type of separation technique (GC or HPLC) and the detector used (MS, UV, etc.). In some cases it is reported limits in the ng l^{-1} when sensitive detectors are used.

Strong cation exchange SPE (SCX-SPE, Fig 3.d) has been proposed for ametryn, atrazine, propazine, prometryn, simazine, simetryn and terbutryn isolation. The positive charge of the acid form of triazines allows the isolation of the analytes. Elution was performed by adding a 0.07 M KCl aqueous-methanolic solution. The obtained extract was analyzed by HLPC-UV. The LODs reported using spikes water samples was 0.01 μgl^{-1} [24].

In order to increase the selectivity of extraction, the use of immunosorbents was reported (Fig. 3.e). The production of polyclonal antibodies was done by immunization of rabbits with caprolyl-atrazine. The immunosorbent was applied in the analysis of ametryn, atrazine, propazine, prometryn, simazine, simetryn and terbutryn in water samples. The system could be used in off- or on-line modes. The pre-concentration of 50 ml water samples and the use of methanol 70% (v/v) as elution solvent prior to HPLC-UV analysis provided LODs of 1-2 μg l^{-1} [25-26].

A highly specific method for atrazine isolation was developed using a moleculary imprinted polymer (MIP, Fig 3.f). The MIP was synthesized using atrazine : methacrylic acid: ethylene glycol dimethyl methacrylate in a molar ratio of 1:4:20. The polymer exhibited a high selectivity to atrazine isolation, achieving LOD of 0.5 μg l^{-1} when it is coupled to HPLC-UV [27].

Multiwalled carbon nanotubes have been evaluated as adsorbent for atrazine and simazine isolation. The atrazines were retained on the solid phase in their neutral form and they were eluted from the solid using acetonitrile or acetone. The methodology was tested using different geometries (disk and cartridge) and volumes (200 and 500 ml) and also different detection methods (GC-MS and HPLC-DAD). The LODs were in the μg l^{-1} order [28-29].

During the last decades, different techniques have been proposed to improve the SPE. Extraction of triazines has been usually carried out by solid phase microextraction (SPME), stir bar sorptive extraction (SBSE) and dispersive solid phase extraction (DSPE).

2.2.1. Solid phase microextraction

SPME (Figure 4) has become popular for the analysis of organic compounds because it combines sampling and pre-concentration in a single step. In this technique a fused silica fiber coated with a polymeric film is immersed into the sample (Fig. 4.a and b). The analytes are adsorbed into the stationary phase and later desorbed for its ulterior analysis (Fig. 4.c and d). SPME has the following advantages: (i) the extraction time is reduced, (ii) it provides good results over a wide range of analyte concentrations and (iii) it can be easily automated. Obviously, the composition of the fibers is a great importance in this methodology.

Atrazine, simazine, terbuthylazine and terbutryn have been extracted from water and soil samples using SPME with a carbowax-divynilbenzene fiber. Extraction was carried out by direct immersion of the fiber into the sample (3.0 ml) containing 10% of NaCl to adjust the ionic force. The mixture was stirred for 30 min and desorption of the herbicides was carried out at 240 ºC in the hot GC-MS injector. The LODs were below 0.1% μg l^{-1} with precision intra-

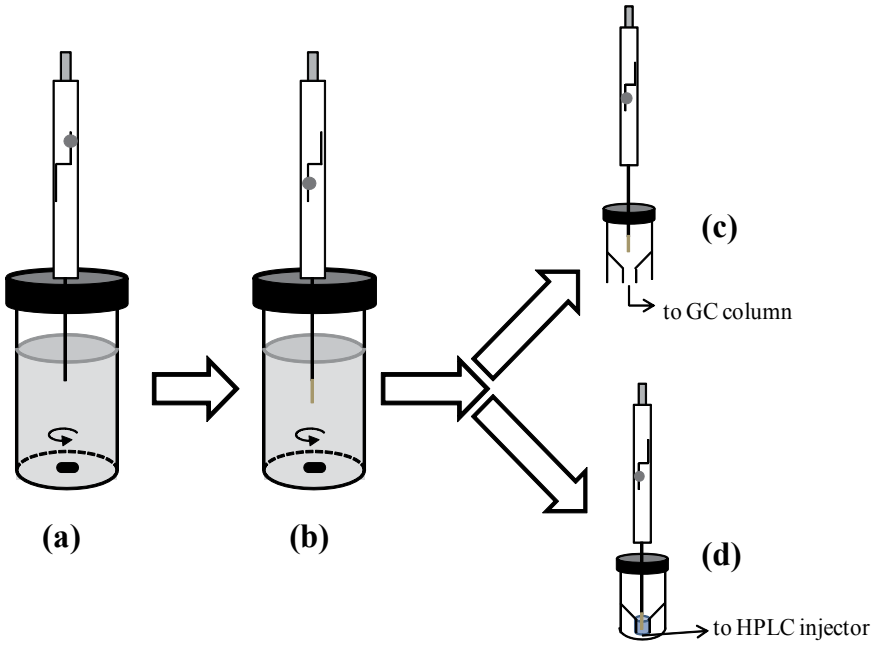

Figure 4. Representation of the solid phase microextraction methodology

and inter-day below 10 and 20%, respectively. The absence of organic solvent during sample preparation was the main advantage of the proposed method [30].

Carbon solids are one of the most important adsorption materials since they exhibit high isolation capacity for organic compounds. Graphene is a novel carbon material with large delocalized π-electron system that can form strong π-stacking interaction with the aromatic ring presented in the triazine structure. Atrazine, ametryn, prometron and prometryn were extracted using iron fibers coated with graphene. The fiber was immersed into 10 ml water sample solution for 30 min with stirring at 950 rpm. Afterwards, the extracted analytes on the SPME fiber were desorbed with 50 μl of acetone. The extract was then analyzed by HPLC with diode array detection (HPLC-DAD). The LODs of the method were in the range of 0.05-0.2 μg l^{-1} with precision <5%, as RSD [31].

2.2.2. Stir bar sorptive extraction

SBSE is a relatively new technique. It has been used with success for the extraction of organic compounds from aqueous, food, biological and environmental samples. In SBSE the sample is stirred for a given time with a stir bar coated with a sorbent (Fig. 5.a), until the analyte reaches equilibrium between the polymer and the aqueous phase according to their distribution constant. The sorbed analytes are then desorbed by high temperatures into the injector port of the GC (Fig. 5b) or by liquid removal for HPLC analysis (Fig. 5.c). The main disadvantage of SBSE is the high extraction time required during sample treatment.

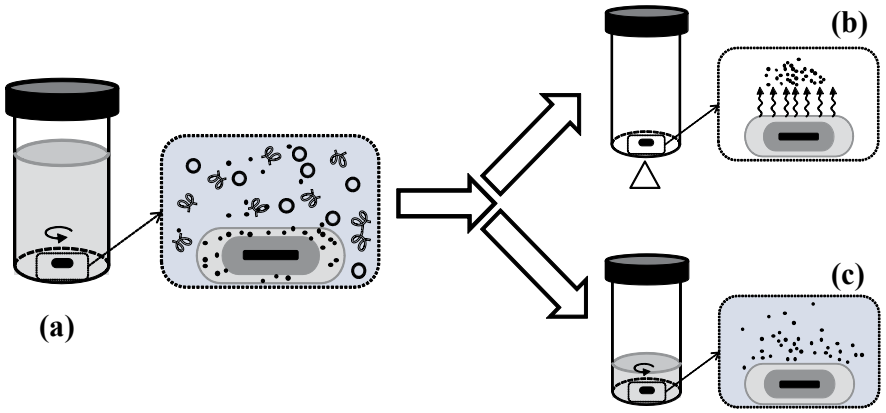

Figure 5. Representation of the solid phase microextraction methodology

A stir bar of 10 mm length and 0.5 mm polydimethylsiloxane was used to extract ten triazines from water samples. The SBSE step was carried out by introducing the stir bar into a vial containing 20 ml of the sample, 30% of NaCl and stirred (31.4 s^{-1}) during 60 min. The stir bar was washed with water and then thermally desorbed and analyzed by GC-MS. The LODs obtained were in the range from 0.2 to 3.4 ng l^{-1} [32].

The use of thermal desorption requires a cold trap during elution process. The hyphenation of SBSE with HPLC-DAD using solvent desorption was applied to atrazine, simazine and terbuthylazine in environmental water samples. A polyethylene bar impregnated with activated carbon (15 mm length and 0.5 mm thickness) was used to extract 10 ml of water samples. The extraction time required was 16 h, followed by desorption of the analytes using acetonitrile as solvent. The reported LODs were around 0.1 μg l^{-1} with precision <15%, as RSD. This method was an alternative to the analysis of analytes with polar characteristics [33].

2.2.3. Dispersive solid phase extraction

DSPE involves a sorbent addition to a water sample to form a dispersion (Fig. 6.a and b). The solid used has been derivatized to produce a bound organic phase (e.g., octadecyl, MIP, etc.) on its the surface similar to those used for packing SPE columns. The contact between the analytes and the support is higher than in traditional SPE, increasing the equilibrium rate and providing higher extraction yields. After centrifuging the suspension, the solid phase sediments are at the bottom of the test tube. An appropriate organic solvent is then used to elute the analytes from the solid sorbent prior to the organic extract analysis (Fig. 6.c).

DSPE has been applied to determine atrazine, prometryn, simazine, terbumeton and terbuthylazine in lettuce and corn acetonitrile extracts. Although this method is not used in water matrices, it is a good example for the isolation of triazine herbicides by DSPE. A MIP was synthesised using methacrylic acid, ethylenglycol, dimethacrylate, dithioester compounds and atrazine. The retention mechanism is based on the electrostatic interaction between the acid monomer and the basic properties of the target molecule. 100 mg of the MIP were dispersed

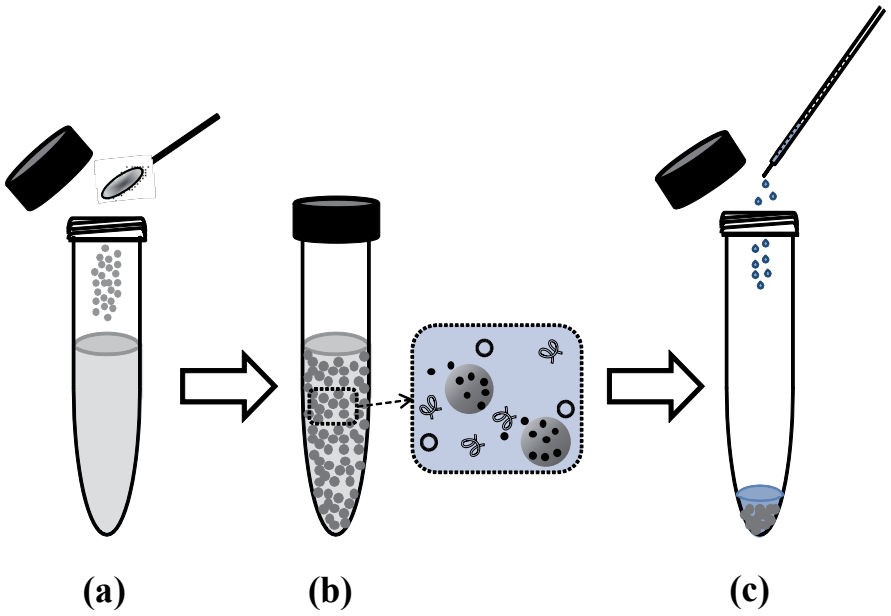

Figure 6. Representation of the dispersive solid phase extraction methodology

in 5 ml of sample solution for 1 hour. Once the extraction was concluded, the solid phase was collected in a membrane filter. The solid was washed with methanol and then the atrazine was eluted with 5 ml of desorption solvent (methanol/acetic acid, 9:1 v/v). The extract was dried and redissolved in acetonitrile before its analysis by HPLC-UV. The LOD reported was 2.8 μg l^{-1} with precisions <10% [34].

2.2.4. Magnetic solid phase extraction

Recovery of the solid phase in DSPE requires the use of filtration or centrifugation techniques that may lead to a solid phase loss and the subsequent decrease in precision and accuracy. The use of magnetic solids is an alternative for the selective preconcentration of different chemical species. It offers adequate surface area, the possibility of functionalization and paramagnetic properties. Their application as dispersed sorbents in liquid samples is so-called magnetic solid phase extraction (MSPE, Fig. 7). This technique has demonstrated several advantages such as: the decrease in sample treatment time, the decrease in solvent use, and the easy treatment of high volume samples. MSPE has been applied for the selective separation of many organic contaminants including antibiotics, anti-inflammatory drugs, pesticides, etc. which are present in different matrices [35].

MSPE has been applied for the extraction of atrazine, prometon, propazine and prometryn in environmental water samples using graphene-Fe_3O_4 nanoparticles. The effect of the amount of magnetic solid, extraction time and pH of the sample were evaluated. 20 mg of the magnetic support was dispersed into a 250 ml aqueous sample solution for 20

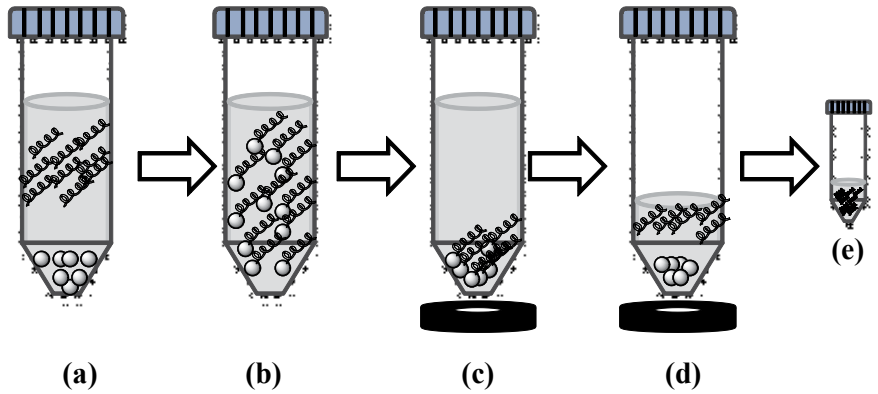

Figure 7. Representation of the magnetic solid phase extraction methodology

minutes. The solid was isolated from the sample solution using a magnet. The liquid phase was discarded and the solid was vortexed with acetone to desorb the analytes prior to its analysis by HPLC-DAD. The LODs of the method ranged between 0.025 and 0.040 μg l^{-1} with reproducilities <5.2% [36].

In this study, the effect of polarity of the solid phase on the interaction between triazines with magnetic supports used as part of a MSPE system coupled to HPLC-UV was evaluated. The developed methodology was used to determine atrazine and simazine in surface water samples.

3. Experimental conditions

3.1. Reagents and solutions

Ferrous sulfate heptahydrate (FeSO4 7H2O), ammonium solution (NH_3 25%, w/w), sodium hydroxide 99%, and hydrochloric acid 36% were purchased from J.T. Baker (Phillipsburg, NJ, USA). Triton X-100, cetyltrimethylammonium bromide (CTAB), tetramethoxysilane (TMOS), phenyltrimethoxysilane (PTMS), octyltriethoxysilane (C8-TEOS), chlorotrimethylsilane (CTMS), and methanol (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine standard 99.5% (atrazine) and 6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine standard 98.5% (simazine) were provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Stock solutions of triazines were prepared at a concentration of 250 mg l^{-1} in deionized water. These solutions were protected from light and stored under refrigeration (4 °C) until use to avoid possible decomposition. Calibration standards were prepared at concentrations of 5-1000 μg l^{-1} by mixing adequate volumes of each standard solution in deionized water.

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3.2. Equipment

Magnetic solids were characterized by X-ray diffraction in a PHILIPS PW1710 (Almelo, The Netherlands) instrument equipped with a Cu anode, automatic divergence slit and a graphite monochrometer under the following experimental conditions: CuKα radiation, 1.54 Å; generator tension, 40 kV; generator current, 30 mA; intensity ratio ($\alpha 2/\alpha 1$), 0.500; divergence slit, 1°; receiving slit, 0.1; start angle (2Θ°), 5; end angle (2Θ°), 70. A JEOL JSM-820 (Tokio, Japan) scanning electron microscope (SEM) was used for obtaining the magnetic solid microphotographs.

The separation and analysis were performed using HPLC equipment consisting of a Gilson (Middleton, WI, USA) model 302 pressure pump, a Rheodyne mod. 7525 injection valve and a UV–VIS diode array HP8453 spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). The absorbance of atrazine and simazine was monitored at 220 nm [37]. The quantification of triazines was made by comparison of peak height with those of the standards. The chromatographic separation was achieved with a Scharlau C18 column (5 μm; 150 mm×4.6 mm i.d.) (Barcelona, Spain). The mobile phase consisted of methanol-deionized water (2:1, v/v). A flow rate of 1.0 ml min^{-1} was established at 25 °C.

3.3. Analytical method

3.3.1. Synthesis and characterization of magnetic supports

The magnetic solids were synthesized by emulsion polymerization. Magnetite particles Fe_3O_4 were synthesized by a co-precipitation method [38] (Figure 8.a). The magnetite obtained was washed three times with 50 ml portions of deionized water and added to a flask containing a mixture methanol/water 3:1, Triton X-100 2%, CTAB 0.05% and the precursors indicated in Table 2. The mixture was heated and refluxed at 120 °C for 16 h with stirring (Figure 8.b). The solids were washed with two portions of 20 ml of deionized water and then a portion of 20 ml of ethanol then were dried at 60 ° C. In order to block superficial silanol groups (–Si–OH), the solids obtained in the previous process were derivatized using a mixture of 0.9 g of chlorotrimethylsilane (CTMS), and 1 ml of pyridine per gram of support in 50 ml of toluene (Figure 8.c). The supports were then washed with 20 ml portions of each of the following solvents: toluene, ethanol and deionized water until the washing liquid was colorless. The obtained magnetic particles were dried at 60 °C for 24 h [39]. Subsequently, all the magnetic solids were characterized by different techniques like SEM, X-ray diffraction and infrared spectroscopy.

3.3.2. Sampling and sample treatment

Surface water samples were collected from an agricultural area in Zamora, Spain in October 2009. Polypropylene bottles previously washed with deionized water and H_2SO_4 solution of 2% (v/v) were used. Once at the sampling site, the bottles were rinsed several times with the water to be collected and the temperature was measured. The samples were stored at 4°C before analysis. They were filtered through 0.45 μm cellulose acetate filters (Sartorius,

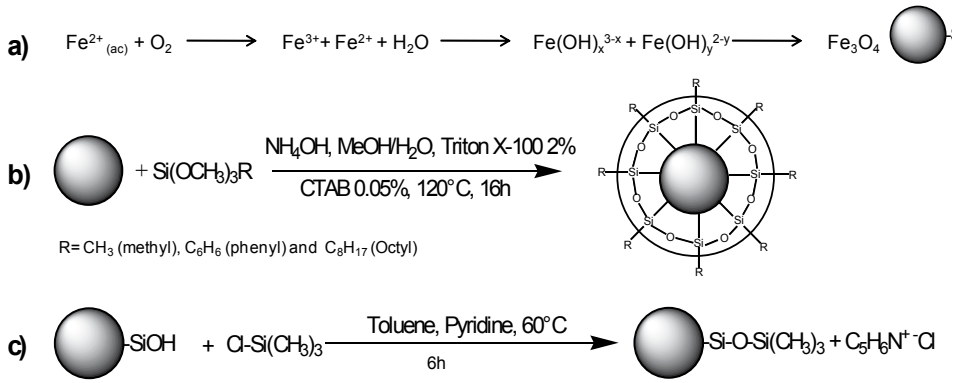

Figure 8. Synthetic methodology for magnetic supports preparation; a) magnetite preparation by co-precipitation method, b) silica polymerization onto magnetite particles by emulsion polymerization, c) silanol block reaction.

Magnetic Solid Name	Ratio SiO_2 monomer-Fe_3O_4 (w/w)
Polar 1 (P1)	1:1 / TMOS
Polar 2 (P2)	1:2 / TMOS
Phenyl 1 (PH1)	1:1 / PTMS
Phenyl 2 (PH2)	1:2 / PTMS
Octyl 1 (C8-1)	1:1 / C8-TEOS
Octyl 2 (C8-2)	1:2 / C8-TEOS

Table 2. SiO2/Fe3O4 ratio (w/w) and functionalized monomers for magnetic supports used such as adsorbents in MSPE for atrazine and simazine isolation

Göttingen, Germany) in a glass filtration device connected to a hand-operated vacuum pump (Sartorius, Göttingen, Germany).

Parameters such as the water samples' pH and the nature of the functionalized magnetic solids (mainly polarity) were modified to find the adequate extraction conditions. All the experiments were carried out with five replicates.

The optimal developed MSPE procedure involves the following steps: First, 1 ml of methanol is added to 0.1 g of the magnetic support (PH1) for activation and the magnetic solid is washed with 5 ml of deionized water. After addition of a known volume of water sample (0.2, 0.5 or 1.0 l) and pH adjustment to a value of 5 with HCl 1 M, the mixture is dispersed in an ultrasonic bath for 10 min. Then, a neodymium magnet is placed on the bottom of the beaker providing the isolation of the magnetic supports with the adsorbed analytes from the solution. The water sample is then eliminated by decantation. After the adsorption process, the solid is rinsed twice with 10 ml of deionized water. Finally, 1 ml of methanol was added to the magnetic solid and dispersed in an ultrasonic bath for 10 min. A neodymium magnet was placed on the bottom

of the beaker, and using a syringe the extract was isolated, dried, redisolved in 50 μl of methanol and injected into the HPLC system for their separation and analysis.

The SPE procedure for comparison was performed as described [40]: C18 SPE cartridges (500 mg, Bound Elut, Varian, Netherlands) were conditioned using 5 ml of ethyl acetate, 5 ml of methanol and 5 ml of deionized water at a flow rate around 2 ml min^{-1}. Water samples (1 l) were flowed through the cartridges with a flow rate between 10-15 ml min^{-1} under vacuum and the loaded cartridges were rinsed with 3 ml of methanol:water (5:95, v/v). The elution was performed with three aliquots (1 ml) of ethyl acetate at a flow-rate of about 1 mL min^{-1}. The combined aliquots were evaporated to dryness by a gentle stream of nitrogen and the residues were dissolved in 50 μl of methanol and injected into the HPLC system.

4. Results and discussion

4.1. Triazine extraction by MSPE

Figure 9 shows the effect of the polarity of the magnetic solid and the pH value of the aqueous phase on the recovery of each analyte. The results obtained demonstrate the high affinity achieved by phenyl based supports. The best extractions were observed when using the PH1 solid at pH 5 with recoveries of 90% and 100% for simazine and atrazine, respectively. Based on this performance, solid PH1 and pH 5 were selected for further experiments.

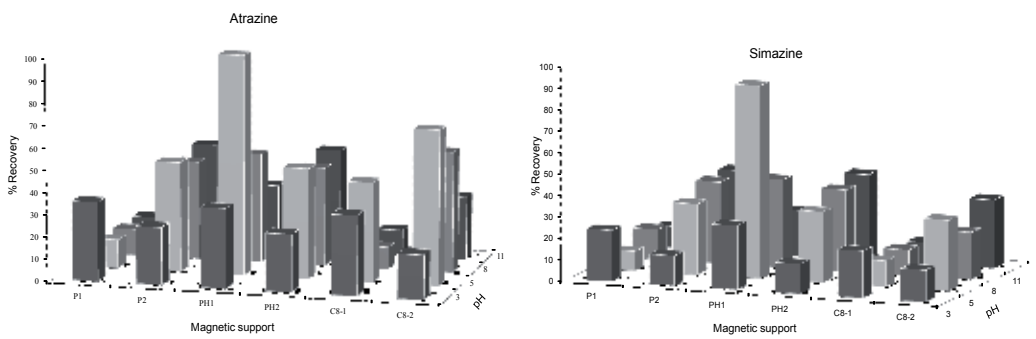

Figure 9. Effect of magnetic solid polarity and sample pH value on the triazine recoveries in spiked surface water (10 ng ml^{-1})

The adsorption of analytes on the solid surface depends on acid-base interactions (hydrogen bonds), π-π interactions, and Van der Waals forces (hydrophobic interactions). However, it has been observed that solid absorbent with aromatic groups improve the adsorption of triazines, due to π-π interactions, improving significantly recovery percentages [41-43]. This type of interaction has been reported for adsorption of triazines on mineral oxides coated with surfactants, the hydrophobic interaction between adsorbents and analytes improve the adsorption [44]. On the other hand, the acid-base equilibrium has an important role during

adsorption, in this case is evident that the better adsorption onto the surface solid is presented at pH 5. In these conditions the triazines are neutral without electric charges increasing the hydrophobic interactions. At pH values >7.0 the remaining surface silanol groups acquire a negative charge, increasing the repulsions between the surface of magnetic solid and the triazines decreasing the percentage recoveries for both analytes [45].

4.2. Characterization of magnetic solids

The magnetic solid characterization has been previously reported [39]. In this paper, we focus on the characterization of the most adequate solid for the selected triazine preconcentration. The magnetic particle morphology is spherical with core-shell type, where the core particle is magnetite, with super paramagnetic properties (20-50 emu g^{-1}) [46]. On the other hand, the shell is formed by silica phase functionalized with phenyl groups. The micrograph shown in Figure 10 confirms the spherical morphology of magnetic particles, with an approximated diameter of 2 μm. The diffraction pattern shows the magnetite line diffraction (m) and a broadband signal between 2Θ° of 10° and 40°, corresponding to the amorphous silica phase. The physiochemical and magnetic properties of the magnetic particles were adequate for their application as adsorbents in MSPE.

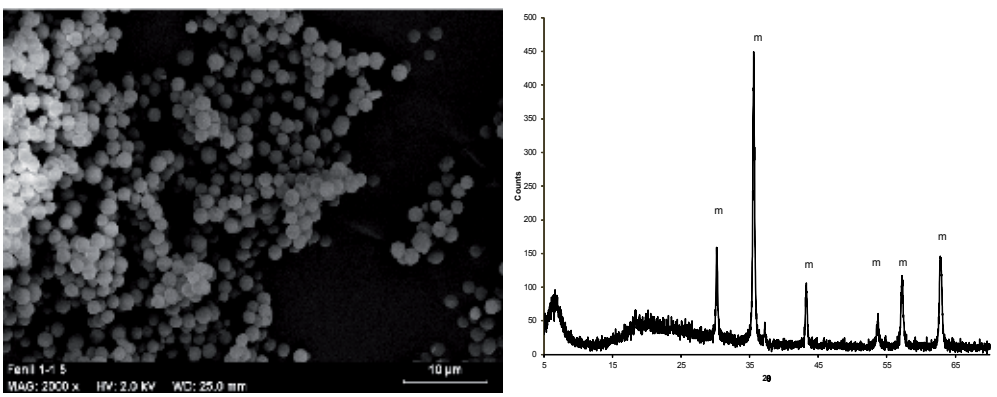

Figure 10. Microphotography and diffraction pattern of PH1 support

4.3. Analytical parameters of the MSPE

In the optimized conditions, the analytical parameters and precision data were determined using spiked tap water samples. Different volumes of spiked surface water samples (0.2, 0.5 and 1.0 l) with an interval concentration of 10-1000 μg l^{-1} were used. The results obtained are listed in Table 3. The limits of detection (LODs) were calculated for a signal/noise relation equal to (S/N = 3.29). The limits of quantification (LOQ) were determined using a signal/noise relation equal to 10 (S/N = 10). The calibration curves were constructed from the high signal versus concentration μg l^{-1}.

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According to the results from Table 3, it is possible to observe that the LODs decrease when higher initial sample volumes are used. The lowest limits of detection were reached between 0.01 and 0.02 μg l^{-1} using 1 l of initial sample. LOD and LOQ obtained by the method are comparable to those reported by other methods.

Sample volume (l)	Analyte	LOD (μg l^{-1})	LOQ (μg l^{-1})	Repeatability		Reproducibility	
50 μg l^{-1}	100 μg l^{-1}	50 μg l^{-1}	100 μg l^{-1}				
0.2	Simazine	4	12	2.8	6.2	4.8	2.8
	Atrazine	3	9	2.3	5.1	3.2	2.6
0.5	Simazine	1	3	2.4	6.7	2.5	2.9
	Atrazine	1	3	1.6	5.6	4.2	2.2
1.0	Simazine	0.01	0.03	1.4	2.8	1.5	2.0
	Atrazine	0.02	0.06	1.6	3.9	1.2	1.5

Table 3. Analytical parameters for different sample volumes, repeatability and reproducibility (%RSD, n = 5) for two concentration levels.

The precision of method expressed as the repeatability and reproducibility values (%RSD < 5%) and the high recoveries obtained make the proposed method a viable alternative to be routinely implemented in the analysis of simazine and atrazine in water samples, without the necessity of expensive or difficult access equipment.

4.4. Analysis of superficial water samples

The developed method was applied to the determination of triazines in surface water samples from agricultural lands in Zamora, Spain. Only one of the four samples analyzed showed contamination by triazines, being this water sample was collected in a waterhole near a corn field, which shows that pesticides applied to crops, migrate to nearby water bodies.

The concentrations found with the MSPE-HPLC (mean and %RSD, n=5) method were 9.9(3.0) and 12.2(2.5) μg l^{-1} for simazine and atrazine, respectively. The concentrations determined using the SPE-HPLC were 9.8(3.0) and 11.8(2.3 μg l^{-1}. The average of each analyte (determined by both methods) was compared by a t-test for comparison of means, assuming comparable variances (verified by a F-test). Calculated t values were compared with the tabulated t value for 8 degrees of freedom and a significance level of α= 0.05 (t = 2.30). Thus, the null hypothesis was accepted meaning that there were no significant differences between the results provided by both methods.

Figure 11 showed the chromatogram of surface water sample collected from Zamora, Spain, extracted with MSPE and SPE and a standard chromatogram. The clean-up process results for

both preconcentration methods are similar, showing that MSPE can be used as an alternative method for the determination of atrazine and simazine.

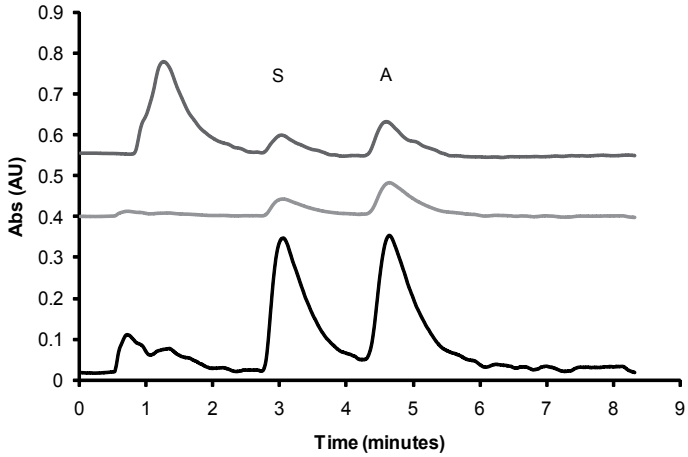

Figure 11. Chromatograms obtained at the optimized conditions: (a) surface water sample obtained by MSPE pre-concentration method, (b) surface water sample obtained by SPE pre-concentration method and (c) simazine and atrazine standard solution 20 μg l^{-1}. S: simazine and A: atrazine.

5. Conclusions

Due to the wide application of herbicides, it is necessary to develop fast and reliable methods for their determination in different analytical matrices providing a correct risk assessment to human health and the environment.

The results obtained by the optimized and validated MSPE method are comparable with other reported methods, concluding that the developed MSPE-HPLC procedure can be used for the screening and quantification of atrazine and simazine in water samples.

Although there are more sensitive methods, they require expensive and inaccessible instrumentation such as mass spectrometry, representing the MSPE-HPLC-DAD a rapid and low cost determination method of atrazine and simazine in water samples.

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References

[1] Crafts AS. The Chemistry and Mode of Action of Herbicides. New York: Wiley; 1965

[2] LeBaron HM, McFarland, Burnside, OC. The Triazine Herbicides. 50 Years Revolutionizing Agriculture. Hungary: Elsevier; 2008

[3] Pacakova V, Stulik K, Jiskra J. High-Performance Separations in the Determination of Triazine Herbicides and Their Residues. Journal of Chromatography A 1996;754 17-31

[4] Mitra S. Sample Preparation Techniques in Analytical Chemistry. New Jersey: Wiley; 2003.

[5] U.S. Environmental Protection Agency, EPA 500 Series, Method 507 (revision 2.0). Determination of Nitrogen- and Phosphorus-Containing Pesticides in Water by Gas chromatography with a Nitrogen-Phosphorus Detector. 1989

[6] Chimuka L, Michel M, Cukrowska E. Buszewski. Advances in Sample Preparation Using Membrane-Based Liquid-Phase Microextraction Techniques. Trends in Analytical Chemistry 2011; 30(11) 1781-1792

[7] Shen G, Lee HK. Hollow Fiber-Protected Liquid Phase Microextraction of Triazine Herbicides. Analytical Chemistry 2002; 74 648-654

[8] Trtic-Petrovic T, Dordevic J, Dujakovic N, Kumric K, Vasiljevic T, Lausevic M. Determination of Selected Pesticides in Environmental Water by Employing Liquid-Phase Microextraction and Liquid Chromatography-Tandem Mass Spectrometry. Analytical and Bioanalytical Chemistry 2010; 397 2233-2243

[9] Wu C, Liu Y, Wu Q, Wang C, Wang Z. Combined Use of Liquid-Liquid Microextraction and Carbon Nanotube Reinforced Hollow Fiber Microporous Membrane Solid-Phase Microextraction for the Determination of Triazine Herbicides in Water and Milk Samples by High-Performance Liquid Chromatography. Food Analytical Methods 2012; 5 540-550

[10] Nagaraju D, Huang S. Determination of Triazine Herbicides in Aqueous Samples by Dispersive Liquid-Liquid Microextraction with Gas Chromatography-Ion Trap Mass Spectrometry. Journal of Chromatography A 2007; 1161 89-97

[11] Zaijun L, Xiulan S, Junkang L. Ionic Liquids as Novel Solvent for Extraction and Separation in Analytical Chemistry. In: Kokoron A. (ed.) Ionic Liquids: Applications and Perspectives. Rijeka: Intech; 2012. p153-180

[12] Wand S, Ren L, Liu C, Ge J, Liu F. Determination of Five Polar Herbicides in Water Samples by Ionic Liquid Dispersive Liquid-Phase Microextraction. Analytical and Bioanalytical Chemistry 2010; 397 3089-3095

[13] Zhong Q, Su P, Zhand Y, Wang R, Yang Y. In-situ Ionic Liquid-Based Microwave-Assisted Dispersive Liquid-Liquid Microextraction of Triazine Herbicides. Microchimica Acta 2012; 178 341-347

[14] Sanagi MM, Abbas HH, Ibrahim WAW, Aboul-Enien HY. Dispersive Liquid-Liquid Microextraction Method Based on Solidification of Floating Organic Drop for the Determination of Triazine Herbicides in Water and Sugarcane Samples. Food Chemistry 2012; 133 557-562

[15] Wang H, Li G, Zhang Y, Chen H, Zhao Q, Song W, Xu Y, Jin H, Ding L. Determination of Triazine Herbicides in Cereals Using Dynamic Microwave-Assisted Extraction with Solidification of Floating Organic Drop Followed by High-Performance Liquid Chromatography. Journal of Chromatography A 2012; 1233 36-43

[16] Mou R, Chen M, Cao Z, Zhu Z. Simultaneous Determination of Triazine Herbicides in Rice by High-Performance Liquid Chromatography Coupled with High Resolution and High Mass Accuracy Hybrid Linear Ion Trap-Orbitrap Mass Spectrometry. Analytica Chimica Acta 2011;706 149-156

[17] Dean JR, Wade G, Barnabas IJ. Determination of Triazines Herbicides in Environmental Samples. Journal of Chromatography A 1996; 733295-355

[18] Molto JC, Pico Y, Font G, Mañes J. Determination of Triazines and Organophosphorus Pesticides in Water Samples Using Solid-Phase Extraction. Journal of Chromatography 1991; 555 137-145

[19] Chavez-Moreno CA, Guzman-Mar JL, Hinojosa-Reyes L, Hernandez-Ramirez A, Ferrer L, Cerda V. Applicability of Multisyringe Chromatography Coupled to On-Line Solid-Phase Extracrtion to the Simultaneous Determination of Dicamba, 2,4-D, and Atrazine. Analytical and Bioanalytical Chemistry 2012; 403 2705-2714

[20] Ollers S, Singer HP, Fassler P, Muller SR. Simultaneous Quantification of Neutral and Acidic Pharmaceuticals and Pesticides at Low.ng/l Level in Surface and Waste Water. Journal of Chromatography A 2001; 911 225-234

[21] Benvenuto F, Marin JM, Sancho JV, Canobbio S, Mezzanotte V, Hernandez F. Simultaneous Determination of Triazines and Their Main Transformation Products in Surface and Urban Wastewater by Ultra-High-Pressure Liquid Chromatography-Tandem Mass Spectrometry. Analytical and Bioanalytical Chemistry 2010; 397 2791-2805

[22] Bester K, Lamani X. Determination of Biocides as Well as Some Biocide Metabolites From Facde Run-Off Waters by Solid Phase Extraction and High Performance Liquid Chromatographic Separation and Tandem Mass Spectrometry Detection. Journal of Chromatography A 2010; 1217 5204-5214

[23] Lissalde S, Mazzella N, Fauvelle V, Delmas F, Mazzellier P, Legube B. Liquid Chromatography Coupled with Tandem Mass Spectrometry Method for Thirty-Three Pesticides in Natural Water and Comparison of Performance Between Classical Solid Phase Extraction and Passive Sampling Approaches. Journal of Chromatography A 2011; 1218 1492-1502

[24] Battista M, DiCorcia A, Marchetti M. Extraction and Isolation of Triazine Herbicides from Water and Vegetables by a Double Trap Tandem System. Analytical Chemistry 1989; 61 935-939

[25] Pichon V, Chen L. Hennion MC, Daniel R, Martel A, LeGoffic F, Abian J, Barcelo D. Preparation and Evaluation of Immunosorbents for Selective Trace Enrichment of Phenylurea and Triazine Herbicides in Environmental Waters. Analytical Chemistry 1995; 67 2451-2460

[26] Pichon V, Chen L, Durand N, LeGoffic F, Mennion MC. Selective Trace Enrichment on Immnunosorbents for the Multiresidue Analysis of Phenylurea and Triazine Pesticides. Journal of Chromatography A 1996; 725 107-119

[27] Muldoon MT, Stanker LH, Moleculary Imprinted Solid Phase Extraction of Atrazine from Beed Liver Extracts. Analytical Chemistry 1997; 69 803-808

[28] Zhou Q, Xiao J, Wang W, Lui G, Shi Q, Wang J. Determination of Atrazine and Simazine in Environmental Water Samples Using Multiwalled Carbon Nanotubes as the Adsorbents for Preconcentraction Prior to High Performance Liquid Chromatography with Diode Array Detector. Talanta 2006; 68 1309-1315

[29] Katsumata H, Kojima H, Kaneco S, Suzuki T, Ohta K. Preconcentration of Atrazine and Simazine with Miltiwalled Carbon Nanotubes as Solid-Phase Extraction Disk. Microchemical Journal 2010; 96 348-351

[30] Hernandez F, Beltran J, Lopez FJ, Gaspar JV. Use of Solid-Phase Microextraction for the Quantitative Determination of Herbicides in Soil and Water Samples. Analytical Chemistry 2009; 72 2313-2322

[31] Wu Q, Feng C, Zhao G, Wand C, Wang Z. Graphene-Cated Fiber for Solid-Phase Microextraction of Triazine Herbicides in Water Samples. Journal of Separation Science 2012; 35 193-199

[32] Sanchez-Ortega A, Unceta N, Gomez-Caballero A, Sampedro MC, Akesolo A, Goicolea MA, Barrio R. Sensitive Determination of Trizazines in Underground Waters Using Stir Bar Sorptive Extraction Directly Coupled to Automated Thermal Desorption and Gas Cromatography-Mass Spectrometry. Analytica Chimica Acta 2009; 641 110-116

[33] Neng NR, Mestre AS, Carvalho AP, Nogueira JMF. Powdered Activated Carbons as Effective Phases for Bar Sorptive Micro-Extraction (BSμE) to monitor levels of triazinic herbicides in environmental water samples. Talanta 2011; 83 1643-1649

[34] Xu S, Li J, Chen L. Moleculary Imprinted Polymers by Reversible Addition-Fragmentation Chain Transfer Precipitation Polymerization for Preconcentration of Atrazine in Food Matrices. Talanta 2011; 85 282-289

[35] Aguilar-Artega K, Rodriguez JA, Barrado E. Magnetic Solids in Analytical Chemistry: A Review. Analytica Chimica Acta 2010; 647 157-165

[36] Zhao G, Song S, Wang C, Wu Q, Wang Z. Determination of Triazine Herbicides in Environmental Water Samples by High-Performance Liquid Chromatography using Graphene-Coated Magnetic Nanoparticles as adsorbent. Analytica Chimica Acta 2011; 708 155-159

[37] Katsumata H, Kaneco S, Suzuki T, Ohta K. Determination of Atrazine and Simazine in Water Samples by High-Performance Liquid Chromatography After Preconcentration with Heat-Treated Diatomaceous Earth. Analytica Chimica Acta 2006; 577 214-219

[38] Barrado E, Rodríguez JA, Prieto F, Medina J. Characterization of Iron Oxides Embedded in Silica Gel Obtained by Two Different Methods. Journal of Non-Crystalline Solids 2005; 351 906–914

[39] Aguilar-Arteaga K, Rodriguez JA, Miranda JM, Medina J, Barrado E. Determination of Non-Steroidal Anti-Inflammatory Drugs in Wastewaters by Magnetic Matrix Solid Phase Dispersion–HPLC. Talanta 2010; 80 1152-1157

[40] Ma WT, Fu KK, Cai Z, Jiang GB. Gas Chromatography/Mass Spectrometry Applied for the Analysis of Triazine Herbicides in Environmental Waters. Chemosphere 2003; 52 1627-1632

[41] Bagheri H, Khalilian F, Naderi M, Babanezhad E. Membrane Protected Conductive Polymer as Micro-SPE Device for the Determination of Triazine Herbicides in Aquatic Media. Journal of Separation Science 2010; 33 1132-1138

[42] Bruzzoniti MC, Sarzanini C, Costantino, Fungi M. Determination of Herbicides by Solid Phase Extraction Gas Chromatography–Mass Spectrometry in Drinking Waters. Analytica Chimica Acta 2006; 578 241-249

[43] D'Archivio AA, Incani A, Mazzeo P, Ruggieri F. Adsorption of s-Triazines onto Polybenzimidazole: A Quantitative Structure-Property Relationship Investigation Analytica Chimica Acta 2009; 650 175-182

[44] Moral A, Sicilia MD, Rubio S, Perez BD. Multifunctional Sorbents for the Extraction of Pesticide Multiresidues from Natural Waters. Analytica Chimica Acta 2008; 608 61-72

[45] Nawrocki J. The Silanol Group and Its Role in Liquid Chromatography. Journal of Chromatography A 1997; 779 29-71

[46] Gupta AK, Gupta M. Synthesis and Surface Engineering of Iron Oxide Nanoparticles for Biomedical Applications. Biomaterials 2005; 26 3995-4021

Chapter 14

Floral Biology and Africanized Honeybee Behaviour in Transgenic (Roundup ReadyTM var. BR-245 RR) and Conventional (var. BRS-133) Soybean (Glycine max L. Merrill) Flowers

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Additional information is available at the end of the chapter

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1. Introduction

Therefore, this research was carried out to evaluate the Africanized honeybee effect on soybean production, mainly, over genetically modified organisms (GMOs). This chapter presents experimental data about floral biology of soybean *Glycine max* L. Merrill, BR-245 RR (transgenic soy – Roundup Read™) and BRS-133 (conventional soy) with and without application of Glyphosate, as well as the honeybee behaviour in those flowers of these varieties.

Soybean (*Glycine max* L. Merrill) is one of the main commodities in the world. Currently, more than 70% of its production is genetically modified. Brazil has a highlighted place concerning about this crop and is the second place in world production. During the 2010/2011 harvest season, Brazil produced 75 million tons of soybean on 24.2 million hectares of planted area with 3,106 $kg.ha^{-1}$ of productivity. The soybean crop represents the main worldwide oleaginous crop produced consumed by animals and human beings. After several decades of searching for alternatives to control weed pests, genotypes of genetically modified (GM)

soybean were developed to be resistant to the herbicide glyphosate, of the substituted glycine chemical group.

Brazil increased soybean hectares more than any other country, with an impressive increase of four million hectares in 2009. Brazil is now second in the commercial cultivation of GM plants, 25.4 million hectares, in the whole word [54]. GM soybean, in 2010, occupied 50% of cultivated global area among genetically modified organisms, reaching 73.3 million ha. Since the beginning of commercialization in 1996 until 2010, the tolerance to herbicides is the dominant attribute [54].

The current thought of scientists is increasing the productivity to meet the demand for soybean meal and soybean oil for animal and human consumption, respectively. Despite intensive research in this area with advances in genetically modified genotypes, new techniques in cultivation, irrigation, machine production, and agricultural implements with excellent performance are available. However, pollination, especially that performed by honeybees is little studied.

Around 70% of the world's most produced crop species depends, to some extent, on insect pollination [30], contributing an estimated €153 billion to the global economy, according to [31], and accounting for approximately 6 to 9% of agricultural production [27, 32]. Some authors [30] claim that about one third of the global food production depends on biotic pollination. However, the generally accepted figures are considerably lower [31, 46]. The estimated value of insect pollination for European agriculture is €22 Billion [33].

The scarcity of information about Africanized honeybees in cross-pollination of soybean indicates the need for more research, and for instance, the soybean crop can benefit greatly from biotic pollination with increased profits and production. Therefore, studies in pollination ecology, with special attention to pollinator-flower interaction may contribute to the success in this crop. Although soybean is auto compatible with auto pollination, auto incompatibility occurs too; the increase of production is possible to achieve with pollinator introduction in agricultural areas. A study in floral biology and honeybee behaviour in soybean is necessary to elucidate the effective importance of honeybees for this crop.

Transgenic can aid in genetic improvement of plants, with an aim on food production, fibers and oil, as well as the production of medicines and other industrialized products [1]. One of the main concerns with these plants is that an unanticipated and cumulative effect of contamination from crossing among genetically modified and conventional plants may occur. The instability and risk of propagating one gene to wild species are critical for environmental conservation.

The Monsanto Corporation has developed Roundup Ready™ soybean cultivars with a tolerance to glyphosate, the active ingredient of the weed killer Roundup®, by the production of enolpyruvylshikimate -3- phosphate synthase (EPSPS; EC 2.5.1.19) [2]. This enzyme is a carboxyvinyl transferase that catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) and forms enolpyruvylshikimate-3-phosphate. It is the sixth key enzyme in the shikimate pathway, which is essential for the production of aromatic amino acids Phe, Tyr, and Trp, as well as chorismate-derived secondary

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metabolites in plants, fungi, and microorganisms [3]. Glyphosate competes with PEP and forms a stable ternary complex with the enzyme and s3p [4, 5]. The safety and efficacy of glyphosate, together with the widely used glyphosate-resistant crop plants containing a transgene for EPSPS, have combined to make glyphosate the most used herbicide in the world [6].

The consequences of what this gene can provoke on plant biology is not known; some studies show that the abortion of conventional soybean flowers can reach up to 80%, depending on the cultivar and from the environmental conditions [6]. [7] found on cultivar BRS-133, an average of 53.31 and 82.90% of flowers aborted on the areas in which Africanized honeybees *A. mellifera* were present, and were not present, respectively.

The correct use of pesticides can arise the productivity of all crops in the world, mainly because pesticides contain biologically active compounds developed for the purpose of protecting plants. Insecticides control pest insect populations; herbicides control weeds, while fungicides are used to control fungal plant diseases [28]. According to Regulation (EC) No.1107/2009, a plant protection product (pesticides) shall be approved only when pesticides "…has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honeybee behaviour" [29]. Neonicotinoid residues in plants and plant parts only become dangerous for bees once they are exposed. Many lethal and sublethal effects of neonicotinoid insecticides on bees have been described in laboratory studies; however, no effects were observed in field studies [34]. Neurotoxic compounds such as neonicotinoids also interfere with the orientation process of honeybees [35]. The toxicity of neonicotinoids may, however, increase by synergistic effects with other compounds as reported by [36].

The sugar concentration of nectar in the flowers determines the frequency of visitors, while the volume determines the quantity of honeybee foragers that will visit [8]. Besides that, flowers have a period of viability for pollination and fecundation.

In the central region of the USA, the production of nectar and the visit of honeybees to soybean flowers occur among 9h00 and 15h00 every day. The peak of these activities and the time in which the flowers will remain open vary between the cultivar and the local environmental condition. The contents of total sugar in nectar varied from 37 to 45%, and the sugar of soybean flowers increased and the volume decreased according to the time of day and temperature [9, 46]. Most likely, the sugar concentration in nectar is associated with the intensity of foraging by honeybees, which is directly related to the nectar quantity and quality [47, 48] or to its sugar composition [49, 50, 51].

The volume of nectar on each flower, which is increased in warm climates, varied significantly between cultivars from 0.2 to 0.5 μL. In another experiment, performed by [10], in Haiti observing the same parameters, the authors verified that the most significant change occurred on the output rate of nectar per flower, which varied from 0.022 to 0.127 μL between cultivars.

A study in state of Parana (Brazil), with cultivar BRS-133 [7] showed that the Africanized honeybees searched the soybean culture to collect nectar and pollen. Just a small amount of pollen was from some other areas; however, more than 50% of the total amount of collected pollen by some colonies could have been from soybean [11].

The structure of the soybean flower assured the foraging of *A. mellifera,* favoring the pollen transfer [12, 46]. The soybean autogamy and that the self-pollination would guarantee good productivity to the agriculturists, not needing insects to do the pollination [13].

Based on the lack of information of the effect of honeybees on soybean production and, mainly, on genetically modified organisms, this research was carried out to study the floral biology of the *Glycine max*, cultivar BR-245 RR (transgenic soy - Roundup Read™) and BRS-133 (conventional soybean) and to evaluate the Africanized honeybee *A. mellifera* behaviour in the flowers of these cultivars.

2. Materials and methods

This research was carried out in the experimental area of Empresa Brasileira de Pesquisa Agropecuária (Embrapa Soja), located in Londrina city (23° 08'47" S and 51º 19'11" W), which is situated in the North region of state of Paraná, Brazil. The planting season, the cultivation, and management of the culture and crop occurred in appropriated time, and followed technical recommendations for soybean plants [27].

A completely randomized design was used with three treatments and six replications each. Three treatments were evaluated: covered soybean area with a colony of honeybees inside during the flowering; covered soybean area without a honeybee colony; and an uncovered soybean area, free for insect visitation. In each area, of 24 m^2 each, soybean planting was in eight lines, of 6 m, interlaced two by two, with cultivars BR-245 RR and BRS-133. The stand used was 0.5 m between lines and 30 seeds by linear metre (Figure 1).

For covered areas, pollination cages were installed, made with nylon screen (two mm), supported by PVC tubes (¾ inch), and iron (3/8 inch), forming cages in a semi-arch four metres wide, six metres long and two metres high, covering an area of 24 m^2 (Figure 2) to prohibit the passage of insects [14].

Each treatment was six parcels, with 24 m^2 for each pollination cage and the planting was carried through in eight intercalated lines, two by two, with cultivars BR-245 RR (transgenic soybean - Roundup Ready™), with and without an application of glyphosate (32 days after the germination) and BRS-133 (conventional soybean). Therefore, in each treatment, there were three parcels with an application of herbicide and three parcels without an application. The culture of soybean was monitored during all periods, with particular attention at bloom, which began on December 31 2003 and extended up to January 28 2004. Harvesting was carried out separately line-by-line on the 18 parcels (Figure 2).

In each line, five floral buttons were randomly marked with labels numbered and followed by comments made periodically, during all anthesis period. The stigma receptivity to the pollen grains was evaluated in five flowers collected at 8h00, 11h00 and at 17h00 in five days during the flowering, from January 6 to January 10 2004, in the transgenic and conventional lines of all parcels, following the method of [15].

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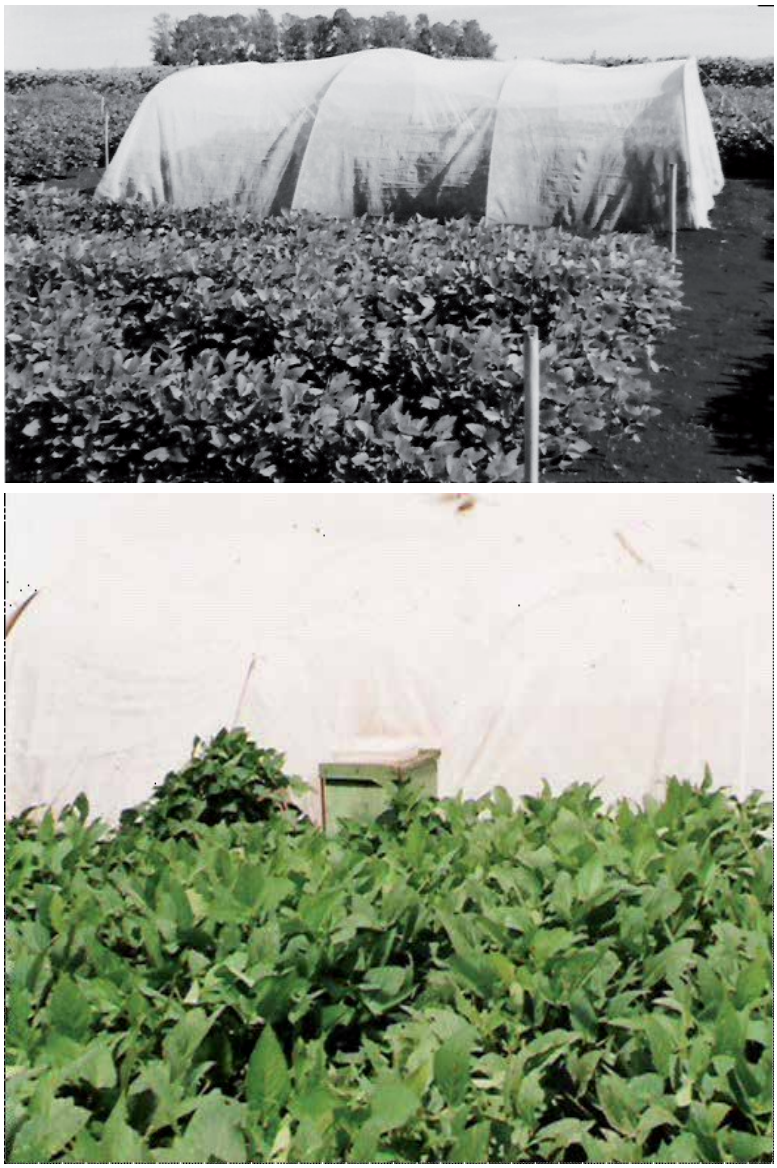

Figure 1. Pollination cage model used in the experiment with dimensions of 24 m^2, below the Africanized honeybee colony inside the cage.

The stigma of each flower was separated and immersed in hydrogen peroxide (20 volumes) and the air bubbles detached observed and evaluated with scores that varied from zero for non-receptive, one for moderate receptivity and two for high receptivity [7, 40]. For the verification of pollen grain viability, withdrawal of the pollen grains was made from the same flowers used to evaluate receptivity; these were deposited in blades, stained with acetic carmine and stored for posterior analysis, following the technique of [16, 17]. The viability of

Figure 2. The soybean harvesting carried out separately line-by-line on the 18 parcels in the experimental area of Embrapa – Soja in Londrina-PR, Brazil.

the pollen grains removed from the honeybee forager corbicula was also verified using this same technique.

The percentage of abortion in the soybean flowers was measured by counting all floral blossoms of three marked plants of each line with ribbons of different colors, in all parcels. In

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the maturation phase, the string beans of each plant were counted to get the percentage of the aborted flowers (Figure 3).

The frequency of the insect visits, during the day, was obtained by counting them, observing during 10 minutes in each schedule (8h00 to 17h00) in the transgenic and conventional lines of the covered area by pollination cages with honeybees and in the area of free insect visitation. The insects most frequent were photographed, filmed, collected with an entomological net, fixed and later, identified by specialists.

The time of nectar and/or pollen collection was evaluated with a chronometer, having followed the honeybee forager in its activity. In addition, the time of permanence of the honeybee in the flower and the number of visits per minute were counted and recorded.

Figure 3. A transgenic soybean plant in flowering, and in zoom showing the abortion of the flowers.

To observe the type of collection (nectar and/or pollen) carried through by the *A. mellifera*, six honeybees were collected each hour (9h00 to 16h00) in all the parcels for three days in the area with honeybees and in the area of free insect visitation. Corbicula content and honey stomach content was recorded with scores that varied from zero for empty (honey stomach and corbicula), one for moderate, two for full and three for maximum load. Evaluation of the total

sugar concentration was performed by placing the crop content in a manual refractometer and recording the brix value.

The statistical analyses of the variables were carried out with Statistical Analysis System [18] software using the following model:

$Y_{ijklm} = \mu + B_i + H_j + T_k + C_l + (BH)_{ij} + (BT)_{ik} + (BC)_{jl} + (HT)_{jk} + (HC)_{jl} + (TC)_{kl}$ + and $_{ijklm}$, where,

Y_{ijklm}= Observed in reference to variants of Block i, Herbicide j, Treatment k, Cultivar l

μ is the effect of general average;

B_i is the effect of Block (i = 1, 2... 6);

H_j is the effect of herbicide (j = 1, 2);

T_k is the effect of treatment (1, 2, 3);

C_l is the effect of cultivar;

$(BH)_{ij}$ is the interaction of Block i and Herbicide J;

$(BT)_{ik}$ is the interaction of Block i and Treatment k;

$(BC)_{jl}$ is the interaction of Block i and Cultivar l;

$(HT)_{jk}$ is the interaction of Herbicide j and the Treatment k;

$(HC)_{jl}$ is the interaction of Herbicide i and the Cultivar l;

eijklm is the mistake associated to the observation ijklm.

The delineation used was of completely randomized blocks and the data submitted to the variance analysis. Tukey's test was used for comparison of the averages.

Frequency of stigma receptivity was analyzed by the NPAR1WAY Wilcoxon procedure and the viability was estimated by the Kruskal-Wallis test.

Data without normal distributions were analyzed using generalized linear models [19], assuming Poisson distribution with logarithmic link function.

For analysis of the type of collection, the model considers the treatment effect as a fixed variable, random for the effect of the interaction treatment versus day and the linear effect, squared and cubical for the hour collection.

3. Results

There was no effect ($P > 0.05$) of cultivar, from the application of the herbicide and the interactions of these variables, therefore floral biology and behaviour of the insects in two cultivars were similar ($P > 0.05$), independent of the herbicide used, but the presence of insects influenced these parameters (Table 1).

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The anthesis period in hours was, on average, 8h04min greater (P=0.0001) in the pollination cages without honeybee colonies than in the pollination cages with honeybee colonies and then in the uncovered area for free insect visitation (P>0.05). In Table 1, there is a summary of the variance analysis and the average comparison of the anthesis period in the three treatments, and two cultivars.

The receptivity of the stigma was not influenced by cultivar (P>0.05) with a general average of 87.43%, and was not influenced by treatments either (P>0.05) which had a general mean of 88.71%. There was no difference (P> 0.05) in the percentage of viable pollen grains in the soybean flowers between treatments and the general average was 89.82% ±7.27 (Table 2).

The percentages of viable pollen grains removed from corbicula honeybee forager in the pollination cages with honeybee colonies and in the areas of free insect visitation were not different (P> 0.05) and the average was 99.95% (±0.14).

In the analysis of the removed pollen grains from corbicula of honeybee forager, 100% were of soybean, showing a high constancy of the honeybees to this crop.

Variation source		Anthesis period in hours
Cultivar	0.13	P = 0.7243
CV % of cultivar	10.24	
Treatments	3.26	P= 0.0104
CV % of treatments	15.27	
Transgenic soy	42.37	a* (±6.83)
Conventional soy	42.89	a (±6.36)
Covered area with honeybees	37.90	b (±4.05)
Covered area without honeybees	48.00	a (±3.72)
Uncovered area	41.98	b (±6.95)

Means followed by different letters, in the same column, are different by Tukey's test (P< 0.05)

Table 1. F values with respective probability (P), coefficient of variation (CV%), means and their standard deviation of anthesis period, in hours, for soybean flowers, *Glycine max*, var. BR-245 RR (transgenic soybean - Roundup Ready™) and BRS-133 (conventional soybean), in experimental area of EMBRAPA Soja, in Londrina-PR, Brazil.

Variation source	Stigma receptivity (%)		Viable pollen grains (%)	
Cultivar	0.52	P = 0.9100	0.53	P = 0.4668
CV % of cultivar	8.48		8.19	
Treatments	0.87	P = 0.4816	0.62	P = 0.6167
CV % of treatments	7.17		8.11	
Transgenic soy	87.65	a (±6.19)	90.14	a (±7.52)
Conventional soy	87.20	a (±6.52)	89.51	a (±7.07)
Covered area with honeybees	89.81	a* (±7.04)	89.22	a (±7.70)

Variation source	Stigma receptivity (%)		Viable pollen grains (%)	
Covered area whithout honeybees	87.50	a (±4.81)	90.38	a (±6.64)
Uncovered area	84.72	a (±6.28)	89.86	a (±7.46)

Means followed by different letters, in the same column, are different by Tukey's test ($P< 0.05$)

Table 2. F values with respective probability (*P*), coefficient of variation (CV%), means and their standard deviation of stigma receptivity and viable pollen grains (%), in soybean flowers *Glycine max*, var. BR-245 RR (transgenic soybean - Roundup Ready™), and BRS-133 (conventional soybean) in experimental area of EMBRAPA Soja, in Londrina-PR, Brazil.

The percentage of aborted flowers was greater (P=0.0001) in the covered area by cages without honeybees in relation to the covered areas by cages with honeybee colony and of free insect visitation; these last treatments did not differ (P>0.05). The averages and the standard deviation of the abortion percentage in the soybean flowers in the treatments are presented in Table 3.

Variation source	Abortion percentage of flowers	
Cultivar	2.55	P=0.1302
CV % of cultivar	6.55	
Treatments	13.21	$P< 0.0001$
CV % of treatments	18.08	
Transgenic soy	73.51	a* (±9.54)
Conventional soy	80.21	a (±7.03)
Covered area with honeybees	50.78	b* (±8.30)
Covered area without honeybees	71.10	A (±3.05)
Uncovered area	55.12	B (±5.84)

Means followed by different letters, in the same column, are different by Tukey's test ($P< 0.05$)

Table 3. F values with respective probability (*P*), coefficient of variation (CV%), means and their standard deviation of abortion percentage of soybean flowers *Glycine max*, var. BR-245 RR (transgenic soybean - Roundup Ready™), and BRS-133 (conventional soybean) in experimental area of EMBRAPA Soja, in Londrina-PR, Brazil.

In the area of free insect visitation, *A. mellifera* was the species most abundant with 97.02% frequency. Other bee species with 1.65%10 frequency had been observed also, and, Lepidoptera with 1.33% (Figure 4). Amongst the collected bees, there were nine species, six from the *Apidae* family, two from *Megachilidae* and one from *Halictidae*.

The behaviour for the type of collection observed in workers of *A. mellifera* foraging the soybean flowers was typical of nectar collecting, although, workers with unique behavior for pollen collection was observed on occasion (Figure 5).

In an accurate evaluation of the honey stomach contents and corbicula, it was observed that, throughout the day, 69.39% of the foragers had collected nectar, 37.05% had collected nectar and pollen and 2.56% had only collected pollen.

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The nectar collection throughout the day varied and differed in the amount of honeybees in the different schedules (P=0.0001), but did not differ (P> 0.05) between the covered area by cages with honeybees present and of free insect visitation. In these two areas, the moment of greatest visitation of the *A. mellifera* was at 12h34min. The frequency of honeybees with a larger amount of nectar in the covered areas by cages with honeybees and of free insect visitation is represented in Figure 6.

In the area of free insect visitation, the time that *A. mellifera* spent collecting nectar was 2.59 ±0.52 seconds/flower which was less (P=0.0001) than the observed time in the pollination cages with honeybee colonies (2.99 ±0.54 seconds/flower). Regardless of treatment, honeybee *A. mellifera* spent, on average, 2.74 ±0.56 seconds/flower in nectar collection and 4.37 ±0.62 seconds/flower in pollen collection and this difference was significant (P=0.0001). *A. mellifera* visited 7.14 (±0.26) flowers/min. collecting nectar and 3.75 (±0.30) flowers/min in pollen collection.

The average concentration of total sugar removed from the honey stomach (called crop) of the honeybees that collected nectar throughout the day was of 41.19% ±3.72, in pollination cages with honeybee colonies inside; this was greater (P=0.0008) than the 38.22% ±3.37 measured from the area of free insect visitation (Figure 7). In these two areas there were also a difference (P=0.0001) between the collecting schedule.

Figure 4. Lepidoptera in soybean flower

Figure 5. The honeybee searching a reward in soybean flower

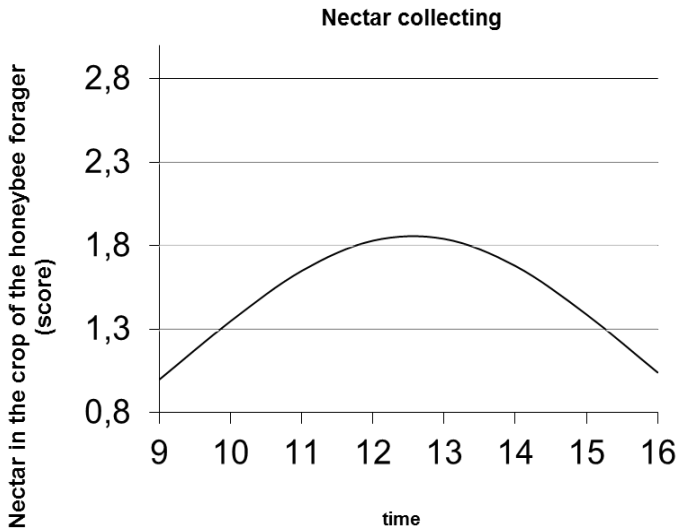

Figure 6. Regression curve obtained through the equation: $Y=exp^{(-7.111+1.231xh-0.049xh2)}$ of number of worker honeybees collecting nectar during the day in covered area with honeybee colony inside, and $Y=exp^{(-7.111+1.318xh-0.049xh2)}$ in the area for free insect visitation, in soybean *Glycine max*, var. BR-245 RR (transgenic soybean - Roundup Ready™) and BRS-133 (conventional soybean) in experimental area of EMBRAPA Soja, in Londrina-PR, Brazil.

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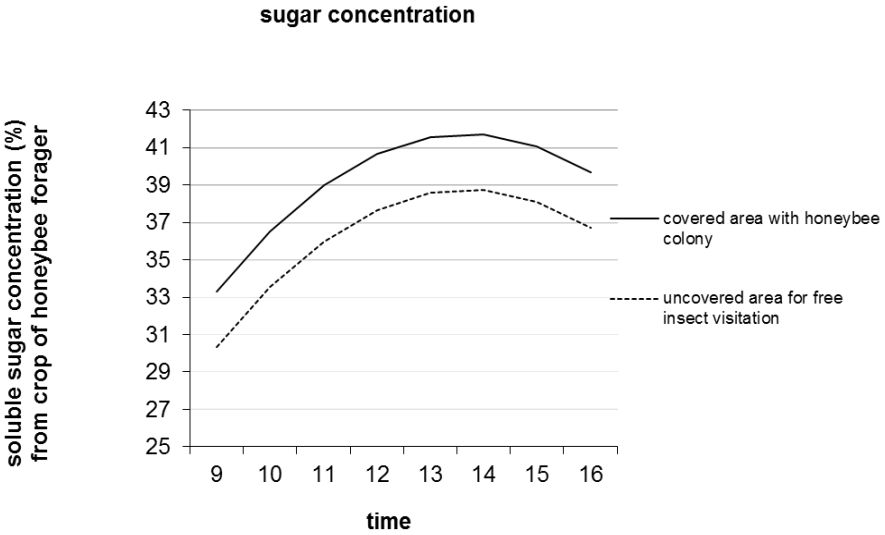

Figure 7. Regression curve obtained through the equation $Y=41.19+0.9127(h-12.5)-0.3836(h-12.5)^2$ in covered area with honeybee colony and $Y=38.22+0.9127(h-12.5)-0.3836(h-12.5)^2$ in uncovered area for free insect visitation showing the total sugar, in Brix, measured from worker crop content of captured honeybees, foraging soybean flowers *Glycine max*, var. BR-245 RR (transgenic soybean - Roundup Read™) and BRS-133 (conventional soybean) in experimental area of EMBRAPA Soja, in Londrina-PR, Brazil.

4. Discussion

The results showed that the genetically modified cultivar, regardless of receiving an application of the herbicide had a similar pattern as the conventional cultivar for the analyzed parameters. This showed that the transgenes did not interfere in the analyzed parameters, like floral biology, and the Africanized honeybee behaviour. The bees, mainly honeybees, are considered the most effective pollinator in nature, principally, in cultivated plants like soybean [39]. The honeybee has become a domesticated animal; they can be managed, moved to a new site, divided, mature, and stimulated to go to a specific crop for a specific time.

When evaluating the outcome of Africanized honeybee presence on soybean culture, the parameters of anthesis and the percentage of aborted flowers decreased just as much on the transgenic cultivar as the conventional one. [37] describes an excellent protocol to be applied to focal crops at the farm-scale level to assess pollinator density and diversity for comparison purposes among different sites. It becomes an important tool for researchers and professors who work in pollination because in several areas, one of the most difficult things is to compare results that are obtained by different methods.

The anthesis period was, on average, 20.49% longer in the area where insects had been prevented from visiting the flowers than in the pollination cages with honeybee colonies and the area for free insect visitation. Similar results have been observed by [7] on BRS-133, which

verified an average increase of 20.38% into the anthesis period in the area that insects were prevented from visiting in relation to the areas where the insects had access. In agriculture and horticulture pollination management, a good pollenizer is a plant that provides compatible, viable and plentiful pollen and blooms at the same time as the plant that is to be pollinated or has pollen that can be used and/or stored when needed to pollinate the desired flowers.

An increase in the anthesis period in areas that insects were prevented from visiting has also occurred with other species of plants. [18] observed an increase of 43.5% into the anthesis period in flowers of siratro (*Macroptilium atropurpureum* Urb.) when they had been prevented from having insect visitation. The outcomes of the above mentioned authors show that the Africanized honeybees have been efficient in the pollination process, since that anthesis period was smaller in areas for free insect visitation than flowers protected from them, which presented a greater anthesis period and when the Africanized honeybees were present, the anthesis period of soybean and *M. atropurpureum* was smaller.

The stigma receptivity was similar between treatments. Similar results had been observed by [18] in the *M. atropurpureum* flowers which had 76.9% of the blossoms and 91.95% of the flowers opened in the receptive stigma to pollen grains.

The high viability of the pollen grains in all treatments, 99.6% (±0.02) on average, suggest that these do not depend on the conditions imposed on the flowers in this cultivar. [18] found 100% of viability in the pollen grains in *M. atropurpureum*. [7] on cultivar BRS-133 obtained 99.86% of viable pollen grains.

In the analysis of the removed pollen grains from corbicula of foragers in the area of free insect visitation, no pollen from other flowers was found. These results are in accordance with [7] that observed 93 to 100% of honeybee foragers collected pollen in one single species. These results have shown that the alimentary resource offered by the soybean flower satisfies the requirements of *A. mellifera* and they did not need to visit other cultures while the samples were being collected.

The number of aborted flowers was 40.02% greater in the covered area by cages without honeybees in relation to the covered area by cages with honeybees, and 28.99% greater than in the area of free insect visitation. These results match the results of [20] that observed high rates of abortion in soybean, varying from 43% to 87%, depending on the cultivar. [9] had 75% of the flowers aborted in some cultivars, [11] had superior rates of more than 75% of abortion and [7] found a rate of abortion 53.66% greater in areas protected of insect visitation to flowers compared with covered area of cages with *A. mellifera*. This biological response of the flower shows that, even if soybean is considered to have autogamy by researchers such as [13], [21], it probably also possesses a mechanism of genetic auto-incompatibility to prevent 100% of self-fertilization, as observed on plants of the genus *Brassica* by [22]. This evidences the importance of honeybees for the reduction in the abortion percentage and, consequently, increases the amount of string beans and seeds in soybean.

The largest frequency of *A. mellifera* (97.02%) in soybean flowers in the area for free insect visitation was similar to the results obtained by [7], which found 95.18% of this species visiting soybean flowers on cultivar BRS-133. Therefore, in this cultivar of soybean, the presence of *A. mellifera* is important not only for honey production but also for pollination, and mainly for an

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increase of productivity. The larger part of agricultural land consists of cultivated areas like fields. However, an amount of space remains that is often quantitatively underestimated, and could be managed to promote plant and biotope diversity. Natural or semi-natural habitat remnants provide nesting sites and reliable food sources for pollinators. Conserving these areas can benefit biodiversity and offer potential for improved crop productivity [28]. Soybean is largely used in Brazilian agriculture in extensive systems called monoculture. Therefore, in these areas it is easier to implant management programs for rational use of pesticides focusing on the reduction of negative effects on the bees [38].

The comments regarding the collecting behavior have shown that *A. mellifera* visits the soybean flowers intensely, mainly, for nectar collection and, in part of the morning, from 9h00 to 11h30, the nectar and pollen collection was intense. On occasion, the behavior of foragers collecting pollen was observed. These results are in accordance with [23] and [24], which observed a great number of worker honeybees visiting soybean flowers. Even with nectar collecting behavior, *A. mellifera* was efficient as a pollinator, strengthening the affirmations of [11] that the honeybee does not necessarily need to be pollen collectors to make an efficient pollinator since they are able to accomplish that as they collect nectar. [7] observed intense visits of *A. mellifera* in the cv. BRS-133 collecting, predominantly, nectar and had an increase of 58.86% in the areas with *A. mellifera* in relation to the area with insect restriction. Honeybee nectar foragers were more frequent (2.28 bees per chapter) than pollen foragers (0.40 bees per chapter) in sunflowers and seed yield was 43% higher ($P < 0.05$) from sunflower plants that were visited by pollinator-insects than plants restricted to pollinators [25].

The evaluation of amount of total sugars present in the crop content of the foragers showed that the harvested nectar awakened the interest of the *A. mellifera* for the visitation of these cultivars of soybean. The consistency of the honeybees to the soybean showed that the flowers of this species satisfies its nutritional requirements and they do not need to visit other species, even if there are other flowers available and some very attractive, such as sunflower (*Helianthus annuus*).

In the research of [41] and [42], the means of sucrose.hexose^{-1} (S/H) per flower for all treatments were: 0.91 $\mu g.\mu L^{-1}$, for covered areas with Africanized honeybee colonies – rich in sucrose; 0.74 $\mu g.\mu L^{-1}$, semi-covered areas with free insect visitation – rich in sucrose; 0.86 $\mu g.\mu L^{-1}$, uncovered areas with free insect visitation – rich in sucrose; and 3.05 $\mu g.\mu L^{-1}$, for covered areas without Africanized honeybee colonies – sucrose dominant. According to [43], this range suggests that sugar concentration in soybean nectar is influenced by other environmental factors independently of pollinator action. [44] reported that edaphic and climatic factors affect the number of flowers and other floral characteristics during soybean growing. Therefore, the environmental conditions that generate an increase in number and size of flowers, longer anthesis period, more intense coloring, and greater nectar production are the factors responsible for making flowers more attractive to honeybees [42]. [45] suggested that the maximum nectar accumulation occurs before or at the beginning of pollination activity.

Technological innovations play an important role in pollinator protection. Application technologies allow for reductions in spray drift; this helps prevent pesticide residues in non-target areas. This is achieved with application nozzles that create spray droplets large enough to be less affected by wind [28].

One of the greatest concerns of Brazilian farmers in relation to GM plants is the unexpected and cumulative effect of cross-contamination between GM plants and conventional plants. The instability and risk of propagation of a gene to wild species are critical to maintenance of the environment [52]. Genetic pollution is inevitable and the transgenic pollen may contaminate conventional or biological fields located several kilometres from GM plantations [55].

In another study [52] in transgenic and conventional soybean, it was reported that the estimate of grain production increased 37.84% in the area where honeybee visits were permitted. However, the cv. BRS-133, not GM [7, 14] was intensively visited by *A. mellifera* Africanized honeybees. The researchers reported an increase of 61.38% in number of pods, and 58.86% in seed production, when compared with plants not visited by insects. [26] reported that Africanized honeybees provided a considerable increase of gene flow from transgenic cv. BR-245 RR to conventional cv. BRS-133 soybean (1.57%). Since these cultivars of soybean were attractive to the honeybees, they performed the cross-pollination in the tested varieties.

The economic value of honeybee service in the USA reported by [57, 58, 59, and 60] was about US $15 billion, and specifically for soybean, the value was US $754 million. [53] using the same table of [57, 58, and 59], estimated the economic value of Africanized honeybee service for soybean culture, using data from [7, 14, and 52] obtained as medium value US $3.561,2 million. These estimates are considerable in Brazil and in other parts of the world, and must not be disregarded.

The Green Revolution has reduced the percentage of the word-wide population that suffers from hunger from 50% in sixty years to 20% at present. In plantations free of pesticides, the loss of production is 10% to 40%. Without this technology, about two billion of the seven billion inhabitants of the planet would be starving. The use of transgenic crops may help to increase the productivity, avoiding more deforestation, and more erosion of the soils [56].

Floral biology and the behaviour of the honeybees in transgenic soybean cv. BR-245 RR did not depend on the application of herbicide and was similar to the verified one in the conventional cv. BRS-133. This implies that it is possible to have cross-pollination between transgenic and conventional soybean and gene flow between them like reported by [26].

General summary

The experiment was carried out to study the floral biology of the *Glycine max* L. Merrill, cultivar BR-245 RR (transgenic - Roundup Ready™) and BRS-133 (conventional) and to evaluate the behavior of the Africanized honeybee *A. mellifera* in the flowers of these cultivars. Three treatments established included: a covered area with an Africanized honeybee colony, a covered area without a honeybee colony and area of free insect visitation. Each treatment was six parcels of 24 m^2 each and the planting of soybean was carried through in this area in eight pared and intercalated lines with tested cultivars, being applied herbicide (glyphosate) in the plants of transgenic cultivars in half of the parcels in the three treatments. The anthesis period was 8h04min longer in the covered area without a honeybee colony than in the covered area

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with a honeybee colony and in the area of free insect visitation. The average of the analyzed stigma receptivity was 87.35% and there was no difference between treatments. The average of viable pollen was 89.82% with no difference between treatments. In the analysis of the removed pollen grains from the honeybee forager corbicula, 100% observed belonging to soybean. The flower abortion rate was 71.10% in covered areas without honeybee colonies, which was greater by 50.78% and 55.12%, respectively, than the covered area with a honeybee colony and area of free insect visitation. *A. mellifera* was the insect that most frequently (97.02%) visited the soybean flowers. The time that *A. mellifera* spent to collect nectar was greater in the covered area with a honeybee colony than in the area of free insect visitation. The average time foragers spent for nectar collection was 2.74 second/flower and 4.37 second/flower for the pollen collection. *A. mellifera* visited, on average, 7.14 flowers/min, collecting nectar, and 3.75 flowers/min for pollen collection. The total sugar concentration in the honey stomach content was 41.19% in the covered area with a honeybee colony, greater than the 38.22% observed for the area of free insect visitation. In the uncovered areas for free insect visitation and covered areas with Africanized honeybee colony, the results were different from the covered area without honeybee colony. There was no difference in the evaluations of floral biology and behaviour of insects between the transgenic and the conventional soybean, independent of the application of the herbicide glyphosate in transgenic soybean.

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References

[1] Nodari R.O., Guerra M.P. Implicações dos transgênicos na sustentabilidade ambiental e agrícola, História Ciências e Saúde 2000; 7(2) 481-491.

[2] Avaliação da segurança alimentar e ambiental da soja Roundup Ready™. Evento 40-3-2 (2001) [online]. http://www.monsanto.com.br/biotecnologia/estudos/terceiros/pdf/avaliacao.pdf (Accessed 20 June 2005).

[3] Herrmann K.M., Weaver L.M. The Shikimate Pathway. Annual Review of Plant Physiology and Plant Molecular Biology 1999; (50) 473–503.

[4] Marzabadi M.R., Gruys K.J., Pansegrau P.D., Walker M.C., Yuen H.K., Sikorski, J.A. An EPSP synthase inhibitor joining shikimate 3-phosphate with glyphosate: synthesis and ligand binding studies. Biochemistry 1996; (35) 4199–4210.

[5] McDowell L.M., Schmidt A., Cohen E.R., Studelska D.R., Schaefer J. Structural constraints on the ternary complex of 5-enolpyruvylshikimate-3-phosphate synthase from rotational-echo double-resonance NMR. Jounal of Molecular Biology 1996; (256) 160–171.

[6] Peng, Ri-He; Tian, Yong-Sheng; Xiong, Ai-Sheng; Zhao, Wei; Fu, Xiao-Yan; Han, Hong-Juan; Chen, Chen; Jin, Xiao-Fen; Yao, Quan-Hong. A Novel 5-Enolpyruvylshikimate-3-Phosphate Synthase from *Rahnella aquatilis*with Significantly Reduced Glyphosate Sensitivity. PLoS One 2012; 7(8): e39579.

[7] Chiari W.C., Toledo V.A.A., Ruvulo-Takasusuki M.C.C., Oliveira A.J.B., Sakaguti E.S., Attencia V.M., Costa F.C., Mitsui M.H. Floral biology and behavior of Africanized honeybees *Apis mellifera* in soybean (*Glycine max* L. Merril). Brazilian Archives of Biology and Technology 2005; 48(3) 367-378.

[8] Free J.B., Williams I.H. The pollination of hybrid kale (*Brassica oleracea* L.). Journal of Agricultural Science 1973; 81(3) 557-559.

[9] Erickson E.H. Soybean pollination and honey production a research progress report. American Bee Journal 1984; (124) 775-779.

[10] Severson D.W., Erickson Jr. E.H. Quantitative and qualitative variation in floral nectar of soybean cultivars in Southeastern Missouri. Environmental Entomology 1984; 13(4) 1091-1096.

[11] Free J.B. Insect pollination of crops. London: Academic Press, 1993.

[12] Erickson E.H., Garment M.B. Soya-bean flowers: nectary ultra-structure, nectar guides, and orientation on the flower by foraging honeybees. Journal of Apicultural Research 1979; 18(1) 1-11.

[13] Rubis D.D. Breeding insect pollinated crops. Arkansas Agricultural Extension Service 1970; (127) 19-24.

[14] Chiari W.C., Toledo V.A.A., Ruvulo-Takasusuki M.C.C., Oliveira A.J.B., Sakaguti E.S., Attencia V.M., Costa F.C., Mitsui M.H. Pollination of soybean (*Glycine max* L. Merril) by honeybees (*Apis mellifera* L.). Brazilian Archives of Biology and Technology 2005; 48(1) 31-36.

[15] King J.R. The peroxidase reaction as an indicator of pollen viability. Stain Technology 1960; (36) 225-227.

[16] Radford, A.E, Dickison, W.C, Massey, J.R., Bell, C.R. Vascular Plant Systematics. New York: Harper & Row Publishers, 1974.

[17] Vitali M.J., Letizio-Machado V.L. Entomofauna visitante das flores de *Tabebuia chrysotricha* (Mart.) Standl. (Bignoniaceae). Anais da Sociedade Entomológica do Brasil 1995; 24(1) 77-88.

[18] Vieira R.E., Kotaka C.S., Mitsui M.H., Taniguchi A.P., Toledo V.A.A., Ruvulo-Talasusuki M.C.C., Terada Y., Sofia S.H., Costa F. M. Biologia floral e polinização por abelhas em siratro *(Macroptilium atropurpureum Urb*.). Acta Scientiarum 2002; 24(4) 857-861

[19] McCulloch C.E., Scarle S.R. (2001) Generalized, Linear, and mixed models, Wiley Inters Sci. Publ. New York. pp. 321.

[20] Shaik P.H., Van Probst A.H. Effects of some environmental factors on flower production and reproductive efficiency in soybeans. Agronomy Journal 1958; (50) 192-197.

[21] Ahrent D.K., Caviness C.E. Natural cross-pollination of twelve soybean cultivars in Arkansas, Crop Science 1994; 34(2) 376-378.

[22] Gaude T., Cabrillac D. Self-incompatibility in flowering plants: the *Brassica* model. C. R. Acad. Sci. 2001; (324) 537-542.

[23] Jaycox E.R. Ecological relationships between honeybees and soybean. American Bee Journal 1970; (110) 306-307, 343-345, 383-385.

[24] Erickson E.H. Effect of honeybees on yield of three soybean cultivars. Crop Science 1975; 15(1) 84-86.

[25] Chambó, E.D., Garcia, R.C., Oliveira, N.T.E., Duarte-Júnior, J.B. Honey bee visitation to sunflower: effects on pollination and plant genotype. Scientia Agricola 2011; 68(6) 647-651.

[26] Chiari, W.C., Ruvolo-Takasusuki, M.C.C., Chambó, E.D., Arias, C.A., Hoffmann-Campo, C.B., Toledo, V.A.A. (2012). Gene flow between conventional and transgenic soybean pollinated by honeybees. In: Hasaneen, M.N.A. (ed.) Herbicides- mechanisms and mode of action. Rijeka: InTech, 2012. p.137-152.

[27] Embrapa. (2003). Tecnologia de produção de soja – Paraná. In: *Embrapa Soja*, 2003, Available from http://www.cnpso.embrapa.br/download/recomendacoes_parana.pdf (accessed 03 March 2005).

[28] Künast, C., Riffel, M., Whitmore, G. Pollinators and agriculture. Brussels: Elo and ECPA, 2011.

[29] Council of the European Communities (2009). Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50.

[30] McGregor, S.E. Insect pollination of cultivated crop plants. s.l.: United States Department of Agriculture. 1976. 411 p. Agriculture Handbook, nº 496.

[31] Teeb (2010). The Economics of Ecosystems and Biodiversity: Mainstreaming the Economics of Nature.

[32] Aizen, M., Harder, L. (2009). The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current Biology 19, 1-4.

[33] Gallai, N., Salles, J.-M., Settele, J., Vaissière, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics, (2009). 68, 810-821.

[34] Decourtye, A., Devillers, J. (2010). Ecotoxicity of neonicotinoid insecticides to bees. In: Thany, S.H. (ed.) Insect nicotinic acetylcholine receptors. New York: Springer, 2010. p.85-95.

[35] Blacquière, T., Smaggle, G., van Gestel, C.A.M., Mommaerts, V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology, (2012), 21, 973-992.

[36] Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, M.L. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection, (2004). 23, 371-378.

[37] Vaissière, B.E.; Freitas, B.M.; Gemmill-Herren, B. Protocol to detect and assess pollination deficits in crops. Roma: FAO, 2009. 26 p.

[38] Freitas, B.M.; Pinheiro, J.N. Polinizadores e pesticidas: princípios e manejo para os agroecossistemas brasileiros. Brasília: MMA, 2012. 112 p.

[39] Malerbo-Souza, D.T.; Toledo, V.A.A.; Sene Pinto, A. Ecologia da polinização. Piracicaba: CP 2, 2008. 31 p.

[40] Dafni, A.; Kevan, P.G.; Husband, B.C. Practical pollination biology. Cambridge: Enviroquest, 2005. 590 p.

[41] Alves, E.M. Polinização e composição de açúcares do néctar de soja (*Glycine max* L. Merrill) variedade Codetec 207. Master dissertation, Universidade Estadual de Maringá, Maringá, Brasil. 2004. 57p.

[42] Alves, E.M., Toledo, V.A.A., Oliveira, A.J.B., Sereia, M.J., Neves, C.A., Ruvolo-Takasusuki, M.C.C. Influência de abelhas africanizadas na concentração de açúcares no

néctar de soja (*Glycine max* L. Merrill) var. Codetec 207. Acta Scientiarum – Animal Science, (2010). 32(2), p.189-195, ISSN 1807-8672.

[43] Toledo, V.A.A., Ruvolo-Takasusuki, M.C.C., Oliveira, A.J.B.O., Chambó, E.D., and Lopes, S.M.S. Spectrophotometry as a tool for dosage sugars in nectar of crops pollinated by honeybees. In: UDDIN, J. (Ed.), Macro to nano spectroscopy. Rijeka: InTech, (2012). p.269-290 ISBN: 978-953-51-0664-7, Available from: http://www.intechopen.com/books/macro-to-nano-spectroscopy/spectrophotometry-as-a-tool-for-dosage-sugars-in-nectar-of-crops-pollinated-by-honeybees (accessed 20 October 2012)

[44] Robacker, D.C., Flottum, P.K., Sammataro, D. Effects of climatic and edaphic factors on soybean flowers and of the subsequent attractiveness of the plants to honey bees. Field Crops Research, (1983). 6(4), p.267-278, ISSN 0378-4290

[45] Cruden, R.W., Hermann, S.M., Peterson, S. Patterns of nectar production and plant-pollinator coevolution. In: Bentley, B., Elias, T. (Eds.). The biology of nectaries. New York: Columbia, (1983). p. 80-125. ISBN 023104461.

[46] Delaplane, K.S.; Mayer, D.F. Crop pollination by bees. New York: CABI Publishing, 2000. 344p.

[47] Heinrich, B. Resource heterogeneity and patterns of movement in foraging bumblebees. Oecologia, (1979). 40, p. 235-245, ISSN 00298549

[48] Hagler, J.R. Honeybee (*Apis mellifera* L.) response to simulated onion nectars containing variable sugar and potassium concentrations. Apidologie, (1990). 21, p. 115-121, ISSN 12979678

[49] Waller, G.D. Evaluating responses of honeybees to sugar solutions using an artificial flower feeder. Annals of the Entomological Society of America, (1972). 65, p. 857-862, ISSN 0013-8746

[50] Abrol, D.P., Kapil, R.P. Foraging strategies of honeybees and solitary bees as determined by nectar sugar components. Proceedings of the Indian National Academy of Sciences, (1991). 57-B, p. 127-132, ISSN 0019-5588

[51] Abrol, D.L. Pollination biology – Biodiversity conservation and agricultural production. New York: Springer, 2012. 792p.

[52] Chiari, W.C., Toledo, V.A.A., Hoffmann-Campo, C.B., Ruvolo-Takasusuki, M.C.C., Arnaut de Toledo, T.C.S.O., Lopes, T.S. Polinização por *Apis mellifera* em soja transgênica *Glycine max* L.Merrill, Roundup Ready™ cv. BRS 245 RR e convencional cv. BRS-133. Acta Scientiarum, (2008). 30(2), p.267-271.

[53] Costa-Maia, F.M., Lourenço, D.A.L., Toledo, V.A.A. Aspectos econômicos e sustentáveis da polinização por abelhas. In: Martin, T.N.; Waclawovsky, A.J., Kuss, F., Mendes, A.S., Brun, E.J. (Ed.). Sistemas de Produção Agropecuária - (Ciências Agrárias, Animais e Florestais). Dois Vizinhos (PR): UTFPR, 2010, Cap.3, p. 45-67.

[54] James, C. (2010). Global Status of Commercialized Biotech/GM Crops: 2010. lsaaa Brief No.42, ISBN 978-1-89245649-4, Ithaca, NY, USA

[55] Scottish Crop Research Institute. (1999). Gene flow in agriculture: relevance for transgenic crops conference, Proceedings of a Symposium Held at the University of Keele, pp. 13-21, ISBN 190139672, Staffordshire, UK, April 12-14, 1999.

[56] Souza, L. (2006). Liberação da soja transgênica no Brasil, vantagem ou não? In: AN-Bio, 7/03/2006, Available from http://www.anbio.org.br/noticiasflucia.htm (accessed 29/09/2012).

[57] Robinson, W.S., Nowogrodzi, R., Morse, R.A. The value of honey bees as pollinators of U.S. crops. Part I. American Bee Journal, 1989a. 129, p.411-423.

[58] Robinson, W.S., Nowogrodzi, R., Morse, R.A. The value of honey bees as pollinators of U.S. crops. Part II. American Bee Journal, 1989b. 129, n.7, p.477-487.

[59] MORSE, Roger A.; Calderone, Nicholas W. The value of honey bees as pollinators of U.S. crops in 2000. Bee Culture, 2000. 132, n.3, p.1-15.

[60] Losey, J.E.; Vaughan, M. The economic value of ecological services provided by insects. BioScience, 2006. 56, n.4, p.311-323.

Chapter 15

Herbicidal Activity of Mimosine and Its Derivatives

Tran Dang Xuan, Shinkichi Tawata and
Tran Dang Khanh

Additional information is available at the end of the chapter

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1. Introduction

Mimosine [β-[N-(3-hydroxy-4-oxypyridyl)]-α-aminopropionic acid] is a non-protein amino acid, and is a major compound present in all plant parts of *Mimosaceae*, which includes Leucaena (*Leucaena leucocephala*), *Leucaena glauca*, and other legumes belonging to *Mimosa* spp.. Structurally, mimosine is an analog of dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxy-phenyl ring (Fig. 1). Although Leucaena has a rich protein content and high annual yield, the presence of mimosine has limited the wide use of this plant as animal feed. This compound causes alopecia, growth retardation, cataracts and infertility in animals [1]. Mimosine can be degraded to DHP [3-hydroxy-4(4H)-pyridone] (Fig. 2) by microorganisms in the rumen and bacteria in rhizome nodules of Leucaena, by endogenous enzymes in the Leucaena plants, or by HCl hydrolysis. Although DHP is also toxic, it exerts lower toxicity than mimosine [2]. Mimosine possesses antimitotic activity that blocks the cell cycle in the large G1 phase [3] and inhibits DNA synthesis, which prevents the formation of the replication fork by altering deoxyribonucleotide metabolism [4]. The amino acid may also act as a tyrosine analogue which incorporates biologically vital proteins and, in turn, causes hair loss [1].

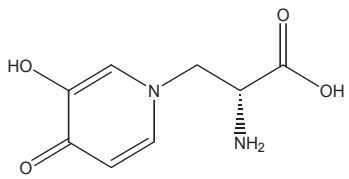

Figure 1. Chemical structure of mimosine

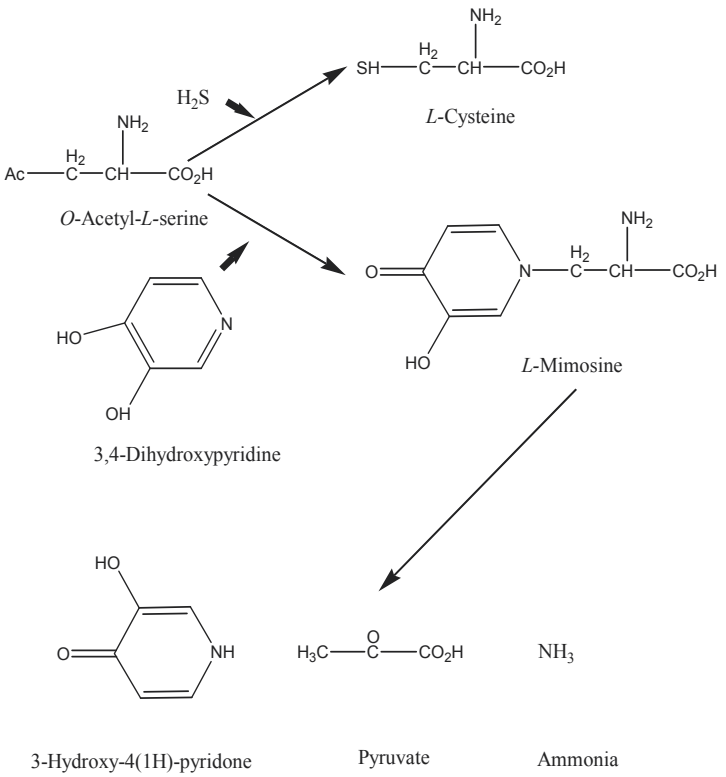

Figure 2. Metabolic relations between cysteine, mimosine and DHP [3-hydroxy-4(1H)-pyridone] [41]

Mimosine has been known as an allelochemical, which is responsible for the strong allelopathic activity of Leucaena and *Mimosa* spp., by suppressing growth of plants and plant fungi [2,5]. Our previous work examined the possibility of exploiting higher plants selected from the plant ecosystems in Southeast Asia for paddy weed control, of which *Leucaena glauca* was among those which reduced paddy weeds up to 70% and increased rice yield by 20% [6]. We concluded that mimosine was responsible for significant weed reduction of *L. glauca* in paddy fields, and rich nutrients contained in plant materials of the legume also contributed to the increase of the rice yield.

Because mimosine is a compound that is responsible for many interesting biological activities as mentioned above, many works have been conducted to purify mimosine, typically from Leucaena because it contains higher mimosine content than other species of Mimosaceae. However, most methods published so far have been too complicated and costly to successfully

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yield mimosine in highly pure grade. Our group has already developed a simple method to purify mimosine at an industrial scale by the use of ion exchange resin. Although mimosine shows herbicidal and antifungal activity, the synthesis of mimosine-derived compounds is indispensable because of the need to find mimosine derivatives with stronger suppression against pests and fungi. Synthesized compounds exerting strong activity and require a simple process for synthesis will be selected for development of novel bioactive pesticides. However, very little success with synthesis of mimosine derivatives has been reported.

This paper describes the allelopathic interaction of mimosine as well as its efficacy in application for biological control of weeds and pests. The analytical and purified methods of this compound developed in our laboratory and the synthesis of its derivatives and their suppressive activities against plants and fungi are also demonstrated.

2. Discovery of mimosine

Mimosine was first isolated from the sap of *Mimosa pudica* by Renz [7] and was given the name "mimosine". Later, minosine was biologically characterized from *M. pudica* by Nienburg and Taubõck [8]. From the extraction of ground *Leucaena glauca* seeds, Mascré [9] successfully isolated an optically inactive crystal line solid, m.p.287°C named "leucenol". Its empirical formula was elucidated to be $(C_4H_5O_2N)\chi$, and further experimental research has shown that it contains an α-amino acid including a phenolic hydroxyl. Bickel and Wibaut [10] named leucaenol and concluded the formula of mimosine was $C_8H_{10}N_2O_4$; it was chemically synthesized by Adams and Johnson [11]. Wilbaut and Klipool [12] isolated mimosine from *Leucaena leucocepphala* which they named "leucaenine" and verified that the three different substances are analogs. The chemical structure of mimosine was determined by Bickel [13] as β-N-(3-hydroxy-4pyridone)-α-amino propionic acid. The structure of mimosine is similar to dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxy-phenyl ring (Fig.1).

3. Content of mimosine

Mimosine exists in large quantities in leaves, pods and seeds of tropical legumes of the genus *Leucaena.* This compound is present in a much greater quantity in Leucaena than in *Mimosa* [14]. The Leucaena hybrid has lower mimosine content than the original *Leucaena leucocephala* [15]. In the non-hybridized Leucaena legume plant, mimosine accounted for 2-5% of fresh weight, and the level of concentration could increase to 10% in young leaves[16]. Small amounts of mimosine were in the nodules and the root exudates of Leucaena as well. The seed, stem, pod, and leaf tissue of different Leucaena species contained 1-12% mimosine, whereas the highest amount of mimosine was found in growing tips of Leucaena [14,16,17]. More recently, Xuan et al. [5] noted that all plant parts of Leucaena contained mimosine; however, the amount of mimosine in the young leaves and mature seeds was the highest, varying from

2.4 to 2.7% of the fresh weight, whereas the lowest mimosine content was in the root xylems and xylems (0.11 to 0.18%, respectively). Our research team did not find mimosine content greater than 5% in any plant parts of Leucaena observed in previous reports [5].

The quantity of mimosine in Leucaena plants is species dependent. *Leucaena leucocephala* has a medium level of mimosine, whereas, *L. collinsii, L. diversifolia, L. escuienta, L. greggii*, and *L. pallida* have low mimosine content [18]. In addition, leaf size also showed different mimosine concentrations, with smaller-leaved species having lower mimosine content [18]. The aforementioned evidences suggest that mimosine concentration may be related to the genetic variation in Leucaena species. Among ecotypes, content of mimosine and DHP in the dry season were higher than in the rainy season [19,20]. The main difference may involve the anti-nutritional factors. The concentration of mimosine contained in Leucaena plants also fluctuates with the time of year and is proportionally related to growth rate. For example, better growth of Leucaena leads to higher mimosine content [19]. Moreover, some abiotic factors of environmental stress such as drought and moisture stress can dramatically increase mimosine levels in both new and old leaves [21].

4. Purification of mimosine

Although mimosine levels are high in Leucaena, it is not easy to isolate pure mimosine. The determination of mimosine via extracting solvents and analytical instruments and spectrophotometrics was conducted [22,23]. However, it is complicated to separate mimosine from other amino acids in Leucaena, the cost of mimosine purchased from chemical companies is rather high. In our laboratory, mimosine can be easily purified at an industrial scale by the use of ion exchange resin. By this process, 6 kg of freshly harvested Leucaena leaves were immersed in 30 L of boiling water for 10 min. The water extract was cooled to room temperature and filtered with 300 mesh sieve. Ultrafiltration was carried out at 4 atm, 30°C and 700 rpm using a Filtron Miniset Omega equipped with the cassette system membrane. The filtrate was passed through a column packed with acid form Amberlite IRA (technical grade). The resin was washed with 1 L of 2N NH_4OH. About 30 g of relatively pure mimosine was obtained after adjusting the pH to 4.5~5.0. We have examined various conditions for mimosine purification and observed that the type of ion exchange resin and adjustment of pH are crucial conditions to obtaining the maximum quantity and high purity of mimosine (5 g per 1 kg fresh Leucaena leaves, purity>95%).

5. Mimosine acts as an allelochemical

Mimosine is considered as an allelochemical and is responsible for the allelopathic activity of the *Leucaena* genus and other species belonging to *Mimosa* spp. Leucaena is popular in intercropping with annual crops, using as a hedgerow, and alley cropping for yield promotion and weed control [24]. In bioassays, this compound exerted inhibition against seedlings of

mung bean (*Phaseolus aureus*) [25,26], lettuce [27,28]; hemp sesbania (*Sesbania exaltata*), ryegrass (*Lolium perenne* L), sicklepod (*Senna obtusifolia*), wheat (*Triticum aestivum*)[29], and rice (*Oryza sativa*)[28,30]. Similar to other phytotoxins, effects of mimosine against plant germination and growth are proportional to applied doses. Chou and Kuo [28] indicated that at 20 ppm, mimosine significantly suppressed growth of lettuce, rice and ryegrass; however, *Miscanthus floridulus* and *Pinus taiwanensis* were not inhibited by the mimosine at 200 ppm. Mimosine exhibited selective influence against the germination and growth of certain indicator plants including hair beggarticks (*Bidens pilosa* L), creeping grass (*Mimosa pudica* L), cabbage (*Brassica rapa*), Italian ryegrass (*Lolium multiflorum* L), and kidney bean (*Phaseoulus vulgaris* L) at 50-100 ppm. However, the effect of mimosine was the lowest against plants which are mimosine producers (*M. pudica* and *L. leucocepphala*) [5].

Mimosine also shows selective influence against certain bacteria and fungal growth. Some bacteria were inhibited, whereas growth of several bacteria was promoted by mimosine. Soedarjo and Borthakur [31] reported that growth of some root nodule bacteria was inhibited by mimosine. In contrast, some Leucaena-nodulating *Rhizobium* strains could utilize mimosine as a source of carbon and nitrogen. *Rhizobium* sp. strain TAL 1145 is such a strain that can catabolyze mimosine, which provides it a competitive advantage for nodulation of Leucaena [17]. Tawata et al. [32] revealed that *Escherichia coli* Crooks (1222) growth was inhibited by mimosine, but increased by DHP. *Aerobacter aerogenes* (1232) growth was increased by both mimosine and DHP. *Coryne bacterium psudodiphterium* (1471) growth was inhibited by DHP, but increased by mimosine.

There were 38 unknown microorganisms collected from the Leucaena population growing around Campus of University of the Ryukyus, Okinawa, Japan, including 12 from roots, 13 from top soil, and 8 from deep soil, and the remaining was from Leucaena stems; they were examined against mimosine and DHP. Among the unknown microorganisms, fungus D6-31 growth was inhibited by DHP, but increased by mimosine, whereas that of fungus D6-30 was inhibited by mimosine, but increased by DHP. The population of fungus D6-27 was dramatically increased by both mimosine and DHP, however, that of fungus D3-6 was inhibited by both mimosine and DHP. These four unknown fungi were selected for future research [32]. Other reports such as Murugesan and Radha [33] demonstrated that mimosine inhibited growth of bacteria and fungi, including *Alternaria* sp., *Cercospora canescens, Colletotrichum indemuthianum, Diplodia natalensis, Sclerotium rolfsii, Dreschlera oryzae*, and *Rhizoctonia solani*. Anitha et al. [34] noted that mimosine was toxic against fungi rather than bacteria.

On the other hand, mimosine released from rhizomes and foliated leaves to soil caused inhibition of plants in the vicinity of Leucaena [5,20]. Soils amended with mimosine retarded growth of *Brassica rapa* [5]. Hong et al. [6] evaluated the potential of weed suppression of various plants collected from plant ecosystems in Southeast Asia. Several species showed the potential for weed suppression up to 70% and increased rice yield to 20%, including *Leucaena glauca*. Because of its weed suppression and rich nutrients as well as the wide adaptation of Leucaena in the tropics, the biomass of this plant is useful for weed control and serves as a source of natural fertilizer.

6. Synthesis of mimosine and its derivatives

Mimosine toxicity is ascribed to the presence of –OH and –O in the pyridine ring and known to suppress iron-containing enzymes and compete with tyrosine [35]. The characteristic activity of growth inhibitory properties of mimosine is a hydroxyl group α to the oxo function of the pyridone ring (Fig.1). The location of the amino acid side chain seems to be less critical and an isomer (Fig.1). The synthesis of two mimosine isomers with the position of the α-hydroxy-oxo function in the pyridine ring of mimosine was at least as active *in vitro* and *in vivo* as the natural amino acid [36]. The constituent properties of the α-hydroxy-oxo group are involved in the biological activity of mimosine and other systems and may play a key factor in growth suppression [25,37-39]. The structure of the heterocyclic ring in mimosine is possible to modify the chelate properties of the molecule and their biological activity which could lead to the design of a mimosine analogue [36].

Even though mimosine shows a great potential as an allelochemical, it is difficult to apply this amino acid as a natural herbicide because it may be unstable in natural conditions. Mimosine can be easily degraded in soil by soil factors such as nutrients, minerals, pH and microorganisms. Therefore, synthesis of mimosine derivatives with stronger activity and greater stability is needed. Although many interesting experiments on mimosine have been conducted, very sporadic work on the synthesis of mimosine-derived compounds has been carried out and reported. This is the first synthesis of propionates as mimosine derivatives and was carried out in our laboratory [32].

6.1. Synthesis of propionates as mimosine derivatives

Each of 2-hydroxypyridine (material A) and 4-hydroxypyridine (material B) were well blended with each 12 different acrylates (Fig. 3), at 90-110^{o}C for 4-6 h to receive oily substances with deep yellow and brown color (Fig. 4). The reactive products were applied to TLC for purification. Solvents of TLC were benzene: methanol (1:1 or 1: 2, v/v). Yielded compounds were recovered by methanol and subsequently subjected to ^{1}H-NMR, ^{13}C-NMR and IR to determine their chemical structures. The synthesized propionates are shown in Fig. 5. Herbicidal and antifungal activities of the propionates were examined against growth of *Brassica rapa* and two noxious fungi *Schlerotium dellfinii* and *Rhizoctonia solani* at 100 ppm, respectively.

6.2. Herbicidal activity

Among synthesized propionates, two compounds including A2 and B2 [chloro-3-(2-oxohydoropyridyl) and chloro-3-(4-oxohydoropyridyl) propionates] exhibited the strongest herbicidal activity against growth of *B. rapa* (50-70% of inhibition) (Fig. 6). On the other hand, lengths of radicle and hypocotyl were either promoted or inhibited by the propionates A3, A4, A11, B4, B5, B6, B11, and B12. The other compounds reduced growth of *B. rapa* by lower magnitudes (20-40%). The chloric group in the two propionates A2 and B2 may be responsible for the greater herbicidal activities than other compounds. However, none of these synthesized propionates could exert stronger herbicidal activities than mimosine, which showed a 80-90% inhibition.

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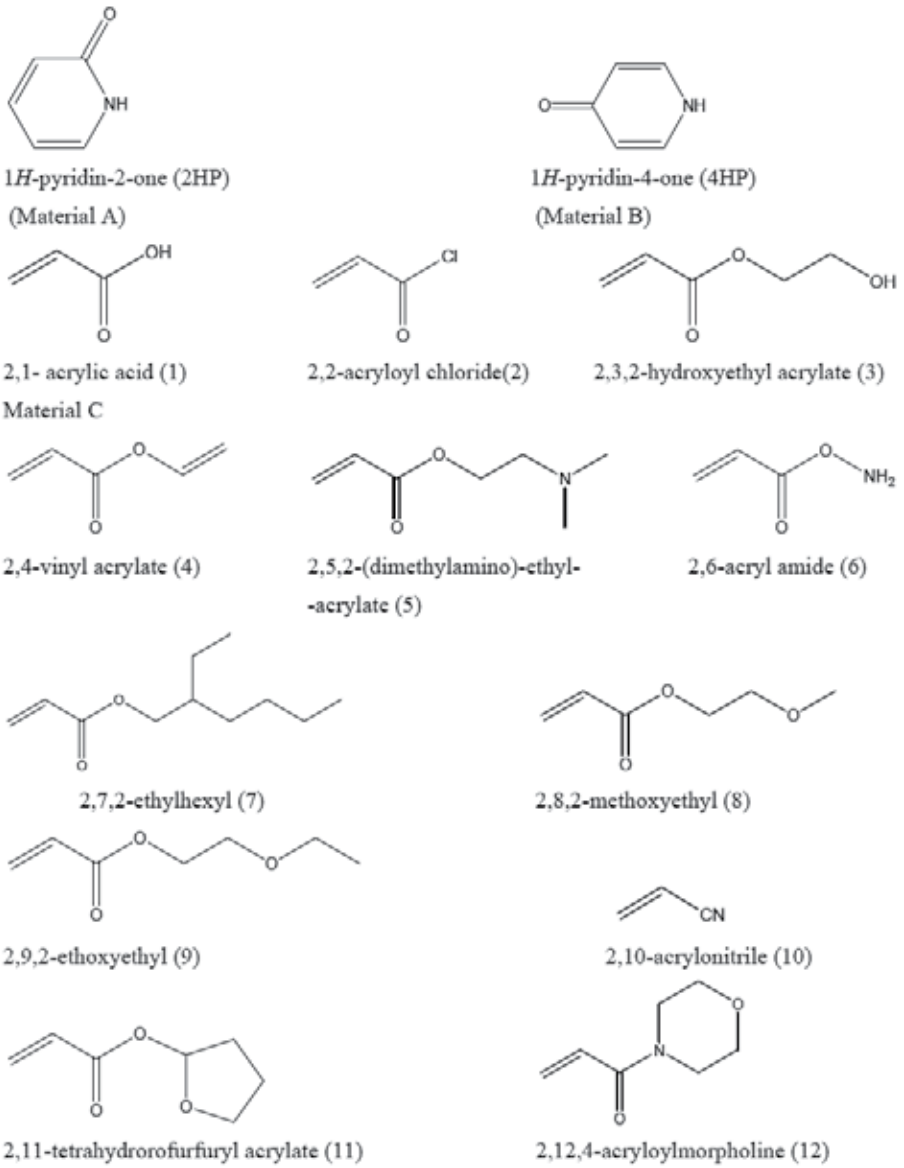

Figure 3. Chemical structures of materials for propionate synthesis

6.3. Antifungal activity

The fungal activity varied among the mimosine derivatives. The compounds A1, A2, A11, B6, and B8 were the most inhibitive against both *R. solani* and *S. dellfinii* (50-70% inhibition) (Fig. 7), whereas there were 5 propionates B3, B4, B5, B11, and B12 that stimulated growth of the two fungi up to 20%. Growth of *R. solani* and *S. dellfinii* were either stimulated or suppressed

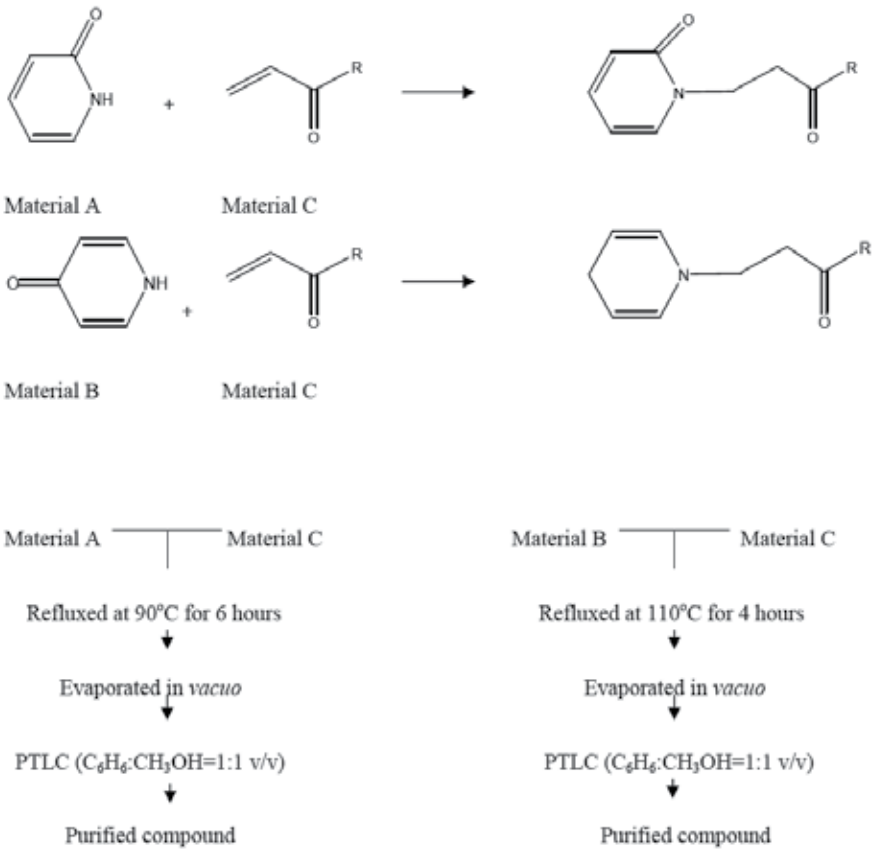

Figure 4. Synthesis route of propionates

by A3 and A4. The other propionates exerted fungal activity by 10-40%. The compounds chloro-3-(2-oxohydoropyridyl) and chloro-3-(4-oxohydoropyridyl) propionates (A2 and B2) showed good antifungal activity, whereas the chloro-3-(4-oxohydoropyridyl) propionate exhibited weak suppression of *R. solani* (about 10% inhibition). The two compounds, A2 and B2, were the most potential among synthesized propionates for obtaining herbicidal and antifungal activities. Mimosine did not show any effects against *S. dellfinii*, but inhibited growth of *R. solani* by about 30%. The antifungal strength of these synthesized propionates was greater than that of mimosine, with the exception of compounds A4, A8, B4, B5, B6, B11, and B12 (Fig. 7).

Several compounds among the synthesized propionates from this research showed good herbicidal and antifungal activities. In general, antifungal activity of these propionates was greater than their herbicidal activity. The most promising compounds were chloro-3-(2-oxohydoropyridyl) and chloro-3-(4-oxohydoropyridyl) propionates.

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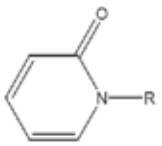
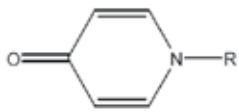


A



B

Synthesize propionates

A1, B1: R= 2,1- acrylic acid
A2, B2: R= 2,2-acryloyl chloride
A3, B3: R= 2,3,2-hydroxyethyl acrylate
A4, B4: R= 2,4-vinyl acrylate
A5, B5: R= 2,5,2-(dimethylamino)-ethyl-acrylate
A6, B6: R= 2,6-acryl amide
A7, B7: R= 2,7,2-ethylhexyl
A8, B8: R = 2,8,2-methoxyethyl
A9, B9: R= 2,9,2-ethoxyethyl
A10, B10: R = 2,10-acrylonitrile
A11, B11: R= 2,11-tetrahydrorofurfuryl acrylate
A12, B12: R= 2,12,4-acryloylmorpholine

Figure 5. Chemical structures of synthesized propionates

7. Analytical determination of mimosine

Paper and thin layer chromatography were used to identify mimosine [28]; however, mimosine content could not be quantified. Gas-liquid chromatography, liquid chromatography, and reversed-phase ion-pair high-performance liquid-chromatography were also applied for mimosine determination. However, these methods require elaborate preparation of samples, but with no appreciable improvement in the range of sensitivity [23]. Other methods were the coupling of mimosine with *p*-nitroaniline [22] or mimosine with N-1(naphthyl)ethylenediamine (NEDA) forming a pink-colored azodye with an absorbance of 540 nm [23], and the use of indirect spectrophotometricity which is based on its reaction with diazotized sulfanilamide (DZSAM). These methods were reported to increase the sensitive estimation of mimosine. A useful HPLC system to determine mimosine and DHP contents that influenced *Rhizobium* isolates was reported by Soedarjo et al. [40]. They applied a C18 HPLC column, UV detection at 280 nm, a solvent system of 0.2% orthophosphoric acid to detect mimosine and DHP at 2.7 and 4.8 min, respectively.

Our laboratory also developed a simple method using HPLC to determine mimosine and DHP. This method is nottime consuming, uses simple reagents and procedures, and has a high level of accuracy. Of which, the HPLC system includes an 880-PU pump and column (Fine pak Sil C18, Nihonbunko company). The mobile phase employed was a mixture solution of 10 mM potassium-dihydrogen phosphate, 10 mM phosphoric acid, acetonitrile (45:45:10), and finally, 0.1% sodium 1-octanesulfonate was added to the mixture as the surface active agent. The flow rate was 1.5 mL per min. Mimosine and DHP were detected at a wave length of 280 nm. The fresh samples from Leucaena (leaves, stems, or roots) were boiled for 10 min, cooled at room temperature, centrifuged, filtered and injected into HPLC at 2-5 μL. The peaks of mimosine

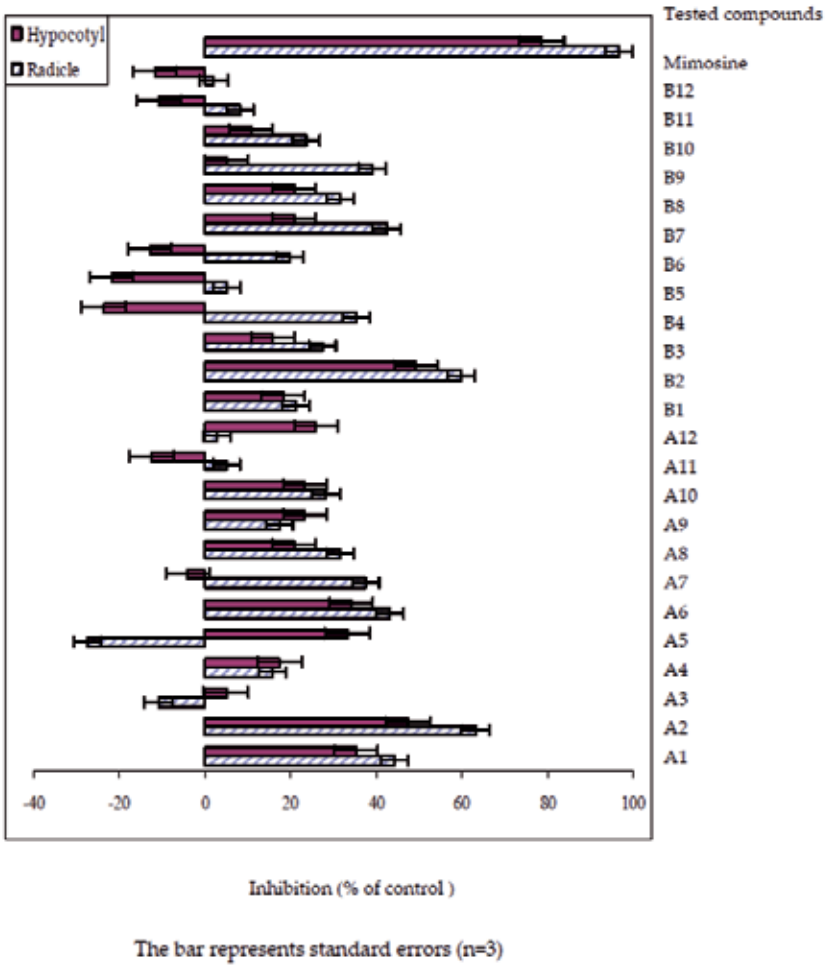


Figure 6. Herbicidal activity of mimosine and its propionate derivatives against *Brassica rapa* (100 ppm)

and DHP appear at 2.5 min and 7.5 min retention time, respectively. However, these retention times varied among columns and HPLC conditions.

8. Conclusion

Mimosine is a major secondary metabolite in Leucaena and Mimosaceae plants and is responsible for the biological activities of these plants. Its allelopathic interaction includes both inhibition of plants, fungi, and bacteria, and stimulation of several strains of bacteria. The biomass of Leucaena in the tropics is a potential source for reduction of weed emergence in paddy fields and simultaneous utilization as green manure. Although we have synthesized several propionates which exerted potent antifungal activity, further synthesis should be

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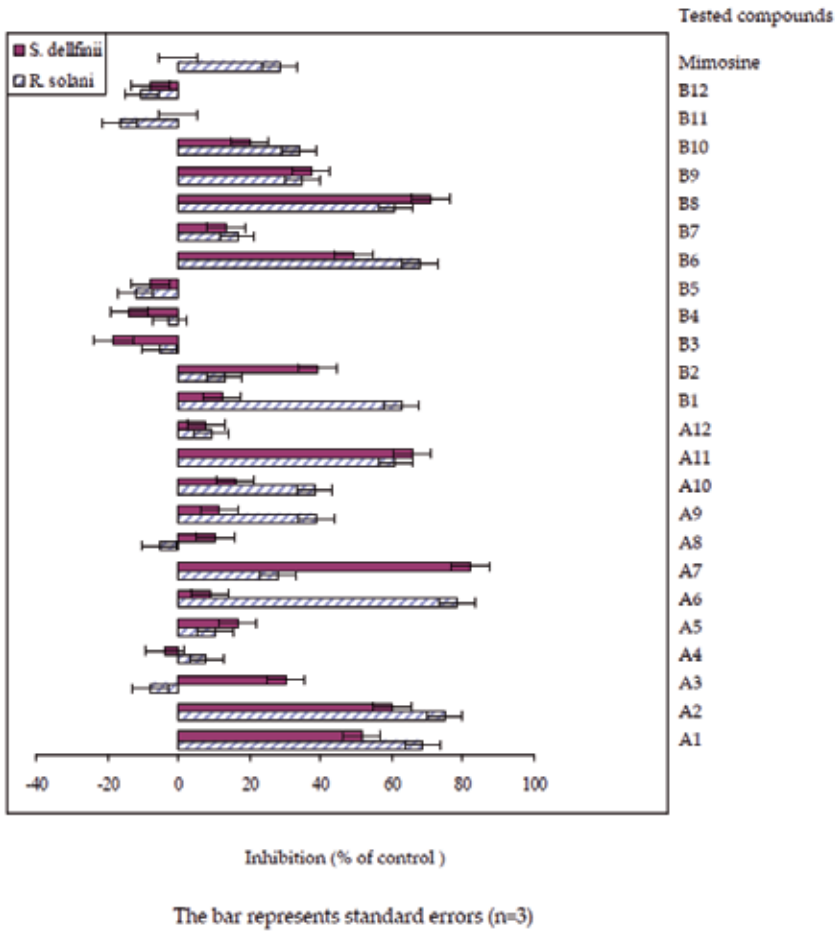

Figure 7. Antifungal activity of mimosine and its propionate derivatives (100 ppm)

continued to yield novel derivatives of mimosine which can obtain stronger inhibition on plant growth than their parent, mimosine.

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References

[1] Crounse RG, Maxwell RD, Blank H. Inhibition of growth of hair by mimosine. Nature 1962; 194: 694-695.

[2] Tawata S. Effective reduction and extraction of mimosine from Leucaena and the potential for its use as a lead compound of herbicides. In: Casida, J.E.(ed.). Pesticide and Alternatives. Elsevier Science Publishers, Amsterdam, pp. 541-554. 1990.

[3] Khanna KK, Lavin MF. Ionizing radiation and Uv induction of P53 protein by different pathways in ataxia-telangiectasia cells. Oncogene 1993; 8(12): 3307-3312.

[4] Gilbert D, Neilson A, Miyazawa H, Depamphilis ML, Burhans WC. Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. Journal of Biological Chemistry 1995; 270 (16): 95957-9606.

[5] Xuan TD, Elzaawely AA, Deba F, Fukuta M, Tawata S. Mimosine in Leucaena as a potent bio-herbicide. Agronomy for Sustain Development 2006; 26(2): 1-9.

[6] Hong NH, Xuan TD, Tsuzuki E, Khanh TD. Paddy weed control by higher plants from Southeast Asia. Crop Protection 2004; 23(3): 255-261.

[7] Renz J. Uber das mimosin. Zeitschrift für Physiologische Chemie 1936; 244(3-4): 153-158.

[8] Nienburg H, Taubõck K. Zur constitution des mimosine und uber einige substituierte phenyl-aminoessigsauren. Zeitschrift für Physiologische Chemie 1937; 250: 80-86.

[9] Mascré. Structural of leucenol. Comptes Rendus 1937; 204: 890.

[10] Bickel AF, Wibaut JP. On the structure of Leucaenine (leucaenol) from *Leucaena glauca* Bentham. Rescueil des Travaux Chimiques des Pays-Bas 1946; 65(1): 65-83

[11] Adams R, Johnson JI (1949) Leucenol VL total synthesis. Journal of the American Chemical Society 1949; 71 (2): 705-708.

[12] Wilbaut JP, Kilpool RJC. On the structure of leucaenine (leucaenol) 3rd communication. Rescueil des Travaux Chimiques des Pays-Bas 1947; 66 (1): 24-28.

[13] Bickel AF. On the structure of Leucaenine (leucanol) from Leucaena glauca Bentham. IV. Journal of the American Chemical Society 1948; 70 (1): 328-329.

[14] Jones R J. The value of *Leucaena leucocephala* as a feed for ruminants in the tropics. World Animal Review 1979; 31: 13-23.

[15] Ruskin FR. Leucaena, proving forage and tree crop for the tropics (ed) in Leucaena. National Academy of Science, Washington, D.C: 1-66. 1977.

[16] Brewbaker JL, Hylin JW. Variation in mimosine content among Leucaena species and related Mimosaceae. Crop Science 1965; 5(4): 348-349.

[17] Soedrjo M, Borthakur D. Mimosine, a toxin produced the tree legume Leucaena provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains. Soil Biology and Biochemistry 1998; 30(12): 1605-1613.

[18] Bray RA. Possible of developing low mimosine Leucaena opportunities and limitations. Proc.Workshop, Indonesia, January, 24-29, 2004, p 199-124. 1994.

[19] Fortes D, La OO, Chongo B, Scull I. A note on the chemical composition of six ecotypes of *Leucaena leucocephala*. Cuban Journal of Agricultural Science 2003; 37(3): 299-306.

[20] Arora SK, Joshi UN. Lignification of Leucaena leaves during growth and its relationship with mimosine content. Leucaena Research Report 1986; 7: 34.

[21] Bray RA, Hoekstra J. The effect of moisture stress on mimosine content of *Leucaena leucocephala*. Proc. XV. Inter. Grasslands Cong., Kyoto, Japan, 356-375. 1995.

[22] Lalitha K, Vargheese CM, Balasubramanian N. Spectrophotometric determination of mimosine and 3-hydroxy-4(1H)-pyridone-the toxic principle of *Leucaena leucocephala*. Analytical Biochemistry 1993; 213(1): 57-62.

[23] Lalitha K, Kulothungan SR. Determination of mimosine by a sensitive indirect spectrophotometric method. Talanta 2004; 63(3): 635-640.

[24] Siaw DEKA, Kang BT, Okali DUU. Alley cropping with *Leucaena leucocephala* (Lam.) de Wit and *Acioa barteri* (Hook.F.). Agroforestry System 1991; 14(3): 219-231.

[25] Smith IK, Fowden L. A study of mimosine toxicity in plant. Journal of Experimental Botany 1966; 17(4): 750-761.

[26] Ling KH, Wen WH, Ling W I. Study on the mechanism of the toxicity of mimosine. Journal of Formosa Medicinal Association 1969; 68(10): 510-517.

[27] Wilson MF, Bell EA. Amino acid and β-aminopropionitrile as inhibitors of seed germination and growth. Phytochemistry 1978; 17(3): 403-406.

[28] Chou CH, Kuo YL. Allelopathic research of subtropical vegetation in Taiwan. III. Allelopathic exclusion of understory by *Leucaena leucocephala* (Lam.) de Wit. Journal of Chemical Ecology 1986; 12(6): 1431-1448.

[29] William R, Hoagland R. Phytotoxic of mimosine and Albizziin on weeds and crops. Proceedings of Southern Weed Science Society; 53: 204. 2000.

[30] Prasad MNV, Subhashini P. Mimosine-inhibited seed germination, seedling growth and enzymes of *Oryza sativa* L. Journal of Chemical Ecology 1994; 20(7): 1689-1696.

[31] Soedarjo M, Borthakur D. Mimosine produced by the tree-legume Leucaena provides growth advantages to some *Rhizobium* strains that utilize it as a source of carbon and nitrogen. Plant and Soil 1996; 186(1): 87-92.

[32] Tawata, Xuan TD, Fukuta M. Herbicidal lead compound mimosine and its degradation enzyme. In: Fourth World Congress on Allelopathy (Ed.). Wagga Wagga, NS, Australia, 21-26 August, 2005.

[33] Murugesan K, Radha A. Biochemical mechanism of mimosine toxicity to fungi. International Journal of Tropical Plant Disease 1994; 12: 171-176.

[34] Anitha R, Jayavelu S, Murugesan K. Antidermatophytic and bacterial activity of mimosine. Phytotherapy Research 2005; 19(11): 992-993.

[35] Vickery ML, Vickery B. Compound derived from amimo acids, pp, 220- 252, in Vickery ML, Vickery B. Secondary plant metabolism, London.1981.

[36] Harris RLN, Teitei T. Potential wool growth inhibitors 2(1H)-pyridone analogues of mimosine. Australian Journal of Chemistry 1977; 30(3): 649-655.

[37] Matsumoto H, Smith EG, Sherman GD. The effect of elevated temperatures on the mimosine content and toxicity of koa haole (*Leucaena glauca*). Archives of Biochemistry and Biophysics 1951; 33(2): 201-211.

[38] Lin JY, Ling KH. Studies on free amino acids in the free seeds of *Leucaena glauca* Benth. III. Biological study on mimosine. Journal of Formosa Medical Association 1961; 60: 657-664.

[39] Tsai WC, Ling KH. Toxic action of mimosine. I. Inhibition of mitosis and DNA synthesis of H. Ep-2 cell by mimosine and 3,4-dihydroxypyridone. Toxicology 1971; 9(3): 241-247.

[40] Soedarjo M, Hemscheidt TK, Borthakur D. Mimosine, a toxin present in leguminous tree (Leucaena spp.), induces a mimosine-degrading enzyme activity in some *Rhizobium* strains. Applied and Environmental Microbiology 1994; 60(12): 4268-4272.

[41] Vestena S, Fett-Neto A, Duarte, R.C, Ferreia AG. Regulation of mimosine accumulation in *Leucaena leucocephala* seedlings. Plant Science 2001; 161(3): 597-604.

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Herbicides represent one of the most widely used groups of pesticides worldwide for control of weed species in agricultural and non-crop settings. Due to the extensive use of herbicides and their value in weed management, herbicide research remains crucial for ensuring continued, effective use of herbicides while minimizing detrimental effects to ecosystems. Presently, a wide range of research continues to focus on improved herbicide use, environmental impact of herbicides, and even medicinal application of herbicide chemistries. In Herbicides - Advances in Research, authors cover multiple topics concerning current, valuable herbicide research.

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