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Citrus Pathology

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Meet the editors

Harsimran "Rosie" Gill earned her PhD degree (Entomology) from the University of Florida, USA, and studied organic and inorganic (soil solarization) mulches and their role in pest management. She has published 53 articles that include 16 peer-reviewed research articles, 7 nonrefereed articles, 5 book chapters, 19 extension articles, 6 newspaper and magazine articles, and 1 edited book, and has delivered 26 oral and poster presentations at national and international levels. She has reviewed more than 80 articles and is serving as an editor for many national and international journals. She has won several research and travel awards, and has won awards from the University for being the best student at the Entomological Society of America for her active participation. She has been working on pest management research, extension, and teaching for the last 15 years. Currently, she is working as a freelance editor and researcher at Cornell University, USA.

Dr. Harsh Garg has received her PhD degree from the University of Western Australia and examined host-pathogen interactions at cellular and molecular levels. She then worked as a NSERC visiting fellow at the Saskatoon Research and Development Centre (Canada) and as an assistant professor in the Punjab Agriculture University (India). She has received many scholarships and awards for her academic achievements. She has published more than 25 articles including peer-reviewed research papers, review articles, conference papers, etc. Her areas of interest include plant pathology and plant breeding, specifically, the mechanisms of host resistance (both at molecular and histology levels) against plant pathogen and breeding for resistance. She has now expanded her learning horizon to the photosynthesis area and is currently working as a postdoctoral research associate at the University of Sydney examining biosynthesis and biochemistry of chlorophylls.

Contents

Preface

Citrus (*Citrus* L.) is one of the world's largest fruit crops, both in terms of production and international trade. *Citrus* is a genus of flowering trees and shrubs in Rutaceae family. It is a common term, which includes many important crops of economic importance such as oranges, lemons, grapefruit, pomelo, and limes. A rapid increase in the area under citrus production has been reported over the past few decades due to increased awareness of the therapeutic value of its fruit besides being a very important source of vitamin C and other essential nutrients. Citrus has also been associated with the reduction of breast cancer, stomach cancer, scurvy, and other diseases as well. However, a number of diseases, viruses, and insects confront the citrus crop under both field and storage conditions. It is important to have an understanding and knowledge of such challenges to maximize the yield and quality of the produce. The branch of science that focuses on diseases in plants caused by pathogens (organisms) is called plant pathology or phytopathology. The book *Citrus Pathology* is intended to provide an overview of citrus diseases, host-pathogen interactions, and pre- and postharvest management of diseases.

Many leading researchers across the world have contributed to the publication of this book. We aimed to compile information from a wide diversity of sources in a single volume in the form of this book. We have focused on different areas of citrus: citrus diseases, host-pathogen interactions, preharvest management of diseases and insects, postharvest management of diseases, and application of citrus and its compounds. The book covers a detailed overview of various major endemic and emerging fungal diseases of citrus followed by a special emphasis on a lesser known pathogen, i.e., *Spiroplasma* spp., that can be a threat to citrus industry in the future. Besides having the knowledge of various diseases, it is important to understand the host-pathogen interactions to design effective management strategies for diseases. The book covers the two emerging aspects in this area where metabolic changes in citrus plants against various stresses and the role of quorum sensing in virulence and symptom development are broadly discussed. The rest of the chapters offer information on preharvest and postharvest management of diseases and insects and application of citrus and its compounds. Various aspects of pre- and postharvest disease and insect management practices covered in this book can be of special interest to researchers and growers in order to increase the quality of produce and to increase the shelf life of the citrus. A comprehensive detail of advantages of citrus and its compounds is provided at the end of this book to increase our knowledge of its various applications such as for managing neurogenerative diseases.

The inclusion of citrus diseases, host-plant interactions, and different management options and its applications will make this book of great significance to researchers, scientists, graduate students, growers, policy makers, and other professionals who can make use of compiled information from this particular book. We hope that this book will continue to meet the expectations and needs of all interested to know more and understand citrus pathology in detail.

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Section 1

Citrus Diseases

Chapter 1

Major and Emerging Fungal Diseases of *Citrus* in the Mediterranean Region

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Santa Olga Cacciola

Additional information is available at the end of the chapter

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Abstract

This chapter deals with major endemic and emerging fungal diseases of citrus as well as with exotic fungal pathogens potentially harmful for citrus industry in the Mediterranean region, with particular emphasis on diseases reported in Italy and Maghreb countries. The aim is to provide an update of both the taxonomy of the causal agents and their ecology based on a molecular approach, as a preliminary step towards developing or upgrading integrated and sustainable disease management strategies. Potential or actual problems related to the intensification of new plantings, introduction of new citrus cultivars and substitution of sour orange with other rootstocks, globalization of commerce and climate changes are discussed. Fungal pathogens causing vascular, foliar, fruit, trunk and root diseases in commercial citrus orchards are reported, including *Plenodomus tracheiphilus*, *Colletotrichum* spp., *Alternaria* spp., Mycosphaerellaceae, Botryosphaeriaceae, *Guignardia citricarpa* and lignicolous basidiomycetes. Diseases caused by *Phytophthora* spp. (oomycetes) are also included as these pathogens have many biological, ecological and epidemiological features in common with the true fungi (eumycetes).

Keywords: *Plenodomus tracheiphilus*, *Colletotrichum* spp., *Alternaria* spp., greasy spot, Mycosphaerellaceae, Botryosphaeriaceae, *Guignardia citricarpa*, Basidiomycetes, *Phytophthora* spp

1. Introduction

Citrus are among the ten most important crops in terms of total fruit yield worldwide and rank first in international fruit trade in terms of value. More than seven million hectares are planted with citrus throughout the world. The term “citrus” indicates a complex of species and hybrids of the genera *Citrus*, *Eremocitrus*, *Fortunella*, *Microcitrus* and *Poncirus*, subfamily

Aurantioideae (family Rutaceae). It is assumed that all presently cultivated citrus species originate from three ancestral "true" species, *Citrus medica* (citron), *Citrus reticulata* (mandarin) and *Citrus maxima* (pummelo). Although citrus are native to East Asia, citriculture has expanded in tropical, subtropical and Mediterranean climatic regions, and presently Mediterranean countries are the leading producers for the international fresh market. The term "fungal" diseases referred to citrus pathologies includes both diseases caused by "true" fungi or fungi sensu stricto (eumycota) and those caused by oomycetes. In fact, although Oomycetes are part of a distinct kingdom (Chromista or Stramenopiles), they are traditionally considered fungi sensu lato as they have in common with Eumycota some ecological and morphological (e.g. filamentous hyphae) features. This chapter is not intended to be a complete review of fungal diseases reported in the Mediterranean citrus belt. It deals only with major endemic and emerging fungal diseases of citrus reported in Italy and Maghreb countries, as well as with exotic fungal pathogens potentially harmful for citrus industry in the Mediterranean region. The aim is to provide an update of the taxonomy of the causal agents and their ecology and diagnosis based on a molecular approach as a preliminary step towards developing or upgrading integrated and sustainable management strategies. Postharvest fungal diseases of citrus are not within the scope of this brief review.

2. General considerations

Any rational disease management strategy is based on accurate diagnosis and prevention. Fungal diseases of citrus showing specific symptoms can be easily diagnosed visually, while for diseases with no typical symptoms, laboratory tests are needed. A limit of symptomatic diagnosis is that some symptoms are visible only at certain times of the year or appear on organs distinct from those colonized by the pathogen. Moreover, secondary parasites or opportunistic pathogens can overgrow primary pathogens or colonize senescent or necrotic tissues. Typical examples are *Colletotrichum* species such as the cosmopolitan *Colletotrichum gloeosporioides*, the most common *Colletotrichum* species on citrus and *Colletotrichum karstii. C. karstii* has recently been described as a separate species of the *Colletotrichum boninense* complex, using a multilocus molecular phylogenetic analysis [1]. It is a widespread and polyphagous species; however, so far it has been reported on citrus only in Italy and China [2, 3], very probably reflecting a sampling bias. *Colletotrichum* species are associated with citrus as endophytes, saprobes as well as pre- and postharvest anthracnose pathogens. They can switch their lifestyle from endophytes to saprobes or opportunistic pathogens and produce acervuli on necrotic tissues as a consequence of biotic and abiotic stresses, such as mal secco disease, frost, wind, hail and any type of mechanical injury.

The most effective control method of fungal diseases is prevention, especially for diseases caused by soil-borne pathogens. Most of the rootstocks used in commercial citrus orchards, e.g. are resistant to *Phytophthora* trunk gummosis and root rot. Genetic resistance has also been used in citriculture for prevention of vascular and canopy diseases such as mal secco disease of lemon and brown spot of tangerines, respectively. However, the choice of the scion is primarily conditioned by commercial requirements. The genetic susceptibility to fungal diseases is a limiting factor to the widespread diffusion of some citrus cultivars ['Fortune' mandarin, e.g. is not planted in humid areas as it is very susceptible to Alternaria brown spot (ABS)]. Prevention methods include selection of the plantation site, surface levelling of the ground to avoid waterlogging, soil drainage and rational management of irrigation. Irrigation systems that wet the trunk favour *Phytophthora* gummosis, but risk is reduced if trees are irrigated during the morning in order to allow the bark to dry quickly. Sprinkler irrigation under the canopy is conducive to *Phytophthora* infections on trunk and fruits, while overhead sprinkler irrigation favours brown spot epidemics in orchards of susceptible tangerine cultivars as well as *Septoria* spot and mal secco in lemon orchards. Generally speaking, localized irrigation methods, such as drippers, are less conducive to leaf and fruit diseases. Usually, fungal infections occur on citrus trees irrespective of their vigour. However some diseases attack only weakened trees, while others develop preferentially on vigorous trees. Alternaria brown spot, e.g. is more severe on trees with dense canopy and copious spring vegetation as the causal agent, *Alternaria alternata* (*A. alternata*), sporulates only on young leaves and with high relative humidity. As a consequence this disease is favoured by intensive plantings and high amounts of nitrogen fertilizers.

Mediterranean climate is not conducive to epidemic infections of fungal diseases of the tree canopy, and as a consequence, chemical control of these diseases in the Mediterranean region is economically justified only in few cases. The choice of fungicides is restricted to active ingredients registered for citrus. In Italy, only copper derivatives (oxychloride, hydroxide and sulphate tribasic) are allowed for field treatments against fungal diseases. Systemic fungicides, metalaxyl M and ethyl-phosphytes (Al ethyl-phosphite and K ethyl-phosphite) are available for chemical control of diseases caused by *Phytophthora*. Metalaxyl M can be applied as soil drench or trunk paint as it is translocated through the plant apoplast. The derivates of phosphorous acid are translocated through the symplast so they can be applied to the trunk (as paints or sprays) or the tree canopy (as leaf sprays). In some commercial products, cupric and systemic fungicides are blended together.

Hereafter we illustrate major fungal diseases of citrus already established or potentially harmful for the citrus industry in the Mediterranean region. Two quarantine fungal pathogens of citrus presently are included in the A1 list of the European and Mediterranean Plant Protection Organization (EPPO), *Guignardia citricarpa* (*G. citricarpa*) (anamorph, *Phyllosticta citricarpa*), the causal agent of fruit black spot, and *Pseudocercospora* (*Phaeoramularia*) *angolensis* (*P. angolensis*), the causal agent of angular leaf spot also known as Phaeoramularia leaf and fruit spot. However, only for *G. citricarpa* an official diagnostic protocol is available. The EPPO A1 list includes pathogens and parasites whose introduction in the territory of EPPO would cause severe phytosanitary risks. Import of citrus fruits and propagative material from areas where these pathogens are present is subject to customs restrictions. Geographic distribution of *G. citricarpa* includes Asia, Africa, Australia, South America and Florida. A real risk for the citrus industry in Italy is that this fungal pathogen can be imported with citrus from South Africa to South America. In the last years, this pathogen has been intercepted several times on citrus imported from South Africa, Brazil and Uruguay. For more details on citrus black spot, refer to the EPPO diagnostic protocol PM7/17 (http://www.eppo.int) [4]. The protocol reports official molecular diagnostic methods to identify the causal agent of black spot and distinguish it from the non-pathogenic species *Guignardia mangiferae*. *P. angolensis* causes leaf spots and necrotic lesions on fruits. Geographic distribution of this pathogen is restricted to the warm and humid areas of central Africa, at altitudes between 80 and 1500 m, and Yemen, in Asia. All species of cultivated Citrus appear to be susceptible.

3. Mal secco

The mal secco, an Italian name meaning "dry disease", is a vascular wilt disease (**Figure 1**) caused by the mitosporic fungus *Plenodomus tracheiphilus* (*P. tracheiphilus*), formerly *Phoma tracheiphila*. Symptoms include strands of salmon-pink to orange-red discoloration visible in stem xylem (**Figure 2**) as well as veinal chlorosis (**Figure 3**), wilt and shedding of leaves, dieback of twigs and branches. The disease is particularly destructive on lemon (*Citrus limon*) in Mediterranean countries and the Black Sea region. So far, however, it has not been reported in Spain, Portugal and Morocco, as well as other major citrus-growing regions of the world, even though there is no obvious climatic or cultural factor limiting its establishment in uninfested areas.

Figure 1. Wilting, leaf shedding and defoliation on a young lemon tree affected by mal secco (courtesy S.O. Cacciola, G. Magnano di San Lio, A. Pane).

Figure 2. Pink-salmon discoloration of the wood associated to mal secco disease (courtesy S.O. Cacciola and A. Pane).

Figure 3. Clearing and chlorosis of a sour orange leaf affected by *Plenodomus tracheiphilus* (courtesy S.O. Cacciola and A. Pane).

P. tracheiphilus is a quarantine pathogen of great concern to many international plant protection organizations; it is listed on the list A2 of EPPO and the lists of quarantine pathogens of Asia and Pacific Plant Protection Commission (APPPC), Caribbean Plant Protection Commission (CPPC), Comité Regional de Sanidad Vegetal del Cono Sur (COSAVE), North American Plant Protection Organization (NAPPO) and Inter-African Phytosanitary Council (IAPSC). Moreover *P. tracheiphilus* was included in a list of microorganisms that have to be regarded as potential biological weapons as they cause destructive diseases of economically relevant crops [5]. Although lemon is the principal host, other species of citrus and related genera (*Fortunella*, *Poncirus* and *Severinia*) may be infected [6]. In several Mediterranean countries, including Greece, Israel, Italy and Tunisia, severe infections have been sporadically observed, also in commercial orchards of bergamot, citron, sweet oranges, tangerines, mandarins and mandarin hybrids. Many of these reports on sweet oranges, tangerines, mandarins and mandarin hybrids refer to the chronic *facies* of the disease known as "mal nero" (**Figure 4**), very probably originating in the nursery. Young seedlings of common citrus rootstocks such as sour orange (*Citrus aurantium*), citranges (*Citrus sinensis* 'Washington' sweet orange × *Poncirus trifoliata*) and citrumelo 'Swingle' (*Citrus paradisi* 'Duncan' grapefruit × *P. trifoliata*) proved to be susceptible to natural infections in nursery.

Figure 4. The 'mal nero' *facies* of mal secco disease (courtesy S.O. Cacciola, G. Magnano di San Lio, A. Pane).

Alemow (*Citrus macrophylla*) is extremely susceptible, and trees on alemow rootstock may die as a consequence of root infections. This *facies* of the disease associated to root infections is known as "mal fulminante", an Italian name meaning "sudden death".

The mal secco fungus was originally classified among the Deuteromycota as *Deuterophoma tracheiphila*. Later, it was reclassified as *P. tracheiphila* and considered a member of the subgenus *Plenodomus* due to the presence of thick-walled cells in the pycnidial scleroplectenchymatous tissues [7]. Many species in this subgenus have *Leptosphaeria* teleomorphs and/or *Phialophora* synanamorphs [8]. Although a molecular phylogenetic study has shown a relationship between *P. tracheiphila* and *Leptosphaeria* species [9], the teleomorph of the mal secco fungus has not yet been identified.

Recent molecular phylogenetic studies on the genus *Phoma* demonstrated its polyphyly and lead to the definition of the genus *Plenodomus*. *P. tracheiphila* and many other fungi of the section *Plenodomus* were allocated within this genus [10–12]. Presently, therefore the accepted scientific name of the causal agent of mal secco disease of citrus is *P. tracheiphilus*. Identification of *P. tracheiphilus* is currently based on morphological and molecular methods as described in the OEPP/EPPO diagnostic protocol PM7/048(3) (http://www.eppo.int) [13]. In the last years, numerous molecular techniques based on conventional polymerase chain reaction (PCR) and real-time PCR (rtPCR) have been developed, allowing fast and sensitive detection and quantification of the pathogen from infected tissues [9, 14–17]. Some of these techniques have been applied in breeding programmes to monitor the progress of the fungus in the host xylem and test the susceptibility of lemon cultivars to mal secco. As in other tracheomycoses, in fact, there is a direct correlation between the rate and extent of xylem colonization by the pathogen (i.e. the amount of the fungus in the vessels) and the susceptibility of the cultivar to the disease as determined by symptom severity.

P. tracheiphilus can infect citrus hosts all year round, but most infections occur from September to April. Pycnidiospores produced within pycnidia and phialoconidia produced on mycelium are the infective propagules. Pycnidia are present throughout the year on withered twigs (**Figure 5**) and branches; pycnidiospores are extruded when the weather is wet. Phialoconidia are produced on the surface of infected tissues exposed by wounds or on woody debris in soil. Penetration occurs through wounds. Penetration through natural opening has been hypothesized but never demonstrated. After penetration, the mycelium grows in the lumen of xylem vessels producing both phialoconidia and blastoconidia that are translocated by the ascendant lymph and invade the wood. The pathogen can be detected distantly from its penetration site, by molecular tools, before the expression of the disease symptoms [17]. Systemic invasion by the pathogen leads to the loss of xylem functionality and the appearance of water stress symptoms typical of tracheomycoses. Impairment of water transport is proved by the increase of hydraulic resistance of the stem and leaves as well as the closure of stomata [18, 19]. Although the optimum temperature for pathogen growth is about 25°C, optimum temperature for symptom expression and xylem colonization is 20–22°C.

Infection occurs between 14 and 28°C, whereas at temperatures above 28°C, fungal growth ceases and symptoms are not expressed. As a consequence disease progress is temporarily inhibited during the hot or cold temperature extremes.

Figure 5. White-red 1–2-year-old twig of lemon with pycnidia of *Plenodomus tracheiphilus*. Pycnidia appear as scattered black spots on the dried portion of the twig (courtesy S.O. Cacciola and A. Pane).

While in Sicily most infections occur from autumn to early spring; in Israel, mid-November to mid-April was the most conducive time for infection, coinciding with the rainy season, although no correlation was found between the amount of rain, the number of rainy days and the percentage of infected plants. No infection was observed after the rain ceased, so it appears that the rain affects inoculum dissemination rather than infection [20]. Length of the incubation period varies according to season, and in young trees, it ranges between 2 and 7 months, whereas it can last several years in the "mal nero" form of the disease because this chronic infection could remain confined to the heartwood over a long time. Expression of symptoms is therefore a poor selection criterion for phytosanitary inspection of propagation material. This aspect has practical relevance as the use of disease-free propagation material helps to reduce the dissemination of mal secco and its introduction into disease-free areas.

Like for other tracheomycoses, fungicide treatments are not effective against mal secco, and research of newly resistant genotypes remains the only effective strategy to control this disease. Lemon cultivars with various degrees of resistance to mal secco, such as 'Monachello', 'Interdonato', 'Feminello Zagara Bianca', 'Femminello Continella' and 'Cerza', have been selected in Sicily. However, 'Monachello' has a poor yield, 'Interdonato' does not bloom several times, and its juice has low acidity, and the tolerance to mal secco of the other cultivars is not comparable to that of 'Monachello'. Two new cultivars with high yield potential, 'Femminello Siracusano 2Kr', a mutant nucellar clone obtained with cobalt γ-radiation, and the triploid hybrid 'Lemox' (European patent number 20040073), have been included in the official list of lemon cultivars whose use is recommended in Italy for new plantings. However they proved to be extremely susceptible to mal secco disease. The goal of obtaining tolerant cultivars with competitive yields and satisfactory bio-agronomic characteristics remains one of the primary objectives of lemon-breeding programmes in the Mediterranean region. Additional and more detailed information on mal secco disease can be found in two recent comprehensive reviews [20, 21].

4. Emerging and endemic foliar and fruit diseases

Despite the Mediterranean climate is not conducive to epidemic outbreaks of fungal diseases of the canopy of citrus trees, being characterized by long periods of drought and high temperatures in summer as well as cool winters, in the last years, some citrus-growing areas of the Mediterranean region have experienced the emergence or resurgence of new and endemic fungal diseases of leaves and fruit.

4.1. Alternaria brown spot

Alternaria brown spot (ABS) is one of the most important diseases of tangerines and their hybrids worldwide. It is caused by the tangerine pathotype of the fungus *A. alternata* [22, 23]. *A. alternata* is a typical necrotrophic pathogen. It produces a host-specific (hs) toxin named ACT-toxin (ACTT), which induces necrotic lesions on fruit and young leaves, defoliation and fruit drop in susceptible citrus genotypes. There are several pathotypes of *A. alternata* characterized by host specificity [24]. The chemical structure of ACT-toxin is similar to those of other hs-toxins such as AK- and AF-toxin, produced by the Japanese pear and strawberry pathotypes, respectively [25, 26]. The tangerine pathotype of *A. alternata* carries a gene cluster (ACTT) located in a small chromosome which is responsible for the biosynthesis of ACT-toxin [27]. There is also indirect evidence suggesting the presence of toxin receptors in susceptible citrus genotypes. In addition, recent studies confirm that the ACT-toxin is a pathogenicity factor and indicate that the mitigation of reactive oxygen species (ROS) produced by the host plant is essential for the pathogenicity of *A. alternata*. A significant correlation was found between pathogenicity on citrus leaves and ACTT gene expression in isolates of *A. alternata* from citrus of various geographic origins [28]. ACT-toxin is released during the germination of conidia. It induces necrotic areas of the leaf blade and may be translocated to the vascular system inducing chlorosis and necrosis along the veins.

ABS is prevalent in citrus production areas with a Mediterranean climate, characterized by cool, humid winters and hot, arid summers. It was first reported on 'Emperor' mandarin in Australia in 1903, and subsequently it was detected in the Americas, the Mediterranean basin, South Africa, Iran and China affecting mainly 'Fortune' and 'Nova' mandarin hybrids [22, 29, 30]. In Europe, it has been reported in Greece, Italy and Spain. Its appearance in Italy coincided with the diffusion of the mandarin 'Fortune'. Warm temperatures and prolonged wetness are required for infection. However the disease causes severe epidemics in both humid areas and semi-arid regions provided a susceptible citrus variety is present. Fruits can get infected in all development stages but are more susceptible during the first four months following petal fall. Spring infections on young fruits may lead to premature fruit drop. Early fruit drop is common, especially if infection has occurred shortly after petal fall. Symptoms on fruits are necrotic brown circular lesions that may vary in size (**Figures 6** and **7**). Mature

Figure 6. Typical symptoms of Alternaria brown spot on 'Fortune' mandarin (courtesy S.O. Cacciola, G. Magnano di San Lio, A. Pane).

Figure 7. Alternaria brown spot on 'Nova' tangelo hybrid (courtesy S.O. Cacciola, G. Magnano di San Lio, A. Pane).

lesions have a corky appearance, and in older lesions, the centre may dislodge leaving tan-coloured pockmarks. Brown to black lesions surrounded by yellow halos and veinal necrosis appear on young leaves, which often are deformed due to necrosis of the margin. On highly susceptible cultivars abundant defoliation, abscission of young shoots and twig dieback may occur. Conditions of persistent humidity (fog or dew), which provide a wetting period of 8–12 h, are conducive for the development of infections; the optimum temperature is 20–27°C, but infections can occur between 17 and 32°C. The disease incubation period is 16–36 h. Conidia are produced on necrotic lesions in young leaves but not on fruits and are dispersed by air currents and rain splash. The presence of the disease is a limiting factor for the diffusion of highly susceptible mandarin or tangerine-like cultivars such as 'Fortune', 'Dancy', 'Minneola', 'Orlando', 'Nova', 'Guillermina', 'Clemenpons', 'Esbal', 'Page', 'Lee', 'Sunburst', 'Encore', 'Murcott', 'Michal', 'Winola', 'Ponkan', 'Emperor', 'Tangfang' and 'Primosole'. Even some varieties of pomelo are susceptible, while orange cultivars, with very few exceptions, are resistant.

Lemon and lime cultivars are not susceptible, with the exception of Mexican lime (*Citrus aurantifolia*) which is slightly susceptible.

Generally speaking, hybrids with 'Dancy' and 'King' mandarins as a parent are very susceptible. In many countries, such as Italy, Israel, Spain and the USA, ABS is a strong concern for triploid breeding programmes aiming at producing seedless mandarin cultivars. From diploid progeny analysis, it has been proposed that the inheritance of ABS susceptibility in citrus is controlled by a single gene with two alleles, one dominant (S) and the other recessive (r) which transmit susceptibility and resistance, respectively [31]. Therefore, resistant cultivars are considered to be recessive homozygous for this locus, whereas susceptible cultivars could be heterozygous or homozygous dominant. Cultivars like 'Minneola' and 'Dancy', which are homozygous (SS), transmit susceptibility to all the descendants. Most susceptible commercial cultivars like 'Fortune', 'Nova' and 'Murcott' are heterozygous, and both resistant and susceptible hybrids can be found in their progeny.

Resistant oranges, mandarins and clementines are recessive homozygous (rr), so when they breed with each other, all descendants are resistant. The single-locus dominant inheritance of susceptibility was corroborated by the analysis of triploid progenies. Recently, in Spain two new ABS-resistant hybrids of 'Fortune', 'Garbi' ('Murcott' × 'Fortune') and 'Safor' ('Kara' × 'Fortune') have been released.

Currently on susceptible cultivars, ABS control is based on the application of fungicides [32, 33]. Sprays must be scheduled to protect susceptible organs during the critical period for infection. Depending on the climate and the susceptibility of the cultivar, between four and ten fungicide sprays per year may be needed to produce quality fruit for the fresh market. On susceptible cultivars, foliar applications with copper fungicides are requested every 10–15 days in periods of high susceptibility. Despite this large number of sprays, disease control is not always satisfactory, and cultivation of very susceptible cultivars such as 'Fortune' in Mediterranean countries and 'Minneola' in Florida has declined significantly.

An integrated approach can reduce the risk of ABS infections and the disease severity [22]. In the nursery, it is recommended to grow susceptible citrus cultivars indoors, to avoid infections on young shoots and prevent inoculum dissemination in new commercial citrus plantings. New plantings of susceptible cultivars should be established in ventilated sites where environmental conditions are unfavourable for infections and sporulation of the causal agent on young leaves. Similarly, dense planting is not recommended for susceptible cultivars. Orchards of susceptible cultivars should be monitored frequently to detect the presence and prevent epidemic outbreaks of the disease.

4.2. *Septoria* spot

Septoria is a genus of plant pathogenic fungi with a wide geographic distribution, commonly associated with leaf spots and stem cankers of a broad range of host plants. Species of *Septoria* are among the most common and widespread leaf-spotting fungi worldwide. The causal agent of *Septoria* spot of citrus has been identified as *Septoria citri* [34]. This disease has been found in many citrus-producing countries of the Mediterranean basin, South Africa, South America, Australia (including Tasmania, Eastern and Southern Australia), India and California. Lemon (*C. limon*) and grapefruit (*C. paradisi*) are the most frequently damaged *Citrus* species worldwide, but all commercial citrus cultivars are susceptible. In Australia grapefruit, lemon and sweet orange (*C. sinensis*) 'Washington navel' are regarded as the most susceptible hosts. 'Valencia' oranges for juice production can also be affected although this cultivar is considered less susceptible than 'Washington Navel'. In California, Septoria spot affects 'Valencia' oranges, late-season navel oranges and occasionally lemons and grapefruits. It occurs in the San Joaquin Valley and interior districts of southern California during cool, moist weather. In Italy, *Septoria* spot has been reported on lemon, clementine and bergamot. Surprisingly, *S. citri* is a quarantine organism for Western Australia and South Korea. In 2004, Korean National Plant protection and Quarantine Service (NPQS) detected and rejected citrus fruits infected with Septoria spot imported from California. In most citrus-producing countries, *Septoria* spot is generally considered a disease of minor significance, except for fruit produced for the fresh market as rind blemishes reduce fruit quality aesthetically and affect saleability. Symptoms on fruit are small (1–2 mm in diameter), round, light tan-coloured lesions (pits) with a narrow green margin on the outer rind. As the fruit matures, they become reddish to pale brown (**Figure 8**) and contain small black spots (*S. citri* pycnidia) barely visible to the naked eye. When frost occurs or during storage fruit, lesions may enlarge (3–10 mm in diameter) and merge to form brown-to-black sunken blotches. These may be several centimetres in diameter and extend to the inner rind (albedo) and occasionally into the fruit segments. In severe infections, fruits develop an off flavour and drop prematurely. Symptoms may not appear until fruit is in storage. Leaf symptoms incited by *S. citri* are initially confined to the lower surface of the leaf and consist of small, blister-like brown to black spots, 1–4 mm in diameter, surrounded by a yellow halo. After leaf fall the spots turn brown with a dark margin and the pycnidia of the fungus form on necrotic tissues. Where under-canopy irrigation is used, infection may result in severe defoliation of the lower part of the tree (canopy skirt).

Septoria spot is more severe in years when rainfall levels are high and temperature fluctuates. The causal agent survives in infected orchards as a saprobe. Inoculum is constituted by pycnidia forming on dead twigs and leaves. Conidia, the infective propagules, are dispersed by water splash. Infections usually occur during cool, damp weather in late summer or autumn and when the fruits are still green. They may remain latent for up to six months, and fruit symptoms generally appear as the fruit starts ripening in late winter and early spring, after

Figure 8. Typical symptoms of *Septoria* spot on a lemon fruit (courtesy S.O. Cacciola and A. Pane).

cool, frosty or cold windy weather. The susceptibility of fruits is related to the maturity of the rind at the time of infection. Management practices to prevent or reduce the disease incidence and severity include tree skirting and canopy pruning to improve air circulation, early fruit harvesting and the removal of withered twigs from the tree canopy and fallen leaves from the soil under the tree canopy to reduce the amount of inoculum. Copper sprays in late fall or early winter to control fruit brown rot caused by *Phytophthora citrophthora* are also effective against *Septoria* spot.

The traditional taxonomy of *Septoria*, accommodating more than 2000 species, is confused as it has been based on few and conserved morphological characters. Moreover, it has been largely dependent on host data, and most species are not restricted to a single host. However, during the last years, the taxonomy of *Septoria* has been revisited using a polyphasic approach including both multilocus DNA sequencing and morphological characters. A more robust classification system is now available [35, 36]. In view of the worldwide distribution of *S. citri* and its status as a quarantine pathogen in some countries, it would be interesting to examine the genetic variability of *Septoria* populations associated to *Citrus* in different citrus-growing areas of the world in the frame of this new classification system.

4.3. Greasy spot and other cercosporoid diseases

Several species of cercosporoid fungi have been associated with leaf and fruit spot diseases of *Citrus* spp. Two of these diseases are particularly serious, Greasy spot, caused by *Zasmidium citri (Z. citri)-griseum* (sexual morph *Mycosphaerella citri*), and Phaeoramularia fruit and leaf spot, caused by *P. angolensis*, a fungus of quarantine concern for the European and Mediterranean Region.

Symptoms of greasy spot appear as yellow to dark brown to black lesions occurring first on the underside of mature citrus leaves. As the lesions develop on the underside of the leaves, they become darker, and a corresponding chlorotic spot appears on the upper leaf surface. Symptoms differ among citrus species. On highly susceptible species, such as lemon, spots are diffuse and tend to remain yellow, while on grapefruit, which is somewhat less susceptible, lesions are less diffuse, more raised and darker. On mandarins and 'Valencia' oranges, which are much more tolerant, lesions are smaller, brown to black and much more raised. Affected leaves fall prematurely from the tree during the fall and winter, resulting in reduced tree vigour and yield. Beside defoliation, the disease causes a rind blemish on fruit which has been referred to as greasy spot rind blotch. Greasy spot rind blotch significantly reduces the marketability of fruit for fresh consumption and is a serious problem especially on grapefruit but can also occur on oranges and other citrus. Greasy spot was first reported in Florida and is now endemic in all citrus-growing areas of the Caribbean Basin [37]. It also occurs in Texas but does not cause serious damage, probably because of a drier climate. Similar diseases of citrus have been observed in Argentina, Australia, China, Brazil, Egypt, Japan, Korea, Morocco, Spain and Italy. However the causal agent is not always *Z. citri-griseum* or has not identified. In a recent study, four *Zasmidium* species have been recognized on *Citrus*, namely, *Z. citri-griseum*, which has a worldwide distribution and a wide host range, and the three Asian species *Zasmidium fructicola*, *Zasmidium fructigenum* and *Zasmidium indonesianum* [38].

During the last years, an epidemic outbreak of a foliar disease closely resembling greasy spot has been observed in some citrus-growing areas of western Sicily (Italy). Symptoms appear on mature leaves and range from those typical of greasy spot (**Figures 9** and **10**) to black dots. Premature leaf drop occurs and causes heavy defoliation of the tree.

Figure 9. Symptoms of foliar greasy spot on the upper leaf surface of sweet orange (courtesy S.O. Cacciola and A. Pane).

Figure 10. Symptoms of foliar greasy spot on the lower leaf surface of sweet orange (courtesy S.O. Cacciola and A. Pane).

The analysis of the fungal community using an amplicon metagenomic approach has revealed that Mycosphaerellaceae were the dominant group of fungi, in both symptomatic and asymptomatic leaves, and were represented by the genera *Ramularia*, *Mycosphaerella* and *Septoria*, with about 44, 2.5 and 1.7% of the total detected sequences [39]. The most abundant sequence type was associated to *Ramularia brunnea*, a species originally described to cause leaf spot in a plant of the family Asteraceae. Surprisingly, none of the detected sequences clustered with reference species currently reported as possible causal agents of greasy spot. Results are not conclusive and the aetiology of this emerging disease is still unresolved.

5. Bleeding cankers caused by Botryosphaeriaceae and *Phomopsis/Diaporthe*

Botryosphaeriaceae and *Phomopsis/Diaporthe* spp. are known to cause cankers on a variety of woody hosts including citrus. Formerly this disease of citrus was known as Dothiorella canker or Dothiorella gummosis because the pathogens most often isolated were *Dothiorella* spp. However, recent studies have shown that the disease is caused by a complex of fungal species, of which the most common belong to the family Botryosphaeriaceae and to a lesser extent the genera *Phomopsis/Diaporthe*.

On citrus trees, cankers are found prevalently on trunk and main branches. The canker exudes a reddish gum, giving it a bleeding, water-soaked appearance. Symptoms may also include wilt of shoots and branches, sometimes with dead leaves still attached. Two types of fruiting bodies (perithecia and pycnidia) can be found on cankers, which are the sexual and asexual stage of these fungi, respectively. They produce the infective spores and appear as tiny black bumps protruding from the bark. Pycnidial spores that are far more frequently observed in nature than perithecial spores ooze out in a ribbon-like gelatinous matrix and are usually disseminated by rain splash. Botryosphaeriaceae and *Phomopsis*/*Diaporthe* gain entrance into the host through both wounds and natural gaps in bark continuity.

Species of Botryosphaeriaceae (*Botryosphaeriales*, *Dothideomycetes*) are cosmopolitan. Most of them have a wide host range. On citrus, like other fruit trees, Botryosphaeriaceae are found especially on stem and woody branches. In recent studies, the taxonomy of this group of fungi has been radically revised using multigene phylogenetic analysis, and presently the family comprises 22 recognized genera [40], including *Diplodia*, *Botryosphaeria*, *Neofusicoccum*, *Dothiorella*, *Neoscytalidium*, *Macrophomina*, *Lasiodiplodia* and *Sphaeropsis*, just to cite a few of them. Also the taxonomy of *Diaporthe* and its asexual morph *Phomopsis* has been revised on the basis of phylogenetic and molecular data, and new species associated as endophytes with citrus have been described [41, 42]. The report as a new disease of shoot blight, associated with sooty cankers and gummosis, caused by *Neoscytalidium dimidiatum* in top-worked 'Tarocco Scirè' trees on sour orange rootstock is an example of the radical change in classification system and nomenclature of these groups of fungi. The same disease, in fact, was already known as Hendersonula branch wilt as the causal agent was originally identified as *Hendersonula toruloidea* [43]. Botryosphaeriaceae and *Phomopsis*/*Diaporthe* spp. are associated with citrus not only as endophytes but also as saprobes as well as latent pathogens.

Biochemical and genetic stimuli, resulting from environmental changes inside the hosts (changes in host physiological conditions or microbial equilibrium) or outside the host (climatic changes or extreme environmental events), trigger these fungi to change their lifestyle from endophytic to pathogenic. Therefore these fungi can be regarded as opportunistic fungal pathogens, and the management of diseases they cause is based essentially on preventing environmental stresses. In particular, an agronomical means to prevent the disease is to avoid water stress by reducing the time intervals between irrigations. Surgical removal of infected bark does not restrict the expansion of cankers, and copper treatments are only partially effective to reduce the inoculum on the tree. They can be recommended to protect top-worked stumps and prevent shoot blight.

6. Wood rots

Wood rots are caused by a wide variety of wood-degrading microorganisms and are characterized by decay and discoloration of wood of the trunk, large branches and main roots. Most wood-degrading fungi are Basidiomycetes that on living trees can cause two major kinds of decay: brown and white rots. Although wood-decay fungi play an ecologically important role as primary biotic decomposers of wood in forest ecosystems, they can cause economic losses in cultivated orchards by contributing to the premature ageing and the structural failure of the trees. It can also affect young trees as a result of abiotic stress, such as severe frosts and sun burning of branches exposed by heavy pruning. Most wood-decay fungi penetrate through wounds, although a few of the root-infecting species can enter the unwounded surface directly.

Citrus wood rot is a chronic disease occurring endemically on old trees in most citrus-growing areas of the world. Although this disease is not a major constraint for the citrus industry, it can contribute to the deterioration of orchards because affected trees show a premature ageing, a progressive decline in vigour and reduced productivity. A direct effect of wood decay is the breakage of scaffold branches due to loss of wood strength. Moreover trees show symptoms of leaf chlorosis and twig dieback. The incidence of the disease is high in more than 40-year-old orchards, and its severity depends on environmental conditions and susceptibility of the citrus species and cultivars. In particular, lemon trees are significantly more susceptible to wood decay than other types of citrus, including orange, grapefruit and tangelo. In addition, the disease incidence seems to be correlated with the intensity and pattern of precipitations. As far as the Mediterranean region is concerned, *Fomitiporia mediterranea* (*F. mediterranea*) was found to be the most common white wood-rotting fungus of citrus [44–46], whereas *Fomitopsis* sp. was associated with brown rot [47]. Other nonidentified basidiomycetous fungi showing genetic affinity with *Phellinus* and *Coniophora* were occasionally recovered from decayed wood.

F. mediterranea is a ubiquitous and polyphagus species, commonly found also on other fruit, ornamental and forest tree species, such as hazelnut, olive, kiwi, locust tree and privet. On grape, it is associated to 'Esca' disease. It is assumed that most infections in citrus orchards originate from airborne basidiospores germinating on large pruning wounds of trunk and main branches. Basidiospores produced by basidiomata germinate with relative humidity over 90% and are dispersed by wind. Usually, basidiomata (**Figure 11**) emerge from the bark after the wood of trunk or branches has been extensively colonized by the fungus (**Figure 12**). In Southern Italy, the analysis of *F. mediterranea* internal transcribed spacer (ITS) sequences revealed a high level of genetic variability, with both homozygous and heterozygous

Figure 11. Symptoms of wood rot caused by *Fomitiporia mediterranea* on *Citrus* sp. (courtesy S.O. Cacciola and A. Pane).

Figure 12. Basidiocarp of *Fomitiporia mediterranea* (courtesy S.O. Cacciola and A. Pane).

genotypes. This and the high frequency of basidiomata in old commercial orchards confirm the outcrossing nature of reproduction in *F. mediterranea* and the primary role of basidiospores in the dissemination of inoculum. Prevention is the only way to manage wood rots. Trees should be kept healthy and vigorous. Large pruning cuts, or other wood-exposing injuries, should be avoided, especially during wet periods. Proper management guidelines include sanitation of pruning cuts with mastics to prevent the penetration of the pathogen.

7. *Phytophthora* diseases

The all-inclusive term "*Phytophthora* diseases" indicates a complex pathology which is caused by soilborne species of *Phytophthora* and is recognized as a major fungal disease of citrus almost universally. *Phytophthora* species attack citrus plants at all stages and may infect all parts of the tree, including roots, stem, branches, twigs, leaves and fruits. There are several forms (*facies*) of *Phytophthora* diseases including root rot, foot rot (**Figure 13**) (also known as *Phytophthora* gummosis, trunk gummosis or collar rot), fruit brown rot (**Figure 14**), twig and leaf dieback (often indicated collectively as canopy blight) and rot of seedlings (better known as damping off of seedlings). Trunk gummosis and root rot are the most serious *facies* of this group of diseases, and after the pandemic outbreak of the nineteenth century and the consequent widespread use of resistant rootstocks, they are regarded as endemic diseases in all citrus-growing areas of the world.

Figure 13. Trunk gummosis caused by *Phytophthora citrophthora* on a citrus tree (courtesy S.O. Cacciola and A. Pane).

At least ten species of *Phytophthora* have been reported to attack citrus in the world, but the commonest species in commercial citrus orchards of the Mediterranean region are *P. citrophthora* and *P. nicotianae* [48]. The latter is the dominant species and is usually associated to root rot, while *P. citrophthora* is frequent in old plantings and is commonly associated to trunk

Figure 14. Sweet orange fruit with symptoms of brown rot caused by *Phytophthora citrophthora* (courtesy S.O. Cacciola and A. Pane).

gummosis. Other species found occasionally include *Phytophthora citricola*, *Phytophthora cactorum*, *Phytophthora hibernalis* and *Phytophthora syringae*. The last two species are found during winter months solely because of their low-temperature requirements.

Recently, *Phytophthora meadii*, which is new for the Mediterranean region, was recorded in rhizosphere soil of potted ornamental citrus plants in southern Italy, using a very sensitive amplicon metagenomic approach with genus-specific primers [49, 50]. Both *P. citrophthora* and *P. nicotianae* are polyphagous.

Interestingly, however, genetic analyses of a worldwide collection of *P. nicotianae* isolates from different hosts, including agricultural crops and ornamentals, revealed that isolates from citrus clustered together and constituted a separate group regardless of their geographic origin [51–53]. This result is indicative of host specialization and suggests that *P. nicotianae* population associated to citrus has been very likely spread worldwide with infected nursery plants.

Temperature is a major ecological factor affecting seasonal fluctuations of *P. citrophthora* and *P. nicotianae* and their distribution. *P. nicotianae* prefers warmer temperatures, and in the Mediterranean region, it is not active in winter, producing chlamydospores which allow the pathogen to survive in unfavourable conditions. By contrast, *P. citrophthora* is not inhibited by low temperatures, and during fall and winter, it can cause brown rot outbreaks. Another epidemiological difference between the two species is the ability of *P. citrophthora* to produce sporangia on fruits, which are the sources of the secondary inoculum. This ecological feature explains sudden epidemic explosions of brown rot, following persistent rainfall. Neither of the two species forms sporangia on the gummy cankers at the base of the trunk. As far as it is known, *P. citrophthora* does not reproduce sexually, and *P. nicotianae* reproduces sexually only occasionally, since only A1 mating type is found in most citrus orchards.

Sporangia produced in the most superficial soil layer (0 to about 30 cm depth), on contact with air, are the main source of inoculum. Natural infections are most frequently caused by zoospores and occasionally by direct or indirect germination of sporangia through a germ tube or by releasing zoospores, respectively. Production and germination of sporangia are influenced by temperature and soil water potential. Their dissemination is mostly by water splash and occasionally by wind, within water droplets. The zoospores are motile and can swim short distances by flagellar movement or can be carried over longer distances by soil water. They swim towards roots, as they are attracted by root exudates, and encyst upon contact, germinate and penetrate fruits, leaves, shoots and green twigs directly.

Grafting on resistant rootstocks, such as sour orange, is the most practical and widely used means to control *Phytophthora* gummosis. The rootstocks that are substituting sour orange in the Mediterranean region, following the epidemic spread of *Citrus tristeza virus* (CTV), to which sour orange is very susceptible, are mainly citranges, hybrids of trifoliate orange (*P. trifoliata*) and sweet oranges, such as 'Troyer', 'Carrizo' and 'C-35' citranges. Generally speaking, rootstocks resistant to *P. citrophthora* and *Phytophthora* gummosis are also resistant to *P. nicotianae* and root rot, with few exceptions (e.g. 'Carrizo' citrange is resistant to *Phytophthora* gummosis but susceptible to root rot, while trifoliate orange is resistant to both *facies* of the disease).

Brown rot is both a preharvest and postharvest decay of citrus fruit. Infected fruit shows a typical leathery brown rot with indistinct edges and has a characteristic rancid smell. With high moisture in the environment, white furry mould forms on the fruit surface. Infections cause the fruit to drop prematurely and occur especially on fruits hanging in the lower part of the tree canopy, with rain splash. Epidemic explosions are more frequent in citrus orchards where trunk gummosis is endemic. The incubation period of the disease is 7–10 days at 10°C but may be longer with lower temperatures. Asymptomatic infected fruits can infect healthy fruits even after harvesting, during transportation and storage.

Two major breakthroughs in the implementation of integrated management strategies of *Phytophthora* diseases were the launch of systemic fungicides with specific activity against Oomycetes [54–58] and the development of selective media for direct isolation of *Phytophthora* spp. from soil and infected tissues. Serial dilutions of soil suspensions on a selective medium became a very popular method for monitoring the amount of *Phytophthora* inoculum in the soil. Monitoring was used to study seasonal fluctuations of *Phytophthora* populations to manage the irrigation as well as to schedule chemical treatments and evaluate their effects [59–62]. The rationale of these strategies is the assumption that a direct correlation exists between the amount of inoculum and the incidence and severity of root rot. Although a very sensitive molecular method based on real-time PCR with specifc primers was developed for the detection of *P. citrophthora and P. nicotianae* in soil [63], soil dilution on a selective medium in Petri dishes still remains the preferred and most widely used standard method for quantitative determination of *Phytophthora* inoculum. The quantity of inoculum determined with this microbiological method is expressed in terms of colony-forming units (CFU)/g or cm^3 of soil. The threshold intervention level in bearing citrus orchards is between 10 and 30 CFU/cm^3 of soil, but ideally a zero-tolerance threshold would be requested for nursery stocks to be sold. More details on *Phytophthora* diseases may be found in many comprehensive reviews [48, 64–66].

8. Concluding remarks

Over the last decade, there have been a number of publications dealing with molecular studies on fungal pathogens of citrus, focusing particularly on their identification and genetic diversity. In particular, new species of *Colletotrichum*, Botryosphaeriaceae and Mycosphaerellaceae have been described, and significant progress towards a new, unified phylogenetic classification system of these groups of fungi has been achieved, for which substantial advances were obtained by multigene sequencing and phylogenetic analysis. However, there have been fewer publications related to other molecular aspects such as the mechanisms underlying the host-pathogen interactions. With the development of next-generation sequencing techniques and the availability of whole-genome sequences, molecular studies are expected to get insight into the biology, pathogenicity and ecology of well-identified pathogens as well as the aetiology of diseases whose causal agent is still undetermined. Moreover, diversity studies will provide epidemiological information such as distribution, virulence and genetic structure of pathogen populations. The results of preliminary applications of these new molecular techniques to the study of citrus diseases, such as *Phytophthora* diseases and greasy spot-like diseases, are very promising.

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Chapter 2

Spiroplasma spp.: A Plant, Arthropod, Animal and Human Pathogen

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Additional information is available at the end of the chapter

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Abstract

Mollicutes is a class of smallest and free-living bacteria. They have no cell wall and their plasma membrane contains cholesterol; nevertheless, cellular organization does not differ from that of other prokaryotes. They are used as simple model systems for studying general biological problems, such as those concerning membrane structure and functions, symbiosis between arthropods and microrganisms, animal and plant pathogens. Mollicutes includes the family of *Spiroplamataceae*, which contains *Spiroplasma* genus, a group of species associated, in different manner, with arthropods (insects, mites, crustaceans). *Spiroplasma* species can be commensals or parasites and even be involved in more close symbiosis, such as synergism or mutualism. Out of 38 described *Spiroplasma* species, only three have been associated with plant diseases and three with arthropod diseases. Moreover, some species have been related to animal diseases, such as transmissible spongiform encephalopathy (TSE), and their role in human disease has been assessed. The chapter describes the taxonomic situation of the genus and reports the most important diseases due to the presence of *Spiroplasma* in different living organisms with special emphasis on citrus in which it causes one of the most economically damaging infectious diseases in a number of citrus growing areas worldwide.

Keywords: *Spiroplasma*, citrus, disease, crab, transmissible spongiform encephalopathy, insect vector

1. Introduction

Mollicutes is a class of microorganisms composed by the smallest and free-living bacteria, which have no cell wall. Their cellular organization does not differ from that of other prokaryotes: plasma membrane, which, however, contains cholesterol, cytoplasm, and ribosomes;

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their metabolic pathways are simpler than those of other eubacteria and their DNA has a low GC content. The cell biology of these organisms is interesting to many researchers who use *Mollicutes* as simple model systems for studying general biological problems, such as those concerning membrane structure and functions, symbiosis between arthropods and microorganisms, animal and plant pathogens. The genus *Spiroplasma* has arthropods as a peculiar host. *Spiroplasma* species (spiroplasmas) have developed different types of symbiosis with insects and mites, and, as recently shown, with crustaceans. Among the 38 described *Spiroplasma* species, only three have been associated with plant diseases. In this chapter, the relevance of the spiroplasma diseases in citrus is described together with some relevant diseases reported as associated with the presence of spiroplasma in other living organisms, such as arthropods, animals, and humans.

2. Methods

Bibliographical sources have been obtained through a PubMed search, integrating the bibliographic citations from PMC. The used keywords to select bibliographic items were *Spiroplasma*, *Spiroplasma* pathogenesis, *Spiroplasma*, and human infections, *Spiroplasma* taxonomy. As of today, there are 709 bibliographic entries, since 1973, in PubMed and 1140 citations in PMC, since 1981. This discrepancy is due to the fact that PMC also includes sources from the conference proceedings, books, etc., etc. A literature search on *Spiroplasma citri*, *Spiroplasma kunkelii*, and *Spiroplasma phoeniceum* was conducted on the CAB Abstract and web-based search engines, such as Google Scholar. Further references and information were obtained from experts, citations within the references, as well as from gray literature.

3. Classification and taxonomy

The spiroplasmas belong to the Class of *Mollicutes*, Order *Entomoplasmatales*, Family *Spiroplasmataceae*, and Genus *Spiroplasma* [1]. Species of officially recognized *Spiroplasma* are shown in **Table 1**. Most species have mandatory relationships with insects, with whom they develop different types of symbiosis [37, 38], while a number species were reported to be pathogenic for animals, arthropods, and plants and a few has been involved in human diseases.

4. Pathogenetic mechanisms and interactions with insect vectors

Spiroplasma species are mainly transmitted to plants by specific insect vectors; in order to achieve the transmission, they must cross the salivary gland barrier [39]. *S. citri* and *S. kunkelii* invade the hemocoel through the gut epithelium of the insect host by a process of receptor-mediated cell endocytosis [40]. Receptors on leafhopper gut epithelial cells likely recognize specific spiroplasma membrane proteins. Several candidate *S. citri* attachment protein genes have been studied, including spiralin (immunodominant membrane protein) [41], P58 [42], SARP1 [43], P32 of pSci6 plasmid [44–46], and phosphoglycerate kinase (PGK) [47]. It has

Species	**Host species**	**Geographic distribution**	**Authors**
Spiroplasma alleghenense	Common scorpion fly *(Panorpa helena)*	USA (WV)	[3]
Spiroplasma apis	Honey-bee (*Apis mellifera*)	France	[4]
Spiroplasma atrichopogonis	Biting midge (*Atrichopogon* spp.)	USA (MD)	[5]
Spiroplasma cantharicola	Soldier beetle (*Cantharis carulinus*)	USA (MD)	[6]
Spiroplasma chinense	False bindweed *(Calystegia hederacea)*	China (Jiangsu)	[7]
Spiroplasma chrysopicola	Deerfly (*Chrysops* sp.)	USA (MD)	[8]
Spiroplasma citri	*Citrus* spp.	USA	[2]
Spiroplasma clarkii	Green June beetle (*Cotinus nitida*)	USA (MD)	[9]
Spiroplasma corruscae	Lampyrid beetle *(Ellychnia corrusca)*	USA (MD)	[10]
Spiroplasma culicicola	Salt marsh mosquito (*Aedes sollicitans*)	Worldwide	[11]
Spiroplasma diabroticae	Corn rootworm *(Diabrotica undecimpunctata*)	USA (MD)	[12]
Spiroplasma diminutum	Mosquito (*Culex annulus*)	Taiwan	[13]
Spiroplasma eriocheiris	Chinese mitten crab (*Eriocheir sinesis*)	China	[14]
Spiroplasma floricola	Tulip tree (*Liriodendron tulipifera*)	USA	[15]
Spiroplasma gladiatoris	Maryland horsefly *(Tabanus gladiator*)	USA (MD)	[7]
Spiroplasma helicoids	Horseflies (*Tabanus abdominalis-limbatinevris*)	USA (MD)	[7]
Spiroplasma insolitum	Fall flower (*Bidens* sp.)	USA (MD)	[16]
Spiroplasma ixodetis	Black-legged ticks (*Ixodes pacificus*)	USA (OR)	[17]
Spiroplasma kunkelii	Corn (*Zea mays*)	America	[18]
Spiroplasma lampyridicola	Firefly beetle *(Photuris pennsylvanicus)*	USA (MD)	[19]
Spiroplasma leptinotarsae	Colorado potato beetle (*Leptinotarsa decemlineata*)	USA (MD)	[20]
Spiroplasma leucomae	Satin moth larvae, (*Leucoma salicis*)	Poland	[21]
Spiroplasma lineolae	Striped horsefly (*Tabanus lineola*)	USA (GE)	[22]
Spiroplasma litorale	Horsefly (*Tabanus nigrovittatus*)	USA (NC)	[23]
Spiroplasma melliferum	Honey bee *(Apis mellifera)*	worldwide	[24]
Spiroplasma mirum	Rabbit ticks (*Haemaphysalis leporispalustris)*	USA (GE, MD)	[25]

Species	Host species	Geographic distribution	Authors
Spiroplasma monobiae	Vespid wasp (*Monobia quadridens*)	USA (MD)	[26]
Spiroplasma montanense	Tabanid fly (*Hybomitra opaca*)	USA (MN)	[27]
Spiroplasma penaei	Pacific white shrimp (*Penaeus vannamei*)	Colombia	[28]
Spiroplasma phoeniceum	Periwinkle *(Catharanthus roseus)*	Syria	[29]
Spiroplasma platyhelix	Dragonfly (*Pachydiplax longipennis*)	USA (MD)	[30]
Spiroplasma poulsonii	Fruit fly (*Drosophila willistonii*)	South America	[31]
Spiroplasma sabaudiense	Mosquitoes (*Aedes stricticus, Aedes vexans*)	France	[32]
Spiroplasma syrphidicola	Syrphid fly (*Eristalis arbustorum*)	Unknown	[33]
Spiroplasma tabanidicola	Horsefly *(Tabanus abactur)*	USA (OK)	[7]
Spiroplasma taiwanese	Mosquitoes *(Culex tritueniorhynchus*)	Taiwan	[34]
Spiroplasma turonicum	Fly (*Haematopota* sp.)	France	[35]
Spiroplasma velocicrescens	Vespid wasp (*Monobia quadridens*)	USA (MD)	[36]

Table 1. *Spiroplasma* species reported in literature after the genus description by Saglio et al. [2] (in bold strains recognized as pathogenic).

been reported that a spiralin mutant is less effectively transmitted [41]; moreover, the spiralin binds to glycoproteins of its insect vector [48] and it is, therefore, a candidate molecule for insect vector specificity. It was also shown that *S. citri* phosphoglycerate kinase (PGK), a glycolytic enzyme, could bind to actin of its leafhopper vector for the internalization of *S. citri* into leafhopper cells [47]. In addition, the PGK protein or partial PGK peptides were shown to inhibit spiroplasma transmission by leafhoppers [49].

A relationship between plasmids of *S. citri* and insect-transmissibility has been demonstrated for spiroplasmas [44–51]. The plasmid pSci6 confers insect transmissibility to a nontransmissible strain of *S. citri* [44] encoding protein *P32*; however, when only the p32 gene was introduced into the nontransmissible strain of *S. citri*, its insect transmissibility was not restored; therefore, pSci6-encoded determinants other than P32 might be essential for insect transmission [46]. There is no report of transmission through seeds from infected plants [52].

The mechanisms by which spiroplasmas cause disease in plants are poorly understood, and the genetic determinants involved, are unknown. For *S. citri* toxins and lactic acid production seems to play a role in the disease development; spiroplasmas cause also a shortage of auxins, most probably, due to the utilization of sterols [53]. In particular, it was shown that the pathogenicity of *S. citri*, i.e., the ability to induce diseases, correlates with its ability to use fructose [54], indicating that the sugar metabolism is certainly an important factor in the relationships of *S. citri* with its two hosts, the plant and the leafhopper vector. Indeed, carbohydrate partitioning was shown to be impaired in infected plants [55].

5. Diagnosis and epidemiology

Citrus stubborn disease (CSD) caused by *S. citri*, is a relevant threat to the citrus industry in several citrus-growing countries, such as California and Cyprus; however, the knowledge of its epidemiology is mostly anecdotal and in most cases diagnosis was only based on symptoms; in particular on the acorn-shaped fruits. The pathogen is graft-transmissible and vectored by leafhoppers in a persistent and propagative manner [56]. Field diagnosis of CSD is difficult because foliar symptoms can resemble nutritional deficiencies or symptoms induced by other phloem-restricted pathogens, such as, the huanglongbing agents ('*Candidatus* Liberibacter' species). Moreover, symptoms of CSD can vary with season, citrus cultivar, and disease severity. The isolation and *in vitro* culture of *S. citri* are time consuming and technically demanding since it is typically low in concentration and unevenly distributed in citrus tissues, making its reliable detection challenging. Currently, the preferred detection methods are based on polymerase chain reaction (PCR) assays with primers developed from sequences of *S. citri* housekeeping genes [57]. Recent genome sequencing revealed that the bacterium harbors multiple copies of prophage genes; therefore, it was hypothesized that targeting these genes could improve the sensitivity of PCR detection. Several different pathogen detection protocols have been optimized to evaluate the pathogen presence in commercial groves and assess its impact on fruit quality and yield. PCR and quantitative PCR targeting multicopy genes *P89* and *P58* are currently the most sensitive detection systems [54]. Recently, a rapid serological method based on the detection of a protein secreted by *S. citri* has been developed and proven to be as efficient as quantitative PCR [58].

Genetic diversity among *S. citri* strains has been observed; however, in recent surveys, no correlation has been observed between symptom severity and genotypes. Findings suggest that CSD incidence in commercial groves in California could be as high as more than 80% and its impact on yield and fruit quality is significant. The principal economic hosts of *S. citri* are susceptible *Citrus* species, including the major commercial species in the Mediterranean area: grapefruits (*C. paradisi*), lemons (*C. limon*), mandarins (*C. reticulata*), orange (*C. sinensis*), and sour orange (*C. aurantium*). Other citrus hosts are *C. grandis, C. limettioides, C. limonia, C. madurensis*, rough lemons (*C. jambhiri*), satsumas (*C. unshiu*), and tangelos (*C. paradisi* × *reticulata*) [59]. Other rutaceous hosts include *Fortunella* species and interspecific hybrid rootstock, such as citrange (*C. sinensis* × *Poncirus trifoliata*); however, they are considered minor or incidental hosts. Some forms are symptomlessly infected including *P. trifoliat*a [60]. Many other cultivated or wild plants have been found to be naturally infected in South-Western USA. *S. citri* causes a specific disease (brittle root) of horseradish (*Armoracia rusticana*) in Eastern USA.

S. citri is known to be transmitted by seven species of leafhoppers (Cicadellidae Deltocephalinae). In California (USA), its main natural vectors are the leafhoppers *Circulifer tenellus*, *Scaphytopius nitridus*, and *Scaphytopius acutus* [61]. In the Mediterranean area, it is reported to be transmitted by *Neoaliturus haematoceps* [62] and *C. tenellus* [63]. Other

Homoptera may acquire the spiroplasma, but not transmit it [64, 65]. In North America, the distribution of *S. citri* follows rather closely that of *C. tenellus* (primarily a sugarbeet insect). In the Mediterranean area, insects known as vectors are present practically wherever citrus is grown, so that their presence does not appear to be a limiting factor in the spread of CSD to new areas. Spiroplasma develops best in citrus under hot conditions (28–32°C) and may not induce conspicuous symptoms at lower temperatures. Annual plants experimentally infected are rapidly killed at temperatures over 30°C, but may recover at lower temperatures [62].

C. tenellus and *N. haematoceps* are the Mediterranean vectors of stubborn disease and they feed on a wide range of host plants, such as weeds, ornamental plants, and crops. *N. haematoceps* has been found particularly on the ornamental *Matthiola incana* and on wild plants, *M. sinuata* and *Salsola kali* [66]. Neither insect is particularly associated with citrus, on which feeding is incidental. Oldfield et al. [67] reported that *C. tenellus* could not be reared on citrus as sole host. Klein and Raccah [68] reported the presence of two *N. haematoceps* populations in Israel, one polyphagous and the other host-specific. Although *S. citri* naturally infects many other hosts, it is not reported to have any economic impact on those. Their main significance would be as reservoirs of *S. citri* for infection of citrus. Horseradish brittle root is of purely anecdotal interest.

S. kunkelii is transmitted by leafhoppers in the field, mainly by *Dalbulus maidis* (Homoptera: Cicadellidae) that is a subtropical species which occurs throughout the Americas and causes severe production losses in corn cultivation. *S. phoenicium* was only experimentally transmitted from cultures by *Macrosteles fascifrons* on aster and periwinkle plants.

6. Plant diseases

Only three *Spiroplasma* species are reported as agents of plant diseases: *S. citri*, *S. kunkelii*, and *S. phoeniceum*.

Citrus stubborn disease (CSD) was first detected in California, and it is a widespread bacterial disease caused by *S. citri*, mostly found in arid areas of the United States and the Mediterranean Region, where citrus is mainly produced. It causes quite relevant loss in both fruit production and quality. Affected trees are stunted and quite often flat topped, because the stems have shortened internodes and undersized leaves showing mottle and chlorosis. Most fruits drop while very small. The few fruits that could reach maturity are misshapen and abnormally matured with aborted seed. The pathogen induces symptoms by consuming the sap fructose produced through sucrose hydrolysis by the companion cells of phloem [69]. *S. citri* had been identified as a pathogen of several citrus species as above-mentioned and it has a wide plant host range than that of citrus, since it is transmitted by polyphagous leafhoppers. In addition to naturally infected plant hosts, several experimental plant hosts have been infected through the forced inoculation with leafhopper vectors. The disease is considered to be absent in Europe except in the Mediterranean area and it is quite severe in areas where the climate is hot and arid. Only mild symptoms, if any, are recorded where the field temperature does not exceed 28°C. Presently, *S. citri* is a quality pathogen in the European and Mediterranean

Regions, however, it is listed as an harmful organism in Council Directive 2000/29/EC; in addition, the Directive also considers some of its host plants and insect vectors [70].

Corn stunt (CSS) [71] is one of the major diseases of maize (*Zea mays*) in the Americas (CABI Crop Protection Compendium). It was reported that the disease caused in Tucuman province in Argentina severe damages that resulted in reduction of the yield ranging from 50% to 90% (with an average of 70%) with infected plants producing grains with weight less than 3 times. In the USA, the disease was considered sporadic, although it was observed in the California central valley every year, until recently. In particular, in 2001, there was an outbreak with losses of more than 5 million USD. Although CSS is due to a complex of pathogens, *Spiroplasma kunkelii* (**Table 1**) seems to play an important role in the etiology and epidemiology of the disease. *S. kunkelii* is transmitted by leafhoppers mainly *Dalbulus maydi*s (Homoptera: Cicadellidae), a subtropical species which occurs throughout the Americas in all the maize growing areas [72]. The same leafhopper transmits maize bushy stunt phytoplasma (MBSP) and *Maize rayado fino virus* (MRFV). *S. kunkelii* has also been detected in teosintes (e.g., *Euchlaena amexicana, Z. perennis*) [73]. The insect vector, *D. maydis,* feeds on cultivated or wild species of the genus *Zea* and occasionally on species of the closely related genus *Tripsacum*. CSS has been detected in maize crop in Brazil, mainly in the second maize growing season at the warm areas [69]. The mollicute-infected leafhoppers migrate from diseased maize crop fields and infect maize seedlings in other places. CSS symptoms appear after the maize flowering, and are usually leaf reddening, shortness of the internodes, and small ears, with small or a few grains, depending on the cultivar [74]. Based only on the foliar symptoms, the CSS can be identified by the presence in the leaves of chlorotic streaks, almost of white color, that extend from the leaf base to the leaf apex. However, these typical and diagnostic streaks in the leaves are not frequently present, and so, it is difficult or impossible to distinguish the CSS from the MBSP symptoms, at field. The spiroplasma and the phytoplasma can be detected and distinguished mainly by PCR tests. The management efficiency of the CSS through the insecticide control of *D. maydis* leafhopper is limited, since this insect vector can infect the maize seedling before its death. The disease management can be achieved by the use of resistant maize cultivars, associated to the insecticide seed treatment for *D. maydi*s control, together with the choice of best month for the maize sowing. There is little information about the genetic control of the maize resistance to corn stunt *spiroplasma*, although some studies carried out in field indicate that additive and nonadditive effects are present in the maize resistance inheritance to this disease [75]. *S. kunkeli*i appears to be pathogenic also to its insect vectors, in particular it is able to shorten the life of *D. maidis* [76, 77]. Moreover, *S. kunkelii* appears not to be seed transmitted, while the aster yellows phytoplasmas associated with the disease resulted recently to be seed transmitted [78].

Spiroplasma phoeniceum was found to be the causal agent of a periwinkle yellows disease (PYD) and described as a new species of *Spiroplasma* in Syria, in the vicinity of orchards with high incidence of CSD, during a survey aimed at demonstrating the natural spread of the latter disease [29]. Infected periwinkle plants showed yellowing symptoms that were indistinguishable from those associated to infections that were associated with phytoplasma presence or due to *S. citri*. The report of this disease has remained anecdotic.

7. Arthropod diseases

Severe mortalities in the marine shrimp *Penaeus vannamei* were reported in early 2002 in one pond at a shrimp farm in the Colombian Caribbean coast. During May-June 2002, two other shrimp ponds at the same farm also experienced very high mortalities. *S. panaei* was then identified as the causative agent of the outbreak and spread during the next grow-out cycle to a neighboring farm, which suffered mortalities ranging from 10 to 90% [27]. Earlier reports on Chinese mitten crab (*Eriocheir sinensis*) showing a tremor disease (TD) resulted to be infected by *S. eriocheiris* by molecular testing, supporting sever epizootic outbreak mainly in aquaculture [79]. A strain isolated from the hemolymph of a Chinese mitten crab with tremor exhibited a predilection for muscle, nerve, and connective tissues and was found to be transported to various tissues and organs by haemocytes. The pathologic features seen in experimentally infected crabs were similar to those observed in naturally occurring infected crabs [14].

In natural populations of some neotropical species of *Drosophila, s*ingle females were found whose progenies consisted of only daughters [80]. After demonstration that this sex-ratio trait was maternally (vertically) inherited and infectious, with the electron microscopic examination of fixed and negatively stained cells, microorganisms similar to spiroplasmas, were observed. The microorganisms were subsequently grown in culture characterized at the serological level and named *S. poulsoni* [31].

8. Human diseases

In 2002, Lorenz et al. reported the first case of human infection due to *Spiroplasma* in a female child, born prematurely, who was diagnosed with a unilateral cataract associated with anterior uveitis [81]. The microscopic examination, cultures for fungi, aerobic and anaerobic bacteria were negative, as well as molecular tests for the *Toxoplasma gondii*, *Herpes simplex virus* (HSV), and *Varicella-Zoster virus* (VZV). Similarly, the serological tests for HSV-1 and HSV-2, CMV, and VZV were negative. By using PCR and TEM, the presence of *Spiroplasma* spp. was confirmed; however, its speciation was not done.

Two additional cases of human infections due to *Spiroplasma* were reported in 2015 [81, 82]. Both patients had a deficiency of the immune system due to hypogammaglobulinaemia or to pharmacologic immunosuppression, after transplantation. In the first case, a patient with rheumatoid arthritis and hypogammaglobulinaemia presented on admission, clinical signs of infection, no signs of autoimmune disease, and negative tumor makers [82]. The only sign of bacterial infection was a positive blood culture, but the bacteriological examination after Gram staining was negative. The presence of beta hemolysis in blood agar subculture, in the absence of visible bacterial growth, was suggestive for the presence of *Mollicutes*. Out of all the tested media, only the subcultures on Agar A7 [83], a specific medium for *Mollicutes*, showed growth. PCR amplification of the 16S rDNA and sequencing, have demonstrated the presence of a bacterium with high homology to *Spiroplasma turonicum*. The infection has been resolved with doxycycline and levofloxacin therapy. The second case was represented by another transplanted patient hospitalized with signs of liver disease, a diagnosis confirmed by positron emission tomography (PET) [84]. Serological tests for classical agents responsible for liver diseases were negative. Similarly, the parasitological examination of stools was negative as well. The initial therapy, pipera-tazobactam, was replaced by meropenem because of no result. Molecular tests for the detection of 16S rDNA and sequencing, both from blood and liver samples, showed the presence of *Spiroplasma ixodetes*. The therapy with azithromycin and doxycycline led to healing.

9. *Spiroplasma* and transmissible spongiform encephalopathy (TSE)

By entering the query "Spiroplasma and encephalopathy" or "Spiroplasma ad TSE", you get more than 30 citations, in PubMed [85–117]. It is surprising that the first paper dates back to 1979. In fact, in the late 1979, Bastian described membranous structure very similar to *Spiroplasma* spp. cells by the electron microscopy observation, in a brain biopsy from a man with Creutzfeldt-Jakob disease (CJD). Author's conclusion suggested a concurrence of the microorganism with such a disease [85]. One year later, another paper was published, in which, the previous described membranous structures with a spiral shape were found by using the electron microscopy, in axoplasm of brain cells, obtained from a biopsy in a patient with suspected CJD [86]. Two other cases of patients suffering from CJD, in which, brain biopsies showed membranous spiral inclusions resembling spiroplasma cells were reported by Reyes and Hoenig in 1981 [87]. In the same year, Bastian et al. reported other histological evidence of the presence of spiroplasma cells in the brain tissue biopsy from another patient suffering from CJD [88].

An experimental study done on newborn rabbits infected with the *Spiroplasma* spp. responsible for the suckling mouse cataract (SMCA) showed that intracerebral inoculation provoked hemorrhages in the brain, conversely subcutaneous inoculation did not induce any diseases [87]. The intracerebral inoculation of *Spiroplasma* SMCA in newborn Syrian hamster induced severe cerebral damages and death, although adult animals did not develop the disease [90]. The attempt of Leach et al. to isolate either spiroplasmas or mycoplasmas from the brain tissue of 18 patients with CJD was negative; likewise, the detection of antibodies in 15 patients with CJD did not show any positivity [91].

Spiroplasma mirum GT-48 strain has been the subject of two studies on experimental animals conducted by Tully et al. [92] and by Bastian et al. [93], respectively. In the first study, it has been shown that the organism spread and multiplied rapidly in the brain; in the second study, spiraling and membranous inclusions in brain cells similar to those described by the electron microscopy in patients with CJD were observed [85–88]. At the end of 1980s, two experimental studies have investigated the pathogenesis of *S. mirum*. In the first, *S. mirum* has been shown to cause a persistent infection of the brain in the suckling rat. Furthermore, the tropism of this microorganism for the brain tissues was correlated to the presence of sterols, which are necessary for its growth [94]. In the second study, it was observed, by western blot, that antibodies to the fibrils associated with Scrapie interacted with either the brain tissue of patients with CJD either with fibrillar proteins resistant to proteases obtained from *S. mirum* [95].

As previously suggested by Leach et al. [91], even Connolly et al. [96] did not consider that there is a correlation between *Spiroplasma* spp. and CJD, since in their electron microscopy observations they did not found no structures similar to *Spiroplasma* spp. Humphery-Smith and Chastel, whereas have claimed that the results of Gray et al [86] were due to artifacts, reported the finding of *S. sabaudiense* antibodies in a patient with amyotrophic lateral sclerosis and in a patient of the control group [97, 98]. Bastian and Foster by using PCR specific primer for *Spiroplasma* genus and sequencing have investigated the brain tissue from necroscopy of 13 patients suffering from CJD, as well as, 9 scrapie cases, and 50 controls. *Spiroplasma* DNA was detected in 13/13 CJD patients, in 5 out of 9 scrapie brains, but in none of 50 control cases [99]. Successively, 16S rDNA with a homology greater than 99% with *S. mirum* was recovered in most of (>80%) the experimental animals investigated; moreover, 16S rDNA was recovered in two human brains with CJD but not in the control [100]. Moreover, Bastian et al. have shown that there is an association between *Spiroplasma* spp. and scrapie by using an experimental model of sheep suffering from scrapie [101]. Alexeeva et al. in an experimental study on hamster have ruled that either *S. mirum* or other bacteria cannot be considered as causative agents of TSE [102]. Experimental studies have demonstrated that *S. mirum* did not induce TSE-like disease in raccoons [103] and recently suggested that the ability to form biofilms in *Spiroplasma* spp. is the basis of the spread and of the pathogenesis of diseases caused by this *Spiroplasma* [104].

We are far from having clarified the role of *Spiroplasma* in TSE. To date, despite Bastian et al. studies and studies of other authors, there are still many doubts about the role these microorganisms can play in TSE. Probably, in the years to come, more extensive studies on molecular biology and serology may give rise to some answers.

10. Conclusions

CSD and CSS are the only two diseases caused by spiroplasmas of relevant economic importance on a world scale. Although, CSD cause serious damages to the citrus industry in North America and seems widespread in most citrus-producing areas, including North Africa, the Mediterranean basin, and the Middle-East, information on its distribution and incidence, as well as, its impact in citrus orchards are still limited. Routine molecular detection methods have been developed for the identification of *S. citri*; however, robust diagnostic methods remain challenging and most of the reports about this pathogen are not reliable because based only on symptoms. Recently, the European Food Safety Authority (EFSA) has reconsidered the literature on *S. citri* and confirmed that it should be categorized as an harmful organism for the citrus industry in the Mediterranean Region.

In 2008, as the importance of CSS was increasing in several American countries, S. *kunkelii* was added to the EPPO Alert List and it has been included in this list for more than 3 years. During this period, no particular International action was requested by the EPPO member countries and in 2012, it was therefore considered that sufficient alert has been given and the pest was deleted from the list.

Human infections due to *Spiroplasma* spp. are, probably, a new clinical reality: their role as opportunistic pathogens should not be undervalued. Moreover, despite the many evidences on the possible involvement of spiroplasmas in TSE, more studies are needed to definitively associate these bacteria with such a disease.

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Section 2

Host-Pathogen Interactions

Metabolic Changes during Defense Responses against Wound Stresses in Citrus Plants

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Additional information is available at the end of the chapter

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Abstract

Citrus plants are well known as a rich source of functional chemicals; however, metabolites involved in defense responses against environmental stresses are not yet well understood. Among environmental stresses, mechanical wounding is a continuous threat toward the growth and survival of plants. Recent advances in analytical technology and informatics enable comprehensive analysis of primary and secondary metabolites. In this chapter, metabolic profiling of leaf metabolites in seven *Citrus* species during responses against wound stress as well as defense-related phytohormone treatments was described. Moreover, we discussed current metabolomic techniques, application of these techniques to researches on *Citrus* defense responses and metabolic profiling-oriented identification of novel compounds.

Keywords: defense response, wound stress, metabolic changes, GC/MS, *metabolomics*

1. Introduction

Wound stresses such as mechanical injury and herbivore feeding are unavoidable and continuous threats to growth and survival of plants. Damaged tissue allows pathogen invasion and leads to spread of disease into whole plant. Higher plants have evolved defense mechanisms against such attack of natural enemies. For instance, plants accumulate wound-healing compounds such as suberin in response to wounding [1] and prepare defense chemicals including repellents and toxins as well as physical defense reaction such as cell wall reinforcement. Moreover, it has been reported that phytoalexins, antimicrobial compounds produced by plants in response to pathogen infections, are accumulated after wounding [2].

Citrus family is one of the most commercially important horticultural plants and cultivated all over the world. Moreover, *Citrus* fruits have been well known as rich sources of bioactive compounds which exhibit pharmacological activities such as antioxidant, antimicrobial, antitumor and anti-inflammatory activities [3]. Despite well-studied pharmacological properties of this plant family, knowledge about physiological and biological properties during defense responses against environmental stresses including mechanical injury is quite limited. *Citrus* plants are highly diverse, and their taxonomy and phylogeny are very complex and confusing because of asexual seed reproduction and sexual compatibility between *Citrus* and related genera. Thus, it has been difficult to find common physiological behaviors among them. Since *Citrus* plants are commonly grown in fields, it is difficult to survey their physiology under strictly controlled conditions. However, it was reported that *Citrus* plants are seriously suffered from insect pests accompanied with mechanical injury accompanied with post-wounded pathogen infections [4]. It is necessary to provide insight into their physiology during defense responses against wound stresses for cultivation and protection of *Citrus* plants. In this chapter, we describe metabolomic approaches to elucidating wound responses in *Citrus* plants.

2. Metabolomic approach to investigate responses to wound stress

As described above, when higher plants face to wound stresses, plant metabolism is drastically changed in order to defend themselves against stresses. This metabolic change involves accumulation of defense compounds such as phytoalexins and lignin-like compounds, regulation of signaling pathway, up-regulation of substrate supplies and increase or decrease of many other specific compounds. This reconfiguration of metabolic network is highly complex, and therefore, details in plant defense mechanism are still unelucidated. To understand the mechanism, comprehensive perspective of regulation of metabolic network must be needed. Recently, the "omics" technologies have been developed to characterize and quantify all of the molecules leading to the phenotype of an organism in non-targeted and non-biased manner. Among "omics" technologies, the term "metabolomics" has been used to address the analysis of low-molecular metabolites. Recent advances in technologies of mass spectrometry (MS) and nuclear magnetic resonance (NMR) as well as bioinformatics such as multivariate analyses and chemical libraries enable the application of metabolomics to varieties of organisms. Metabolomics has become in the spotlight as a powerful tool to gain comprehensive and collective information of metabolic network and to find out biomarkers related to defense mechanisms [5].

Since *Citrus* plants are one of the most diverse plant families, metabolite profiles are expected to be diverse among species. *Citrus* plants are commonly grown in open fields, and therefore, variations in levels of metabolites especially those involved in defense mechanisms may be significant due to environmental factors such as temperature, humidity, wind and irradiation of sunlight. Thus, investigation of defense mechanisms of *Citrus* plants needs non-biased and comprehensive analyses and minimization of data variation caused by environmental factors. For this aspect, metabolomics must be a strong tool to understand the *Citrus* defense mechanisms, because metabolomics includes comprehensive instrumental analyses as well as multivariate analyses which can find specific valuables from numerous and highly diverse valuables.

3. Metabolomic analysis of leaf volatiles during wound responses

3.1. Volatile compounds in plant defense responses

Plants induce various defense reactions including phytoalexin and/or pathogenesis-related (PR) protein, hypersensitive reaction (HR) and emission of volatile organic compounds (VOCs) in response to wounding [6–8]. Among these, emission of VOCs is involved not only in direct defenses, such as toxins and repellents against herbivores, but also in indirect defenses that include recruitment of natural enemies against herbivores and elicitation of defense mechanisms in intact receiver plants [9, 10]. Plant VOCs consist of two major classes of compounds, that is, terpenoids and C6 green leaf volatiles (GLVs). Terpenoids are one of the most structurally diverse groups of plant metabolites and synthesized from two biological precursors, isopentenyl pyrophosphate and dimethylallyl pyrophosphate. It has been demonstrated that several terpenoids play roles as antimicrobial or antifeedant compounds in direct defense responses [11, 12]. GLVs consist of C6 aldehydes, alcohols and their esters and are synthesized from α-linolenic acid through the lipoxygenase pathway. The compounds *trans*-2-hexenal and *cis*-3-hexenol are typically released in response to wounding and have been reported to mediate plant-plant signaling and intra-plant information transfer in indirect defenses [13, 14]

Citrus fruits are well known as a rich source of VOCs, and several components show pharmaceutical functions such as antimicrobial, anticancer, and anti-inflammatory activities. However, little is known about physiological roles of VOCs released by leaves. To understand the roles of VOCs in wound responses, comprehensive analysis of VOCs during wound defense responses would be useful. In this section, metabolic profiling of VOCs during wound responses was highlighted.

3.2. Method of metabolic profiling of VOCs

To compare responses among species, plant materials used for study should be maintained under the same condition to minimize effects of environmental factors. We usually use plants grown in the same field, and at least five leaves per square meter were used for the study. To investigate the responses against stresses, freshly excised leaves were immediately exposed to stresses, and during the treatments, leaves were placed under strictly controlled condition to avoid influence of environmental factors. The treated leaves were immediately frozen in liquid nitrogen and maintained at −80°C before analysis [16].

For analysis of VOCs, gas chromatography/mass spectrometry (GC/MS) is suitable analytical platform because it separates majority of the components and mass spectral fragmentation pattern of each compounds makes it easier to identify compounds. Sample preparation methods for GC/MS include essential oil extraction such as solvent extraction or headspace extraction. Among them, headspace extraction method using microfiber solid phase (headspace solid phase microextraction, HS-SPME) is most sensitive and useful method for comprehensive analysis of VOCs. Various SPME fibers are now available for analyses, and among them, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber has been well used and recommended for VOCs extraction because this fiber consists of triple phases and thus absorbs wide range of volatile compounds [15]. For chromatographic separation of VOCs, nonpolar CP-SIL 8-CB MS capillary column was used, and helium was used as the carrier gas at a liner velocity of 45.0 cm/s. For annotation of VOCs, the mass spectrum data were compared against spectra in the NIST reference library of GC/MS data system, and retention indices (RIs) from the literature were used for identification of VOCs. Identified VOCs in our previous study using seven *Citrus* species, *C. sinensis*, *C. limon*, *C. paradisi*, *C. unshiu*, *C. kinokuni*, *C. grandis* and *C. hassaku* are listed in **Table 1**.

Chemical group	**Compounds**	**Retention indices**	**Molecular weight**	**Mass spectrum data *m*/*z* (relative intensities)**
GLV aldehydes	Hexanal	802	100	41 (100), 44 (97), 56 (89)
	trans-2-Hexenal	846	98	41 (100), 83 (97), 55 (95)
Fatty aldehydes	Octanal	1005	128	43 (100), 56 (77), 44 (77)
	Nonanal	1106	142	57 (100), 41 (66), 56 (66)
	trans-2-Nonenal	1162	140	43 (100), 55 (95), 41 (92)
Fatty alcohol	1-Octanol	1075	130	56 (100), 55 (77), 41 (65)
Monoterpenenes	α-Thujene	927	136	93 (100), 91 (47), 92 (40)
	α-Pinene	934	136	93 (100), 92 (40), 91 (33)
	Camphene	950	136	93 (100), 121 (69), 79 (35)
	Sabinene	973	136	93 (100), 91 (33), 77 (28)
	β-Pinene	977	136	93 (100), 41 (43), 69 (41)
	β-Myrcene	991	136	93 (100), 41 (82), 69 (71)
	α-Phellandrene	1005	136	93 (100), 91 (44), 92 (32)
	3-Carene	1008	136	93 (100), 91 (37), 92 (32)
	α-Terpinene	1017	136	121 (100), 93 (98), 136 (61)
	Cymene	1025	134	119 (100), 134 (32), 91 (24)
	D-Limonene	1031	136	68 (100), 93 (100), 67 (66)
	cis-β-Ocimene	1039	136	93 (100), 92 (42), 91 (37)
	trans-β-Ocimene	1050	136	93 (100), 80 (40), 91 (39)
	γ-Terpinene	1061	136	93 (100), 136 (59), 90 (58)
	α-Terpinolen	1085	136	93 (100), 121 (86), 136 (82)
Monoterpene aldehydes	Citronellal	1154	154	41 (100), 69 (95), 95 (54)

Chemical group	Compounds	Retention indices	Molecular weight	Mass spectrum data *m*/*z* (relative intensities)
	β-Citral	1241	152	41 (100), 69 (86), 94 (30)
	α-Citral	1271	152	69 (100), 41 (85), 84 (27)
Monoterpene alcohols	Eucalyptol	1033	154	43 (100), 81 (56), 84 (51)
	cis-Sabinene hydrate	1071	154	43 (100), 71 (97), 93 (58)
	Linalool	1101	154	71 (100), 93 (75), 55 (60)
	Terpinen-4-ol	1180	154	71 (100), 111 (50), 93 (47)
	α-Terpineol	1195	154	59 (100), 93 (66), 136 (47)
	Nerol	1223	154	69 (100), 41 (78), 68 (24)
	Geraniol	1253	154	69 (100), 41 (67), 68 (24)
	Thymol	1293	150	135 (100), 150 (35), 91 (15)
Monoterpene ester	Geranyl acetate	1380	196	69 (100), 41 (49), 43 (47)
Sesquiterpenes	α-Cubebene	1347	204	105 (100), 119 (94), 161 (91)
	α-Copaene	1376	204	161 (100), 119 (97), 105 (96)
	β-Elemene	1390	204	93 (100), 81 (91), 68 (67)
	β-Caryophyllene	1421	204	93 (100), 69 (87), 133 (81)
	α-Bergamotene	1435	204	119 (100), 93 (98), 41 (41)
	Aromadendrene	1439	204	161 (100), 93 (91), 91 (89)
	β-Farnesene	1454	204	69 (100), 41 (62), 93 (56)
	Humulene	1457	204	93 (100), 80 (32), 121 (23)
	Alloaromadendrene	1461	204	93 (100), 91 (93), 105 (93)
	γ-Selinene	1475	204	189 (100), 133 (64), 204 (49)
	γ-Muurolene	1482	204	161 (100), 105 (57), 81 (42)
	β-Selinene	1491	204	105 (100), 93 (98), 107 (93)
	α-Selinene	1498	204	189 (100), 93 (86), 107 (74)
	α-Farnesene	1506	204	93 (100), 41 (68), 69 (60)
	β-Bisabolene	1510	204	69 (100), 93 (84), 41 (71)
	δ-Cadinene	1521	204	161 (100), 204 (55), 134 (55)
	β-Sesquiphellandrene	1526	204	69 (100), 41 (52), 93 (49)

Table 1. Volatile organic compounds (VOCs) detected by GC/MS in Asai et al. [16].

Fifty VOCs were identified with our system, and majority of them is terpenoids, that is, monoterpene hydrocarbon and their derivatives and sesquiterpene hydrocarbons. In addition to terpenoids, two GLC aldehydes, three fatty aldehydes and a fatty alcohol were identified. Among VOCs identified, monoterpene hydrocarbons constituted the main part of the leaf VOCs in all species tested according to ratios of each chemical group on the basis of the total ion current peak area measured by GC/MS, but the profiles of VOCs were different among species [16].

3.3. Evaluation of changes in VOCs profiles during wound responses

It has been well known that phytohormones, such as jasmonic acid (JA) and salicylic acid (SA), are involved in the signaling pathway for induction of plant defense mechanisms. Wound stress induces temporal and organ-specific JA accumulation that mediates to activation of defense-related genes and leads to induction of defense responses [17, 18]. In contrast, SA accumulation is caused by insect egg deposition or pathogen infection and results in induction of *PR* genes, systemic acquired resistances and hypersensitive reactions [19, 20]. It has been suggested that JA- and SA-signaling pathways regulate different defense responses and that the JA pathway is involved in responses to necrotrophic pathogens, while SA is primarily activated in response to biotrophic pathogens [21]. However, correlation between VOCs emission and JA- and/or SA-signaling has been reported to vary among plant families [22–24]. To understand wound defense mechanisms in *Citrus* plants, it would be useful to survey the comprehensive dynamic changes in VOC profiles in response to wounding, JA and SA stimuli.

For comparison of VOC profiles among species and treatments, it must be necessary to handle large amount of datasets. To evaluate significant changes in VOC profiles, statistical analysis should be employed. For metabolomics, multivariate analysis is typically employed to process datasets. In our previous report, VOC profiling was performed by application of principal component analysis (PCA) [16]. PCA can provide an overview and clustering of all the applied datasets by projecting each sample.

The results of PCA demonstrated that *C. limon* (common name: lemon) and *C. kinokuni* (kishu) showed separate clusters among different treatment samples, suggesting that metabolism of VOCs in response to wound, JA and SA treatments was independently regulated from one another. In contrast to these two species, *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), *C. unshiu* (unshu), *C. grandis* (pummelo) and *C. hassaku* (hassaku) showed no clear separation among species.

PCA provides an overview of datasets by unbiased and unsupervised manner, and thus, it shows clear clustering only when the variation within each group is sufficiently less than variation between groups. In contrast, since orthogonal partial least square-discriminant analysis (OPLS-DA) is supervised discriminant analysis that relies on the class membership of each treatment, OPLS-DA should be a powerful tool to evaluate treatment-specific changes in metabolites and thus to find biomarkers in defense responses [25]. *Citrus* plants are commonly grown in open fields, and thus, data obtained from them tend to be high variation when open field grown plant materials are used for researches. In order to find wound-related compounds, data were analyzed by OPLS-DA. According to our previous OPLS-DA results, the patterns of VOC profile changes in the seven *Citrus* species studied could be divided into four different groups [16]. First group included *C. limon* and *C. kinokuni* which showed increase in most VOC components under all treatments. The highlighted markers in this group were D-limonene and β-pinene in *C. limon* and linalool and γ-terpinene in *C. kinokuni*, respectively. Second group consisted of *C. paradisi* and *C. grandis* which showed decrease of most VOC components under all treatments, and among the VOCs, linalool and sabinene were suggested to be markers in *C. paradisi*, while β-pinene was highlighted in *C. grandis*. Third group consisted of only *C. unshiu* and showed a different trend from other species. Most of the VOCs increased after wounding and SA treatment, but decreased after JA treatment. Final group included *C. sinensis* and *C. hassaku*. In this group, several VOCs decreased by wound and JA treatment, while only slight changes in VOCs were detected after SA treatment. Consequently, VOC responses to stresses were suggested to be different among *Citrus* species. However, two GLVs, hexanal and *trans*-2-hexenal, and α-farnesene, were clearly affected in many of tested species, and thus, these compounds can be candidates of the common wound stress biomarkers in *Citrus* plants, although details in their physiological roles were still to be elucidated.

4. Metabolomic analysis of primary metabolites during wound responses

4.1. Changes in primary metabolism during plant defense responses

Wound stress elicits not only secondary metabolites including VOCs but also whole metabolic network including primary metabolites. Activation of glycolytic pathway in response to wounding leads to energy production as well as substrate production for various defense compounds including defense-related proteins [26]. It has been demonstrated that accumulation of free amino acids was induced in response to environmental stresses. Branched-chain amino acids were induced against drought stress, while aspartate family amino acids were related to the osmotic stress [27, 28]. Moreover, amino acid biosynthetic pathways such as tryptophan pathway have been well known to be involved in biosynthesis of defense compounds [29]. For insight into defense mechanisms in *Citrus* plants, understanding of changes in whole metabolic network must be needed. In this section, metabolomics focused on primary metabolites is described.

4.2. Method of metabolomics of primary metabolites

For analysis of primary metabolites, various platforms including capillary-electrophoresis/mass spectrometry, GC/MS and NMR have been developed. Among them, GC/MS has been well used because of its sensitivity and availability for wide range of compounds, although appropriate derivatization should be needed. Extraction and derivatization methods of plant primary metabolites were well developed for validation of food and beverage materials, and data processing software such as peak alignment and peak annotation by mass spectrum is now commercially and non-commercially available. GC/MS-based metabolomics has been frequently and routinely used for many metabolomic studies.

In our previous study, extraction and derivatization were carried out according to the method developed by Fukusaki et al. [30]. In this method, a mixture of methanol/water/chloroform (2.5:1:1 v/v/v) was used as an extraction solvent for a wide range of polar compounds including primary metabolites, and derivatization was carried out by adding methoxyamine hydrochloride, followed by silylation with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. GC/MS peak detection and alignment were carried out using MetAlign software (www.metAlign.nl), and AIoutput software [31] was used for peak identification by retention index and mass spectrum. With this procedure, 28 organic acids, 21 amino acids, 13 sugars and sugar alcohols and 7 nitrogen containing compounds were identified [32].

4.3. Profiling of primary metabolites in *Citrus* leaves

Metabolomic analysis of identified primary metabolites in *Citrus* leaves showed that profiles of primary metabolites in leaves of seven *Citrus* species, *C. sinensis*, *C. limon*, *C. paradisi*, *C. unshiu*, *C. kinokuni*, *C. grandis* and *C. hassaku*, were different among species [32]. Among them, *C. limon* formed separate cluster from other species in PCA score plot. It has been suggested that citron-derived *C. limon* is genetically separate species from pummelo- and mandarin-derived species [33]. Although variations in levels of each metabolite were relatively high, this result may reflect the genetic background of each species.

To assess changes in profiles of metabolites, datasets obtained from wound treated leaves and untreated leaves were statistically analyzed. Hierarchical cluster analysis and OPLS-DA showed that levels in amino acids are high sensitivity to stress treatments, indicating that amino acids are involved in *Citrus* defense mechanisms against wounding [32]. Among amino acids, tryptophan and serine were highly sensitive to stress treatments. Tryptophan was up-regulated after wounding and JA treatments, while serine was down-regulated under the same conditions. In tryptophan biosynthetic pathway, tryptophan synthase catalyzed the conversion of serine and indole to tryptophan, and thus, our result indicated the activation of *de novo* synthesis of tryptophan.

5. Identification of novel wound-stress related compounds

5.1. Bottleneck in metabolomics

As described above, metabolomics is a strong tool to find characteristic biomarkers related to genetic and/or environmental factors. However, compound annotation and identification are major bottleneck in metabolomics, especially in mass spectrometry-based metabolomics. Currently, several MS databases have been established, and increasing amount of MS data is now available to facilitate metabolite annotation. It has been estimated that there are hundreds of thousands compounds in plant kingdom, and many of them belong to secondary metabolites. Secondary metabolites are well known as functional metabolites that play important roles in plant physiology and/or ecology, and therefore, many of researches on plant physiology and biology have been focused on the secondary metabolism. Despite of the importance of secondary metabolites, identification of many of them is still to be carried out, and many of them are not commercially available. MS databases depend on literatures and analyses of authentic standards, and thus, spectra of secondary metabolites are quite limited. For better annotation in plant metabolomics and understanding of plant physiology, isolation and identification of metabolites are essential.

5.2. Defense-related compounds in *Citrus* plants

Citrus plants are rich source of secondary metabolites that exhibit pharmacological activities. Phenylpropanoids, flavonoids, terpenoids and alkaloids are major bioactive compounds in this plant family, and some of them are known to be involved in defense mechanisms against pathogens. For example, polymethoxyflavanones, such as nobiletin and tangeretin, have antifungal effects on *Penicillium digitatum* [34]. Activities of phenylalanine ammonia lyase and chalcone synthase, which are the key enzymes of phenylpropanoid and flavonoid biosynthesis, have been reported to increase after mechanical stress or pathogen infection in a resistant species, *Poncirus* (*Citrus*) *trifoliate*, but not in a susceptible species, *C. sunki*, and thus, these biosynthetic pathways are considered to be involved in defense mechanisms [35]. However, defense-induced compounds derived from flavonoid pathway are still unknown. In *Citrus* plants, coumarins have been identified as phytoalexins from fruits [36], but information of metabolites involved in leaf defense responses is quite limited.

5.3. Isolation and identification of wound-induced compounds in *Citurs hassaku* leaves

Hassaku (*C. hassaku* Hort ex. Tanaka) is one of the most popular *Citrus* species in Japan, and the fruits have been consumed not only as fresh fruit and juice but also as a source of traditional medicine [37]. Varieties of flavonoids, limonoids and coumarins have been isolated from this plant. Despite of increasing studies on pharmacological compounds, reports of defense-related compounds in this plant are quite limited. From these backgrounds, we focused on wound inducible metabolites in hassaku leaves and carried out metabolic fingerprinting-oriented identification of induced compounds [38].

For detection of wound inducible compounds, hassaku leaves were cut into 5 mm square segments by surgical knife for mechanical wounding. This treatment is unusual in fields, but significantly facilitates the wound responses. Wounded and intact leaves were grounded and extracted with methanol that is a useful solvent to extract a wide range of compounds. Leaf extracts were then subjected to high performance liquid chromatography (HPLC) analysis, and chromatograms obtained from wounded and intact leaf extracts were compared. The HPLC fingerprinting showed that two peaks were occurred only in the chromatogram of wounded leaf extract but not in that of intact leaf extract, suggesting that these two compounds were related to the defense response against mechanical wounding.

To isolate wound-induced compounds, crude extract of wounded leaves was subjected to normal phase open column chromatography, followed by reverse phase preparative HPLC. Isolated compounds were applied to MS analysis and NMR for structural characterization. Spectral analyses revealed that one of wound-induced compounds was hesperetin, a major flavanone in *Citrus* plants and another was a novel compound. This novel compound was characterized as prenylated furofuran lignan (**Figure 1A**) and suggested to be a dimer of citrusnin-A (**Figure 1B**), which was isolated from *C. natsudaidai* [39]. We named this novel compound as "biscitrusnin-A," and stereochemical analysis revealed that biscitrusnin-A consists of the racemic mixture of two enantiomeric isomers.

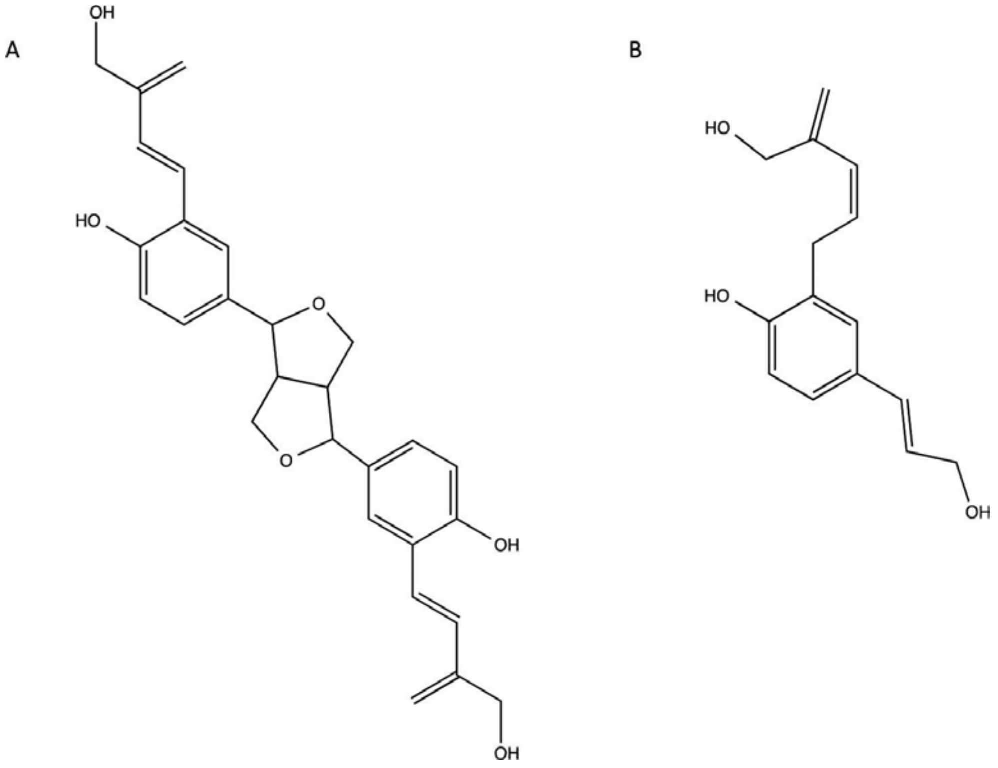

Figure 1. Chemical structures of biscitrusnin-A (A) and citrusnin-A.

To investigate physiological roles of the novel compound, antibacterial activities of these compounds were evaluated against plant pathogens, *Xanthomonas citri*, *X. phaseoli*, *X. oryzae*, *Pseudomonas syringae*, *Clacibacter michiganensis* and *Rhizobium radiobacter* [38]. However, biscitrusnin-A showed no significant activities except for *X. oryzae*. In contrast, citrusnin-A, a monomer of biscitrusnin-A was reported to exhibit high antibacterial activities against almost all of the bacteria tested [38]. It was reported that accumulation of lignans caused by defense responses contributed to cell wall reinforcement rather than direct defense against pathogens [40]. Biscitrusnin-A also might play physical defense roles.

Although prenylated coumarins have been identified from several *Citrus* plants, the report about biscitrusnin-A was a first report about prenylated lignans isolated from *Citrus* plants. Many of prenylated lignans have been isolated from *Zanthoxylum* and *Haplophyllum*, which belong to Rutaceae family as well as *Citrus* [41], and thus, prenylated lignans may distribute in other *Citrus* plants and related genera.

6. Conclusion

In this chapter, metabolic profiling of *Citrus* leaves for investigation of their defense mechanisms is discussed. Metabolomics has been attracting increasing attention as a new and powerful tool for elucidation of complexities of metabolic network. GC/MS-based metabolomics successfully provided the information of metabolites related to wound responses in *Citrus* plants, that is, three VOCs, hexanal, *trans*-2-hexenal and α-farnesene, and two amino acids, tryptophan and serine. In addition, metabolic fingerprinting-oriented isolation resulted in identification of a novel wound-related compound. Although physiological roles of these compounds are still unknown, these compounds can be used as biomarkers in *Citrus* defense responses. Recently, pest management using synthetic pesticides with direct toxicity have been regarded to be undesirable because of their negative effects on ecological environment, and the use of environmentally favorable approaches is required. From this point, plant activators that prime and/or elicit plant defense responses have attracted much attention in crop protection. For development of plant activators, useful biomarkers that indicate defense responses are essential. Various "omics" approaches including metabolomics would be powerful tools to find useful biomarkers of defense responses as well as to elucidate the *Citrus* physiology.

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Chapter 4

Quorum Sensing, Its Role in Virulence and Symptomatology in Bacterial Citrus Canker

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Additional information is available at the end of the chapter

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Abstract

Xanthomonas citri subsp. *citri* (*Xcc*) is the etiological agent of citrus canker, a disease that affects almost all types of citrus crops. Production of *Xcc* virulence factors is controlled by a gene cluster *rpf*, which encodes elements of a cell-cell communication system called quorum sensing (QS). Perturbation of cell-cell signaling systems either by signal degradation or by overproduction significantly reduces symptoms and thereby the severity of the disease. Pathogenicity assays in *Citrus sinensis* showed that some bacterial species natural inhabitants of citrus phyllosphera have the strong ability to disturb QS system mediated by diffusible signal factor (DSF) molecule in Xcc and to reduce disease severity. The lessening of symptoms was associated with alteration in bacterial attachment and biofilm formation. These factors are known to contribute to Xcc virulence in the early stages of disease. The aim of this chapter is to review QS system in Xcc, the virulence factors affected by QS disturbing, as well as the main secretion systems that participate actively in virulence and its effect on the symptomatology of citrus canker.

Keywords: *Xanthomonas citri*, DSF, quorum quenching, biofilm, adhesin

1. Introduction

Of the entire bacterial population that comprise the plant microbiome, both phyllosphere and rhizosphere, the vast majority live as saprophyte on the plant surfaces and a few of them get internalized into the plant tissues, without causing any damage to their host; only about 100 bacterial species has became pathogenic to their host. Pathogenicity of microorganisms is due to the expression of virulence factors, which could be attributed to their genetic, biochemical, or structural traits in the attempt to produce host infection. Quorum sensing (QS) is a cell-

cell communication system, which leads to the regulation of specific genes, which in bacteria control essential biological functions as bioluminescence, antibiotic production, virulence, motility, and biofilm formation. In the bacterium *Xanthomonas citri* subsp. *citri* (Xcc), the etiological agent of citrus canker, the production of particular pathogenicity factors is controlled by a cluster of genes called *rpf* (for regulation of pathogenicity factors), which encodes elements of a QS system mediated by molecules of the diffusible signal factor (DSF) [1]. Interference with cell-cell signaling, also termed quorum quenching, drastically decreases disease symptomatology and is a promissory tool for biological control [2]. The aim of this chapter is to review what is known so far about mechanistic details of QS system in Xcc, the virulence factors affected by QS disturbing in *Xcc*, and its effect on the symptomatology of citrus canker.

2. *X. citri* subsp. *citri* virulence factors

The genus *Xanthomonas* (from Greek xanthos which means yellow) comprises a large group of plant pathogenic bacteria belonging to the group of Gamma proteobacteria. The xanthomonas bacteria infect 124 species of monocot plants and 268 species of dicot plants [3]. They are Gram-negative rod-shaped bacteria with a single polar flagellum, colonies are yellow because of a pigment named xanthomonadin and have a glossy appearance due to exopolysaccharide (EPS) called xanthan [4]. Although the genus itself has a very wide range of hosts, individual members are generally specialized to cause disease in a limited number of taxonomically related hosts. Several specialized virulence factors are employed by *Xanthomonas* bacteria to successfully invade the tissues of their susceptible hosts and multiply within them and cause disease.

The bacterium *X. citri* subsp. *citri* (Xcc) is the etiological agent of bacterial citrus canker (CC), a disease that affects almost all types of citrus crops. The invasion and colonization of the host occur through stomata and wounds in plant tissues, infecting leaves, fruits, and stems. Xcc harbors a wide range of virulence factors as surface attachment structures, cell-wall-degrading enzymes, several secretion systems and their effectors, and a diffusible signal factor-mediated quorum-sensing (QS) system [5].

2.1. Adhesins

A fundamental step in the bacterial colonization of host is its ability to attach to the surface of host cells. Bacterial surface structures anchored in its outer membrane that enables adhesion are termed adhesins, which are mostly of polysaccharidic nature (lipopolysaccharides (LPS) and exopolysaccharides) but may also be of proteinaceous nature (type IV pili, chaperone/usher pili, two-partner secretion) [6].

2.1.1. Lipopolysaccharide and exopolysaccharide

The lipopolysaccharides (LPS) play a role in adhesion, but it is difficult to determine whether it affects directly or indirectly the bacterial adhesion [7]. LPS is a crucial constituent of the outer membrane in Gram-negative bacteria and plays multiple roles in plant-microbe interactions [8]. LPS acts as a barrier for protecting bacteria from adverse environmental factors such as antibacterial compounds produced by the host cell, besides triggering or enhancing plant-defenses responses, because it acts as pathogen-associated molecular pattern (PAMP) identified in a widespread variety of phytopathogenic bacteria [9]. A recent study by Casabuono et al. [10] identified the structure of LPS in Xcc as a penta- or tetra-acylated diglucosamine backbone attached to either two pyrophosphorylethanolamine (PP-EtNH2) groups or to one PP-EtNH2 group and one phosphorylethanolamine group. The core region is composed by a branched oligosaccharide and two phosphate groups, whereas the O-antigen consists of a rhamnose homo-oligosaccharide. Xcc mutants of *wxacO* and *rfbC* genes form an impaired biofilm on glass or host plant leaves reducing resistance to antimicrobial compounds such as polymyxin B and hydrogen peroxide. Both mutants also showed deficiency in virulence and growth on host leaves of susceptible host after spray inoculation [11]. Moreover, additional six LPS genes, that is, *wzm*, *wzt*, XAC3591, XAC3593, XAC3595, and XAC3597, were described to be involved in biofilm formation [12]. The *wzm* and *wzt* genes encode an ATP-binding cassette (ABC) transporter system that exports the O-antigen polysaccharide of LPS [13]. Mutation of *wzm* and *wzt* decreases the virulence of Xcc and significantly reduces bacterial populations in the host plant [5, 10].

The exopolysaccharide (EPS) in *Xanthomonas* is known as xanthan gum, which is another bacterial polysaccharide released as an extracellular slime in the late stationary growth phase. The EPS in Xcc is involved in biofilm formation that facilitates intimate association of bacteria to abiotic and biotic surfaces [13]. EPS in xanthomonads is composed by a backbone of b-1,4-linked ᴅ-glucose with trisaccharide side chains of mannose-(b-1, 4)-glucuronic acid-(b-1,2)-mannose attached to alternate glucose residues in the backbone by a 1,3 linkages [14]. Xanthan production in *Xanthomonas* is hierarchically regulated by *rpf* (regulation of pathogenicity factors) gene cluster [15]. Biosynthesis and exportation of EPS in Xcc are encoded by *gum* gene cluster (i.e., *gumB* to *gumP*), *gumCDEFJK* genes also are involved in biofilm formation in biotic and abiotic surfaces [12]. An early study has showed the expression in the plant of *gum* operon of Xcc using a reporter β-glucuronidase [16]. The authors found that bacteria isolated from leaves and subsequently inoculated into mesophilic tissue have an increased expression of *gum* gene in the later stages of the bacterial growth, suggesting a requirement for EPS production at later stages of infection. X*cc gum*B deletion mutant has shown a significant reduction in the EPS production as well as an impaired ability to form a well-structured biofilm, leading to a deep reduction in severity disease [17]. Nevertheless, the defective mutant *gumD* remains fully pathogenic when it has been inoculated by infiltration pressure to the susceptible host, but displayed impaired survival capacity in citrus leaves and a reduction in symptoms when abaxial surfaces of leaves citrus were sprayed with a solution containing the deletion mutant *gumD*, suggesting a significant role of biofilm formation in the epiphytic fitness of Xcc, critical event in the early stages of pathogenicity development of citrus canker [18]. EPS also acts as plant-defense suppressor by preventing the callose deposition in the plant cell wall, because it operates as a chelator of divalent calcium ions present in the plant apoplast [19].

2.1.2. Proteic attachment structures

Pili (fimbriae) are filamentous appendices linked to cell surface; it has been categorized by their potential to induce hemagglutination, their anchoring site in bacteria (i.e., polar or omnidirectional) and their size. Several types of pili in *Xanthomonas* genus bacteria have been demonstrated or hypothesized to exist such as type IVpili, chaperone/usher pili, and pili linked to different protein secretion systems [20].

Homopolymeric ensemble of the pilin proteins termed PilA and PilE is the main constituent of *type IV pili* whose structure is associated to the type II secretion system [21]. Completely assembled structure projects from the bacterial surface to contact biotic or abiotic surfaces and once firmly attached retracts to bacterial cell wall, leading to bacterial cell movement known as twitching motility [22]. Type IV pili-related genes are consistent in the vast majority of sequenced strains of *Xanthomonas*; these genes are highly diverse and do not have any association with the phylogenetic relationship of strains. In Xcc, a tandem duplication of pilin has befallen and its consequences remain uncertain (i.e., whether there is an antigenic switch due to differential expression of the two genes) [23]. The changeability among major pilins could be related with specific interactions with their hosts, evocative of the situation in human and animal pathogens. The role of type IV in attachment and pathogenicity to their host plant is crucial in *Xanthomonas* bacteria. Studies using scanning electron microcopy (SEM) have shown that defective PilA mutants of several *Xanthomonas* species compromised its adhesion capacity, cell aggregation, and decrease its survivor and colonization of leaves [23].

Genes that encode for type I pili or CU pili (chaperone/usher) were first demonstrated in *Xylella fastidios*a [24] in *Xanthomonas* there is a conserved gene cluster for type I pili. In *Xanthomonadaceae* family type I pili play a central role in cell attachment, cell aggregation, and biofilm formation [25].

2.2. Pigment xanthomonadin

Members of the group of *Xanthomonas* genus produce a yellow pigment where chemical structure is mono- or di-bromo-aryl polyene. This pigment is bounded to the outer membrane and is termed xanthomonadin. A group of genes *pigA-pigG* are required for the biosynthesis of pigment and were first identified in *X. campestris* pv. *Campestris* [26]. The xanthomonadin's main function is to protect bacteria against photobiological damage. Actually, there are no conclusive studies on the protective effect of xanthomonadin against UV radiation injury. Comparison between *pigB*-defective mutants and wild-type strain *X. campestris* pv. *Campestris* does not display differences in sensitivity to UVA and UVC radiation damage. Xanthomonadin provides defense against visible light injury, because the pigment protects the lipid component of cell membrane from reactive oxygen species generated by the exposure to visible light [27]. Defective mutants of *pigB* gene in *X. campestris* pv. *Campestris* were unable to survive on the surface of the leaves and also the infection of leaves was severely reduced when the infection is through estomatos and hydathodes. Interestingly, the same mutant does not display variation in virulence after inoculation by infiltration pressure or spray in wounded leaves, indicating that the pigment is necessary to epiphytic infection, but is not required for growth in the inner tissue of susceptible host plant [28]. In addition to the loss of xanthomonadin production, defective mutants of pigB strains were also impaired in EPS production [28].

2.3. Virulence-related protein secretion systems and their effectors

Bacteria belonging to *Xanthomonas* genus display at least six different types of protein secretion system (i.e., T1SS to T6SS), which diverge in their arrangement, function, and in a recognition of secretion substrates [21]. As numerous Gram-negative phytopathogenic bacteria, Xcc employs mainly T2 secretion system (T2SS), T3SS, T4SS, and T5SS and their effectors as effective tools in an attempt to invade and to multiply in a susceptible host.

2.3.1. T2 secretion system (T2SS)

The T2SS was initially discovered in *Shigella flexneri*, a human pathogen bacterium [29]. T2SS is the principal secretion system that enables protein transport from bacterial periplasm to the extracellular milieu. It secretes extracellular enzymes as lipases, proteases, and cell-wall-degrading enzymes. It is feasible that type II-secreted enzymes cooperate to the degradation of the plant cell wall, which is the foremost difficulty for plant pathogenic bacteria. T2SS apparatus is made up of 12–15 components, most of which are linked with the bacterial inner membrane [30]. A member of secretin protein family forms a transmembrane channel in the outer membrane. It is expected that the secretion across the outer membrane depends on a predicted periplasmic pilus that is uninterruptedly assembled and disassembled and thus impulses T2SS substrates through the secretin channel [31]. It is assumed that the substrates transported by T2SS could enable the assemblage of extracellular appendages of virulence associated with T3SS, T4SS, and T6SS systems that are dedicated to effector protein translocation, thereby synthesis of T2SS is co-regulated with T3SS expression genes; it suggests a functional interplay between both secretion systems [32]. Genome sequence analysis disclosed that Xcc is outfitted with two predicted T2SS systems, which are encoded by *xcs* and *xps* gene clusters [20]. Remarkably, T2SS besides associated to bacterial virulence also can induce plant-defense response such as the deposition of callose in the cell wall; it has been evidenced in *X. oryzae* pv. *oryzae*. Induction of basal plant-defense response T2SS dependent is suppressed by *X. oryzae* pv. *oryzae* that contains a functional T3SS system [33]. This allows us to assume that T3SS effectors proteins act against the basal plant-defense response elicited by T2SS.

2.3.2. T3 Secretion system (T3SS)

Pathogenic bacteria employ the T3SS secretion system termed "needle," in order to provide virulence factors (effectors) directly into host cells and consequently influence cell host activities [20]. Initially, the T3SS has been identified and studied in pathogenic animal bacteria, such as *Yersinia* spp., *Shigella* spp., and *Salmonella*, which were found to secrete a number of virulence determinants called Yops (Yersinia Outer Proteins), IPAS (Invasion Plasmid Associated Protein), and SIPS (Salmonella Invasion Proteins) into host cells. An early study has reported the presence of a homolog of T3SS in *Ralstonia solanacearum*, a phytopathogen bacterium of Solanaceae family as well as in several phytopathogenic bacteria including Xcc [34]. Xcc includes a *hrp* (hypersensitive response and pathogenicity) gene cluster, which comprises 26 genes from *hrpF* to *hpa2*, which encodes the T3SS proteins [35]. Deletion mutant strains *hrpB*, *hrpB4*, *hrcV*, and *hrcN* in Xcc completely abolished the bacterial ability to cause citrus canker symptoms on susceptible citrus host [5], thus confirming the critical role of T3SS in virulence of Xcc.

In the Xcc genome, 24 known and putative effectors have been identified [36]. One of the principal effectors carried by the T3SS in Xcc belongs to the family AvrBs3/PthA. These effectors contain functional domains characteristic of eukaryotic transcriptional activator and are thus named TALE (Transcription Activators Like Effector) [37]. Particularly, TALE contains a central repeat domain that recognizes the host DNA in a highly specific fashion. Each repeating unit contains 34 amino acids, with 12 and 13 hyper-variable amino acids termed VRD (Variable Repeat Di-residue). Thus, the composition and arrangement of different VRDs provide TALEs with tremendous capacity to recognize DNA host, and binding occurs with a high degree of specificity to a particular region at the promoter of target gene known as EBE (Effector Binding Element). Bioinformatics and experimental approach sophisticatedly demonstrated that each VRD type acts as a code recognizing a specific nucleotide [38]. Xcc contains four genes (*PthA*) that encode TALE, of which *pthA4* is known to be necessary for the formation of citrus canker lesions. Hypothetically, the TALE in Xcc encoded by *pthA4* gene induces a gene in the susceptible host resulting in the formation of erumpent lesions. Research done by Yang Hu et al. [39], on susceptible host gene expression, under TALE effect encoded by gene pthA4 in *Xanthomonas* species, revealed a remarkable and constant searching for vulnerabilities in the host physiology. Based on these findings, it was proposed that erumpent lesions involve recruitment and expression of a single gene in the host, termed CsLOB. Induction of this gene by the effector encoded by the pthA4 gene was assessed in sweet orange and grape, two susceptible host species to Xcc infection [39]. Although specific functions of CsLOB1 gene are unknown, previous studies have revealed that LOB protein domains are involved in the regulation of development of lateral organs, nitrogen, and anthocyanin metabolism. CsLOB1 gene also responds to plant hormones such as auxin, cytokinin, and gibberellin, as well as to environmental stimuli [40].

2.3.3. T4 secretion system (T4SS)

The translocator apparatus designated as type IV secretion system (T4SS) is an important virulence factor in several animals and plants bacterial pathogens, T4SS involving the secretion of proteins or DNA into the host cells [41]. The T4SS apparatus extends from the bacterial inner membrane through the periplasm; it ends at the outer membrane into a pilus-like structure, which protrudes from the surface of the bacterial cell. Several studies made on the *Agrobacterium tumefaciens vir B* locus revealed that 11 proteins form the T4SS apparatus [42]. Xcc has two sets of genes that individually are potential encoders for T4SS components [35], one placed in chromosome and the other on the plasmid pXac64. The T4SS translocator apparatus consists of a core 12 proteins, called VirB1-VirB11 and VirD4. Structural organization of T4SS currently is well elucidated: (i) a set of three cytoplasmic ATPase (VirB4, VirB11, and VirD4), which energizes the secretion process; (ii) a complex periplasmic core consisting of 14 repetitions of a trimer: VirB7-VirB9-virB10, in which virB10 is inserted in both inner and outer membranes and VirB7 is an outer membrane lipoprotein; (iii) an inner membrane complex that includes VirB3, VirB6, and VirB8; (iv) an extracellular pili formed by VirB2 and VirB5; and (v) VirB1 which is a periplasmic transglycosylase [43]. A differential feature of T4SS in Xcc is the presence of one protein called VirD4 (VirD4XAC2623), VirD4 recruit effectors for

T4SS secretion. A set of uncharacterized proteins termed VirD4-interacting proteins (XVIPs) is recruited by VirD4. A recent study by Souza et al. [44] has shown that XVIPs proteins secreted by the carrier apparatus T4SS of Xcc are toxins. These toxins kill contender bacteria in the niche. Peptidoglycan in bacterial cell wall is the target of XVIPs proteins.

3. Citrus canker, life cycle, symptoms, and types of diseases

One of the most important citrus diseases is citrus canker, affecting almost all types of citrus crops. The disease causes extensive damage to the cultivars and severity of infection varies with different bacterial species and the predominant weather conditions. The geographical origin of citrus canker is a matter of controversy. Lee et al. [45] reported that citrus canker may have emerged in southern China. However, several authors believe that the disease had its origin in particular regions of India and Java [46]. These reports suggest, therefore, that the origin of the disease has occurred in tropical areas of Asia, where it is assumed that citrus species have originated and been distributed to other areas through vegetative propagation material. Currently, citrus canker occurs in over 30 countries in Asia, Indian and Pacific Ocean islands, South America, and Southeast of USA [47]. Copper-based products are routinely used as a standard control measure for citrus canker.

The invasion and colonization of the host occur through natural openings of the leaves (the stomata) and wounds in the plant tissues. The pathogen multiplies within the intercellular spaces, inducing cell hyperplasia, leading to rupture of the leaf epidermis and resulting in raised corky and spongy lesions surrounded by a water-soaked margin, that is, the characteristic canker lesion. Yellowish chlorotic rings are also often observed on leaves and fruits and, when conditions are highly favorable to disease development, could produce general defoliation, tree decline, and premature fruit drop [48, 49].

There are three different forms of citrus canker produced by two species of *Xanthomonas*: citrus canker types A, B, and C. The differentiation of these forms is principally based on geographical distribution and a variety of pathogen hosts [50]. Asian form of citrus canker is caused by Xcc [48]. It is the most common and widespread, and its geographical distribution continues to increase. Disease is endemic in more than 30 countries: Asia, India, Pakistan, the islands of the Indian Ocean, Southeast Asia, South America, and Southeast China and Japan. Xcc has extended its host range, producing disease in most citrus species, that is, *C. paradisi*, *C. aurantifolii*, *C. sinensis*, and *C. reticulate*. Citrus canker type B is caused by *X. fuscans* subsp. *aurantifolii* type B (XauB) [36]. It has similar symptoms to symptoms present in type A citrus canker; nevertheless, the symptoms take longer to appear as a consequence of XauB slower growth. Host range is restricted to *C. limon*; however, it has also been sporadically isolated from *C. sinensis* and *C. paradisi* [51]. Type B citrus canker was first isolated in Argentina and a few isolated infection cases have been reported also in Uruguay and Paraguay [51]. Type C citrus canker has only been identified in the state of São Paulo, Brazil [52]. It presents the same symptoms as type A citrus canker and is produced by *X. fuscans* subsp. *aurantifolii* type C (XauC). Its host range is restricted to *C. aurantifolii* [36].

4. Quorum sensing in Xcc

Recognition of altruistic behavior, as those actions that increase the adaptation of another individual, their own cost, is a major challenge for evolutionary biologists because natural selection seems to favor selfish and uncooperative individuals [53]. Nevertheless, there are many examples in the animal kingdom, where this form of cooperation was successfully demonstrated. However, it is only recently that social behavior in microorganisms has been studied in relation to evolutionary theory [54]. Research over the last 20 years has expanded the view of bacteria as unicellular organisms having the ability to participate in complex social and cooperative behavior.

The development of an intercellular communication system is a hallmark characteristic that enables bacteria to colonize new habitats, adapt to environmental changes, resist host defense and antibiotic action, strengthen competitiveness, and take advantage of new food sources [55]. This talent for cooperative multicellular behavior depends on the implementation and recognition of diffusible signal molecules by a system known as quorum sensing (QS). Functionally, QS is a signal translation mechanism to coordinate the expression of genes at the population level. The process of QS relies upon the production, release, and detection of small signaling molecules termed auto-inducers (AIs). Each bacterial cell produces a basal amount of AIs, which are exported to the extracellular environment and reflect bacterial population density. At high cell densities, the AIs reach a critical concentration, at which point they are recognized by their cognate receptor, triggering a cascade of biological functions [56]. Bacteria within the genus *Xanthomonas* encode a cell-cell signaling or QS system which uses as AI, molecules from the diffusible signal factor (DSF) family. The DSF family are cis-2-unsaturated fatty acids, of which the paradigm is DSF itself, first identified in *X. campestris* pv. *campestris* and characterized as cis-11-methyl-2-dodecenoic acid. Detection of the DSF molecule activity as AI was first reported for over two decades by MJ Daniels Research Group [15], when these researchers were analyzing a cluster of genes in *X. campestris* called *rpf* (regulation of pathogenicity factors). It was found that the activity of the protease and endoglucanase enzymes in *rpfF* deletion mutant could be restored when this mutant was grown in the vicinity of its parental wild strain [1]. Observation of this effect led to speculation that *X. campestris* wild strain could produce a diffusible factor (DSF), which induced the production of protease and endoglucanase, and that the protein encoded by *rpfF* gene was linked with DSF biosynthesis. The signaling molecule DSF has a cis-unsaturated double bond at the two positions. It is a key structural feature for its activity; this motif is considered as signature for the DSF family. Additionally, it is believed that methyl branching plays an important role in signaling, for example, unbranched cis-2 dodecenoic acid and cis-3 tridecenoic acid; they are between 60 and 120 times less active than cis-11-methyl-2-dodecenoic acid [57].

The QS system in Xcc as well as in other species that comprise the *Xanthomonas* genus is encoded by *rpf* gene cluster. Biosynthesis of DSF auto-inducer in Xcc is dependent on *rpfF* and *rpfB* genes; these two genes encode, respectively, a putative enoyl-CoA hydratase (RpfF) and an acyl-CoA ligase of fatty acid long chain (RpfB); however, their catalytic mechanisms and corresponding substrates, as well as the reaction products, require further investigation [1]. A recent analysis of RpfF-crystallized structure has shown that it is structurally similar to members of crotonase superfamily [58]. Sequences alignment and structural analysis enabled the identification of two putative catalytic glutamate residues (Glu141 and Glu161), which are preserved in enoil-CoA hydratase/dehydratase. Cheng et al. [58] demonstrated that the substitution of these two residues in RpfF completely abolished the DSF production, emphasizing its critical role in DSF biosynthesis.

DSF detection and transduction in bacteria involve a complex kinase sensor positioned in the cytoplasmic membrane, which is associated to a cytoplasmic regulator. The best studied of these systems is RpfCF/RpfG of *X. campestris*, which is shared by all *Xanthomonas* species [57]. RpfC is a complex sensor kinase composed of (i) sensory domain, which contains five transmembrane helices with periplasmic and endoplasmic loops, which has the function to sense DSF levels, (ii) a histidine kinase domain (HisKA) coupled to an ATPse, (iii) a CheY-like two components domain (REC), and (iv) histidine phosphotransferase domain (HPT) (**Figure 1**). The regulator RpfG comprises (i) an REC domain and (ii) a HD-GYP domain, which is a phosphodiesterase involved in the degradation of the second messenger cyclic di-GMP.

Responsible residues for DSF bond are not known yet. It is assumed that the binding of signal molecule triggers RpfC auto-phosphorylation, within the histidine-kinase domain, followed by phosphorelay, involving an aspartic acid residue in the REC domain and a histidine residue in HPT domain, and finally the transfer of one phosphate group to the REC domain in

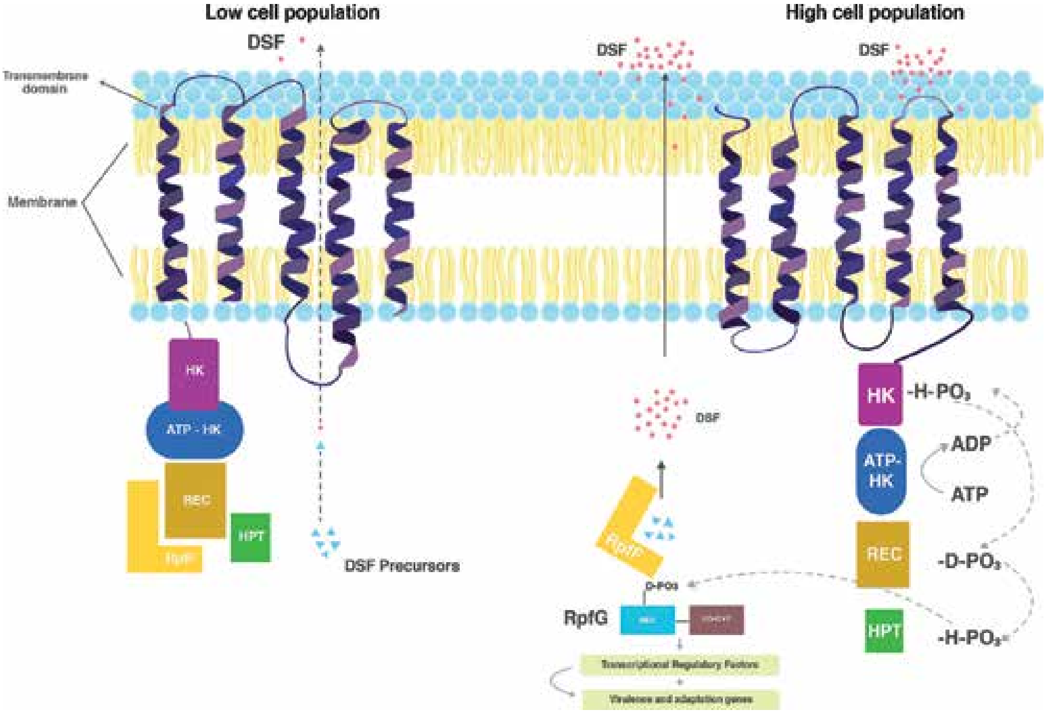

Figure 1. At low cell density, the sensor RpfC maintains a compact conformation, forming a complex with DSF synthase RpfF. At high cell density, DSF binds to RpfC, inducing a conformational change, which initiate the autophosphorylation and phosphorelay to RpfG and release RpfC.

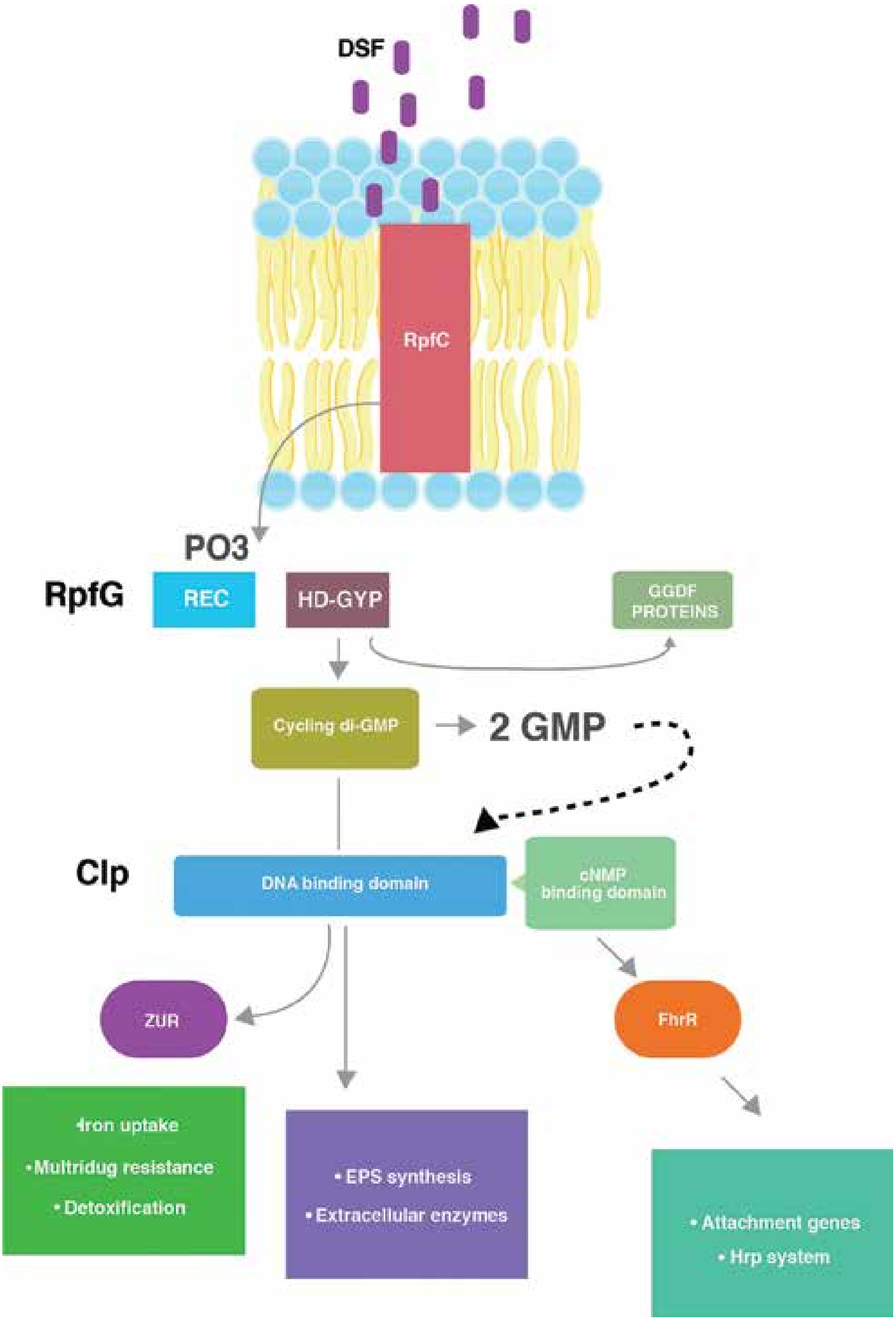

Figure 2. Perception of DSF by RpfC leads the phosphorylation of RpfG, it triggers the activation of RpfG as a cyclic di-GMP phosphodiesterase reducing the level of cyclic di-GMP and releasing Clp that promotes the synthesis of extracellular enzymes and EPS.

RpfG regulator [59]. The phosphorylation of RpfG enables its activation as a cyclic di-GMP phosphodiesterase, resulting in modifications in the level of cyclic di-GMP in the cell affecting the synthesis of virulence factors such as extracellular enzymes, EPS, biofilm dispersal, and motility [60].

Mechanistic detailed studies have been revealed that RpfG the regulator of DSF signaling cascade, it has Che-Y like receiver domain (REC) connected to a HD-GYP domain, which has phosphor diesterase activity, which acts on the degradation of the second messenger cyclic di-GMP (**Figure 2**). As outlined above, the perception of DSF in a RpfC Xcc is bound to the phosphorylation of HD-GYP domain in the RpfG regulator, and the consequent changes in intracellular second messenger cyclic di-GMP trigger major changes in bacterial phenotype [61].

Diverse pathways subsequently act to control different subgroups of virulence functions under the regulation of Rpf. The physical interaction of RpfG with two proteins with a diguanylate cyclase (GGDEF) domain acts to control motility but does not influence extracellular enzyme synthesis or biofilm formation; these two events require the conserved GYP motif in the HD-GYP domain of RpfG and are determined by DSF signaling [59]. The effect of RpfG in the synthesis of extracellular enzymes and biofilm formation could be carried on through the cyclic di-GMP influences on the global transcriptional activator Clp (cAMP receptor-like protein), which contains nucleotide- and DNA-binding domains [62]. At physiologically relevant levels of cyclic di-GMP, the global transcriptional activator Clp remains bound to cyclic di-GMP by nucleotide, avoiding the binding of Clp to the gene promoters that encode for several virulence and adaptation factors. However, when the HD-GYP domain is phosphorylated as a consequence of DSF perception, the HD-GYP domain starts its phosphodiesterase activity over cyclic di-GMP and relieves the Clp inhibition. Therefore, Clp binds to the gene promoters of several virulence factors, that is, extracellular enzymes, iron uptake, and adhesion synthesis [57]. Two transcription factors were identified to be directly regulated by Clp, that is, FhrR which regulates the expression of genes that encode flagellar, Hrp, and ribosomal proteins, the second transcription factor identified was Zur (Zinc uptake regulator), which is implicating in the regulation of multidrug resistance, iron uptake, and detoxification [63]. DSF also regulates the expression of *clp* gene; this suggests a more complex regulatory network of DSF regulon.

5. Virulence factors affected by quorum quenching in Xcc

Quorum sensing helps to coordinate bacterial behavior based on community, but it is not essential for the survival of bacteria. Therefore, the inhibition of QS only disrupts phenotypic traits that could be targets for the control of diseases, such as virulence, biofilm formation, and bacterial resistance to several antibiotics. QS interference could involve signal degradation (quorum quenching) or signal overproduction (pathogen confusion) [64, 65]. Quorum quenching is a mechanism adopted by a number of bacteria to disrupt QS signaling of competitors, affording these organisms an advantage within a particular habitat. A recent study has shown that bacterial members of the autochthonous microbiota of citrus leaves displayed a great ability to disrupt quorum sensing in Xcc, thus drastically reducing the symptoms and severity disease of citrus canker in susceptible host [66].

The bacterium Xcc has evolved a regulatory system to adapt the expression of virulence factors. As mentioned above, the *rpf* gene cluster, which encodes components of cell-cell communication system termed QS, rules a complex and hierarchic regulatory network, which enables the bacteria to express virulence and adaptation genes in a coordinated fashion in accordance with a population density. DSF/Rpf system regulates the expression of almost 180 genes. The biological functions performed by the gene products include chemotaxis and motility, adhesion, stress tolerance, transport, and detoxification [67]. DSF/Rpf system plays a major role in the initial attachment and fitness of Xcc to leaf surface. The leaf surface is considered a restrictive and hostile habitat for the bacterial colonist. Nutrient limitations, sudden temperature changes, and relative humidity are some of the factors that determine the leaf microbiota [68]. Attachment to the leaf surface and colonization is critical aspects of the early stage of pathogenesis [49]. Intriguingly, DSF signaling in Xcc positively regulates five genes encoding cell surface attachment structures such as adhesins (hmsHR) and fimbria (pilM); it plays a crucial role in a biofilm formation [67]. In our recent work, we have shown that the *rpfF* deletion mutant strain of Xcc 306 exhibited an inability to form a well-established biofilm in abiotic surfaces. Moreover, the scanning electron microscopy (SEM) showed direct evidence about the impaired bacterial attachment ability and lack of biofilm formation of Xcc 306 to the leaf surface. Because of this, a drastic reduction of citrus canker symptoms was displayed in susceptible hosts [66]. SEM assay similarly showed that more wild-type cells are taken the depressions between epidermal cells and around stomata than the QS mutants.

The DSF/Rpf QS system is also prerequisite for the full virulence of Xcc after entering the host, at the mesophyll tissue. Deletion mutants rpfF, *rpfC*, and *rpfG* genes displayed reduction in the symptom development in Duncan grapefruit leaves, when these strains were inoculated by infiltration pressure at a final concentration of 10^4 CFU/ml. The QS mutants displayed also impairment in motility, and extracellular protease production [67]. DSF/Rpf QS system physically interacts with proteins enclosing a GGDEF domain [69]; this domain possesses diguanylate cyclase activity associated to the synthesis of bacterial second messenger cyclic-di-GMP [70]. GGDEF domain controls motility but not other DSF-mediated phenotypes in *X. campestris* pv. *campestris* [59]. Finally, DSF/Rpf QS system radically regulates the expression of several components of protein secretion systems T2SS and T3SS through direct effect in the cellular concentration of cyclic-di-GMP and its consequent bind/release of the global transcriptional activator Clp [63].

6. Conclusions

Inhibition of quorum sensing can lead to certain phenotypic alterations, such as virulence reduction, reduced biofilm formation, and increased bacterial sensitivity to treatments. All these traits have implication in a severe symptomatology reduction. Interruption of DSF signaling in *X. citri* subsp. *citri* may lead to a downregulation of genes that encode cell surface attachment structures such as adhesins and fimbria, disturbing biofilm formation and epiphytic fitness, factors that are critical in the early stages of pathogenicity. Besides, other bacterial traits essential for disease development are directly or indirectly regulated by DSF/Rpf QS system in Xcc, and such traits are the production of extracellular enzymes and effectors.

Because QS or cell-cell signaling control processes associated with virulence of many pathogens, interference with these processes may afford a route toward disease control. Such interference could involve signal degradation (quorum quenching) or signal overproduction (pathogen confusion). Design and implementation of strategies to disrupt QS in Xcc may represent a highly valuable tool in the process of biological control and offer an alternative to the traditional copper treatment currently used for the treatment of citrus canker disease, with significant environmental, economic, and health implications worldwide.

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Section 3

Pre-Harvest Management of Diseases and Insects

Chapter 5

Synergistic Effect of a Mixture of Benzimidazole and Iminoctadine Triacetate for the Preharvest Control of Benzimidazole-Resistant *Penicillium digitatum*, a Causal Agent of Citrus Green Mold in Japan

Nobuya Tashiro and Mizuho Nita

Additional information is available at the end of the chapter

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Abstract

Green mold, caused by *Penicillium digitatum*, is the leading cause of citrus decay in Japan. Due to a ban on the post-harvest fungicide application in Japan, the preharvest application of benzimidazoles has been used and demonstrated good efficacy since 1971. A benzimidazole resistant *P. digitatum* strain was first isolated from a packinghouse in 1974, and more cases were reported in subsequent years. On the other hand, very few cases were reported from the grove for two decades. However, by the mid-1990s, when the field incidences of benzimidazole resistant strain started to increase, the effect of benzuimidazoles became unstaible. An alternative to a benzimidazoles, iminoctadine triacetate, exhibited good antifungal activity against *P. digitatum in vitro*, but its efficacy was inconsistent in the field. We examined the efficacy of a mixed application of iminoctadine triacetate and benzimidazoles against each fungicide by itself based on five years of data from multiple locations. The results indicated a synergistic suppression on green mold, where the efficacy of the mixture was consistently greater than treatments with either fungicide alone. The improved efficacy was considered acceptable for a practical use by the industry, and lead to a development of a pre-mixed commercial product, Beftopsin flowable in 2006.

Keywords: benzimidazole, benzimidazole-resistant, citrus green mold, iminoctadine triacetate, preharvest control, synergistic effect

1. Introduction

Postharvest diseases of citrus, particularly on the Satsuma mandarin orange (*Citrus unshiu*), including two very popular cultivars the mid-to-late-maturing citrus (Siranuhi; ((*C. unshiu* × *C. cinensis*) × *C. reticulata*) and Kiyomi-tangora (*C. unshiu* × *C. cinensis*)), cause serious problems at the production, distribution, and retail in Japan. Nowadays, Japanese consumers have very high expectations when it comes to food safety, and they have almost zero tolerance toward "rot" and "mold" on Satsuma mandarine oranges or any other fruit crops. Moreover, the identity of the production location (it could be as big as a region or as small as a town or individual farm) is often used as a part of marketing tools to ensure the quality of agricultural commodities in Japan. For example, it is not uncommon to see a picture of the grower on the package of fruits or vegetable. This works in favor, if the production location is producing high-quality products, but on the other hand, even a single rotten fruit in a shipment box can result in an unfavorable evaluation directly toward the production location. This may result not only in the price reduction of all fruits shipped from this location but also in the worst-case scenario result in a halt of the entire transactions and shipments of fruits from the region associated with the location. Therefore, it is not an exaggeration to say that a single rotten fruit can affect the future of a whole production region. Thus, if any rotten fruits are included among those shipped to the market, the particular shipment is often not distributed. In order to achieve this level of stringent quality control, robust countermeasures against postharvest diseases are needed.

In this chapter, the problem of fungicide-resistant strains associated with the occurrence of citrus green mold (caused by *P. digitatum*) in Satsuma mandarin orange, which causes the worst damage among Japanese postharvest diseases, is discussed based on the data from a previous study conducted by the author [1]. Then, the effectiveness of a tank-mix combination of fungicides as a preventative application against citrus green mold is explained based on another study conducted by the author [2]. Some of data from these previous studies were reanalyzed to meet the current standard.

2. Postharvest diseases in citrus cultivated in Japan

Several postharvest diseases affect citrus fruits (**Figure 1**). Among these diseases, green mold (caused by *P. digitatum*) [3], sour rot (caused by *Geotrichum candidum*) [4], blue mold (caused by *Penicillium italicum*) [3], Aspergillus rot (caused by *Aspergillus niger*, etc.) [5, 6], and anthracnose (caused by *Colletotrichum gloeosporioides*) [7] cause the greatest damage. Damage by Aspergillus rot of Satsuma mandarin orange becomes the serious problem in vinyl greenhouse in which the temperature may rise to more than 30°C in high summer season between July and September. Outbreak of sour rot is common in very early maturing Satsuma mandarin orange (VEMS) that is harvested in September when temperature is still high. Anthracnose is a common issue with very early maturing and early maturing Satsuma mandarin orange, which are harvested during the late-September to mid-October. The mid-maturing Satsuma mandarin orange or mid-to-late-maturing citrus are usually harvested during the late-November to mid-January and then stored in a room temperature (5–15°C) until shipment. When these fruits are stored for more than 3 months, Phomopsis stem-end rot (caused by *Diaporthe citri*) [8], whisker mold (caused by *Penicillium ulaiense*) [9], and Rhizopus rot (caused by *Rhizopus stolonifer*) [10] may occur. When it rained heavily or the tropical storm (typhoon) hit the production area before the harvest period, brown rot (caused by *Phytophthora* spp.) [11, 12] may occur frequently. Green mold and blue mold become the problem in all the Satsuma mandarin oranges and the mid-to-late-maturing citruses. Green mold tends to appear as fruits matured, and during storage period, and rate of blue mold outbreak can be very high during the middle to the late period of the storage, e.g., after 3–4 months in the storage.

Figure 1. Symptoms of citrus fruit rots mainly occurred in Japan; A: green mold caused by *Penicillium digitatum*, B: green mold (left) and blue mold (right) caused by *Penicillium italicum*, C: blue mold, D: whisker mold caused by *Penicillium ulaiense*, E: aspergillus rot caused by *Aspergillus niger*, F: sour rot caused by *Geotrichum candidum*, G: anthracnose caused by *Colletotrichum gloeosporioides*, H: brown rot caused by *Phytophthora palmivora*, I: black rot caused by *Alternaria citri*, and J: rhizopus rot caused by *Rhizopus stolonifer*.

Excluding anthracnose and brown rot, each of these pathogens infects plants through wounds on rind tissues, causing the fruit to rot. Development of anthracnose can be promoted by a damage on rind tissues, but wounds were not necessary to cause infection. The increase in problems associated with postharvest diseases of citrus in recent years is partially due to a strong consumer preference for high sugar and low acid contents. This demand has driven the production of ripe fruits with a thinner, bruise-prone rind. Because many postharvest citrus diseases take advantage of damages on rind tissues, the prevention of postharvest diseases on ripe fruits becomes more difficult.

3. Factors influencing the occurrence of postharvest diseases and prevention

The occurrence of postharvest diseases, such as green mold, is related to three major factors: (i) ripe fruit that rots easily, (ii) the presence of pathogens and its infection condition, and (iii) insufficient efficacy of fungicides. Several environmental and cultivation conditions affect these factors (**Figure 2**). The market demand for riper fruit results in more green mold susceptible fruits; furthermore, these fruits are more susceptible to damage to the surface of fruits, which can be caused by rough handling, contaminations in a harvest container such as dead twigs, cut peduncles, and very small pebbles [13] or by peduncles attached to fruits that were cut too long [14].

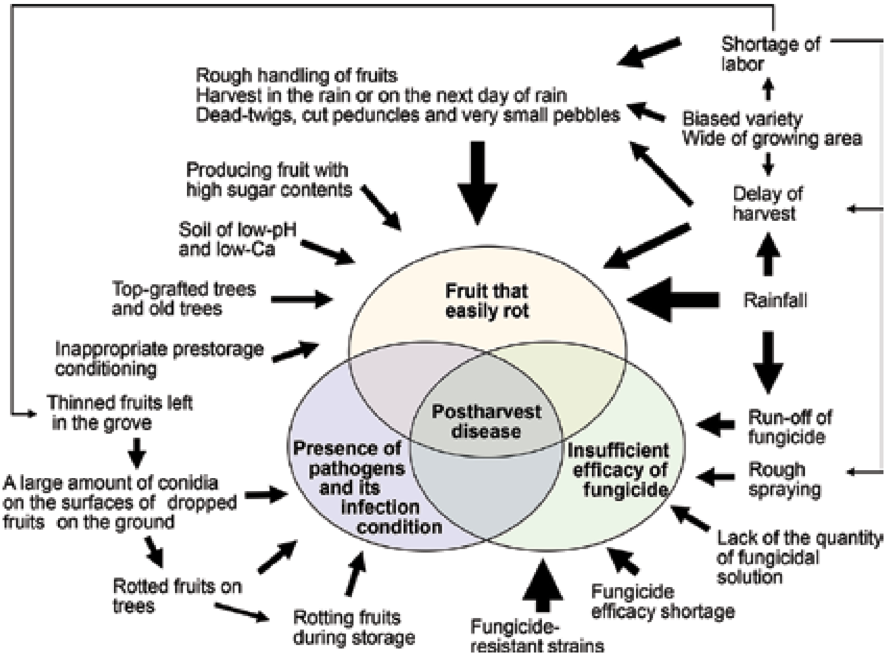


Figure 2. Factors influencing the occurrence of postharvest disease, especially citrus green mold in Japan.

It is safe to assume *P. digitatum* is a common pathogen in groves; thus, we may not have means of control over the presence of the pathogen; however, conditions to promote production of conidia that differ among groves may also differ among locations. For example, if sanitation measurements were not practiced, thinned fruits and fruits dropped by the animal and insect feedings can be the inoculum source, which can produce a large number of conidia in early autumn prior to harvest. Insufficient efficacy of fungicides maybe due to the emergence and increase of fungicide-resistant strains, lack of fungicide application and/or coverage, compromised spray coverage due to precipitations, and/or uneven adhesion of fungicides.

Presence of all three of these major factors is required for fruit rot development. For example, even if the surface of a fruit is damaged by rough handling, the fruit would not rot in the absence of a pathogen. Even if a damaged fruit is exposed to a pathogen, an effective fungicide would prevent rot. Therefore, if the effect of one of these three factors can be eliminated, postharvest diseases can be prevented. However, it is difficult to completely eliminate any of these factors because not only these three factors are influencing each other, but also each is highly depended on so many other external factors to form complex relationships (**Figure 2**). Therefore, rather than aiming for the elimination, it is important to aim for sufficient reduction of these three factors so that incidences of rotten fruits can be suppressed.

4. Basis for the implementation of cultural measures against postharvest diseases

4.1. Relationship between precipitation and postharvest diseases

Precipitation during harvest sharply increases the number of rotten fruits both at harvest and postharvest [15]. Precipitations not only make fruits more susceptible to rot [16], but it also washes fungicides off plants [17] to limit their function. In addition, many plant pathogens require water for their dissemination and penetration into plant tissues. However, when plants are grown outdoors, precipitation cannot be artificially controlled; thus, other than site selection and cultural practices to increase airflow in groves, our options against the precipitation are very limited.

4.2. Relationship between fruit cultivar, tree age, and postharvest diseases

Based on an analysis of fruits shipped from approximately 3200 groves, postharvest diseases tend to occur more frequently with fruits harvested from older trees than relatively young trees, irrespective of cultivar. The rate of rotten fruits did not differ between seedlings and top-grafted trees younger than 30 years but was higher with top-grafted trees 30 years or older [15]. These findings indicate the influence of age and type of grafting, and stricter countermeasures are probably necessary for top-grafted grove of 30 years or older. The other potential tools for green mold management may be derived from understandings of tree properties and soil conditions that promote the occurrence of rotten fruits, but further researches are needed.

4.3. Importance of the source of infection and the control of disease via the removal of the source

The quantity of spores from green mold fungus scattered in groves can be substantially reduced by removing fruits affected by green mold prior to the harvest. By thinning fruits and removing all fruits with sign of spore formation prior to the harvest, the occurrence of rotten fruits can be reduced [13]. For example, following the final thinning of fruits, some growers completely remove debris from their groves. This is a cultural measure that needs to be actively promoted in the future.

5. Importance of fungicide control

Damage to the surface of fruits, which often happen during the processes of harvest, transportation, and sorting, is the only means of infection for *P. digitatum* and *P. italicum*. Careful handling of fruits minimizes damage and can prevent rot. However, despite the awareness of this issue and availability of instructions for the careful handling of fruits to pickers, good cultural practices are not commonly implemented. All the tree fruit harvests in Japan are performed by manual labor, and the lack of skilled labor when they are needed is generally the cause for rough handling. It can be due to a lack of manpower to cover large growing areas and/or uneven distribution of workload owing to a concentrated production of a specific market-favored variety. Furthermore, autumn is a season for tropical storms in Japan. These adverse weather conditions during or close to the harvest period can cause delays, and then force harvesting to be completed within an extremely short time frame, resulting in very little care during fruit handling. Therefore, to account for realistic conditions at harvest time, control measures must be developed based on the assumption that the rind will be damaged; hence, preharvest fungicide sprays become important.

6. Fungicide use to prevent postharvest diseases and the development of resistant strains

The fungicide with a superior effect against the green mold and the blue mold has not existed until the latter half of 1960s. Thus, by 1971, soon after the introduction of benzimidazoles, it became common to apply a benzimidazole (benomyl or thiophanate-methyl) immediately prior to harvest in Japan. In Japan, the use of fungicide to harvested fruits is prohibited by the Agricultural Chemicals Control Act. Therefore, unlike countries in which harvested fruits are drenched or dipped in fungicides in packinghouses in order to prevent the rotting of fruits during storage or transportation [3,18–20], the only chemical control measure available in Japan is fungicides that are sprayed in groves prior to harvest. The preharvest spray of benzimidazoles was shown to have a favorable suppressive effect on many postharvest diseases, particularly green mold [21–30]. Furthermore, iminoctadine triacetate [31, 32], which has a different mode of action from that of benzimidazoles, became available in 1985. Presently, seven preharvest fungicides have been registered to prevent postharvest diseases in Japan, but these two fungicides (i.e., iminoctadine triacetate and benzimidazoles) are the only ones in use. Compared to other mode of action groups, their effects are consistent, and costs are relatively low. However, compared to many other diseases of citrus or other crops that are typically managed using four or five modes of action, the use of just two fungicides for citrus postharvest disease increases the risk of resistant strain development.

Benomyl, (methyl 1-(butylcarbamoyl) benzimidazol-2-yl-carbamate, is a systemic benzimidazole fungicide discovered by DuPont in the early 1960s (rates and other information are summarized in **Table 1**). It prevents cell division in sensitive fungi by binding to tubulin and inhibiting the formation of spindle fibers at metaphase and is highly active at low concentrations against spore germination and mycelial growth. Further, locally systemic properties enable the fungicides to penetrate the host tissue and provide post-infection curative action. It was first registered under the brand name Benlate (50% wettable powder) by DuPont in Japan in 1971 and Sumitomo Chemical Co., Ltd. (Tokyo, Japan) acquired the business in 2002. Benomyl is a very broad-spectrum fungicide with low phytotoxicity and controls many fungi in the classes Ascomycetes, Deuteromycetes, and Basidiomycetes. It has long been used for wide range of crop groups, notably for citrus as a postharvest diseases control agent with its strong rainfastness and residual activity.

Generic name	Trade name	FRAC code	Active ingredient (%)	Company, city, country	Rate applied (μg/mL)
Benomyl	Benlate wettable powder	1	50.0	Sumitomo Chemical Co., Ltd., Tokyo, Japan	125
Thiophanate-methyl	Topsin-M wettable powder	1	70.0	Nippon Soda Co., Ltd., Tokyo, Japan	350
Iminoctadine triacetate	Befran 25 liquid	M7	25.0	Nippon Soda Co., Ltd., Tokyo, Japan	125

The spray volume for preharvest application was 500 L/10 a.

Table 1. Common name of each fungicide tested, their corresponding trade names, active ingredient percentages, manufacturers, and preharvest application rates.

Thiophanate-methyl, dimethyl 4,4′-(*o*-phenylene) bis(3-thioallophanate), is a methyl benzimidazole carbamate fungicide with preventive and curative effects (FRAC group 1). Thiophanate-methyl is converted to MBC (methyl benzimidazol-2-ylcarbamate) on the plant surface and in tissues, and MBC inhibits β-tubulin assembly during mitosis in fungi. Its spectrum includes Basidiomycota, Ascomycota, and imperfect fungi. It was first registered in Japan in 1971 under the brand name Topsin-M (70% wettable powder; Nippon Soda Co. Ltd. (Tokyo, Japan) as a pre-harvest spray targeting blue mold, green mold, and stem-end rot on citrus. Long-lasting effects are high because they exhibit penetrative and systemic activities.

Iminoctadine triacetate, 1,1′-iminodi (octamethylene) diguanidinium triacetate, is a bis-guanidine with preventive effects (rates and other information are summarized in **Table 1**). It is classified as a multi-site contact activity fungicide and is generally considered to have a low resistance risk according to the FRAC (group M7). Its mode of action involves cell membrane transport and sterol biosynthesis at different sites via C14-demethylase in sterol biosynthesis inhibitors. Owing to these unique mechanisms of action, it is a good tool to manage resistance to fungicides with various modes of action, e.g., benzimidazoles, dicarboximides, C14-demethylase inhibitors, Qo inhibitors, and succinate dehydrogenase inhibitors. Its spectrum includes Ascomycota and imperfect fungi, and it inhibits spore germination, germ tube elongation, and appressorium and infectious hypha formation in the life cycle of pathogens. It was first registered in Japan in 1983 under the brand name Befran (25% liquid; Nippon Soda Co., Ltd.) as a preharvest spray targeting blue mold, green mold, Alternaria rot, and sour rot on citrus. However, the efficacy of this chemical on stem-end rot is lower than benzimidazoles, which shows a superior effect. Since it is a contact fungicide, spraying just before harvest is optimal.

6.1. Occurrence and spread of green mold fungus resistant to benzimidazoles

The extensive usages of benzimidazoles have led to the development of benzimidazole-resistant strains (BRS) of *P. digitatum* and *P. italicum*, which was first discovered in Kanagawa and Shizuoka prefectures in 1974 [33, 34]. Since then, BRS have been found in Saga prefecture [35] and other major producing areas [36, 37]. Concern about a decline in control effects owing to BRS has prompted many studies.

6.2. Despite BRS incidences, the reduction of benzimidazoles' efficacy was not reported prior to 1990s

These studies found that the incidence of BRS in groves sampled immediately after spraying with was either very low or nondetectable [37, 38]. Among early maturing Satsuma mandarin orange, which is shipped immediately after harvest or after a short period of storage, there was no decrease in control efficacy even after the development of BRS [37, 38]. In some varieties, such as mid-maturing Satsuma mandarin orange and mid-to-late-maturing citrus that are shipped after storage, the incidence of BRS tends to gradually increase during storage [35]. However, if the damages on the fruit surface are limited, the disease incidence does not increase quickly [37]. Moreover, there were reports of BRS in producing areas nationwide [39], but these occurrences were not severe enough to cause actual damage. Thus, despite reported cases of BRS, the efficacy of benzimidazoles was considered to be maintained at the acceptable level [39]. Therefore, it was concluded that the efficacy of benzimidazole-based chemical management was not decreased by BRS [39].

6.3. BRS is already present in groves prior to harvest

High numbers of BRS isolates were observed after an outbreak of green mold in harvested greenhouse Satsuma mandarin orange fruits in Saga prefecture in late August of 1993. Subsequently, a survey of fruits from packinghouses in Saga was conducted [1] and resulted in a large number of thiophanate-methyl–resistant strains (**Table 2**). Without the host, *P. digitatum* conidia can survive in the packinghouse for 1 month or more in the winter; however, they can only survive for about 5 days during summer months [40]. Thus, it is less likely that packinghouses were contaminated when new crops were brought in a packinghouse in late August to mid-October. This further confirms that resistant fungi were already present at the harvest whether they came from greenhouses or groves.

Location of packinghouse	Source of isolate[2]	Rate of resistant strains[3]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–200	>1600
Hamatama	SGPG	14/14	0	0	14
Chinzei	VEMS	5/5	0	0	5
Tara	VEMS	11/11	0	0	11
Omachi	VEMS	13/14	1	0	13
Ogi	VEMS	5/15	10	2	3
Total		48/59 (100)[4]	11 (19)	2 (3)	46 (78)

[1] The investigations were performed from end August to beginning of October in 1993. Original data for the table are from [1], adapted with a permission from the authors.

[2] SGPG, Satsuma mandarin orange grown in a vinyl greenhouse; VEMS, very early maturing Satsuma mandarin orange.

[3] No. of resistant strains (MIC > 1.56 μg/mL)/(no. of strains tested).

[4] Values in parentheses are the percentages of the total strains in the category.

Table 2. Thiophanate-methyl sensitivity of *Penicillium digitatum* strains isolated from Satsuma mandarin orange at various packinghouses in Saga prefecture in Japan.[1]

6.4. Confirming cases of decreased control effects by resistant strains

In order to determine the presence of BRS, a series of experiments were conducted from 1993 to 1995 [1]. Fruits were artificially damaged just before harvest to promote development of green mold. Throughout the experiment, benzimidazoles were not sprayed. *P. digitatum* was then isolated from diseased fruits and examined for sensitivity to a benzimidazole (Thiophanate-methyl). Overall, highly resistant strains were detected at a high frequency at vinyl greenhouse (**Table 3**) and at grove (**Table 4**).

Year investigated	Location of grove sampled	Rate of resistant strains[1]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–100	>1600
1993	Hamatama-1	15/18	3	0	15
	Hamatama-2	23/23	0	0	23
	Total	38/41 (93)	3 (7)	0 (0)	38 (93)
1994	Hamatama-1	15/28	13	0	15
	Hamatama-2	15/26	11	0	15
	Total	30/54 (56)	24 (44)	0 (0)	30 (56)
1995	Hamatama-1	18/52	34	0	18
	Hamatama-2	11/32	21	0	11
	Total	29/84 (35)	55 (66)	0 (0)	29 (34)

[1] Number of resistant strains (see **Table 2**)/total number of strains tested. Values in parentheses are the percentages of the total for each category. Original data for the table are from [1], adapted with a permission from the authors.

Table 3. The number of thiophanate-methyl resistant *Penicillium digitatum* strains isolated from Satsuma mandarin orange grown in plastic greenhouses.

Year investigated	Location of grove sampled	Rate of resistant strains[1]	Number of strains for each MIC range (μg/mL)		
			≤0.78	1.56–200	>1600
1993	Tara	22/25	3	10	12
	Oura	40/46	6	7	33
	Kashima	26/26	0	5	21
	Ogi	10/10	0	6	4
	Yamato	24/38	14	11	13
	Kagami	29/39	10	9	20
	Total	151/184 (82.1)	33 (17.9)	48 (26.1)	103 (56.0)
1994	Tara	2/9	7	0	2
	Oura	2/24	22	0	2
	Kashima	6/13	7	3	3
	Ogi-A	7/14	7	0	7
	Hamatama-A	14/23	9	3	11
	Total	31/83 (37.3)	52 (62.7)	6 (7.2)	25 (30.1)
1995	Tara	5/15	10	0	5
	Oura	11/16	5	7	4
	Kashima	5/12	7	1	4
	Yamato	0/12	12	0	0
	Kitahata	18/45	27	0	18
	Ogi-B	8/29	21	0	8
	Hamatama-A	2/22	20	0	2
	Hamatama-B	3/13	10	0	3
	Kyuragi	2/17	15	0	2
	Total	54/181 (29.8)	127 (70.2)	8 (4.4)	46 (25.4)

[1] Number of resistant strains (see **Table 2**)/total number of strains tested. Values in parentheses are the percentages of the totals for each category. Fruits were artificially wounded prior to applications of treatment. Original data for the table are from [1], adapted with a permission from the authors.

Table 4. The number of thiophanate-methyl resistant *Penicillium digitatum* isolated from Satuma mandarin orange grown in groves.

Furthermore, another experiment was conducted in a total of six groves to examine the effect of wounding and fungicide treatments [1]. Three groves, each consisted of the one cultivar, were examined in 1994 and 1995. The experimental design was a split-plot design where the main block consisted of two wound treatments (artificial wounding or not) and the subplots consisted of three fungicide treatments (thiophanate-methyl, iminoctadine triacetate, and non-treated control). The experimental unit was a fruit, and 50 fruits per subplot were examined. Based on the data from this study [1], a generalized linear mixed model (GLMM) [41] (PROC GLIMMIX, SAS ver 9.4, SAS Institute, Cary, NC, USA) was utilized to reexamine the effects of grove, wounding, and fungicide treatments on the probability of green mold disease incidence per fruit. Grove, wounding, and fungicide were considered as a fixed effect, and a logit was used as a link function.

The number of rotted fruit ranged from 0 to 40 out of 50 fruits examined per subplot (**Table 5**). The results from a GLMM showed significant grove ($F = 37.5$, $P < 0.01$), wounding ($F = 117.8$, $P < 0.01$), fungicide ($F = 12.1$, $P < 0.01$), and wounding × fungicide interaction ($F = 5.6$, $P = 0.01$). The interaction between wounding and fungicide treatment showed that all the fruits with wounding treatment resulted in higher probabilities ($P \leq 0.05$) of rotted fruit than nonwounded fruits; however, the effect of fungicide treatment differed between wounding and nonwounding treatments. With wounding treatment, fruits treated with thiophanate-methyl and nontreated control resulted in significantly higher probabilities of rotted fruit ($P < 0.05$) than iminoctadine triacetate treatment (**Table 6**). On the other hand, with nonwounding treatment, both thiophanate-methyl and iminoctadine triacetate treatments resulted in significantly lower probabilities of rotted fruit ($P < 0.05$) than nontreated control (**Table 6**). The results indicated the importance of a wounding event to the development of green mold, as well as the lack of efficacy provided by thiophanate-methyl.

Treatment	Fungicide and rate	Number of rotted fruit/number of fruit investigated					
		Kashima	Ogi-1	Oura	Kyuragi	Tara	Ogi-2
No-wound	Control	0/50	14/50	0/50	4/50	2/50	0/50
	Thiophanate-methyl 350 μg/mL	0/50	3/50	1/50	1/50	0/50	0/50
	Iminoctadine triacetate 125 μg/mL	0/50	1/50	0/50	0/50	1/50	0/50
Wound[1]	Control	12/50	40/50	7/50	34/50	8/50	4/50
	Thiophanate-methyl 350 μg/mL	15/50	15/50	6/50	40/50	38/50	5/50
	Iminoctadine triacetate 125 μg/mL	5/50	4/50	3/50	30/50	7/50	0/50
	Number of resistant strains	**(6/13)**[2]	**(7/14)**	**(2/24)**	**(2/17)**	**(5/15)**	**(8/29)**

[1] The fruits were damaged at three places per 1 fruit to 2 mm depth of the rind of a fruit using the inoculation tool which three setting pins were bundled up. Original data for the table are from [1], adapted with a permission from the authors.

[2] Boldfaced ratios: number of resistant strains (MIC ≥ 1.56 μg/mL, see **Table 2**)/number of strains tested. Kashima, Ogi-1, and Oura were tested in 1994, and Kyuragi, Tara, and Ogi-2 were tested in 1995.

Table 5. The efficacy of a preharvest application of thiophanate-methyl and iminoctadine triacetate on green mold fruit rot in groves of very early maturing Satsuma mandarin orange, and the detection frequency of BRS from each grove.

These findings differed from previous results indicating that the incidence of BRS is extremely low in groves prior to the harvest [37, 38]. When harvesting greenhouse Satsuma mandarin orange, very early maturing Satsuma mandarin orange, and early maturing Satsuma mandarin orange, BRS were present at a high rate and thus many cases of postharvest disease were observed.

Wound	Fungicide and rate	Mean probability of rotted fruit[1]	Standard error
No-wound	Control	0.042 C	0.010
	Thiophanate-methyl 350 μg/mL	0.010 D	0.004
	Iminoctadine triacetate 125 μg/mL	0.004 D	0.003
Wound	Control	0.320 A	0.031
	Thiophanate-methyl 350 μg/mL	0.380 A	0.033
	Iminoctadine triacetate 125 μg/mL	0.118 B	0.019

[1] The analysis of wound and fungicide treatment effects on the mean probability of rotted fruit was conducted using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4). A significant wounding and fungicide treatment interaction was observed ($F = 5.6$, $P = 0.01$). The estimated mean probabilities of rotted fruit and standard errors from the model are shown in the table. The different letters following two numbers indicate these two treatments are significantly ($P \leq 0.05$) different from each other, based on Fisher's LSD. The original data from [1] were re-analyzed with a permission from the authors.

Table 6. The effect of wounding and preventative application of thiophanate-methyl and iminoctadine triacetate on the probability of green mold fruit rot in groves of very early maturing Satsuma mandarin orange using the pooled data from **Table 5**. Values followed by different letters differed significantly.

The high rate of BRS during the harvest of greenhouse Satsuma mandarin orange and very early maturing Satsuma mandarin orange may be explained by the continuous use of fungicides over 20 years. BRS and benzimidazole-sensitive strains of *P. digitatum* did not differ with respect to virulence, lesion expansion, and sporulation [38]. This lack of competitive fitness cost most likely promoted increase in the number of BRS under continuous uses of benzimidazoles. In addition, recent increases in the use of the vinyl-greenhouse for production of Satsuma mandarin oranges changed seasonal availability of *P. digitatum* spores. With the traditional outdoor growing practices, Satsuma mandarin orange fruits were not ripe enough for *P. digitatum* to produce spores during the hot summer. However, the vinyl-greenhouse system allows a long harvest period of Satsuma mandarin oranges from April to September; thus, when very early maturing Satsuma mandarin orange in the outdoor field are ready for harvest in the mid-September, they will be exposed abundant spores produced in the vinyl-greenhouse production. Moreover, these isolates from the vinyl-greenhouse production are most likely exposed to a benzimidazole; thus, fruits from the outside field can be exposed to a high number of BRS even prior to the application of a benzimidazole.

6.5. Increasing of BRS after benzimidazole spraying in groves

Another experiment was conducted in 1995 using three groves where detection frequencies of BRS were examined before and after application of fungicide treatments [1]. Prior to the experiment, the detection frequencies of resistant strains were low (12–33%, **Table 7**) and not significantly different among groves ($\chi^2 = 2.3$, $P = 0.3$, reanalyzed using the data from [1], PROC FREQ, SAS ver. 9.4) (**Table 7**). Five trees were treated with each treatment, and fruits with symptoms of green mold were examined for the detection of BRS. The effect of grove and fungicide treatment on the probability of BRS detection after fungicide treatment application was reexamined using a GLMM with a logit as the link function. A significant effect of fungicide on the probability of detecting BRS was found ($F = 20.9$, $P = 0.01$; **Table 8**), but the grove effect was not significant ($F = 1.9$, $P = 0.26$). Thiophanate-methyl treatment resulted in a higher probability of detecting BRS than that of iminoctadine triacetate or nontreated control treatments. The high recovery rate of BRS despite the small number of samples indicates the abundance of BRS conidia in these groves.

Grove[2]	Fungicide	Rate applied	Detection ratio of resistant strains[3]	
			Before spraying[4]	After spraying
Kyuragi	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	10/12
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	1/16
	No fungicide	–	2/17 A	0/10
Tara	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	31/31
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	2/11
	No fungicide	–	5/15 A	4/10
Ogi-2	Thiophanate-methyl 70% wettable powder	350 μg/mL	–	8/8
	Iminoctadine triacetate 25% soluble concentrate	125 μg/mL	–	0/1
	No fungicide	–	8/29 A	1/4

[1] Investigation was performed in 1995. Original data for the table are from [1], adapted with a permission from the authors.

[2] See **Table 5** regarding the preventive effect of fungicides in the groves.

[3] The number of the resistant strains (see **Table 2**)/the number of strains tested. Fruits of very early maturing Satsuma mandarin orange were artificially wounded to promote fungal infection.

[4] There is no significant effect of grove on the detection ratio of resistant strain ($\chi^2 = 2.3$, $P = 0.3$, PROC FREQ, SAS ver. 9.4) prior to the application of fungicide treatment. Values followed by same letters show not significantly different.

Table 7. Detection frequency of thiophanate-methyl–resistant strains of *Penicillium digitatum* isolated at before and after the fungicide treatment from fruits of very early maturing Satsuma mandarin orange.[1]

Fungicide and rate	Mean probability of BRS[1]	Standard error
Control	0.327 B	0.102
Thiophanate-methyl 350 μg/mL	0.961 A	0.031
Iminoctadine triacetate 125 μg/mL	0.105 B	0.067

The analysis of fungicide treatment effect on the mean probability of BRS was conducted using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4). The expected mean probabilities of BRS and standard errors from the model are shown. The different letters following two numbers indicate these two treatments are significantly ($P \leq 0.05$) different from each other, based on Fisher's LSD. The original data from [1] were re-analyzed with a permission from the authors.

Table 8. The effect of application of thiophanate-methyl and iminoctadine triacetate on the probability of detection ratio of BRS, using the pooled data from **Table 7**.

Results from these series of experiment consistently show the lack of efficacy provided by thiophanate-methyl (**Tables 5** and **6**), as well as the high probability of detecting BRS from thiophanate-methyl–treated fruits (**Tables 7** and **8**). These results triggered growers to use iminoctadine triacetate, which also shown to be effective against green mold in our study (**Tables 5** and **6**).

6.6. Problems with iminoctadine triacetate

Although the use of iminoctadine triacetate shows strong antifungal activity *in vitro* against *P. digitatum* and *G. candidum*, its efficacy on green and blue mold in the field is shown to be inconsistent. As shown in our study, its efficacy in the field may not be sufficient to achieve a high level of protection, especially when fruit surfaces are damaged (**Table 6** and **Figure 3**). The same concern was discussed in studies by Koga et al. [42] and Miyoshi et al. [43].

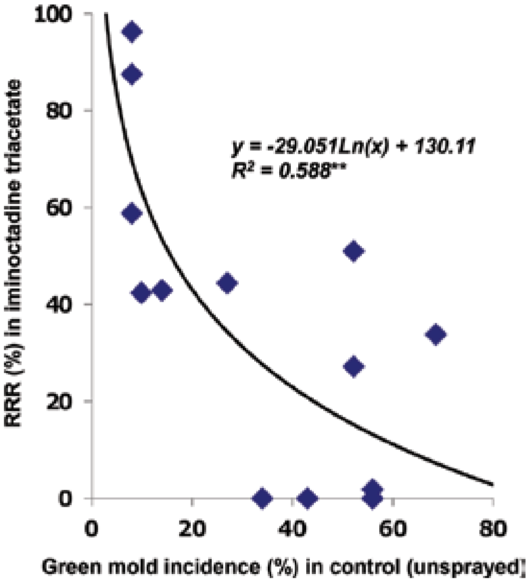


Figure 3. Relationship between the occurrence of green mold in control conditions and the preventive effect of iminoctadine triacetate. Preventive effects are based on the relative risk reduction (RRR).

7. Tank-mix combination of fungicides for resistant strains

As discussed above, there are loss of efficacy provided by benzimidazoles, and the efficacy of iminoctadine triacetate, are found to be relatively inconsistent. Alternative fungicides to replace these materials are in need; however, there is no such alternative available in Japan at 1990s.

Combining fungicides with different modes of action can be more effective compared to the separate use of a single fungicide [44–51]. There are four main reasons for considering the use of fungicide mixtures [52, 53]. (1) Broadening the target spectrum: If the aim is to control two target pathogens that differ in sensitivity to fungicide modes of action with one application, it can be useful to spray mixtures of fungicides. (2) Improved disease control: if the target pathogen is susceptible to two modes of action, a use of two effective modes of action should increase the efficacy. (3) As an insurance against resistance: if a certain population of the target pathogen develops resistance to one of the mixture components, the other mode of action in the mix can act against the resistant population. (4) Resistance management: with a combination of reasons #2 and #3, the rate of resistance development among pathogen populations can be delayed, thus, the effective life of a fungicide can be extended. For our situation, the mix of a benzimidazole and iminoctadine triacetate is considered because both are still somewhat effective against green mold. For example, benzimidazoles sometimes show preventative effects even when resistant strains are detected at a high frequency, as shown in one of our experiments (**Table 5**; Ogi-1). In addition, antifungal activity of iminoctadine triacetate *in vitro* against *P. digitatum* is high [32], and our experiment also showed a significant reduction of green mold (**Table 6**). Thus, a series of experiments were conducted to examine the efficacy of a mixture of a benzimidasole and iminoctadine triacetate [2].

7.1. Improved effects using a tank-mix combination of benzimidazole and iminoctadine triacetate

Table 9 shows the results of the effects of the combined use of 125-ppm benomyl and 125-ppm iminoctadine triacetate on incidence of green mold from different studies [2]. The preventive effect of treatments was compared based on relative risk reduction (RRR). RRR is the percent reduction in risk in the treated group (e.g., benomyl application) compared to the control group (e.g., no application). RRR = (1 − risk ratio: RR) × 100. RRR values showed that a tank-mix combination of benzimidazole and iminoctadine triacetate improved the preventive effect substantially in 11 of 12 studies. Using a meta-analysis, the total relative risk ratio was 0.23 (95% confidence interval, 0.15–0.33), as shown in **Figure 4**. Including the error range, the risk ratio was 1.0 or less; thus, the onset of disease is significantly less than that in unsprayed plants (by approximately 15–33%).

Incidences of diseased fruits in unsprayed grove block was not associated with those in the block of the combined spray, as shown in **Figure 5** ($R^2 = 0.171$). In the other words, even with the cases with higher incidences of diseased fruits in the unsprayed grove block, the preventive effect in the combined-sprayed area did not decrease, indicating that the efficacy of the combined spray was not affected by the background level of green mold rot. Moreover, regardless of the BRS detection frequency, a stable preventative effect was obtained (**Table 9**). Harmful effects, such as brown discoloration and/or delayed color development, using the combined benomyl and iminoctadine triacetate spray were not confirmed in any of the experiments.

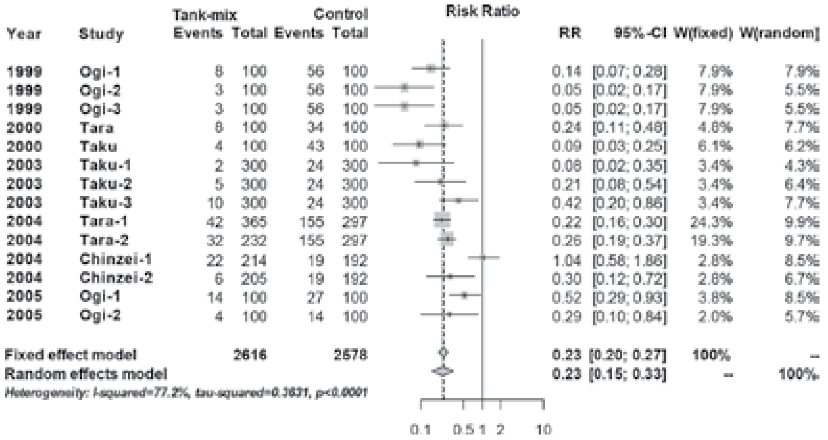
Year	Study	Spray timing (days to harvest)	Rate of BRS[1]	Fungicide and rate						Incidence of control (%)[3]
				Benomyl 125 μg/mL		Iminoctadine triacetate 125 μg/mL		Iminoctadine triacetate 125 μg/mL + benomyl 125 μg/mL		
				Incidence (%)	RRR (%)[2]	Incidence (%)	RRR (%)	Incidence (%)	RRR (%)	
1999	Ogi-1	21	4/15	55.0	1.8	55.0	1.8	8.0	85.7	56.0
1999	Ogi-2	14	4/15	18.0	67.9	60.0	0	3.0	94.6	56.0
1999	Ogi-3	7	4/15	7.0	87.5	65.0	0	3.0	94.6	56.0
2000	Tara	14	9/16	54.0	0	72.0	0	8.0	76.5	34.0
2000	Taku	7	6/10	23.0	46.5	52.0	0	4.0	90.7	43.0
2003	Taku-1	21	4/11	5.0	37.5	3.3	58.8	0.7	91.3	8.0
2003	Taku-2	14	4/11	7.7	3.8	0.3	96.3	1.7	78.8	8.0
2003	Taku-3	7	4/11	0.7	91.3	1.0	87.5	3.3	58.8	8.0
2004	Tara-1	7	–[3]	–[3]		25.6	51	11.5	78.0	52.2
2004	Tara-2	21	3/11	24.6	52.9	38.0	27.2	13.8	73.6	52.2
2004	Chinzei-1	21	–[3]	14.3	0	24.9	0	10.3	0	9.9
2004	Chinzei-2	7	–[3]	–[3]		5.7	42.4	2.9	70.7	9.9
2005	Ogi-1	7	3/11	22.0	18.5	15.0	44.4	14.0	48.1	27.0
2005	Ogi-2	7	2/14	10.0	28.6	8.0	42.9	4.0	71.4	14.0

Benzimidazole-resistant strain detection frequency before benzimidazole application; the number of the resistant strains/investigated strains.

The preventive effects are based on relative risk reduction (RRR). RRR is the percent reduction in risk in the treated group (thiophanate-methyl application) compared to the control group (no application). RRR = (1 − risk ratio: RR) × 100. Risk ratio is shown in **Table 6**. In evaluating the effect, a high RRR indicates high effectiveness.

Examination and investigations were not enforced. Original data in the table are from [2], adapted with a permission from the authors.

Table 9. Efficacy of preharvest application of benomyl, iminoctadine triacetate, and tank mix of two materials against green mold on very early maturing Satsuma mandarin orange. Harvested fruits were subjected to an artificial wounding treatment where fruits were rolled on a sloped concrete surface for 5 m.



Year	Study	Tank-mix Events	Tank-mix Total	Control Events	Control Total	RR	95%-CI	W(fixed)	W(random)
1999	Ogi-1	8	100	56	100	0.14	[0.07; 0.28]	7.9%	7.9%
1999	Ogi-2	3	100	56	100	0.05	[0.02; 0.17]	7.9%	5.5%
1999	Ogi-3	3	100	56	100	0.05	[0.02; 0.17]	7.9%	5.5%
2000	Tara	8	100	34	100	0.24	[0.11; 0.48]	4.8%	7.7%
2000	Taku	4	100	43	100	0.09	[0.03; 0.25]	6.1%	6.2%
2003	Taku-1	2	300	24	300	0.08	[0.02; 0.35]	3.4%	4.3%
2003	Taku-2	5	300	24	300	0.21	[0.08; 0.54]	3.4%	6.4%
2003	Taku-3	10	300	24	300	0.42	[0.20; 0.86]	3.4%	7.7%
2004	Tara-1	42	365	155	297	0.22	[0.16; 0.30]	24.3%	9.9%
2004	Tara-2	32	232	155	297	0.26	[0.19; 0.37]	19.3%	9.7%
2004	Chinzei-1	22	214	19	192	1.04	[0.58; 1.86]	2.8%	8.5%
2004	Chinzei-2	6	205	19	192	0.30	[0.12; 0.72]	2.8%	6.7%
2005	Ogi-1	14	100	27	100	0.52	[0.29; 0.93]	3.8%	8.5%
2005	Ogi-2	4	100	14	100	0.29	[0.10; 0.84]	2.0%	5.7%
Fixed effect model			2616		2578	0.23	[0.20; 0.27]	100%	--
Random effects model						0.23	[0.15; 0.33]	--	100%

Heterogeneity: I-squared=77.2%, tau-squared=0.3631, p<0.0001

Figure 4. The forest plots represent the integrated meta-analysis [54–58] of the effect of the tank-mix application of benomyl and iminoctadine triacetate on green mold of very early ripening Satsuma mandarin orange in 14 field trials. Data were analyzed by a meta-analysis in a random effects model using the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0). Each gray square marks the value of the risk ratio (RR) [61] of each study, and the size of the square indicates the weight of each field trial. The horizontal line indicates the 95% confidence intervals of the effect estimate of individual studies. The diamond at the bottom of the graph shows the integrated risk ratio, and the width of the diamond shows the confidence intervals for the overall effect estimate.

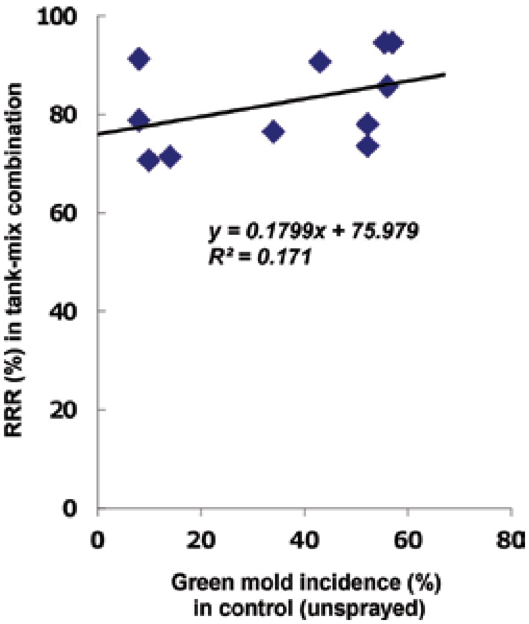


Figure 5. Relationship between the occurrence of green mold in the control and the preventive effect of the tank-mix combination of benomyl and iminoctadine triacetate.

In the comparison between the combined spray of benomyl and iminoctadine triacetate and the individual fungicides, the preventative effect increased greatly. Combined spraying with another benzimidazole, thiophanate-methyl, and iminoctadine triacetate, also substantially decreased the occurrence of rotten fruits (**Figure 6** and **Table 10**).

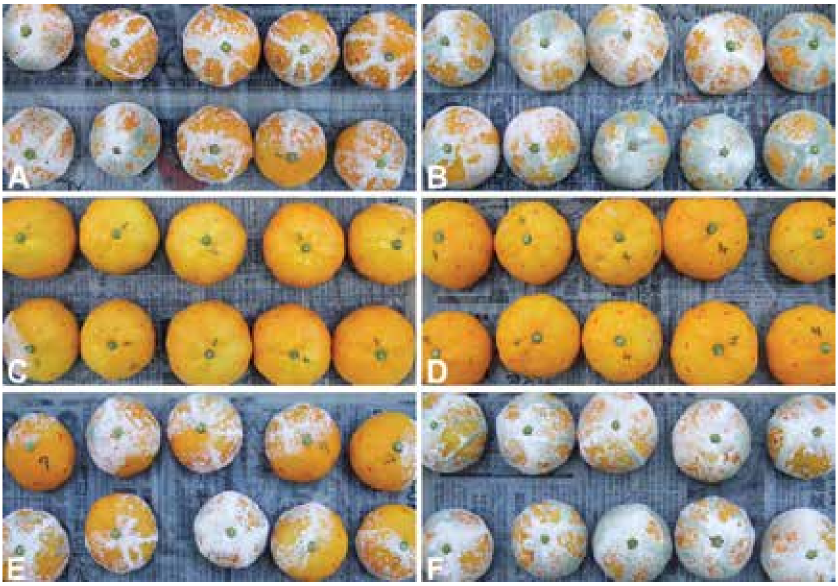


Figure 6. The efficacy of the tank-mix combination of benzimidazoles and iminoctadine triacetate for the control of green mold in very early ripening satsuma mandarin orange. A, benomyl 125 μg/mL; B, thiophanate-methyl 350 μg/mL; C, benomyl 125 μg/mL + iminoctadine triacetate 125 μg/mL; D, thiophanate-methyl 350 μg/mL + iminoctadine triacetate 125 μg/mL; E, iminoctadine triacetate 125 μg/mL; F, control. Fruits were harvested 3 days after spraying and artificially inoculated with a conidial suspension (10^6/mL) of benzimidazole-resistant *Penicillium digitatum* (EC50 of benomyl = 6.25 μg/mL, mutation in β-tubulin codon 200 of phenylalanine to tyrosine). Green mold incidence was evaluated after storage for 1 week under 98% relative humidity.

7.2. Synergistic effects of the tank-mix combination

The control effects of the combined spray were superior to the effects of each individual fungicide. Whether the effects were additive or synergistic was examined using the Colby method [62], which compared theoretical values based on an additive model and measured values. Effects are additive if the total efficacy of multiple fungicides is similar or equal to the sum of the efficacies of individual fungicide. Synergistic effects are inferred when a total efficacy of multiple fungicides is greater than the sum of the efficacies provided by two individual fungicides.

As shown in **Table 11**, eight out of 12 tests (excluding Taku-2 and Taku-3 (2003), Chinzei-1 (2004), and Ogi-1 (2005)) showed the (actual RRR/expected RRR) values larger than 1. Thus, the observed RRR value was greater than the theoretical RRR values, indicating the synergistic effect of the combined spray. In Taku-2 and Taku-3 (2003), good control was achieved in not only the combined treatment but also benomyl only and iminoctadine triacetate only

Fungicide and rate	The number of the tests	Combined risk ratio (95% CI)[2]	
Benomyl 125 μg/mL	12	0.60	(0.41–0.89)
Iminoctadine triacetate 125 μg/mL	14	0.80	(0.62–1.04)
Benomyl 125 μg/mL + iminoctadine triacetate 125 μg/mL	14	0.23	(0.15–0.33)
Thiophanate-methyl 350 μg/mL + iminoctadine triacetate 125 μg/mL	11	0.43	(0.30–0.63)

[1] Data in **Table 9** were analyzed by a meta-analysis [54–58] in a random effects model by the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0).

[2] Risk ratio (RR) was defined as: RR = $(a/A)/(b/B)$, where a is the number of rotted fruits in the fungicide application, A is the total number of fruits in the fungicide application, b is the number of rotted fruits in the control, and B is the total number of fruits in the control. RR is the ratio of the probability of disease development in the exposed group to that of the unexpected group [61]. In evaluating the effect of a fungicide application, a low RR indicates high effectiveness. The variance of RR (V) and the 95% confidence interval (95% CI) were defined as follows: $V = (1/a-1/A + 1/b-1/B)$, 95% CI = RR × exp $(\pm 1.96 \times V^{1/2})$. A prevention effect is recognized if the 95% CI of the combined risk ratio is <1.0.

Table 10. The efficacy of benzimidazoles, iminoctadine triacetate, and the tank-mix of two materials for the control of green mold in very early maturing satsuma mandarin orange.[1]

treatment. Only exception is Ogi-1 (2005), where efficacies of all treatments, including the combined spray, were low. Given the lack of efficacy of benomyl treatment, further examination of the BRS population may need for this location.

7.3. Tank-mix combination improves resistance to precipitation and aftereffects

If the growing area is large (i.e., several hectares or more), application of preharvest fungicide for postharvest rot control can be difficult. Maturity timing among outdoor Satsuma mandarin orange cultivars varies; thus, harvest can start from mid-September for very early maturing cultivars and continue to late November for the others. Therefore, a long residual effect is often a desired aspect of the preharvest fungicide. The combined spray of benomyl and iminoctadine triacetate applied 3 weeks prior to the harvest showed a sufficient level of green mold rot prevention (**Figure 7**). Also, it appeared that the effect of the combined treatment seemed not to be affected by rain. A sufficient level of efficacy was achieved even with the case where cumulative precipitation after spraying was high, e.g., about 150 mm (**Figure 8**). Thus, this combined spray has desirable a long residual activity and rain fastness. Further studies are needed to examine the residual effect under artificial precipitation to examine rain fastness of the combined spray.

7.4. Increased cost of fungicides using the tank-mix combination

As of 2016 in Saga prefecture, the costs of 500 L of benomyl is 1225 yen, and that of iminoctadine triacetate is 1662 yen, thus, the combined application costs 2887 yen. The cost increases for the combined application; however, as noted previously, the presence of rotten fruits will result in a substantial negative impact on the responsible grower as well as the related production area. Therefore, the benefits of risk mitigation far exceed the relatively small increase in the cost of fungicides. Moreover, the synergistic effect reported in this study should support growers' decision to apply these two fungicides in combination, even with the increased cost.

Year	**Study**[1]	**Actual RRR (%) in green mold compared with the control**			**Expected RRR (%)**[2]	**Actual RRR (%)/ expected RRR (%)**[3]
		Benomyl 125 μg/mL	**Iminoctadine triacetate 125 μg/mL**	**Iminoctadine triacetate 125 μg/mL+ benomyl 125 μg/mL**		
1999	Ogi-1	1.8	1.8	85.7	3.6	23.8
1999	Ogi-2	67.9	0	94.6	67.9	1.4
1999	Ogi-3	87.5	0	94.6	87.5	1.1
2000	Tara	0	0	76.5	0	∞
2000	Taku	46.5	0	90.7	46.5	2.0
2003	Taku-1	37.5	58.8	91.3	74.3	1.2
2003	Taku-2	3.8	96.3	78.8	96.4	0.8
2003	Taku-3	91.3	87.5	78.0	98.1	0.8
2004	Tara-2	52.9	27.2	73.6	65.7	1.1
2004	Chinzei-1	0	0	0	0	Not estimable
2005	Ogi-1	18.5	44.4	48.1	54.7	0.9
2005	Ogi-2	28.6	42.9	71.4	59.2	1.2

[1] See **Table 9**. Original data in the table are from [1], adapted with a permission from the authors.

[2] Determined using the formula $E = X + [Y(100-X)]/100$, where X = the percent control in plots treated with benomyl and Y = the percent control in plots treated with iminoctadine triacetate.

[3] Actual relative risk reduction (%) of iminoctadine triacetate 125 μg/mL + benomyl 125 μg/mL related to the expected relative risk reduction (%).

Table 11. Comparison of the expected and actual relative risk reduction percentages among benomyl, iminoctadine tiacetate, and a tank-mix of two materials.

7.5. Tank-mix combination is effective against green mold in varieties other than Satsuma mandarin orange

Superior disease-control effects of combined agents on green mold have been confirmed not only in Satsuma mandarin orange but also in mid-to-late-maturing citruses, such as Shiranui ((*C. unshiu* × *C. cinensis*) × *C. reticulata*) cultivated both outdoors and indoors [63].

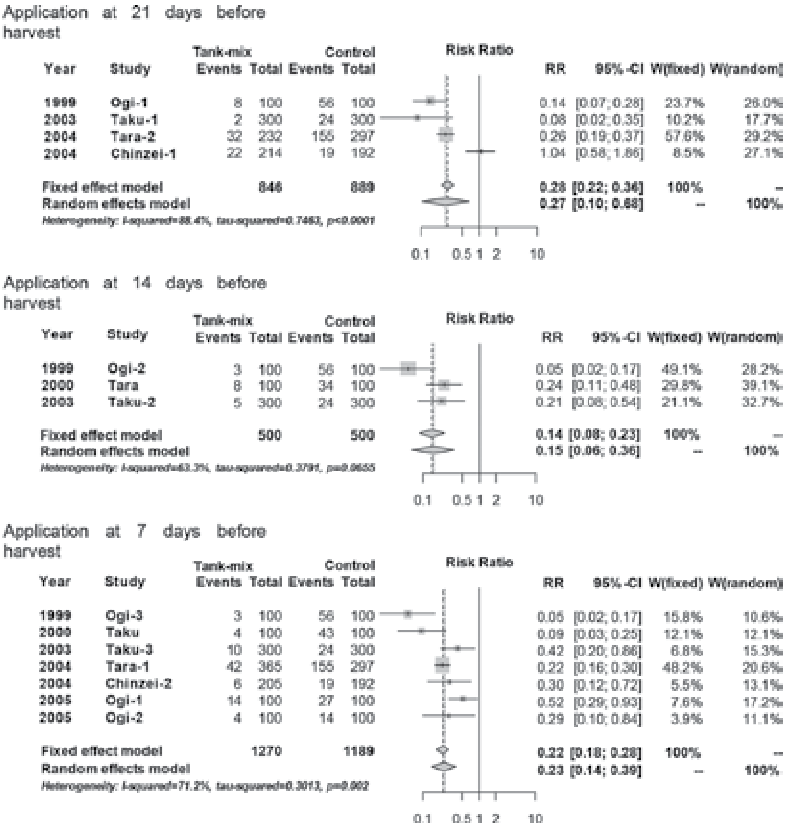


Figure 7. The forest plots (see **Figure 5**) represent the integrated meta-analysis of effect of the tank-mix application of benomyl and iminoctadine triacetate on green mold of very early ripening Satsuma mandarin orange different times. Data were analyzed by a meta-analysis in a random effects model using the DerSimonian-Laird method [56, 59]. The meta-analysis was performed using the EZR [60] graphical user interface for R software (The R Foundation for Statistic Computing, version 2.14.0).

7.6. The tank-mix combination is also effective against other postharvest diseases

Presently, in addition to green mold (*P. digitatum*), BRS of *Aspergillus* isolates are known, which is a serious disease-affecting greenhouse Satsuma mandarin oranges in warm temperatures. Against BRS of *Aspergillus* and anthracnose (*Colletotrichum* species), which results in rapidly increasing damage in September and October, an application of benzimidazoles is not sufficient. However, similar to observations with green mold, benzimidazoles combined with iminoctadine triacetate shown some favorable results [64–66].

7.7. Development and evaluation of a mixed iminoctadine triacetate/thiophanate-methyl agent

For some growers, properly mixing two fungicidal materials can be challenging; thus, the production of an easily administered premixed agent can be beneficial. In fact, the outcomes discussed in this chapter resulted in a development of a premixed product of iminoctadine triacetate and thiophanate-methyl (flowable), which was registered in December 2006.

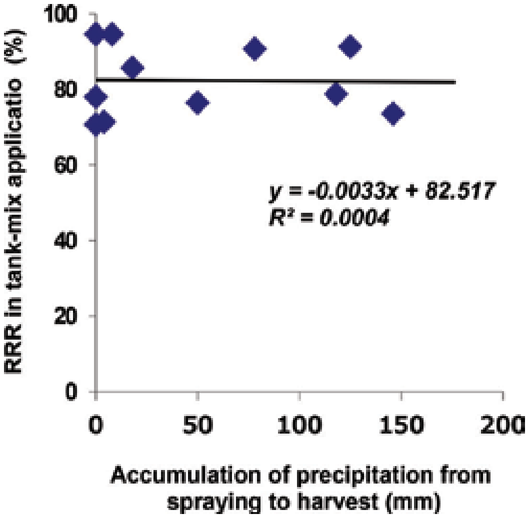


Figure 8. Influence of the tank-mix combination of benomyl 125 μg/mL and iminoctadine triacetate 125 μg/mL on the control of green mold in very early ripening Satsuma mandarin with respect to the accumulation of precipitation.

A pre-mixed product of 15.7% iminoctadine triacetate and 26.2% thiophanate-methyl is available in Japan. It was first registered in 2006 under the brand name Beftopsin flowable (Nippon Soda Co., Ltd.). Even in conditions of resistance, and as shown in the previous studies, synergistic effect is observed. Citrus diseases covered on the label are green mold, blue mold, stem-end rot, Alternaria rot, Aspergillus rot, sour rot, and anthracnose.

Limited studies have compared this pre-mixed product with the combined use of benzimidazoles and iminoctadine triacetate, but representative cases are described in **Table 12**. This mixed product had a favorable effect on green mold and as well as on anthracnose. Based on the RRR value comparison, its effectiveness was higher compared to that of the combined spray of iminoctadine triacetate and thiophanate-methyl. This may be attributed, at least in part, to the miniaturization of component particles by creating a flowable agent as well as the impact of an added auxiliary agent. Irrespective of the cause, the mixed product was a case of successful integration of two modes of action, which resulted in better efficacy than a combination of two solo materials and exhibiting an expanded range of target diseases.

Since this mixed product has better penetration and locally systemic effects, addition of acaricides, pesticides, and even fungicides other than benzimidazoles as a tank mix partner raised a concern on a coloring disorder in Satsuma mandarin orange fruit. However, none of mixtures did not result in symptoms of phytotoxicity (**Table 12**). Although the sample size is small, the combined use with other chemicals did not decrease the control effects (**Table 12**). In the future, we will continue to evaluate the effectiveness of this combined product on a number of crop varieties, both outdoors and indoors. Additional work is needed to determine the most effective use of this agent, such as determination of the optimum timing of spraying.

Mixed agent/+other compound	**Active ingredient (%)**	**Rate applied**	**Investigated fruits**	**Rotted fruit**		**Total rotted fruit (%)**	**Discoloration on the fruit surface**
				Green mold	**Anthracnose**		
Control			271	43	33	28.0	–
Tank-mix combination of iminoctadine triacetate and thiophanate-methyl			220	8	8	7.3	–
Mixed agent of iminoctadine triacetate and thiophanate-methyl (flowable)			113	1	0	0.9	–
+Milbemectin wettable powder (acaricide)	2	10 μg/mL	306	1	2	1.0	–
+Etoxazole flowable (acaricide)	10	50 μg/mL	232	1	2	1.3	–
+Spirodiclofen flowable (acaricide)	30	75 μg/mL	291	0	3	1.0	–
+Chlorfenapyr flowable (pesticide)	10	25 μg/mL	322	1	9	3.1	–
+Acetamiprid water soluble powder (pesticide)	20	100 μg/mL	301	3	4	2.3	–
+Acetamiprid liquid formulation (pesticide)	18	90 μg/mL	325	2	4	1.8	–
+Bifenthrin wettable powder (pesticide)	2	20 μg/mL	188	0	1	0.5	–
+Fosetyl wettable powder (fungicide)	80	2000 μg/mL	287	2	3	1.7	–
+Kresoxim-methyl wettable powder (fungicide)	50	10 μg/mL	234	0	4	1.7	–

[1] Chemicals were sprayed on October 6th, 2006, and fruits were harvested on October 16th. Harvested fruit were artificially damaged by rolling treatment at a slope of 5 m on concrete. Rotted fruit incidence was evaluated during storage for 30 days under the 98% relative humidity.

Table 12. The effect of the mixture of iminoctadine triacetate, thiophanate-methyl (flowable), and another material on postharvest diseases of very early maturing Satsuma mandarin orange.[1]

8. Conclusions

In this chapter, current understandings on increased cases of BRS of *P. digitatum*, the causal agent of green mold of citrus, and the synergestic efficacy of the tank-mix combination of two fungicides, benzimidazole and iminoctadine triacetate, against postharvest development of green mold based were summarized. Using the tank-mix combination of fungicides, two independent selection pressures by two fungicides are simultaneously applied to pathogens. To the best of our knowledge, iminoctadine triacetate–resistant *P. digitatum* [67] has not been found in Japan. However, it is necessary to carefully monitor how these selection pressures result in the changes of the sensitivity of pathogens to benzimidazoles and iminoctadine triacetate.

Because of superior effects on postharvest rot shown in various studies, the tank-mix of benzimidazole and iminoctadine triacetate is widely used in citrus-producing areas of Japan. Additionally, the usage of a newly developed iminoctadine triacetate + thiophanate-methyl wettable powder (flowable) has increased since its introduction. Although this chapter focused exclusively on the effectiveness of fungicides, the mechanism of observed synergistic effects is not clear and warrant further investigations. In addition, other factors that can influence the effectiveness of fungicide application, such as the spray volume and nozzle selection need to be evaluated. As shown in **Figure 2**, many factors are associated with postharvest diseases and the preharvest spray of a fungicide alone cannot control the onset of disease. Precise verification of causal factors for rot is necessary to develop comprehensive and appropriate countermeasures against these factors. The use of technology to maintain the freshness of harvested fruits is another future challenge [68].

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Aleurocanthus woglumi (Hemiptera: Aleyrodidae) in *Citrus*: Opportunities and Challenges to Implement a Sustainable Management

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Additional information is available at the end of the chapter

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Abstract

Citrus BlackFly (CBF) always represented a threat to Brazil. The impact of the introduction in Brazil of the CBF has led to serious economic and environmental consequences. In this chapter, we will show relevant information on biological aspects, history of occurrence, and impact of CBF on *Citrus* in Brazil; data about dynamics populations and spatial distribution patterns and dependence will be presented. We are intending to emphasize in this chapter the main challenges and opportunities of some important tactics to promote sustainable management of CBF in citrus, such as: (i) biological control, (ii) chemical and others methods, and (iii) induced resistance.

Keywords: citrus blackfly, sampling, biological control, insecticides, induced resistance, integrated management

1. Introduction

In the southern hemisphere, Brazil dominates a great part of the orange production [1]. Besides the production of fruit, the main destination is orange juice industry, Brazil being one of the largest producer and exporter of that drink in the world [2]. Nevertheless, there are several problems associated with some stages of the production chain, but in plant production the main obstruction is the occurrence of Citrus BlackFly (CBF) *Aleurocanthus woglumi* (Hemiptera: Aleyrodidae) [3]. Though they can be seen in a wide range of plants, their main hosts are the genus *Citrus*, which is economically important [4].

In Brazil, CBF is considered as one of major pest introduced in *Citrus* and has spread surprisingly over the various regions of the country [3]. CBF adults and nymphs are phloem feeders, which cause direct damage to plants by ingesting large quantities of plant sap. In addition, they produce copious amounts of honeydew, which facilitate the growth of sooty mold on leaves and fruits. This causes a reduction in the photosynthetic capacity of the plant, and fruits are rendered unmarketable [5]. Production losses are estimated in the range of–80% [6].

CBF was first detected in the Nagpur region of Maharashtra (India) in 1910 by Woglumi. In 1915, it was reported in the rest of Asia by Ashby. [7]. In 1913, it was discovered in the New World and in West Indies in 1913 from where it spread out to other islands and Central and South America [8]. On the American continent, it was first discovered in Jamaica in 1913. Between the years 1934 and 1935, it was detected in Cuba, Florida, and Mexico. In Brazil, this insect was first detected in the state of Pará, in 2001 [9]. In 2007, CBF was officially included in the quarantine pest list of Brazil. But due to the extensive spread, this insect was excluded from pest quarantine list Brazil, after losing its quarantine character (Normative Instruction (NI) no. 42, the Ministry of Agriculture, Livestock and Supply (MAPA)). Register of CBF occurrence already was realized in these following Brazilian states: Amazonas, Bahia, Ceará, Espírito Santo, Goiás, Mato Grosso do Sul, Maranhão, Pará, Paraíba, Paraná, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Norte, Rondônia, Roraima, São Paulo, Sergipe, and e Tocantins [10].

The Plant Transportation Permission–PTV is an official document issued to monitor the transit of starting plants, parts of plants, or plant products produced in accordance with the standards of plant health protection in order to prevent the spread of pests regulated, as stated in the Normative Instruction 54, of December 4, 2007, of the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA). In Brazil, CBF restricted transport of more than 31 vegetable species considered hosts to the pest, which required the issuance of PTV for the transit of these products when transported from a state where the pest is present to another state that did not had its occurrence. Since it is wide spread, on February 20, 2015, the NI no. 2 of MAPA established that currently there is no restriction on the interstate transit of plants and their parts. Transportation of fruit seedling and infested leaves is the main way for the spread of this pest to long distances [11]. The fast spread must have been facilitated by river and road transport, especially on *Citrus* fruits from occurrence areas of the insect to juice industry concentrated areas like São Paulo State [3]. After detection in the state of São Paulo, CBF population became a prominent pest on infested citrus orchards, including sweet orange (*Citrus sinensis*), mandarin (*Citrus* spp.) and Tahiti acid lime (*Citrus latifolia* Tanaka) [11].

Since there are a large number of arthropod pests infecting *Citrus*, some vectors of destructive diseases, chemical control has been used in Brazil, often without technical criteria, causing undesirable side effects. In general, *Citrus* in Brazil, mainly in big areas like in São Paulo State, is highlighted as it is both a major source of income and jobs and due to the difficulty in the control of pests and diseases that occur in cultivation [4]. In some parts of the world, CBF has been successfully controlled using alternative control methods, especially biological control [5]. In some cases, biological control is more effective than chemicals to control CBF populations. In Brazilian *Citrus* orchards, there are some candidates for the biological control of the

CBF. In addition, others strategies like induced resistance and natural insecticides are promising. In this chapter, we are emphasizing the main challenges and opportunities to use some important tactics to promote management of CBF in *Citrus* with sustainability, in addition we are addressing some perspectives focusing in what, when and/or how use each one of tactics.

2. Dynamics populations and dispersal pattern

The knowledge about major infestation potential of CBF and its seasonal dynamics is essential for the orientation of management strategies, which may result in the minimization of production losses [12]. Because with use of CBF sampling, it is possible to prevent outbreaks of the pest and to decide only when necessary. In others words, the tactics of control such as use of chemicals or similar or release of natural enemies will be realized only on the recommended thresholds. The variation in climatic variables, especially temperature and rainy season, is important on infestation potential of CBF [13]. In general, the highest population levels of CBF occur in the low-precipitation season [14, 15]. But in Municipality of São José de Ribamar, State of Maranhão (Brazil), in a commercial orchard of *C. latifolia*, the average total number of citrus blackflies was higher during the rainy season than in dry season [16].

In a bioclimatic simulation in the North region of Brazil, the optimum bioclimatic zone was established between October, November, and December. In general, summer is favorable for the occurrence of CBF in the South Hemisphere; that is, winter is unfavorable [12]. In Minas Gerais and north of São Paulo, the optimum time is in December and unfavorable months are July, August, and September, a similar pattern may favor the spontaneous migration of the insect between these sites [12]. In Municipality of Artur Nogueira, State of São Paulo (Brazil), with one year of evaluation the peak occurrence of egg was observed in the spring (August) and for nymphs in the autumn (from March to May) in a commercial orchard of *C. latifolia* [11].

Females of CBF prefer oviposition sites of the canopy with high humidity [11]. But the distribution pattern intratree shows difference between geographical regions and between years and seasons. In an experiment conducted in São Paulo State, the trees were divided in four quadrants (north, south, east, and west), the western quadrant showed more CBF egg masses than the northern, but no difference was observed in the southern and eastern quadrants. Western and eastern quadrants showed highest quantities of CBF nymphs [11]. However, in another experiment in State of Maranhão, Brazil, it was observed that during rain period the insects (eggs and nymphs) were distributed homogeneously on the trees canopies in a commercial orchard. In addition during the period without rain, the north and south quadrants showed less clutches/plant, eggs/plant and nymphs/plant in a non-commercial orchard and clutches/plant, eggs/plant in a commercial orchard [15]. The infestation level may vary in accordance with the crop system, because there is evidence that infestation of CBF is different in agroforestry and conventional system [13].

Upon adjusting the calculated variograms to the spherical model in the dry and rainy seasons [16], it was concluded that the spatial distribution of CBF in the orchard is aggregated, but the level of average aggregation depends on the weather especially during the season, during the rainy season the average aggregation is 162,092 m^2, and 9615 m^2 in the dry season. They recommend to obtain a reliable estimate of citrus blackfly populations, at least one trap should be used for each 17 hectares during the rainy season and one trap per hectare in the dry season. Silva et al. [17] used a similar approach of spatial dependence described by the spherical model; that model has a simple polynomial expression and its shape had an almost linear growth up to a certain distance and then stabilized. Silva et al. [17] confirmed that spatial distribution of CBF in citrus orchard in the agroforestry field in Pará State was predominantly aggregate, forming clusters from 15.5 to 34 m. This aggregation behavior of CBF increased the initial damage in newly infested orchards [11].

In a general way, some studies have showed evidence of an aggregation behavior of CBF on a spatial scale. In terms of sampling methods to implement this component of CBF IPM, it is interesting to know how the population dynamics is in intratree distribution and in spatial scale of different landscape structures, because some differences of results found about the occurrence of CBF should be considered according to the kind of pest management and the degree of landscape heterogeneity. We would like to encourage future studies about dynamics populations of CBF in a period longer than 2 years of evaluations that may consider spatial mathematical modeling and/or use of statistical models as generalized linear mixed models with overdispersion for helping to understand this cluster behavior and temporal and spatial dispersal patterns according to the landscape structure of *Citrus* orchards.

3. Biological control

The use of beneficial organisms as a component of integrated pest management (IPM) is relevant for most crops [18, 19]. The search about natural enemies associated with CBF has enabled the use of biological control worldwide. One of the most effective parasitoids of the CBF is *Encarsia perplexa* Huang & Polaszek (Hymenoptera: Aphelinidae) [20]. *E. perplexa* was originally misidentified as *Encarsia opulenta* (Silvestri) (Hymenoptera: Aphelinidae) but was later identified to be *E. perplexa*. When the adult parasitoid emerges from the blackfly pupal case, it leaves a roundish black hole. Normal emergence of a CBF adult would leave a T-shaped split in the pupal case [20]. Biological control of CBF, especially with predators of genus *Ceraeochrysa*, *Chrysopa*, *Chrysoperla*, and *Delphastus*; and parasitoids of the genus *Calles*, *Encarsia*, and *Amitus* have been indicated to maintain the CBF population below of the economic threshold [21–23]. *E. perplexa* already was released for the control of CBF in Mexico [24], Texas [25], and Florida [26]. The parasitoids *Amitus hesperidum* Silvestri (Hymenoptera: Platygasteridae) and *E. perplexa* were introduced in the United States in 1996 by the Ministry of Agriculture of the United States, in collaboration with the United States Department of Agriculture. Nearly 49,000 *A. hesperidum* and 165,000 *E. perplexa* were released in 1996–1997 [5]. Continued control of CBF requires sustained releases of *A. herperidum* in South Texas, because it is unable to survive the severe summers of that region [27].

The parasitoids *A. hesperidum* and *E. perplexa* have kept CBF populations under effective biological control in Dominica [5, 28]. In effective control program of the CBF, with field multiplication and redistribution, a total of 573,000 parasitoids were produced and released in various *Citrus*-growing areas of Dominica [28]. In another release program of *A. hesperidum*, *E. perplexa* in Dominica, after 4 years of the release for the classical biological control of the CBF, these two parasitoids were found at the site where CBF was encountered and pest populations were declining [5]. In addition, Lopez et al. [5] believed that there is no evidence of any nontarget effects on other Aleyrodidae or their natural enemies, because the two parasitoids were not among the several species collected on nontarget Aleryodidae and Hemiptera.

The solitary endoparasitoid *E. opulenta* is also a promising biocontrol agent for population regulation of CBF in several countries [29]. However, the assessments of the different aspects of the biology and behavior as well as its effectiveness to be released are still necessary. Satisfactory parasitism levels were observed with *A. hesperidum* in inundative releases in Trinidad and Tobago [30]. In Pará, there has been an increase in parasitism by microwasps which was not identified, with parasitism reported up to 90% [31]. In Rio de Janeiro, for the first time, the association of *Encarsia pergandiella* Howard (Hymenoptera: Aphelinidae) acting as a parasitoid of *A. woglumi* nymphs infesting *Citrus latifolia* leaves was recorded [32].

The natural enemies of Chrysopidae family, known as lacewings, are predators that play a significant role for controlling the population of the blackfly on various crops of agricultural importance such as cotton *Gossypium hirsutum* preying *Heliothis* spp. (Lepidoptera: Noctuidae) and on tomato *Solanum lycopersicum* preying *Bemisia tabaci* B biotype. The potential of these predators to reduce the population of many pests is a factor, which has been reported by several researchers as promising on control of CBF [23, 33]. As well as the occurrence of species *Chrysopidae* (Neuroptera) [32] was common, and the species of *Coccinellidae* (Coleoptera) was also recorded as the natural biological control of CBF in *C. latifolia* orchards in Brazil. In fact, species of *Chrysopidae* and *Coccinelidae* (Coleoptera) are important in the control of CBF, for example in the north region of Brazil, 11 species of *Chrysopidae* and *Delphastus pusillus* (Le Conte) (Coccinelidae) were observed preying CBF [11, 34, 35].

In the last few years, a strong effort has been made to improve techniques for rearing of natural enemies of CBF in laboratory by some Brazilian University Laboratories. The main challenge to release natural enemies on a large scale to biologically control CBF in Brazil is the absence of commercial availability of these natural enemies yet. But a promising perspective of applied biological control will probably happen with government partnerships like Bahia State, Brazil for mass production of CBF's natural enemies [37].

4. Chemical insecticides and other methods

Chemical insecticides have been used to control its infestation; however, this strategy reduces the insect pest of infestations only temporarily triggering the imbalance in the environment that in turn, poses threat to nontarget organisms [19, 36]. Evaluations about the effect of Dursban 4E [chlorpyrifos] in two different nursery locations by Ref. [37] showed that they observe only limited control, and this product was not phytotoxic to nursery citrus. Monocrotophos (0.05%) was effective to promote nymphal mortality (range from 75.10 to

85.50%) of CBF [38]. They believe that application of monocrotophos (0.05%) during early nymphal stages followed by neem oil (1%) during later stages may be effective and also safe to parasitioids.

In Brazil, four insecticides are registered to control this pest in *Citrus*, three neonicotinoids with the active ingredient imidacloprid and a technical mixture based on anthranilamide [chlorantraniliprole] with lambda-cyhalothrin [pyrethroid] [39]. Synthetic insecticides should not be sprayed when much of the blackfly population is in the adult stage; therefore, it is recommended to wait 10–12 days for the decrease in adult population allowing the young stages (egg and nymphs) to emerge which can be controlled before they cause damage [40].

In the Texas (USA), it is common that organic growers apply pesticides such as sulphur, oils, and microbials to CBF control [27]. In particular, in the region of Borborema, State of Paraiba, the farms consist of highly diversified systems, with high abundance of natural enemies. The citrus areas are usually in a highly diversified landscape along with the annual crops (e.g., *Manihot* spp.), others fruit species or agroforestry, in the intercropping system or in the neighboring. The landscape diversification is an important cultural practice in pest management and is based on the principle of reducing insect pests by increasing the diversity of an ecosystem by the presence of more than one plant species. Studies indicate that diversification practices such as intercropping in the northeast region are beneficial because these practices reduce pest damage [41]. But when applied to *Citrus*, it is still necessary to understand how the occurrence of natural enemies associated with CBF differs in a landscape with the diversification system compared to the monocrop system in the northeast region. In addition, the most families in the region do not use pesticides. The use of chemicals insecticides must be analyzed judiciously, especially in small areas because according to the reduced size of the property and proximity of homes with orchards, it is possible that the drift of chemical insecticides can reach locations other than target of applications such as the vicinity of houses, animals, small and local food supply, and water reservoirs [42].

The use of mineral oil, vegetable oils, or derivatives may result in improved control strategies for agricultural pests and associated diseases and can cause minimal adverse effects on populations of natural enemies and other non-target species [43, 44]. Therefore as an alternative to chemical control, potential alternative products have been the subject of study by the group of researchers from the Federal University of Paraíba–UFPB [43, 44]. The interesting result is that some vegetable oils were effective and promoted ovicidal activity [42], for example, it is observed that cottonseed oil provided 100% egg mortality. Oils from *Eucalyptus globulus* Labill, *Allium sativum* L., *Ricinus communis* L. are a promising alternative to control *A. woglumi*, especially between 10 and 15 days after application of these products, the mortality of eggs is near to 90–100% [44]. These and other results are summarized in **Table 1**, indicated the effect of some oil from plants applications on the mortality of the CBF. We could separate in some intervals of mortality observed. Other treatment like gamma irradiation [45] is also showed.

In general, the use of chemical control for of CBF mentioned in this section should ultimately be used because it is too costly and inefficient [19], especially when performing on the clutches

Activity on insect stage	Treatment (concentration or dose) [source]
Treatment with product from plants	
Mortality on egg (90–100%)	Rott Nim® (1.5%), oils from *Glycine max* (0.5–1.0%), *Helianthus annuus* (0.5–1.0%) and *Gossypium hirsutum* (1.5%) [43]. Oils from *Eucalyptus globulus* (4–6%), *Allium sativum* L. (4–6%), *Sesamum indicum* L. (6%) and *Ricinus communis* L. (6%) [44]
Mortality on egg (60–89%)	*Zea mays* oil (0.5%) [43]. *Dianthus caryophyllus* L. [44]
Mortality on egg (40–59%)	Extract from *Piper aduncum* L. [63]
Mortality on nymph (90–100%)	Oils from *Glycine max* (1.5%), *Zea mays* (1.5%), *Helianthus annuus* (1.5%), *Gossypium hirsutum* (1.5%) and Rott Nim® (1.5%) [43]
Mortality on nymph (50–70%)	Neem oil (1%) [38]
Treatment with irradiation	
Mortality on egg (90–100%)	Gamma irradiation (200 Gy) [45]

Table 1. Summary of some alternative treatments from plants and with irradiation on mortality of citrus blackfly stages.

of this insect. In addition, high-dispersion by means of the adult flight favors the fast infestation of plants and orchard and cross infestation among citrus and other hosts and between neighboring groves [10]. This probably has hindered the effectiveness of chemical control because of the ease of reinfestation, especially in abundance of host sites. In Brazil, many are the hosts of the blackfly [4]. In Rio de Janeiro, for example, recently–three new host plants for *A. woglumi* were identified: *Artocarpus heterophyllus* (Moraceae), *Pouteria caimito* (Sapotaceae), and *Struthanthus flexicaulis* (Loranthaceae) [32]. In spite of the use of various substances as alternative to the synthetic insecticides, insect pests are destructive especially in small farms that produce fruit [46, 47], but for a precise recommendation the use of these substances, future studies should validate their efficiency in field and their compatibility with others tactics as biological control.

5. Induced resistance in citrus with silicon

Resistance induction corresponds to activation of the latent defense system in plants when they come in contact with compounds called elicitor agents. Among the elicitors, silicon has attracted the attention and interest of researchers. In addition to providing resistance, it may also provide nutritional benefits and increase the production and quality of agricultural products. The resistance induced by silicon is expressed in various ways, such as cell wall lignification, papillae formation, or induction of various defense proteins [48].

The use of silicon for the induced resistance of plants is a potential strategy in the integrated pest management, however this substance has not being considered as an essential nutritional element to the plants [49], but as potassium silicate, calcium silicate, and sodium silicate from other sources has determined the tolerance of many plant species to insects [50–53]. Silicon promoted cuticle thickening and accumulation of crystals on the leaf stomata in sugarcane [54]. The action of silicon may not be only restricted to resistance constitutive or induced but may also involve induced plant chemical defense [51].

Inducing agents sensitize the plant to activate their defense mechanisms in response to the presence of pests. These mechanisms may involve enzymes such as peroxidase, β-1,3-glucanase, chitinase, phenylalanine ammonia lyase, and polyphenol [55]. The peroxidase activity has been implicated in a variety of processes pertaining to the protection of plants, including hypersensitivity reaction, lignification, and suberization [56]. Hypersensitivity reaction is characterized as a fast and localized response. Among the main characteristics of the possible responses are the rapid and localized collapses of plant tissue around the site of infection, caused by the release of toxic compounds, which also act in some cases, directly on the pathogen, causing mortality. Structural barriers may involve lignification and suberisation and can be seen as physical defenses that restrict the development of insect pests. The lignification is a biochemical process that covers monolignol biosynthesis, transport, and polymerization in the cell wall, which in the first stage is highly mediated by enzymes intrinsic to the formation of the forerunners in the cytoplasmic compartments. The second stage is the formation of lignin in the cell wall. The oxy-reducing enzymes such as peroxidases and corresponding isoenzymes, act in the polymerization of lignin in the cell wall, forming a coordinate complex with hydrogen peroxide [57]. The deposition of lignin increases the resistance to the cell wall digestive enzymes of the insect pests. This resistance is also enhanced with presence of suberin or deposition of suberin lamella covering the cell wall of this process is called suberization.

Peroxidases participate in various physiological processes by catalyzing the oxidation and polymerization of hydroxycinnamic alcohol in the presence of hydrogen peroxide, resulting in lignin, an important physical barrier of plant defense [58], which contributes to strengthening the cell walls of the host. Changes in peroxidase activity by treatment with elicitors may indicate their involvement in resistance in plant induction [59]. Phenylalanine ammonia-lyase plays a fundamental role catalyzing the conversion of L-phenylalanine to trans-cinnamic acid, a deamination reaction. This reaction is considered an essential step in the phenylpropanoid pathway producing many products, including lignin, involved in plant defense reaction. The polyphenol oxidases are enzymes that often increase their activity in response to stress, and one of its main roles seems to be to promote the protection of the cell [60].

With hypotheses that silicon could to be an elicitor that potentiates the defense mechanisms of *Citrus reticulata* to CBF, Vieira et al. [61] used silicon in the form of potassium silicate (K_2SiO_3) to assess the activity of enzymes peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. Results obtained by Vieira et al. [61] showed that silicon is not only involved in mechanical restraints against insect feeding but also with biochemical changes. In their study, they concluded that the peroxidase and polyphenol activities indicated strong induction of plant defense against CBF.

Using principal components analysis–PCA, it is possible to see a clear characterization of the different patterns of peroxidase, polyphenol oxidase, and PAL activity in response to time after infestation of citrus blackfly. In addition, an isolated activity in relation to peroxidase and polyphenol oxidase may be observed, but there was overlap of activities between polyphenol and peroxidase activity (**Figure 1**).

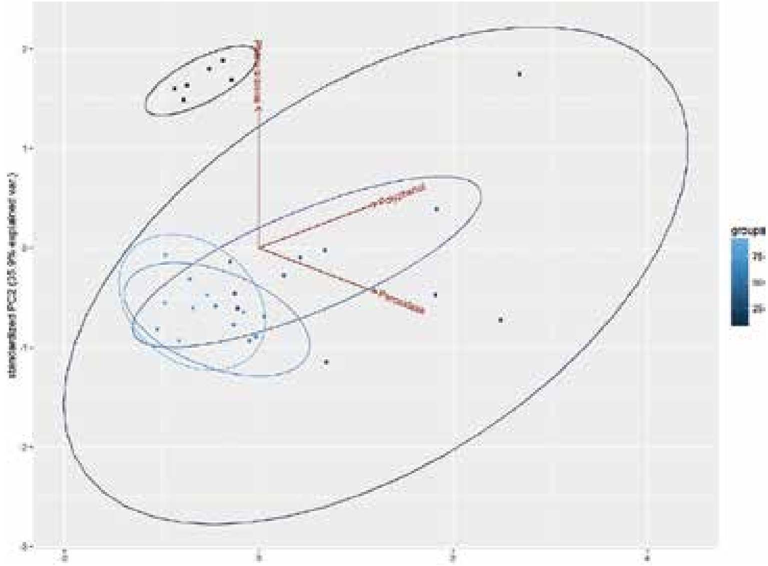

Figure 1. Biplot of enzymes activity mediated by silicon on seedlings of *Citrus reticulata*. The dots represent the mean activity, whereas the arrows represent the vector of each variable. Original data are showed in Fruits (2016) [61]. Copyrights (2016) with permission from EDP Sciences.

There is evidence that the increase in peroxidase and polyphenol oxidase activity revealed the induction of synthesis of compounds for plant defense against CBF, but this effect depended on the time of *A. woglumi* feeding and on the concentration of silicon. No effect of silicon as an activator of the PAL was observed [61].

Silicon probably triggers the natural defense mechanisms of plants such as the production of phenolic compounds, chitinases, peroxidases, and lignin, which can interfere with the physiology and development insect pests, and consequently silicon can reduce the oviposition preference and provide sublethal effects such as extending the development time and nymphal mortality [50]. A positive correlation between peroxidase activity and the development of *A. woglumi* was registered after use of silicon [61].

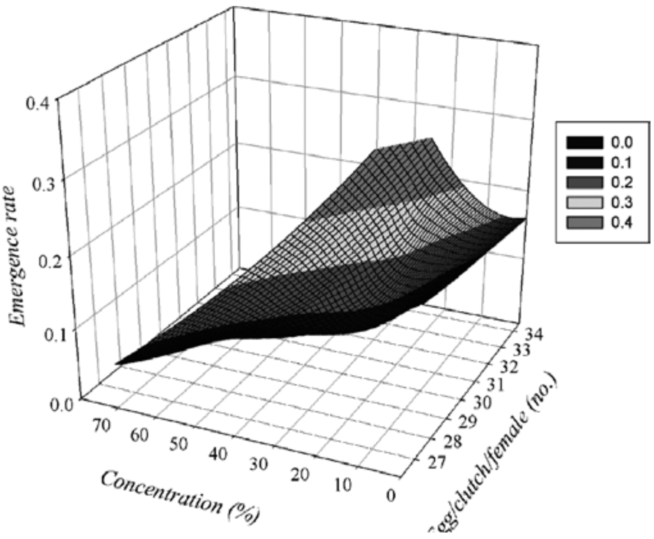

Figure 2. Effect of doses of potassium silicate on the average number of egg / clutch/ female and adult emergence rate of citrus blackfly. Original data are showed in [62]. The elicitor silicon may cause reduction in population growth rate of CBF, providing greater mortality, stimulating growth and plant development that is coenfirmed with the increase or the simple presence of defense substances, which are the peroxidase enzymes and polyphenol. Therefore, resistance induction is a promising alternative for the management of CBF, activating the synthesis of plant defense compounds.

Our results expressed in **Figure 2**, reveal that silicon doses promote low emergence rate. In the control treatment, an emergence rate was recorded as approximately 40%, significant reduction was observed with increasing of silicon doses with an emergence rate near to 5% in all doses used of silicon. But, it is clear that there is no great influence on eggs/clutch/female (**Figure 2**).

6. Final considerations

In Brazil, citrus is frequently affected by various pests. CBF has been causing severe damage, impacting the economy and reducing the citrus production. Mapping allows spatial visualization of the pest in the agroecosystem, allowing rational control with targeted applications, reduce production costs and decrease the negative impacts of pesticides, population fluctuation and spatial dependence of CBF in citrus, trapping as a representative sample of CBF is a tool to promote management of CBF, to decision making only when necessary. Conservative and release of natural enemies like Chrysopidae are potential to control CBF populations. Besides, to reduce populations of CBF ovicidal action based in some products such as oils from cotton seed, *E. globulus*, *A. sativum*, *R. communis* are valuable (please see **Table 1**). The use of silicata to induce citrus defense mechanisms with the increased activity of peroxidase and polyphenol oxidase presents a promising tool.

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Section 4

Post-Harvest Management of Diseases

Chapter 7

Advance in Citrus Postharvest Management: Diseases, Cold Storage and Quality Evaluation

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Francesco Genovese and Giovanni C. Di Renzo

Additional information is available at the end of the chapter

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Abstract

Citrus is a fruit crop grown in different Mediterranean countries. Generally, harvested fruits are used for fresh consumption or are processed (mainly to produce juices). In this chapter, the authors discuss the state of art on citrus postharvest with a scientific approach, evaluating the current knowledge about the physiology and pathology of citrus fruits and the main causes of deterioration. In addition, the authors explain the main facilities for the cold storage of citrus fruit with particular reference to the rapid-cooling techniques and treatments needed prior to shipment of citrus fruits (refer shipment). In the last part of the chapter, the non-destructive methods for the quality evaluation are presented.

Keywords: citrus fruit, physiological disorders, postharvest treatments, non-destructive methods

1. Introduction

In 2015, the world citrus production was around 121 million tons, with about 24 million tons in the Mediterranean Region (primarily Spain, Egypt, Turkey, Italy, Morocco and Greece), mainly destined for the fresh fruit market [1]. In Italy, the areas of citrus cultivation are mostly in the Southern regions, characterized by a Mediterranean climate where oranges (*Citrus sinensis* (L.) Osbeck), clementines (*C. clementina* Hort. ex Tan.), mandarins (*C. reticulata* L.) and lemons (*C. limon* (L.) Burm.) are the most cultivated varieties. The Eastern coast of Sicily, in the foothills of the Etna volcano (**Figure 1**), has a long-standing tradition in pigmented oranges

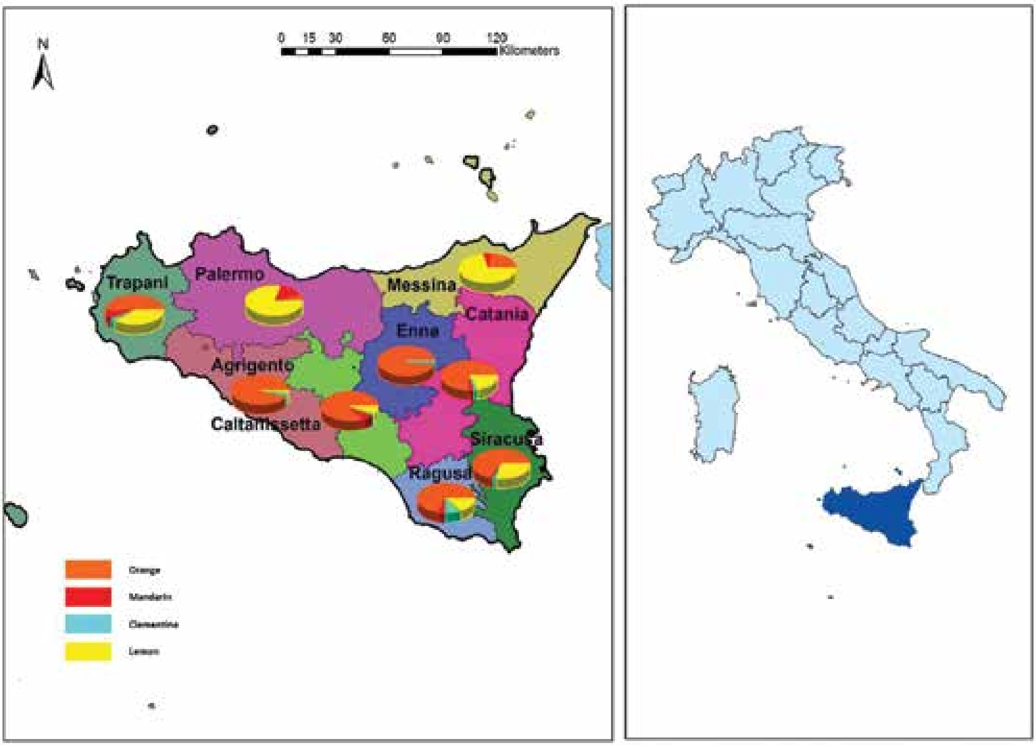

Figure 1. Citrus production is a fundamental sector of agriculture in Sicily, the largest of the Italian islands, located in the South of Italy.

growing, such as 'Tarocco', 'Moro' and 'Sanguinello'; along the coast is located the 90% of the Italian lemon production, based on three major cultivars: 'Femminello', 'Monachello' and 'Interdonato'.

Different species of citrus are cultivated all over the world, especially oranges, mandarins, lemons, grapefruits (*C. paradisi* Macf.), limes (*C. aurantifolia* Swing), pummelos (*C. maxima* (Burm.) Merrill), kumquats (*Fortunella* spp.), citrons (*C. medica* L.) and many other hybrids also commercially important.

During the last decade, citrus production increased significantly, which oriented the commercialization of the fruit towards distant markets. Therefore, increased consumers' concern regarding fungicides residues in the fruit has prompted the research into finding safer and effective alternative strategies as well as the development of adequate postharvest-handling technologies. New eco-friendly solutions would offer the opportunity to eliminate chemically synthesized fungicides currently used with the aim to preserve the natural qualities of fresh citrus fruits after harvest and to extend their shelf-life.

In addition, further development of non-hazardous postharvest management practises and technologies to preserve the quality of freshly harvested citrus fruit would also permit to obtain a high value-added product, with the possibility to reach distant markets.

2. Postharvest issues

Citrus fruits (genus *Citrus*; family *Rutaceae*) are specialized form of berry, named hesperidium, characterized by a juicy pulp made of vesicles within segments. The size range varies from nearly 2.25 cm for kumquats to more than 20 cm in diameter for pummelo. The shape is also variable going from spherical to oval in sweet oranges, oblate in grapefruit and mandarins, and oblong in lemons. Citrus rind (pericarp) comprises two regions: the exocarp or flavedo and the mesocarp or albedo. The flavedo is the outer, pigmented part containing oil glands; the albedo is the inner white section. The outermost constituent of the flavedo is the cuticle, a thin continuous polymer comprising waxes that plays an important role as the primary barrier between the fruit and its environment, before postharvest wax application. Moreover, it is mainly responsible for gas exchange and helps in maintaining high water content within tissues necessary for normal metabolism [2].

Citrus fruits are highly appreciated by consumers not only for their taste but also for the positive health values, representing a rich source of bioactive substances that include vitamin C, phenolic compounds such as hydroxycinnamic acids and flavonoids. The major classes of flavonoids in citrus are flavanones and anthocyanins, which are largely present in the pigmented varieties. The anthocyanins give an important sensory input and they have a very important role for their pharmacological and antioxidants properties. The advantage of fresh fruit consumption is due to some salutistic properties of these components, associated with the reduction of cardiovascular, stroke and cancer risks [3].

Citrus fruits are non-climateric, and thus different from climateric fruit (e.g. apple, pear, melon, tomato), are characterized by the lack of a ripening-associated increase in respiration and in ethylene production. They have a relatively long shelf-life compared to other tropical fruits; however, if not properly handled and stored, the fruit would become unsuitable for marketing. The perishability of fruits is generally proportional to their respiration rate and energy released as heat affects postharvest technology considerations such as estimations of refrigeration and ventilation requirements. Transpiration is a physical process characterized by the evaporation of water from fruit tissues, having as consequence fruits deterioration because of the loss in appearance (wilting and shrivelling), textural (softening) and nutritional quality [2]. Transpiration rate is influenced by rind injuries, maturity stage and environmental factors, such as temperature, relative humidity and air movement. The application of surface coatings and the manipulation of the storage environment (low temperatures, high relative humidity levels and control of air circulation) allow the management of the process. Diseases, physiological disorders, fruit senescence and physical damages represent the major causes of postharvest losses. Postharvest citrus decay is the most severe cause of wastage and quality deterioration since it renders fresh fruit unsuitable for consumption, causing consequently heavy economic losses. Losses can reach 30% of the total production and 50% in less developed countries. Physiological disorders and fruit senescence depend on inadequate temperatures used for citrus fruit storage, high rate of respiration and transpiration, and the stress caused by harvesting and postharvest handling. Rind injuries and impact bruising are the major contributors to fruit deterioration, since they accelerate water loss, stimulate higher respiration and ethylene production rates and help pathogens development [4].

2.1. Diseases and physiological disorders

Fungal pathogens are considered the main cause of citrus diseases, severely affecting postharvest management. Pre-harvest infections include Brown rot (*Phytophthora* spp.), Alternaria rot (*Alternaria* spp.), stem-end rot (*Diplodia natalensis* Pole-Evan, *Phomopsis citri* Fawcett), Grey mould (*Botrytis cinerea* Pers.), Anthracnose (*Colletotrichum gloeosporioides* Penz.), whilst postharvest infections include Green mould (*Penicillium digitatum* Sacc.), Blue mould (*P. italicum* Weh.) and Sour rot (*Geotrichum candidum* Link). Green and blue moulds represent the most common and serious diseases, and cause significant economic losses during fruit storage and marketing. *P. digitatum* and *P. italicum* particularly attack blood oranges, and infection takes place only through rind wounds, where nutrients are available to stimulate spore germination. The incidence of other pathogens is generally low but become a serious problem in warm, wet years [5].

Postharvest physiological disorders are a consequence of environment, handling, treatments, and storage conditions to which fruits are subjected. In particular, losses of citrus fruits stored for several months or shipped for long distances are a consequence of the low temperatures required for cold storage. Chilling injury (CI) represents the major disorder of citrus fruit that occurs when fruits are held at low non-freezing temperature storage (0–10°C) and it depends on species, cultivars, season, maturity stage and location of the crop. The severity of CI is related to the temperature and time of exposure. Recent findings indicate that oxidative stress is related to CI [6]. This physiological disorders manifested in a variety of symptoms, most often characterized by areas of the rind that collapse and darken to form pit (not targeted to the oil glands) and brown colour areas. As a consequence of CI, it is possible to observe an increase in mould development on fruit surface. Ageing is a physiological disorder indicated by the shrivelling and collapse of the button tissue, caused by prolonged storage at low temperature.

2.2. Sanitation

As studied [7], population of *Penicillium* spp. on fruit surface and in the packinghouse atmosphere fluctuated along the packingline, reaching a peak at "bin emptying" step. An accurate sanitation of packingline and environment is provided to reduce the inoculum density of the spore, with positive consequences for fruits.

Sanitizers, used for fruit surface sterilization, have the aim to reduce the initial high level of inoculum present on the products. Chlorine solutions are the current products used in packinghouses. Cleaning is usually achieved by spraying sodium hypochlorite solutions (100–150 ppm) and washing on brushes followed by a potable water rinse [7]. However, this method has some negative aspects due to the constant adjustment of the available chlorine and the monitoring of pH of the solution for a correct treatment. Moreover, chlorine-releasing compounds can produce toxic breakdown by-products (halogenated) when reacting with organic materials [2].

The use of different sanitizers (peroxyacetic acid (PAA), ozone and electrolyzed water (EW)) has been evaluated, for water disinfection in packinghouses operations of citrus fruit [8].

Peroxyacetic acid is a strong oxidizer formed from hydrogen peroxide and acetic acid, effective against a wide range of microorganisms. Decomposition products of PAA are acetic acid and oxygen, which are not toxic and no rinse step is necessary. PAA applications at 800 μg/ml for 1 min significantly reduced green mould incidence on lemon [9, 10]. Ozone is a strong naturally occurring oxidizing agent, known for its efficacy to kill various microorganisms (bacteria, cysts of protozoa, viruses and fungal spores). Moreover, a significant advantage of ozone in water is related to the quick decomposition of ozone molecule to oxygen, with no residues. Electrolyzed water, generated by passing a diluted salt solution through an electrolytic cell, has shown a reduction of the microbial pathogen population in citrus process water [11].

2.3. Conventional and alternative treatments

Current postharvest decay control strategies are based on the application of synthetic chemical fungicides which are relatively inexpensive, easy to apply, with preventive and curative action against established and new infections, respectively. The most widely used methods of application are by adding fungicides [e.g. imazalil (IMZ)] to liquid commercial wax that is sprayed onto the fruit, before fruit storage. This method simplifies packinghouse operations, because the improvement in the packing line is inexpensive and straightforward, and, unlike dipping, there are no problems due to drainage and water treatment. Moreover, several problems arise when spraying IMZ in mixture with wax, as obstruction of nozzles with reduction in treatment efficacy and uniformity of distribution; dispersion and loss of fungicide and wax on brushes and along the packing line; accumulation of fungicide on brushes leading to waste treatment problems; accumulation of spores in the brushes requiring their regular cleaning [8]; and the chemical composition of wax, as it contains several alkaline soluble materials which may cause more IMZ partition in the wax and hence a reduced amount in the aqueous phase to penetrate the peel. Consequently, all these problems require an increase in fungicide concentrations.

Fruit dipping is more effective than wax spraying with regard to decay control, because pathogens basically develop in small punctures on the fruit's peel where the water solution penetrates better than the more viscous wax. Moreover, the fungicide concentration gradually decreases [12] and the number of spores increases in water emulsion, with possible problems related to the uniformity of treatment, the cross contamination of fruit and the stability of fungicide in the presence of sanitizing agents. IMZ, thiabendazole (TBZ) and sodium-orthophenylphenate (SOPP) are the only authorized fungicides for postharvest applications, and their use is regulated by government authorities and differs from one country to another [5]. These treatments, although carefully tested for side effects and tightly regulated, leave a residue on fruit as well as in the environment. For these reasons, there is an increasing interest to develop and implement alternative eco-friendly, nontoxic strategies effective and not reliant on conventional fungicide applications, for postharvest diseases control of fresh commodities.

In recent years, several low-risk fungicides (Trifloxystrobin, Azoxystrobin, Fludioxonil, Cyprodinil and Pyrimethanil) classified as a minimal risk to human and environmental health have been registered, also for the control of citrus postharvest decay. Effectiveness of

Fludioxonil tested on Tarocco orange fruit inoculated 24 h before with *P. digitatum* showed high curative activity in the fruit of various cultivars, harvested at different degrees of maturity [13].

Finding alternatives that are widely accepted and commercially viable has been a challenge [14]. Alternative methods to fungicide treatments include the application of physical treatments, generally recognized as safe (GRAS) compounds, biological control agents and natural antimicrobial compounds. Their inhibitory effect on decay control is mainly a consequence of direct inhibition of pathogens. However, some of these strategies have shown the ability to enhance defence mechanisms on citrus fruit tissues, inducing disease resistance and making a significant contribution to *Penicillium* decay control. Nevertheless, due to low persistence and lack of preventive effect on pre-existent diseases, the alternative strategies should be used in combination, as part of an integrated management programme, able to further minimize postharvest decay and to extend the fruit's shelf-life.

2.3.1. Physical treatments

Physical treatments are considered an interesting and feasible alternative to chemicals, because of the total lack of residues on treated products and the potential to develop induction of resistance against future infections. They appear as a cheap solution, when compared to other heat treatments, for organic crops and for markets requirement of minimal or no chemical postharvest fruit treatments. Moreover, an increase of resistance to chilling injury during storage was observed in some fruits. However, some negative effects could occur affecting fruit quality, when not properly applied and technological problems can be better studied to permit applications on a commercial scale. Heat is the physical treatment most employed in postharvest applications, used in the form of hot water dip (HWD), short hot water brushing (SHWB), curing, hot dry air and vapour heat [15].

HWD is based on the use of water at temperature above 40°C, and consists in a complete immersion of fruit for 2–3 min. Many authors found that HWD on organic citrus fruits reduced rot development, without detrimental effects on fruit appearance and quality traits [16]. However, the period of fruit immersion represents an obstacle to adopting this method in packinghouses, where it is necessary to process large volumes of product quickly. Treatments at 56°C for 20 s are shown to have higher effectiveness in inhibiting *P. digitatum* spore germination than at 52°C for longer exposure time [17].

SHWB treatment consists on the employment of tap water using a spray nozzle system. Water is sprayed above fruits rolling over brushes on sorting line, followed by pressurized hot water rinse at higher temperature (60°C) and short exposure time (20–60 s), and final forced-air drying [15]. It provides a more effective cleaning than HWD, thanks to its capacity to remove heavy dirt and fungal spores on fruit pericarp, while maintaining fruit quality. SHWB is commercially adopted in Israel, Egypt, Indonesia and Morocco, for various commodities, such as melon (*Cucumis melo* L.), mango (*Mangifera indica* L.), grapefruit (*Citrus paradisi* M.) and pepper (*Capsicum annum* L.) [18]. In Europe, the use of hot water treatments currently regards organic apples (*Malus domestica* Borkh.). The application of SHWB at 60°C for 20 s has been reported to reduce consistently green mould on citrus fruit [8, 10].

Curing is a treatment consisting on the exposure of fruits for several days to an air atmosphere at 30–36°C and high relative humidity (RH > 90%). As reported by different authors, the exposure of citrus fruit to this treatment evidenced the healing of rind wounds. The application of curing against citrus green and blue moulds has shown satisfactory disease reductions in a variety of citrus cultivars [10, 19], less for blue mould when the fruit was cold stored for long periods after treatment [20]. As reported by Pérez et al. [21], an intermittent curing treatment of two cycles of 18 h at 38°C completely controls *P. italicum* on mandarin stores under ambient conditions.

In recent years, different promising technologies have acquired increasing interest for the control of fruit postharvest diseases as the use of the radio frequency or microwave heating, hypobaric and hyperbaric pressure and ultraviolet-light therapy (UV-C irradiation) that have also shown the potential in inducing resistance in the fruit [22–25]. Some studies have been carried out to control the most common pathogens of stone fruit, small fruit and berries, obtaining variable results. Further studies are carried out to evaluate the influence of these treatments on fruit quality.

2.3.2. Microbial biocontrol agents

In the past 30 years, impressive progress has been achieved since the first publication on biological control by bacteria [26]. Different products reached advanced stages of development and commercialization to manage key postharvest pathogens. AspireTM (*Candida oleophila*), PantovitalTM (*Pantoea agglomerans*) and BiosaveTM (*Pseudomonas syringae* Van Hall) were originally registered in the USA and Spain as commercial products for the control of several postharvest diseases of pome and citrus fruit, such as *Penicillium* rot of apples (*P. expansum*), Green mould (*P. digitatum*), Blue mould (*P. italicum*), Rhizopus rot (*Rhizopus stolonifer*) and Grey mould (*Botrytis cinerea*). Aspire is not currently commercialized and Biosave was later extended to cherries (*Prunus avium* L.), potatoes (*Solanum tuberosum* L.) and sweet potatoes (*Ipomoea batatas* (L.) Lam.). ShemerTM (*Metschnikowia fructicola*), initially registered in Israel for both pre- and postharvest application on various fruits and vegetables, was later acquired by Bayer CropScience (Germany) and recently sublicensed to Koppert (Netherlands) [27].

Research work is currently carrying out to develop new microbial antagonists that occur naturally on fruit surfaces, able to avoid infections in wounded fruits, also studying the several possible mechanisms involved in a tritrophic host-pathogen-antagonistic interaction system [27, 28].

Despite the progress obtained with microbial biological control agents (BCAs), their application in packinghouses is still limited, mainly due to the difficulty in obtaining adequate results under commercial conditions.

Among the antagonistic microorganisms used as BCAs, for postharvest applications, yeasts resulted the most effective on fresh fruits, due to their ability to adapt and to grow on particular fruit micro-environment and environmental postharvest conditions and to compete with the fungal pathogen for nutrients and space at the wound site. Penicilli and minor wound pathogens, parasite the citrus fruit throw rind wounds, where nutrients are more available and utilized for germination and host colonization. Yeasts need the same environmental conditions, and if present before pathogen infections are able to immediately develop and colonize the wound, by formation of an extracellular polysaccharide capsule that can promote adhesion to fruit surface forming biofilms covering the entire wound area. However, competition becomes an effective biocontrol mechanism when the antagonist is present in sufficient amounts at the right time. In the case of pre-established infections, the efficacy of antagonistic microorganisms is lower. Often, pathogens' growth is inhibited, but they leave alive [29].

Results obtained on citrus fruits applications of yeasts showed no commercially acceptable value, when antagonist stand-alone treatments were carried out [9]. For this reason, the integration of antagonists with different alternative methods has been proposed regarding different combined application of antagonists followed by generally recognized as safe (GRAS, for food contact applications) [30], hot water treatments [31] and elicitors of resistance. Most of the substances used (sodium bicarbonate) resulted in a delay in spore pathogen germination, with a competitive advantage obtained for antagonistic development. Recent study about mechanisms of action of microbial antagonists is a promising chance for a better utilization of microbial antagonists for postharvest treatments [27, 32, 33]. They effectively can be useful in improving knowledge about the quadritrophic interactions taking place among the antagonist, the pathogen, the host and the resident epiphytic microflora, on wound site, offering the opportunity to improve yeast's efficacy, when applied on fruits under commercial conditions [34, 35].

2.3.3. Generally recognized as safe

Owing to rapid degradation on the host surface, GRAS leaves low or no detectable residues in the commodity.

Carbonate and bicarbonate salts resulted as the most effective substances, on reducing *Penicillium* rots on citrus fruit immersed in 3% (wt/vol.) of sodium carbonate and bicarbonate solutions at ambient or at high temperature [36]. Ozone has successfully proven to be suitable for fruits and vegetable preservation, due to its antimicrobial and antioxidant activity (increased vitamin C and phenolic content). In addition, ozone has been used for the reduction of volatile compounds and ethylene present in the storage room or during shipping, in order to slow the senescence of the fruit, to reduce the incidence of rots conferring greater resistance to some physiological disorders, and it is fundamental to extend the shelf-life of citrus fruit during long-term storage [37].

During the postharvest treatments of fresh fruit and vegetable, ozone can be used for short periods in air or washing water, or it can be added continuously or intermittently into the environment during the storage period. Ozone diluted in water can be used as a hypochlorite substitute for disinfection and sanitation purpose.

Di Renzo et al. [12, 38] studied a prototype in order to control ozone/air mixture during washing and storage of citrus fruits. A feedback control system, equipped with high-precision-measuring sensors, was set up to control the active concentration. The obtained results showed a low efficacy of control when using ozone in the washing water, probably due to the variable level of contamination due to the impurities deriving from citrus fruits. Whangchai et al. [39] demonstrated the synergistic effect of ultrasonic irradiation in combination with ozone in reducing residual ethion of tangerine (*C. reticulata* Blanco cv. Sai Nam Pung) fruit after harvest. Ethion concentration was reduced to 75.43% after ultrasonic irradiation at 1000 kHz and ozone exposure for 60 min. Palou et al. [40] studied the ozone gas penetration and its effectiveness in controlling the sporulation of *P. digitatum* and *P. italicum* within commercial packages of oranges (cv. 'Lanelate') during cold storage at 12.8°C. However, discrepancies in results were found in the literature [41] due to variables that may influence ozone efficacy (O_3 generation and application methods, O_3 concentration and duration time, method of O_3 exposure, storage conditions and varieties). In recent years, a large variety of natural compounds with antifungal activity from plants, animal-derived materials and microorganisms have been evaluated, and much literature have been reported [42, 43]. The effectiveness at low concentrations of some aroma components was found of particular interest. In vitro fungal inhibition was obtained by some isothiocyanates, trans-2-hexenal, carvacrol, thymol, citral and trans-cinnamaldehyde [44]. An obstacle to their practical application has been the off-odours absorbed by commodities, able to alter their flavour, the phytotoxicity and humans' safety issues, when used at high concentrations and the possible spore germination stimulation when used at low concentrations [45, 46]. Recent studies have been carried out employing a pomegranate peel extract (PGE) for the control of postharvest rots [47].

2.3.4. *Integrated treatments*

Effectiveness of alternative methods can be improved by the combination of different approaches, such as GRAS, physical methods, biocontrol agents and ultraviolet light [20, 40, 48]. This strategy could result in a synergic effect on disease control and two or more alternative approaches need to be combined to reach commercially acceptable effectiveness for postharvest decay control comparable to the synthetic fungicide treatments.

2.3.5. *Coatings*

Synthetic coatings are generally anionic microemulsions containing resins and/or waxes (shellac, wood rosin, candelilla, carnauba, beeswax, polyethylene and petroleum). The main purpose of coatings is to reduce fruit weight loss, and chilling injuries and shrinkage by reducing transpiration and respiration, and improve fruit appearance, providing gloss. Alternative coatings known as edible coatings and films have been developed as an eco-friendly technology. Located on food surface as a coating or placed on the environment of a packaged food, edible coatings or films provide a semi-permeable barrier to water vapour, oxygen and carbon dioxide between the food and the surrounding atmosphere, prevent physical damages, chemical and microbiological deteriorations, and prolong the shelf-life of products [49]. Due to the high economical value of worldwide citrus trade, the development of novel antifungal edible coatings for citrus fruit is a very active research field and a considerable number of studies are available, including biopolymers, cellulose derivatives with generally regarded as safe (GRAS) salts and plant, pectin and commercial waxes with essential oils [40, 50–52]. Chitosan, the cationic deacetylated derivative of chitin, is a biopolymer owing to biocompatibility, biodegradability and absence of toxicity, studied for its antimicrobial activity against a variety of bacteria and fungi and as chemical elicitor, able to enhance the protection of host plant tissue through induced/acquired resistance.

Despite the substantial progress that has been accomplished in evaluating new antifungal edible coatings, their implementation is still limited mainly because of general limitations associated to the edible nature of food-grade- coating components.

3. Cold storage

Temperature and relative humidity are considered the key factor in the control of deterioration rate during the postharvest-handling chain of citrus fruit. Several authors have shown that the maintenance of an optimal temperature level during the postharvest storage is the main strategy in order to extend the shelf-life. Optimal temperature and high relative humidity levels (RH 90–95%) represent the best strategy for citrus fruit storage. RH prevents moisture loss from the host tissues and consequent shrivelling.

The optimal storage temperature is variable with reference to citrus species and varieties. Most citrus cultivars are able to tolerate low-temperature levels during prolonged storage while other cultivars such as 'Fortune' and 'Nova' mandarins, lemons and grapefruit are most sensitive to low temperature so values above 9°C are recommended to avoid the insurgence of chilling injuries [53]. Common chilling injuries are the formation of brown pitlike depression in the flavedo (mandarins and grapefruits) and superficial scald in the flavedo of some oranges ('Navelate').

Rapid cooling allows lowering the temperature to the levels applicable during storage, cold treatment and shipment to market, resulting in a substantial reduction in both weight loss and decay. Different systems for rapid cooling are available, in relation to the heat-removal system: room cooling (by chilled air), forced air cooling, hydrocooling (by chilled water) and vacuum cooling. The air-cooling system (especially using a forced airflow through the fruits stacked in a pallet) is the most efficient method for the removal of heat in citrus fruits [54].

In the 'room-cooling' system, the heat exchange between fruits (packed in cartons, sacks, or bins) and cold air takes place directly in cold storage rooms, by means of air fans blowing the cold air (air speed between 1 and 2 m/s). Once the final temperature has reached (depending on species requirements), the air velocity is reduced to about 0.05–0.1 m/s. The room cooling system is still the most widely adopted system, mainly for economic reasons. However, this system leads to both inefficient cooling (very slow) and excessive water loss (associated with an over-drying of citrus skin). The space between stacks of boxes inside the storage room is fundamental in order to reduce the cooling time. A distance of about 10–15 cm (4–6 inches) is enough to allow cold air to circulate around the boxes or pallets. Furthermore, using vented boxes leads to a more efficient cooling process with respect to unvented boxes. For these reasons, it is clear that the use of traditional cold storage room is adequate only for the storage of fruits already chilled, because the mechanism of cooling is too slow and it is not suitable for the quick removal of the 'fruit heat' (thermal level of fruit when harvested in field). Therefore, in order to quickly cool fruit after harvesting specific equipment (pre-cooling plant) is needed, characterized by very high cooling capacity to make possible the removal of the 'field heat' in medium-short times. In order to improve the uniformity of the temperature and cooling in citrus storage rooms, the forced ventilation system was developed. The rapid cooling with forced air provides for the removal of heat by means of an airflow which, chilled on an evaporator of a mechanical refrigeration system, is forced through the mass of the produce by means of a fan, working generally in pressure. Examples include a fixed unit equipped with a fan housed inside the wall of a cold room, or a portable fan unit that can be moved around inside the cold room. Furthermore, the alignation of boxes vents is fundamental in order to allow the cold airflow to pass through the pallets. Forced air cooling is able to reduce the cooling time from more days (room cooling) to few hours, depending from the availability of adequate cooling power.

After pre-cooling, it is important to maintain high relative humidity levels around the fruits. Among the techniques applicable in commercial distribution and for the preservation of citrus fruit, the use of plastic films is particularly considerable. Such films are able to lead to an accumulation of carbon dioxide and a reduction of oxygen, slowing both the respiratory activity and degradation of the reserve substances. Furthermore, the transpiration of the fruits favours the establishment of high moisture conditions, which typically slows down the ageing phenomena and the weight losses, restricting the production of ethylene and extending the "shelf-life" [54].

3.1. Citrus shipment

The global citrus fruit market had recently seen a great development in export activity, hence requiring both the respect of fruit quality standards and the restriction of the parasites spread (Mediterranean fruit fly, *Ceratitis capitata* Wiedemann; Mexican fruit fly, *Anastrepha ludens* (Loew)) in fruits. With this aim, it is mandatory for the importer to verify the thermal history (in terms of temperature variations) of fruits during the shipment, before the produce acceptance, and the respect of the cold treatment.

The cold treatment consists in low-temperature storage of fruits for a specific time (**Table 1**), carried out in cold storage rooms prior to shipping or during shipping ('in transit'). After a partial abandonment in favour of the methyl bromide fumigation, cold treatment was definitively introduced once the effect of methyl bromide (phased out on January 2005) was demonstrated on the atmospheric ozone layer depletion [55].

Core temperature (°C)	Treatment time (days)
0°C or less	12
0.6°C or less	13
1.1°C or less	14
1.6°C or less	16
2.2°C or less	18

Table 1. Protocol for the cold treatment adopted in the United States (USDA – APHIS 2002) for the control of the Mediterranean fly (*Ceratitis capitata*) spread during import of citrus fruit (orange, clementine, grapefruit and lime).

Both empirical and theoretical studies have been carried out to study the thermal variations and airflow during perishable goods shipping, showing that, in almost any transport situation, local temperature deviations are always present. From the literature reports, deviations of roughly 5°C or more depending on the transport conditions [56] are indicated.

Tauriello et al. [57] carried out experimental trials to simulate a cold treatment during a refrigerated transport of citrus fruits at industrial scale, monitoring the fruit temperature distribution inside the reefer in order to reduce the temperature difference in the load.

Several approaches have been used to predict both the airflow rate and temperature distribution, nevertheless giving only qualitative information on the air circulation rate. For this reason, computational fluid dynamics (CFD) tools are widely used [58]. CFD is a method that using numerical, physical analysis and computational software solves and analyses problems related to fluid flows (air mixtures and liquids). Recently, Defraeye et al. [59] suggested an innovative and promising cold-chain protocol alternative to the commonly used forced-air pre-cooling (FAC) of citrus fruit prior to shipping. The theoretical approach, by means of computational fluid dynamics (CFD), explored ambient (warm) loading of citrus fruit into refrigerated containers (reefer) for cooling by vertical airflow during marine transport. Results suggested that the optimization of ambient-loading protocol is strictly related to the improvement of both box design and stacking on the pallet, in order to reduce the airflow circuits between the pallets, but authors concluded that a synergy between numerical and full-scale experiments could contribute to improve further model.

4. Non-destructive methods for fruit quality evaluation

Fresh citrus fruit required external and internal quality from the harvest until reaching the consumer. The fruit quality could be defined as the combination of fruit attributes or characteristics that have significance in determining the degree of consumer acceptance. For the sake of quality control, fresh citrus fruits are generally inspected for adherence to both minimum maturity (internal quality) and grade (external quality). Minimal acceptable grade standards are prescribed in various laws and minimum maturity standards are specific for each citrus type. Based on these standards, quality inspectors judge the quality and decide its utility and marketability. Quality evaluation and control is also essential for deciding fruit price. Quality attributes can be evaluated by both subjective and objective methods. The objective methods are precise and involve the use of instruments, while subjective methods make use of human senses. In addition, most acceptable citrus fruit quality classification systems, whether manually operated or automated, are based on some traditional techniques and methods well established for evaluating fruit quality. These methods are normally sample-destructive, laborious and time-consuming, thereby limiting their utility in online/in-line quality monitoring. Moreover, industry demand increased for innovative tools: rapid with cost-effective for the evaluation and monitoring of citrus fruit quality. Therefore, several researches were focused on the application of new non-destructive methods in citrus fruit quality detection, including hyperspectral imaging (HSI), electronic noses (e-noses) techniques and nuclear magnetic resonance (NMR) [60–62].

Consequently, the advantage of non-destructive methods is that they can be used after harvest to make sure that every fruit sent to the market meets the quality norms, in contrast to the destructive methods, in which a representative sample is lost during analysis. Furthermore, citrus fruit quality attributes may significantly vary among citrus species and cultivars within the same species or even within the same cultivar grown in diverse climatic conditions or under different cultural practices.

4.1. Measurement of external quality

4.1.1. Firmness

Fruit firmness is roughly estimated by consumers' touch in the supermarket or by a Magness-Taylor (MT) tester in the laboratory [63]. However, MT testers tend to cause operation; consequently, such tests were replaced with others capable of assessing the mechanical properties of citrus fruits in a more objective and reproducible way [64]. A universal testing machine (UTM) [65] was used to sort on-line only the fruits with tension in the range of 375–445 N m^{-1}, in order to limit or vanish Sicilian Tarocco orange fruit rejection after long shipping in foreign markets and guaranteeing their longer shelf-life. In addition, the firmness of two kinds of orange was estimated using an HIS system [66], with utilization of partial least square regression (PLSR) to build prediction model of orange firmness [67].

4.1.2. Detection of contaminants and defects of fruit

The presence of common defects or disorders is insufferable for consumers and the presence of skin defects is one of the most influential factors in the price of fruit. The challenge is significant regarding citrus rind disorders that do not manifest during harvest grading and postharvest treatments but is developed 1–5 weeks after harvest. Consequently, the main task is to develop non-destructive technology to determine rind quality in the packing line to assist in sorting and segregation of fruit into quality grades.

4.1.3. Fruit contaminants

Various non-invasive and rapid technologies were investigated for the automatic detection of decay in citrus fruit as alternatives to human inspection. The great potential of HSI technique was evidenced to detect an early-infested mandarin with *P. digitatum* from sound ones [68]. Moreover, the potential of Near InfraRed (NIR) reflectance spectroscopy in combination with linear discriminant analysis was proved for the automatic detection of the early symptoms of decay caused by *P. digitatum* fungus in mandarins [69]. Furthermore, e-nose was able to identify different volatile organic compounds (VOCs) surrounding oranges infected by *P. digitatum*, representing an early indication of the up-coming deterioration [4]. In addition, several researches reported that a commercial e-nose was able to differentiate between lemon and oranges non-contaminated and contaminated with *P. digitatum* spores [70, 71]. Moreover, the high specificity and sensitivity of e-nose sensors in combination with a PLS discriminant analysis was evidenced for the early detection of low VOCs production in infected citrus fruit placed in controlled environment [62].

4.1.4. Fruit defects

In general, fruit defects can be divided into two categories. The first occur before the harvest without degeneration after the harvest, while the second occur in the whole process from fruit growth to their postharvest marketing. Traditional detection methods of fruit defects such as visual inspection, computer vision and spectroscopic technique are only able to detect defects occurring on fruit surface or under peel [72]. HSI technique is increasingly introduced to simultaneously estimate defects on fruit surface and under the peel. In addition, Near InfraRed (NIR) region of the spectrum can improve the inspection by detecting specific defects or allowing the detection of non-visible damages. NIR in the absorbance/reflectance mode has been used successfully to detect drying internal disorder in Tangerine citrus [73], and to predict oleocellosis sensitivity in citrus fruit [74]. Furthermore, multispectral inspection system was developed to detect and sort citrus fruits according to 11 different types of external defects including some morphological features of defects [75]. In addition, species and cultivars of citrus present a high rate of unpredictability in texture and colour, which makes it difficult to develop a general unsupervised method able to perform this task. In this context, a novelty detection technique was performed by using unsupervised method, based on a multivariate image analysis strategy in combination with PCA for the detection of new unpredictable defects in oranges and mandarins [76]. This unsupervised method needs only a few samples to be trained and could be suitable for the task of citrus inspection.

4.1.5. Measurement of internal quality

Citrus internal quality parameters can be assessed using NIR methods by determining the concentration of organic molecules on citrus fruit. Many studies have been focused on evaluating the internal quality attributes using reflectance measurements acquired with visible NIR spectroscopy technology on citrus fruit [77, 78]. Currently, the NIR technique has significantly greater accuracy for determining solid soluble content (SSC represents the amount of solids present in fruits, and soluble in tannin or water extracts, correlated to sugar content) in citrus than any other quality parameters such titratable acidity (TA represents the acidity of fruits due to the presence of organic acids, mainly citric acid). Consequently, the low success for the remaining internal parameters could be attributed to the fact that organic acids concentration on intact fruit is relatively low (about 10%); for that reason, calibration of this attribute is likely to represent secondary correlations to the parameters related to fruit maturity [79].

5. Conclusions

Citrus fruits are cultivated all over the world due to their positive health values, especially bioactive substances, vitamin C and phenolic compounds. The perishability of fruits is generally due to wrong temperature and relative humidity, responsible for increased respiration rate, physiological disorders and fungal pathogens. In order to optimize temperature and relative humidity during cold storage and shipping of citrus fruits, both experimental and theoretical methods are used, also by means of numerical methods (computational fluid dynamics). Fungal pathogens affect severely the postharvest life of citrus fruits, especially if stored for several months or shipped for long distances. Sanitizers (chlorine solutions) and fungicides [imazalil (IMZ), Thiabendazole (TBZ) and sodium-orthophenylphenate (SOPP)] are used for fruit surface treatment. In recent years, several low-risk fungicides (Trifloxystrobin, Azoxystrobin, Fludioxonil, Cyprodinil and Pyrimethanil) classified as a minimal risk to human and environmental health have been proposed.

A fundamental focus in the postharvest sector is the development and application of innovative methods for citrus fruit quality detection. A particular attention is paid to the nondestructive systems, as image vision systems (hyperspectral imaging), spectrophotometric methods (based on visible and infrared light source), sensing technologies (electronic-noses) and nuclear magnetic resonance (NMR).

On the basis of the current knowledge about citrus postharvest and in order to extend the shelf-life of fruits, the following future challenges could be identified: reduction of mechanical damages during handling and packing operations; research to find new alternative methods for fruits treatment to reduce the use of chemical compounds; and optimization of both cold storage room and refrigerated container for citrus shipping.

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Chapter 8

Organic and Inorganic Salts as Postharvest Alternative Control Means of Citrus

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Additional information is available at the end of the chapter

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Abstract

Several postharvest disease control means alternative to conventional chemical fungicides, such as organic and inorganic salts, will be highlighted in the proposed chapter. In particular, it will comprehensively cover different aspects of the use of salts against postharvest *Penicillium* decay of citrus. It will be an essential resource for the graduate and postgraduate students, researchers, professionals, supply chain players, citrus industries, and retailers. Organic and inorganic salts have a broad spectrum of activity against a wide range of fungi. In addition, they are easy to apply, inexpensive, safe for humans and the environment, and suitable for commercial postharvest handling practices. Different application strategies of salts, before and after harvest, and combined application (with wax, natural compounds, and fungicides, etc.) will be also discussed. The present chapter attempts to highlight how the use of organic and inorganic salts as alternative postharvest disease management technologies has developed from the laboratory to the market.

Keywords: citrus, salts, postharvest pathology, GRAS

1. Introduction

Citrus world production has reached 71 M tons in 2013 (http://faostat3.fao.org/), and the Egyptian production reached 2.9 M tons, representing 30% of the total Egyptian fruit production. Italy ranked at ninth place in the world producers chart with 1.7 M tons. Citrus fruit suffers from postharvest diseases caused by several fungal pathogens. The major postharvest diseases can be divided into two classes based on their initial infections. Rots from field infection include *Alternaria* rot, *Phytophthora* rot, *Phomopsis* and *Diplodia* stem-end rot, and Anthracnose rot. Rots from postharvest infection include *Penicillium* rots, *Aspergillus* rots, Rhizopus rot, Sour rot, and Fusarium rot [1].

Green, blue, and whisker moulds caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., *P. italicum* Wehmer, and *P. ulaiense* (Hsieh, Su & Tzean), respectively, are the most important and destructive postharvest diseases of citrus fruit, especially if grown under Mediterranean climate conditions. The control of postharvest diseases is generally exerted by chemicals. However, many fungal pathogens have developed resistance to the active ingredients of a wide range of fungicides. In addition, the problems associated with their use (i.e., waste disposal), as well as the public increasing awareness about health and environmental risks, have promoted the search for new and safer alternatives. Replacement of synthetic fungicides by salts, which are nontoxic for consumers and for the environment, is gaining considerable attention.

During last two decades, because of the consistent increase in the area under citrus cultivation, the production has grown dramatically. However, the interest in protecting the fruit against postharvest losses caused by the pathogens has not gained the same spur in the implementation of the available technologies. Despite international organizations responsible for monitoring food resources have been recognized that the most economically feasible and expedient means to increase food supply are to reduce postharvest losses [2].

The surface of fruits or vegetables is covered by fungal and bacterial propagules; however, most of them do not cause decay, even when conditions suitable for penetration and development are present because of natural resistance. However, natural resistance to disease of fruits generally declines after harvest, due to ripening and senescence, leading to decay and physiological impairment. The preharvest factors (soil conditions, moisture, temperature, relative humidity, and nutrient availability, etc.) that affect the postharvest life of a fresh produce were discussed [3].

2. Penicillium decay

The genus *Penicillium* includes 150 species, but only few species are economically important plant pathogens [4]. In Mediterranean climate areas, green and blue moulds caused by *P. digitatum* and *P. italicum*, respectively, are the most important postharvest diseases of citrus fruit. Both fungi are necrotrophs that feed on the dead matter and require wounds to enter the fruit [5]. Blue mould is generally of lesser overall importance, but may become a major problem under prolonged storage conditions, which suppress development of green mould [6]. At room temperature, green mould invades fruit much more rapidly than blue mould and predominates in mixed infections [7]. Currently, another species of *Penicillium*, *P. ulaiense* causing whisker mould, has being considered important on citrus, especially in packinghouses. It is a pathogenic fungus described as a member of the series Italica [8], together with *P. italicum*. In the past, the fungus was mistaken for *P. italicum* and its unique features dismissed as variations due to particular environmental conditions [9]. Recently, this fungus is becoming a serious concern for citrus packinghouses in several countries, because of its resistance to the commonly used preservatives. Rots are differentiated from those of *P. italicum* by the presence of coremia on the fruit [10]. Initially isolated from decaying oranges in Taiwan [11], *P. ulaiense* has been described on citrus fruit in the United States [9, 10], Australia [12], Argentina [13], and recently in Egypt [14] and Tunisia [15]. Besides the taxonomic and phytopathological aspects [8, 10], there is little or no information about this organism [16].

The optimal growth conditions for *Penicillium* involve a warm and moist environment. Although infection may take place in the field, blue and green moulds are essentially postharvest pathogens. The fungus can spread during storage, since the airborne spores are long-lived and may easily survive from season to season on contaminated bins. Inoculation can also occur during fruit handling, for example, in water contaminated with the pathogen spores in packinghouses [17]. A single decayed fruit may contain enough spores to contaminate water of the entire packing line [18]. The pathogen can penetrate the host tissues also through openings in the peel, and can spread from one fruit to another by simple contact (nesting). When the host is first infected, a stain appears on the peel; this rapidly forms a slight indentation, after which the fruit decomposes partially or totally within few days.

3. Disease management of citrus fruits

The best and most effective management strategies to control citrus fruit diseases would be to avoid or minimize the predisposing factors responsible for origin and development of the diseases through an integrated approach. These management strategies include improved cultural practices, regular monitoring of the disease appearance and weather conditions, improved postharvest handling, control agents, timely use of effective fungicides, transport, and storage conditions.

3.1. Accurate cultural practices

Efficient removal of infected fruit and minimizing fruit injury are effective ways to control blue and green moulds. Disinfectants such as chlorine, quaternary ammonium compounds, formaldehyde, and alcohol are useful for preventing inoculum buildup. Spore populations are kept low in packing areas by removing infected fruit promptly, using exhaust fans, and keeping dump areas well away from packing facilities [19]. Harvest following rain is discouraged because wet fruits are more prone to injury. However, sanitary practices should be applied to prevent sporulation on diseased fruit and the accumulation of spores on equipment surfaces as well in the atmosphere of packing and storage facilities. Efficient packinghouses systematically segregate spoiled fruit in storage, distribution or repack, effectively reducing the disease [20, 21]. Moreover, effective sanitation practices during pre and postharvest handling, avoiding possible inoculum sources and preventing contamination, can significantly reduce the incidence of decay.

3.2. Chemical control means

Control of green and blue moulds varies among different countries' legislation but is mainly based on the use of fungicide treatments, principally thiabendazole and imazalil, sprayed alternately or simultaneously, on fruit during waxing operation in the packinghouse. The control of postharvest fungal pathogens on citrus depends on the prompt application of a suitable fungicide at its recommended concentration. If the fungicide is not applied soon after the rind injury, which usually occurs during harvesting and subsequent handling, infection establishes and the organism escapes the protective activity of the compound being applied. The time allowed between the beginning of an infection and the application of a fungicide to completely inhibit or control infection depends on the growth rate of the germinating organism, which in turn depends on the fruit temperature [22]. Fungicidal dips and sprays, with thiabendazole, imazalil, thiophanate-methyl, sorbic acid, guazatine or sodium o-phenylphenate (SOPP) are used to control the disease [23]. Several years after the introduction of fungicides as thiabendazole, imazalil, and SOPP, resistant biotypes of *P. digitatum* were reported in packinghouses in many citrus production areas [24] and terminal markets [25]. These fungicides are used in a manner that is highly conductive to the selection and proliferation of resistant strains of *P. digitatum* and *P. italicum*. O-phenylphenol and thiabendazole have been used routinely on citrus fruits over the past three decades, resulting in a serious problem of resistance by the late 1970s [26]. It was reported that imazalil, successfully used in Mediterranean citrus production areas for several years, could be an effective treatment for control of thiabendazole-resistant isolates of *P. digitatum* and *P. italicum* [27]. It was investigated that all *P. digitatum* and *P. italicum* isolates collected in a citrus orchard were sensitive *in vitro* to imazalil and thiabendazole [28]. This result was consistent with earlier studies [29–31] suggesting that naturally occurring resistant isolates of *P. digitatum* and *P. italicum* are rare or absent in citrus orchards, especially in those orchards without a prior history of fungicide usage. Nevertheless, isolates of *P. digitatum* resistant to thiabendazole, imazalil, or both fungicides were detected in all packinghouses sampled. Pyrimethanil (PYR) has been registered in USA against citrus green and blue moulds. A 90% reduction of green mould by PYR applied at ≥500 mg/l by dipping or drenching the fruit, and a 65% reduction applying PYR at 1000 or 2000 mg/l in wax over rotating brushes were observed. Indeed, PYR in aqueous solutions controlled sporulation better than when applied in wax, but it was less effective than imazalil, although an imazalil-resistant *P. digitatum* isolate was controlled by PYR. The sodium bicarbonate addition proved to improve PYR performance [32].

3.3. Nonchemical control means

Although synthetic fungicides are the primary means for controlling postharvest diseases of fruits and vegetables, the growing concern for the human and environmental health [33], the cost of developing new pesticides to overcome pathogen resistances [34, 35], and the lack of continued approval of some of the most effective fungicides motivated the search for alternative approaches [36]. Several alternative means have been proposed to control postharvest diseases of fruits and vegetables: biocontrol agents [37], natural substances [38], physical treatments [39], and organic or inorganic salts [40–42]. Although all these approaches proved to be effective on a large number of hosts, they do not always offer a control level comparable to that provided by synthetic fungicides. However, an economically sufficient control extent can be obtained by the use of two or more alternative means in an integrated approach [37].

3.3.1. Generally recognized as safe (GRAS) substances

"Generally recognized as safe (GRAS)" is a category of the American Food and Drug Administration (FDA), stating that a chemical is considered safe to humans and animals, and thus considered food-grade. As such, carbonic acid salts are GRAS additives allowed with no restrictions for many applications (including food industry) in Europe and North America. The antimicrobial activity of these chemicals has been described *in vitro* [43] and in a wide range of substrates as well. Sodium bicarbonate ($NaHCO_3$) has been used as a disinfectant for citrus fruit since 1920 [44–49]. Treatments with sodium carbonate (Na_2CO_3) and $NaHCO_3$ that reduced the incidence of postharvest decay on lemons proved to be as effective as higher concentrations of calcium chloride ($CaCl_2$) in preserving tissue firmness during storage [50–52]. Inversely, Ca salts at high concentrations (187.5 mM) caused symptoms of phytotoxicity on the fruit surface, in terms of skin discoloration and superficial pitting, leading to further chemical changes and reduced tissue firmness [53]. Concerning strawberries, $CaCl_2$ dips in combination with heat treatment or storage in modified atmosphere and refrigeration proved to increase calcium content, fruit firmness, and delay postharvest decay [54]. Organic calcium salts are an alternative calcium source and calcium lactate has been described in the literature as a firming agent for several fruit. According to Lawles, the bitter and salty tastes associated with calcium chloride are largely suppressed when calcium is combined with larger organic ions such as lactate, gluconate, or glycerophosphate [55].

In Egypt, a complete inhibition was observed in the linear growth of *G. candidum*, *P. digitatum*, and *P. italicum* when exposed to benzoic, citric, and sorbic organic acids at concentrations of 4% and 2% of either sodium benzoate or potassium sorbate, respectively [56]. The efficacy of potassium sorbate and ammonium bicarbonate as possible alternatives for controlling soilborne pathogens *Fusarium oxysporum* f. sp. *melonis*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* was evaluated [57]. Authors summarized that potassium sorbate had higher toxicity to all fungi compared to ammonium bicarbonate in soil tests. Both ammonium bicarbonate and potassium sorbate increased the pH of soil. The rate of pH increase was higher in ammonium bicarbonate.

More than 20 food additives and GRAS compounds were tested to control major postharvest diseases of stone fruit by Ref. [58]. Overall, the best compounds were 200 mM potassium sorbate, 200 mM sodium benzoate, 200 mM sodium sorbate, 100 mM 2-deoxy-D-glucose, 400 mM sodium carbonate, and 250 mM potassium carbonate. Moreover, potassium sorbate was successfully tested in mixture with commercial fungicides against the major postharvest pathogens of citrus fruit, in particular, combined low concentrations of potassium sorbate with imazalil, thiabendazole, pyrimethanil, and fludioxonil [59]. Potassium sorbate was not only compatible with these fungicides, but also enhanced their effect against *P. digitatum* and *G. citri-aurantii*. In other studies [40, 60–62], potassium sorbate solutions applied at room temperature or moderately heated for relatively long immersion times (2–3 min) resulted effective to control green mould [59].

3.4. Integrated control options and strategies

Alternative control means alone are often less effective compared with commercial fungicides or provide inconsistent control. Therefore, to achieve a similar level of efficacy provided by conventional fungicides, an integration of commercial chemicals at low doses [63], hot water [64], chloride salts [56, 65], carbonate salts [40], natural plant extracts [66], and other physical treatments such as curing and heat treatments [67], is recommended [68–71].

3.4.1. Citrus fruit wax combined with salts

Generally, citrus fruits are waxed in order to improve their shine [72] and reduce water loss during postharvest storage. Previous studies showed that, the application of shellac-based waxes reduced internal O_2 levels, and increased internal CO_2 and ethanol levels [73]. According to Waks, waxes minimize stem-end rind breakdown and other collapses of rind tissue, and can protect the fruit from the entrance of pathogens. The effect of waxing on the incidence of postharvest rots apparently is not unique to citrus fruits [74]. For example, it is reported for Starking apples attacked by *Gloeosporium* sp., the cause of apple bitter rot [75]. Coating (mainly with wax, shellac, and sucrose ester, etc.) is not recommended for long-term storage to prevent off-flavor [76].

Waxes may also serve as carriers of fungicides or growth regulators, because these chemicals are less effective when applied in waxes than when applied in water; therefore, higher concentrations are generally used in waxes than in water [77]. According to Taverne, when combined in wax and applied at label rate (150 μL/100 g), imazalil reduced the decay levels. In particular, when fruits were treated with wax containing ≥3000 ppm of imazalil, sporulation was reduced, since residues of 2–3 ppm were achieved. Doubling the wax volume did not significantly improve the sporulation control. Combinations of imazalil applied in water followed by imazalil in wax might be required to ensure high decay and sporulation control [78].

It evaluated the effect of organic acids (ascorbic, benzoic, citric, and sorbic) as well as organic salts (potassium sorbate and sodium benzoate) at different concentrations (0, 0.5, 1.0, 2.0, and 4.0%) on the growth of *Geotrichum candidum*, *P. digitatum*, and *P. italicum in vitro* and *in vivo*. A complete inhibition was observed in the linear growth of all tested fungi when exposed to all organic acids concentrations and to 4% and 2% of either sodium benzoate or potassium sorbate salts, respectively. The various tested organic acids and salts showed different extent of protective or therapeutic effect on the coated lemon fruit against mould infection, whatever the time of their artificial inoculation. All treated citrus fruit showed reduced rate of sour rot, green and blue moulds when compared with untreated fruits. A complete inhibition of mould incidence was obtained in coated lemon fruits with 4% of sodium benzoate and potassium sorbate in water or wax mixtures 24 h before inoculation. In addition, high reduction in mould incidence was observed in lemon fruits coated with the same salt concentration 48 h after inoculation under the same conditions [56].

In general, the most practical combination of solution temperature, chemical concentration, and treatment duration for optimal decay control must be determined for each chemical and each host-pathogen systems [68]. Heat and the integration of certain physical, chemical, or biological treatments have been evaluated for postharvest decay control of peaches and nectarines [79, 80]. According to our knowledge, a limited research has been conducted to elucidate the ability of a mixed application of wax and food additives on the development of postharvest diseases of citrus during storage [41].

3.4.2. Electrolyzed water in combination with salts

Sanitizing is considered a fundamental component of processing the fresh fruit and vegetables. Typically, chlorine compounds and fungicides are the main active elements in sanitation and postharvest processing of fresh fruits. The corrosive effect of chlorine and toxicity of fungicide residue are considered a serious issue in processing fresh products [81, 82]. Development of alternative methods for sanitizing fresh products and controlling postharvest diseases is derived by economic and consumer demand motivations [83].

Electrolyzed water (EW) was introduced as decontaminating agent [84], and its application was approved in Japan and USA as a food additive and sanitizing agent [85, 86]. EW has shown its potential to inactivate pathogenic microorganisms such as bacteria [87–89] and fungi [17, 90–93]. One of the main advantages of EW is the less adverse effect on environment and human health because no hazardous materials are used during its production [85].

The electrolysis of diluted solution of salts (e.g., NaCl, KCl, and $MgCl_2$) leads to dissociation of salt ions, and formation of anion and cations at anode and cathode, negative and positive electrodes, respectively [94]. The physical properties and chemical composition of EW vary depending on the concentration of salt solution (e.g., NaCl), electrical current, length of electrolysis, and water flow rate [95]. Most previous studies assessed electrolyzing parameters (flow rate, current intensity, and time for electrolyzing) effect on free chlorine, electric conductivity, and pH of the resulted EW [96–98], while very few studied the effect of electrolyzing parameters on the ability of electrolyzed water to deactivate pathogen unit [17]. Although salt solutions used in electrolysis play a major role in the effectiveness of EW, few salts were studied for their effect on EW efficiency: sodium chloride [93, 96–100], potassium chloride [101], and sodium bicarbonate [102].

Anions of Cl^- are targeting polypeptides and carbohydrates in the cell wall, in addition to destruction of nucleic acids—DNA and RNA—which impairs the process of DNA replication and gene expression, therefore, halts the cell division and stops the essential biological function leading to cell death. Oxidation Reduction Potential (ORP) has shown moderate biocide effect following free chlorine level. ORP indicates the tendency of ions to accept/donate electrons (reduction/oxidation), and was suggested as alternative stronger sanitizing factor compared to free chlorine [91, 103, 104]. It was found that in case of long exposure time, decrease of ORP to −300 mV reduced coliform bacteria population to 10 fold, and at ORP −400 mV, coliform bacteria population decreased 100 fold, and decreasing ORP to below −600 mV will completely inactivate all coliform bacteria [105]. Thus, it was possible to improve the biocide activity by manipulating the ORP regardless the anion present. Using salts with different anion radical than Cl^-, it is possible to still achieve the same level of disinfestations or even improve the biocide activity targeting multiple sites in the pathogen biological system [105].

The values of pH represent the level of hydrogen ion activity governing the solution acidity/alkalinity. The solution acidity/alkalinity has a biocide effect against pathogen cells, and although it came third to free chlorine and ORP, it still shows significant effect on the pathogen count. The role of pH is believed to make the cells more sensitive to active chlorine by erupting their outer membrane and facilitating the entry of HOCl [106]. Previous papers have studied the effect of electrolyzing parameters on the electrolyzed water characteristics [17, 95]. Recently, acidic electrolyzed water has shown higher biocidal activity toward *P. digitatum*, *P. italicum*, and *P. ulaiense* causing green, blue, and whisker moulds, respectively. When compared to the effect of alkaline electrolysis water [107], the author suggested that using amended salt solutions would improve the biocidal effect of electrolyzing. Further studies have suggested the higher biocide effect of acidic water and also suggested alkaline water to be used in cleaning and degreasing before application of acidic electrolyzed water [108–112].

3.5. Preharvest treatment for controlling postharvest citrus decay

Citrus requires about 5–9 months for its maturity on trees, and during this long maturity period, the fruit remains exposed to the attack of preharvest fungal pathogens such as *Colletotrichum gloeosporioide*s, *C. acutatum*, *Botryodiplodia theobroma*e, *Alternaria citri*, and *Phomopsis citri*, etc. The incipient infection of preharvest pathogens subsequently manifests in the form of postharvest diseases, besides the attack of the main postharvest pathogens such as *P. digitatum*, *P. italicum*, and *G. candidum*, etc., during postharvest handling, transport, storage, and marketing. A number of nonconventional alternatives have been trailed as standalone treatments. It was showed that, potassium sorbate was effective when used on oranges, grapefruits, mandarins, and tangerines, but later research found that the effectiveness was influenced by citrus species and cultivars [60]. Potassium sorbate efficacy is variable when used as commercial treatment for citrus fruits, limiting its use as a standalone treatment [113].

In this perspective, the optimization of some promising alternative methods, like the use of salts, is a great interest. Since injuries sustained by citrus fruit during harvest, strongly favorite wound pathogens such as *P. italicum* and *P. digitatum*, the reduced efficacy of alternative control means could be partially ascribed to the lack of curative effect against already established infections. Based on this consideration, a near-harvest treatment could be an appropriate strategy to prevent the colonization by pathogens during harvesting and postharvest handling of fruits [42, 114].

The effectiveness of different salt solutions (2%, w/v), sodium carbonate and bicarbonate, potassium carbonate and bicarbonate, and calcium chloride, in controlling postharvest *Penicillium* rot of "Hernandina" clementine were reported [115]. Preharvest sprays and the combination of pre and postharvest application of salts were in average more effective, in suppressing *Penicillium* rot, than the individual postharvest dipping [42]. A detailed study of the ability of sodium carbonate and bicarbonate to induce resistance in oranges has been performed and published [116].

4. Conclusion

The book chapter aims at presenting salts as a sustainable approach for controlling postharvest diseases of citrus fruit to researchers and industries, graduate, and postgraduate students. The use of organic and inorganic salts was reviewed since the pioneer studies in the early 1920s of the last century until the most recently published works. Several salts confirmed their efficacy as affordable, safe to consumers and operators, and already used in the food industry. More studies concerning their mode of action might contribute to expand their application in the field and/or packinghouses.

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Section 5

Application of Citrus and Its Compounds

Chapter 9

Citrus: A Perspective for Developing Phytomedicines for Neurodegenerative Diseases

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Additional information is available at the end of the chapter

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Abstract

The antioxidant and anti-inflammatory properties of *Citrus* flavonoids can play a key role in their activity against several degenerative diseases and particularly brain diseases. In Brazil promising studies deposited in the patents "pharmaceutical formulation" form was obtained from the inclusion of *Citrus sinensis* L. (orange) essential oil with β-cyclodextrin and *Citrus limon* (lemon) compounds and their applications in therapy of Alzheimer's disease. In this chapter, we report activities of active compounds present in the genus *Citrus*, which include antioxidant, anti-inflammatory, anxiolytic, insecticidal, and anti-cholinesterase activities. These activities are associated with some neurodegenerative diseases such as Alzheimer's disease (AD). Pharmaceutical formulations containing such compounds (for example, inhibitors such as acetylcholinesterase (AChE) of *C. limon* (5,8-dimethoxy-psoralen and 5,7-dimethoxycumarin) and essential oil from *C. sinensis* oils are reported in this chapter. These results indicate that the effects of the essential oil and substances of *Citrus* species are very interesting for further isolation of AChE inhibitors that can be used in the formulation of natural products for neurodegenerative diseases.

Keywords: citrus, *Citrus sinensis*, *Citrus limon*, acetylcholinesterase, neurodegenerative diseases

1. Introduction

Citrus fruits make up the largest sector of the global production of fruits, with more than 100 million tons produced every season [1]. *Citrus* is one of the most important genera of Rutaceae family because its fruits are estimated primarily as articles of diet [2]. The aromatic oils from

Citrus are used as flavoring agents in a variety of foods, drinks and confectionery and for fragrance applications. There are reports of the *Citrus* genus with important biological activities, namely antioxidant [3, 4], antimicrobial [5], anti-inflammatory [6], insecticidal [7, 8], anxiolytic [9], and anticholinesterase [3, 10]. In traditional Chinese medicine, the dried peel of *Citrus reticulata* (mandarin) has been widely used for centuries as a remedy for treating indigestion and fighting respiratory tract inflammatory syndromes such as asthma and bronchitis [1]. *C. aurantium* L., commonly known as sour orange bitter, is popularly used as a medicine in Brazil and other countries to treat anxiety and insomnia and used as an anticonvulsant, suggesting itcan act as a central nervous system (CNS) depressent [11]. *Citrus limon*, popularly known as lemon, was described possesesing variuos biological activities such as antifungal [12], antimicrobial [13], antioxidant [14, 15], antinoceptive [14], and larvicidal [16]. *C. limon* comprises flavonoids, volatile oils, and coumarins; however, their effect on the CNS is not well known [17].

In this chapter, we report activities of active compounds present in the genus Citrus, including antioxidant, anti-inflammatory, anxiolytic, insecticidal, and anticholinesterase activities, which are associated with some neurodegenerative diseases such as Alzheimer's disease and we report the pharmaceutical formulation of *C. limon* and *Citrus sinensis*.

2. Acetylcholinesterase activity (AChE) of Citrus species

Alzheimer's disease (AD) is a multifactorial disease that affects a significant portion of the population and its incidence has grown over the years due to the increasing proportion of elderly people in the world population. Factors such as formation of senile plaques and neurofibrillary tangles, reduction of acetylcholine levels (by inhibiting the enzyme acetylcholinesterase) and oxidative phenomena are related to the development and/or progression of AD [18, 19]. The acetylcholinesterase (AChE), an enzyme inhibitor associated with AD, is widely detected by Ellman's test. According to the principle of the method of Ellman et al. [18], the reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme and in the concentrations used does not inhibit the enzymatic hydrolysis [18, 19].

Some AChE inhibitors are found naturally in medicinal plants. Reversible cholinesterase inhibitors are currently used in clinical trials for treatment of Alzheimer's disease [4]. The treatment is based on the inhibition of AChE, which hydrolyzes acetylcholine, increasing their availability to cholinergic transmission [20].

The anticholinesterase activity of extracts from *C. limon* (lemon) leaves compared to galantamine, a drug widely used in the treatment of AD, and other species used in popular medicine in Northeast Brazil are reported in a phytochemical screening [21]. Another study described inhibition of a fraction of the ethyl acetate extract from the leaves of *C. limon,* which was isolated from the active fraction named two coumarins: 5,8-dimethoxy-psoralen and 5,7-dimetoxi-coumarin (**Figure 1**). *In vitro* studies indicate IC_{50} of 5.8 μg mL^{-1} (95% confidence) and *in vivo* studies with male Swiss mice showed inhibition of 30.09–30.06% for the enzyme AChE compared to neostigmine, a drug used in the treatment of AD [11].

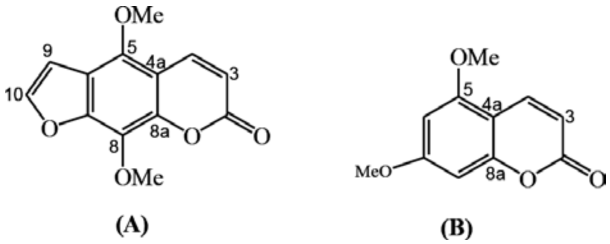

Figure 1. Inhibitors AChE of *C. limon* (A) 5,8-dimethoxy-psoralen (B) 5,7-dimethoxycumarin. Molecular structures were drawn with ChemDraw13 (Perkin Elmer).

In vitro and *in vivo* assays with the essential oil of *C. sinensis* (L.) Osbeck (orange) against AChE enzyme indicated that there was a significant decrease in AChE activity in the hippocampal region of mice in *in vivo* tests. *In vitro* testing of *C. sinensis* essential oil showed a value inhibition concentration with IC_{50} = 63 μg mL^{-1} whereas for the standard (neostigmine) was obtained as IC_{50} value = 1.87 μg mL^{-1}. For the antioxidant, activity was a significant 20% reduction in the hippocampus of mice treated with 150 mg kg^{-1}on lipid peroxidation, thereby reducing oxidative stress and nitrite content, these doses showed a significant reduction in all groups, suggesting a neuroprotective effect against brain injury [4].

Studies on the species as *Citrus medica* L. cv. Diamante (cidra) demonstrated anticholinesterase activity, which can be explained by the high amount of monoterpenes present in the skin of the fruit [10]. According to studies 17 monoterpenoids with *p*-methane skeleton was reported, the AChE inhibitory activity of compounds such as γ-terpinene and terpinen-4-ol arrive at 22.6 and 21.4% at 1.2 mM, respectively. Other terpenes such as limonene and linalool present in 164 μg mL^{-1} concentration, inhibition of the 27 and 37%, respectively. The same activity is presented to study the species such as *Citrus hystrix* (Combava), which caused 10% inhibition of AChE enzyme and that this action was related to the presence of acyclic and monocyclic monoterpenes such as citronellal and β-phellandrene, respectively, present in the essential oil extracted from the leaves of this plant [22].

The structural diversity of terpenoids that exert inhibitory activity of AChE is difficult to predict the potential structure-activity relationship. But it is known that some features, such as the presence of a hydrophobic ligand, may be associated with greater effectiveness in the inhibition, since the active site of AChE is known to be susceptible to hydrophobic interactions. The monoterpenes consist of a hydrocarbon skeleton that can be cyclic (α-pinene) or acyclic (linalool), a feature that may also influence their AChE inhibitory activity. For a bicyclic monoterpene skeleton pinene or carene, the potential of AChE inhibition was associated with the position of the double bond [10]. The presence of terminal olefins ($H_2C=CH_2$) resulted in decreased inhibition of AChE, as well as the presence of an oxygenated functional group [20].

Experiments that assess memory evaluate the effects of acute treatment with the essential oil of leaves (EOL) from *C. sinensis* in the acquisition of spatial memory in rats using the paradigm of the Morris water maze. In Morris assay, the acquisition of memory space is evaluated by time the animal takes to locate the platform after having been trained [23].

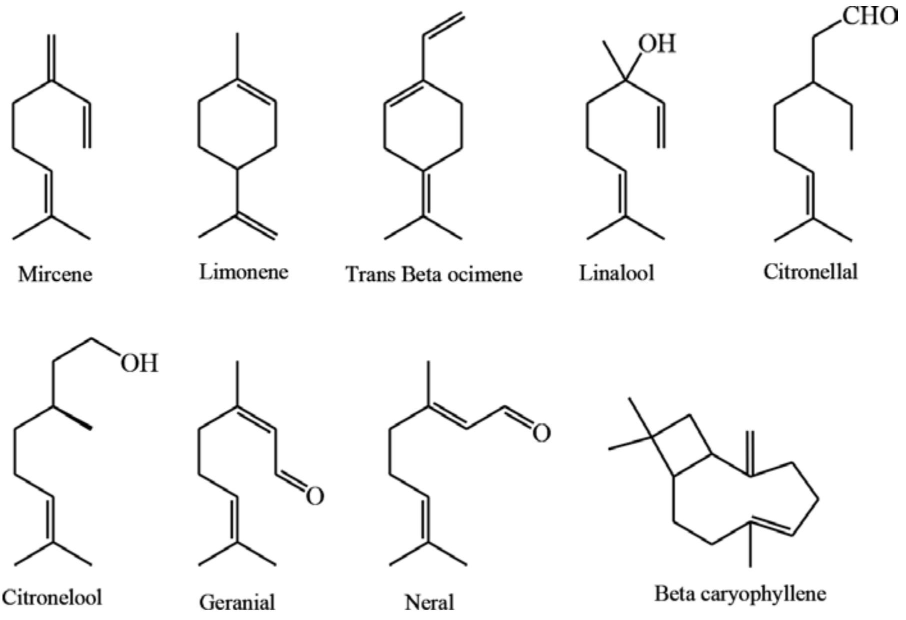

Figure 2. Constituent chemicals of the *C. sinensis* essential oil. Molecular structures were drawn with ChemDraw13 (Perkin Elmer).

The composition of EOL of *C. sinensis* is mainly composed of the class of monoterpenes such as limonene (24.14%), citronellol (30.42%), and geranial (31.42%) (**Figure 2**). The animals were previously untreated with doses of EOL of *C. sinensis* 50, 100, and 200 mg kg^{-1} and the open field test conducted and the Morris water maze task [23].

The acquisition of memory space is evaluated by time the animal takes to locate the platform after having been trained. The results of the open field were demonstrated that animals do not exhibit motor stimulus when treated with the essential oil of *C. sinensis* and the results of water maze were significantly lower to find the submerged platform than the negative control group ($p < 0.01$) indicating an increased memory capacity in the treated animals, but must be reinforced by other memory tests recommended by the literature. These results indicated that the effects of the EOL of *C. sinensis* may involve the cholinergic system and produce a reversal of memory impairment, caused by over activity of AChE [4].

Activities of active compounds present in the genus Citrus, which include activities such as antioxidant, anti-inflammatory, anxiolytic, insecticidal, and anticholinesterase. These activities are associated with some neurodegenerative diseases such as Alzheimer's disease [24].

3. Citrus antioxidants

Oxidative stress produced by free radicals has been implicated in the pathogenesis and progression of a wide variety of clinical disorders such as cancer, cardiovascular disease, inflammation, epilepsy, diabetes, and Alzheimer's disease [25]. Oxidative stress is the result of natural deficiency of antioxidant defenses, or by increased levels of reactive species derived from oxygen [26]. Reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•) are produced as a result of many biochemical reactions and can be considered the main cause of oxidative damage, as protein denaturation and lipid peroxidation mutagenesis [10, 27, 28].

The plants of the Citrus genus are rich in compounds that have antioxidant properties [22]. Phenolic compounds, particularly flavonoids, have shown an important antioxidant activity, which is mainly based on their structural characteristics and other chemical characteristics due to the number and position of phenolic hydroxyl groups [29].

Natural antioxidants from fruit juices offer an alternative source of dietary ingredients to promote healthy life. A recent study on the juice of *C. sinensis* (L.) Osbeck suggested the influence of C-and O-glycosyl flavonoid antioxidants and anticholinesterase properties [24, 30]. From the juice, 12 individual components were identified for the first time, namely, four C-glycosyl flavones (lucenin-2, vicenin-2, stellarin-2, lucenin-2 40-methyl ether, and scoparin), three flavonol derivatives (quercetin-3-O-(2-rhamnosyl)-rutinoside, quercetin-3-O-hexoside, quercetin 3-hydroxy-3-methylglutaryl-glycoside), O-triglycosylflavanone (narirutin 4-O-glucoside), and a flavones O-glycosides (chrysoeriol 7-O-neoesperidoside). Moreover, the influence of the identified C- and O-glycosyl flavonoids on the antioxidant and acetylcholinesterase activity of these juices has been evaluated [30].

Thirty-four types of essential oils of Citrus and its components were investigated for their antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH). The activity of components compared with a standard antioxidant, the trolox showed effects on DPPH scavengers ranging between 18 and 64%. One possible explanation for the difference in efficiency found in this study may be substantial variation in the compounds of the essential oils of citrus. Among the 34 types of essential oils, the radical scavenging activity of the lemon (*C. ichangensis*) species was the largest compared to oils Tahiti limon (*C. aurantifolia*) and eureka lemon (*C. limon*). Higher antioxidant activity of the high amount of terpenes with the exception of limonene and myrcene are reported [5]. Generally, the high radical scavenging activity was found when large quantities of terpenes include γ-terpinene and terpinolene. In Mexican lemons, Tahiti, eureka, and lisbon, the combined percentage of neral and geranial varied from 1.7 to 3.5%. It was also found that the components neral and geranial contribute to its effect sequestering these samples. *C. sinensis* and *C. reticulata* most effective compounds such as γ-terpinene and terpinolene were present in small amounts [5]

The antioxidant effect of α-terpinene, nootkatone, citronellal, citral, γ-terpinene, terpinolene, and geraniol substances result showed greater than trolox ($p < 0.05$). According to calculations based on the height of the peak of DPPH, γ-terpinene (84.7%), terpinolene (87.4%), and geraniol (87.7%) had radical scavenger effect 3.5 times that of trolox. Significant differences ($p < 0.05$) were not found between decanal and geranyl acetate. The antioxidant activity of linalool, citronellol, α-pinene, and octanal (18.7–22.4%) was higher than that of α-terpineol, octanol, myrcene, decanol, β-pinene, terpen-4-ol, *p*–cymene, and *d*-limonene (8.8 to 16.5%) [3]. Subsequent studies demonstrated a significant *in vitro* antioxidant activity of α-terpineol against lipid peroxidation inhibition of nitrite ions and hydroxyl radical [5].

The low-density lipoprotein plasma (LDL) plays a significant role in the development of atherosclerosis. The antioxidant activity of the essential oil of three Citrus species (*C. natsudaidai*, *C. hassaku*, and *C. unshiu*) was evaluated and showed promising effects. Studies have shown the antioxidative effect to evaluate the effect of citrus essential oil components on human LDL *in vitro*. Among the authentic volatile compounds, tested gamma-terpinene showed the strongest antioxidative effect, and inhibited both the Cu^{2+}-induced and AAPH-induced oxidation of LDL. Gamma-terpinene added after 30 min (mid-lag phase) and 60 min (propagation phase) of incubation of LDL with Cu^{2+} inhibited LDL oxidation [31].

4. Citrus anti-inflammatory activity

The inflammation is typically characterized by an increase in tissue permeability and endothelial leukocyte influx of blood into the interstitium, causing edema. Different mediators influence each step of the cascade of inflammation and characteristically inflammatory agents exhibit therapeutic properties by blocking the action or synthesis of these mediators. While inflammation is a normal response to tissue injury, often it is uncontrolled in chronic autoimmune diseases such as rheumatoid arthritis and Crohn's disease, or when related to allergic response such as asthma and anaphylactic shock. In these cases, anti-inflammatory compounds are administered therapeutically to control the inflammatory response [32].

Plants rich in certain flavonoids have traditionally been used for its anti-inflammatory properties, being increasingly reported the isolation of flavonoids, including the Citrus genus with anti-inflammatory potential [6]. Citrus flavonoids appear to impact blood and microvascular endothelial cells [33].

Studies reported that nobiletin (flavone) is a major component in juice from *Citrus depressa*, it inhibits the invasive activity of human fibrosarcoma HT-1080 cells not only by suppressing the expression of matrix metalloproteinases but also by augmenting metalloproteinases-1 production [34, 35]. Also, nobiletin prevents tumor-cell invasion due to a decrease of metalloproteinase-9 production in the peritoneal dissemination of human gastric carcinoma in severe combined immunodeficient mice [36].

Therefore, these results further support the notion that nobiletin is likely to be a candidate for characterization as a novel immunomodulatory and anti-inflammatory drug [34].

Citrus peels, the dominant residue, possess a large variety of bioactive compounds; they are considered as potential sources of functional components [37]. In traditional herbal medicine in Korea, the dried fruit peels of *Citrus reticulata* have been widely used for centuries as a remedy to treat indigestion and to improve inflammatory syndromes of the respiratory tract such as bronchitis and asthma [38].

5. Citrus insecticidal

The insecticidal properties have been recognized in the essential oil of many species of the Citrus genus and various products containing (+)-limonene, linalool, and crude extract of some species of *Citrus* are already been sold [39].

Analyses were performed with the potential insecticidal activity by spraying volatile extracts from the bark of species two of orange–*C. sinensis* and *C. aurantifolia* [7]. Both exhibited, to varying degrees, insecticidal activity against mosquito, fly (*Musca domestica* L.) and cockroach. Insecticidal activity was better in the longer period of exposure (60 minutes) compared to 30 minutes of spraying. The volatile extracts of *C. sinensis* showed best potential insecticide and cheap. *Blattella germanica* L) was the species most susceptible to the effects of orange peel among the three insects studied. Later studies examined the ability of the insecticide essential oil *C. sinensis* in flies [7].

The observed effect of oil extracted from *C. limon* on the larvae *Cule*x *pipiens* (house mosquito) and found a positive relationship between the exposure time and the percentage of mortality of larvae [8]. The extract from the bark of *C. aurantium* (L.) was evaluated for its toxicity against fly of olive (*Bactrocera oleae*), and fly of mediterranean (*Ceratitis capitata*). The flies of the species *B. olea*e to extract were more likely than *C. capitata* in residual contact bioassays. Both sexes of *B. oleae* were equally susceptible in both tests. However, males of the *C. capitata* were more likely than females, which can be best explained by their ability to metabolize chemical insecticides [39].

The herbal extracts to inhibit AChE activities are promising for the symptomatic treatment of Alzheimer's disease, a neurodegenerative disease that initially affects memory and thinking ability. Research indicates that in the literature, there is a growing search for new inhibitors of AChE activity in plant extracts [40]. This search is directed mainly to plants already used in traditional medicine for the treatment of insomnia, amnesia, depression, and anxiety, or to extend longevity and improve memory and cognitive function. Some plants, such as *Centella asiátic*a L. (centelha-asiática) (Umbeliferae) and *Ginko bilob*a L. (ginkgo) (Coniferae), used in traditional Indian and Chinese medicine, demonstrated in studies of pharmacological activities, relevant results in the treatment of cognitive disorders, anticholinesterase, anti-inflammatory, and antioxidant actions. In light of these results, these plants have been indicated for therapeutic use in the treatment of Alzheimer's disease [24].

The inhibitory activity of AChE is a significant effect induced by coumarins and particularly monoterpenes such as *d*-limonene. It is also due to the presence of *d*-limonene from Citrus extracts that showed toxic actions in insects, representing a potential alternative to chemical insecticides and pharmacological promising to search for inhibitors of the enzyme AChE [41].

6. Terpenes acetylcholinesterase inhibitors as perspective for the production of herbal medicines

Pharmacological treatments most commonly employed for Alzheimer's disease (AD) stands out the use of materials whose action increases central cholinergic function, increasing levels of acetylcholine in the brain through inhibition of the enzyme acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These enzymes are responsible for the hydrolysis of acetylcholine to acetate and choline, which prevents its action to the neurotransmitter. The inhibition of the enzyme AChE has been investigated for the treatment of various neurological

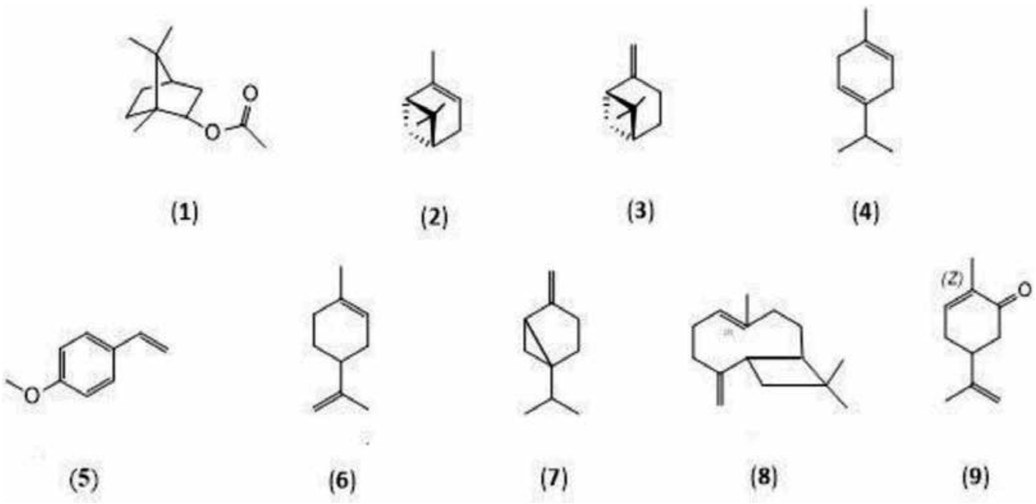

Figure 3. Terpenes AChE and/or BuChE inhibitors (ethyl bornila (1) α-pinene (2), β-pinene (3), γ-terpinene (4), trans-anethole (5), (+) - limonene (6) and (+) - sabinene (7) α-caryophyllene (8) and (-)- - carvone (9). Molecular structures were drawn with ChemDraw13 (Perkin Elmer).

diseases, and are therefore as the most suitable strategy for the treatment of AD and other diseases such as: senile dementia, ataxia, myasthenia gravis, and Parkinson's disease [42].

Various terpenes present in plant essential oils are reported in the literature showing inhibitory activity against AChE and BuChE for example: ethyl bornila (1) α-pinene (2), β-pinene (3) γ-terpinene (4), *trans*-anethole (5), (+)-limonene (6), (+)-sabinene (7), α-caryophyllene (8), and (-)-carvone (9) [24] (**Figure 3**). In promising studies it was shown that *trans*-anethole (5) compound exhibited the strongest activity against AChE and BuChE, with IC_{50} values of 134.7 and 209.6 ng mL^{-1}, respectively [43].

Promising studies show that terpenes can have their activity potentiated by complex with cyclodextrins making it a promising source of possible pharmaceutical formulations. The inclusion complex obtained between β-cyclodextrin and *p*-cymene terpene potentiated analgesic and anti-inflammatory activities of this monoterpene [44]. The thermal instability, poor solubility in water, and the highly volatile compounds are some of the properties of essential oils or terpenes that by complexing with cyclodextrins improves its technological applicability [45, 46].

7. Pharmaceutical formulation of citrus

The cyclodextrins (CDs), for example β-cyclodextrins (**Figure 4**), are composed of complex carbohydrates glucose units (α-D-glucopyranose) joined by α-1,4 linkages type, with a structure similar to a trunk cone. In 1903 Franz Schardinger identified cyclodextrins, as products resulting from the degradation by the action of amylase enzyme. Cyclodextrin starch glycosyl is a compounds of CDs that have played an important role in Medicinal Chemistry with regard to controlled release technology of drugs, currently represents one of the boundaries of science

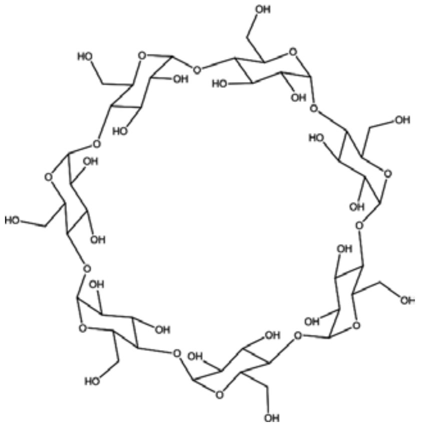

Figure 4. Structure of β-cyclodextrin. Seven units of D – (+)-glucopyranose. The CDs have a hydrophobic central cavity and a hydrophilic surface. Molecular structures were drawn with ChemDraw13 (Perkin Elmer).

involving different aspects multidisciplinary knowledge. The delivery systems, often described as a "drug delivery systems," offer numerous advantages when compared to other conventional dosage [47].

In the pharmaceutical industry, the CDs are used mainly to improve stability and bioavailability of the active principle and its organoleptic properties such as taste and smell in pharmaceutical formulations. The CDs can also be used to mask the unpleasant smell and taste of certain drugs, transforming solid liquid compounds, reduce volatility, and avoid undesirable mismatches. The increased solubility, dissolution, modification of the pharmacokinetics, and controlled release of drugs are other CD applications. The CDs have been used successfully in the management and drug delivery by various routes and/or administration sites such as oral, vaginal, rectal, nasal, ophthalmic, pulmonary, dermal, and transdermal [47]. **Figure 4** shows the structure of β-cyclodextrin is widely used as an excipient in pharmaceutical industry at low cost and the size of the cavity, which is suitable for encapsulating most substances

In Brazil a pharmaceutical formulation from the essential oil inclusion complex of *C. sinensis* was obtained with β-cyclodextrin and their applications in therapy of disease Alzheimer's (AD). The aim of the present study was to explore the essential oil from leaves of *C. sinensis* (EOLCS) and inclusion complex with the cyclodextrin complexing and their use as a candidate drug in the formulation pharmaceuticals for the prevention and/or treatment of AD. These properties were determined from experimental models where biological application of essential oil (EOLCS) and inclusion complex with cyclodextrin complexing. Inclusion complex with (EOLCS) was proved experimentally by evaluating the inhibitory effect on the activity of the enzyme AChE *in vitro* and *in vivo* (deposit patent number BR 10 2016 0018552).

For the characterization inclusion complex of cyclodextrin and EOLCS was held the infrared spectroscopy and differential scanning calorimetry with three proportions, β-CD, and EOLCS, denominate of misture physical. It proved the formation of EOLCS complex with β-cyclodextrin through the analysis of differential scanning calorimetry, infrared spectra disappearance of the groups, and thermogram and it can be concluded that the ratio is 6:94 IC greater stability. It was shown in preliminary studies that the EOLCS significantly inhibits acetylcholinesterase a total of 73% in group 50 mg kg^{-1}, 83% in group 100 mg kg^{-1}, and 76% 200 mg kg^{-1} and significantly improves memory of the animals in promising preclinical studies.

The *Citrus sinensis* results show a significant pharmacological effect of the compound isolated in the inhibition of AChE enzyme activity, and this action of great interest in the development of new phytomedicine for the prevention and/or treatment of AD. The results of this effort were so promising that we decided to deposit (BR 10 2016 0018552 deposit number) a request for a patent, aiming to protect the innovations developed.

The composition of the active fraction in *C. limon* (L.) Burm leaves is composed of a mixture of two coumarins, 5,8-dimethoxy-psoralen and 5,7-dimethoxycoumarin (**Figure 1**), identified by ^{1}H and ^{13}C NMR data analysis, and others experiments. It was also demonstrated that this mixture presents qualitative and quantitative AChE inhibition. *In vitro* studies indicated a CE_{50} value of 340 μg/mL with 95% of confidence. *In vivo* studies (10 and 25 mg/kg) revealed inhibition of 30.09 and 30.06% of AChE activity in relation to neostigmine, respectively. The deposited formulations suggest that the isolated fraction *C. limon* (L.) containing (5,8-dimethoxy-psoralen and 5,7-dimethoxycoumarin) can demonstrate inhibitory results of the AChE activity *in vitro* and *in vitro*, with potential applications in neurodegenerative diseases dependent on the modulation of this enzyme, including the Alzheimer's disease [28]. The sample containing the mixture of isolated components showed no toxic properties in rodents, since none of the animals treated with the doses of 10 and 25 mg/kg died during the 24-hour observation period. Suggesting that the said compound can be used in lower dose and may increase more effectively cholinergic stimulation.

Citrus species are very interesting for further isolation of AChE inhibitors which can be used for neurodegenerative diseases for example Alzheimer's disease application (**Figure 5**).

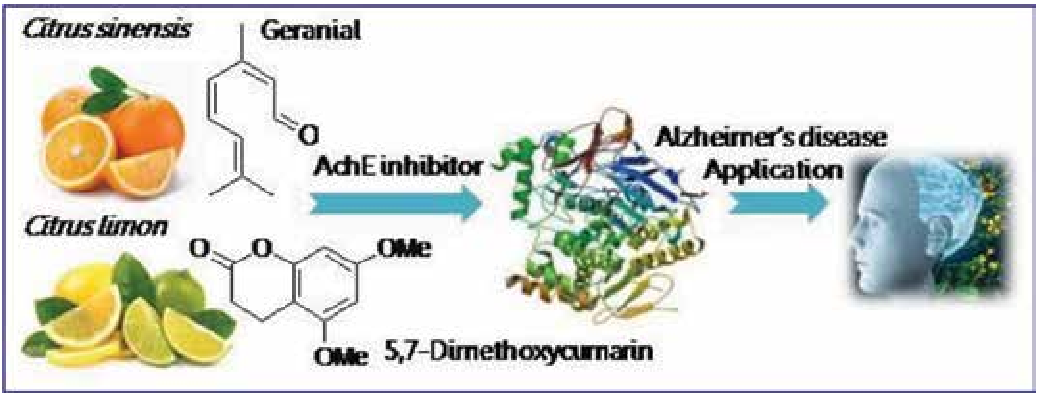

Figure 5. Pharmaceutical formulations were obtained from compounds and essential oils of *C. limon* (e.g., 5,7-dimethoxycoumarin) and *C. sinensis* (e.g., geranial). These compounds and essential oils were evaluated in preclinical trials on mice by memory tests and enzyme acetylcholinesterase inhibitors and proved to be very promising for potential applications in the treatment of neurodegenerative diseases such as Alzheimer's disease. Molecular structures were drawn with ChemDraw13 (Perkin Elmer).

Promising preclinical studies were conducted with compounds isolated from some of these Brazilian species others namely *Platonia insignis* (bacurizeiro), *Mangifera indica* (mango), and *Kalanchoe brasiliensis* (courama branca), for the prevention or progression of Alzheimer's disease [48].

8. Concluding remarks

The results confirm that AChE inhibitors as alternatives for preparation of phytomedicines are used in therapeutic treatment of AD, being plants the principal source of these inhibitors. Recent studies show that the terpenes may have intensified activities through inclusion complexes with cyclodextrins excipient pharmaceutical, making them a promising source for potential pharmaceutical formulations.

In general, the formulations reported in this chapter from essential oils or their constituents is in progress, as they contribute to various activities of plants and open perspectives regarding the chemical composition of bioactive metabolites of the studied species and are considered promising alternatives to discoveries of new chemical compounds of pharmaceutical interest.

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Chapter 10

Pharmacological Actions of *Citrus* Species

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Abstract

The genus *Citrus* belongs to family Rutaceae, which is characterized by trees and bushes. *Citrus* species are extensively cultivated throughout the world because of their multiple health benefits for humans and their applications in pharmaceutical and food industries. This chapter is a survey covering *in vitro* and *in vivo* studies that demonstrate the pharmacological activities of various *Citrus* species. The species *Citrus aurantium*, *Citrus sinensis*, *Citrus limon*, and *Citrus bergamia* are well known and several studies have been carried out to evaluate the pharmacological effects of their extracts, essential oils, and isolated constituents. These studies have found that they possess anxiolytic, anti-inflammatory, hypoglycemic, anthelmintic, anticancer, and anti-infective properties.

Keywords: *Citrus*, Pharmacology, *Citrus aurantium*, *Citrus sinensis*, *Citrus limon*, *Citrus bergamia*

1. Introduction

The genus *Citrus* belongs to family Rutaceae, which is characterized by trees and bushes. Fruits of this genus, such as oranges, lemons, and tangerines, are widely cultivated in the tropical and subtropical areas of the world [1].

In medicine, *Citrus* fruits are used in the treatment of various diseases. Research shows that the intake of *Citrus* fruits can reduce the incidence of gastric cancer [2]. In addition, some isolated compounds from these fruits have effects on the central nervous system. For example, limonene, which is present in high concentrations in *Citrus aurantium*, showed a strong anxiolytic effect when tested in both animals and humans [3, 4].

To further understand the therapeutic potential of different *Citrus* species, we carried out a survey of *in vitro* and *in vivo* studies demonstrating their pharmacological actions, and summarized them in this chapter.

2. *Citrus* species with pharmacological activity

2.1. *Citrus aurantium* L

The species *C. aurantium* L., popularly known as bitter orange, *laranjeira-amarga*, or *laranjeira-cavalo*, is a native plant of Southeast Asia, a region that corresponds to China and India. It was first introduced to Syria and Egypt by the Arabs and was subsequently brought to Europe [1]. In the medieval times, it was widely used in the Mediterranean region as a cardiac and vascular stimulant, digestive, sedative, tranquilizer, appetite stimulant, general tonic, and antidote against poisons [5]. In traditional Chinese medicine, bitter orange is used as a gastrointestinal stimulant and general tonic [6].

Ethnopharmacological studies in Brazilian communities (documentation of the empirical uses of medicinal plants in traditional communities) describe the popular use of *C. aurantium* L. fruit peels, flowers, and leaves in the treatment of the central nervous system disorders such as insomnia, anxiety, and hysteria [7, 8]. Another study reports that tea made from the leaves of *C. aurantium* L. can relieve stomach cramps and constipation, combat stomach acidity, and relieve fever, while tea from the seeds is reported to control diabetes [9].

Studies have focused on the investigation of *C. aurantium* L. effects on the central nervous system, especially its anxiolytic effect. Several studies on animals and humans have demonstrated the anxiolytic effect of this species. For example, the essential oil obtained from *C. aurantium* L. peels was found to show an anxiolytic effect on rats after a single dose, denoted by an increase in the residence time in the open arms of the elevated plus maze [10]. In addition, anxiolytic activity was observed in experimental models of generalized anxiety and obsessive-compulsive disorder. At the same time, the mice did not show any signs of motor impairment, even after 15 consecutive days of treatment [7].

In a study conducted by Leite et al., 12 rats were evaluated in two models of anxiety: elevated plus maze and open field maze models. The rats were previously made to inhale the essential oil from *C. aurantium* L. at concentrations of 1.0, 2.5, and 5.0% for 7 min in an acrylic box. The authors observed a decrease in the emotional reactivity of the animals in both experimental models, suggesting a possible central action [11]. Moreover, the results of another study strongly suggest the involvement of 5-HT 1a receptor, a subtype of serotonin receptors, in its anxiolytic activity, suggesting a potential mechanism by which *C. aurantium* L. affects the central nervous system [12].

Clinical trials testing the anxiolytic effect of *C. aurantium* have also demonstrated satisfactory results. In a certain study, preoperative patients received distilled *C. aurantium* L. flower 2 h before the procedure, then the STAI scale was used for anxiety evaluation. In comparison to the control group, the patients from experimental group showed a reduction in preoperative anxiety [13]. In another study, patients with chronic myelogenous leukemia inhaled essential oil of *C. aurantium* L. before the procedure of medullary material collection. The results indicated that the patients subjected to this intervention showed a decrease in the anxiety levels and remained relaxed during the procedure. In addition, *C. aurantium* L. oil, even at a single exposure, showed an activity comparable to that of the anxiolytic used as the positive control. This ensures its efficacy in controlling anxiety in patients subjected to unpleasant diagnostic procedures [3].

Its anxiolytic effect was also tested before labor. In a certain study, 18–35-year-old primiparous women were subjected to aromatherapy with *C. aurantium* L. oil during labor, and the results showed a significant reduction in the anxiety during labor in those women. Another clinical trial conducted on 126 primiparous women investigated the effects of aromatherapy with *C. aurantium* L. on pain during the first stage of labor. The women received 4 ml of *C. aurantium* L. in distilled water soaked in gauzes every 30 min. The results showed pain relief in the women subjected to this procedure. When asked whether aromatherapy using *C. aurantium* L. was satisfactory or not, 88.1% of the participants said that they were satisfied with the method applied and 92.1% said that they would use this method in future procedures. These studies show that aromatherapy with *C. aurantium* L. is a simple, inexpensive, and noninvasive intervention that can be beneficial for pregnant women [14]. However, because vaginal birth is a painful and serious process that is often feared by primiparous women, further studies are still required [15].

Several studies have been conducted to identify other possible actions of *C. aurantium* L. constituents, other than their effects on the central nervous system. Researchers isolated the flavonoids such as nobiletin, naringin, and hesperidin from *C. aurantium* L. and evaluated their anti-inflammatory effect on rat cells *in vitro*. Results demonstrated the suppression of proinflammatory mediators, which confirms the anti-inflammatory action of the isolated flavonoids [16]. Flavonoids isolated from *C. aurantium* L. were also evaluated for possible anticancer activity in murine lung cells. They demonstrated an anticancer effect through the regulation of apoptosis and cell migration, providing scientific support for the use of *C. aurantium* L. flavonoids in the treatment of human lung cancer [17].

Using a rat model of diabetes, researchers evaluated the possible hypoglycemic effect of neohesperidin derived from *C. aurantium* L. Treatment with neohesperidin was shown to increase glucose tolerance and insulin sensitivity, and to decrease blood glucose levels in the rats of the experimental group. These results demonstrate its hypoglycemic effect, and thus, its potential application for the prevention of diabetes and its complications [18].

Finally, although *C. aurantium* L. has been known for millennia, and a large number of studies have been focusing on its effects in the last decade, further research is needed to elucidate new mechanisms of action and therapeutic properties.

2.2. *Citrus sinensis*

The species *C. sinensis*, popularly known as sweet orange, also belongs to family Rutaceae. This fruit is of Asian origin, where it has been known for about 4000 years. With the expansion of trade and shipping routes, orange was introduced to various regions of the world for cultivation. Information from 1471 to 1472 reported the presence of sweet orange in Liguria, a region in Italy, before its introduction by the Portuguese to the Iberian Peninsula in 1498. In the eighteenth century, there were reports about varieties of sweet orange in Palestine, possibly introduced by an Armenian monk. In the Americas, Portuguese colonists introduced it to Bahia/BR in the sixteenth and eighteenth centuries, and Jesuits introduced it to California/USA, where they settled and started subsequent plantations [19].

An ethnobotanical survey in a Brazilian community identified the species *C. sinensis* as the most cited by women in attention to basic health, indicating the use of its leaves prepared in the form of tea as a soft tranquilizer for mild cases of anxiety and insomnia [20]. In addition, the flower and fruit are used by the population as tranquilizers and for relief of headache, migraine, fever, flu, allergy, and cough. We can conclude that sweet orange is among the main species of medicinal plants commonly used by the Brazilian community [21].

The traditional use of *C. sinensis* as a sedative can be attributed to hesperidin, which was identified in a study as the active principle in this plant responsible for sedation. Hesperidin performs its sedative action through interaction with and stimulation of adenosine receptors. Its effect is opposite to that observed after consumption of coffee and tea, which antagonize the adenosine receptors and thus maintain the wakefulness state [22]. Researchers investigated the properties of hesperidin extracted from *C. sinensis* through *in vitro* experiments, revealing its strong cytotoxic activity against human carcinoma cell lines and its antioxidant activity against DPPH free radical. According to these results, hesperidin can be considered a promising future drug. Orange peels are a cheap and available source from which hesperidin can be extracted in an efficient way and with high purity [23].

C. sinensis extracts have shown potent anthelmintic properties *in vitro* when compared with the positive control, indicating that it is equipotent to conventional anthelmintic drugs. However, *in vivo* studies should be conducted to establish this efficacy and to identify the active components responsible for the anthelmintic activity [24].

Researchers induced liver cirrhosis in rats for 16 weeks and treated the animals with *C. sinensis* dried peel extract orally for 9 weeks. After the completion of the treatment, the animals were euthanized to evaluate the biochemical and histopathological changes associated with liver cirrhosis. The histopathological examination of the liver tissue and the biochemical findings demonstrated the curative effect of the extract, suggesting its potential therapeutic application in liver fibrosis and cirrhosis [25].

Another study investigated the effects of the *C. sinensis* peel extract when administered orally to rats at a concentration of 25 mg/kg and revealed its antidiabetic potential. The researchers administered a single dose of alloxan (120 mg/kg) to the rats, which triggered an increase in serum glucose levels and α-amylase activity. Then, a group of the rats was administered *C. sinensis*, which normalized all the adverse changes induced by alloxan. This reinforces the antidiabetic properties of the orange peel extract, which might be applied in the development of therapies for diabetes control [26].

In 2010, Faturi et al. demonstrated that the essential oil from *C. sinensis* had an anxiolytic effect when inhaled by rats. The animals were subjected to the elevated plus maze test, and the residence time of each rat in the open arms was measured. The possibility that the anxiolytic effect could result from any other inhaled aroma was discarded, since no anxiolytic activity was observed with *Melaleuca alternifolia* essential oil. The authors also reported that the animals were not previously exposed to the essential oil of sweet orange or any solutions that could contain the essence [27].

In a clinical study conducted on patients waiting for dental care, a group of the patients inhaled sweet orange essential oil in the waiting room, a second group listened to music, and the third group, which was the control group, did not receive any intervention. The results showed that the patients who inhaled the essential oil were calmer and had lower anxiety levels than the patients of the other groups [28].

Results obtained by Goes et al. in 2012 demonstrated the anxiolytic effect of the *C. sinensis* essential oil on healthy individuals while performing an anxiogenic task. The anxiolytic activity was evidenced by the significant difference in the level of anxiety between the group exposed to *C. sinensis* and the control group, providing scientific support for the use of this essential oil as a tranquilizer in aromatherapy [29].

The results presented here support the use of *C. sinensis* as an aid to combat insomnia and anxiety. Research also indicates that the natural constituents of sweet orange can be used in the pharmaceutical industry, as natural cytotoxic agents, anthelmintics, antidiabetic agents, or for the treatment of liver cirrhosis.

2.3. *Citrus bergamia*

Citrus bergamia, commonly known as bergamot [30], is a plant belonging to family Rutaceae, subfamily Esperidea, and is defined as a hybrid between bitter orange (*Citrus aurantium*) and lemon (*Citrus limon*) [31]. The botanical and geographical origins of this plant are still unknown. The name "bergamot" seems to be derived from Berga, a Spanish city from which the plant was transported to Calabria in Southern Italy. *C. bergamia* is cultivated almost exclusively along the southern coast of Calabria; over 90% of its world production comes from this region. However, it is also cultivated to a lesser extent in other countries, such as Greece, Morocco, Iran, Ivory Coast, Argentina, and Brazil [32].

The fruit of *C. bergamia* is mainly used for the extraction of its essential oil, which is used in perfumes, drugs, cosmetics, food, and clothing [33]. Throughout the cultivation and the processing of *C. bergamia*, tons of wastes of low commercial value, but of great industrial potential, are generated. These wastes contain high levels of nutrients, pigments, and bioactive components with low toxicity [34].

There are evidences that *C. bergamia* contains antibacterial and antifungal active constituents, in addition to its anti-inflammatory, antiproliferative, neuropsychopharmacological, neuroprotective, and analgesic effects, [35] as well as its cardiovascular properties in rodents [32]. Bergamot juice, which is obtained from the endocarp after the extraction of the essential oil, was found to exhibit hypoglycemic and hypolipidemic activities, as well as anti-inflammatory [36–38] and anticancer properties [39].

The anti-infective properties of *C. bergamia* derivatives can be observed from the action of its essential oil against bacteria, mycetes, and larvae, and the action of its juice against *Helicobacter pylori*. Compounds derived from its fruit peel extract have antimicrobial properties. Studies report that its essential oil has antibacterial and antifungal activities against *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and dermatophytes. The essential oil of *C. bergamia* shows *in vitro* activity against *Candida* species, which suggests its potential application in the topical treatment of fungal infections by Candida. It also demonstrates *in vitro* activity against dermatophytes [40].

In addition, favorable results were obtained regarding its pharmacological properties in nervous system disorders. Sometimes, the essential oil from *C. bergamia*, usually extracted from the peel, is used in aromatherapy to relieve stress and anxiety [41].

In a study carried out on rats to investigate the anxiolytic activity of *C. bergamia*, the essential oil was administered to the rats at different concentrations and its effects were compared with those of diazepam. The results of this study indicated that *C. bergamia* showed an anxiolytic action, which was observed when the rats were subjected to the elevated plus maze and hole-board tests. The researchers observed a reduction in the activity of the hypothalamic-pituitary-adrenal axis, reducing corticosterone response to stress [42].

Clinical studies have also reported beneficial effects of *C. bergamia* essential oil in cardiac control, blood pressure reduction, and stress response management, in addition to its effect on the central nervous system [43]. Although the mechanisms by which the effects on the central nervous system are mediated have not yet been fully elucidated, it has been suggested that this action might be mediated by the release of amino acids that modulate the synaptic plasticity [32].

Some studies demonstrated that the essential oil from *C. bergamia* also shows anticancer activity. For example, Berliocchi et al. demonstrated *in vitro* antiproliferative activity of *C. bergamia* essential oil against SH-SY5Y human neuroblastoma cells. In this work, the lethal effect of *C. bergamia* was mediated by activating multiple pathways that lead to cell death by both necrosis and apoptosis [44]. Compounds derived from bergamot oil, such as limonene, monoterpenes related to limonene, alcohol, and perillic acid, were also found to inhibit the proliferation of breast cancer cells, and to show chemopreventive and chemotherapeutic effects in models of mammary tumors [45].

However, the poor water solubility, weak stability, and limited bioavailability of essential oils have prevented their use in cancer therapy. Nevertheless, due to the favorable results regarding the anticancer action of the essential oil from *C. bergamia*, some attempts have been made to use bergamot in cancer therapy. For example, in 2013, Celia et al. developed liposomes of *C. bergamia* essential oil, which improved its water solubility and increased its *in vitro* anticancer activity against SH-SY5Y human neuroblastoma cells [46].

In order to elucidate the mechanisms by which the essential oil from *C. bergamia* has anticancer activity, Russo R et al. conducted a study in 2013 to identify the components involved in the cell death process. The results of the study suggested an important role of the combined action of monoterpenes in the process of cell death [35].

Regarding the protective cardiovascular properties, Di Donna et al. established a rat model in 2014 to investigate the hypocholesterolemic effect of 3-hydroxy-3-methyl-glutaryl flavanones enriched fraction (HMGF), extracted from *C. bergamia* fruits, in comparison with that of statin (simvastatin). Both HMGF and simvastatin reduced total cholesterol, triglycerides, very low density lipoproteins (VLDL), and low-density lipoproteins (LDL). However, only HMGF caused an increase in high-density lipoprotein (HDL). In addition, HMGF showed no genotoxic effects and was cytotoxic only at high concentrations. Thus, the authors concluded that daily supplementation of HMGF in the diet can be very effective against hypercholesterolemia, featuring the cardiovascular protective properties of bergamot [47].

Although all the pharmacological effects of *C. bergamia* indicate potential clinical applications in the future, only clinical studies investigating its anxiolytic effects in aromatherapy were published heretofore [32].

2.4. Citrus limon

The Latin name of lemon is *C. limon (Linnaeus) N. Burman.* It belongs to family Rutaceae, and it is sometimes called limoeiro or limoeiro-azêdo [48]. It originated in Southeast Asia and is believed to have been introduced to Europe by Muslims across the Iberian Peninsula and Sicily [5]. Currently, Spain is considered the main producer country of this genus in the Mediterranean region. Lemon is considered the third most important species of the genus *Citrus*, as it contains many relevant natural chemical compounds, including citric acid, ascorbic acid, minerals, and flavonoids [49].

Recently, some of the therapeutic properties of *C. limon* have been recognized in the literature. Studies have found that the use of lemon helps neutralize the acidity of the gastric environment, by stimulating the production of potassium carbonate, indicating its protective effects on the gastric mucosa. It was also found to have analgesic, anti-anemic, anti-sclerotic, antipyretic, antiseptic, emollient, and moisturizer properties. The recognized actions of lemon cellulose are that it is anti-diarrheal, diuretic, intestinal mucosa protector, local hemostatic, vascular stimulant and protector, and vitamin [5].

Production networks of lemon generate large amounts of wastes and by-products, which are an important source of bioactive compounds with potential applications in animal feeding, processed foods, and health care [49]. Although its health benefits are always attributed to its vitamin C content, recently, it has been found that flavonoids also play an important role [48]. Some authors suggest that flavonoids present in lemon have different biological functions, including antioxidant, anti-inflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticancer activities [48].

Hesperidin, which is the main flavonoid in *C. limon*, influences the vascular permeability, increases the capillary resistance, and has analgesic and anti-inflammatory properties [49]. It is also an effective antioxidant, since it is capable of scavenging free radicals that are involved in cancer. Some studies have shown that flavonoids present in lemon juice also have hypocholesterolemic properties [50].

Trovato et al. conducted a study on rats in 1996 and found that *C. limon* had a significant effect on the levels of cholesterol and triglycerides, suggesting that the prolonged consumption of its juice might offer significant protection from hypercholesterolemia [51].

Another study investigated the protective effect of the essential oil from *C. limon* against acute hepatic and renal damage induced by a high dose of aspirin in Wistar albino rats. The data obtained in this study demonstrated that the treatment with *C. limon* protected the liver and kidney from damages induced by aspirin [52].

Regarding the pharmacological effects on the central nervous system, a recent study conducted by Khan and Riaz evaluated the effects of lemon on the behavior of rats, using three different doses (0.2, 0.4, and 0.6 ml/kg), considered low, moderate, and high doses, respectively. The anxiolytic and antidepressant activities were evaluated twice, for 15 days, using the open field, elevated plus maze, and forced swimming tests. In the open field test, *C. limon* revealed an increase in the distance traveled, the number of central entries, and the number of rearing at moderate dose, while in the elevated plus maze, the number of open arm entries was found to be increased. Whereas in the forced swimming test, there was a decrease in duration of immobility and an increase in the duration of climbing. Thus, results suggest that *C. limon* at moderate dose has an anxiolytic effect [53].

It has been noted that disorders such as anxiety and depression can be managed through healthier variations in dietary patterns, since there is evidence that a diet rich in antioxidants and vitamins reduces these symptoms. Accordingly, a study was performed in order to evaluate the behavioral effects of *C. limon* and *Punica granatum* in rats. In this study, two combinations of doses were used: 0.4 + 5 ml/kg and 0.2 + 8 ml/kg of *C. limon* and *P. granatum*, respectively. As in the previous study, the antidepressant and anxiolytic effects were evaluated twice, for 15 days, using forced swimming, open field, and elevated plus maze tests. In the open field test, the use of *C. limon* and *P. granatum* showed an increase in the distance traveled and the number of central entries at the dose combination of 0.4 + 5 ml/kg. In the elevated plus maze test, the number of entries was increased at the highest dose combination (0.2 + 8 ml/kg). In the forced swimming test, there was a decrease in the immobility duration and an increase in the climbing duration at both dose combinations: 0.4 + 5 ml/kg and 0.2 + 8 ml/kg *C. limon* and *P. granatum*, respectively. Based on these results, the authors suggested that *C. limon* and *P. granatum*, at a combination of 0.4 + 5 ml/kg, show anxiolytic and antidepressant effects [54].

In another study conducted by Riaz et al. in 2014, the effect of *C. limon* and pomegranate juice on rats' memory was evaluated. It is known that memory is greatly influenced by factors such as diet, stress, and sleep quality. The results of this study indicated that *C. limon* contains essential phytochemicals and nutrients that improve the memory, particularly the short-term memory. They also concluded that flavonoids in these juices could be responsible for this effect [55].

Hesperidin extracted from *C. limon* was found to play an important vasodilator action. In 2016, Dobias et al. evaluated the effect of hesperidin on vascular responses in normotensive and hypertensive rats. Fifteen-week-old healthy Wistar rats and spontaneously hypertensive (SHR) rats were randomly assigned to receive either hesperidin (50 mg/kg/day) or a corresponding volume of water orally for 4 weeks. The vascular responses of isolated femoral arteries were studied using a myograph under control conditions and during the NO-synthase inhibition. Although hesperidin had no effects on blood pressure, it significantly improved endothelium-dependent vasodilation in Wistar and SHR rats. The contraction responses were increased in all the groups to a similar extent, but the relaxing responses were significantly attenuated in the SHR group only. The inhibition of the potassium channels (Kv) significantly reduced the endothelium-dependent vasodilator response only in SHR rats administered hesperidin. This indicates that hesperidin can improve endothelium-dependent vasodilation during hypertension [56].

Regarding the anti-inflammatory activities described for *C. limon*, in 2016, Amorim et al. conducted a study to confirm the hypothesis that essential oil (EO) from *C. limon*, *C. latifoli*a, *C. aurantifoli*a, and *C. limonia* have antinociceptive effects. Thus, they were tested each one on the formalin-induced licking behavior. This model is a widely used pain model in evaluating antinociceptive and anti-inflammatory drugs. The results of this study suggest that EO from *C. limon*, *C. aurantifoli*a, and *C. limonia* have an anti-inflammatory effect because they reduced the second phase response to formalin. This may occur through a reduction in inflammatory mediator liberation in mice paws or a direct action on one or more mediator receptors [57].

Since *C. limon* is widely used in traditional medications in India and China, we sought to explore the importance of flavonoids that are abundant in citrus fruits on platelet function. Nobiletin is a highly abundant flavonoid present in these and has been shown to reduce the adhesive properties of platelets. In 2015, Vaiyapuri et al. conducted a study to verify the pharmacological effects of a polymethoxy flavonoid, nobiletin, in the modulation of platelet function. Nobiletin was shown suppress a range of well-established activatory mechanisms, including platelet aggregation, granule secretion, integrin modulation, calcium mobilization, and thrombus formation. This study provided insight into the underlying molecular mechanisms through which nobiletin modulates hemostasis and thrombus formation. Therefore, nobiletin may represent a potential antithrombotic agent of dietary origins [58].

In conclusion, *C. limon* presents numerous curative properties, and thus it has been widely used as a traditional medicine for the treatment of various diseases [52]. However, more studies still need to be conducted in order for its application in clinical practice to be established and disseminated [59].

3. Conclusion

Citrus species are well known and have been commonly used by populations for hundreds of years for various purposes. This knowledge of their therapeutic potential has led to several studies that proved the pharmacological effects of the above-mentioned species (**Table 1**). The effect of *Citrus* species on the central nervous system was highlighted as the study objective in most of the publications. However, research has advanced in seeking other pharmacological actions, especially of isolated constituents of the species.

Citrus species	Pharmacological action
Citrus aurantium L.	Gastrointestinal stimulant and general tonic. Treatment of central nervous system disorders like insomnia, anxiety, and hysteria. Relieve stomach cramps and constipation, combat stomach acidity. Hypoglycemic effect. Anti-inflammatory. Anxiolytic effect.
Citrus sinensis	Sedative action. Anthelmintic properties. Treatment of liver cirrhosis. Antidiabetic properties. Anxiolytic effect.
Citrus bergamia	Antibacterial. Antifungal. Anti-inflammatory. Analgesic. Antiproliferative and anticancer properties. Neuropsychopharmacological. Neuroprotective. Anxiolytic activity. Hypoglycemic and hypolipidemic activities.
Citrus limon	Analgesic. Anti-anemic. Anti-sclerotic. Antipyretic. Antiseptic. Emollient and moisturizer properties. Anti-diarrheal. Diuretic. Intestinal mucosa protector. Local hemostatic. Vascular stimulant and protector. Antioxidant. Antiallergic. Antiviral. Anti-inflammatory. Antiproliferative, antimutagenic, and anticancer activities.

Table 1. Pharmacological action of different citrus species (studies *in vitro* and *in vivo*).

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This book is an attempt to compile different aspects of citrus pathology to provide an overall knowledge to those who are interested in it, so that they may identify the bottlenecks to improve it further. The book chapters detail about citrus diseases, metabolic changes in citrus plants against various stresses, quorum sensing and its role in symptom development, preharvest and postharvest disease management, and application of citrus and its compounds. The goal of this book is to provide the most up-to-date review on information available on pathological aspects of citrus. Therefore, this book will equip academia and industry people with adequate basic knowledge of citrus diseases and management options.

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