FUNGICIDES – SHOWCASES OF INTEGRATED PLANT DISEASE MANAGEMENT FROM AROUND THE WORLD

Edited by **Mizuho Nita**

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Fungicides –
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Edited by Mizuho Nita

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Preface

Welcome to the fourth series of"Fungicides"book from InTech. It is titled"Fungicides - Showcases of Integrated Plant Disease Management from around the World."As the title implies, many of our chapters are about varieties of IPM programs implemented on a wide array crops grown in different countries. However, this book won't stop there. We have a section on natural anti-fungal products, and this book finish strongly with two excellent chapters on fungicide-fungal interactions that could affect the implementation of IPM practices.

This book starts off with two excellent chapters on wheat disease managements in Argentina and in the United State of America. From Argentina, Simón et al. discuss about the effect of fungicide applications on yield and quality when the major foliar diseases affect the wheat, and they also extend discussion of effects of fungicides on mycorrhizae. From the USA, Wegulo et al. provide a review of a very important wheat disease, Fusarium Head Blight, and then discuss integrated management with an emphasis on combined use of fungicides and host resistance. The following chapter is also from the USA, where Woodward et al., describe a development of fungicide application strategies that utilize the strengths of different chemistries to control both foliar and soilborne pathogens of peanut grown in southeast part of the USA.

The next chapter is from India. Kumar et al. provide an overview of rice diseases and history of fungicide usages in India. The next three chapters are from Brazil. Juliatti et al. explain fungicide application technologies and economic analysis on fungal disease management on corn. Survey results of fungal species associated with cotton ball disease complex, and management options were described by Zancan et al. Masson et al. explore an interesting production of eucalyptus in Brazilian forest, and management of their major disease, eucalyptus rust. This section finishes with another wood production, which is pine seedling production in Serbia, described by Rajkovic et al. They discuss management of Lophodermium needle cast.

The second section is about exploration of fungicide or fungistatic materials derived from natural sources. An overview of natural products from plants of fungus that can be used as fungicides was provided by Soković et al., followed by a chapter by Patiño et al. who discuss uses of natural products to control various Fusarium species.

The last section is composed of two excellent chapters about interactions between fungicides and fungus. Various types of fungicide resistance mechanisms, and methods to detect and describe fungicide resistance were discussed by Beckerman. Garzon and Flores provide a wonderful review of hormesis, which is a toxicological concept characterized by low-dose stimulation and high-dose inhibition, and its application in the field of plant pathology.

The variety of crops and IPM practices displayed throughout this book, as well as discussions on the effects of fungicides on pathogens, would be wonderful assets to the field of plant pathology and beyond. I hope you will enjoy the book.

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Section 1

Integrated Plant Disease Management Showcases from Around the World

Chapter 1

Recent Advances on Integrated Foliar Disease Management with Special Emphasis in Argentina Wheat Production

María Rosa Simón, María Constanza Fleitas and Santiago Schalamuk

Additional information is available at the end of the chapter

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1. Introduction

Wheat (*Triticum aestivum* L.) is grown in most regions of the globe due to its importance as a food source, and its enormous genetic variability in phenological response to photoperiod and temperature including vernalization [1]. Argentina is one of the countries with the largest wheat-growing area with more than 5 million ha spread all over the country.

Most of the Argentinean wheat is produced in the Pampean region. This region has a temperate humid climate without a dry season and with a warm summer. Precipitation is higher in summer than in winter. The rainfall distribution is close to monsoonal in the north-west of the Pampas and it tends to an isohigrous pattern at the southeast of Buenos Aires, which means that excess or defect of precipitation could appear at any time. The temperature regimen for the region shows that June and July are the coldest months and January is the hottest. Mean monthly temperatures rarely fall below 7°C and the period of free frost ranges between 180 and 260 days. Temperature indices decrease along a north-south direction, but thermal amplitude also increases from east to west; the frequency and intensity of frost increase westward.

It has an annual rainfall of approximately 600-1000 mm and a mean temperature of 15-17 ° C depending on the region, with some differences between the east and the west. Soils in the region are mainly mollisols including argiudolls, hapludolls and haplustolls developed on a deep mass of Pampean loess [2]. Wheat crops are sown from the second half of May to the first half of August. Varieties are classified as long or short season. Long season varieties have higher requirements of long photoperiod or days with low temperatures, although their requirements in vernalization are not as high as in winter varieties cultivated in other countries. Short season varieties have in general low requirements in photoperiod or days

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with low temperatures, and are similar to some spring varieties. Risk of frost damage at flowering is the main climatic factor determining optimum sowing dates for particular varieties in the various regions. Optimum seeding rates for long-season varieties may vary between 200 to 250 established plants per m^2, while short-season varieties tend to be sown with seeding rates between 250 to 350 established plants per m^2 [3].

During grain production, plant species are rotated following different patterns depending on the region but the most common cropping system tends to be the double-cropped full-season-wheat and soybean [*Glycine max* L. (Merr.)]. The doubled-crop system is usually stable and financially convenient, since wheat crop provides a financial return during summer and the soybean during autumn and winter.

The grain production region has experienced severe tillage changes in the past twenty years, mostly due to the increased interest in maintaining soils covered with plant residues. This has led to implement no tillage systems to restore soil structure in large areas cultivated with double-crop sequences such as wheat/soybean; corn (*Zea mays* L.) - wheat/soybean; or wheat monoculture. No tillage is also desirable because of its positive effect on soil organic matter, for maintaining soil humidity and to prevent soil erosion [4].

No tillage can reduce costs by decreasing fuel consumption required to produce a crop. However, in the wheat/soybean system under no tillage, as in wheat following wheat, the inoculum of necrotrophic fungi may survive until the next wheat season. Therefore, the use of fungicides is essential to decrease the severity of necrotrophic diseases.

On the other hand, nitrogen (N) fertilization is necessary to achieve high yield and grain quality. Even in high soil fertility conditions, N uptake is important because is positively correlated to grain protein content [5]. However, N availability may also enhance the development of some foliar diseases caused by fungi. Fungicides are usually applied on foliage to control diseases but they are also used for seed treatments to prevent seed decay (since soil fungicide applications are not a common practice in Argentina).

2. Wheat yield and quality as affected by foliar diseases

Foliar diseases caused by fungi are the major biotic limitation on yield and quality on wheat [6, 7]. Foliar pathogens reduce yield through reductions in the photosynthesis rate, increasing the rate of respiration, and decreasing translocation of photosynthates from infected tissue [8, 9]. Photosynthesis of diseased plants is reduced due to the destruction of the photosynthetic area. Infected plants usually produce fewer tillers and set fewer grains per spike and the grains are smaller, generally shriveled and of poor milling quality. Shriveled grains occur because the diseases reduce the dry matter destined to the grain but also because the fungi induces earlier maturity of the plant, resulting in decreased time available for the grain to fill [10]. Shriveled grains can contribute to impurities, reduced flour extraction rates and lower contents of metabolizable energy [11].

Foliar pathogens include three diverse groups ranging from poorly specialized necrotroph to highly specialized biotroph parasites. The leaf blights are caused by necrotroph and

hemibiotroph parasitic fungi that cause tissue death. The most important leaf-blights in wheat are tan spot [(*Pyrenophora tritici-repentis* (Died.) Drechs., *Drechslera tritici-repentis* (Died.) Shoemaker)] and Septoria leaf blotch, caused by *Septoria tritici* Rob. ex Desm., teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn. Tan spot symptoms include tan lesions surrounded by a yellow halo on leaves (Fig. 1a), and Septoria leaf blotch produce yellowish specks leaf spots that later enlarge, turn pale brown and finally dark brown, usually surrounded by a narrow yellow zone (Fig. 1b). Both fungi mentioned before can be grown in laboratory conditions. The control of these foliar diseases by genetic resistance strategies has been difficult because the pathogens have a high variability partially caused by the presence of both asexual and sexual reproduction and because the pathogens show a high degree of specialization. Cultivars in Argentina generally are moderately susceptible to susceptible with only a few with moderate resistance. Therefore, integrated disease management including cultivars with acceptable levels of resistance, crop rotation, seed treatments, different cropping and tillage systems, N fertilization management and fungicides has been used by growers. Tan spot and leaf blotch can be managed by cultural practices such as crop rotation with non-hosts, removal or destruction of infested residue, or tillage, which buries infested residue. Seed treatments are usual since tan spot and leaf blotch can be seed-transmitted, therefore treating seed with fungicide before planting can reduce seed-borne inoculum.

Together with some other pathogenic fungi (mainly *Bipolaris sorokiniana* (Sacc.) Schoem., teleomorph *Cochliobolus sativus* (Ito & Kuribayashi) Drechsler ex Dastur and *Alternaria* spp.), tan spot and Septoria leaf blotch form a leaf spot disease complex in Argentina. The proportion of each fungus in this complex may vary depending on the environment and geographic location [12, 13, 14].

Figure 1. (From left to right): **a.** Tan spot symptoms caused by *Drechslera tritici-repentis* on wheat leaves [15]. **b.** Leaf blotch symptoms caused by *Septoria tritici* [16]. **c.** Leaf rust symptoms caused by *Puccinia triticina* [17].

On the other hand, leaf rust (*Puccinia triticin*a Eriks) is the main foliar disease in Argentina caused by a biotroph fungus. It is a very-specialized obligated parasite, thus it cannot be cultivated in laboratory conditions. This foliar disease attacks all the aboveground parts of wheat plants, especially leaves, and causes numerous rusty, orange spots that rupture the

epidermis on wheat leaves (Fig. 1c). Leaf rust may reduce the grain number per plant [18] and the grain produced may be of extremely poor quality, as it may be devoid of starch [10]. Chemical control is a common practice complemented with cultivars with different levels of resistance, usually with a short durability.

3. Different types of fungicides: Its control mechanisms

Planting resistant cultivars is one of the least expensive and most effective management strategies to prevent diseases. However, cultivars with an adequate genetic resistance level to necrotroph foliar diseases are scarce, and usually resistance to leaf rust is complete, conditioned by one or a few genes and has low level of durability in Argentina. Therefore, chemical protection together with cultural practices is a common method of control. In addition, fungicides are also important because Argentinean wheat region combine high yield potential cultivars with high infection pressure, both deriving from adequate temperature and moisture levels, large application of N fertilizers and rotations dominated by cereals, which promote progression of some foliar diseases.

However, the response varies depending not only on the fungicide but also on the N fertilization level, tillage system, foliar disease type and characteristics of the genotypes. The relationship between yield loss and disease severity can differ widely between crop genotypes [9] and some of them exhibit a smaller yield loss under a given severity of infection than others. On the other hand, mechanisms of fungicides to control foliar diseases on wheat may vary according to the active ingredient they have.

Recently, varieties with French germplasm have been introduced or crossed with local germplasm to produce new cultivars in Argentina. These cultivars are characterized by high yield potential but lower resistance to foliar pathogens as tan spot, leaf blotch and leaf rust than the traditional ones. However the increasing adoption by growers of French germplasm varieties susceptible to foliar diseases is leading to a higher use of fungicides.

Triazoles and Strobilurins are the most common systemic fungicides used to control foliar diseases on wheat in Argentina. Statistics shown by Campos [2] indicate that 50% of the products used in Argentina are triazoles and the remaining 50% consists in mixtures of formulations containing triazoles and strobilurins (Fig. 2). Systemic fungicides are absorbed through the foliage or roots and are translocated within the plant through the xylem. These types of fungicides generally move upward in the transpiration stream and may accumulate at the leaf margins [19].

Triazoles are characterized by being an active ergosterol inhibitor, which is the major sterol in fungi. Sterols derivate from terpenes, and they are an essential part of the fungal cell membrane. These molecules are rigid and flat and in its association with the cell membrane give them stability, making it less flexible and allowing the permeability control. Ergosterol Biosynthesis Inhibitors (EBIs) have become one of the most important groups of fungicides, however they may not be effective in controlling Oomycetes because they do not possess the ergosterol synthesis via [19].

The EBIs can be divided into: 1,4 α-demethylase inhibitors (DMIs), which includes the azole (triazole, imidazole) and pyrimidines; the Δ 8,7 isomerase and Δ1,4 reductase inhibitors (morphines and piperazines) and 3-ceto reductase sterol inhibitors (hydroxyanilide).

The triazoles have been useful to control many foliar diseases. They inhibit the fungus dependent enzyme cytochrome P-450 called 1,4α-demethylase involved in the ergosterol biosynthesis and consequently affect the permeability of the membrane. However, the mode of action may vary relatively between the different active principles within this group. One of the most common chemicals commercialized in Argentina containing triazoles is Tebuconazole, which is used for seed treatment and foliar and spike applications in cereals [19].

The fungi-resistance genetic basis to triazoles is not well known. In many cases it seems to be polygenic and observed decreasing effectiveness does not always imply loss of yield performance. The triazol group has many benefits such as high antifungal activity, low toxicity to other organisms, curative properties, and they are compatible with an integrated disease management; however its preventive action is low. That is why they are usually used in mixed formulations with other chemical groups to compensate this deficiency.

TRADE NAME	COMPANY	ACTIVE INGREDIENTS
Caramba	Basf	Metconazole 9
Duett	Basf	Epoxiconazole + Carbendazim
Duett Plus	Basf	Epoxiconazole + Metconazole
Folicur 25%	Bayer	Tebuconazole
Artea	Syngenta	Cyproconazole + Propiconazole
Tilt	Syngenta	Propiconazole

TRADE NAME	COMPANY	ACTIVE INGREDIENTS
Allegro	Basf	Kresoxim Methyl + Epoxiconazole
Opera	Basf	Pyraclostrobin + Epoxiconazole
Nativo	Bayer	Trifloxystrobin + Tebuconazole
Amistar Xtra	Syngenta	Azoxystrobin + Cyproconazole
Sphere	Bayer	Trifloxystrobin + Cyproconazole
Planet Xtra	Dow	Azoxystrobin + Cyproconazole
Stinger	Dupont	Picoxystrobin + Cyproconazole

Figure 2. Most common fungicides used in Argentina.

On the other hand, strobilurins are a chemical group which act as mitochondrial respiration inhibitors (MRIs). The strobilurins are an important class of agricultural fungicides, the discovery of which was inspired by a group of natural fungicidal derivatives of β-methoxy-

acrylic acid [20]. Strobilurins are synthetic derivatives of the Basidiomycete fungus *Strobilurus tenacellus*, which grows on pine wood producing decomposition. This chemical group reduces or eliminates competition with other microorganisms that uses wood as a source of food. Strobilurins have become a valuable tool for disease management, as this group controls Oomycetes, Ascomycetes and Basidiomycetes, the three major groups of plant pathogenic fungi in crops. However, strobilurins vary in their levels of activity against the different plant diseases and not all of them give high levels of control of all three major groups of plant pathogenic fungi [20].

Strobilurins mode of action was not considered generating resistance initially; but in recent years resistance to this group has been found in different countries on several diseases, therefore it is essential to achieve an appropriate disease management to avoid these kind of problems [21].

Strobilurins are mesostemic compounds (except Axozystrobin which is partially systemic), which means they possess strong adsorption and cuticle-waxes penetration on leaves. Most of the strobilurins are lipophilic and therefore, the active ingredient is moved into the leaf and may enter through the cuticle of the lower leaf surfaces. Consequently, the fungicide may be found on both leaf surfaces even if only one was treated. This movement may take one or a few days and it may move in vapor phase in the air layer adjacent to the leaf surface as well. These processes might be especially important in crops with dense canopy as in the case of wheat in advanced development stage [19]. Moreover, strobilurins are excellent preventive fungicides because they can kill spores. Nevertheless, they are not curative fungicides, since strobilurins binds tightly to the leaf cuticle and therefore the amount of active ingredient present into the leaf tissue would be lower than in the cuticle, being insufficient to control the fungus once it has entered in the plant. Furthermore, the germinative spores are more sensitive to the strobilurins than the mycelium and consequently the best use of the strobilurins is when they are applied before the infection takes place. With this new mode of action the strobilurins are an important addition to the existing fungicide range, particularly for cereals in which recent broad-spectrum fungicide products have been largely based on sterol biosynthesis inhibitors (EBIs) [22]. Therefore, they are generally used in mixtures with triazole fungicides which provides curative power. Finally, strobilurins has an ethylene-synthesis-inhibition-property that cause a delay in leaves senescence and it may causes higher increases in crop yield than other types of fungicides. Wu & von Tiedemann [23] suggested that the fungicide-induced delay of senescence is due to an enhanced antioxidative potential protecting the plant from harmful active oxygen species. A longer period of photosynthetic active green leaf area has been suggested to be the main factor for yield increases obtained with strobilurin fungicides, because the increased photosynthetic period would increase the quantity of assimilate available for grain filling [22].

Strobilurins fungicides have become an integral part of disease-management programs on a wide range of crops in many countries of the world. The major reasons for the success of strobilurins have varied between individual active ingredients, but have consisted of one or more of the following: broad-sprectrum activity, control of fungal isolates resistant to other fungicides mode of action, low use-rates and excellent yield and quality [20].

4. The use of fungicides in the integrated foliar disease management to enhance wheat yield and quality

Crop potential yield is defined as that attainable yield, when no nutrient or water limitations occur, i.e. when incident radiation, temperature and physiological crop genotype characteristics determine yield [24]. On the other hand, grain quality has several definitions depending on the users; therefore, the end-use quality is vastly diverse [25]. Several factors have influence on the severity of the main foliar diseases of wheat, among them resistance of the cultivars, tillage systems, N fertilization and fungicide applications.

Genetic resistance is the basis of the integrated disease management. Plant disease resistance can be classified into two categories: qualitative resistance, conferred by a single resistance gene (also termed as race non- specific or slow rusting resistance) and quantitative resistance, mediated by multiple genes or quantitative trait loci (QTLs) (also termed as race non-specific or slow rusting resistance) with each providing a partial increase in resistance [26]. Considering the main foliar diseases in wheat during the last decade, 18 major genes conferring resistance to the pathogen have been identified for resistance to *Septoria tritici*. They were: *Stb1* located on the chromosome 5BL [27], *Stb2* on the chromosome 3BS [28], *Stb3* on the chromosome 6DS [29], *Stb4* on the chromosome 7DS [30]; *Stb5* on the chromosome 7DS [31]; *Stb6* on the chromosome 3AS [32]; *Stb7* on the chromosome 4AL [33]; *Stb8* on the chromosome 7BL [34]; *Stb 9* on the chromosome 2B [35], *Stb10* on the chromosome 1D [36]. *Stb 11* on the chromosome 1BS [37], *Stb12*, on the chromosome 4AL [36], *Stb13* on the chromosome 7BL [38], *Stb14* on the chromosome 3BS [38], *Stb15* on the chromosome 6AS [39], *Stb16* on the chromosome 3D [40], *Stb17* on the chromosome 5A [41] and *Stb 18* on the chromosome 6DS [42]. In addition, several QTL were also found. Eriksen *et al*. [43] found some on chromosomes 2BL, 3AS, 3BL, 6B and 7B. In Argentina resistance was localized in several foreign lines [41]

Considering resistance to tan spot eight races of the pathogen has been characterized based on their ability to cause necrosis and/or chlorosis in differential wheat lines [44]. In Argentina and in general around the world cultivars with acceptable levels of resistance to tan spot and Septoria leaf blotch are scarce.

Considering leaf rust, more than sixty genes for leaf rust resistance (*Lr*), most of them major or race specific genes, have been catalogued to date in wheat [45, 46]. However, the gene-for-gene interaction between host resistance genes and pathogen virulence genes combined by virulence shifts in pathogen populations have reduced the effectiveness of a significant number of major leaf rust resistance genes [47, 48]. Replacement of highly variable land races by higher yielding, pure-line varieties in many parts of the world, including the South Cone, has further reduced the wheat gene pool and favored virulence shifts events in pathogen populations.

In Argentina using molecular markers, a set of 66 adapted cultivars previously evaluated by gene postulation for presence of 15 *Lr* genes was screened, and eight genes were detected: six seedling genes (*Lr9, Lr10, Lr19, Lr24, Lr26, Lr47*) and two adult plant resistance genes (*Lr34, Lr37*). Genes *Lr20, Lr21, Lr25, Lr29, Lr35* (adult plant resistance gene) and *Lr51* were

not detected in tested cultivars [49]. Resistance in most Argentinean cultivar and around the world is conditioned by one or a few genes.

In the Rolling Pampa region of Argentina, conservation management practices such as no tillage are increasing as alternative cropping systems. No tillage systems have been implemented to restore soil structure in large areas cultivated with double-crop sequences such as wheat (*Triticum aestivum* L.)/soybean (*Glycine max* L. (Merr.); corn (*Zea mays* L.) - wheat/soybean; or wheat monoculture [50]. Annual wheat/soybean double-crop sequences using conventional tillage are considered less desirable because of the effect on soil organic matter and the reduced quantity of residue that soybean crops leave after-harvest [51]. In the semiarid region of Argentina, conservation management techniques are also necessary to prevent soil erosion and effectively store and use the limited amount of precipitation for crop production [52]. No tillage can also reduce costs by decreasing fuel consumption required to produce a crop [53].

However, in the wheat/soybean system under no tillage, as in wheat following wheat, the inoculum of necrotrophic fungi usually survives until the next wheat season; typically, a minimum of one to two years between wheat crops is required to reduce populations of these organisms [54]. In no tillage systems, crop residue mineralization is slow. It requires 14 to 16 months in Brazil [55] but approximately 18 to 32 months in Argentina and Uruguay due to lower average temperatures than in Brazil [56, 57]. No tillage may have a different effect on plant diseases depending on the soil type, geographic location, environment, and the biology of the particular disease-causing organism [58].

Tan spot and Stagonospora blotch [*Phaeosphaeria avenaria* (G.F. Weber) O. Eriksson f. sp. *triticea* T. Johnson, anamorph *Stagonospora avenae* (A. B. Frank) Bissett f. sp. *tritica* T. Johnson] increased in no tillage systems in wheat monoculture or wheat following fallow, although the opposite occurred when wheat followed other crops [59, 60, 61, 62, 63]. In some studies, conventional tillage increases crop residue mineralization, reducing fungal inoculum [61, 64]. However, others [23, 58, 59, 61, 65, 66, 67, 68, 69] reported contrasting results regarding the effect of no tillage on necrotrophic wheat diseases, depending on the environment and the crop growth stage evaluated (early or late in the season).

Fungicides are widely used to manage foliar wheat diseases in Argentina and several countries [70]. The response to fungicide application depends on the severity of specific foliar diseases, cultivar disease resistance or tolerance, management practices, and environmental conditions [71, 72, 73]. Fungicides applied at flag leaf and spike emergence of winter wheat increased mean grain weight and grain yield when they extended canopy life [74]. The green area duration of flag leaf is important because is the last leaf senescing, it intercepts more light than lower leaves and it is in closer vascular proximity to spikes than lower leaves [75]. Strategies to protect flag leaf and delay the senescence process are therefore important to assure not only higher yield but also higher grain quality [76]. Gooding [74] found that the effect of fungicides increasing green area duration of the flag leaf was associated with increases in yield, thousand grain weight and specific weight. Fungicides containing strobilurins to control foliar diseases in wheat are associated in some

cases with higher increases in grain yield and grain weight comparing with triazoles. Dimmock & Gooding [77] reported that strobilurins prolonged green flag leaf area duration and increased mean grain weight significantly more than triazoles.

Jorgensen and Olsen [67] reported wheat yield increases following fungicide treatments ranging from 0.8 to 4.4 Mg ha^{-1}, depending on the amount of infested straw on the soil surface, disease severity and fungicide strategy (type of active ingredient, timing or number or applications, rates and method of application). Severe foliar infections before or at flowering stage of wheat are extremely damaging and may cause important yield losses, whereas when serious infections occur later, the damage to yield is much smaller.

Increased yields disease management are associated mainly with an increase in thousand grain weight [72, 73, 78, 79, 80], while other yield components such as number of $spikes.m^{-2}$ [72] or $grains.spike^{-1}$ [72, 79, 80, 81] are usually not affected by disease severity. However, Simón *et al*. [82] reported that preventing early wheat infection by *Septoria tritici* could result in an increase of $spikes.m^{-2}$ and $grains.spike^{-1}$.

In Argentina, Serrago *et al.* [83] determined that grain number was not affected by foliar diseases when they appeared after anthesis. Grain weight was strongly, poorly or not affected by foliar diseases and was not associated individually with both, the sink size and the source size. However, when the grain weight increment due to fungicide application was plotted against the healthy area absorption per grain, a significant negative association was found for the Argentine experiments [83]. When the healthy absorption area per grain was corrected by the grain weight potential all experiments conducted in Argentine and in France fit well to a common negative linear regression for the relationship between grain weight variation and grain weight potential demonstrating that grain weight potential is an important feature to consider in diseases control programs [83]. Foliar diseases forced the crop to use the accumulated reserves increasing the utilization rate of the water soluble carbohydrates , depleting as a consequence the water soluble content at physiological maturity in all experiments. The association between water soluble carbohydrates and the healthy area absorption per grain corrected by grain weight of healthy crops suggests that foliar diseases in wheat cause source limitation, forcing to the crop to use the water soluble content reserve which could be insufficient to fill the grains previously formed [83].

Management practices such as N fertilization can also affect the expression of wheat foliar diseases [82, 84] and the effectiveness of foliar fungicide application [72, 82, 84, 85]. Increasing N rates may cause negative, positive or neutral effect on foliar disease severity, depending on the geographic location [86] and the type of disease. The magnitude and direction of the influence of N supply on Septoria leaf blotch severity has been studied with contrasting results [85, 87, 88, 89]. Simón *et al*. [82, 84, 90] found that in conducive conditions, N fertilization increases the severity of Septoria leaf blotch and discussed the effect of different factors affecting the influence of N supply. Increasing N rates retarded tan spot development [66, 69, 73, 91, 92, 93, 94]. However, Bockus and Davis [95] suggested that N applications do not directly affect tan spot severity, but rather appear to reduce disease impact through delayed leaf senescence or that high N rates increase Septoria leaf blotch or

tan spot severity due to an increase in crop biomass production, which creates a micro-environment conducive to fungal development in humid regions [82, 84, 85, 96, 97]. In addition, experiments carried out in Argentina indicated that yield increase and increase in yield components due to application of tebuconazole was similar in fertilized and non fertilized conditions, despite the increase in the area under disease progress curve under N fertilization [82].

Biotrophic pathogen such as leaf rust also causes important diseases in wheat. N fertilization usually increases the severity of this disease [98, 99, 100].

Using cultivars with good behavior to tan spot, optimizing N rates and fungicide applications would reduce yield losses compared to non fertilized plots planted with susceptible cultivars. Results of some experiments carried out in Argentina addressing this question are presented. Those experiments showed that no tillage often leads to wheat yield losses from diseases caused by necrotrophic foliar pathogens. Conventional tillage reduced foliar disease severity caused mainly by tan spot at GS 23 [101] by 46 and 56% and the area under disease progress curve (AUDPC) [102] by 20 and 14% for each season, respectively compared with no tillage (Table 1). Fungicide and N application reduced disease severity at GS 23 by 35 and 34% respectively, on average over two seasons (Table 1) Disease was less severe in no tillage plots which received a fungicide compared to conventional tillage plots that were not treated with fungicide. Application of 160 kg ha^{-1} N increased crop biomass by 71% at GS 23 and 57% at GS 83 averaged over two seasons compared to plots that received no nitrogen. N fertilization treatments decreased the AUDPC 17.2% and 23.5%, and fungicide input reduced the disease severity 37.6% and 24.7% in each season. It is remarkable that AUDPC was reduced with N160 as much as with fungicide applications in one of the years (Table 1).

Fungicides increased yield by 9% on average of both years. The increased yield resulted from increases in spikes.m^{-2} and thousand grain weight in two seasons, and also from grain.$spike^{-1}$ in one season [94] (Table 2).

Experiments were also carried out in Argentina with artificial early inoculation with *Septoria tritici* to investigate how N supply influences the disease severity, yield and yield components. In one of the years, with weather conditions conducive to the disease, AUDPC values were higher in the fertilized treatment. In another year with insufficient rain immediately after inoculation, the disease only progressed faster under N fertilization in the flag leaf, which was exposed to conducive environmental conditions from its appearance. The effect of N fertilization was influenced by the cultivar characteristics, climatic, and agronomic conditions (Table 3). Knowledge that N fertilization promotes the development of *Septoria tritici* blotch in conducive conditions will be useful for deciding management strategies of the cultivars and for optimizing conditions for the selection in breeding programmes. Considering yield and yield components, additional N increased yield, spikes.m^{-2} and grains.$spike^{-1}$, but not thousand kernel weight or test weight. The percentage reduction in yield, yield components and test weight due to inoculation was similar in fertilized and non-fertilized conditions, despite the increase in the AUDPC values by N fertilization (Table 4).

	Year 1								Year 2							
	Conventional tillage				No tillage				Conventional tillage				No tillage			
	0N	80N	160N	Average	0N	80N	160N	Average	0N	80N	160N	Average	0N	80N	160N	Average
	Disease severity GS 23 (%)															
Without fungicide	8.2	7.3	8.4	8.0	18.4	18.5	13.4	16.8	14.6	13.0	7.3	11.6	30.9	22.9	19.5	24.4
With fungicide	7.8	7.3	5.8	7.0	14.3	9.1	8.3	10.6	6.9	6.2	6.1	6.4	21.2	15.6	11.9	16.2
Averages	8.0	7.3	7.1	7.5	16.3	13.8	10.9	13.7	10.8	9.6	6.7	9.0	26.1	19.3	20.3	21.9
	AUDPC															
Without fungicide	1364	1197	1189	1250	1610	1569	1456	1545	1920	1590	1525	1678	2098	1885	1728	1904
With fungicide	943	751	610	768	1102	934	897	978	1450	1171	1074	1232	1742	1465	1184	1464
Averages	1153	974	899	1009	1356	1251	1176	1261	1685	1380	1299	1455	1920	1675	1456	1684
	Biomass (g) GS 23															
Without fungicide	66.7	84.4	109	86.7	61.9	82.2	106	83.4	70.2	85.7	94.1	83.3	42.4	60.3	86.9	63.2
With fungicide	72.1	110	115	99.0	59.4	104	105	89.5	65.1	83.7	117	88.6	43.8	80.7	90.1	71.5
Averages	69.4	97.5	112	92.9	60.7	93.1	105	86.4	67.6	84.7	106	86.0	43.1	70.5	88.5	67.4
	Biomass (g) GS 83															
Without fungicide	688	1090	1113	964	629	951	1082	887	911	1100	1197	1069	561	882	1153	865
With fungicide	762	1202	1416	1127	683	1069	1309	1020	898	1464	1509	1290	802	1047	1169	1006
Averages	725	1146	1265	1045	656	1010	1196	953	904	1282	1353	1179	682	964	1161	936

AUDPC; area under disease progress curve, GS, growth stage

LSD (P=0.05) for significant interactions:LSD interaction T x F severity GS 23, 2002=5.82

Table 1. Means for the interactions of cultural practices on foliar disease intensity and wheat biomass over two season at Los Hornos, La Plata, Argentina

	Year 1								Year 2							
	Conventional tillage				No tillage				Conventional tillage				No tillage			
	0N	80N	160N	Average	0N	80N	160N	Average	0N	80N	160N	Average	0N	80N	160N	Average
	Yield (kg.ha^{-1})															
Without fungicide	3918	5684	6347	5316	3469	5223	6005	4899	2617	4639	5045	4100	2180	3314	5602	3699
With fungicide	4040	6037	7192	5756	4063	5964	6235	5421	3278	5397	5399	4691	2384	4220	5720	4108
Averages	3979	5861	6769	5536	3766	5593	6120	5160	2948	5018	5222	4396	2282	3767	5661	3903
	SPM2 (nº)															
Without fungicide	356	426	506	429	323	419	439	394	307	411	444	387	293	345	450	363
With fungicide	342	442	512	432	353	472	460	428	312	450	444	402	298	366	470	378
Averages	349	434	509	431	338	445	449	411	309	430	444	394	295	355	460	370
	KPS (nº)															
Without fungicide	29.1	35.5	33.9	32.8	30.7	34.7	36.0	33.8	23.5	31.5	31.6	28.9	21.2	28.1	33.9	27.7
With fungicide	30.9	34.6	37.0	34.2	30.2	32.5	34.8	32.5	26.3	31.7	32.7	30.2	25.1	31.8	32.8	29.9
Averages	30.0	35.0	35.5	33.5	30.5	33.6	35.4	33.3	24.9	31.6	32.1	29.6	23.2	29.9	33.3	28.8
	TKW (g)															
Without fungicide	37.6	38.2	37.4	37.7	34.6	35.9	37.1	35.9	35.3	36.4	36.6	36.1	35.4	34.3	37.0	35.6
With fungicide	38.9	39.1	38.2	38.7	37.1	37.7	38.8	37.9	39.9	39.0	38.7	39.1	34.9	36.1	37.0	36.0
Averages	38.2	38.6	37.8	38.2	35.8	36.8	38.0	36.9	37.6	37.7	37.7	37.7	35.1	35.2	37.0	35.8

Only significant LSD values are given, SPM2 TxN, 2002=74, 2003=96, KPS: LSD T × F, 2002= 3.8; C × N, 2003= 8.4
TKW: LSD T × N, 2002=2.7; C × N, 2002=2.8
Yield: LSD T × N, 2003= 1742

Table 2. Means for the interactions of cultural practices on yield and yield components of wheat over two seasons, at Los Hornos, La Plata, Argentina

	Year 1			Year 2		
	----------AUDPC----------					
Cultivar	**With fertilizer**	**Without fertilizer**	**Average**	**With fertilizer**	**Without fertilizer**	**Average**
Buck Ombú	723 a[y] (812)[x]	634 a (702)	679[z] E (757)	343 a (351)	370 a (380)	356 B (365)
Don Ernesto	428 a (459)	237 b (273)	332 B (366)	362 a (370)	286 a (295)	324 AB (333)
Klein Centauro	505 a (330)	466 a (272)	486 C (301)	420 a (489)	374 a (445)	397 B (467)
Klein Dragón	265 a (231)	85.3 b (96)	175 A (163)	222 a (246)	258 a (268)	240 A (257)
PROINTA Federal	313 a (343)	169 b (205)	241 A (274)	382 a (391)	332 a (342)	357 B (366)
PROINTA Verde	721 a (778)	423 b (472)	572 D (625)	406 a (292)	336 a (225)	371 B (258)
Averages	492 a	336 b		357 a	326 a	

Means are adjusted by heading date as a covariant.
[x] Unadjusted values. [y] Means followed by the same letter in the same row within the same year are not significantly different, LSD (P=0.05). [z] Means followed by the same letter in the average columns within the same year are not significantly different, LSD (P=0.05).

Table 3. Means of the AUDPC of *Septoria tritici* blotch on six wheat cultivars under two nitrogen fertilisation treatments in two years.

	Year 1				Year 2					
	With fertilization		Without fertilization		With fertilization		Without fertilization			
Cultivar	With inocula-tion	Without inocula-tion	With inocula-tion	Without inocula-tion	With inocula-tion	Without inocula-tion	With inocula-tion	Without inocula-tion	**Average 1996**	**Average 1997**
	Kg.ha^{-1}									
Buck Ombú	5305 (38.1)†	8579	4501 (44.9)	8166	5097 (31.3)	7423	4822 (30.6)	6951	**6638**	**6074**
Don Ernesto	6521 (26.3)	8852	4949 (32.0)	7251	5157 (27.0)	7062	4176 (29.5)	5925	**6888**	**5580**
Klein Centauro	6835 (18.6)	8400	5836 (20.8)	7371	7413 (19.5)	9213	5961 (22.0)	7644	**7111**	**7558**
Klein Dragón	9325 (16.6)	11175	6974 (17.7)	8474	6512 (20.8)	8223	5798 (23.5)	7508	**8987**	**7029**
PROINTA Federal	6524 (25.3)	8744	4713 (31.6)	6888	5035 (28.2)	7015	4760 (27.0)	6525	**6717**	**5834**
PROINTA Isla Verde	6550 (31.4)	9542	5252 (31.5)	7661	4950 (28.0)	6879	4550 (28.0)	6321	**7251**	**5675**
Average	6843 (25.7)	9215	5367 (29.7)	7635	5694 (25.4)	7636	5011 (26.6)	6824	**7265**	**6291**
Cultivar Average fertilization										
With	8029				6665					
Without	6501				5918					
Average inoculation										
With	6185				5353					
Without	8425				7230					
LSD cultivars	249.0				906.8					
LSD fertilization	522.9				598.6					
LSD inoculation	721.0				306.6					

† Percentage of reduction relative to the non-inoculated control are given in parenthesis.

Table 4. Means of yield per hectare for six wheat cultivars under two nitrogen fertilization conditions and two inoculation treatments with *Septoria tritici.*

Further experiments were also carried out in Argentina comparing the effect of N fertilization and fungicides on the severity caused by tan spot, Septoria leaf blotch and leaf rust and on the yield of wheat in the same environment [103]. Results indicated that there was a three way interaction pathogen × N fertilization × fungicide. This interaction was caused by the fact that tan spot severity decreases with N fertilization, but increases for Septoria leaf blotch and leaf rust (Fig. 3, 5, 7). The application of N fertilization did not reduce severity of tan spot as much as fungicide application. Fungicides (Nativo: combination of triazoles and strobilurins) were effective in controlling the three foliar diseases, but mainly leaf rust. In addition the control produced by the fungicide was higher when the severity increases. With similar severity values, the control produced by the fungicides was similar for all N treatments. Yield was increased by fungicide application 20% and by N fertilization by 27.5% when the pathogen inoculated was *Septoria tritici* (Fig. 4) and by 10.3% and 18.6% when the pathogen inoculated was *Drechslera tritici-repentis* (Fig. 6) On the contrary, when the pathogen inoculated was *Puccinia triticina*, fungicides caused the higher increase in yield (19.2%), whereas the increase due to N fertilization was 9.2% (Fig. 8).

Grain quality in wheat is a complex of different traits deeply influenced by genotypic and environmental factors. The baking market requires flour for different types of products, e.g. mechanized bread, artisan bread, baguette, flat breads, steamed bread, biscuits, crackers, pasta, noodles, etc. Although varieties are assigned to quality groups when they are registered to be commercialized, the final product after growing and harvesting is not always adequately classified for commercialization.

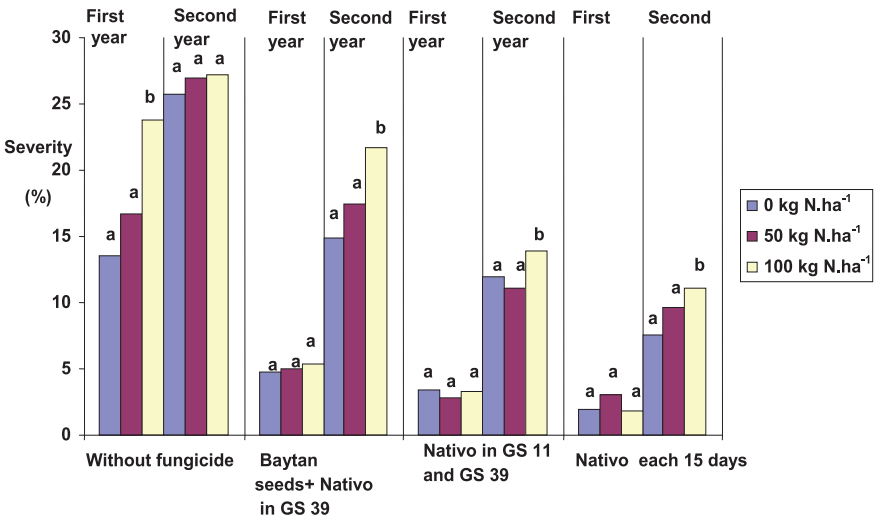

Figure 3. Means of fungicide x fertilizer interaction of disease severity (%) on a trial inoculated with *Septoria tritici* with three nitrogen levels, four fungicide treatments and two cultivars in two years.

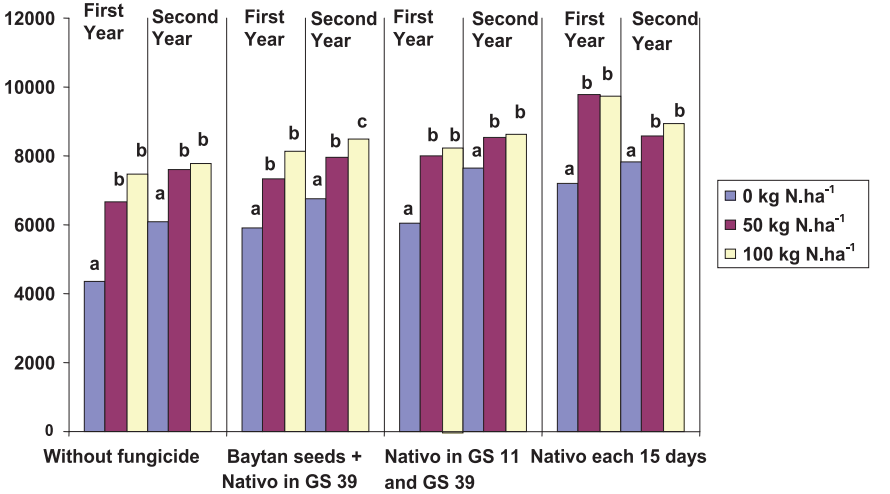

Figure 4. Means of fungicide x fertilizer interaction of grain yield in wheat (kg.ha^{-1}) on a trial inoculated with *Septoria tritici* with three nitrogen levels, four fungicide treatments and two cultivars in two years.

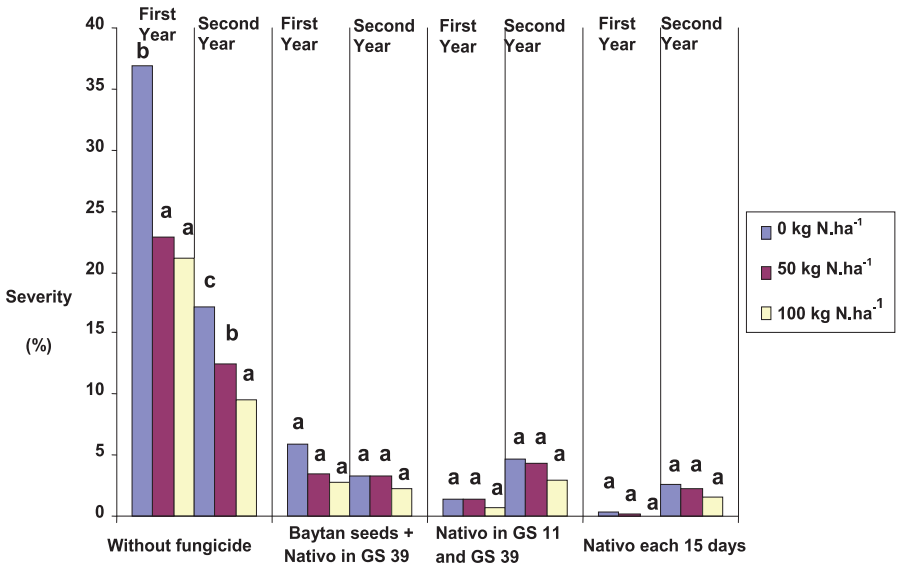

Figure 5. Means of fungicide x fertilizer interaction of disease severity (%) caused by *Drechslera tritici-repentis* in GS 82 on a trial with three nitrogen levels, four fungicide treatments and two wheat cultivars in two years.

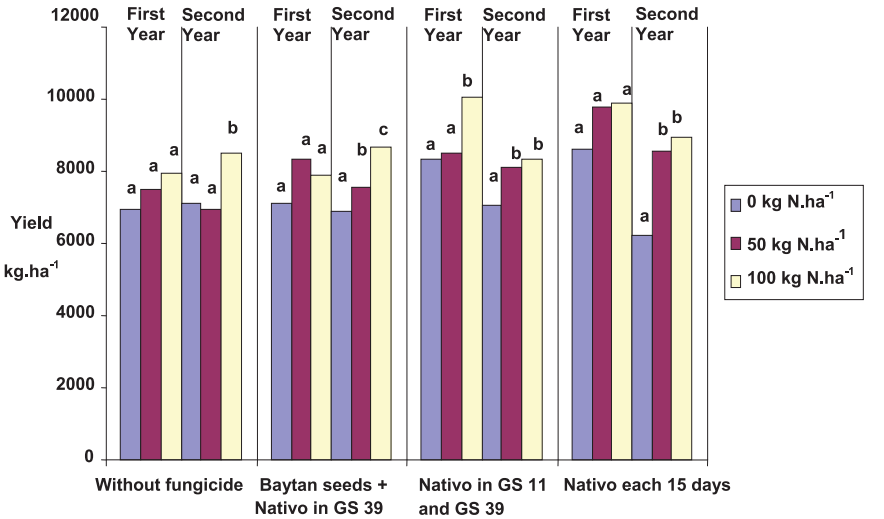

Figure 6. Means of fungicide x fertilizer interaction of grain yield in wheat (kg.ha-1) on a trial inoculated with *Drechslera tritici-repentis* with three nitrogen levels, four fungicide treatments and two cultivars in two years.

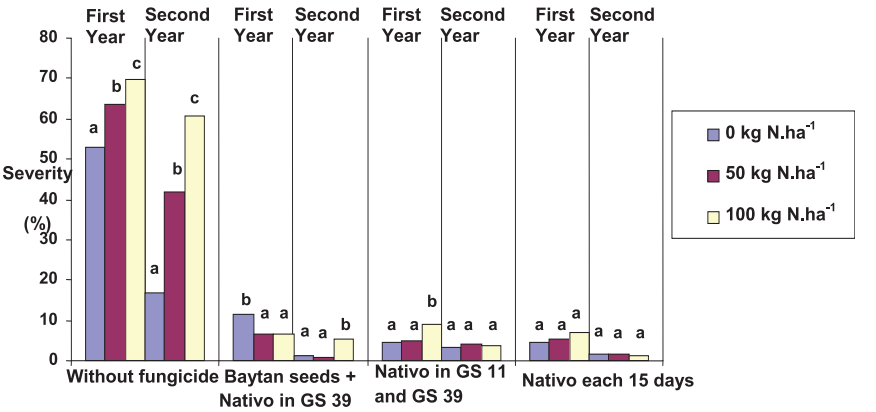

Figure 7. Means of fungicide x fertilizer interaction of disease severity (%) caused by *Puccinia triticina* in GS 82 on a trial with three nitrogen levels, four fungicide treatments and two wheat cultivars in two years.

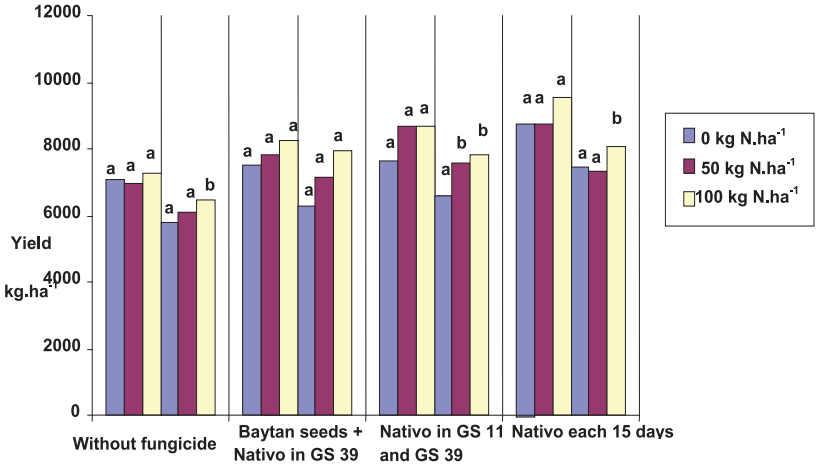

Figure 8. Means of fungicide x fertilizer interaction of grain yield in wheat (kg.ha-1) on a trial inoculated with *Puccinia triticina* with three nitrogen levels, four fungicide treatments and two cultivars in two years.

The main quality characteristics for the wheat utilization are flour extraction (milling yield), flour protein concentration and rheological-breadmaking properties. The behavior of dough is strongly linked to the type and amount of protein present in flour, and hence the concentration of protein in the wheat grain at harvest. Grain protein concentration is positively associated with breadmaking quality, particularly to loaf volume [104]. In most production systems there is a negative relationship between yield and grain protein concentration. Nevertheless, this does not imply that higher grain protein cannot be obtained at high-yield levels. At low N rates of fertilization (Fig. 9), yield increases asymptotically, i.e. the response of starch accumulation is greater than protein content (zone 1) [105]. The first increments of N tend to increase yield but decrease protein percentage, resulting in the frequently reported negative relationship between grain yield and protein percentage (zone 1). After a certain level of N is attained, the response of starch and protein accumulation has a different response (zone 2). At these N fertilization levels, additional N results in a lower yield increase regarding the previous N doses (but still positive), and a comparatively higher increase in protein percentage. Finally, with higher amounts of N, the crop reaches a third region of response (zone 3), where maximum yield may be attained. At this point, additional fertilizer does not affect the amount of starch in the grain, but increases protein content (Fig. 9). On the other hand, different genotypes generate different protein concentrations in grain, depending on N rates fertilization and how efficiently they absorb and use N for yield generation . The increase in grain protein content under high N fertilization conditions results in greater synthesis and accumulation of storage protein (gliadins and glutenins), which are the gluten forming proteins [106]. Gluten proteins are the major determinant of the processing properties of wheat dough, by conferring viscoelasticity, which is essential for breadmaking process.

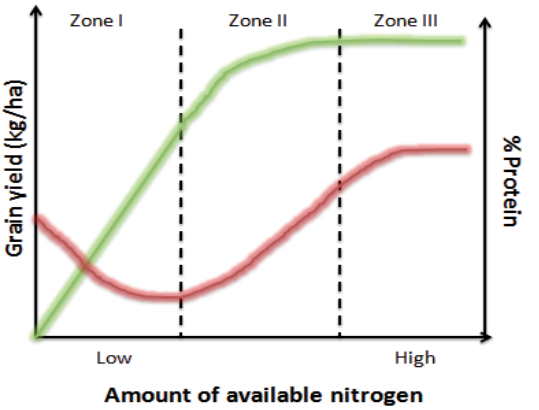

Figure 9. Diagrammatic representation of the response of yield and protein percentage to nitrogen fertilizer [105].

Little attention has been given to foliar diseases impact on milling and baking quality and to the interactions of disease severity × cultivar on the wheat quality. These effects are more significant when strobilurins are applied due to the prolongation of the green flag leaf area duration compared with triazoles. Flag leaf photosynthesis in wheat contributes about 30-50% for grain filling [77], and longevity of the flag leaf promoted by strobilurins affects concentration of protein in the grain.

Gooding [74] reported fungicide effects on crude protein concentration depending on cultivar and disease control. The effect of foliar diseases on protein content may vary depending on foliar disease type. When biotrophic fungal pathogens such as leaf rust affects wheat, the protein concentration usually decreases, (i.e. the pathogen causes more damage on the accumulation and partitioning of N in the grain than on the accumulation and partitioning of dry matter) leading to a modification of the rheological properties of flour [74, 79, 107]. On the other hand, when wheat is affected by necrotrophic pathogens as tan spot, protein concentration increases [108]. Finally, hemibiotrophic pathogens such Septoria leaf blotch may cause both effects, depending on the genotype and environmental conditions. Controlling Septoria leaf blotch usually reduced protein concentration [79]. Liaudat [109] found increases in protein concentration when severity of Septoria leaf blotch increases. In the same study, the disease control with fungicide produced decreases in protein concentration and this reduction was more significant when strobilurins were applied.

5. The effect of fungicides on mycorrhizae

Arbuscular mycorrhizal fungi (AMF), which form symbiotic associations with root systems of most agricultural species, have been suggested as widespread potential bioprotective agents, inducing local and systemic resistance to some diseases. The knowledge of these fungi populations could also be an interesting contribution for the integrated disease management. Arbuscular mycorrhizae are associations between fungi that belong to the

phylum Glomeromycota [110] and most plant species [111]. Whereas there are numerous studies on the biocontrol effect of arbuscular mycorrhizae, there are relatively few on the effects of fungicides on these beneficial associations.

Arbuscular mycorrhizae are considered beneficial to plants, although their positive effects are variable because mycorrhizal symbioses reflect complex interactions among the plant, the fungi, and the environment [112, 113]. In agriculture, research dealing with mycorrhizal fungi is valuable both for determining appropriate management strategies and as a background to achieve successful inoculations [114]. The interaction between the fungus and its host plant mainly consists of nutrient transfer (the plant provides the arbuscular mycorrhizal fungi with photosynthates while the fungus delivers nutrients to the plant). The increased nutrient uptake from the soil, particularly of phosphorus and nitrogen, is the main benefit attributed to mycorrhizal symbiosis [115, 116]. However, other benefits are enhancement of resistance to root parasites [117], improvement of drought tolerance [118] and mitigation of environmental stresses such as salinity [119]. Another important role attributed to arbuscular mycorrhizal fungi is improving soil stability, which may diminish erosion [120, 121, 122, 123]. Recent studies have found evidences of bioprotectional effect of arbuscular mycorrhizal fungi against fungal pathogen, mainly those causing soil-borne diseases [124, 125, 126]. Arbuscular mycorrhizal fungi may control plant pathogens or contribute to activate plant defence responses through direct or indirect mechanisms, such as: improving plant nutrition and damage compensation [115], anatomical alterations in the root system [127], microbial changes in the rhizosphere and enhancing the attenuated plant defence responses by altering the host's signalling pathways [128]. Nevertheless, the knowledge about the induction of plant defence responses, the genetic, biochemical and signalling factors, their mechanisms and pathways involved, is still low [129].

The studies related to the effect of arbuscular mycorrhizal fungi on reduction of root diseases produced by fungi have mainly focused on those rots produced by species of *Phythium*, *Phytophtora*, *Fusarium*, *Verticillium*, *Pyrenochaeta*, *Gaeumannomyces*, *Sclerotium*, and *Rhizoctonia* [130]. Regarding foliar diseases, Gernns *et al*. [131] reported a compensation mechanisms between mycorrhizal plants and biotrophic fungal diseases. They found that mycorrhizal barley-plants were more susceptible to the obligate biotrophic shoot pathogen *Erysiphe graminis* f. sp. *hordei*, however, mycorrhizal plants suffered less than non-mycorrhizal plants in terms of grain number, spikes yield and thousand-grain weight. As mentioned before, other bioprotective effect of arbuscular mycorrhizal fungi on wheat is that found against take-all disease caused by *Gaeumannomyces graminis* [132, 133].

On the other hand, little is known about the effect of fungicides on mycorrhizal colonization, sporulation or spore germination. The effect of fungicide on arbuscular mycorrhizal fungi may be direct on the fungal growth or indirect, through changes in the physiology of the host plant, reductions in the disease levels and/or modifications in the soil environment. Considering the fungal component of mycorrhizal plants, is reasonable to infer that some fungicides might affect mycorrhizal colonization. Fungicides comprise a huge variety of compounds that differ in their effect on the host physiology, mode of action, spectrum of activity, application methods and formulation. Several studies have shown that fungicides

can affect mycorrhizal associations in a negative, neutral or even in a positive manner [134]. Consequently, it is difficult to generalize about the effects of fungicides on arbuscular mycorrhizal fungi. It is fundamentally important to distinguish the foliage fungicide applications, to those which are directed to the soil, or those which are applied on seeds.

In field crops, in the Pampas region, the application of fungicides to the soil is not usual. However the so-called "seed treatment" make contact with soil, and then, direct effects of fungicides on the external hyphae and / or spores impacting the functionality of the symbiosis are expected. Thiram is one of the classic fungicides used for seed treatments, with preventive and contact action, belonging to the dithiocarbamate group. Inhibitory effects on root colonization and spore production of dithiocarbamates applied as soil or seed treatments have been widely reported in the literature [135, 136, 137, 138]. Among the triazole compounds, triadimenol is widely used for seed treatments in wheat. Triazoles act as inhibitors on the biosynthesis of ergosterol, a major component of fungal membranes. Since the relative amount of ergosterol in the Glomeromycota is low compared to other groups of fungi, the negative effect of triazole application on arbuscular mycorrhizal fungi is generally low [139, 140]. The active ingredient metalaxyl is a widely used systemic seed treatment used for different crops. It has been found that metalaxyl applications increased mycorrhizal colonization and plant growth [141, 142]. This fungicide is specific controlling plant pathogenic oomycetes, and has no effects on other groups of fungi. Therefore, it has been suggested that its favorable effect on mycorrhizal colonization is primarily indirect, through reductions in populations of antagonistic organisms to arbuscular mycorrhizal fungi [143]. However, Giovannetti *et al*. [137] documented direct effects of this fungicide, since the application of metalaxyl stimulated spore germination and hyphal growth in the pre-symbiotic phase of Glomeromycota *in vitro*. Although these studies show interesting trends, conditions of sterile culture media are markedly different to those occurring in field soil, because of a large number of factors, including fungicide absorption by the soil. Within the classical fungicides for seed treatment, which are being gradually replaced by modern ones, there are those belonging to the group of benzimidazoles such as benomyl and carbendazim. Benomyl and other benzimidazoles decompose to methyl benzimidazole carbamate (carbendazim), and the latter compound interferes with the division of the nuclei of sensitive fungi. The deleterious effect of benomyl or carbendazim (the latter still used in seed treatment) on the arbuscular mycorrhizal fungi is widely known. Benzimidazoles specifically bind to beta-tubulin, thereby inhibiting the tubulin function, which is crucial for fungal growth [144, 145, 146, 147, 148]. Venedikian *et al*. [149] found that mycorrhizal colonization may be less inhibited by carbendazim applications than spore germination and hyphal growth in agar medium. This suggests that different growth phases of these fungi can tolerate different fungicide concentrations [150, 151, 152].

Regarding fungicide foliar applications, negative effects of triazole at high doses or repeated applications on mycorrhizal colonization have been reported [153, 154]. However, in a wheat crop in Argentina, Schalamuk *et al*., 2011 (unpublished) found that triazole applications did not reduce mycorrhizal colonization. When considering the evaluation of the effects of foliar fungicides on arbuscular mycorrhizal fungi it should be taken into

account not only the effect of the compound *per se*, but also the reduction in disease generated by increasing green leaf area and photosynthate supply to the roots . On the other hand, the strobilurins group, with mesostemic and trans-laminar action, is rapidly spreading in the Argentinean agricultural region. Fungicides of this group possess a broad-spectrum action, inhibiting mitochondrial respiration. Diedhiou *et al*. [154] found that strobilurins, despite its broad spectrum, did not negatively affect mycorrhizal colonization of crops when applied to control foliar pathogens at recommended doses. Schalamuk *et al*. [155] found similar results in wheat. Since the mode of action of this group of foliar fungicides is not fully systemic, it is questionable if strobilurin applications would present a detrimental effect on arbuscular mycorrhizal fungi.

Concerning the effect of fungicide application on the diversity of Glomeromycota, the information on this topic is low, although it is recognized that there are differences in sensitivity to fungicides among different groups or isolates among Glomeromycota taxa [150].

6. Conclusions

The grain production region has experimented severe tillage changes in the past twenty years in Argentina, mostly due to the increased interest in maintaining soils covered with plant residues and the increase used of N fertilization necessary to achieve high yield and grain quality.

In the wheat/soybean system under no tillage, as in wheat following wheat, the inoculum of necrotrophic fungi usually survives until the next wheat season. Therefore, the use of fungicides is essential to decrease the severity of necrotrophic diseases.

The results of experiments carried out in Argentina indicates that sowing wheat following wheat in no tillage is possible without significant yield losses if effective disease management practices including moderately resistant cultivars, N fertilization and fungicides are applied.

N fertilization increases the severity caused by leaf rust whereas decreases the severity caused by tan spot

Increased yields by disease management are associated mainly with an increase in thousand grain weight while other yield components such as number of spikes.m^{-2} or grains.spike^{-1} are usually not affected by disease severity. However, preventing early wheat infection by *Septoria tritici* could result in an increase of spikes.m^{-2} and grains.spike^{-1}.

Some studies determined that grain number was not affected by foliar diseases when they appeared after anthesis. Grain weight was strongly, poorly or not affected by foliar diseases and was not associated individually with both, the sink size and the source size. However, when the grain weight response due to fungicide application was plotted against the healthy area absorption per grain, a significant negative association was found for the Argentine experiments.

Further experiments carried out in Argentina with wheat cultivars inoculated with the causal agent of tan spot or Septoria leaf blotch or leaf rust determined that there was an interaction pathogen × N fertilization × fungicide. This interaction was caused by the fact that tan spot severity decreases with N fertilization, but increases for Septoria leaf blotch and leaf rust. Fungicides (combination of triazoles and strobilurins) were effective in controlling the three foliar diseases, but mainly leaf rust. In addition the control produced by the fungicide was higher when the severity increases.

It is difficult to generalize about the effects of fungicides on arbuscular mycorrhizal fungi, because they may have positive, negative or neutral effects. In a wheat crop in Argentina it was found that neither triazole nor strobilurins applications reduce mycorrhizal colonization.

Further studies should be done with different cultivars to determine the effect of tolerance and its control mechanisms, in addition to N fertilization and fungicide applications on yield and quality when wheat is affected by necrotrophic or biotrophic pathogens. Furthermore, field experiments on the effect of fungicides on mycorrhizal fungi in wheat in Argentina are recent and should be intensified.

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Chapter 2

Integration of Fungicide Application and Cultivar Resistance to Manage Fusarium Head Blight in Wheat

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Additional information is available at the end of the chapter

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1. Introduction

Fusarium head blight (FHB), also known as scab, is a destructive disease of wheat and other small grain cereals. Losses are compounded by the associated mycotoxin deoxynivalenol (DON) which contaminates grain. This chapter provides a brief review of FHB of wheat in North America including occurrence, symptoms, life cycle, economic importance, and integrated management with an emphasis on use of fungicides and host resistance. The review is followed by a presentation of selected research results from experiments conducted by the authors to determine the effects of integrating fungicide application with cultivar resistance on FHB. These results indicate that combining cultivar resistance with fungicide application is a more effective strategy for management of FHB and DON than using a single approach. In North America, a slow but steady progress has been made during the last decade in the development of wheat cultivars with improved resistance to FHB and DON. These cultivars are replacing or complementing older, FHB-susceptible cultivars. Availability of moderately resistant cultivars and new fungicide chemistries coupled with improved fungicide application technology has led to greater farmer adoption of an integrated strategy in the management of FHB and DON.

In North America, FHB occurs mainly in the eastern half of the United States (McMullen et al., 1997) and in eastern Canada (Gilbert & Tekauz, 2000), although surveys by the Canadian Grain Commission have increasingly found it in western Canada (Clear & Patrick, 2010). Several species of *Fusarium* and its allies are among the causal agents of FHB. They include *F. culmorum, F. avenaceum, F. graminearum, F. poae*, and *Microdochium nivale* (Liddell, 2003). Worldwide, *F. graminearum* is the major cause of FHB (Liddell, 2003; Parry, 1995) and predominates in North America (Parry, 1995; Sutton, 1982). Stack (2003) reviewed the

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history of FHB with emphasis on North America. The disease has occurred sporadically since the 1880s, with several major epidemics documented worldwide to date. In the U.S., major epidemics occurred in 1917, 1919, 1928, 1932, and 1935 (Stack 2003). More recently, the disease re-emerged in the early 1990s and since then outbreaks of varying intensity have been common and widespread in the U.S., particularly in areas with high moisture and abundant maize culture (McMullen et al., 1997).

In wheat, FHB symptoms are recognized by the premature bleaching of one or more spikelets on a head (spike). This bleaching can continue until the entire head is whitened. Bleached heads appear suddenly and are readily visible in a wheat field. *F. graminearum* sporulates on infected spikelets and glumes during prolonged wet weather, resulting in pink to salmon-orange spore masses which are a diagnostic feature of FHB. Infection of the stem (peduncle) immediately below the head may also occur, causing a brown or purple discoloration. If the peduncle is infected early, the entire head becomes sterile. Bleached spikelets are sterile or contain shriveled and/or chalky white or pink kernels commonly referred to as *Fusarium*-damaged kernels (FDK), scabby kernels, or "tombstones." Apparently healthy kernels also may be infected, especially if infection occurred late in kernel development.

F. graminearum overwinters as chlamydospores or mycelia in the soil or in host crop residues which serve as a source of primary inoculum in the spring (Dill-Macky, 2010). FHB primary inoculum consists mainly of ascospores produced in perithecia, which form on crop residues in the spring as temperatures warm up. Maize and wheat residues are particularly suitable for survival of the fungus. Khonga and Sutton (1988) observed perithecia formation on *F. graminearum*-inoculated maize and wheat residues placed on or above the soil surface for up to two years. Dill-Macky & Jones (2000) reported up to 45 and 94% recovery of *F. graminearum* from maize and wheat residue, respectively, in a single cropping cycle. In the spring, ascospores and/or conidia are released from crop residues and are spread by wind or splashing water. They land on wheat heads and during wet, warm weather they germinate and infect glumes, flower parts, or other parts of the head.

Infections occur mostly during anthesis. Wheat heads are susceptible from head emergence until harvest (Dill-Macky, 2010). Infections that occur during anthesis are the most damaging. During warm temperatures (25°C to 30°C) and wet conditions, blight symptoms develop within 2 to 4 days after infection. Therefore, an apparently healthy crop can show symptoms suddenly. Later in the growing season or after harvest, perithecia may form on wheat heads. FHB is considered a monocyclic or one cycle disease, that is, after the initial or primary infection, little or no secondary infection occurs by conidia formed on infected heads. FHB is favored by prolonged wet, warm weather prior to and during anthesis. Excessive rainfall during the growing season and especially during a one to three week period prior to anthesis can lead to severe epidemics of FHB. The disease usually is more severe in fields with corn and/or wheat residue on the soil surface and in irrigated fields.

In addition to lowering grain yield and quality, *F. graminearum* produces mycotoxins, primarily the trichothecenes deoxynivalenol (DON), nivalenol (NIV) and T-2 toxin

(McCormick, 2003; Gale, 2003). The sterol zearalenone (ZEA) is also commonly encountered (Gale 2003). These mycotoxins are harmful to humans and livestock. In North America, DON, also known as vomitoxin, is the most common and economically important mycotoxin found in *Fusarium*-infected wheat. Its acetylated derivatives, 3-ADON and 15-ADON, are commonly detected in contaminated grain. Grain with high concentrations of DON often is discounted or rejected at the elevator, which exacerbates the losses incurred by the farmer.

DON has been shown to be positively correlated with both FHB intensity (incidence, severity, or index) and FDK (Paul et al., 2005; Wegulo et al., 2011). In replicated field studies conducted over two years (2008 and 2009) in Manhattan, KS, USA (Wegulo et al., 2011), the authors of the current chapter generated different levels of FHB intensity by applying or not applying the fungicide Prosaro (prothioconazole + tebuconazole) to six cultivars differing in susceptibility to FHB. Grain samples from treated and check plots were ground to flour and submitted to the North Dakota Veterinary Diagnostic Laboratory at North Dakota State University for DON content determination using gas chromatography with electron capture detection (GC/ECD) (Tacke and Casper, 1996). Regression of DON concentration on FHB index revealed a strong positive linear relationship between the two variables. For every percent increase in FHB index, DON concentration increased by 0.31 ppm (Fig. 1).

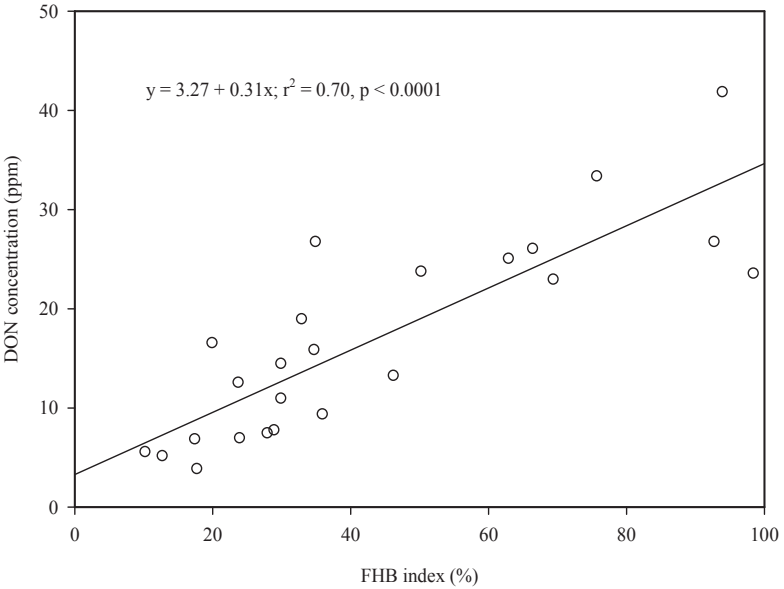

Figure 1. Relationship between Fusarium head blight (FHB) index and DON concentration determined from field experiments in which the fungicide Prosasro (prothioconazole + tebuconazole) was applied or not applied to six winter wheat cultivars differing in susceptibility to FHB. The experiments were conducted in Manhattan, Kansas, USA in 2008 and 2009.

In a laboratory study to demonstrate the relationship between FDK and DON, the authors of the current chapter mixed FDK collected from winter wheat fields and grain elevators in 2007 and 2008 (when there were severe epidemics of FHB in Nebraska, USA) with healthy grain in 5% (by weight) increments from 0% FDK, 100% healthy grain to 100% FDK, 0% healthy grain. Samples were ground to flour and submitted for DON content determination as described above. Regression of DON on FDK revealed a strong linear relationship between FDK and DON in both years (Fig. 2). For every percent increase in FDK, DON concentration increased by 0.33 and 0.53 ppm in 2007 and 2008, respectively.

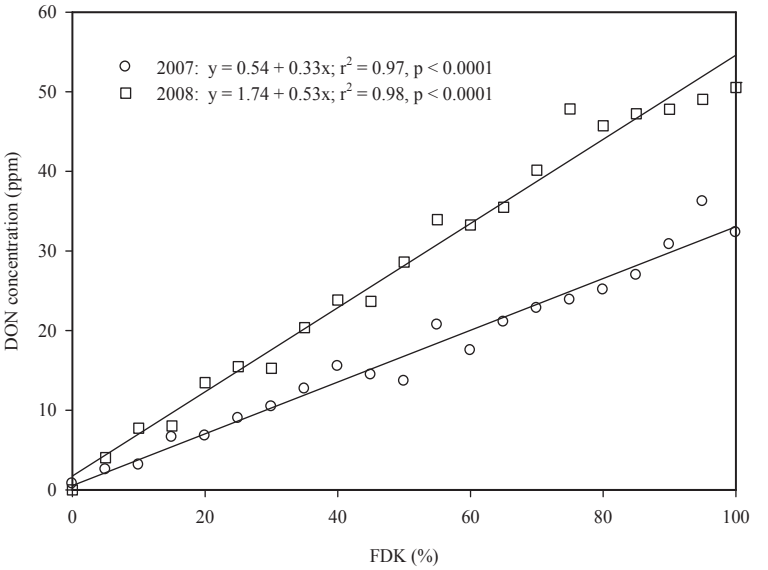

Figure 2. Relationship between *Fusarium*-damaged kernels (FDK) and deoxynivalenol (DON) concentration in grain samples with increasing proportions (5% increments) by weight of FDK from 0% FDK, 100% healthy grain to 100% FDK, 0% healthy grain. FDK were collected from fields and grain elevators in 2007 and 2008 when severe epidemics of Fusarium head blight occurred in winter wheat fields in Nebraska, USA.

2. Economic importance of FHB

FHB can cause substantial economic losses. In 1917, FHB caused losses estimated at 288,000 metric tons (10.6 million bushels) in 31 out of 40 states surveyed (Atanasof, 1920). Dickinson and Mains (1929) reported that the 1919 epidemic in the U.S. resulted in a loss of 2.18 million metric tons (80 million bushels) of wheat. Mains et al. (1929) estimated that the 1928 epidemic caused a 15% yield loss in wheat in Indiana. Significant additional losses occurred in the 1930s (Dickinson & Mains, 1942). Major economic losses occurred again in the 1980s (McMullen et al., 1997), with total losses in U.S. wheat production totaling 2.72 million

metric tons (100 million bushels) in 1982 alone (Boosalis et al., 1983). Johnson et al. (2003) estimated that FHB caused direct losses in wheat and barley totaling more than $1.3 billion in the U.S. during the period from 1991 to 1997. They estimated the total economic impact in rural communities and businesses related to grain production and marketing to be three to four times this amount. To date, FHB continues to cause significant economic losses in the U.S. and other parts of the world.

3. The use of fungicides to control FHB in the United States

In the U.S., a less than desirable number of current commercial wheat cultivars have moderate resistance to FHB and this resistance can be overwhelmed in years with high disease intensity. Fungicides are often applied to control FHB when favorable conditions for disease development are forecast. In North America, the most commonly used fungicides are in the triazole class. They include metconazole, prothioconazole, tebuconazole, prothioconazole + tebuconazole, and propiconazole. Strobilurin fungicides are generally not recommended for control of FHB because some studies have shown them to be associated with elevated levels of DON in grain (Blandino et al., 2006; Mesterházy et al., 2003c; Zhang et al., 2009).

Although the triazole fungicides have been tested extensively in university trials, not all farmers apply them specifically for FHB control, in part due to the sporadic nature of the disease. There are several reasons why some farmers do not apply fungicides specifically for FHB control. Firstly, to be effective, fungicide application usually is timed to coincide with anthesis. By this time those farmers who apply fungicides will have treated their crop at least once to control foliar fungal diseases, making it economically unfeasible to apply a second spray. Secondly, the application window for effective control of FHB is only a few days during anthesis. Unfavorable weather conditions during this time will prevent timely fungicide application. Thirdly, most farmers who apply fungicides do so by contract with commercial applicators. Often these contracts are made long before it is known whether FHB will occur at epidemic proportions. Because of this uncertainty, risk-averse farmers do not sign the contracts. Fourthly, some farmers are discouraged by less than adequate control of FHB by fungicides.

3.1. Variability in fungicide efficacy

Studies have shown that results from fungicide application to suppress FHB are highly variable. This variability has been attributed to various factors including improper timing of application, inadequate coverage of wheat heads due to inefficient application technology, and poor fungicide efficacy (McMullen, 1997; Mesterházy, 2003b). In North Dakota, USA, Ransom and McMullen (2008) found that in a year with high FHB intensity, tebuconazole did not reduce FHB to acceptable levels in most of the winter wheat cultivars in the field trial. However, in a year with low disease intensity, tebuconazole + prothioconazole achieved almost 100% FHB control in all but the most susceptible cultivars. Paul et al. (2008)

analyzed over 100 FHB uniform fungicide studies across 11 years and 14 U.S. states. In these studies, metconazole, propiconazole, prothioconazole, tebuconazole, and prothioconazole + tebuconazole were applied at anthesis to suppress FHB. The analysis showed that although all fungicides significantly reduced FHB index and DON, there was substantial between-study variability. A given fungicide can vary in its efficacy in controlling FHB versus DON. Paul et al. (2007) showed that tebuconazole was more effective in controlling FHB than DON. The same study (Paul et al., 2007) showed that tebuconazole controlled FHB and DON more effectively in spring wheat compared to winter wheat.

3.2. Prospects for control of FHB with fungicides

Over the last two decades, there has been considerable improvement in the effectiveness of fungicides in controlling FHB and DON. This improvement is attributable in part to improved fungicide chemistries and greater knowledge gained through research on fungicide application rates, timing, and technology. A review of fungicide trials conducted over the last two decades clearly demonstrates this improvement. In trials conducted in 1992 and 1993 in Arkansas, USA, Milus and Parsons (1994) found that the fungicides benomyl, chlorothalonil, fenbuconazole, flusilazole, myclobutanil, potassium bicarbonate, propiconazole, tebuconazole, thiabendazole, and triadimefon + mancozeb) applied to the soft red winter wheat cultivar Florida 302 at the heading stage had no effect on FHB incidence, DON, yield, or test weight. The investigators concluded that prospects for chemical control of FHB were poor. Similar trials conducted between 1994 and 1997 by Jones (2000) in Minnesota led to the conclusion that although benomyl and tebuconazole significantly reduced FHB, FDK, and DON in the hard red spring wheat cultivars Norm and 2375, prospects for chemical control of FHB remained limited.

With the realization that the triazole fungicides are more effective than other fungicide classes in controlling FHB, and with newer chemistries and refinements in application timing, rates, and technology, the majority of fungicide trials conducted over the last decade have demonstrated improved effectiveness of triazole fungicides in controlling FHB and DON. In Minnesota, USA, Hollingsworth et al. (2006) showed that the then experimental products (now registered) metconazole and tebuconazole + prothioconazole significantly reduced FHB severity and FDK compared to tebuconazole. These results indicate that the prospects for chemical control of FHB and DON in wheat have improved over the last decade and continue to improve with the development of new fungicide chemistries and improvements in application timing, rates, and technology.

4. Management of FHB with host resistance

Genetic resistance is the most cost-effective management strategy for FHB (Ruckenbauer et al., 2001). Five categories of resistance to FHB have been described (Shroeder & Christensen, 1963; Wang & Miller, 1988; Mesterházy, 1995; Mesterházy, 2003a). They are resistance to initial infection (Type I), resistance to pathogen spread in infected tissue (Type II), resistance to kernel infection (Type III), tolerance (Type IV), and resistance to toxins (Type V).

Challenges to breeding for resistance to FHB include the quantitative nature of resistance to the disease (Ruckenbauer et al., 2001), the fact that there are up to five categories of resistance, the lack of well adapted and complete resistance sources, and confounding environmental effects (Anderson et al., 2001). In addition, because only a few sources of resistance (mainly Sumai 3 and its relatives) are widely used, the potential exists for *F. graminearum* and other FHB-causing pathogens to overcome this resistance that relies on a narrow genetic basis (Ruckenbauer et al., 2001). Recent progress in breeding for resistance to FHB is attributable to a combination of traditional breeding methods and molecular breeding techniques such as marker-assisted selection (Anderson, 2007). In the U.S., there are now several cultivars in most wheat classes with moderate resistance to FHB (Scab Smart, http://www.scabsmart.org/). These cultivars have been released as a result of concerted efforts in greenhouse and field screening of germplasm. In addition, many research programs at universities and private companies are devoted to screening commercially released cultivars whose reaction to FHB and DON was previously unknown.

5. Forecasting FHB

To facilitate the judicial and economical use of fungicide applications to control FHB and DON, several forecasting systems have been developed. In the U.S., the Fusarium Head Blight Risk Assessment Tool (http://www.wheatscab.psu.edu/riskTool_2011.html) is an Internet-based forecasting system deployed in at least 23 states. It was developed based on logistic regression models for FHB using information from 50 location-years in four states and three different wheat production regions (De Wolf et al., 2003). The system uses combinations of temperature, relative humidity, and rainfall during seven days before anthesis to calculate the risk of occurrence of FHB. Specifically, the predictor variables used are duration (hours) of precipitation 7 days before anthesis, duration (hours) when temperature is between 15 and 30°C 7 days before anthesis, and relative humidity greater than or equal to 90%. Based on these variables for a particular location, the system outputs a risk category of low, moderate, or high. Farmers can then decide whether to apply a fungicide at early anthesis based on the risk predicted for their respective locations. In Canada, the DONcast® model was developed for use by wheat farmers to predict DON accumulation. Hence, farmers can make fungicide spray decisions more efficiently. The model uses weather forecast data supplemented by actual data from additional weather stations to make site-specific DON predictions based on wheat cultivar, crop rotation, tillage, heading date, and local weather conditions. The DONcast® prediction tool is Internet-based and is available on the weathercentral.ca website. In Switzerland, FusaProg is an Internet-based decision support system which provides information about local and regional risks of FHB outbreaks (Musa et al., 2007). In addition, it forecasts field-specific DON contamination of winter wheat. FusaProg uses a model that takes into account the effects of cropping factors, previous crops, soil and straw management, and cultivar susceptibility as driving variables which are combined with growth stage (anthesis) and prevailing weather conditions to predict DON in specific wheat fields. Hence, farmers can

optimize the timing of fungicide applications to control FHB and DON. In Argentina, one of the forecasting systems for FHB index was recently modified into a new forecasting system that has potential to forecast annual DON content in mature wheat grain using primary meteorological daily data from surface stations (Martinez et al., 2012).

6. Integrated management of FHB

The best approach to managing FHB is to integrate multiple strategies (McMullen et al., 2008) including host resistance, fungicide application, crop rotation, residue management, and forecasting. A combination of two or more of these strategies can significantly reduce losses caused by FHB. Few studies have been done to determine the effect of multiple management strategies on FHB and DON. In Germany, Koch et al. (2006) found that tillage type, cultivar, and application of the fungicide tebuconazole had a significant effect on DON accumulation. Reduced tillage resulted in higher DON content in both a moderately resistant and a highly susceptible cultivar compared to clean tillage, with the highly susceptible cultivar accumulating more DON than the moderately resistant cultivar. Fungicide application reduced DON concentration only slightly in the moderately resistant cultivar, but significantly in the highly susceptible cultivar. In Hungary, Mesterházy et al. (2003c) found that fungicide efficacy in controlling FHB and DON accumulation was higher in the more resistant than in the more susceptible winter wheat cultivars. In Minnesota, USA, Hollingsworth et al. (2008) reported that in spring wheat, fungicide application resulted in higher economic returns in moderately susceptible cultivars than in moderately resistant cultivars when disease intensity was low. However, when disease intensity was moderate, economic returns did not differ between moderately susceptible and moderately resistant cultivars. In the same study (Hollingsworth et al., 2008), fungicide application reduced FHB intensity in moderately resistant cultivars, but had no effect on DON accumulation in both moderately resistant and moderately susceptible cultivars. McMullen et al. (2008) reported that in North Dakota, USA, FHB severity was reduced by 50, 80, and 92% with rotation, rotation + a tolerant cultivar, and rotation + a tolerant cultivar + fungicide application, respectively. Recently, Willyerd et al. (2012) used multivariate analysis to evaluate the integration of host resistance and application of the fungicide prothioconazole + tebuconazole in wheat using data from over 40 trials in 12 U.S. states. They found that the best control of FHB was provided by a combination of fungicide application and moderately resistant cultivars.

7. Experiments conducted to determine the effects of integrating cultivar resistance and fungicide application on FHB

From 2007 to 2009, the authors of the current chapter evaluated the effects of integrating cultivar resistance and fungicide application in hard winter wheat in two sets of experiments (Wegulo et al., 2011). In the first set (experiments 1-3) the fungicide Prosaro (prothioconazole + tebuconazole) was applied or not applied to three cultivars (Harry, 2137,

and Jagalene) at full heading to early anthesis in 2007 to 2009. In the second set (experiments 4 and 5), the same two fungicide treatments were applied to six cultivars (Truman, Heyne, Roane, Karl 92, Overley, and Tomahawk) at full heading in 2008 and 2009. From these experiments we demonstrate the effects of combining cultivar resistance and fungicide application on FHB index, DON, FDK, and yield using four moderately resistant (based on FHB phenotype only) and four susceptible cultivars selected from the two sets of experiments in location-years that had high FHB intensity (Manhattan 2007, Mead 2008, Manhattan 2008, and Manhattan 2009).

7.1. Methods

The methods used in the experiments have been published previously (Wegulo et al., 2011). Briefly, the experiments were conducted near Mead, Nebraska and in Manhattan, Kansas, USA from 2007 to 2009. The experimental design was a split plot in randomized complete blocks with four to six replications with cultivars as the main plots and fungicide treatments as the subplots (Table 1). The fungicide treatments consisted of Prosaro (prothioconazole + tebuconazole) not applied (check treatment) or applied at a rate of 0.475 liters/ha at Zadoks growth stage 59 (GS 59) or 2 days before GS 65 (mid anthesis). Fungicide was applied at a rate of 187 liters/ha of spray volume and a pressure of 207 kPa using a back-pack sprayer equipped with flat-fan nozzles angled forward about 30°. Plots were inoculated with corn kernels colonized by *F. graminearum* two to four weeks before anthesis. At Mead, plots were additionally spray-inoculated with spores of *F. graminearum* (1×10^5 spores ml^{-1}) at GS 65. Plots were over-head irrigated at Manhattan but not at Mead.

FHB index was assessed as the percentage of spikelets blighted in a plot or as (incidence (%) x severity (%))/100 three to four weeks after fungicide application. Plots were harvested with a small-plot combine and subsamples from the harvested grain were used to determine the percentage of FDK visually or with an automated single kernel near-infrared (SKNIR) system (Perten Instruments, Stockholm, Sweden) (Dowell et al., 2006). Ten-gram subsamples were ground to flour and sent to the North Dakota Veterinary Diagnostic Laboratory at North Dakota State University for DON content determination using gas chromatography with electron capture detection (GC/ECD) (Tacke and Casper, 1996).

The GLM and GLIMMIX procedures of SAS (SAS Institute, Cary, NC, USA) were used to analyze data. Treatments were considered significantly different at $p \leq 0.05$. Fungicide efficacy for index, DON, and FDK was calculated as

$$[(C - F)/C]*100$$

where C is the check treatment value and F is the fungicide treatment value. Fungicide efficacy for yield was calculated as

$$[(F - C)/F]*100$$

where C and F are as previously defined.

7.2. Results and discussion

7.2.1. Main effects and their interactions

In experiments 1-3, the effects of location-year, cultivar, and fungicide were significant for index, DON, FDK, and yield (Table 1). The effect of location-year by cultivar interaction was also significant for all four variables. However, the effect of location-year by fungicide interaction was significant only for index and the effect of cultivar by fungicide interaction was not significant for any of the variables. The effect of the three-way interaction was significant for index, DON, and FDK, but not yield.

In experiments 4 and 5, the effects of location-year, cultivar, and fungicide were significant for index, DON, FDK, and yield (Table 1). The effect of location-year by cultivar interaction was significant for all four variables whereas the effect of location-year by fungicide interaction was significant only for yield and the effect of cultivar by fungicide interaction was significant for index and DON. The effect of the three-way interaction was significant for index and yield.

These results indicate that location-year, cultivar, and fungicide significantly affected FHB index, DON, FDK, and yield. The only interaction effect that significantly affected all four variables in both sets of experiments was that between location-year and cultivar, implying that the resistance or susceptibility of a given cultivar to FHB, DON accumulation, and *Fusarium* damage can be influenced by environmental conditions during the growing season. The location-year by fungicide and cultivar by fungicide interaction effects were inconsistent between the two sets of experiments and among the measured variables, suggesting that environment and cultivar did not always affect fungicide performance.

7.2.2. FHB index, DON, FDK, yield, and fungicide efficacy in moderately resistant and susceptible cultivars

We selected from experiments conducted under high disease intensity four moderately resistant (based on FHB phenotype) (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tamahawk, 2137, and Jagalene) for analysis and presentation in this chapter. Overall, FHB index was lower in the moderately resistant than in the susceptible cultivars in both the Prosaro and check treatments and Prosaro reduced index in both moderately resistant and susceptible cultivars (Fig. 3). For three of the moderately resistant cultivars (Truman, Heyne, and Roane), index in the check treatment was lower than index in the Prosaro treatment in all the susceptible cultivars whereas in Harry, index in the check treatment was similar to index in the Prosaro treatment in the susceptible cultivar 2137. These results indicate that the resistance in the moderately resistant cultivars was effective under high FHB intensity even in the absence of fungicide application. This implies that under low FHB intensity, resistance alone may be sufficient and no fungicide application may be needed in the moderately resistant cultivars.

Cultivar response to DON was less clear-cut compared to the response to FHB index. In the Prosaro treatment, cultivars Harry and Heyne (moderately resistant) had similar levels of

DON as cultivars 2137 and Jagalene (susceptible), indicating that some cultivars with a moderately resistant FHB phenotype may be susceptible to DON accumulation (Fig. 4). Within the check treatment, the four moderately resistant cultivars accumulated DON amounts similar to those in the susceptible cultivars 2137 and Jagalene. Overley and Tomahawk accumulated more DON than all other cultivars regardless of fungicide treatment. Overall, DON reduction due to fungicide application was greater in the moderately resistant than in the susceptible cultivars.

The response of both susceptible and moderately resistant cultivars to DON accumulation within the two fungicide treatments highlights the complexity of managing FHB and DON by integrating fungicide application and cultivar resistance. Fungicide efficacy for FHB intensity does not necessarily mirror efficacy for DON in a given cultivar. The authors of the current chapter have consistently observed the cultivar Harry to have a moderately resistant FHB phenotype. However, this cultivar appears to be susceptible to DON accumulation (Figs. 3 and 4; Wegulo et al., 2011; Hernandez Nopsa et al., 2012). These observations call for a consensus among FHB scientists to standardize the criteria by which to classify cultivars as resistant or susceptible to FHB, as well as the criteria for classifying fungicide efficacy. Should resistance to FHB refer to resistance to FHB intensity, DON, and FDK combined, or should resistance refer to each variable separately?

Among the moderately resistant cultivars, Truman, Heyne, and Roane had fewer FDK than the susceptible cultivars regardless of fungicide treatment (Fig. 5). Harry, on the other hand, had FDK levels similar to those in the susceptible cultivars. This result indicates that Harry, despite having a moderately resistant FHB phenotype, is susceptible when evaluated based on FDK. Because FDK and DON are positively related (Fig. 2), this result also suggests that the higher DON in Harry may be due to the cultivar's susceptibility to *Fusarium* damage.

Prosaro generally increased yield in both moderately resistant and susceptible cultivars (Fig. 6). There was no clear distinction in yield between the moderately resistant and susceptible cultivars, with Heyne (moderately resistant) having low yield and 2137 (susceptible) having high yield. The insignificant yield response to fungicide application and the inconsistency in yield response between moderately resistant and susceptible cultivars may be due to differences in genetics and the fact that yield, unlike the other three measured variables (index, DON, and FDK), is influenced by other factors in addition to FHB. These factors include other diseases (including foliar and root and crown diseases), nutrients, and weeds. This result indicates that among the four measured variables, yield may be the least accurate to use in assessing fungicide efficacy in controlling FHB.

Fungicide efficacy for index was generally higher in moderately resistant than in susceptible cultivars (Fig. 7). It was highest in Truman and lowest in Jagalene. Similarly, fungicide efficacy for DON was generally higher in moderately resistant than in susceptible cultivars except for Harry (moderately resistant) in which the efficacy was as low as in the susceptible Tomahawk and Jagalene (Fig. 7). The finding in this study that overall fungicide efficacy for index and DON was higher in moderately resistant than in susceptible cultivars is in agreement with the results of Mesterházy et al. (2003c). The finding suggests that integrating

cultivar resistance with fungicide application can be an effective management strategy for FHB and DON.

Source of variation	d.f.[a]	Index[b] (%)		DON (ppm)		FDK (%)		Yield (kg ha^{-1})	
		MS[c]	*P > F*	MS	*P > F*	MS	*P > F*	MS	*P > F*
Experiments 1-3[d], 2007-2009									
Location-year (Y)	2	27,296	<0.0001	2,319	<0.0001	2,512	<0.0001	14,988,93	<0.0001
Rep (Y)	14	199	<0.0001	13	0.0353	103	0.5199	629,384	0.0058
Cultivar (C)	2	4,559	<0.0001	80	<0.0001	833	0.0014	4,163,646	<0.0001
Y * C	4	2,794	<0.0001	26	0.0066	1,101	<0.0001	2,881,204	<0.0001
Error (a)	28	71		9		163		265,965	
Fungicide (F)	1	2,417	<0.0001	89	0.0005	3,108	<0.0001	5,697,574	<0.0001
Y*F	2	2,032	<0.0001	15	0.1060	44	0.6690	13,733	0.9419
C*F	2	68	0.2295	15	0.0974	127	0.3209	11,395	0.9515
Y*C*F	4	138	0.0249	21	0.0166	471	0.0050	232,551	0.4107
Error (b)	42	44		6		108		229,164	
Total	101								
Experiments 4 and 5, 2008-2009									
Location-year (Y)	1	11,484	<0.0001	1,900	<0.0001	7,368	<0.0001	12,747,54	<0.0001
Rep (Y)	6	76	0.0021	36	0.0973	48	0.7694	385,074	0.0061
Cultivar (C)	5	7,935	<0.0001	1,072	<0.0001	6,517	<0.0001	14,500,24	<0.0001
Y * C	5	1,336	<0.0001	181	<0.0001	732	<0.0001	3,420,870	<0.0001
Error (a)	30	72		28		132		135,553	
Fungicide (F)	1	6,970	<0.0001	678	<0.0001	823	0.0041	6,816,701	<0.0001
Y*F	1	24	0.2474	4	0.6467	27	0.5819	493,848	0.0367
C*F	5	69	0.0059	51	0.0309	63	0.6121	251,029	0.0569
Y*C*F	5	186	<0.0001	28	0.2064	92	0.4042	308,975	0.0252
Error (b)	36	17		18		88		104,686	
Total	95								

[a]Degrees of freedom.
[b]Index was assessed as the percentage of spikelets blighted in a plot or calculated as [FHB incidence (%) x FHB severity (%)]/100.
[c]Mean square.
[d]Experiments 1-5 were conducted at Manhattan, KS in 2007; Mead, NE in 2008; Mead, NE in 2009; Manhattan, KS in 2008; and Manhattan, KS in 2009, respectively. There were 6, 6, 5, 4, and 4 replications in experiments 1-5, respectively.

Table 1. Analysis of variance from experiments conducted to determine the effect of combining cultivar resistance and fungicide application on *Fusarium* head blight (FHB) index, deoxynivalenol (DON) concentration, *Fusarium* -damaged kernels (FDK), and yield in winter wheat, 2007-2009

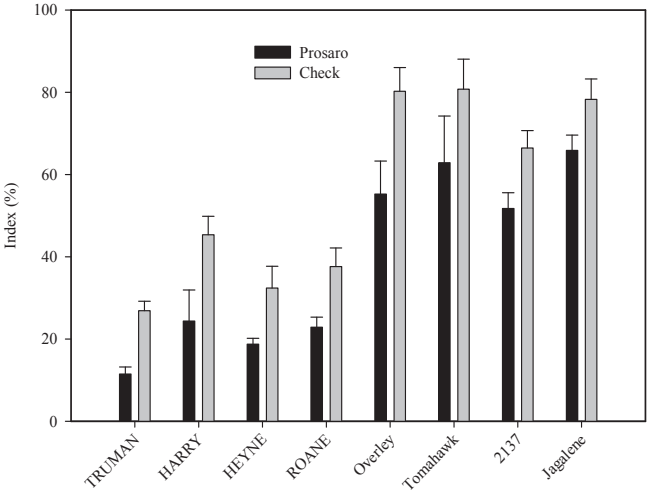

Figure 3. Fusarium head blight (FHB) index (percentage of blighted spikelets) in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading or not treated (check). Each mean was calculated from eight replications (four replications from each of two years). Error bars represent the standard error of the mean.

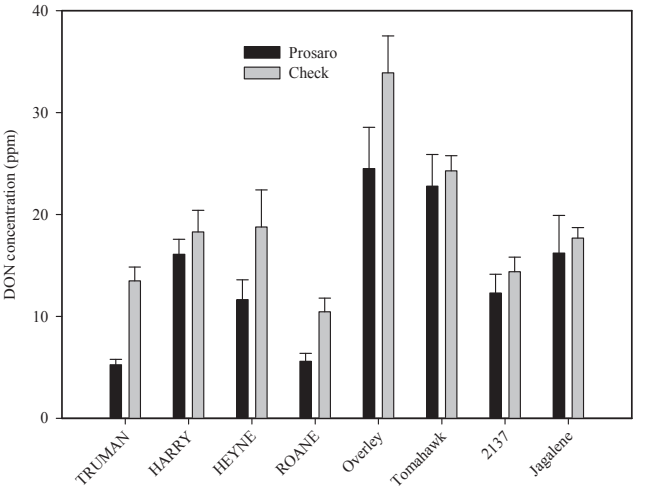

Figure 4. DON concentration in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading or not treated (check). Each mean was calculated from eight replications (four replications from each of two years). Error bars represent the standard error of the mean.

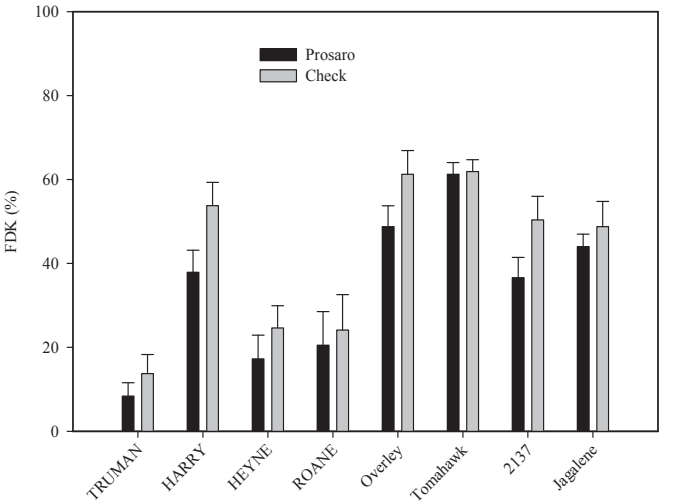

Figure 5. *Fusarium*-damaged kernels (FDK) in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading or not treated (check). Each mean was calculated from eight replications (four replications from each of two years). Error bars represent the standard error of the mean.

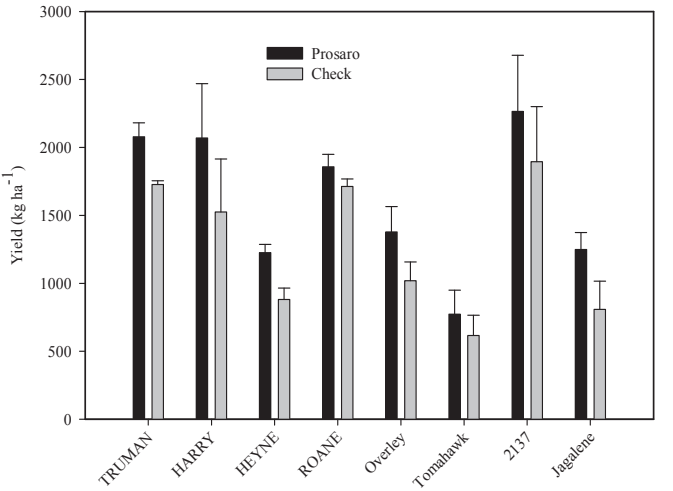

Figure 6. Yield in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading or not treated (check). Each mean was calculated from eight replications (four replications from each of two years). Error bars represent the standard error of the mean.

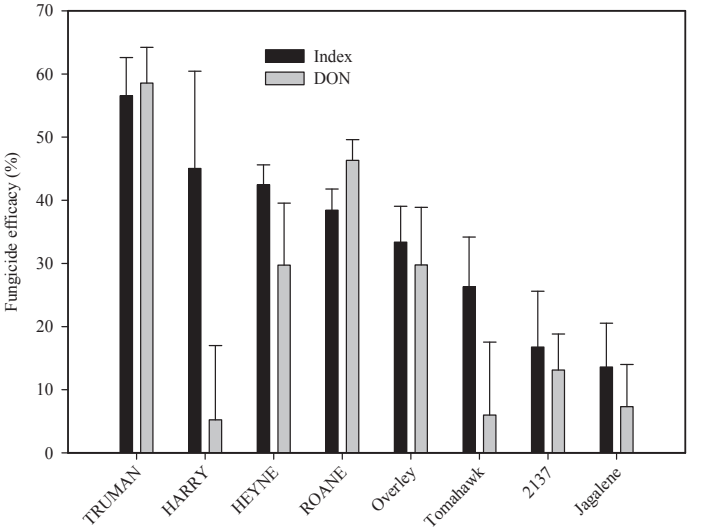

Figure 7. Fungicide efficacy for index and DON in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading. Error bars represent the standard error of the mean.

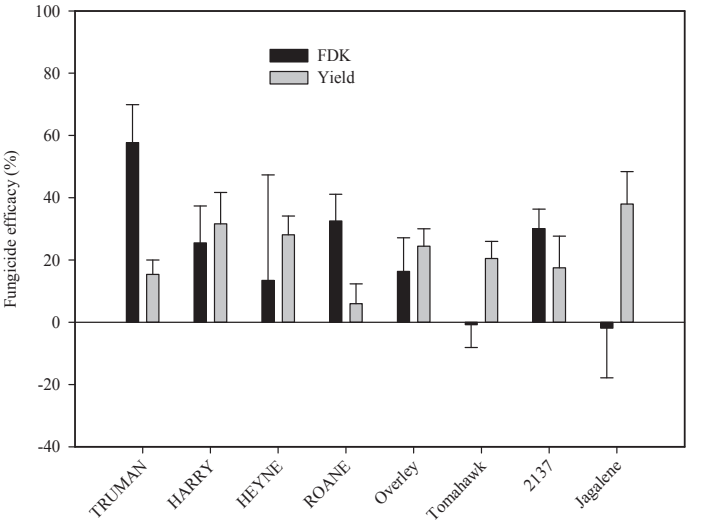

Figure 8. Fungicide efficacy for FDK and yield in four moderately resistant (based on FHB phenotype) winter wheat cultivars (Truman, Harry, Heyne, and Roane) and four susceptible cultivars (Overley, Tomahawk, 2137, and Jagalene) treated with the fungicide Prosaro at full heading. Error bars represent the standard error of the mean.

In contrast to index and DON, fungicide efficacy for FDK and yield was similar between moderately resistant and susceptible cultivars (Fig. 8) except for Truman in which fungicide efficacy for FDK was higher than that in the rest of the cultivars and Tomahawk and Jagalene in which fungicide efficacy for FDK was lower than that in all other cultivars. The similarity between moderately resistant and susceptible cultivars in fungicide efficacy for FDK and yield may be due in part to the fact that in contrast to index and DON which are more directly affected by fungicide treatment, FDK and yield are indirectly affected. In addition, the loss of significant quantities of FDK during machine harvesting can lead to inaccurate measurements of FDK and yield.

8. Conclusions

FHB continues to be an economically devastating disease of wheat in the U.S. and other parts of the world. Management strategies include the use of fungicide application timed at anthesis, planting resistant/tolerant cultivars, crop rotation, and residue management. Forecasting systems can facilitate the judicial and economical use of fungicides to control FHB and DON. Recent progress in the development of new chemistries of fungicides and improvements in fungicide application technology have improved the prospects for chemical control of FHB. Development of new cultivars with resistance using traditional and molecular breeding techniques has led to commercial availability of cultivars with moderate resistance and desirable agronomic characteristics. The best approach to managing FHB is to integrate available management strategies. Research has shown that integrating cultivar resistance with fungicide application can be an effective management strategy for FHB.

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Chapter 3

Chemical Control of Peanut Diseases: Targeting Leaves, Stems, Roots, and Pods with Foliar-Applied Fungicides

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Additional information is available at the end of the chapter

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1. Introduction

Peanut (*Arachis hypogaea* L.) is an important food crop with high levels of proteins, carbohydrates, vitamins and minerals contained within seeds (Moss and Rao, 1995). While the cultivation of peanut may occur over a wide range of climatic conditions, close attention should be paid to soil type, temperature range, and rainfall amount and distribution. Well-drained, sandy soils are best suited for peanut production (Beasley et al., 1997). Williams and Boote (1995), found the optimal temperature range for peanut production to be between 27 and 33°C. Furthermore, peanut plants require large amounts of rainfall, 50-75 cm, during production to optimize growth, yield and seed maturity (Beasley et al., 1997). If ample water and optimum temperatures are available after planting, peanut plants will emerge within 2 weeks of planting. These plants form self-pollinating flowers approximately 30-40 days after emergence and may continue to produce new flowers throughout the growing season until harvest. Fertilized flowers will form pointed needle-like carpophores (commonly referred to as "pegs"), that grow geotropically. The tissue at the tip of the peg becomes lignified, thus protecting the fertilized ovaries located behind the tip. Pegs grow into the soil to a depth of 2-7 cm (Porter, 1997). Peanut pod growth is initiated as the tip of the peg becomes horizontally oriented. The mature pods are oblong and may contain as many as five seeds. There are four market types of peanuts: runner, spanish, valencia and virginia. Runner and virginia types are most commonly grown throughout production regions in the United States; however, spanish and valencia market types are grown in the Southwest. The aforementioned market types differ in growth habit, days to maturity, yield potential, as well as susceptibility to diseases.

The worlds leading peanut producing countries include India, China and the United States. In 2011, approximately 444,500 hectares of peanuts were harvested in the United States

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(NASS, 2011); with largest production region being the southeastern states of Alabama, Florida, Georgia, and South Carolina. Production is concentrated in this region due to the semi-tropical temperate climate conditions. Unfortunately, these environmental conditions are conducive for many pests, including weeds, insects and diseases. Other production regions in the United States include the southwestern region (New Mexico, Oklahoma, and Texas), as well as the Virginia-Carolina region (North Carolina and Virginia), each of which has their own disease issues.

1.1. Peanut leaf spot

Several fungal diseases are known to affect peanut leaves. Most notibly are early and late leaf spot, caused by *Cercospora arachidicola* (Hori), (Teleomorph: *Mycosphaerella arachidis* Deighton), and *Cercosporidium personatum* (Berk. & Curt.) Deighton, (Teleomorph: *Mycosphaerella berkeleyi* Jenk.), respectively. Either disease may be present within a given area or year. While both pathogens are destructive on leaves, they are also capable of causing lesions on petioles, pegs, main stems and lateral branches (Shokes and Culbreath, 1997). Leaf spot symptoms are initially seen as small necrotic flecks that appear approximately 10 days after spore deposition. Over several weeks, the lesions will enlarge from 1-10 mm in diameter and sporulate. The physical appearance of the two diseases is similar (**Fig. 1**); however, early leaf spot can be distinguished from late leaf spot based on lesion characteristics; the most noteworthy is the color of the lesion on the adaxial surface. Light to dark brown lesions are characteristic of *C. arachidicola*; while *C. personatum* lesions have more of a black appearance (Smith and Littrell, 1980; Sholar et al., 1993; Shokes and Culbreath, 1997). The orientation of sporulation may also be used in distinguishing between the two diseases. *Cercospora arachidicola* sporulates on the adaxial leaf surface; whereas *C. personatum* sporulates on the abaxial surface of the leaf. Microscopic examination of conidia may be required to further differentiate the two pathogens. Conidiophores of *C. arachidicola* are dark at the base, unbranched, and septate; giving rise to curved, subhyaline, septate conidia (15-45 × 3-6 μm). Conidia (20-70 × 4-9 μm) of *C. personatum* are typically straight, rounded at the apex and not constricted, and are produced on smooth, brown conidiophores (Shokes and Culbreath, 1997).

Optimal environmental conditions for infection and reproduction for the two pathogens are quite similar; 16-24 °C and 20-26 °C for *C. arachidicola* and *C. personatum*, respectively, and both require long periods of relative humidity greater than 90% (Shokes and Culbreath, 1997). Primary inoculum for either pathogen originates from infected residue in the soil from previous peanut crops (Shokes and Culbreath, 1997). Both *C. arachidicola* and *C. personatum* overwinter as dormant stromata on infected residue until environmental conditions are conducive for sporulation and dispersal. Initial inoculum is responsible for the onset of leaf spot epidemics, and subsequent sporulation increases the disease. If left unmanaged, yield reductions as great as 70% may be incurred (Nutter and Shokes, 1995; Shokes and Culbreath, 1997).

Several other diseases including pepper spot, caused by *Leptosphaerulina crassiasca* (Sechet) C.R. Jackson & D.K. Bell, web blotch, caused by and peanut rust, caused by *Puccinia arachidis*

Speg., are also capable of infecting peanut foliage with the latter causing substantial losses throughout many production areas around the world. Peanut rust occurs sporadically in the southeastern United States and is generally considered a late season disease. Widespread use of chlorothalonil for managment of leaf spot is believed to have kept problems with rust to a minimum (Hagan, 1998).

Figure 1. Early and late leafspot of peanut.

1.2. Diseases of peanut stems, roots, pegs and pods

Numerous other fungal diseases are known to affect peanut stems, roots, pegs and pods. Diseases such as stem rot (Fig. 2), Rhizoctonia limb and pod rot (Fig. 3), Pythium pod rot (Fig. 4), Cylindrocladium black (Fig. 5) rot and Sclerotinia blight (Fig. 6) are among the most difficult to manage.

Stem rot, caused by the soilborne fungus *Sclerotium rolfsii* Sacc., is a very destrucive disease. *Sclerotium rolfsii* has a worldwide distribution and is capable of infecting a wide variety of row crops including crucifers, grasses and legumes (Aycock, 1966; Punja, 1985). Although the sexual stage of *S. rolfsii*, the basidiomycete *Athelia rolfsii* (Cruz) Tu & Kimbrough, has been identified, it is rarely seen under field conditions (Backman and Brenneman, 1997). *Sclerotium rolfsii* does not produce conidia and is classified as a Deuteromycete in the group

'Mycelia Sterilia' (Alexopolous et al., 1992). The fungus overwinters in the soil as hard, round, brown sclerotia (Backman and Brenneman, 1997). Mature sclerotia have a melanized outer layer, the rind, which allows the fungus to survive periods of adverse environmental conditions and remain viable for up to 3 years (Punja, 1985).

Upon germination of sclerotia, *S. rolfsii* may survive saprophytically as mycelium in organic matter in the soil or directly infect a susceptible host plant (Aycock, 1966). After an infection site is established, the fungus becomes necrotrophic, meaning an external energy source is needed to breach host defenses (Punja, 1985). Initial symptoms of infection include chlorosis and/or wilting of a lateral branch; however, if main stems become infected, the entire plant may appear wilted or chlorotic (Backman and Brenneman, 1997). Infected leaves typically have a water-soaked or necrotic appearance. Symptoms may appear rather quickly if temperatures are favorable. In very young pods the rot is clear and watery. As the pods mature, the damage on the pod is white or brownish in color and white mycelia is present. Sclerotia may be found in the vicinity of the plant stem or pods as well. The incubation period typically ranges from 2 to 4 days; however, wounding of plants may decrease the time required (Aycock, 1966).

Figure 2. Stem rot of peanut, caused by *Sclerotium rolfsii*.

Rhizoctonia solani (Kühn) anastomosis group 4 (AG-4) is capable of causing seed decay, pre- and post-emergence damping-off, as well as hypocotyl and root rot; however, it is most

devastating on mature plants causing a rot of pegs, pods, and stems. Although variable from year to year, Rhizoctonia limb rot is considered a major disease of peanut in the southeast (Brenneman, 1997; Thompson, 1982) and Texas (*personal observation)*. Substantial losses due to limb and pod rot can be experienced (Kemerait, 2003). Limb rot is more severe during cool wet periods and may be exacerbated by excessive nitrogen fertility (*Brenneman, unpublished*). Generally limb rot symptoms are first observed on lower branches that are in contact with the soil surface. Circular lesions, yellow to dark brown in color, occur at infection sites and have distinct target spot appearance. As lesion development progresses, infected limbs become girdled and die (Franke, 1999). The fungus may produce irregularly shaped sclerotia within host tissue as nutrient sources become depleted (Brenneman, 1997). Hypae of *R. solani* are typically white to brown in color, 4-15 μm thick, septate and branched at right angles (Taber and Pettit, 1970). During infection, hyphae quickly invade the epidermis and advance intracellularly (Christou, 1962). Studies conducted by Bateman (1970) suggest that *R. solani* produces various phytotoxins and degradative enzymes to kill host tissue, resulting in the release of nutrients that promote fungal growth. The disease is somewhat sporadic in nature (Thompson, 1982; Barnes et al., 1990), and cannot easily be assessed until after digging. *Rhizoctonia* spp. are comonly found associated with peanut pods that are left in the soil, and may also actively colonize and rot developing pods. Symptoms of Rizoctonia pod rot consist of a dry-rot, where the reticulations of the pods are exposed, having a skeletonized appearance. Cream to brown colored mycelia may be observed on diseased kerenls.

In addition, Pythium pod rot can also be responsible for considerable losses and frequently occurs throughout Texas, Oklahoma and North Carolina. A severe pod rot in Nicaragua was also recently determined to be caused by *Pythium myriotylum (*Augusto et al., 2010, II) Several *Pythium* spp., including *P. myriotylum*, *P. ultimum*, *P. irregulare*, *P. vexam*, and *P. dimorphum*, have been found to incite pod rot (Wheeler et al., 2005). Frank, 1972 reported that pod rot in Israel results from synergistic interactions between *P. myriotylum* and *Fusarium solani*. Pythium pod rot can be characterized by the appearance of wet, greasy pods which often exhibit a very unpleasant odor. White mycelial growth may be observed on decaying pods. Depending on the stage of infection and species involved, the examination of infected tissue may reveal the presence of spherical oospores. Due to the nature of peanut pod rot and similarity of symptoms laboratory diagnosis is often required to differentiate the causal agents

Cylindrocladium black root rot, caused by the fungus *Cylindrocladium parasiticum*, is a disease of economic importance in Georgia, Florida, Alabama, North and South Carolina, and Virginia (Phipps, 1990). The disease is of particular concern in Virginia where 20% of peanut fields are thought to be infested. Under favorable conditions, overwintering microsclerotia of the pathogen germinate and infect roots, causing decay (Fig. 5). Dark, red perithecia of the fungus are produced on the stems of infected plants; however, the sexual stage of the fungus does not appear to play a role in the disease cycle. Various studies have proven that *C. parasiticum* can be seedborne (Glenn et al., 2003). This mechanism is thought to have played a role in the spread of the pathogen from Georgia where it was first reported

in the 1960's (Bell and Sobers, 1965). Recent studies suggest that populations in Georgia are mainly clonal and genetically homogeneous (Wright, et al., 2010)

Figure 3. Peanut pod rot, caused by *Rhizoctonia solani*.

Sclerotinia blight, caused primarily by *Sclerotinia minor* Jagger and to a lesser extent *S. sclerotiorum*, is a destructive and economically important disease throughout areas of North Carolina and Virginia (Porter and Beute, 1974), Oklahoma (Sturgeon, 1982) and Texas (Wadsworth, 1979). Under favorable conditions, sclerotia of the pathogen eruptively germinate at the soil surface and initiate direct infections, with the resulting yield loss ranging from 10 to 50% (Comp). Symptoms consist of wilting and yellowing of the lateral branches. Dense mats of white mycelia develop on diseased areas, and small water-soaked lesions may be apparent near the soil line. Lesions become bleached due to the production of oxalic acid and have a distinct shredded appearance (Woodward et al., 2006). Small, black, angular sclerotia are produced on and within infected tissues. Infected peanut seed and crop debris may serve as initial inoculum (Woodward et al., 2006). Porter et al. (1989) found that disease incidence was correlated to discoloration, indicating that infestations of seed lots were restricted to mycelial infections on the seed testa. The sclerotia are easily capable of surviving 3-4 year crop rotations with non-hosts, and are spread primarily by soil movement through equipment or farming operations.

Figure 4. Peanut pod rot, caused by *Pythium myriotylium*.

Figure 5. Peanut plants with perithecia of *Cylindrocladium parasiticum*.

Figure 6. Sclerotinia blight of peanut, caused by *Sclerotinia minor*.

2. Chemical management of peanut diseases

Numerous chemical fungicides are available for control of the aforementioned diseases of peanut (Table 1). Applications of these products are made for the management of both foliar and soilborne diseases. These products have traditionally been the second largest variable expense in peanut production, behind seed cost. In the United States, management tactics vary among production regions; however, multiple applications of fungicides are typically required to minimize disease-associated losses within a given growing season (Melouk and Backman, 1995; Shokes and Culbreath, 1997). In the southeastern United States, applications of fungicides are typically made on calendar-based schedule; with initial applications beginning approximately 30 days after planting (DAP) and subsequent applications made on 14-day intervals. Due to the long growing season and high disease pressure in this region, a total of six to eight applications may be warranted. Whereas, two-to-three applications may be made in more arid production regions, such as west Texas. In the Virginia/Carolina region, several weather-based spray advisories have been developed and are currently being used to properly time applications (Phipps et al., 1997).

2.1. Management of peanut leaf spot with fungicides

Copper and sulfur dusts, were among the first fungicides used in peanut production for management of foliar diseases (Smith and Littrell, 1980). Most inorganic copper and sulfur

compounds are relatively insoluble, thus, preventative applications create a protectant barrier on leaf surfaces. Small quantities are absorbed by fungal spores, and accumulations result in their lethal effect. Dust formulations are no longer being utilized due to high usage rates, poor plant coverage, and the potential contamination of non-target locations (Backman et al., 1975; Backman, 1978). Some of the early liquid fungicides, such as benomyl and chlorothalonil were or are used to manage *C. arachidicola* and *C. personatum*. Benomyl was very effective at controlling leaf spot (Porter, 1970); however, widespread resistance to benomyl occurred in both *C. arachidicola* and *C. personatum* shortly after use began (Smith and Litrell, 1980).

Chlorothalonil, a broad-spectrum fungicide, is among the most effective fungicides registered for leaf spot control and has been the standard fungicide for leaf spot management since the 1970s (Smith and Litrell, 1980; Culbreath et al., 1992). Unfortunately, chlorothalonil is not active against *S. rolfsii* or *R. solani*, thus other fungicide chemistries are required. The registrations of tebuconazole and azoxystrobin in 1994 and 1997, respectively, greatly expanded fungicide options for peanut since they have excellent efficacy on both foliar and soilborne diseases. Other fungicides, primarily triazoles, strobilurins and carboximides, have been subsequently registered which provide peanut growers numerous options for broad spectrum disease management (Table 1). Although these new fungicides are generally quite active against both foliar and soilborne diseases, they have site specific modes of action, and therefore pose a significant risk for resistance development (Bertrand and Padgett, 1997). Therefore these products have been used as spray blocks or as tank mixes in combination with other chemistries in accordance with FRAC guidelines (www.frac.info). Field trials to evaluate the effects of ergosterol biosynthesis inhibiting fungicides in combination with chlorothalonil demonstrated that using reduced rates of chlorothalonil tank mixed with either propiconazole or cyproconazole improved the control of leaf spot over that of a full rate of chlorothalonil alone (Culbreath et al., 1992; Culbreath et al., 1995). However, tank-mix combinations of fungicides may result in added cost. Culbreath et al. (2001) evaluated the efficacy of various alternations and combinations of chlorothalonil and benomyl for managing benomyl-resistant *C. arachidicola* and *C. personatum* populations. Results of that study showed that full-season tank mixes of the compounds provided leaf spot control comparable to the standard chlorothalonil program, suggesting that tank-mixing is a valid resistance management tool where fungicide resistance is already a problem.

Brenneman and Culbreath (1994) studied various application schedules of chlorothalonil and tebuconazole for leaf spot and stem rot.. They evaluated different application schedules and found that a block of four applications of tebuconazole beginning at the third spray, reduced the severity of both foliar and soilborne diseases, and increased pod yields and kernel quality when compared to the full-season chlorothalonil program. Similar trends were observed when less than four tebuconazole applications were made (Brenneman and Culbreath, 1994). Recommendations in eastern production regions call for chlorothalonil to be added to tebuconazole due to the development of tebuconazole insensitive populations of *C. arachidicola* and *C. personatum* (Stevenson and Culbreath, 2006). It is interesting to note that later generation triazoles such as prothiconazole still maintain field control of leaf spot populations resistant to tebuconazole (Culbreath et al 2008).

Mode of action	Target site and FRAC codes[1]	Group name	Common name	Trade name(s)	Mobility
Nucleic acid synthesis	A1 (4)	Phenylamide	mefenoxam or metalaxyl	Ridomil Gold EC, Ridomil Gold GR, Ridomil Gold SL	locally systemic
Mitosis and cell division	B1 (1)	Benzimidazole	thiophanate-methyl	Topsin M	locally systemic
Respiration	C2 (7)	Carboxamide	penthiopyrad	Fontelis	locally systemic
			boscalid	Endura	systemic
			flutolanil	Artisan (+ propiconazole), Convoy, Moncut	systemic
	C3 (11)	Strobilurin - Quinone outside inhibitor (QoI)	azoxystrobin	Abound	locally systemic
			fluoxastrobin	Evito	locally systemic
			pyraclostrobin	Headline	locally systemic
			trifloxystrobin	Absolute (+tebuconazole), Stratego (+propiconazole)	locally systemic
	C5 (29)	Dinitroanaline	fluazinam	Omega	protectant
Lipids and membranes	F1 (2)	Dicarboximide	iprodione	Rovral	locally systemic
	F3 (14)	Aromatic hydrocarbon	dichloran	Botran	protectant
			PCNB	PCNB	protectant
Sterol synthesis	G1 (3)	Demethylation inhibitor - DMI	cyproconazole	Alto	systemic
			metconazole	Quash	locally systemic
			propiconazole	Tilt, Propiconazole, Propimax, Artisan (+ flutolanil), Stratego (+ trifloxystrobin)	locally systemic
			prothioconazole	Proline, Provost (+ tebuconazole)	systemic
			tebuconazole	Folicur, Muscle, Orius, Tebuzole, Trisum, Absolute (+ trifloxystrobin)	locally systemic
Multi-site activity	M1 (M1)	Inorganic	copper salts	Kocide, Copper-Count-N	protectant
	M2 (M2)		sulfur	*numerous*[2]	protectant
	M3 (M3)	Dithiocarbamate	mancozeb	Mancozeb	protectant

Mode of action	Target site and FRAC codes[1]	Group name	Common name	Trade name(s)	Mobility
			maneb	Maneb	protectant
	M4 (M4)	Phthalimide	captan	Captan	protectant
	M5 (M5)	Chloronitrile	chlorothalonil	Bravo, Equus, Echo	protectant
	M7 (M7)	Guanadine	dodine	Elast	protectant
Unknown	unknown (33)	Phosphonate	phosphorous acid	Phostrol, AgriFos	systemic
			potassium phosphite	Fosphite, Prophyt	systemic
	n/a	n/a	Chlorpyrifos[3]	Lorsban	n/a

Table 1. Peanut fungicides registered in the United States grouped by mode of action

2.2. Management of diseases caused by soilborne pathogens with fungicides

Peanut producers have more options now than ever when it comes to fungicides. While many of the products currently on the market have activity against diseases caused by both foliar and soilborne pathogens, flutolanil was registered in 1995 and is only active against *S. rolfsii* and *R. solani*. Therefore it must be used in combination with products with leaf spot activity (Hagan et al., 2004). However, to effectively use any fungicide for management of soilborne pathogens, the technical difficulties of getting the fungicide to the lower stem and around the pegs and pods must be considered. The most active fungicides will fail to control soilborne diseases if they cannot be placed appropriately. Pentachloronitrobenzene (PCNB), an organochlorine fungicide, was the first fungicide used extensively against stem rot; however, high costs and inconsistent field results limited producer usage (Csinos, 1989). This fungicide was applied as a granule, the logic being that granules were needed to filter down through the canopy to the soil surface for control of soilborne diseases (Csinos, 1989).

This same strategy was applied to newer fungicides, such as the ergosterol biosynthesis inhibitors as they were evaluated in peanut. Granular formulations of diniconazole and tebuconazole were examined, but results were inconsistent (Csinos, 1987). Suppression of diseases caused by soilborne pathogens was observed when liquid formulations of these compounds were applied to foliage in leaf spot studies (Backman and Crawford, 1985; Csinos et al., 1987; Brenneman and Culbreath, 1994; Besler et al., 2003). By mixing dyes with the foliar-applied fungicides and applying irrigation, Csinos (1988) documented how these materials were delivered to the soil. He demonstrated that the architecture of the peanut plant served to funnel rain or irrigation water along the stems and increase deposition of fungicides at the plant crown and pegs. This redistribution is important since these structures serve as primary infection courts for several pathogens (Melouk and Backman, 1995).

Various factors are known to affect fungicide deposition and efficacy. Differences in the leaf cuticle can influence the retention of fungicides (Neely, 1970; Neely, 1971), and changes in the composition of the cuticle have been attributed to different environmental factors (Skoss,

1955). Pesticide deposition is also greatly affected by canopy density. Researchers have found that higher levels of chlorothalonil are deposited on the upper plant canopy, compared to the lower canopy (Brenneman et al., 1990; Hamm and Clough, 1999). Zhu et al. (2004) demonstrated that spray deposits in the upper and lower peanut canopy differed significantly, and deposits in the lower canopy decreased as plants aged. The deposition and retention of chlorothalonil may differ within the peanut canopy layer and volume of water used for application (Brenneman et al., 1990). O'leary et al. (1997) found that both formulation and application method of flutolanil resulted in significant increases in chemical residues on subterranean plant parts and the lower canopy, respectively, characteristics that impacted managment of stem rot.

2.3. Improving fungicide deposition and efficacy via application method

Thorough coverage of foliage or the ability of fungicides to reach target organisms is essential in maximizing disease control. Environmental conditions such as relative humidity, wind speed, temperature and rainfall can greatly affect fungicide deposition. Changes in nozzle type, carrier volumes or pressure may also improve deposition. Application method is known to affect the deposition of fungicide by influencing penetration within the the plant canopy (Brenneman et al., 1990). Fungicides can be applied to peanut through various ground sprayers, fixed wing aerial applicators, or injected through irrigation systems (chemigation). Brenneman and Sumner (1990) reported that cholorthalonil applied via chemigation provided a similar level of leaf spot control as ground applications under low to moderate levels of disease; however, control was not sufficient with severe epidemics. Chemigation with propiconazole (Brenneman et al., 1994) or tebuconazole (Brenneman and Sumner, 1989) in place of foliar applications of chlorothalonil resulted in increased leaf spot incidence. Chemigation wets the entire leaf surface and residues may be displaced from the tissues due to the cuticle (Neely, 1970; Neely, 1971; Skoss, 1955). Johnson et al. (1986) found that only 10% of chlorothalonil applied was retained on the foliage after chemigation. Backman (1982) speculated that the displacement of PCNB and carboxin due to chemigation led to improved efficacy of stem rot in Alabama. A subsequent report evalauting tebuconazole found that Rhizoctonia limb rot was less severe where the fungicide was applied via chemigation (Brenneman and Sumner, 1989). Chemigation is permitted on several fungicide labels including azoxystrobin, metalaxyl and mefenoxam which are used predominantly for pod rot in in Texas where the majority of peanut acres are irrigated (Woodward and Black, 2007). In greenhouse studies simulating chemigation with mefenoxam, Wheeler et al. (2007) found that the chemical should be applied in an appropriate volume of water that places the fungicide at a depth where pods are developing. Higher irrigation rates led to increased concentrations at depths of 10 and 20 cm; however, excesive irrigation can leach the fungicide from the zone completely and compromise efficacy.

Fungicide penetration and deposition may also be affected by canopy density and architecture. Older peanut plants tend to have a more dense canopy, thus reducing deopsits to the lower canopy (Zhu et al., 2003). Much research has been conducted to evaluate

methods of improving fungicide penetration into the lower canopy for control of soilborne diseases. The application of benomyl in conjuntion with the pruning of peanut vines increased stem rot control (Backman et al., 1975). Likewise, the application of iprodione following pruning has improved control of Sclerotinia blight (Bailey and Brune, 1997; Butzler et al., 1998). Implements designed to open the canopy have been used to concentrate fungicides near the crown area. Grichar (1995) found that use of an A-sweep boom attachment improved the efficacy of several fungicides towards stem rot. Targeting applications of fluazinam using a canopy opener allowed for reduced rates to be used in the control of Sclerotinia blight in Oklahoma (Damicone and Jackson, 2001).

More recently, Augusto et al. (2010a) found that fungicide applications made at night (when peanut leaves are folded) rather than the day (when peanut leaves are unfolded) were more effective for the control of stem rot and increased yields. While stem rot control was enhanced, incidence of early leaf spot was not affected by application timing with systemic fungicides, but protectants such as chlorothalonil were less effective for leaf spot when sprayed at night. Additional studies found that early morning applications (applied betwen 3:00 and 5:00 A.M.) of pyraclostrobin and prothioconazole plus tebuconazole decreased stem rot compared to day-time or evening (between 9:00 and 10:00 PM) applications (Augusto et al., 2010b). In that study, applications of systemic fungicides applied prior to sunrise increased yields compared to day applications. This resulted from increased spray coverage, density and droplet size in the lower canopy, as well as improved redistribution downward with movement in dew that was present in the morning applications.

2.4. Redistribution of fungicides via irrigation

Historically, suppresion of soilborne pathogens was achieved through applications of granular fungicides banded over the center of the row (Csinos, 1987). These formulations were thought to sift through the canopy ultimately arriving at the soil; however, control using these materials was costly and inconsistent. The registration of the flutolanil has provided producers with a more effective means of managing soilborne diseases (Hagan et al., 2004). Furthermore, the registration of tebuconazole and azoxystrobin, has greatly improved both stem rot and leaf spot management over the past decade (Brenneman and Culbreath, 1994; Brenneman and Murphy, 1991; Grichar et al., 2000). In contrast to granular fungicides, broadcast-spray applications of these compounds are made to peanut foliage. Fungicide deposition within the canopy contributes to efficacy for leaf spot, but the management of stem rot is more difficult since the target of spray deposition for stem rot control is at the base of the plant or even below ground (Punja, 1985). The mechanism by which foliar-applied fungicides affect stem rot is not fully understood. It is believed that initial deposits of fungicides within the upper canopy are washed on to stems and pegs at the base of the plant via dew, rainfall, or irrigation (Taylor, 1996). This hypothesis was tested by Csinos and Kvien (1988), by using methyl-blue dye to demonstrate fungicide redistribution with irrigation. As a result of these studies and observations of sporadic reductions in efficacy of foliar-applied fungicides in non-irrigated fields, producers in

Georgia are advised to administer irrigation following fungicide applications in order to maximize stem rot control (Kemerait et al., 2006). It is recognized that administering irrigation too quickly may compromise leaf spot control, but the timings needed to optimize control of diseases caused by foliar and soilborne pathogens are not well documented.

There is currently limited information available regarding the redistribution of fungicides from rainfall or irrigation. Most of what has been reported pertains to the influence of rainfall and the rainfastness of protectant compounds in vegetables or fruit crops (Smith and MacHardy, 1984; Neely, 1971; Kudsk et al., 1991). Information regarding mechanisms of suppressing soilborne pathogens with foliar applied fungicides is even more limited. Csinos and Kvien (1988) suggested that initial fungicide deposits applied to peanut foliage are washed to the base of the plant, thus improving contact with soilborne pathogens. Presumably, fungicides were redistributed from the foliage to crowns and pegs.

Using *S. rolfsii* to bioassay peanut tissues, Woodward (2006) was able to quantify the redistribution of azoxystrobin, flutolanil and tebuconazole applied to foliage using irrigation, and to examine the effects of different irrigation timings (0-96 hours after application). In that study, irrigation timing was found to affect the efficacy towards both foliar and soilborne pathogens. Leaf spot was more severe when irrigation was administered immediately after fungicides were applied, whereas, a significant reduction was was observed following a 6 to 12 hour delay in applying irrigation. Maximum leaf spot control was obtained when fungicides were allowed to dry for 24 hours. Inversely, pod colonization (indicating potential for pod rot) increased significantly as irrigation was delayed. Overall, pod colonization was similar for all the fungicides evaluated; however, supression was greatest for tebuconazole at earlier timings. Smaller differences between timings were observed for azoxystrobin. Differences in physiochemical properties of these fungicides, such as affinity to the leaf surface, permeability, and the rate of uptake could have attributed to these differences.

Flutolanil (Araki, 1980) and tebuconazole (Taylor, 1996) are rapidly absorbed by the leaf, whereas, azoxystrobin remains on the leaf surface for a longer period (Bartlett et al., 1995). The persistence of azoxystrobin on the leaf surface may help explain the differences in the pod colonization for the non-irrigated controls. Earlier irrigation timings led to maximum stem rot control, while longer drying times were required to maximize leaf spot control. In the study conducted by Woodward (2006), a period of 18 hours drying time was required between the application of select fungicides and admistering an irrigation event. More recently, Augusto and Brenneman (2011) evaluated the interactive effects of fungicide timing and subsequent irrigation. Leaf spot control was not effected by irrigation, which was applied approximately 24 hours after fungicide applications. Overall, the application of irrigation was less effective at reducing stem rot incidence compared to nighttime applications of fungicides; however, effects of neither fungicide timing or subsequent irrigation were the same for all fungicides evaluated. This could be attributed to differences in retention, absorbtion or systemicity of the fungicides. Systemic fungicides used to manage leaf spot and stem rot move acropetally within the plant; however, applications of

prothioconazole, or prothioconazole plus tebuconazole have been shown to reduce disease in the lower non-treated areas of the plant (Augusto and Brenneman, 2012). A better understanding of fungicide systemicity is needed to maximize foliar and soilborne disease control in peanut. Furthermore, the increased residual activity of newer peanut fungicides has led to changes of commercial fungicide regimes under reduced disease pressure.

2.5. Use of extended interval fungicide programs and forecasting models

While fungicides are typically applied on a 14-day schedule to manage fungal diseases, the use of extended spray intervals could certainly be beneficial to producers by reducing production costs if they could maintain similar yields. In a study conducted by Brenneman and Culbreath (1994), fungicides applied on a 14-day schedule and 21-day schedule provided similar levels of leaf spot and stem rot suppression. Disease suppression decreased in plots treated on a 28-day interval; however, leaf spot and stem rot suppression was lower than what was observed in the non-treated control. A similar trend was observed for yield, where 3-year averages for the non-treated control, 14-day and 21-day intervals were 2914, 5153, and 4704 kg per hectare, respectively. Additional studies have shown that fungicides applied on 21- or 28-day intervals are capable of providing sufficient control of diseases and provide yields comparable to those achieved by the standard 14-day applications interval (Brenneman et al., 2001; Culbreath, 1993; Culbreath et al., 1992; Monfort, 2002; Phatak et al., 2002). Results of one study in particular showed that plots receiving as few as four chlorothalonil applications applied on a 28-day interval had yields as high as plots treated with seven applications made on a 14-day interval (Culbreath et al., 1992). Chandra et al. (1998), found that one properly timed application provided adequate control of leaf spot; however, timings differed within years. More recently Culbreath et al. 2006 also demonstrated excellent leaf spot control with pyraclostrobin applied at more extended intervals, and even when the initial sprays were greatly delayed. Delayed initial applications with this fungicide are now widely used by growers in the southeastern United States with good results.

By better defining the environmental conditions that favor disease development, peanut producers can improve disease control by timely application of fungicides. Forecasting models use environmental data such as temperature, rainfall and relative humidity, to predict when conditions are favorable for pathogen and disease development (Campbell and Madden, 1990). Over the past 40 years, various forecasting models have been developed and successfully implemented for peanut diseases. Jenson and Boyle (1966) and Phipps and Powell (1984) are credited with developing some of the first forecasting models to manage peanut leaf spot. More recently, an early leaf spot spray advisory, developed in Virginia, was effective in reducing number of sprays required for satisfactory disease control and has been highly accepted by growers (Cu and Phipps, 1993; Phipps, 1993). Spray advisories for late leaf spot have been implemented in other peanut producing states, such as Georgia, Alabama, North Carolina and Oklahoma (Nutter and Brenneman, 1989; Davis et al., 1993; Bailey et al., 1994; Damicone 1994).

In Georgia, AU-Pnut is the predominant leaf spot advisory used in research; however, it is not widely used by producers. This model was developed in the late 1980s, and is based solely on precipitation (the number of precipitation events and the five-day forecasted probability of precipitation) (Davis et al., 1993). Studies to evaluate the AU-Pnut advisory for timing applications of fungicides aimed at soilborne fungi have shown suppression of stem rot, but the results have been inconsistent (Brenneman and Culbreath, 1994; Rideout, 2003).

Several spray advisories based on the environmental conditions that incite Sclerotinia blight have been developed in Virginia and North Carolina (Phipps, 1995, Langston, 1998, Langston et al., 2002). Such advisories have been shown to improve disease control when compared to calendar applications. These advisories are based on air and soil temperatures, precipitation, relative humidity, vine growth, and canopy closure. Adaptations of these models have been evaluated for the control of stem rot. Rideout (2003) demonstrated that fungicide application timing has a significant effect on stem rot control and yield in Georgia. Furthermore, he concluded that the application of fungicides according to advisories based on soil temperature, precipitation and host growth provided similar or better disease control than the typical calendar-based programs.

3. Conclusions

Peanut is susceptible to various foliar and soilborne pathogens. Currently there is a wide range of fungicides labeled for management of peanut diseases (Table 1). Standard fungicides, such as chlorothalonil or tebuconazole, commonly comprise fungicide regimes designed to control leaf spot and stem rot, respectively. Other diseases, such as pod rot and Sclerotinia blight are managed with fungicides such as azoxystrobin and fluazinam, respectively. Several other fungicides with different modes of action are avaliable for use in peanut. While some fungicides, such as pyraclostrobin have post-infection activity, efficacy is typically greatest when applications are made in a preventative manner. Utilization of integrated disease managment strategies that incorporate factors such as field history, cultural practices and partially resistant cultivars may be used to reduce disease pressure and increase profitability. Resistance to several classes of fungicides used in peanut have been identified in populations of leaf spot pathogens. Most recently, resistance to triazole fungicides, such as tebuconazole, have been reported in eastern production regions of the United States. Furthermore, the potential exists for resistance to develop in other fungicide classes, primarily the strobilurin; therefore, it is imperative that producers rotate chemistries to ensure the sustainability and longterm use of these fungicides. Future research evaluating aspects of peanut fungicides, such as initial application timing, systemic and residual activity and interactive effects of tank-mixtures are warranted. For diseases caused by soilborne pathogens, a better understanding of spatial and temporial aspects of the pathogen could allow for more precise applications of fungicides.

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Chapter 4

Impact of Fungicides on Rice Production in India

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Additional information is available at the end of the chapter

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1. Introduction

Rice is the most economically important staple food crop in India, China, East-Asia, South East Asia, Africa and Latin America catering to nutritional needs of 70% of the population in these countries (FAO, 1995). In several developed countries such as North America and European Union (EU) also, rice consumption has increased due to food diversification and immigration (Faure and Mazaud, 1996). Worldwide, rice is grown on 161 million hectares, with an annual production of about 678.7 million tons of paddy (FAO, 2009). About 90% of the world's rice is grown and produced (143 million ha of area with a production of 612 million tons of paddy) in Asia (FAO, 2009). Rice provides 30–75% of the total calories to more than 3 billion Asians (Khush, 2004; von Braun and Bos, 2004). To meet the global rice demand, it is estimated that about 114 million tons of additional milled rice needs to be produced by 2035, which is equivalent to an overall increase of 26% in the next 25 years. The possibility of expanding the area under rice in the near future is limited. Therefore, this extra rice production needed has to come from a productivity gain (Kumar and Ladha, 2011). Maximum yields per unit area of land can be achieved and sustained only if along with high yielding crop varieties there is also a provision for protection of the crop against its enemies (Srivastava *et al*., 2010). Amongst the various biotic factors affecting rice production and productivity, rice diseases are one of the most important ones. The annual losses due to rice diseases are estimated to be 10-15% on an average basis worldwide. Therefore, judicious management of rice diseases can result in improved productivity and additional grain harvested. Rice diseases are caused by wide variety of pathogen including fungus, bacteria, virus and nematodes (Ling, 1980). In the pre-war period, diseases of rice were practically unimportant in Tropical Asia where ancient varieties were traditionally grown on soils of low fertility (Areygunawardena, 1968). However, with the increasing demand for world rice supplies and advent of green revolution resulting in use of improved varieties, high fertilization, irrigation and intensive cultural practices have resulted in great increase in the occurrence and severity of diseases infesting rice in several countries (Teng,

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1990). The major rice diseases that often cause great economic losses are rice blast (*Magnaporthe grisea*), sheath blight (*Rhizoctonia solani*), Bacterial blight (*Xanthomonas oryzae*) and Tungro virus disease especially in South and South East Asia (Ling, 1980).

The various methods used for managing rice disease includes, use of resistant varieties, cultural practices, biological and chemical control. All these methods have varied degrees of success in managing rice diseases. The most important control tactics used worldwide includes use of resistant varieties and chemical control. Breeding for disease resistant varieties has been long used for managing the rice diseases and is one of the most economical methods which contributed immensely to world's rice productivity (Mew, 1991; Bonman, *et al.* 1992). But, most varieties are resistant only to a few major diseases that are the subjects of intensive breeding efforts. The rice production environments, particularly in the tropics, are habitats of many rice pathogens causing varying degrees of damage. Even the "minor" diseases collectively could pose a significant threat to production (Mew, 1992). Moreover, the pathogen often develops new biotypes resulting in breaking down of resistance in the resistant varieties. Therefore, chemical control provides great opportunity for controlling rice diseases and over last two decades a lot of focus has been shifted towards developing new molecules that can be used for controlling rice diseases. As the most destructive rice diseases prevalent across the globe are caused by fungus (Ling, 1980), fungicides are an important tool to control them. This chapter discusses about importance of different fungicides for the control of major rice diseases.

2. Rice diseases dynamics and fungicide market in India

Rice blast and brown spot were the major diseases noticed during pre independent India and before introduction of high yielding varieties. After introduction of HYV, along with them, BLB, tungro and sheath blight have become major diseases. Recently diseases like sheath rot, false smut, stem rot and grain discolouration which were minor and occurring sporadically are emerging and causing considerable yield loss. This is primarily due to climate change, crop intensification and changes in practice. Out of the total yield loss due to diseases in rice, 35% is by blast, 25% by sheath blight, 20% by BLB, 10% by tungro and remaining 10% by other diseases.

The market share of fungicide used on rice in India during 2010-11 is Rs 380 crores, of which blast and sheath blight fungicides alone constitute 280 crores and the share of fungicides used against brown spot, BLB, grain discoloration, stem rot and false smut is 100 crores.

3. Rice fungicides

Fungicides prevent rice diseases which can result in severe damage to the crop in terms of both quality and quantity. Globally 8.4 % of fungicides market share is for rice (Collins, 2007). Synthesizing and characterizing a new molecule to be used as fungicide involves several steps. Initially the new lead molecule is tested in-vitro for its efficacy against the target pathogen and then it is characterized under field condition to ascertain its efficacy against the target disease and to finalize the most effective dose/rate that can be used for the control of the target disease. Several fungicides belonging to different groups have been

synthesized and evaluated for use in rice ecosystem. More than 30 fungicides have been registered for use in rice (Table 1) and several new molecules are under testing. The rice fungicides can be broadly classified in two categories.

Technical Name	Trade Name	Chemical group	Mode of Action	Target pest	Formulation Dosage/ha	g.a.i/ha
Azoxystrobin23%SC	Amistar 25EC	Strobilurin	protectant, curative, eradicant, translaminar and systemic properties	Sheath blight	500	125
Carbendazim 50 WP	Bavistin	Benzimadozole	Preventive &Curative	Brown spots, Blast, Sheath blight	500	250
Carpropamide 27.8%SC	Protega	Carbamate	Preventive	Blast	500	139
Chlorothalonil 75%WP	Kavoch,Bravo	Aromatic fungicide	Preventive & Curative	Sigatoka,Rusts	1000	750
Copper oxychloride 50 WP	Blitox	Copper fungicides	Protective and Eradicants	BLB,	1250	625
Copper Hydroxide 77% WP	Kocide	Copper fungicides	Protective and Eradicants	False smut	2000 gm	1000 gm
Difenconazole25%EC	Score 25EC	Triazole	Preventive & Curative	Sheath blight	250-500	62.5-125
Ediphenphos50%EC	Hinosan	Organophosphatic	Preventive & Curative	Blast and Brown leaf spots	500-600	250-300
Epoxiconazole 7.5%EC	Opus 7.5EC	Triazole	Preventive & Curative	Sheath blight	1.5ml/lit	
Eprobenfos 48%EC	Kitazin48%EC	Organophosphatic	Protective &Curative	Blast,Sheath blight	500	240
Fenbuconazole 24 SC		Triazole	Preventive & Curative	False smut & grain discoloraion	520	125
Flusilazole 12.5 +Carbendazim25%	Lustre 37.5%SE	Triazole +Bemzimidazole	Preventive & Curative	Sheath blight	2ml/lit	
Flusilazole40%EC	Cursor 40EC	Triazole	Preventive & Curative	Sheath blight	300	120
Hexaconazole 5 EC	Contaf	Triazole	Preventive &Curative	Sheath blight	1000	50
Hexaconazole 5 SC	Contaf plus	Triazole	Preventive &Curative	Sheath blight	1000	50
Iprodione 50%WP			Proventiv &Curative	Sheath blight	1125	2250
Iprodione25%+Carbendazim 25%WP	Quintal 50%WP	Dicarboximide	Preventive&Curative	Sheath blight, Blast	500	250
Isoprothiolane 40%EC	Fuji-one	Triazole	Preventive &Curative	Blast	750	300
Kasugamycin 3%SL	Kasu-B	Aminoglycoside Antibiotic		Blast	1000-1500	30-50

Technical Name	Trade Name	Chemical group	Mode of Action	Target pest	Formulation Dosage/ha	g.a.i/ha
Kresoxim methyl 44.3%SC	Ergon	Strobilarin	Preventive & Curative	Sheath blight,Blast	500	250
Mancozeb 35 SC	Eurofil-NT 35% SC	Mn Eth-Bishithiocarbamate	Protective &Eradicants	Brown spots, Blast, Sheath blight	2500	875
Mancozeb 63 + Carbendazim 12 WP	Companion-75%WP	Dithiocarbamate+Benzimidazole	Preventive & Curative	Blast ,Sheath blight	750	375
Mancozeb 75 WP	Dithane M 45	Dithiocarbamate	Protective	Broad spectrum	1000	750
Mancozeb 75WG	Manfil 75%WG	Dithiocarbamate	Protective	Broad spectrum	1000	750
Metominostrobin 20SC		Strobulurin	Preventive &Curative	Blast and sheath blight	500	100
Pencycuron 22.9%SC	Monceren 25 SC	Urea fungicides	Preventive & Curative	Sheath blight	750	187.5
Propiconazole 25 EC	Tilt 25% EC	Triazole	Preventive & Curative	Sheath blights	500	125
Pyroclostrobin20%WG	Headline	Strobulurin	Preventive & Curative	ELB	375-500	75-100
Propineb 70% WP		polymeric ainc propylenebis(dithiocarbamate)	protective	Brown leaf spot	2000	1400
Tebuconazole 25.9%EC	Folicure 25EC	Triazole	Preventive & Curative	Balst, Sheath blight, false smut	750	187.5
Thifluzamide 24% SC	Spencer	Carboximide	Preventive & Curative	Sheath blight	90	375
Thiram 75%WS				Seed borne diseases	25-30	18.8 - 22.5
Tricyclozole 76 WP	Beam 75%WP	triazolobenzothiazole	Preventive	Blast and Brown leaf spots	300-400	225-300
Trifloxystrobin25%+Tebuconazole 50%	Nativo 75%WG	Strobilurin+Triazole	Preventive & Curative	Sheath blight	350	262.5
Validamycin 3%L	Sheathmar 3L	Antibiotics	Preventive & Curative	Sheath blight	2000	60
Zineb 75%WP	Phytox	Zinc ethylenebis-(dithiocarbamate)	protective and curative	Blast	2000	1500

Table 1. Rice fungicides registered in India (Source: Central Insecticide Board, Govt. of India)

3.1. Seed treatment fungicides

These fungicides have narrow to moderate spectrum of control and are highly specific. These are applied on the seed surface before sowing. The seed is dressed with either a dry formulation or wet treated with a slurry or liquid formulation. Low cost earthen pots can be used for mixing pesticides with seed or seed can be spread on a polythene sheet and required quantity of chemical can be sprinkled on seed lot and mixed mechanically by the farmers (http://agritech.tnau.ac.in/seed_certification/seed_treatment_Insecticides%20&%20Fungicides.html). The major advantage is that it provides high level of control at low dose and with low residue. In the greenhouse study, tricyclazole at 0.2g/kg of seed effectively controlled leaf blast upto 21 days after planting and resulted in 8.2 μg/g tricylazole in the leaves at that time according to GLC assay (Froyd *et al.*, 1976). Anwar and Bhat (2005) evaluated few fungicides *viz.*, Isoprothiolene, Tricyclazole, Edifenphos, Hexaconazole and Mancozeb as seed treatment at two varying doses. Isoprothiolene and Tricyc1azole 75WP were most effective in controlling the nursery blast disease, exhibiting no incidence and severity at both the doses tested at 35 DAS. The list of fungicides for use as seed treatment from the IRRI website is shown in table 2.

% Active Ingredient(s)	Rate	Additional Information
Metalaxyl 28.35%	0.75 - 1.5 fl. oz. per 100 lbs. of seed.	For *Pythium* caused seed rot and damping-off control. For use as a commercial seed treatment.
Trifloxystrobin 22%	0.32 - 0.64 fl. oz./cwt	For *Rhizoctonia solani* control
Mefenoxam 33.3%	Apply 0.0425 to 0.085 oz. per 100 lbs. of seed for *Pythium* seed rot and damping-off control in rice when applied in combination with Vitavax-200, 42-S Thiram, or RTU-Vitavax-Thiram at labeled rates.	For *Pythium* seed rot and damping-off control. For use as a commercial seed treatment.
Thiram 42%	1.5 fl oz/bu	For seed decay, damping off, and seedling blights caused by Pythium and Rhizoctonia
Mancozeb 50%	4 oz. per 100 lbs. of seed.	For control of damping-off, seed rots, and seedling blights caused by Drechslera and Pythium. Drill box treatment.
Mancozeb 37%	3.4 to 6.7 oz. per 100 lbs. of seed.	For control of soil borne and seed borne fungi causing seed rot and reduced seedling vigor. Apply before, during, or after soaking in water.

% Active Ingredient(s)	Rate	Additional Information
Carboxin 10% + Thiram 10%	5 to 6.8 fl. oz. per 100 lbs. of seed.	For control of various seed and seedling diseases. The higher rate is recommended for control of *Helminthosporium oryzae*. Ready to use seed treatment which may be applied as a commercial seed treatment or as a pour-on hopper box application.
Carboxin 5.7% + Thiram 5.7%	9 to 12 fl. oz. per 100 lbs. of seed.	To control various seed and seedling diseases, especially effective against *Rhizoctonia solani* and *Helminthosporium oryzae*. The higher rate is recommended for control or *Helminthosporium oryzae*. Apply as a pour-on treatment or by machine.
Carbendazim 50 WP	4 g/kg of seeds	To control blast, brown spot and udbatta disease of rice
Tricyclazole 75 WP	3 g/kg of seeds	To control rice blast disease

Note: 1 oz.= 29.57 ml; 1 lb = 0.45 kgs
(Source: http://www.knowledgebank.irri.org/qualityseedcourse/index.php/module-2-production-of-rice-seed-in-irrigated-mainmenu-103/2-seed-cleaning-and-treatment-before-planting-mainmenu-47/162-table-rice-seed-treatment-fungicides)

Table 2. Rice Seed treatment fungicides:

3.2. Foliar fungicides

These fungicides are applied as spray using power or back pack sprayers directed towards the plant foliage. These fungicides may be contact (surface acting) or systemic (translocated inside plant) in action. They are highly effective in controlling foliar rice diseases with good residuality. Based on their chemical class and mode of action, rice fungicides can be further grouped into following categories;

a. *Melanin biosynthesis inhibitors (MBI) [FRAC CODE – 16]*: This group of fungicides are only effective against rice blast disease. They prevent melanin biosynthesis in appressoria of *Pyricularia oryzae* and penetration of rice plants via appressoria by inhibiting either polyhydroxynapthaline reductase (eg Tricyclazole, Pyroquilon, Chlobenthiazone etc) or scytalone Dehydratase enzymes (Carpropamid, Dichlocymet etc) (Kurahashi, 2001). Tricyclazole inhibits the NADPH-dependent reduction of 1,3,6,8-tetrahydroxynaphthaline to scytalone and 1,3,8-trihydroxynaphthaline to vermelone (Wheeler, 1982). While, carpropamid inhibits the enzyme scytalone dehydratase essential for the synthesis of the melanin precursors 1,3,8-trihydroxynaphthaline and 1,8-dihydroxynaphthaline (Kurahashi et al., 1998).
b. *Benzimidazole [FRAC CODE – 1]:* This group fungicide was introduced for plant disease control in the 1960s and early 1970s as foliar fungicides, seed treatments and for use in post harvest applications. They possess unique properties not seen before in the

protectants. These included low use rates, broad spectrum and systemicity with post-infection action that allowed for extended spray interval. All these qualities made them very popular with growers but also subject to misuse, such as poor spray coverage and curative spraying. These fungicides are single site inhibitors of fungal microtubule assembly during mitosis, via tubulin-benzimidazole-interactions (Smith, 1988). The current ranking of global sales is: carbendazim, thiophanate, thiabendazole.

c. *Strobilurins [FRAC CODE – 11]:* The first fungicides in this family were isolated from wood-rotting mushroom fungi, including one called *Strobilurus tenacellus*. The name strobilurin was coined for this chemical family of fungicides in recognition of the source of the first compounds of this type. These fungicides are now more properly referred to as QoI fungicides (Vincelli, 2002). They were first launched in 1996 and now include the world's biggest selling fungicide, azoxystrobin (Bartlett *et al.,* 2004). Some of the other commonly used strobilurins against rice diseases are fenamidone, kresoxim methyl, pyraclostrobin and trifloxystrobin either as stand alone or mixed with other multi-site inhibitor fungicides or triazoles like propiconazole. They have broad spectrum activity and are effective against most of the pathogens with few exceptions (Vincelli, 2002). In rice they are used against blast, sheath blight and other foliar diseases.

d. *Triazole fungicides [FRAC CODE – 3]:* This is the largest class of fungicides. They are highly systemic with mobility through xylem. They are known to have low resistance development risk and broad spectrum activity against major diseases except oomycetes. In rice they are used for the control of sheath blight, grain discoloration and other foliar diseases. Some of the commonly used triazoles in rice are propiconazole, tebuconazole, hexaconazole, difenconazole etc. They are good mixture partners with other fungicides and are used in combination with other single site/specific fungicides for increased disease control and resistance management.

e. *MET II inhibitors [FRAC CODE – 7]:* Inhibit succinate dehydrogenase in fungi (examples are thifluzamide and flutalonil). Very low resistance risk and highly effective towards sheath blight. These fungicides are systemic (Xylem mobile) and have good residue.

f. *Antibiotics:* Antibiotics are compounds produced by one micro organisms and toxic to other micro organisms. Chemical formulae of antibiotics are complex and are not related to one another. Antibiotics used for plant disease control are generally absorbed and translocated systemically by plants to a limited extent. They may control plant disease by acting on the pathogen or the host (George, 2005). Kasugamycin **[FRAC CODE – 24]**, blasticidin **[FRAC CODE – 23]** and validamycin **[FRAC CODE – 26]** are some of the common antibiotics which are used for the control fungal diseases in rice**.** Kasugamycin and blasticidin have been used for blast control while validamycin provides effective control of sheath blight. Kannaiyan and Prasad (1979) carried out a field study to evaluate several antibiotics for the control of sheath blight disease of rice and found that all the antibiotics were effective in controlling the disease and achieving higher yields as compared to control.

Some of the new fungicides as per the AgroProjects and Agranova database are mentioned below:

Prothioconazole: Prothioconazole is considered particularly effective for the control of stem rot and sheath rot diseases of rice with C-14 demethylation inhibitor (DMI) mode of action

Dimoxystrobin: This fungicide is the second analogue developed which is structurally similar to the first, metominostrobin. Dimoxystrobin is known to control *Pyricularia oryzae, Rhizoctonia solani*. QoI (MET III, Strobilurin).

Pyraclostrobin: belongs to QoI (MET III, Strobilurin). Pyraclostrobin provides a broader spectrum of disease control against many diseases including important diseases of rice *Pyricularia grisea* and *Rhizoctonia solani*. Pyraclostrobin provides excellent curative activity, whereas the existing strobilurins are primarily protectant.

Orysastrobin: Provides systemic and protectant activity with a long residual effect against rice blast (*Pyricularia oryzae*) and sheath blight (*Rhizoctonia solani*). This acts as QoI (MET III, Strobilurin).

Isotianil: In 2010, Bayer CropScience introduced the rice fungicide isotianil (Routine®) in Japan and Korea to control the plant disease rice blast. This new fungicide is known to stimulate the natural defense mechanisms of rice plants, thus boosting their resistance. This is the first time that a substance which combines low application rates with what is termed a resistance-inducing effect. This fungicide can also play an important role in future seed treatment portfolio in rice.

4. Timing of fungicide application

Fungicide timing is a very critical component in disease control and management. The disease needs to be present in order to justify any fungicide application and its effectiveness. This is not the case in every field and the variety grown greatly influences the disease impact, even if present. The geographical and sometimes micro-climatic conditions of the cropping area also greatly influence the incidence and intensity of any plant disease. Thus scouting and sound decision-making are worthwhile, compared to "blanket" preventative fungicide applications (Cartwright *et al,* 2004). Application of right chemical (Hexaconazole 5SC) at a right time (maximum tillering stage) was very important in control of sheath blight (Swamy *et al*., 2009). While, Pencycuron 250 EC was very effective under Punjab and West Bengal rice growing conditions against sheath blight when sprayed at maximum tillering stage(Lore *et al*., 2005; Biswas, 2002).

Several studies have revealed that many new fungicides have been identified for managing sheath blight in rice which differs in their efficacy from place to place and time of application. Dithane M-45 (Das and Mishra, 1990), Carbendazim and Mancozeb (Thangaswamy and Ranagswamy, 1989; Roy and Saikia, 1976) Iprodione (Izadyar and Baradaram, 1989) Triazole (Suryadi *et al*., 1989) and Carbandazim + Mancozeb (Prasad *et al*., 2006) were found effective when applied at maximum tillering stage. Groth and Bond (2006) showed that application of azoxystrobin between panicle differentiation and 50% heading stage reduced sheath blight severity and incidence, resulting in higher yield and high head rice milling yield compared with inoculated but nonsprayed plots. Similarly, previous

studies as reported by Groth (2005), demonstrated that fungicides can be applied over a range of growth stages and obtain satisfactory control of sheath blight. Therefore, in designing an effective disease management program it is essential to understand the most vulnerable stage of the crop for disease incidence, level of disease incidence/severity, pathogenecity, crop micro-climatic condition and suitable fungicide molecule.

5. Fungicide resistance

The major consideration for the design of fungicide use strategies is the threat of fungicide resistance. Fungicide resistance can occur when a *selection pressure* is placed on the fungal pathogen population. Characteristics of both the fungicide and the pathogen play a role in the magnitude of the selection pressure and the risk of resistance occurring. Fungicides that have a single site of action tend to be more at risk for resistance developing compared to those that have multi-site activity. Fungal pathogens that regularly undergo sexual reproduction are more likely to have greater *variability* in the population, which increases the chances of developing a strain that is less sensitive to a fungicide. When diseases have a repeating stage (polycyclic disease), such as blast, the fungal pathogen may also be more likely to develop resistance to a fungicide partially due to the high number of spores that are produced within a season.

Considerable efforts have been made by industry to conduct research in the areas of mode of action, resistance risk, field monitoring for baseline sensitivity and sensitivity variations in treated fields. Numerous pathogens that attack the highly maintained grasses, such as those found on golf courses, frequently require weekly spray applications through out the summer. This has led to resistance development in *Magnaporthe grisea* (gray leaf spot) to strobilurins. Some of the fungicides used to control rice blast disease (e.g. probenazole, isoprothiolane and tricyclazole) have retained effectiveness over many years of widespread use (Brent and Hollomon, 2007).

The first report of practical resistance to fungicides in rice crop was recorded in 1971 on blast pathogen (*Magnaporthe grisea*) against Kasugamycin due to altered target site (ribosomes) and in 1979 for Phosphorothiolates by metabolic detoxification (Kato, 1988). Kaku *et al*. (2003), reported resistance to carpropamid (Melanin Biosynthesis Inhibitors (Dehydratase) (MBI-D)) with altered target site (scytalone dehydratase) as possible mechanism of resistance. Resistance to carpropamid was confirmed in the strains of *Magnaporthe grisea* due to V75M mutation that causes low sensitivities of SDHs of the carpropamid –resistant strains, and strongly suggests that the V75M mutation confers resistance of these strains to carpropamid (Takagaki *et al*., 2004).

Bennett (2012), reported a suspected mutation of the *Rhizoctonia solani* fungus that has been found to be resistant to azoxystrobin (strobilurin fungicide). Following a series of major tests, the pathologists from Louisiana came up with (fungicide) tolerance levels for *Rhizoctonia solani*. Brent and Hollomon (2007) reported the mechanism of resistance of QoIs (strobilurins) which is due to altered target site (ubiquinol-cytochrome c reductase). This decrease in sensitivity to a fungicide was certainly not unprecedented.

Kim *et al*., 2010, reported on Bakanae disease pathogen *Fusarium fujikuroi* strain CF245 which completely degraded 1.0 mg/L of prochloraz in 5 days after incubation, whereas no degradation of prochloraz was observed by the strain CF106 at the same treatment level under in-vitro conditions. Liquid chromatography Q-TOF MS detected N-(2-(2,4,6-trichlorophenoxy)ethyl)propan-1- amine as a major degradation product of prochloraz by the strain CF245. These results indicated that the degradation of prochloraz may account for the reduced sensitivity of the strain CF245 to prochloraz. All living organisms are constantly mutating and evolving, and changes in sensitivity to pesticides are common not only in fungicides but also in insecticides and herbicides.

Studying the case histories of resistance development by considering the genetic, biochemical and epidemiological process which explains the complex interaction and changing factors determining the rate and severity of development of fungicide resistance

6. Fungicide resistant management

Acquired fungicide resistance is a major threat to plant disease control by chemicals. Pathogens respond to fungicides by evolving resistance against them. Fungicides which provide maximum control also create maximum selection pressure on the pathogen to acquire resistance. Resistance results from one or more changes at genetic level of pathogen population due to mutations occurring in nature. Fungicide itself does not induce resistance. It selects resistant propagules already present at low frequency in natural population of pathogen.

Fungicides may be categorised based on resistance development by the pathogen as low resistant risk fungicides –dithiocarbamate which are protectant fungicides and have multisite action, medium risk fungicedes- SBI's where mutation of several genes is required and high risk fungicides – benzimidazole and strobulins where resistance is controlled by single gene. Thus, a major consideration for the design of fungicide use strategies is the threat of fungicide resistance. There have been considerable efforts by industry to conduct research in the areas of mode of action, resistance risk and field monitoring.

7. Efficacy of fungicides against important rice diseases

7.1. Blast

Blast is the most important fungal disease of rice and occurs in all the rice growing regions of the world. Fungicidal control is largely practised for blast disease in temperate or subtropical rice cultivation, mainly in Japan, China, South Korea, Taiwan and, increasingly, Vietnam. The majority of the fungicides used in blast control are protectants. In early years, copper and mercury compounds were recommended against blast but were found not suitable because of phytotoxicity and mammalian toxicity. Current major products are mainly systemics with a residual activity of at least 15 days, although older organophosphorous products such as edifenphos are still widely used. The modern rice fungicides include isoprothiolane, probenazole, pyroquilon and tricyclazole (Anon., 1992; Filippi and Prabhu, 1997), and are applied as foliar sprays, as granules into water or seed-box treatments (irrigated lowland rice),

or as seed dressings for upland rice. In recent years, newer melanin biosynthesis inhibitors such as carpropamid (Motoyama et al., 1999; Thieron et al., 1999) or broad-spectrum fungicides like azoxystrobin (strobilurin)(Lee and Beaty, 1999) have gained favour.

According to Kapoor and Singh (1982) benomyl seed treatment (1:400 w/w) gives protection to seedlings in nursery for 24-25 days. It inhibits spore germination and appressorium formation. Venkata Rao and Muralidharan (1983) found benomyl, carbendazim, MBC, edifenphos (all 0.1%) and 0.25% mancozeb effective against the blast in the order listed, and significantly better than other fungicides. Tewari and Kameshwar Rao (1983) applied carbendazim through mud balls, soil drench and foliar spray at the rate of 0.5 kg a i/ha and found effective control of the disease. Three sprays were given at the tillering stage at 10 day interval and two sprays at the neck emergence stage at 5 days interval. Saikia (1991) has confirmed the same number and timing of sprays of edifenphos, thiophanate methyl and carbendazim at 0.1% effectively reducing the leaf blast by 71.3-81% and neck blast by 60-65% with corresponding increase in yield. Studies on efficacy of fungicides indicated that tricyclazole and isoprothiolane are highly effective resulting in 87.9 and 83.8% reduction in neck blast and 33.8 and 29.9% increase in grain yield over check, respectively (Sachin and Rana, 2011). Sood and Kapoor (1997) evaluated seven fungicides and found that tricyclozole 75 WP was most effective and reduced the leaf and neck blast by 89.2% and 94.5% respectively. Muhammad Saifulla *et. al.* (1998) confirmed that chlorothalonil and hexaconzole were comparatively more effective in controlling the disease. Tsuda *et. al.* (1998) and Thiron (1999) reported that root application systemic fungicides pyroquilon and carpropamid respectively were effective against rice blast.

Prasanna Kumar *et. al.* (2011c) evaluated three new QoI fungicides (Kresoxim methyl, Metaminostrobin and Trifloxystrobin) in combinations with other groups for two seasons against against blast and sheath blight of rice. All the QoI group fungicides were very effective in controlling leaf and neck blast and also improved the growth of the plant in terms of height, test weight and yield. Kresoxim methyl 40% + Hexaconazole 8% SC @ 200+40 g ai/ha was effective against leaf blast (5.18% and 11.11%) and neck blast (11.11% and 11.85%) with highest yield of 45.75 and 53.42 q/ha respectively during Kharif 2010 and summer 2011. Similar effectiveness was recorded in Kresoxim methyl 50% @ 200 g ai/ha against leaf blast (5.18% and 11.11%) and neck blast (11.85% and 11.11%) which was found on par with tricyclozole @ 225 g ai/ha. Application of Metaminostrobin 20% SC + hexaconazole 5% SC and Metaminostrobin alone gave higher grain yield 41.26 and 41.23 q/ha respectively and was on par with tricylcozole 75%WP. The combination was effective against leaf blast (21.11 and 18.89%) and neck blast (25.56 and 33.89%) during Kharif 2009 and summer 2010.

Nine combinations of fungicides and insecticides were tested for their efficacy and compatibility on major pests and disease of rice (Prasanna Kumar *et. al.*, 2011a). The combination treatments involving the insecticides and fungicide treatment recorded moderate severity ranged from 12.1 to 18.5%. Combination of tricylcazole 75% WP + Fipronil 5% SC recorded least disease severity and insect infestation (17% neck blast) and highest yield of 5190 kg/ha, followed by isoprothiolane 40% EC + fipronil 5% SC compared to untreated check which recorded highest neck blast incidence(34%).

Similar results regarding the efficacy of various fungicides have been reported by different researchers globally. Varier *et al.,* (1993) used eight fungicides for management of rice blast and observed that seed treatment with tricyclazole @ 4kg/kg seed proved effective after 40 days of sowing. Dubey (1995) conducted field trials of eight fungicides for control of *Pyricularia oryzae*, Topsin M + Indofil M-45 was proved to be most effective against leaf blast disease of rice. Minami and Ando (1994) reported that probenazole induce a resistant reaction in rice plants against infection by rice blast fungus. Probenazole pre-treatment increased accumulation of salicylic acid and pathogenesis related (PR) proteins in the eighth leaves of adult rice plants at the 8-leaf stage, resulting in the formation of hypersensitive reaction (HR) lesions (HRLs). Enyinnia (1996) evaluated two systemic fungicides Benomyl and Tricylazole on Faro / 29, a rice cultivar, at full booting stage and reported good control of natural infection of rice leaf blast. Filippi and Prabhu (1997) reported that propagation fungicide (40 g a.i. per Kg of seed) was effective in controlling leaf and panicle blast. Moletti *et al*., (1998) conducted field trial against *Pyricularia oryzae*, and found that pyroqulion granules or wettable powder 2 kg / ha once or twice gave good results against leaf blast. Tirmali and Patil, (2000) conducted field experiment on susceptible rice cultivar E. K. 70 with 5 new fungicide formulations *viz*. Antaco 170, Carpropamid 30 SC, Fliqiconazate 25 WP, Ocatve 50 WP and Opus 15.5 SC. These fungicides were sprayed at tillering, booting and heading stages of crop. The new formulation reduce neck blast incidence by 16.27% to 29.23%, Opus 15.5 SC was highly effective in controlling neck blast (29.23%) and increasing grain yield. Tirmali *et al*. (2001) reported the efficicacy of new fungicides in controlling rice neck blast caused by *Pyricularia oryzae* on rice cultivar Ek- 70 (blast susceptible) treated with Capropamid 30 SC, Folicur 250, WE Swing 250 EC and Beam 75 WP at maximum tillering, panicle initiation and at heading stage of the crop and found that all these new fungicides have significantly reduced neck blast. Ghazanfar *et al*., 2009 evaluated several fungicides on a highly susceptible rice variety Basmati C-622 and observed that Tetrachlorophthalide 30 WP @ 3g/litre, Tebuconazole + Trifloxystobin @ 0.8 g/litre of water and Difenoconazole 250 EC @ 1.25 ml/litre proved effective in reducing the disease percentage. The control of disease in case of neck blast was shown by Tetrachlorophthalide 30 WP @ 3g/litre, Difenoconazole 250 EC and Tebuconazole + Trifloxystobin @ 0.8 g/litre of water to the tune of 12.81%, 14.24% and 17.01%, respectively.

7.2. Sheath blight

Sheath blight is one of the most important rice diseases worldwide and ranks number two position after blast disease. Common fungicides used earlier against sheath blight were copper, organomercury and organo-aresenic compounds (Ou 1985). Carbendazim, benomyl, ediphenfos and kitazin have been reported to be the most effective chemicals recorded by various Indian workers (Premalatha Dath 1990). The fungicidal control of sheath blight in India was attempted by Kannaiyan and Prasad (1976) and Bhaktavatsalam *et. al*. (1977). The rhizosphere population of the pathogen of rice seedlings was drastically reduced through foliar sprays of the fungicides such as kitazin, edifenphos, benomyl, carboxin and carbendazim. The efficacy of benomyl (Das and Panda, 1984) and carbendazim (Bhaktavatsalam *et. al*., 1977; Rajan and Alexander, 1988) in the management of sheath blight

was studied. Benomyl and captan at 0.2% were highly effective in reducing the seedling infection while soil drenching with edifenphos, kitazin and benomyl during tillering stage was also effective in controlling the disease. Seed treatment with carbendazim, chloroneb, chlorotholonil, carboxin, benomyl and phenyl mercury acetate (PMA) reduced the seed borne infection of the pathogen and improved the seed germination, shoots and root growth, seedling vigour and prolonged the viability of the seeds. Again, 0.2% sprays of benomyl, kitazin, edifenphos and chlorotholonil were highly effective in controlling sheath blight and increased the grain yield in field trials. Roy and Saikia (1976) obtained the best control of sheath blight with carbendazim or by benomyl sprays (0.05%) both in green house and field tests. In field trials with six fungicides, kitazin granule was the most effective in reducing the disease severity, followed by edifenphos (Verma and Menon, 1977) but Mathai and Nair (1977) showed that edifenphos was the best as it increased the yield.

Flutolanil, a new systemic fungicide developed with both protective and curative properties is very effective to control various Rhizoctonia groups of fungi including rice sheath blight (Araki, 1985; Hirooka *et. al*., 1989). However, sheath blight disease was effectively controlled by 80% at low concentration of 1.6 to 3.2μg/g of plant. Foliar spray, soil drenching and seed treatment have been tried effectively under green house and field studies. Sundravadana *et al*., 2007, reported that for controlling sheath blight disease, the optimum rate of azoxystrobin was 125 g/ha. Field trials in 2008 and 2009 conducted by Parsons *et al*., (2009) showed that a newly formulated mixture of azoxystrobin and propiconazole called Quilt Xcel™ was highly effective in controlling sheath blight and protecting rice yield and milling quality.

PrasannaKumar *et. al.* (2011c) evaluated three new QoI fungicides (Kresoxim methyl, Metaminostrobin and Trifloxystrobin) and combinations with other groups were evaluated for two seasons against blast and sheath blight of rice. All the QoI group fungicides were very effective in controlling sheath blight and also improved the growth of the plant in terms of height, test weight and yield. Kresoxim methyl 40% + Hexaconazole 8% SC @ 200+40 g ai/ha was effective against sheath blight (12.59% and 20.74%) with highest yield of 45.75 and 53.42 q/ha respectively during Kharif 2010 and summer 2011. During summer 2010, application of Metaminostrobin 20% SC+hexaconazole 5% SC and Metaminostrobin alone gave higher grain yield 41.26 and 41.23 q/ha respectively. The combination was effective against sheath blight (25 and 16.11%) during Kharif 2009 and summer 2010. They also found that Trifloxystrobin 50% WG @ 200 g ai/ha recorded higher yield (47.66 q/ha and 50.17 q/ha) in both the seasons (Kharif 2010 and summer 2011). The stand alone formulation of trifloxystrobin 50% WG @ 200 g ai/ha was effective against sheath blight with PDI of 15 and 11.11% during Kharif 2010 and summer 2011 respectively.

PrasannaKumar *et. al*. (2011b) also reported that application of hexaconazole 75% WG @ 50g ai/ha, tetraconazole 11.6% w/w ME @ 1.0 L/ha and thifluzamide 24% SC @ 110 ai/ha were found highly effective in controlling sheath blight with increased yield when compared to untreated check. Thifluzamide a new fungicide group of carboxynilide was tested for its efficacy against sheath blight in three seasons (PrasannaKumar *et. al*., 2012). They found that among different concentrations, thifluzamide 24% SC at 110 g ai/ha was effective in reducing the disease severity [12 % (2005), 19.33% (2006) and 21.33 (2009)] when compared

to uncontrolled check [47% (2005), 62.33 (2006) and 59.67 (2009). Carboxynilide group fungicide was both preventive and curative in effect without phytotoxicity.

A combination of fungicide and insecticide were evaluated against important diseases and insects in rice during kharif 2007, 2008, 2009 and 2010 (PrasannaKumar *et. al*., 2011a). They found that during 2009, the ready mix formulation of flubendiamide 3.5% + hexaconazole 5% WG @ 85 g/ha were effective in controlling rice pests to maximum extent. The combination treatment recorded least sheath blight severity of 13.9% with the yield of 4190 kg/ha when compared to the standard check (40.6% and 2409 kg/ha).

Swamy *et. al*. (2009) reported that new fungicide formulations tricyclozole 400g + propiconazole 125g @ 0.25% and trifloxystrobin 25g + tebuconazole 50g @ 0.04% was on par with the standard checks hexaconazole 5% EC @ 0.2% and validamycin 3L @ 0.25%. Similarly, a new formulation Captan 70% + Hexaconazole 5% WP @ 0.2% was significantly effective in reducing the sheath blight of rice (Kiran Kumar and PrasannaKumar, 2011).

Foliar sprays of fungicides such as validamycin A in Vietnam, Thailand, Korea, Malaysia and Japan and pencycuron in Malaysia have been widely used (IRRI, 1993). First spray is applied between the stage of early internode elongation and the development of 2.5- to 5-cm panicle in the boot, and the second on 80-90% of emerging panicles from 10-14 days later. The best time to apply chemicals was at the jointing stage, during which time the percentage tiller infected was highly correlated with sheath blight at wax ripeness stage: percentage yield loss depended on disease index at wax ripeness (CPC, 2005).

7.3. Brown spot

Brown spot is one of the most important rice diseases in India. The disease affects the yield and milling quality of the grain. Sulpha drugs like sulphanilamide and antibiotics like nyastatin and griseofulvin have been used for seed treatment to control brown spot in rice. Spraying with captafol, edifenphos and zineb was also found to be effective (Chakrabarthi et. al. (1975). A new formulation Captan 70% + Hexaconazole 5% WP @ 0.2% was significantly effective in reducing the brown spot of rice (Kiran Kumar and PrasannaKumar, 2011). According to Sunder *et. al*. (2010), among six fungicides evaluated, propiconazole (2ml/l) proved most effective and reduced the brown leaf spot with significant increase in yield.

7.4. Sheath rot

Sheath rot of rice occurs in most rice-growing regions of the country. Chemical control of sheath rot has been intensively studied in India. Murty (1986) found that carbendazim, edifenphos and mancozeb (seed treatment and two foliar sprays around the booting stage) reduced sheath rot incidence significantly. Benomyl and copper oxychloride have also been reported to be effective in the field. However, studies by Lewin and Vidhyasekaran (1987) indicated that all fungicides they tested (captafol, carbendazim, carboxin, copper oxychloride, edifenphos, iprobenfos, iprodione, mancozeb, tridemorph, thiophanate-methyl and validamycin) were ineffective. Seed treatment with benlate and panoctine improves germination of sheath rot infected seeds (Alagarsamy and Bhaskaran, 1987). For field control of the disease, hinosan, bavistin, and

dithane M-45 proved to be effective. Fungicides like kitazin, benlate, difolatan, miltox, NF-48 and deconil were sprayed @ 0.2% separately on plants twice at 10 days interval during the flowering stage, could control the disease effectively. According to Raina and Singh (1980) and Chinnaswamy *et. al.* (1981), the most effective fungicide for the control of sheath rot was carbendazim followed by MBC, aureofungin and difolatan.

Effective combinations of fungicides (carbendazim) and insecticides (monocrotophos) to control sheath rot and leaf-folder, Cnaphalocrocis medinalis (Raju et al., 1988) resulted in lower incidence of sheath rot. Combined spraying of monocrotophos with any of the fungicides edifenphos, mancozeb and carbendazim resulted in reduced sheath rot and highest yields. Tridemorph + phosphamidon followed by carbendazim + phosphamidon and tridemorph + neem oil provided the best control and increased yield against sheath rot and rice mealybug, Brevennia rehi, (Lakshmanan, 1992). Two sprays of either thiophanate-methyl (Das et al., 1997), carbendazim (Das et al., 1997; Dodan et al., 1996) or propiconazole (Dodan et al., 1996) were highly effective in controlling rice sheath rot and significantly increased grain yield.

7.5. False smut

False smut has recently become an important disease of rice in India. Hybrids are more prone for this disease and fungicides have been extensively tested to manage the disease. Singh (1984) identified aureofungin, captan, captafol, fentin hydroxide, furcarbanil, mancozeb, and thiocyanomethylthiobenzothiozole to be effective in inhibiting conidial germination. Seed treatment with fungicides did not check the disease, but spraying the rice crop with carbendazim and copper fungicides at the time of tillering and pre-flowering effectively controlled the disease and yields increased (Anon., 1990). Copper oxychloride was most effective in decreasing disease incidence by 95.5 and 96.1% on the basis of infected tillers and grains, respectively, with a corresponding increase of 7.2% in grain yield (Dodan *et al*., 1997). Propiconazole or azoxystrobin applied during the boot stage of rice reduced the number of false smut balls in harvested rice grain by 50-75% but yield was not affected. Copper hydroxide fungicides reduced false smut balls in harvested rice by 80% but yield was also often reduced significantly. Barnwal (2011) observed that two sprays of propiconazole (0.1%) was found effective which recorded least false smut disease with number of affected florets panicle (1 of 4.13) with disease severity of 22.2 per cent and disease control over check of 77.6 per cent.

Singh and Singh (1985) have reported that 0.4% Bordeaux mixture or 0.25% COC sprayed thrice at 10 days interval starting when the crop is 60-65 days old gave about 90% reduction in disease incidence.

7.6. Stem rot

Stem rot disease is becoming more serious mainly in rain-fed crop. In India under field conditions, foliar application of mercurial fungicides like merculin and agrosan GN were found to be better than non mercurial like captan and thiram (Kang *et. al*., 1970). Hinosan, kitazin and brassicol were also found effective in reducing the disease with corresponding increase in yield (Jain, 1975).

7.7. Foot rot

Bakanae or foot rot disease is not widely distributed in all rice-growing areas of the country. However, the disease more serious in some endemic areas. Seed treatment with organo-mercury compounds is highly effective in controlling *G. fujikuroi* infection, but the use of these chemicals is no longer advisable due to ban on these groups. Benomyl, thiram and thiophanate-methyl have replaced organo-mercury seed disinfectants; these chemicals are now used extensively in Japan, Taiwan and Korea. These chemicals are highly effective against bakanae, rice blast and brown spot; with the exception that benomyl is not effective against brown spot (Okata, 1981).

Seed treatment with wettable powder containing ipconazole offered protection against seedborne diseases including bakanae (Tateishi *et al*., 1998). Seed treatments with benomyl + thiram and thiophanate methyl + thiram were more effective than carboxin + thiram. A drench treatment on seedlings did not provide significant control of the disease (Padasht *et al*., 1996). Seed soaking for 8 h in a suspension of emisan alone or emisan + streptocycline gave effective control of soil microflora including *G. fujikuroi*, followed by carbendazim + thiram (Sharma and Chahal, 1996).

7.8. Narrow brown spot

Narrow brown spot is generally not considered economically important, and no crop loss information is available on the disease. The commonly used fungicides *viz*., Benomyl and propiconazole controlled narrow leaf blight (*C. oryzae*) when applied as foliar sprays (Groth *et al*., 1990). In pot trials, fungicides that are effective against this disease include carbendazim (Singh, 1988).

From past three decades several fungicedes have been tested at All India Co-ordinated Research Centres in India for their efficacy against important diseases of rice (Table 3).

8. Future of fungicides

The ability of the pathogens to adopt to intensive cultivation of cereals and need to feed the increasing population will lead to increase in area of intensive cropping along with increase in consumption of fungicides. The key change in fungicide use have usually been associated with changes in the spectra of pathogens as well as in crop intensities, practices or prices. Shift in pathogen spectra could not be predicted and will continue to occur in the future due to increase in free trade. The R&D expenditures of major Agro companies on fungicides is > 60 % as against 40% for seed and traits. This ensures that new fungicides will continue to be developed to protect the cultivars species with no genetic disease resistance. Efforts are made develop a new strategy for environmentally friendly control of fungal plant diseases with the development of proteomics-based fungicides.

The trend towards a more judicious use of fungicides in combination with disease forecasting done would be continued which will help reduce the risk of adaptation by the target pathogen and at the same time will reduce residues in the environment and on the produce. The efforts

of breeding for disease resistance will increase along with tools of genetic engineering. Both genetic resistance and selective fungicides are prone to adaptation by the pathogen. Another new area of research is the use of antimicrobial peptides (AMP) for improving resistance to pathogens using transgenic plants as bio-factories for fungicides or bactericides. The balance between genetic and chemical control will continue and research on both areas will complement each other to assure the availability of effective combinations of host resistance and fungicides for crops to produce higher and quality produce.

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Chapter 5

Technology of Pesticide Application in Corn – Nozzles, Sprays Volume, Economic Analysis and Diseases Control

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Additional information is available at the end of the chapter

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1. Introduction

Brazil is the third largest producer, following by U.S. and China, respectively. According CONAB (2011); the annual corn production in Brazil was approximately 50 million tons in an area equivalent to 15,2 million hectares. About 45 % of this area is sowed in the second-crop season (winter), and 70% is sowed in regular (summer) season. The Brazilian corn yield (average summer and winter) is around 4,3 tons per hectare, while the U.S. average is 9.6 tons per hectare. One of the explanations is due to lower climate potential for yeld in second crop season (winter), low application technology, which turns out to be a limiting factor for achieving the crop genetic potential. In U.S., there is a more favorable climate, suitable for larger productions. However, the lack of technology investments in Brazil is presented as a major deterrent. This lack of investment reflects a serious problem, while the demand for food is increasing and areas for expansion of agriculture are being reduced.

According to Agrianual (2011), the harvest of 2010/2011 season showed that the world's leading producers of corn are: the United States (331 million tons), China (148 million tons), Brazil (50 million tons), Argentina (24 million tons) and Mexico (23 million tons), which respectively, with 42.9%, 19.2%, 6.5%, 3.4% and 2.9% of world production (771 million tons). Nationally, the corn area is approximately 14.640 million hectares and total production about 49.848 million tons in the 2010/2011 season, placing it among the major grain yield. In early 2000, the use of fungicides has been intensive in order to promote productive lower losses, caused by foliar diseases. Nowadays, many corn-producing regions are used to spray fungicides within their technological package. Besides the introduction of new products registered by the Ministery of Agriculture and Food Supply (MAPA), there was a significant increase in application technology, especially by the application of chemicals through

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aviation. The success of the plant protection depends largely on: identification, quantity and location of the target to be reached. The size, shape, nature of the surface and other characteristics influence on the retention of sprayed droplets (JULIATTI, NASCIMENTO, REZENDE, 2010). According to the characteristics of the target and environmental conditions, should be select the most effective equipment to reach it by finding the lowest possible pesticide waste and greater biological effect (BOLLER; FORCELLINE, 2007).

Mato Grosso is the warmest and biggest corn production state in Brazil. In this region, a technique known's as aircraft with "low volume oil" (substitution of water for another means) (Monteiro, 2003, Ozeki, 2006) has been used to reduce the sprays volume and also the drop's evaporation. This technique has been successfully used in the control of several corns' disease in different production regions.

2. Diseases evolution on maize fields in Brazil

From the 90's (JULIATTI et al., 2007) foliar fungal diseases had increased it's incidence and severity and causing significant qualitative and quantitative reduction in maize production. These diseases are: a white leaf spot caused by a combination of Pantoea ananatis, and fungi,

Photos. F.C. Juliatti

Figure 1. Fungicides sprays in maize after blossom starting

Phoma sorghina and Phaeosphaeria maydis, Cercospora leaf spot (Cercospora zeae-maydis), rusts caused by Puccinia sorghi, and Puccinia polysora, Phyzopella zeae, and leaf spot by turcicum (Exserohilum turcicum). Frequency of outbreak by Stenocarpella macrospora has been increased with high plant density, spaced by 0,45 or 0.50 cm between rows. In Brazil, hybrid resistance and fungicides sprays used in different stages (V6 to V8, R1 (figure 1), are the most important tools for diseases management The main diseases in these stage are: cercospora and stenocarpella leaf spot, rusts and white or phaeospaheria spot (figure 2)..

Juliatti et al 2007, studied fungicides residual in corn and established the period of control in 25-30 days. The timing of application depends on: initial inoculums and environment conditions. The hybrid response depends on: disease inoculums levels, environment conditions and genetic response (resistance level) (Figure 3). The figure 2 show the main diseases in Brazil

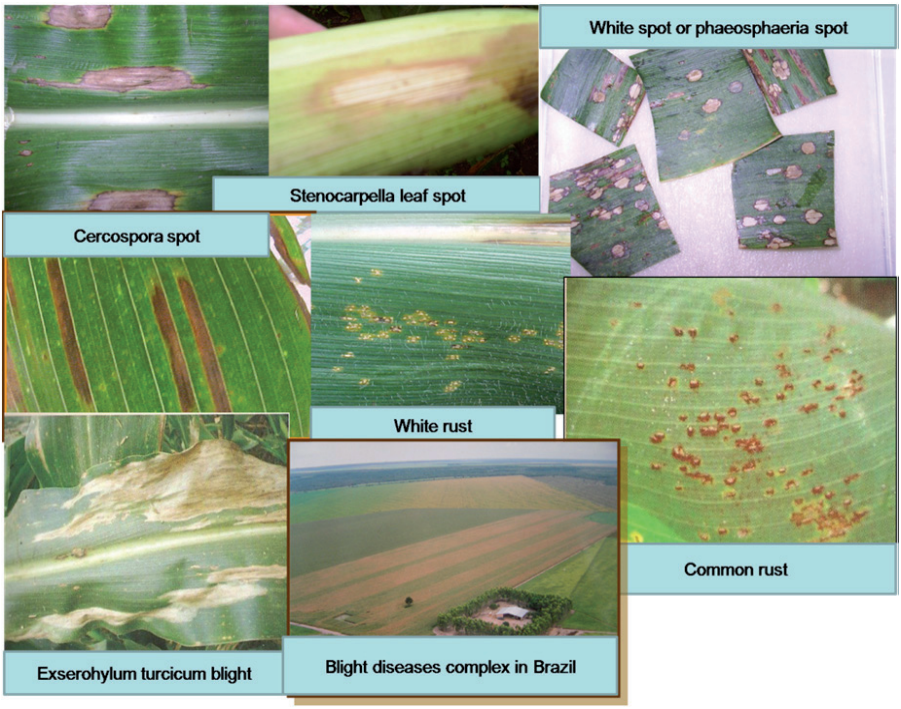

Figure 2. Mainly diseases in Brazil for fungicides spray on leaves

In 2011/2012 crop season, the majority of commercial hybrids in Brazil presented highly susceptibility to *Puccinia polysora* (rust), following by *Exserohilum turcicum*, grains healthy quality, *Fusarium* and Stalk rot diseases [Jaccoud Filho, 2011] (Table 1).

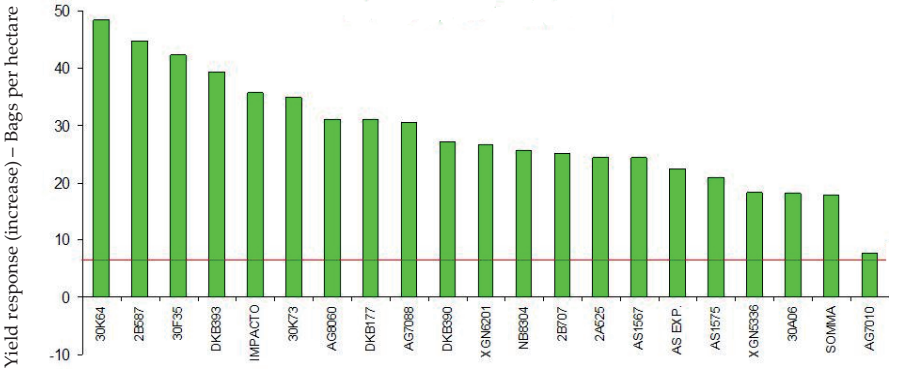

Figure 3. Hybrid response in Brazil from two sprays (V8 and R1)

	Percentages					
DISEASES	**HS+S**	**MS+MR**	**HR+R**	**LT+MT**	**T+HT**	**NI**
Fusarium xylarioides *Fusarium graminearum*	1,63%	31,90%	2,45%	17,79%	6,33%	39,87%
Puccinia sorghi	2,04%	59,10%	6,33%	14,11%	13,08%	5,31%
Physopella zeae	7,15%	40,49%	5,93%	14,72%	3,47%	27,60%
Puccinia polysora	17,38%	56,23%	5,52%	8,17%	8,17%	5,11%
Phaeosphaeria maydis and White leaf spot complex	7,15%	59,30%	4,49%	13,49%	11,65%	3,88%
Stunting	7,77%	36,40%	5,11%	8,38%	13,49%	28,83%
H. turcicum	2,04%	63,80%	3,27%	12,47%	12,06%	6,33%
H. maydis	4,29%	34,76%	2,86%	11,86%	7,56%	38,85%
Cercospora zeae-maydis	6,74%	54,60%	4,08%	13,70%	8,99%	11,86%
Stalk rot	1,63%	53,57%	6,33%	10,83%	16,97%	10,63%
Grains´ healthy quality	2,24%	54,39%	8,17%	10,83%	16,15%	8,17%

Source: Jaccoud Filho et al., 2011

Legends: HS: Highly Susceptible; S: Susceptible; MS: Moderate Susceptible; MR: Moderadate Resistant; HR: Highly Resistant; R: Resistant; LT: Low Tolerance; MT: Moderate Tolerant; T: Tolerant; HT: Highly Tolerant; NI: No information available.

Table 1. Susceptibility levels of commercial corn's hybrids in the crop season 2011/2012, in relation with the main pathogens and diseases.

Several factors has been contributing to the diseases incidence increase in corn: cultivated area expansion, hybrids with high differences on resistance level, inadequate management

of water in crops under pivot irrigation, direct sowing system, row spacing reduced (0,45-0,5 m) and low crop rotation practice, increasing the pathogens initial inoculums (PINTO et al., 1997, JULIATTI, et al. al., 2007).

According to EMBRAPA (2005), the grains can be damaged by fungi at pre-harvest (ear rot fungi with the formation of damaged kernels), during the post-harvest processing, storage and transport (musty or moldy grains). In the process of colonization of grains, many species of fungi known are toxigenic (*Fusarium* spp., *Aspergillus* spp., *Penicillium* spp.) The most common damages are: discoloration of grains, reductions in the contents of carbohydrates, proteins and sugars and production of toxic substances called micotoxins.

3. Spray technology, nozzles, droplet diameter, fungicides action and disease control

The correctly fungicides use can reduce the amount of damaged kernels combined with an appropriate spraying technology. In this context, the staff of the UFU plant, management, in partnership with the Club of Friends of the Earth (Clube amigos da Terra- CAT) in Uberlândia - MG (Growers Council from Uberlândia, Minas Gerais state), has been developing over the past six years, studies on the appropriate response of maize hybrids in relation with disease's evolution, estimates of losses, responses to fungicides (DMI's, QoIs and mixtures of QoIs and DMI's), spray technology (adjusting equipment for the best volume, aircraft and terrestrial equipments, evaluating the type ends flat fan, cone) and impact in the disease's control. Were studied volumes of ranging from 100 to 200 $L.ha^{-1}$, for spray by self-propelled land and air ranging from 10 to 30 $L.ha^{-1}$.

The droplet diameters are one of the most important and represents a spray droplet size expressed in (mm). It depends on the type of target, flow rate, pressure spray and equipment conditions. As the size of droplets produced in a spray is not uniform, the diameter is represented by a number, which may be the volume median diameter (DMV), or the number median diameter (DMN). The DMV is the droplet diameter that divides the spray volume into two halves, while the DMN is the droplet diameter which divides the number of drops of a spray into two halves, which are placed in ascending or descending order of size (BOLLER; FORCELINE, 2007; JULIATTI, NASCIMENTO, REZENDE, 2010). Thus, according to Boller and Forceline (2007), for application of pesticides, the ideal wind speed is between 3.2 to 6.5 $Km.h^{-1}$. However, the absence of winds may be associated with the occurrence of convective air currents, which can keep the drops of a finer spray suspension, leaving them susceptible to wind action. The droplet size generated by the machine depends of the spray solution properties, the type and size of the tips orifice and the pressure that the liquid is subjected to pass for their tips. The factors that makes more influence in the application technology are: target distance and environment conditions (humidity, temperature, wind speed). Is essential to control these factors to insurance the quality of application. According Forceline and Boller (2007), the air-induced droplets, is not recommended for fungicides application, because usually diseases epidemics starts on the lower plant dossel, and air induced droplets concentrate the fungicide in the middle to the top of plant dossel. Although, these technology

generates larger droplets, that can travel the distance between the generating source and the target in less time and can settle this or suffer driftage.

Drift is one of the most serious problems that may occur during a pesticide application. The drops generated by ground sprayers, agricultural aircraft among others, can be carried by wind or air currents reaching upward causing losses and unwanted places, contaminating areas near or distant of the target, off-site sprays. Miller (2006), in the case of aerial application, the higher flying height increase the distance that the drop has to go to reach the target and the longer it will be prone to meteorological factors, thus being more prone to suffer (drift) wind action. According to Christofoletti (1996 and 1999), very fine droplets (diameters smaller than 100 microns) are still hovering in the air for a long time and may evaporate or be carried by air currents away from the biological target, making losses due to drift and contaminating the environment. The smaller the droplet diameter, the greater susceptibility to drift. The air resistance to the free fall of a drop is inversely proportional to this diameter, as can be seen in Table 2.

Diameter of drops (μm)	Classification	Drift distance
500	Light rain	2 m
200	Drizzle	5 m
100	Fog	15 m
30	Clould	150 m
15	Aerosol	610 m

Source: Cristofoletti, 1999; Boller et al. 2007, Juliatti et al. 2010, Santos 2012.

Table 2. Classification of drops by size and horizontal drift distance of droplets released into free fall, the 3 m in height and crosswind of 5 km h^{-1}.

As the distribution of droplets size, coming from a hydraulic power machinery, can be very heterogeneous, some of these will be more susceptible to drift, making the potential risk of drift (PRD). It is understood by the PRD, the percentage of spray volume composed of droplets smaller than 150 micron, which can be lost through drift and evaporation. International standardizing societies, as BCPC (British Council Crop Protection) and ASAE (American Society of Agricultural Engineering) established the limit of six categories of "spray quality", based on droplet size (Table 3).

Categories of spray (Quality approximate)	Aproximate DMV (ASAE standard)	DMV (BCPC standard)	PRD (BCPC standard)
Very thin	<150	<119	57 %
Slim	150 – 250	120 – 216	20 – 57 %
Average	250 – 350	217 – 352	5,7 – 20 %
Thich	350 – 450	353 – 464	2,9 – 5,7 %
Very Thick	450 – 550	>464	<2,9 %
Extremely thick	>550	------	------

Source: Adapted from Brown-Rytlewski and Staton (2006).

Table 3. Categories of drop size standards of a second spray ASAE and BCPC and potential risk of drift (PRD) and the respective sizes of the droplets.

The table 4 presents some results in corn compared aerial and terrestrial applications in disease control. According to the results from this table, the best control of main maize diseases in the field from savanna conditions in Brazil was the terrestrial application with the combination of the 150L.ha^{-1} volume and the flat fan nozzle deflection double droplets. In the other hand, terrestrial application is not practicable when the crop achieve the blossom stage. In this case aerial technology is more effective. According to table 4, the aircraft technology was more effective with 15 L.ha^{-1} volume.

Treatments	White leaf spot* AUDPC (* Phaeospahaeri*a* leaf spot)	Cercospora leaf spot AUDPC
1 – Air (30 L ha^{-1})	808.75b	242.12b
2 - Air (15 L ha^{-1})	195.75 a	308.75bc
3 - Terrestrial (150 L ha^{-1}, deflection nozzles with air induction)	257.12 a	323.25bc
4 –Terrestrial (150 L ha^{-1}, the flat fan nozzle deflection double)	368.75 a	30.37 a
5 -Terrestrial (100 L ha^{-1},nozzes deflection with air induction)	220.87 a	277.37bc
6 - Terrestrial (100 L ha^{-1}, the flat fan nozzles deflection double)	679.37 a	390.75c
7 -Check (Untreated)	1250.62c	593.75d

Means followed by different letters in columns differ significantly at 5% probability by t test (LSD). Juliatti et al 2010.

Table 4. AUDPC - area under the disease progress curve for White and Cercospora leaf spot in corn.

Cunha et al 2010, evaluated the effect of the aerial and ground application of fungicide in the control of corn diseases and in the spray deposition on the canopy. The hybrid AG7010 was used in this study and the spray was laid up on the bottom, middle and upper canopy of the crop. The disease severity and yield were evaluated after the application of the fungicide (pyraclostrobin + epoxiconazole), at the V8-V10 stage. The aerial application was accomplished with spray volumes of 15 and 30 L ha^{-1}, using flat-fan spray nozzles, and the ground one with 100 L ha^{-1}, using turbo twin flat-fan and air induction turbo flat-fan spray nozzles. An additional treatment that received no fungicide was also evaluated. The study of the deposition was achieved using water sensitive papers. It was concluded that the conventional treatments presented larger droplet density in the corn canopy; however all of the treatments provided the minimum deposition recommended for fungicide application. The aerial application using spray volume of 30 L ha^{-1} provided similar yield to the conventional treatments, showing to be technically feasible to use. Although AUDPC and economic analysis weren't assessed in this study.

The definition of parameters such as droplet size and spray volume depends directly on the relation target/pesticide. Systemic products can be sprayed at lower droplet density, allowing the use of larger droplets.This facilities the adoption of drift reduction techniques, improving safety on spraying and increasing their operational efficacy. When used

correctly, larger droplets assure a good deposition level (amount or volume deposited on the target), it's not necessary the best coverage.Then the results from table 4 confirmed this hypothesis and objective. By the way contact fungicides and those limited systemicity require the use the smaller droplets and or greater volumes, due to the greater dependence on better target coverage. For example, when the spray target includes the lower or inner parts of a plant, such as spraying for Cercospora leaf spot, rusts and white spot (phaeospaheria leaf spot), a good droplet penetration cloud is required an, therefore, smaller droplets should be desired.

An important characteristic for the definition of maize diseases control strategies, in relation to spray technology, is the mode of plant movement of the systemic fungicides after spraying and absorption. Most fungicides used in the fields in the market today possess leaf movement only from the base to the tip of each leaf, with minimum chance of translocation from one leaf to another. This means that, although these products are classified as systemic, application technology must provide a good coverage and penetration of the droplets through the leaf mass. In most cases, to obtain good control adequate leaf coveraged is needed, with emphasis on the lower parts of the plant, where the disease starts.

Still in the case of plant directed spraying, a study of target characteristics should include leaf movement (wind or air assistance), development stage, wax layer, hair layer, roughness, leaf surface (upper/lower) and plant architecture. These factors are fundamental for the definition of leaf droplet retention (choice of thick or fine droplets) and plant tissue penetration by the fungicide. Similarly, differences are expected on the spray technology requirements for different maize hybrids (figure 3).

The application technology is a major factor for crops success, because it determinates the correct application of pesticides. The experiment was conducted at Fazenda Mandaguari (Indianópolis-MG-Brazil). The experimental design was randomized in blocks with 13 treatments and 4 replications. The treatments were 4 points (TT, AD / D, ADIA / D, Cone Empty) and 3 volumes of solution (100, 150 and 200 L ha^{-1}) and the control. The objective was to develop the study of different types of nozzles and spray volume for the rational diseases control in corn. We evaluated the severity of disease, drops cm^{-2}, %green area, weight of 1000 grains and yield, being held in an economic analysis. Relative to Spot Stenocarpela, all treatments proved superior to the control. Nozzle ADIA received the least amount of drops cm^{-2} bottoms' of the plant. All treatments were superior to the control in relation to %green area. All treatments showed an increase in 1000 grain weight compared to control treatment, showing the direct control of diseases with the grain filling. The highest yield was obtained when we used the volume of 100 L ha^{-1} in all points evaluated. The economic analysis demonstrated the feasibility of a fungicide application to ensure sustainability of maize yield (figure 1 and table 5,6,7, 10 and 11).

The spray volume is on of fundamental parameters for spray success. The definition of spray volume depends on the type of target to be reached, required coverage, mode action of fungicide and spray technique, among others factors (table 5).In maize is very important a reduction of spray volume and increase the of fungicide concentration on the leaves (upper,

middle and lower parts of plants - canopy) (table 5)The spray volume also affects the operational efficacy of spraying, since the time spent n loading significantly changes the sprayer operational capacity (number of hectares treated per hour). Antuniassi (2006) reported average spray volume for rust or late cycle diseases control, tractor spraying can use from 100 to 300 L ha^{-1}, depending on the region. Base in the recent data(tables 5,6 and 7) growers can use 100-200 L.ha^{-1} to control maize disease. In airplane spraying the values for different kinds of pesticide vary, on average, from to 5 to 30 L.ha^{-1}. For maize diseases control the best spray volume was 15 L ha^{-1} (Table 4). Usually spraying very small volume is done with very fine droplets, which increases the risk of losses, especially due to evaporation or drifting. In contrast large volume can cause spray saturation of leaves and dripping. In general, it is recommended that very low or ultra low volumes be sprayed with methods that control water evaporation, or even the substitution of water for another means. An example of this technique is the use of oil as a surfactant to reduce evaporation during low volume spraying. (aircraft with "low volume oil")

Treatments	Volume (L ha^{-1})	Nozzles
1	100	AD/D 11002
2		ADIA/D 11002
3		TT 11002
4		Empty Cone MAG 02
5	150	AD/D 11002
6		ADIA/D 11002
7		TT 11002
8		Empty Cone MAG 02
9	200	AD/D 11002
10		ADIA/D 11002
11		TT 11002
12		Empty Cone MAG 02
13	Untreated	---

Table 5. Treatments nozzles and spray volume).

Nozzles	Drop	Drift distance Risk	PRD*
Empty Cone MAG02	Very thin - Thin	Hight - Average	MF = >57% F = 20-57%
TT 11002	Thin - Average	Average - Low	F = 20-57% M = 5.7-20%
AD 11002	Thin - Average	Average -Low	F = 20-57% M = 5.7-20%
ADIA 11002("Air indution")	Thick – Very thick	Very Low	G = 2.9-5,7% MG = <2.9%

* PRD=Drift risk potential

Table 6. Nozzles characteristics and drop drift distance risk classification. UFU, Uberlândia, 2007.

Treatments	Means*
Untreated (Check)	15.94 b*
TT – 100 L ha^{-1}	4.86 ab
TT – 150 L ha	4.68 ab
TT – 200 L ha	2.46 a
ADIA/D – 100 L ha^{-1} ("Air indution")	8.00 ab
ADIA/D – 150 L ha	10.32 ab
ADIA/D – 200 L ha	9.32 ab
AD/D – 100 L ha	8.32 ab
AD/D – 150 L ha	2.32 a
AD/D – 200 L ha	4.92 ab
Empty Cone – 100 L ha	4.26 a
Empty Cone – 150 L ha	3.66 a
Empty Cone – 200 L ha	5.48 ab

Means followed by the same letters not are different by Tukey test by LSD 5 %.

Table 7. Severity of Stenocarpela leaf spot (*Diplodia maydis* and *Diplodia macrospora*) after 28 days spray in different treatments.

In the tables 8.9,10 and 10 showed the economic analyses for the maize diseases control after different spray and nozzles.

Nozzles	Means*
Empty cone (empty cone spray)	56,33 a
TT (plain spray)	47,38 ab
AD (plain spray)	24,88 ab
ADIA (plain air induction)	21,22 b

*Means followed by the same letters not are different by Tukey test by LSD 5 %.

Table 8. Means of number drops in the leaves from lowest of maize plants

Another important parameter for a good spraying is droplet density, generally expressed as drops.cm^{-2} (table 8). The efficacy of a greater or lower droplet density is related to mode of action of the pesticide (systemic, contact, etc.). For fungicides spray Matthews (2000) recommended 30 to 70, herbicide (20 to 40) and insecticide (20 to 30). In this case Empty cone (empty cone spray) and TT (plain spray) showed the best values (table 8).

The droplet size class affects the ability of a spraying to cover the target (figure 1). And penetrate through the canopy. Smaller droplets have better coverage capacity (empty cone, table 8) (offer greater number of droplets/cm^2), as well as better penetration are

required. However, small droplets can be more sensitive to evaporation and drift processes. Thick droplets are preferred in the soybean production system for spraying herbicides with major systemic action, which are used for dessecation, such as glyphosate, while fine droplets are more used for insecticides and fungicides. The same response it's true in maize fields.

Adequate droplet size is very important for a good fungicides deposition on the target , and at the same time, avoid drift losses. Each type of nozzle produces a spectrum of droplet sizes, depending on operational pressure. Fine or thick droplets have different capacities for each spraying situation, as illustrated on the tables 5, 6 ,7 and 8.

Treatments*	LHA- Green leaves (%)	Grain storage – Heavy of 1000 grains(g)	Yield (sc ha^{-1})
Testemunha	40.32 b	280.35 b	136.3 b
TT – 100 $L.ha^{-1}$	63.34 a	321.25 a	171.6 a
TT – 150 $L.ha^{-1}$	59.66 ab	304.20 ab	153.2 ab
TT – 200 $L.ha^{-1}$	64.66 a	317.45 ab	152.5 ab
ADIA/D – 100 $L.ha^{-1}$	56.66 ab	314.35 ab	153.0 ab
ADIA/D – 150 $L.ha^{-1}$	54.00 ab	295.25 ab	142.8 ab
ADIA/D – 200 $L.ha^{-1}$	58.00 ab	302.85 ab	145.1 ab
AD/D – 100 $L.ha^{-1}$	60.32 a	322.90 a	167.1 ab
AD/D – 150 L. ha^{-1}	60.32 a	310.30 ab	146.7 ab
AD/D – 200 L. ha^{-1}	56.00 ab	298.25 ab	145.8 ab
Empty Cone – 100 L. ha^{-1}	67.32 a	325.60 a	157.6 ab
Empty Cone – 150 L. ha^{-1}	54.66 ab	311.30 ab	155.7 ab
Empty Cone – 200 L. ha^{-1}	57.98 ab	306.70 ab	146.1 ab

*Means followed by the same letters not are different by Tukey test by LSD 5 %.

Table 9. LHA – Leaf Health foliar area (%), grain storage – One thousand grain heavy (g) and yield in bags.ha^{-1} from different nozzles and volumes.

Source of variation	Price US$ (Liter)*	Dose (L.ha^{-1})	Coast US$. ha^{-1}
Fungicide (Azoxistrobina + Ciproconazol)	100.00	0.3	30.00
Maquinery operation	---	---	6.00
Total			36.00

• Notation in 06/13/2005

Table 10. Economic analyses by hectar in relation fungicide and machinery

Volume	Nozzles	Increment in bags*	US$ by maize bag**	Increment in US$ - by ha^{1} (Two sprays R1 and R3)	Net Return – Superavit (US$ ha^{-1})
100 L ha^{-1}	TT	35.3	10.00	364.45	353
	AD	308	10.00	317.62	308
	ADIA	16.6	10.00	171.19	166
	Empty Cone	2.,3	10.00	219.65	213
150 L ha^{-1}	TT	16.9	10.00	174.28	169
	AD	10.4	10.00	107.25	104
	ADIA	6.5	10.00	67.03	65
	Empty Cone	19.3	10.00	199.03	193
200 L ha^{-1}	TT	16.1	10.00	166.03	161
	AD	9.4	10.00	96.94	94
	ADIA	8.7	10.00	87.72	87
	Empty Cone	9.8	10.00	101.06	98

** Bags of 60 kg in notation at 06/13/2005 – Uberlandia board of trade

* Increment = Maize bags.ha^{-1} in relation the untreated (Check) – Net Yield

Table 11. Net return or superavit after economic analyses by héctar in relation fungicide and maquinery.

It is important to highlight that even when a nozzle producing mostly thick droplets is used, a fraction of the volume sprayed will be formed by fine droplets (sensitive to drift process). This means that a given nozzle does not produce all droplets of the same size, but in a range of droplet sizes (known as spraying spectrum). For a given nozzle, the greater the percentage of fine droplets taking part of the spectrum, the greater the drift risk . This concept has bean used in several countries to standardize a ne w nozzle classification, in which the "drift risk" is evaluated. This classification is based in a comparison of the drift reduction percentage of the nozzle evaluated with that of a standard nozzle. In the countries where this concept was implemented (mostly Europe), some pesticides have a package label recommendation for the type of nozzle to be used, as a function of its drift reduction potential.

4. Environmental conditions

Besides the spray volume another fundamental parameter for treatment success is the adaptation of the technology to the environmental conditions at the spraying time. In most cases spraying should be avoided when air relative humidity is below 50 % and air temperature is above 30º C. In the presence of wind, it is ideal that spraying should be done at wind speed between 3 and 10 $Km.ha^{-1}$. The lack of wind also could be harmful, since there is a chance of ascending hot air, hindering the deposition of small droplets.

Early in the morning, late afternoon or in the evening, when air relative humidity is higher and temperature lower, are considered more adequate for spraying. From a practical standpoint, it is possible and advisable to use fine droplets at these times table 3 – DMV 150 – 250).However, it is necessary to monitor environmental conditions throught the day, for in the case of a considerable rise temperature (with a drop of air moisture), the droplet pattern needs to be changed (using larger droplets).In this case, the spray volume should be incresead to avoid a negative effect on target overage. Rainfall and dew are weather factors that also require attention when planning spraying. In the case of rainfall, care should be taken on noting the minimum time interval between spraying and rainfall, providing the minimum time interval between spraying and rainfall, providing the minimum time required for production action. In the case of dew, the presence of water in the leaves during spraying at night (dawn) and/or early I the morning can interfere on spraying technique. In such case, problems can be found either by product dilution or by eventual dripping, due to excess water and the action of surfactants in the spray mixture. However, there are situations, depending on the technique used and type of pesticide used, where dew can be beneficial. A night spraying should also consider the existence of technical limitations in relation to the pesticides, such as efficacy and absorption speed in the absence of light or low temperatures.

5. Spray surfactants

The use of spray surfactants has become very popular despite the little knowledge about the function of each type of substance. Listed below are the most commonly used surfactants,

according to some of their expected functions: oils (vegetable or mineral) – reducing evaporation and easing penetration, urea – absorption, ammonium sulfate (pH adjustment), spreaders (increasing contact area), adhesives (increasing product adhesion to the plants), chelating (reduce ion reactivity, facilitating the joint spray of foliar fertilizers and/or use of hard water), dispersers (reducing settling); moisturizers – reduce evaporation, emulsifiers (facilitates mixing) and drift reducers (some thickeners decrease the formulation of very small droplets). Most of the problems related to the use of surfactants in tank mixtures come from the lack of knowledge of their mode action and the implication of their use. As an example, the process of droplet formation from the nozzle can be significantly altered by a change on mixture physical characteristics, especially by the use of some formulations and by adding surfactants.Thus, basic factors as droplet size and spectrum can be altered in a more significant way by varying the mixture than by changing spray nozzles. Therefore, the use of surfactants should be preceded by a rigorous study of the real needs of the spraying system. Butler-Ellis (2004) characterized the process of droplet formation for a plane jet nozzle with the use of surfactants. The author considered the change in shape of the liquid film during droplet formation of different mixtures under the same conditions of spray pressure and outflow. These characteristics affect the final size of generated droplets. In this case, the use of an emulsion led to the increase on droplet size, with an opposite effect of spraying with surfactant.

6. Tractorized spraying, airborne for maize diseases control

The use of different droplet sizes and mixture volumes can result in situations with greater or lesser leaf coverage, potentially affecting fungicide performance on control. Table 2 to 11 show results of leaf surface coverage obtained with tractorized spraying in Inidianópolis, Minas Gerais state from Brazil. It was used different nozzles and droplet sizes, for each spray volume. In general, leaf coverage level was affected by droplet size, with lower coverage intensities for very thick droplets produced by air induction nozzles. This characteristic was more important when the leaf evaluated was on the lower part of the plant, where significant differences were found. In the case of very fine droplets there was a trend of better coverage for hollow cone jet, although there were no differences in comparison with double flat and flat nozzles.

In general the results obtained indicate that for situations where greater droplet coverage and penetration are fundamental for spraying success, typical of maize diseases control, air induction nozzles should be avoided and those forming fine or very fine droplets should be preferred. Among these nozzles, a clear trend of better coverage and penetration performance for the hollow cone jets was observed. However, it is important to note that this study Was done on normal weather conditions for spraying (temperature, air humidity and wind speed within the maximum recommended limits).In the case of spraying under less favorable weather conditions, the use of very fine droplets should be avoided due the great risk of drift or evaporation., and nozzles producing fine or medium droplets should be preferred. If somewhat larger droplets are used, the coverage potential can be

compensated by an increase in spray volume. It is important to remember, still, that leaf coverage values observed in the medium and lower thirds of the plants indicate a need for attention about coverage differences generated by different nozzles (or droplet standards), since these differences can have a greater or lesser meaning depending on the type of disease and fungicide used for its control. Still in the case of tractorized spraying, several new technologies are becoming available. The use of low volume became popular, specially with rotation disk atomizers, using volumes as low as 25 $L.ha^{-1}$ with additional of oil in the mixture. Although the existing research is not enough to safely base maize diseases control, field data made available by several users indicate that such a system has good performance. On the same line as low volume spraying is the use of the electrostatic system, which has also become an option for spraying. About 20 $L.ha^{-1}$. Other technologies such as bars with air assistance and the use of nozzles with angulations relative to machinery movement, also have been evaluated for their potential for maize diseases control.

7. Airborne X tractorized application

The increasing demand for quick and effective spraying is one of the major characteristics of the agricultural market nowadays, considering the importance of the spraying time on the success of maize diseases control. Therefore, there is an especial interest on airborne spraying (table 4).Similarly to tractorized sprayers; there are several technologies available for airplane spraying. The conventional systems (flat or conical jet nozzles) are used to spray volumes of 20 to 40 $L.ha^{-1}$. Low or ultra low volume spraying (up to 15 $L.ha^{-1}$) requires electrostatic systems (Spectrum) and rotative atomizers (Micronair, Turboero, etc.). A good part of these reduced volumes spraying is done with surfactants, such as oils (vegetable or mineral) to reduce droplet evaporation and improve product absorption.

Airborne spraying is an activity that demands important investment on system management. Even if the spraying technology choice is correct, several others factors are extremely important for success on phytosanitary control. Factors as flight height, work width, wind position, temperature and moisture and navigation systems (GPS). Usually, applying larger volumes without adding oil, demands lowers flights (about 3 m high), while spraying low or ultra low volumes (with oil) require higher flights (5 meters, for example). Similarity, the work width has to be adjusted for each case. Spraying with volumes between 20 and 40 $L.ha^{-1}$ employ widths of 15 meters, while spraying with lower volumes use widths larger than 20 meters. Wind position is one of the most important factors to assure good coverage of spraying bands. The airplane should always be positioned across the predominant wind, favoring band coverage. In contrast, a narrowing of the spraying bands can occur, as a super positioning error and control failure. Another important factor of airplane navigation system reports (GPS), to detect eventual failures of sprayed bands. Finally, air temperature and relative humidity should be adequate at spraying time to reduce risks of loss and drift. In airborne spraying with low volume and oil added (LVO, for example), special care should be taken to avoid working during high

temperature and lack of wind, avoiding convective currents that hinder droplet deposition on the culture and considerably increase risk of failure and drift. Summarizing in management terms, the decision by airborne or tractorized spraying should be take several factors into account. Among the major ones are the operational capacity (number of hectares per hour), the cost per treated hectare (table 10 and 11), the predominant weather conditions, availability of service in the area, eventual regional traditions (some regions use tractorized or airborne by tradition) and the potential for mechanical damage to the culture (some authors estimate up to 3 % losses by culture lodging in the case of tractorized spraying at the end of maize cycle.

8. Conclusion

When we look Brazil maize yield some years ago (less than 3,000 $Kg.ha^{-1}$), fungicides were not necessary but, nowadays, if we consider a range of growers harvesting more than 10,000 $Kg.ha^{-1}$, the use of fungicides and spray technology is essential to improve grain production. DMI and strobilurins and DMI plus strobilurins fungicides are needed for maize production sustainability in Brazil. It happens because Brazil has a tropical pathosystem conditions and it has several polycyclic diseases (rusts, blights, cercospora leaf spot or GLS, stem rot, ears rot, bacterial blight, etc.).The number of sprays, and the technology cost depends on many factors, such as is leaf area, plant architecture, hybrid resistance, environmental conditions and grain diseases. However the diseases complex increases in a high speed in tropical conditions, we can observe that the problem also advances in subtropical countries in North America and Europe. In the future, Brazil experience on maize disease control can be used in the others countries in all continents.

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Chapter 6

Chemical Control of Eucalyptus Rust: Brazilian Experiences

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Additional information is available at the end of the chapter

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1. Introduction

Eucalyptus (*Eucalyptus* spp.) naturally occurs in Australia, Indonesia and neighboring islands such as Flores, Alor and Wetar. The genus *Eucalyptus* belongs to the Myrtaceae family, with around 600 species and sub-species, and shows high plasticity and worldwide dispersion, growing satisfactorily under different edaphoclimatic conditions and surpassing those of the regions of origin. Less than 1 % of these 600 species have been used for industrial purposes. In essence, the use of eucalyptus in the worldwide industry is based on two species, mainly: *E. globulus*, *E. grandis* and their hybrids with *E. urophylla*, and hybrids between *E. saligna* and *E. camaldulensis*, planted in large scale in Brazil. These Eucalyptus trees are used for the production of paper, cellulose, wood, coal, cluster, sawmill, furniture, oils for pharmaceutical industries, honey, windbreak, and in civil construction and ornamentation.

The importance of eucalypt plantation for Brazil can be assessed based on the participation of the forest sector in the economy's country. Initially supported by governmental tax incentives for reforestation and later by the National Programs for Steel Industry and Charcoal and for cellulose and paper production. Currently, the estimated area of eucalyptus crops in Brazil is of 4.5 million hectares, occupying 66% of the Brazilian reforested area in 2010 [1].

Eucalyptus was considered a genus practically free of diseases until 1970's. However, the progress of reforestation areas to warmer and wetter regions, with the planting of more susceptible species and the repeated use of the same area for planting, created favorable conditions to the occurrence of diseases. Among the latter is rust disease caused by *Puccinia psidii* [2, 3]. Besides the eucalyptus, the pathogen infects other species of *Myrtaceae* [4]. Severe disease infection may cause deformation and necrosis in the shoot of the host reducing the volumetric growth [5-7].

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Eucalyptus rust caused by *Puccinia psidii* Winter is currently a very common and severe disease affecting crops of eucalyptus, which is highly susceptible to the disease when it is younger than two years old [8]. Native of South America, rust was first reported in Brazil in 1929 [9] and formally described in 1944 [10]. Nowadays, it is one of the most important diseases of *Eucalyptus* in the country. It affects both seedlings in the nursery and young plants, up to two years old, in the field, reducing the culture productivity and sometimes leading the most susceptible species to death. It may also infect shoots after clear-cutting and clonal gardens and mini-gardens. The first considerable damages were caused in Espírito Santo in the 1970's to crops of *Eucalyptus grandis*, which were less than two years old and imported from South Africa (IPEF). In São Paulo State, the first cases of this disease were found in commercial crops of this same species in the 1990's. High infection rates were also detected for both nurseries and crops in the regions of Vale do Rio Doce, Minas Gerais, Espírito Santo and South of Bahia.

According to a survey carried out by Furtado & Marino (2003), *P. psidii* was found in 14 eucalyptus species and 23 native and exotic Myrtaceae species in Brazil. Besides eucalyptus, this pathogen infects other species of Myrtaceae such as guava, myrtle, Brazilian grape, strawberry guava, Surinam cherry and jambul trees. In these Myrtaceae hosts, besides meristematic vegetative tissues, the fungus infects flowers and growing fruits and may lead to significant losses [11]. Myrtaceae is considered as Eucalyptus rust's original host.

Puccinia psidii is a serious threat to eucalyptus crops in different parts of the world, especially in Australia, to where eucalyptus is native. Occurrences have also been reported in some South American countries such as: Argentina, Colombia, Ecuador, Paraguay, Uruguay and Venezuela; Central America, in the following countries: Cuba, Dominican Republic, Jamaica, Puerto Rico and Trinidad; and North America in South Florida [12]. *Puccinia psidii* incidences were also reported in Japan [13] and Hawaii [14], both for the species *Metrosideros polymorpha.*

There are reports of *Puccinia psidii* attacks to plant species endemic to Australia such as *Melaleuca quinquinervia*in Florida [15] and *Acmena smithii* in Brazil [16]. Recently, [17] found *Uredo rangelii* (morphologically different from *P. psidii*) parasitizing the species *Agonis flexuosa*, *Callistemon viminalis* and *Syncarpia glomulifera*.

Eucalyptus rust is no longer a disease that causes considerable damages only in rare occasions [11]. During the surveys of *Eucalyptus* plantations in Mozambique in May and July 2009, typical rust disease symptoms were observed on eucalyptus trees in several localities in Maputo Province, as well as in Niassa Province. Subsequently, the rust disease has also been found in KwaZulu-Natal in South Africa. These were disturbing findings given the importance of eucalyptus or guava rust fungus, *Puccinia psidii*. Thus far, *P. psidii* has been the only known rust fungus associated with *Eucalyptus* species, and it is one of the greatest threats to *Eucalyptus* forest plantation and to *Myrtaceae* in natural forest ecosystems. Urediniospores have been found and shown to be distinct from *P. psidii* [18].

Data related to damage are shown in Table 1. It considers 30 m^3/ha/year, seven years for harvesting, 20% of medium damage, and USA 700/ton of pulp. The losses of eucalyptus wood account for more than 2 million dollars per year in Brazil.

STATE	TOTAL AREA (hectares)	RISK AREA (%)	DISEASED AREA	PRODUCTION (*m3*)	DAMAGE (*m3*)	LOSSES (US$)
BAHIA	550.127	7	38.509	8.086.867	1.617.373	404.343
ESPÍRITO SANTO	207.687	10	20.769	4.361.427	872.285	218.071
MATO GROSSO DO SUL	208.819	5	10.441	2.192.600	438.520	109.629
SÃO PAULO	813.372	15	122.006	25.621.218	5.124.144	1.281.060
MINAS GERAIS	1.105.961	5	55.298	11.612.591	2.322.518	580.629
PARANÁ	123.070	7	8.615	1.809.129	361.826	90.456
SANTA CATARINA	74.008	7	5.181	1.087.918	217.584	54.395
RIO GRANDE DO SUL	222.245	5	11.112	2.333.573	466.715	116.678

Table 1. Damages and losses estimated for different Brazilian States, according to the risk area per state.

2. Symptomatology

This disease is characterized by production of urediniospores, pulverulent and yellow, in the affected tissues. When it infects highly susceptible varieties, it causes malformations, necroses, hypertrophy, minicankers and death of the growing portions of growth. Although the uredinia phase is the most common and the major form of dissemination of this disease, in warmer periods, teliospores can be produced. As it is a biotrophic pathogen, its growth and multiplication require live tissues of the host, making impossible to culture "in vitro" in routinely employed media. This fungus extracts nutrients by means of haustoria formed inside live cells of the host [19].

Symptoms of Myrtaceous rust on various hosts are shown in Figure 1.

The primary symptoms of this disease occur in the young tissues of developing leaves and stem. They start with chlorotic spots that transform into pustules or wounds, where they become exposed with the rupture matter of epidermis, pulverulent urediniospore of bright yellow coloration. These pustules may coalesce, covering the surface of eucalyptus shoots when the attack is intense. Consequently, the affected tissues die and become dry, obtaining a dark coloration as if they were burnt.

Depends on the environmental conditions, the plant may react to the infection and produce new shoots. With the development of leaves and stem, the yellow spores disappear; giving rise to salient, rough, brown lesions. In the leaves, these lesions are spread on both leaves surfaces and sometimes on the midrib. They are commonly delimited by a dark and

purplish halo. In the branches, the verrucous characteristic of lesions becomes highly typical. As the attack occurs before the leaves complete their development, they frequently become distorted. Development of the disease can compromise highly susceptible plants, resulting in atrophy when severely affected. These plants may be outcompeted by adjacent ones that are less affected or healthy and continue to grow normally.

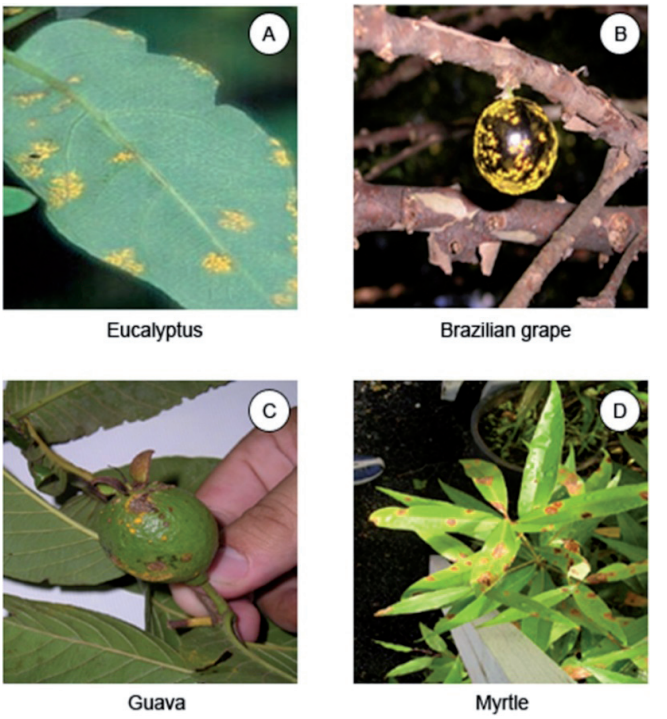

Figure 1. Some hosts of Myrtaceous rust. A: *Eucalyptus*; B: Brazilian grape (*Myrcia* sp.); C: Guava (*Psidium guajava*); D: Myrtle (*Syzygium jambos*).

3. Disease biology

Puccinia psidii produces two types of spores: urediniospores and teliospores.

Urediniospores, which are formed during the favorable phase to the fungus development, show a variable form predominantly globose, elliptical, pyriform and angular, measuring 14-20 x 18-27 micra. They are echinulate with hyaline episporium.

Teliospores are rare, and formed under conditions that are unfavorable to the pathogen. It is found frequently in the same injuries where urediniospores are formed. Teliospores are bicellular, of variable form, with predominance of elliptical and oblong-oval forms. Alternate hosts for this pathogen are unknown.

Spores are spread by the action of wind, rain, insects and birds. However, young developing tissues and favorable environmental conditions are needed for the infection to occur. The existence of young tissues is related to the phenology of the host, as were observed by Ferreira (1989), and the favorable environmental conditions consist in temperatures between 18 and 25ºC, and rather high relative humidity.

Such conditions are important for the development of this disease since they act on the pathogen, allowing the propagation and germination of its infective structures, as well as on the host phenology and consequently on the interaction pathogen against host [20].The most severe attacks occur in young crops, aged between 3–12 months, under favorable environmental conditions. Although there are no specific studies about the effect of the environment on the disease in eucalyptus, based on the reports of other crops, mild temperatures and high air relative humidity indexes are critical factors leading to even more severe attacks.

Rust affects young plants in the nursery and in the field. Temperatures within the range of 18-25 °C (optimal = 23ºC), prolonged periods of leaf wetness (nocturnal dew or drizzle for periods longer than 6 h per 5-7 consecutive days) are favorable to the infection. Mature organs, absence of wetness and temperatures above 30ºC and below 10ºC disfavor the infection [19-22].

Urediniospore directly penetrates epidermal cells, through the cuticle and the epidermis, forming the appresorium. The fungus colonization is intercellular, with the formation of intracellular haustoria. The latter are specialized structures to absorb nutrients inside the host cells. Depends on the use of fungicides in areas where the severity of rust disease is high, the pathogen may change phases since its number of healthy tissue is reduced, the telial phase being predominant in case of adversities.

In general, when the plants reach the phenological stage B [8] (at around 3-4 m height) they escape the disease, probably due to the decreased favorable conditions to the infection in young susceptible plant parts.

Climate analyses using mathematical models to predict rust disease are considered important tools to understand the disease in the field, pointing to epidemiological scenarios in regions where the climate consists of mild temperatures, with daily averages around 20ºC and, daily average relative air humidity of 90% or more.

4. Management of the fungus *P. psidii*

Rust can be controlled by applying fungicides, harvesting susceptible genetic materials in periods unfavorable to the disease (escape by period) and planting resistant materials. Planting *Eucalyptus* with rapid growth genotypes is another recommended measure. The use of genetic resistance is the most ideal control measure since it has the lowest cost, is easy to conduct, and reduces the impact of fungicides on the environment. Resistant *Eucalyptus* species, different origin of seed progenies or clones can be selected for commercial crops or to be used in breeding programs. In the breeding program, candidate lines can be selected from natural infections in the field, in areas where the disease is severe or by means of

artificial inoculation of the pathogen into seedlings. Although the selection of resistant materials has been successful in forest companies, the resistance inheritance in the pathosystem *P. psidii - Eucalyptus*, is not well known, however, essential to determine crossing strategies in breeding programs.

The use of fungicides to control eucalyptus rust is in an important tool for integrated management. The chemical groups of Triazole and Strobilurin are mostly used for disease control and the first group presents better efficacy results. Depending on the severity of the disease, different chemical groups may be used in nursery and in the field. The disease severity should be verified based on numerical criteria, since subjective scales are normally adopted [6, 41]. The first five pairs of leaves (10 leaves) apical leaves of all juvenile branches of each plant should be analyzed, using a diagrammatic scale, in order to include all the parts susceptible to the infection by *P. psidii*. The severity of the rust disease is obtained as percentage of the injured leaf area [23].

The fungicide efficacy may be compromised if the assessment is performed wrongly or without epidemiological criteria, such as quantification of plant diseases.

For assessment of rust in the field, a diagrammatic scale (Figure 2) can be used.

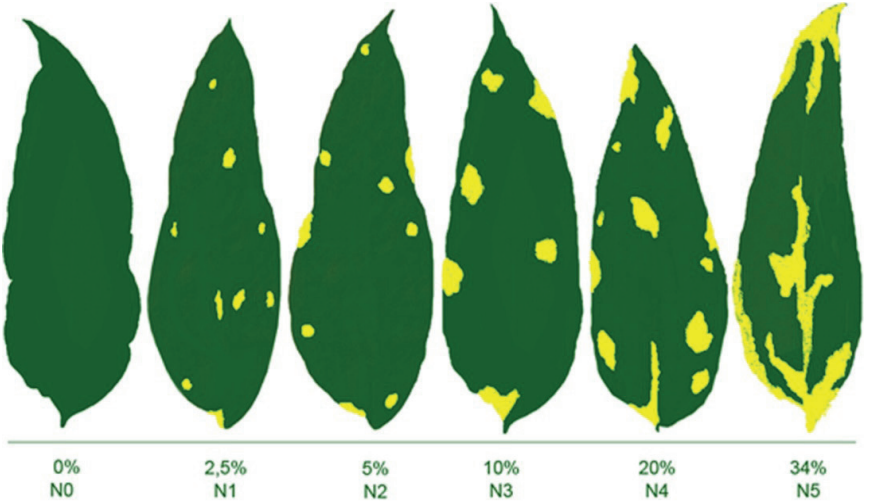

Figure 2. Diagrammatic scale for eucalyptus rust (percentage of Leaf Area with rust).

Correct quantification of diseases is one of the most important factors to be considered in the efficacy of the fungicide in experiments or crops, in the nursery or in the field, and thus shall be performed with extreme criteria. Incidence or severity can be estimated based on the rust intensity in the field. Data collected in the south of BahiaState, Brazil, to quantify rust disease of eucalyptus with two diagrammatic scales, %LAR (Leaf Area with Rust- Fig. 2) [23], and the field scale proposed by Takahashi (2002), modified by Zamprogno et al (2008),

were used to obtain a mathematical model by linear regression between both, with: $y = 7.2463x - 2.3399$, R^2 of 0.703, where y is the percentage of injured leaf area (%LAR), and x, levels of field scale (Figure 3A). This work assessed 180 plants in 9 treatments, subdivided in 4 replicates of 5 plants each. This model is applied to data for assessment using a field scale modified by Zamprogno et al (2008). A total of 321 plants in different soil and climate regions in the South of Bahia State were assessed and data of incidence and severity of the disease were obtaining, transforming the field severity into %LAR. The use of linear regression resulted in the following model: $y = 0.0776x + 0.0324$, with R^2 of 0.7154, where y is the incidence (decimal number), and x is %LAR.

In studies of fungicide efficacy, [23] assessed the severity of rust disease after application of different fungicides in different levels. Upon 7 days after application of fungicide solutions, the authors obtained 74.23% of relative efficiency, and 92.01% in 15 days, for the level of solution 1.5 mL/L of fungicide tebuconazol + trifloxistrobine, using the "Percentage Rate of Fungicide Efficiency - %EF" [23].

The interpretation of the result obtained by Masson (2009) was: eucalyptus rust is caused by a high infection capability pathogen. When susceptible genetic materials were planted in the field, this disease spread very rapidly along with the direction of the planting line. The disease shows an aggregation pattern with low incidence values in the field, while the level of aggregation ascend with increased incidence over time.

Studies of fungicide efficacy are valuable in programs for eucalyptus crops in Brazil and in the world, not only due to the response obtained against the pathogen, but also due to the host's potential to recover tissues and morphological structures. In fact, in Brazil, the fungicides should be used in experimental character due to the policy of product recording under progress in the country (registration with the Agriculture Ministry, for use in the crop).

5. History of fungicide application to control eucalyptus rust

The use of fungicides in the forest sector is targeted especially to control rust started in seedling production in nurseries. For eucalyptus rust, weekly spraying with mancozeb or copper oxychloride in the levels of 160-200 g/100L water, or triadimenol in 75 mL/100L or triforine in 28 mL/100L were recommended [8]. In laboratory tests, the protective fungicides mancozeb and copper oxychloride protected susceptible leaves when applied up to ten days before inoculation, while the systemic fungicides triadimenol and triforine had the same effect. These materials were assimilated from 30 minutes after spraying and translocated from one leaf blade to the another on the opposite side of the stalk and to the blade immediately above, and also had a kick-back effect when applied up to six days after the inoculation [25-26].

According Ferreira (1989), when resistance measures were adopted, fungicides were rarely recommended under field conditions; this control measure could be used only in special situations such as the commercial planting of highly susceptible to rust, which associated with other management practices, would require one or a few applications to control the

disease in a young crop or in shoots after clear cutting. To prevent fungus resistance, the author mentioned the use of protective fungicide separately or in a protective + systemic mixture, or the alternation of an application of protective fungicide with an application of systemic fungicide.

Until 1989, there had been no practice of fungicide application to control such a disease in the field. These data were documented by Krugner & Auer (2005), who indicated products made from triadimenol and azoxystrobin was used to control rust in nurseries as a curative material and clonal gardens and "exceptionally" in the field for materials of high commercial value. Considering epidemics that occurred in the southeast region, especially in Vale do Paraíba, São Paulo State, in 1991, the first rust outbreaks occurred, initially in Santa Branca Municipality. In 1992, Redenção da Serra and Jambeiro were attacked and finally, in 1996 and 1997, the whole region of Vale do Paraíba was affected, including 3-to-14-month-old crops of several genetic materials of *Eucalyptus grandis*, and the damages concerned a large number of farmers [28].

Some companies opted for chemical control in the field after tests and the publication of the results of a study related to the caused damages (Table 2). [6] Obtained 27.08% of damage to plants aged 19 months.

Region	**Guararema**		**São José**		**Taubaté**	
Farm	Rogemar	Rogemar	Varadouro	S. Pedro I	N.S.Ajuda	Gaspar
Plot	4	6	10	6	9	7
Procedence	Paraibuna	Paraibuna	Salto	Botucatu	Taubaté	Resende
Planting date	7/10/1996	12/23/1996	11/20/1996	8/1/1996	7/18/1996	6/28/1996
Age (years)	4.25	3.83	3.92	4.17	4.33	4.33
% plants with rust	76.15	71.60	79.59	85.19	66.67	71.88
% real production	67.23	74.90	74.18	81.08	67.48	76.69
% damage	32.77	25.10	25.82	18.92	32.52	23.31

Table 2. Percentage of damage caused by eucalyptus rust in Brazil [11].

Chemical control in the forest area remained restrict to nursery for a long time. The application of fungicide in the field can be a feasible method once the disease has reached the whole region of Vale do Paraíba and currently the whole south region of São Paulo State where majority. Preventive and curative activity tests were carried out using the fungicides: Triazoles (propiconazole, triadimenol, tebuconazole and cyproconazole) – FRAC Code 3; Anilides (Oxycarboxin) – FRAC Code 7, phthalonitrile (Chlorothalonil) – FRAC Code M5; Dithiocarbamate (mancozeb) – FRAC Code M3, and cuprous (copper oxychloride and cuprous oxide) – FRAC Code M1, in curative activity and preventive assays in Guararema Municipality, Sao Paulo State. The crops of susceptible genetic materials aged 7 months, with the disease established, and aged 4 months, without the disease, assessing the percentage of shoots with rust. Applications were performed in every 14 days, in a total of 6 applications. In the preventive test (Figure 3), fungicides made from Cyproconazole, Triadimenol and Tebuconazole showed the best results after the last application.

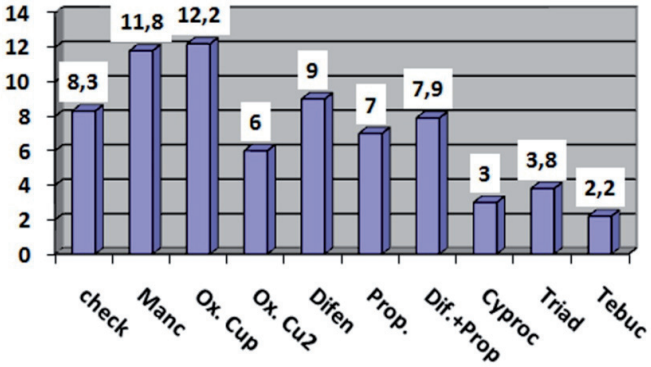

Figure 3. Preventive chemical control of eucalyptus rust. 1997. Vale do Paraiba-Brazil [4].

In the curative activity test (Figure 4), in which plants had more than 70% symptomatic shoots, all treatments showed efficacy, especially fungicides made from mancozeb (preventing the development of new lesions), difenoconazole, tebuconazole, propiconazole and triadimenol, which reduced the disease to less than 10% symptomatic shoots. The last two treatments remained close to zero [4].

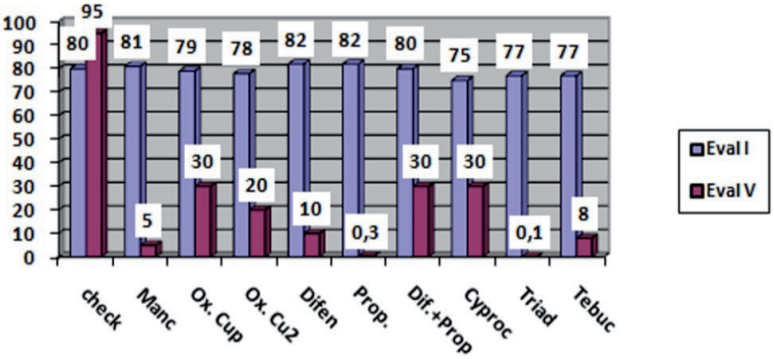

Figure 4. Healing chemical control of eucalyptus rust. 1997. Vale do Paraiba-Brazil [4].

Other field experiments were carried out in northeast region, on the north coast of Bahia State, in the commercial crop areas of Bahia Specialty Cellulose/Copener Florestal Ltda. Sprouting management was used in this assay, based on the larger quantity of young branches and leaf shoots of higher susceptibility to infection by *P. psidii*. The clonal material were used a susceptible hybrid of *Eucalyptus grandis* x *Eucalyptus urophylla* ("urograndis") [23]. To evaluate the efficiency and economic viability of fungicides to control eucalyptus rust, a test was set up in the field. The experimental design adopted for the test was randomized blocks, 3 x 3 (3 products and 3 doses) in factorial arrangement, with 0.5, 1.0 and 1.5 mL or g of commercial product per liter of solution. The treatments were: 1) control; 2) azoxystrobin (strobilurins); 3)

tebuconazole (triazole); 4) tebuconazole + trifloxystrobin (triazole + strobilurins). Four replicates were used to assess plant disease severity based on the percentage of damaged leaf area. Higher fungicide levels led to a greater reduction in the disease in the plants in 7 and 15 days after the application. The fungicide tebuconazole + trifloxystrobin in 1.5 mL / L was most efficient against eucalyptus rust under field conditions. The fungicide tebuconazole was most economically viable at the three tested levels.

TREATMENT	7DAA1*	14DAA1	7DAA2	14 DAA2	7DAA3	14 DAA3	21DAA3
1.Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.Azoxistrobin+ Ciproconazole+ Tiametoxam (250)	44.3	73.3	83.8	83.0	84.5	73.6	62.8
3.Azoxistrobin+ Ciproconazole+ Tiametoxam (330)	49.2	75.9	85.7	86.8	88.4	84.9	72.7
4.Azoxistrobin+ Ciproconazole+ Tiametoxam (400)	49.2	82.3	87.8	87.7	89.5	95.3	81.7
5.Azoxistrobin+ Difenoconazole (300)	55.8	82.2	89.5	88.5	90.9	88.3	82.5
6.Azoxistrobin+ Difenoconazole (400)	77.0	81.0	89.5	96.4	94.5	93.8	91.7
7.Azoxistrobin+ Difenoconazole (500)	68.9	87.3	95.5	96.8	96.7	94.5	92.8
8.Azoxistrobin+ Difenoconazole (300)+mineral oil (600)	62.7	86.1	91.7	94.7	95.5	94.5	90.6
9.Azoxistrobin+ Difenoconazole (400)+mineral oil (600)	80.4	91.1	94.3	94.7	96.0	94.9	91.3
10.Azoxistrobin + Ciproconazole (300) + mineral oil (600)	73.8	81.0	87.4	94.7	95.5	93.8	89.1
11.Piraclostrobin+ Epoxiconazole(500)	67.3	79.7	85.7	85.9	85.0	71.8	63.3
12. Trifloxistrobin+ Tebuconazole(750)	62.4	83.5	87.8	90.3	95.0	85.9	82.5

Table 3. Relative efficiency of fungicides in controlling eucalyptus rust in the field. Itatinga-Sao Paulo State. DAA* - Days After Application (Furtado et al., n.p.).

In the Central-South region of São Paulo State, a field assay was carried out at Primavera Farm, located in Itatinga, São Paulo State, and the used species was *Eucalyptus grandis* under are growth induction and aged 6 months, from March 11 to May 06, 2011, with spacing between plants of 3 x 2 m. Applications occurred in 14-days of intervals. The assay was

carried out in randomized blocks with 5 replicates and 12 treatments, and the plot size was 6x60 m. The application was applied by using a costal sprayer. Five sample plots were established and constituted of 6 plants each. The application volume was 200 L/ha for application with costal sprayer. The used equipment was manual costal of the Jacto's company, model PJH, "JA-2 Inox", under constant pressure of 40 lb.pol-2.

Year	Active	Rate (a.i.)	Where	Reference
1989	Mancozeb Cupric oxid Triadimenol Triforine	160-200 g/100L 160-200 g/100L 70 mL/100L 28 mL/100L	Nursery	[8]
2002	Mancozeb Cupricoxid Triadimenol Difenoconazole Propiconazole Cyproconazole Tebuconazole Difen. + propic.	160 g/100L 352 g/100L 100 mL/100L 100 mL/100L 125 mL/100L 50 mL/100L 125 mL/100L 80 mL/100L	Nursery and field	[4]
2002	Triadimenol	50 mL/100L	Nursery	[42]
2004	Triadimenol Azoxistrobin Mancozeb Cupper Oxicloreto	50 mL/100 L 20 mL/100L 160-200 g/100L 160-200 g/100L	Nursery	[19]
2005	Triadimenol Azoxistrobin	Not described	Nursery	[27]
2011	Azoxistrobin Tebuconazol Tebuconazol + Trifloxistrobin	500-1500 mL/ha 500-1500 mL/ha 500-1500 mL/ha	Field	[23]
2012	Azoxistrobin+ Ciproconazol+ Tiametoxam Azoxistrobin+ Difenoconazole Azoxistrobin + Ciproconazol Piraclostrobin+ Epoxiconazol Trifloxistrobin + Tebuconazol	250 – 400 mL/ha 300 – 500 mL/ha 300 – 450 mL/ha 500 mL/ha 750 mL/ha	Field	Furtado et al. (n.p.)

Table 4. Chronology of the use of fungicides to control eucalyptus rust in Brazil.

The results of the relative efficiency of treatments (Table 3) showed the evidence that the most efficient treatments, in 3 applications, were: azoxistrobin + ciproconazol + tiametoxam

(400 mL/ha), azoxistrobin + difenoconazole (300 to 500 mL/ha, with or without adjuvant), the fungicide azoxistrobin + ciproconazole and the fungicide trifloxistrobin + tebuconazole (750 mL/ha).

The chronology of the use of fungicides to control eucalyptus rust in Brazil is shown in Table 4.

6. Perspectives of fungicide application to control eucalyptus rust

Chemical control of eucalyptus rust is a very useful tool for the management since it may allow the use of clones that are highly productive but susceptible to the disease both under crop conditions and in nurseries [29]. However, the use of fungicides to control this pathogen in eucalyptus is only technically recommended for emergency cases due to the limited number of registered products for this crop.

FSC (Forest Stewardship Council) promotes the management of forests all around the world, in an environmentally responsible, social and economically viable manner, by establishing the Principles and Criteria of Forest Management, renown and respected worldwide. In 2007, a list of prohibited agrochemicals was published, making unviable the control of some pests and diseases affecting *Eucalyptus*. Based on that list, a project was developed to derogate the rule for some products, allowing the control of such eucalyptus pests and diseases. To achieve a more careful assessment that could satisfy the countries interested in the forest sector, a new group of experts from FSC International Commission for Chemicals was formed in order to increase the process transparency and elaborate new criteria for prohibiting the use of pesticides in forests.

Based on these criteria, several studies were developed for the control of eucalyptus rust, considering the criteria elaborated for FSC and the standards in Brazil, resulting in the Prioritization of the Registration of chemical products, given the economic importance of the culture, the increase in areas affected by this disease and in economic losses, the absence of registered products for the culture, and the presence of registered products for other plants cultivated in Brazil, with similar problems.

7. Fungicide application methods to control eucalyptus rust

Rust can cause damages up to 44%, on average, to the production of São Paulo State [30]. Rust can be controlled by means of fungicide application, harvesting of susceptible materials in periods unfavorable to this disease (escape by period) and planting of resistant materials. The use of fungicides to control eucalyptus rust has shown satisfactory control levels, reducing the disease intensity in the field and consequently damages and losses [6, 23, 31, 32-35].

Application technology is the great importance plant disease to control programs. The current concepts of pesticide application have four points that must be considered essential for the successful preservation of harvests and reduction in attacks by pests and pathogens: appropriate period, coverage, level and safety [36]. The influence of biological, meteorological and agronomical factors, not always predictable, must be also considered [37].

Among with the most frequently used application methods, aerial, by tractor and costal, manual or motorized, questions were raised to answer what would be the most efficient application method for the application of agrochemicals in order to have protection against eucalyptus rust. Aerial and terrestrial applications can be complementary, but not necessarily concurrent, due to their peculiarities from a technical and operational point of view, making essential to learn their major differentials to take the decision of adopting one or the other technology [38].

Aerial application has become an appeal to the forest sector, especially due to the shortage of manpower. The use of specialized professionals and complete regulation and supervision of agricultural aircraft activities made aerial application a safe and effective tool for pesticide application with lower risk of environmental contamination. In addition, it allows the treatment of large areas in the appropriate moment in a short period, preventing the increase in areas with and/or new incidences of eucalyptus rust in the field. It has very good application uniformity since the application is not interfered by the ground irregularities, which may occur in application using tractor [32].

The optimal environmental conditions for eucalyptus rust are temperatures around 18 to 25°C and relative humidity above 90%. Considering aerial spraying during rainy periods, the areas can be sprayed as soon as the rain stops, allowing control in the beginning of the epidemics and reducing damages (reduced productivity) and losses (reduced financial value). On the other hand, it will be very difficult to spray using tractors of the rain. The aerial application also does not cause damages to the forest floor due to soil "kneading" and compression. In addition, because of a low spraying volume, each drop tends to have higher product concentration [32].

The aerial application must respect the environmental parameters also adopted as reference by application by tractor, which include: temperature around 27 to 30°C and relative humidity of 55%; fungicides must never be applied when there is no wind, the minimum required is 3 km/h, and the application must be interrupted when the wind is superior to 15 Km/h; ideal drop spectra between 200 um and 250 um. The flight height must be 3 to 4 meters above the crop, considering the application range for fungicides around 18 meters and application velocity of 100 m/h, obtaining a treated area of 4.80 ha/minute [39]. It must be highlighted that everything depends on the crop conditions such as: disease intensity; time requirement of fungicide to be applied soon after infection event, also the distance from the runway. The spraying cost is directly related to the area size, i.e., the larger the area the lower the aerial spraying cost.

The success of any application method is directly associated with use of an effective product, properly adjusted equipment, and right application timing for control. Thus, continuous monitoring of crop areas is required because the interval between applications, the target to be reached, the climate conditions and the disease intensity in the field must be considered.

The study shown below aimed to compare different application methods (manual costal sprayer, tractor turbo atomizer and aerial application) in eucalyptus rust control, using the fungicide azoxystrobin (AZ) + cyproconazole (CCZ) in different levels [40]. The experiment

was conducted in randomized blocks with seven treatments and five replicates, and each replicate consisted of 10 plants. The treatments and their respective levels were: control, costal sprayer (0.3 L/ha of AZ + CCZ + 0.6 L/ha of mineral oil), costal sprayer (0.45 L/ha of AZ + CCZ), Atomizer (0.3 L/ha of AZ + CCZ + 0.6 L/ha of mineral oil), Atomizer (0.45 L/ha of AZ + CCZ), aerial (0.3 L/ha AZ + CCZ + 0.6 L/ha of mineral oil), aerial (0.45 L/ha of AZ + CCZ). The spray volumes were 200 L/ha for the costal sprayer, 350 L/ha for the atomizer and 20 L/ha for aerial application (Figure 5). Natural epidemic conditions were used. Two applications were carried out in a range of 14 days. Evaluations were done within 7 days, in addition to the previous evaluations, 2 after the first application and more 4 evaluations after the second application. For the assessment of rust severity, a diagrammatic scale was used.

Results were analyzed and Relative Efficiency % (RE) and Area Under Disease Progress Curve (AUDPC) were calculated in 28 days after the second application (Figures 6 and 7).

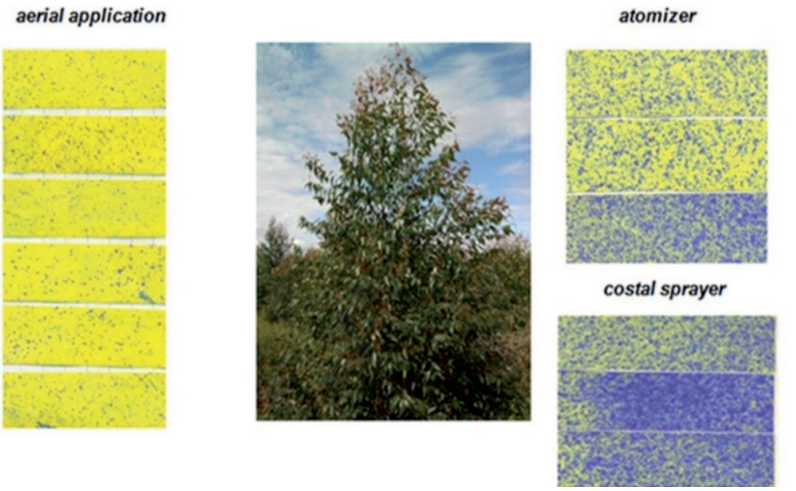

Figure 5. Application Coverage in aerial application, atomizer and costal sprayer [40].

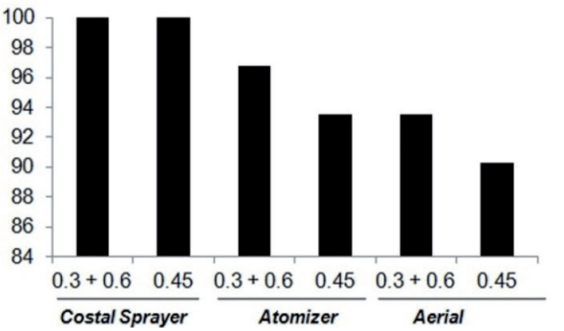

Figure 6. Relative Efficiency % (RE) of different treatments in eucalyptus rust control [40].

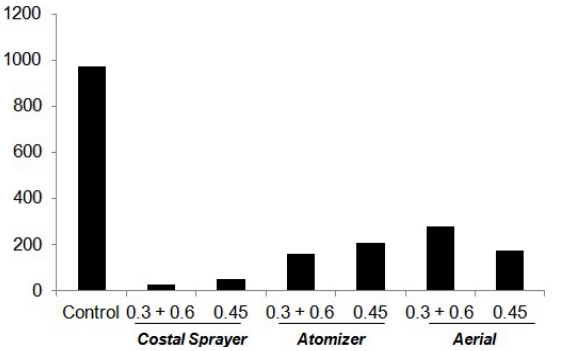

Figure 7. AUDPC (Area Under Disease Progress Curve) of different treatments in eucalyptus rust control [40].

All used application methods and levels were effective in controlling eucalyptus rust; ER was above 90% in 28 days after the second application, providing a reduction in the disease severity over time. No anomalies were observed regarding the effect of phytotoxicity.

The viability of eucalyptus rust control can be exemplified as follows:

A eucalyptus forest has a mean annual increase (MAI) of 30 m^3/ha/year. In 7 years, productivity will be 210 m^3/ha, considering 20% of damage = 42 m^3/ha and the price of R$ 76.00 m^3 of wood; the estimated loss will be R$ 3192.00/ha. An application for the control of the same area would require 0.45 L product=R$ 52.50/ha and aerial application cost of R$ 22.00/ha, with an estimated expense of R$ 74.50/ha which corresponds to 2.37% of the estimated loss for the same area [23].

8. Conclusions

The use of chemical control of eucalyptus rust, in the field, in Brazil is relatively recent. It has grown in importance in the last years since the number of epidemics is increasing, eucalyptus has becoming more like a agronomic crop and less of a forest tree, and due to restriction of genetic basis of breeding programs. Resistance sources have become scarcer and easily overcome by the diversity and the variability of pathogens, including eucalyptus rust. Therefore, chemical control has become a component of great importance in integrated management.

The materials for chemical control have been evolved rapidly, from older products such as cuprous and dithiocarbamates to strobilurins, and recently, the mixture of the latter with triazoles become common approach. This trend has been seen in other pathosystems such as rusts of wheat and soybean. There is still much to be done to find the phenological stage and the selection of areas of higher risk to start control, clonal mosaic composition with different resistance genes to prevent the pathogen proliferation, and rotation of different active principles to increase their lifetime, preventing the emergence of resistant isolates.

The chemical control of eucalyptus rust, as well as its application methods, is viable since it reduces damages and losses to eucalyptus crops. It may also allow the maintenance of clones that are highly productive but susceptible to the disease.

Perspectives in the scenario of integrated handling of plant diseases, within the context of epidemiology point, to studies of disease dynamics, geostatistical and climatic, by mathematical modeling. Fundamentally, additional tools such as using fungicides and genetic improvement, from studies of inheriting resistance and hybridization, are complementary and essential for the success of integrated crop handling.

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Chapter 7

Cotton in Brazil: Importance and Chemical Control of Bolls Rot

Willian Luis Antonio Zancan, Luiz Gonzaga Chitarra and Gilma Silva Chitarra

Additional information is available at the end of the chapter

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1. Introduction

Currently, cotton (*Gossypium hirsutum* L. var. *latifolium* Hutch.) is one of the oldest plant fibers cultivated in the world, and it has been used not only for producing natural raw materials for the textile industry, but also by the use of its products for other important purposes, such as edible oil, pharmaceutical and animal feed oil (Chitarra, 2008).

The current world production of cotton is approximately 25.10 million tons and it has being produced by more than 60 countries on five continents. Among the major world producers, China, India, United States, Brazil and Pakistan stand out (United States Department of Agriculture - USDA, 2012). The expansion of areas under cultivation, the monoculture cropping system and especially the use of susceptible cultivars cause a significant increase in the inoculum potential of various pathogens, consequently causing a higher incidence of diseases.

The cotton boll rot can be caused by various pathogens, especially the fungi *Colletotrichum gossypii* South var. *cephalosporioides* Costa, *Colletotrichum gossypii* South, *Botryodiplodia theobromae* Pat, *Diplodia gossypina* Cke, *Fusarium* sp, *Ramularia areola* Atk. and the bacterium *Xanthomonas axonopodis* pv. *malvacearum* Smith Dye. These pathogens can directly affect the crop by reducing the productivity, the fiber quality and also by increasing the costs of production. Based on these facts, the knowledge of symptoms of the disease, morphology, physiology and epidemiology of pathogens are critical factors for defining the best control strategy to be adopted, in order to achieve high yield and quality of cotton fiber.

Among the methods of management and control of cotton boll rot, the following are used: crop rotation, tolerant varieties, chemically treated seeds, cultivar specific, spacing and plant population, insecticides (for pests and disease vectors) and fungicides. Fungicides that are belonging to different chemical groups are the most commonly used in commercial fields of cotton.

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2. Cotton crop

Cotton is a plant of the malvaceae family, which originated from tropical and subtropical conditions, currently cultivated in all continents. Among the major producing countries, China is responsible for a production of approximately 6.53 million tons, followed by India with 5.53 million tons, the United States with 3.93 million tons, Brazil with 1.96 and Pakistan, with 1.91 million tons (United States Department of Agriculture - USDA, 2012).

In Brazil, the cotton cultivation began in the 90s, however, at that time, there wasn't adequate technology, i.e., operational infrastructure at the field level, fiber processing, lack of genetic material potentially suitable for the producing regions and non-fulfillment of the international consumer market (Guerra, 2006). Cotton production was mainly concentrated in the South, Southeast and Northeast of the country. The shift of cotton production to the states of the Midwest, known as producers of upland cotton, such as Mato Grosso, Goiás and Mato Grosso do Sul, in the region called "Cerrado", was the result of favorable conditions for crop development and, also, the use of varieties adapted to local conditions, disease tolerant and more productive potential. All these factors were combined with modern cultivation techniques, plus the increased use of growth regulators (Beltrão, 1999), what are used to maintain the balance between the vegetative and reproductive growth, which are essential to ensure adequate fiber production. These significant changes meant that Brazil would compete with countries with high technology, such as Australia and the United States. Other Brazilian states that are also cultivating cotton are Bahia, Maranhão and Piauí in the northeast, whose production systems have similar characteristics to the Midwest (Beltrão, 1999).

Cotton is one of the most important economic products of the group of fibers due to volume and value of production. Its cultivation is also of great social importance, due to the number of jobs generated directly or indirectly. The fiber, the main product of cotton, has many industrial applications. Examples are manufacturing of yarn for weaving of various kinds of fabrics, cotton batting for hospital use, felt clothing, blankets and upholstery, photographic films, plates for radiography among others (Richetti & Melo Filho, 2001).

The cottonseed is rich in oil, with approximately 18 to 25%, and contains 20 to 25% of crude protein. The cottonseed meal is a byproduct of oil extraction, and is used in animal feed because of its high protein content, approximately 40 to 45%. The seed coat is used to make certain types of plastics and synthetic rubber (Carvalho, 1996). The cottonseed, after the removal of the plume, is commonly used as ruminant feed. It is considered a palatable food, with characteristics of dietary fiber with high levels of energy and protein (Savastano, 1999).

The cotton plant is one of the most complex phytosystems found in nature (Oosterhuis, 1999). During most of the cycle of plant, various processes are occurring at the same time, such as vegetative growth, budding, flowering, growth and maturation. It has at least two types of branches (monopodials and sympodial), two types of true leaves (of the branches and fruit) and at least two gem (axillary and extra-axillary) located at the base of each sheet.

The cotton life cycle can be divided into five phases. The first phase covers the period between sowing and emergence, where watering and seed germination occurs as well as

establishment of the cotyledons, with an average of four to ten days. In this stage, in Brazil, usually occurs "damping off", due to the intensive period of rainfall. In the second phase, the first flower bud comes out, which usually occurs 30 days after emergence (DAE) and the main cotton diseases start to appear, such as, ramularia and ramulosis. The third phase is characterized by the appearance of the first flower and it occurs at 45 to 60 days after emergence, with an increase of cotton diseases severity. The fourth step is the opening of the first cotton boll, between 90-120 DAE and, depending on the environmental conditions, treatment to control bolls rot has to be done. The fifth phase includes the harvest period, when the cotton bolls are completely open, which occurs after 120 days after emergence on average, depending on the variety and environmental conditions (Beltrão & Souza, 2001). Therefore, the knowledge of the physiological stages of the plant is of fundamental importance in carrying out the cultural practices.

In cotton crops, for the plant to show its productivity potential, it is necessary to maintain healthy conditions (no disease) in all phenophases. The cotton life cycle, in certain regions, reaches approximately 200 to 220 days and it is considered a long cycle when compared with other crops, such as soybeans and corn, that have a life cycle of around 120 days. For this reason, it is necessary to monitor the crop properly during the whole cycle, so the cotton plants can obtain high productivity. Fungicides are responsible, in Mato Grosso, for about 7% of the cost of production, a similar percentage is destined in the control of aphids, vector of the virus causing blue disease (or Virose Vein Mosaic) (Mehta & Menten, 2006).

The expansion of cultivated area, the monoculture cultivation system and especially the use of susceptible cultivars cause a significant increase in the inoculum potential of various pathogens, and, consequently, a higher incidence of diseases. The disease can affect the production of cotton fiber and the seeds. Of course, the damage is proportional to the destructive power of each pathogen and the severity of each disease.

3. Cotton boll rot

In the main producing regions of the world, especially in Brazil, the losses in cotton crops caused by cotton bolls rot has increased in recent years. This disease is considerably affecting the production chain, either by production losses and/or fiber quality.

According to Hillocks (1992) a great number of microorganisms were isolated from cotton bolls rot, and these pathogens can be divided into three groups: those capable of penetrating intact bolls; those which are introduced by insects; and those are introduced after the boll are damaged by insects or after the suture of the boll lobes are broken. Most of the agents that cause cotton bolls rot penetrate through wounds from insect or pests and / or rupture of the division through the lobes of the bolls. However, primary infection of boll, when the pathogen penetrates directly into the healthy boll, is common in areas with high humidity or in those where the crop has dense vegetative growth.

According to Belot & Zambiasi (2007) there are many pathogens that can cause boll rot, such as *Alternaria* spp., *Ascochyta gossypii*, *Aspergillus flavus*, *Bacillus pumilus*, *Colletotrichum* spp.,

Diplodia gossypina, *Erwinia aroideae*, *Fusarium* spp., *Lasiodiplodia theobromae*, *Myrothecium roridum*, *Pantoea agglomerans*, *Phoma exigua*, *Phomopsis* sp., *Phytophthora* spp., *Rhizoctonia solani* and *Xanthomonas axonopodis* pv. *malvacearum*. Various symptoms may be due to the existence of a complex of pathogens. Commonly, the bolls are soft and blackened, and in some cases, arise from lesions in both the apex and at its base. Fructifications in various colors, from white to purple are also verified.

Zancan et al. (2011) worked with three cotton cultivars BRS Araçá, Delta Opal and FMT 701, using two spaces between the rows of cotton, 0.80 and 0.90 m, two treatments, with and without surface disinfection of cotton bolls with (1%) sodium hypochlorite, and three levels of bolls rot (Figure 1), initial, intermediate and final. Nine fungi associated with the cotton bolls rot were detected in the spacing of 0,80 m and seven, in the spacing of 0.90. Among them, *Alternaria* spp., *Aspergillus* sp, *Botryodiplodia* sp, *Colletotrichum* spp., *Fusarium* spp., *Myrothecium roridum* and *Penicillium* sp. were associated with the cotton bolls rot in different percentages in both row spacings. The fungi *Diplodia* sp. and *Stemphylium* sp. were found only in the spacing of 0.80 m, both with 1.7% of occurrence.

In this study, saprophytic and/or opportunistic fungi detected and associated with cotton bolls rot were *Botrytis* spp, *Cephalosporium* sp, *Cercospora* spp., *Cladosporium* sp., *Curvularia* sp, *Epicoccum* sp, *Graphium* spp., *Mucor* sp, *Nigrospora* sp., *Periconia* sp., *Trichotecium* sp and *Rhizoctonia* sp. Among the cotton cultivars, the NUOPAL had the highest occurrence (%) of fungi associated with cotton bolls rot.

The results obtained by Zancan et al. (2011) confirm reports from Belot & Zambiasi (2007) and Silva et al. (1995), who found fungi associated with cotton bolls rot to be *Alternaria* spp., *Colletotrichum* spp., *Fusarium* spp., *Botryodiplodia theobromae*, *Myrothecium roridum* and *Aspergillus* spp.

Ranney et al. (1971) classified four factors that favor infection of cotton bolls rot: long wet periods (5 to 7 days), long periods with relative humidity above 75%, low light intensity, i.e., long overcast periods and high temperatures. According to Hillocks (1992) long periods with high atmospheric humidity is the main factor favoring the development of an epidemic of cotton bolls rot. Araujo & Goulart (2004) stated that the main predisposing factor for bolls rot is an excess of moisture. In the rainy season or under conditions of high relative humidity, the bolls remain with moisture on their surface for long periods, causing the gradual tissue flooding, and, in this case, promoting the penetration of the primary agents that cause the disease followed by secondary action of saprophytes. The excessive vegetative growth, high planting density and unbalanced fertilization are also factors that can increase the incidence of cotton bolls rot.

In Georgia, in 1968, Ranney et al. (1971) observed yield losses in the order of 1.5% caused by cotton bolls rot, in a particularly dry year, while in the next year, these losses increased to 14%, due to higher humidity and temperature. The same authors concluded that with the cotton plant has vigorous vegetative growth, the moisture retained in the canopy may be sufficient to promote boll rot, even if the relative humidity is lower. This effect was observed

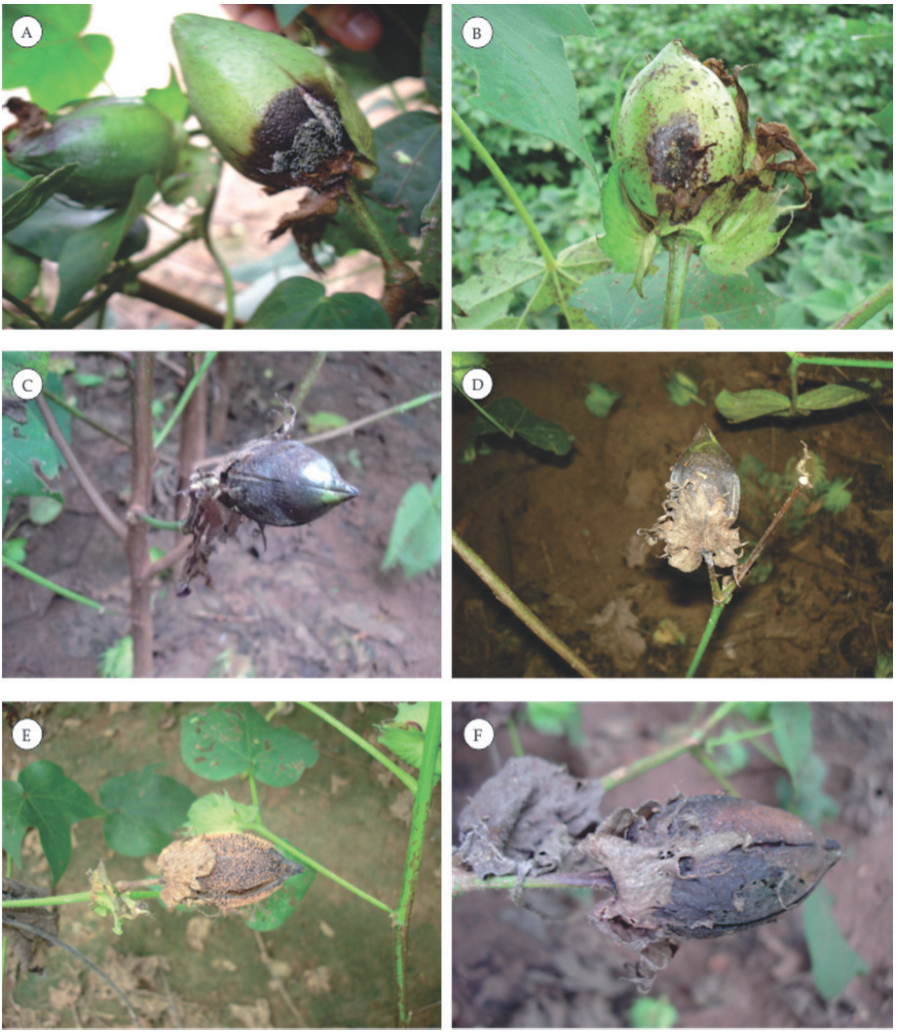

Figure 1. Cotton bolls with symptoms at different stages of decay. A and B) Early Stage, C and D) Intermediate Stage, E and F) Final Stage. IMA - Primavera do Leste - MT, 2011.

in the United States during the 70s, where cotton farmers experienced great productivity losses related to the bolls rot due to the use of high doses of nitrogen fertilize, that favored plant growth and provided a favorable environment conditions for the disease.

Cotton bolls rot can cause 20-30% losses in productivity, (Iamamoto, 2007). In general, affected plants losses the first bolls positions, where the plants produce the best quality of cotton fiber.

The presence of aphids and insects in cotton fields has favored the penetration of various microorganisms mainly due to the damage they cause on the earliest bolls. Moreira et al. (1994) found that the main causes of bolls rot in regions of the state of São Paulo were the attacks of the boll weevil (*Anthonomus grandis*), yellowstriped bugs (*Horcias nobilellus*) and cotton-stainer (*Dysdercus* spp). These insects favor the penetration of fungi and bacteria. The damage related to the attack of these pathogens was between 12.6% and 15.2% of bolls rot.

Ribeiro et al. (2000) found in samples of cotton bolls from Mato Grosso, Mato Grosso do Sul and Bahia showed no visible external symptoms, but all had symptoms of internal decay. Some samples detected external damage caused by stink bugs (migrant populations of *Euschistus heros* and *Piezodorus guildinii* from soybean and striped-bugs from cotton), but internally infected by pathogens. In a study conducted by the same authors, they observed mixed infection among the eleven samples of the variety Ita-90, where the incidence of *Fusarium* sp. was the highest, followed by *Aspergillus* sp. and *Colletotrichum* spp.. With respect to bacteria, *Erwinia* was identified in 2 samples.

Bagga & Laster (1968) reported a simple technique for evaluating the role of insects in cotton bolls rot development and reported that, the tarnished plant bug (*Lygus lineolaris* (Palisot de Beauvois) and fruit fly (*Drosophila melanogaster* Meigen) could cause the infection of cotton bolls rot after their feeding.

Bagga (1970) observed in both laboratory and field that *Bacillus subtilis*, *Diplodia gossypina*, *Glomerella. gossypii*, *M. roridum*, and *Xanthomonas malvacearum* can penetrated the boll valve directly from contact inoculation, and the internal rot was similar to that resulting from injection inoculation. This shows that these organisms are primary pathogens of the cotton bolls rot. The remaining 31 organisms tested in this study did not penetrate the endocarp when applied by contact, but all caused a complete internal rot when the infection method of inoculation was used.

According to Suassuna & Coutinho (2007), symptoms of *Colletotrichum* spp. in cotton bolls are depressed reddish-brown spots that expand and darken over time. In the central part of the lesion, a spore mass may be observed with a pink coloration. The infection causes the bolls to only partially open, leaving the fiber darkened and difficult to remove.

The fungus *D. gossypina* forms small brown lesions on the bolls and cotton bracts, and in high humidity conditions, the spots may expand, affecting the whole cotton boll. With the evolution of lesions, sporulation of the pathogen occurs, and a black coloration to the boll is observed (Paiva et al., 2001). Under these conditions, the boll dries and opens prematurely, exposing blackened fibers and seeds.

In the rot caused by *Fusarium* spp., small necrotic spots ranging in color from dark blue to brown occur in the bracts and in the bolls, and after sporulation of the pathogen, the lesions are covered by a pink color mass (Paiva et al., 2001). In these cases, the capsule does not open (Suassuna & Coutinho, 2007).

Myrothecium leaf spot, caused by the fungus *Myrothecium roridum* Tode, was responsible for losses of 50% in the town of Balsas in Maranhão (crop season 2003/04) and, since then, it has

also been reported in the state of Mato Grosso. The symptoms of the disease can appear on the leaves and cotton bolls (Suassuna et al., 2006). According to Belot & Zambiasi (2007) the first symptoms appear on the bottom leaves, spreading then to the bracts, bolls, petioles and stems. In the cotton bolls, petioles and stems, the lesions are irregularly shaped and have a dark color, surrounded by a violet and red color.

The fungus *Sclerotinia sclerotiorum* was observed infesting cotton plants in areas under central pivot, with symptoms of wilting, necrosis and wet rot of the stem, the petiole of the leaf and the bolls. Inside the capsule was observed a white, cottony mycelium and black and irregular sclerotia, that is, resistance structures of the pathogen (Charchar et al., 1999).

The angular leaf spot (bacterial blight) caused by *Xanthomonas axonopodis* pv. *malvacearum* occurs widely in all cotton producing regions. The wide dissemination and high variability of the pathogen are serious problems for the Brazilian cotton crop. Usually, the bacteria focuses on leaf blades, where we can observe angular lesions, initially green in color with an oily appearance and then brown in color with a necrotic appearance. Commonly, coalescence of the damaged area occurs and over time, tearing of the leaf. According to Belot & Zambiasi (2007) lesions of bacterial blight in bolls are rounded, oily and dark, later becoming black with a small depression on the site. Depending on the phenological stage of the plant, the disease causes fruit drop and premature opening of the new cotton bolls. When the bacterium is installed inside the capsule, infection may reach the seeds, causing yellowing of the fibers and allowing the entry of other pathogens.

3.1. Management of cotton bolls rot

The monitoring of the cotton crop should be performed periodically in order to diagnose early symptoms of diseases, so control measures can be undertaken with the aim of preventing the diseases progress.

Among the tactics of handling the cotton bolls rot can be used crop rotation, tolerant varieties, high quality seeds, adequate spacing and plant population, chemical control with insecticides (pests which are disease vectors that cause damage in cotton bolls) and fungicides. There is no specific recommendation for chemical control; however, the integrated management of diseases, as recommended for foliar diseases, should also reduce the incidence and severity of bolls rot. According to Hillocks (1992), using delinted seeds treated with fungicides is also a practice that reduces the primary inoculum in cultivated areas. The delinting consists in making a chemical reaction using sulphuric acid, which removes partially or totally the lint, which is the residue of the cotton fibers on the seeds. This method improves the performance of cottonseeds by eliminating the superficial microorganisms and, consequently, improving seed germination.

3.1.1. Chemical control of cotton bolls rot

In the integrated management of diseases, the use of fungicides is a major method to control plant diseases to be an effective method against pathogens, the facility of

implementation and the immediate results of this method, which makes it useful in many cultures.

The chemical control of plant diseases is practiced with greater intensity in economically developed countries, where agriculture is more technologically advanced with expected higher yields. Alviter & Nita (2011) states that the abusive uses of fungicides can cost not only growers budget, but it also can cost the society and the environment. Therefore, fungicide usages need to be carefully planned with a good understanding of plant disease epidemics, their components (host, environment and pathogen), fungicide mode of action (biochemical, biological, physical), risk of resistance development, and host physiology, among others aspects. Disease risk assessment tools can be very useful to reduce the costs of disease control and increase safety of the producers by helping growers to use fungicides in a timely and more efficient manner (Harwick 2006; Madden et al., 2007).

In the mid-1980s, developing countries accounted for about one-fifth of global consumption of pesticides. Their share in world use of insecticides is relatively high at 50 percent, while this share is 20 percent for fungicides and 10 percent for herbicides. East Asia (including China) accounts for 38 percent of developing countries' use of pesticides, Latin America for 30 percent, Near East/North Africa for 15 percent, South Asia for 13 percent, and sub-Saharan Africa for only 4 percent. Pesticide use is high on deciduous fruits, vegetables, cotton and cereals, and more moderate on citrus fruits, tropical fruits, cocoa, coffee and tea (FAO, 1995).

Currently, there has been an increase in the practice of using fungicides for controlling plant diseases. Major crops such as soybeans, cotton, corn and beans have already adopted this practice to reduce the progress of leaf spots, reducing the losses in yield and grain quality (Juliatti et al., 2001).

The use of fungicides to control boll rot does not prove effective because it is difficult to reach the bolls, due to the dense foliage of cotton, rainy periods are responsible for washing the fungicides from the leaves and the bolls of cotton (Hillocks , 1992).

Baird (1998) states that due to difficulty in controlling bolls rot, the efficiency of fungicides in Georgia has not justified the cost of control, with no increase in production. Numerous tests have been conducted and the results obtained are not accurate or verify no effective control.

Juliatti et al. (2001) evaluated the effectiveness of the inductor resistance acilbenzolar-S-methyl (Bion) at doses of 5, 15 and 25 g of commercial product, in combination with the fungicide azoxystrobin and copper oxychloride to control Ramularia spot, rust and boll rot. The authors observed no difference among treatments in relation to disease severity, but there was a reduced incidence of boll rot, and they acknowledged the potential use of induced resistance as a strategy for controlling this disease.

In work done by Zancan et al. (2011), the authors evaluated the chemical control of cotton bolls rot with the use of fungicides on cotton cultivars NUOPAL, BRS Araça and FMT 701, in two rows spaced at (0.80 and 0.90 m), and found that there was a variation of

approximately 2 to 15% of bolls rot on the cotton cultivars planted in spacing of 0.80 m and a variation of about 7 to 23% with 0.90 m spacing in the three cultivars when submitted to different chemical treatments. The lower incidence of bolls rot was observed in BRS Araça (Figure 2). These results showed that there are variations in bolls rot among cultivars in the southeastern region of Mato Grosso. The incidence of cotton bolls rot was higher in a larger spacing (0.90 m) due to the further crop development, favoring vegetative growth, with higher number of leaves, which favored the retention of moisture inside the plant canopy. The development of the pathogens was also favorable due to the environmental conditions. In this case, the fungicides were difficult to penetrate the leaf canopy, with lower retention of the products and, consequently, reducing the effect of them.

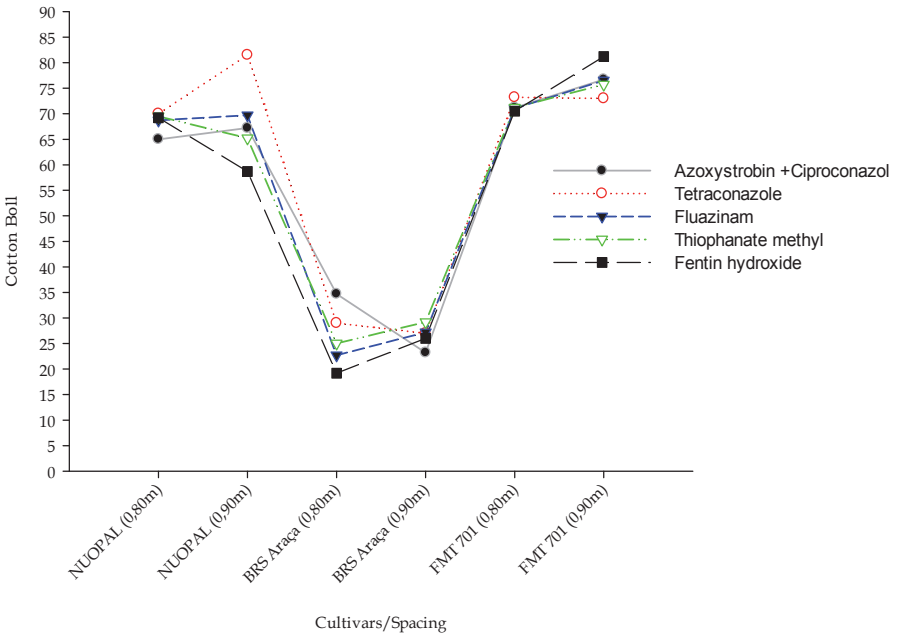

Figure 2. Number of cotton bolls rot in the cultivars NUOPAL, BRS Araçá and FMT 701 treated with fungicides at different spacing between cotton rows (0.80 e 0,90 m). IMA - Primavera do Leste - MT, 2011.

In relation to the fungicides, there were variations in the number of cotton bolls rot. The fungicide Azoxystrobin (strobilurin) + cyproconazole (triazole) reduced the cotton bolls rot in the cultivar NUOPAL (0.80 m) and BRS Araça (0.90 m). Both act differently on the fungi: strobilurins, with mesostemic action, inhibit mitochondrial respiration, while triazoles, with systemic action are related to the inhibition of sterol biosynthesis, and their function is related to the maintenance of the membrane integrity, which is preset in all eukaryotes (Reis et al., 2010).

The fungicide belonging to the strobilurin (Figure 3), groups of chemicals extracted from the mold *Strobilurus tenacellus* are used in agriculture as fungicides. These compounds belong to the group of the quinone outside inhibitors (QoI) or group 11 fungicides, whose toxicity arises from the inhibition of the mitochondrial respiratory chain at Complex III level, preventing the biochemical chain of electron transfer at the site of mitochondria, interfering with respiration of the pathogen (Ghini & Kimati 2000). They are typically absorbed by the cuticle of the fungus, acting as protectant fungicides (Vincelli & Dixon, 2002).

The active ingredient cyproconazole, which acts systematically, belongs to the group of triazoles, inhibitors of sterol synthesis, which are important structural components of fungal cell membranes. Triazoles are referred to as "DMI" (Demethylation Inhibitors) or group 3 fungicides, which is a reference to their unique mode of action (C14-demethylation is sterol biosynthesis), and all triazoles have the same mode of action (Tenuta et al., 2008). The fungicide tetraconazole belongs to the triazole group, which is inhibitor of ergosterol. When this fungicide was used it was observed a great number of bolls rot in NUOPAL cultivars spaced at 0.80 and 0.90 m and FMT 701 spaced at 0.90 m (Zancan et al. 2011). Probably, this fungicide was not efficient enough to control the pathogens associated with bolls rot in these cultivars.

According to Ghini & Kimati (2000) the fungicide fluazinam or group 29, acts as a potent uncoupler of oxidative phosphorylation and this proved to be an intermediary between other fungicides in the control of bolls rot, along with thiophanate methyl. The latter (Figure 3) specifically affects cell division, as it has selective activity for tubulin fungi, and binds to the protein, preventing the occurrence of polymerization of microtubules forming the mitotic spindle (Kendall et al., 1994; Wheeler et al., 1995).

In relation to organostannic compounds or group 30 fungicides, a common example is the fentin hydroxide based fungicide, that when used in controlling bolls rot, observed a reduction in the number of bolls rot in the cultivars NUOPAL (0.90 m), BRS Araça (0.80 m) and FMT701 (0.80 m). This fungicide acts as a multi-site inhibitors, prevents spore germination and inhibits the metabolism of fungi, particularly respiration, inhibits oxidative phosphorylation in mitochondria and induces lipid peroxidation. Low concentrations of organostannic compounds inhibit the translocation of H + bound to the membrane, H +ATPase and ions such as Na + and K + (Papa et al., 1982; Powers & Beavis, 1991).

Lawrence et al. (2007a) while testing the fungicides Quadris ® and 2.08SC Topsin M ® to control bolls rot found that foliar spraying of fungicide 2-4 times during flowering increased the cotton yield.

Roncadori et al. (1975) proposed that the fungicides applied at the flowering stage may be important in controlling *Fusarium*, one of the pathogens of bolls rot. The protection of flowers with fungicides more efficiently reduced bolls rot in relation to fungicide application at the opening bolls. These authors analyzed different methods to control the cotton bolls rot in Georgia, among these, the protectant fungicides captan and none, and found that they were ineffective in controlling this disease, becoming necessary to associate management combinations, such as reduce nitrogen rates and larger plant spacing.

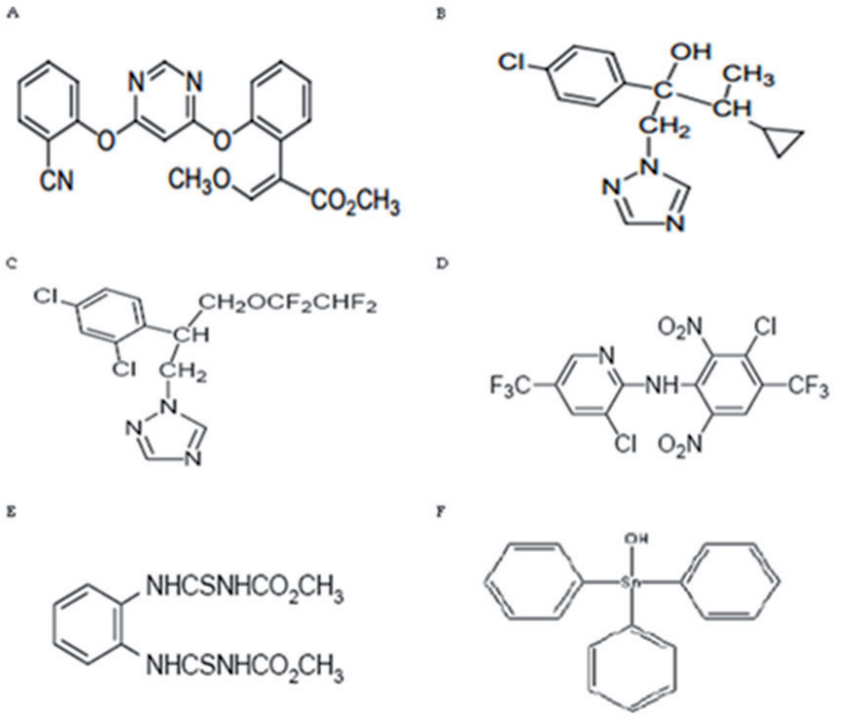

Figure 3. Structural Formula: A) Azoxystrobin, B) Cyproconazole, C) Tetraconazole, D) Fluazinam, E) Thiophanate Methyl F) Fentin hydroxide (Tomlin, 2011).

In a study conducted in western Bahia by Barbosa et al. (2007), the authors found a reduction of bolls rot with the use of two applications of copper tribasic (1.5 kg/ha), obtaining an increase of 330kg of raw cotton by hectare when compared to the control.

In relation to cotton yield in function of spacing, cultivars and fungicides, Zancan et al. (2011) found that the highest yield at the 0.80 m spacing in cotton cultivars were obtained using the fungicide tetraconazole, followed by thiophanate methyl. In the 0.90 m spacing, the fungicides tetraconazole and fentin hydroxide proved effective when compared with other treatments, obtaining higher yields (Figure 4). These authors observed that among the cotton cultivars studied, the highest yield was obtained by the cultivar NUOPAL in both row spacing of 0,80 and 0,90 m when subjected to chemical treatments, followed by FMT 701 and BRS Araça, in the conditions that the experiment was conducted (Figure 4).

More studies are needed to determine the timing and rates of fungicides to control diseases. In the southeastern United States cotton flowers and capsule production reach maturation between 6 to 10 weeks, and it is necessary to protect these flowers over a long period against infection by pathogens. Therefore, the best strategy may be the systemic application of compounds or compounds that induce systemic acquired resistance (Jenkins, et al, 1990).

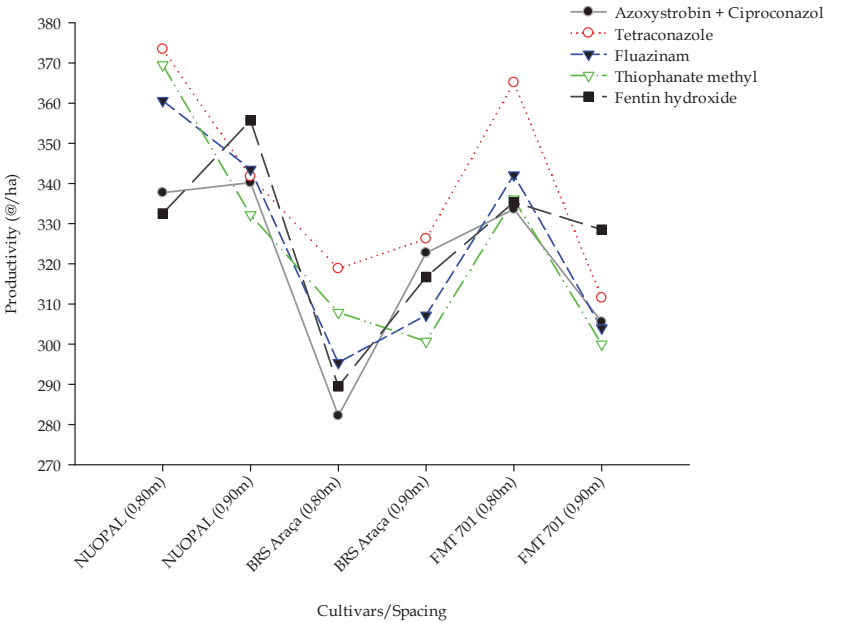

Figure 4. Average cotton yield (@ per ha) in cotton cultivars NUOPAL, BRS Araçá and FMT 701 with row spacing of 0.80 and 0.90 m, submitted to different chemical treatments to control cotton bolls rot. IMA - Primavera do Leste - MT, 2011.

Another management tactic to reduce the rate of cotton bolls rot, especially at the end of the cotton season's is to control pest, with pesticides, in bolls that cause injuries, such as bedbugs. Among them can be cited the brindle (*Horcias nobilellus*), the cotton stainer (*Dysdercus* sp.) and the migratory aphids such as the brown stinkbug (*Euschistus heros*), small stinkbug (*Piezodorus guildinii*), green stinkbug (*Nezara viridula*) and, in addition, *Edessa meditabunda* and *Dichelops melacanthus*, among others. The migratory bugs are so named because in the production systems, cotton remains in the field for long periods of time, longer than other crops such as soya, becoming it the last host in the food chain for these insects. When the soybean crops enter the maturation phase, the migration process of the bugs begins and progresses through the days. Infestations are proportional to the extension of areas planted with soybeans. Attacks occur from advancing into the borders of the blocks (Santos, 2007).

3.1.2. Influency of chemical control on the quality of cotton fiber

According to Zancan et al. (2011) analysis of the results of the High Volume Instrument (HVI), performed to verify the technological characteristics of cotton fibers, showed no variation in the percentages of fiber, fiber length, uniformity, strength and micronaire (fineness of the fiber), among the chemical treatments used to control bolls rot in cotton cultivars, according to data presented in Table 1. These results demonstrate the uniformity

of the fiber characteristics of each cultivar, regardless of fungicides used in the experiment of cotton bolls rot. The results of the technological characteristics of the NUOPAL cultivar obtained in this study are similar to the results reported by MDM (2006), except for the parameter micronaire with values from 4.5 to 5.0, higher than the MDM ranging from 3.8 to 4.5 (cotton fiber thinner), which is more interesting for the industry, because the too low micronaire (<3.8) may mean that fibers are immature, leading to breakages in fibers within the yarn and poor dye uptake during textile processing.

Regarding to BRS Araçá, the values of HVI analyzes obtained for the parameters the percentage of fiber and micronaire ranged from 41.5 to 46.2% and 4.5 to 5.0 respectively, but differ from the values reported by Embrapa (2005), where the BRS Araçá has fiber yield from 37.5 to 38.3% (less percentage of fiber) and micronaire from 3.8 to 4.2. The results regarding the percentage of fiber of FMT 701 subjected to different chemical treatments ranged from 43.5 to 46.2%, higher than the values reported by the Foundation MT (2007), with a yield of 42.5% fiber. For the parameter micronaire, the results obtained in this study for the FMT 701 ranged from 4.8 to 5.1, higher than the value obtained by the Foundation

	High Volume Instruments/Spacing (m)									
Treatments	Fiber		Length		Uniformity		Resistance		Micronaire	
	0,80	0,90	0,80	0,90	0,80	0,90	0,80	0,90	0,80	0,90
Cultivar NUOPAL										
Cyproconazole + Azoxystrobin	43,7	43,2	30,3	31,0	85,5	85,5	30,0	29,2	4,5	5,0
Tetraconazole	44,3	43,2	31,8	32,0	85,5	85,5	29,3	29,2	4,7	4,7
Fluazinam	43,8	43,0	32,5	32,5	86,2	86,0	29,8	30,5	4,6	5,0
Thiophanate Methyl	43,2	43,0	32,4	31,5	86,0	86,2	28,2	30,0	4,5	5,0
Fentin hydroxide	43,9	43,0	31,6	31,7	85,6	85,7	29,5	30,0	4,6	5,0
Cultivar BRS Araça										
Cyproconazole + Azoxystrobin	41,5	46,2	31,2	31,5	84,2	85,0	30,0	28,0	4,6	5,0
Tetraconazole	43,2	45,2	31,6	31,2	85,1	85,2	29,7	29,2	4,7	5,0
Fluazinam	42,3	44,7	31,4	31,5	84,9	85,0	30,7	29,5	4,7	5,0
Thiophanate Methyl	42,5	42,5	31,1	31,5	84,5	85,2	29,2	29,0	4,7	4,5
Fentin hydroxide	42,8	45,0	31,9	32,2	85,5	86,0	30,0	29,0	4,6	4,7
Cultivar FMT 701										
Cyproconazole + Azoxystrobin	45,1	45,7	30,9	30,2	85,8	86,2	30,5	30,2	5,0	5,0
Tetraconazole	45,0	43,5	30,7	31,0	86,0	86,2	31,6	30,5	5,1	5,0
Fluazinam	45,1	45,5	30,7	31,5	86,1	85,7	31,6	31,2	5,0	4,9
Thiophanate Methyl	45,2	45,7	30,5	30,2	85,4	85,7	32,0	30,0	5,1	5,0
Fentin hydroxide	45,3	46,2	30,8	30,7	85,9	85,7	31,4	30,0	5,0	4,8

Table 1. Technological characteristics of cotton fiber from the cultivars NUOPAL, BRS Araçá and FMT 701, submitted to different chemical treatments to control cotton bolls rot with row spacing of 0.80 and 0.90 m. Primavera do Leste - MT, 2011.

MT (2007) of 4.2. Other evaluated parameters such as length, uniformity and strength were similar to those obtained by the Foundation MT (Zancan et al. 2011).

Work done by Meneses (2007), related to physiological seed quality of cotton under water stress induced by polyethylene glycol-600 in the region of Paraíba, found that moisture can or may not improve the quality of the cotton fiber, and water deficit, particularly in stages after flowering can reduce the development of bolls, the strength of the fibers and enhance existing micronaire in the bolls. Moreover, the work done by Rodrigues et al. (2005) to check the quality of cotton fibers soaked in the vegetative phase, it was found that besides the change in resistance, the temporary flooding affect the yield and rate of fiber micronaire, a period of flooding also found in experiments carried out by Zancan et al, (2011).

According to Araújo et al. (2005), the processing is the last stage of cotton production, is the phase that precedes industrialization and consists of cleaning processes, drying, extraction of fiber from the seed and packaging, typically, these operations have a major influence on yield and quality commercial and industrial fiber, a fact that may have caused an increase in the parameter in yield and micronaire cotton cultivars NUOPAL, BRS 701 and FMT Araçá obtained by Zancan et al. (2011).

3.1.3. Resistance of cotton cultivars against bolls rot

The management of bolls rot with the use of resistant cultivars has been considered a strategy to be followed. Ballaminut (2008) states that different cultivars show differences in the incidence of bolls rot, strong interaction with the environment, crop management and the cultivar itself in relation to disease. Lawrence et al. (2007b) evaluated the response of 35 cotton cultivars to bolls rot in Alabama and found a variation from 0.0% to 10.3% among cultivars. The incidence of rot was similar between the flex transgenic cultivars and conventional cultivars. The earliest cultivars had a lower incidence of the disease when compared to those late and were similar to flex transgenic cultivars.

Soomro et al. (2000) observed a lower incidence of cotton bolls rot in Pakistan in Okra leaf cultivars with different cultivars of normal leaves. The percentage of cotton bolls rot varied between 6.0 and 6.9% in cultivars with normal leaves and between 0.5% and 2.2% in cultivars that have Okra leaf. The authors attribute this reduction in the percentage of rot in the resistance of these cultivars. Andries et al. (1972) and Jones (1982) also found that cotton cultivars with Okra leaf type have a lower incidence of cotton bolls rot, probably by promoting greater aeration and penetration of sunlight in the canopy.

In the west of Bahia, Pedroso et al. (2007) found that there are variations in cotton bolls rot among the cultivars in the region. These authors found, among eight cultivars, the cultivar Delta Opal had 50% more yield losses by cotton bolls rot than the cultivar Fibermax 966.

Cultivars 409 and CD 96 IPR have behaved more resistant, while IAN 338 has been the most susceptible with higher more cotton bolls rot. There is a need to develop more studies about this disease, since it is one of the most important occurrences in cotton crops in the state of Mato Grosso (Mehta & Mentem, 2006).

4. Conclusions

The cotton bolls rot causes direct loss in both productivity and quality of cotton fiber, and when the control is not performed properly, can make it impracticable to cultivate cotton in certain regions. Chemical control with fungicides is a rapid and effective tactic in the management of diseases, both through seed treatment and foliar sprays, and it can also be used to control the cotton bolls rot along with others techniques, such as row spacing. The cotton bolls rot can also is related with the region, the planting season and cultivars. Further work should be carried out regarding the use of fungicides to control this disease, since it is one of the most important in cotton crops.

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Chapter 8

Incubation Methods for Forecasting the Occurrence and Development of *Lophodermium seditiosum* Minter, Staley & Millar on Pine

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Additional information is available at the end of the chapter

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1. Introduction

Forest nurseries in Serbia are used to produce seedlings for afforestation, plantation establishment, reclamation of degraded forests and scrub, eroded terrain, barren and therefore it is important their proper growing and protection. Based on the review of nursery production, the data collected in 2011th, Serbia's state and private sectors, for the afforestation of the total produced 2, 238.798 seedlings of black pine (*Pinus nigra*) and 1, 311.160 seedlings of Scots pine (*Pinus silvestris*), aged 1 to 3 years. Of these, container seedlings *P. nigra* produced a total of 698 790, 1, 422.938 classical pieces, and "educated" seedlings is 126 000 pieces. Seedlings of *P. silvestris* are produced in smaller amounts twice, so that the total of 498 570 seedlings of container, in a classic production is 812 590 pieces.

Pine seedlings are produced in Serbia under the Nature Conservation Movement Svrljig and Surdulica, then „Erosion“ of Nis and the private sector (1 site in Sjenica), but to a lesser extent, so that all of the above locations outside the system "Srbijasume" in 2011 . total production amounts of black pine are 326 000, white 30, 000 seedlings aged 1 to 3 years.

In a larger scale, pine seedlings are produced in nine forest management within the public company "Srbijasume" (Uzice, FC, Kraljevo, Krusevac, Despotovac, Pirot, Leskovac, Boljevac and Prijepolje). The greatest production of pine seedlings in the forest management Pirot, which in the past 2011th has had production of 637 760 seedlings of red pine and white pine seedlings 457 500. Woodlands Pirot is a modernized production plant conifer seedlings and is scheduled to be (when the system is a spy) and produces up to 4 million seedlings per year, which would fully satisfy the domestic market and allow export to Bulgaria, Greece, Macedonia and other countries.

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Development of nursery production in neighboring countries, for example. Croatia is linked to the beginnings of reforestation karst. The first nursery for the production of forest trees was established in 1879. in Sv. Michael in Senja. Annual capacity was 1.8 to 2 million seedlings. They were mainly produced by pine trees and some sawmills. Shortly after that were built other nurseries, for example. Chestnut nursery (in Senj prefer), Podbadanj (Crikvenica), etc.. Some of them are still active (Podbadanj). Until the end of World War II forest seedlings were produced in temporary forest nurseries, which are mostly done manually. Most were produced by conifers. Only in karst areas were produced seedlings covered with roots. The largest number of existing nursery was founded in the sixties of the 20th century. They introduced modern production technology. Great attention is paid to the origin and quality of seeds, soil fertility and control safeguards.

According to figures presented Zgela [89], Croatia is the production of pine seedlings in the period since 1991. to 1998. amounted to an average of 253 000 pieces. Planned production of pine seedlings in the period since 1999. to 2003. was an average of 205 000 pieces.

In Bosnia, according to the "Center for Seed - nursery production" in Doboj, the production of conifer seedlings is conducted on an area of 9 ha, of which 6 related to conifers nursery a 3 ha in seminary. The production of conifer seedlings pine accounts for 25% or 2.87 million units, of which the system are not about 145, 000 pieces of plants, which are located in nursery. Production of white pine is about 1, 500, 000 pieces. White Pine also has the most important production in seminary as well as pine, because it is distributed to the field as a two-year seedlings, and less educated as a seedling. The highest production of shortleaf pine seedlings occurred prior to 1987 with at least 2 million seedlings being produced annually. Today less than half a million seedlings are produced at the nursery. Before 1990 most of the seedlings were delivered to the Mark Twain National Forest, but now it is a minor player in artificial regeneration of shortleaf pine. Trends in seed distributed for direct seeding followed those of seedling production [90].

Seed production in natural plant populations vary widely from year-to-year in response to weather variables, insects and diseases, and internal cycles within the plants themselves. Over a 20-year period, for example, forests composed of *Pinus taeda* and *Pinus echinata* produced from 0 to nearly 5 million sound pine seeds per hectare [91]. Over this period, there were six bumper seeds, five poor seeds crops, and nine good seed crops, when evaluated in regard to producing adequate seedlings for natural forest reproduction.

Occurrence of the needle cast disease and the disease agent (historically: *Lophodermium pinastri*) on the territory of Serbia was examinated and documented by the mycologists [6, 7]. Today we know, that: 1) *Lophodermium* needle cast epidemics occur in Serbia almost regularly, but not precisely after definite intervals, and 2) needle cast epidemics are much more frequent in forest nurseries than in plantations, sometimes so frequent that the successive epidemics can not be distinguished from each other.

Species from the genus *Lophodermium* Chevall. (Kingdom: *Fungi*, Division: *Ascomycota*, Subdivision: *Pezizomycotina*, Class: *Leotiomycetes*, Order: *Rhytismatales*, Family:

Rhytismataceae, Genus: *Lophodermium*) have the anamorphic stage *Leptostroma* Fr.:Fr. (*Deuteromycota, Coleomycetes, Sphaeropsidales*), the infectious role of which is unknown [1, 2]. The genus contains 145 species and has a global distribution. The genus infects many different plant families but with a notable concentration in the family Pinaceae. Some are economically important plant pathogens, such as those that cause needlecast disease in European Black Pine, Scots Pine and Red Pine in forestry and christmas tree plantations. In these species, notably *L. pinastri* and *L. seditiosum*, the fungal spores disperse and infect the pine needles in late summer, which turn brown by the following spring and then fall off. It is the most dangerous and the most harmful pathogenic fungus in nurseries and young crops, which causes redness and dispersal of pine needles. It occurs on the needles from the current vegetation of a large number of *Pinus* species [3, 4]. In Serbia, especially susceptible are *Pinus sylvestris* and *Pinus nigra*, although *P. sylvestris* is slightly more sensitive [5].

It is almost impossible to produce healthy seedlings of good quality without control of *Lophodermium spp*.[7]. Protection should be done during the critical period of infection, and only chemical measures are effective. However, outbreaks of this disease continue to occur periodically when control measures are not applied and where climatic conditions are conducive to disease development and pathogen spread. The fungus can also have a longer life cycle lasting for several years [8, 9, 10, 11].

Timing of spray applications is critical. Infected plantings should be sprayed three times, beginning in late July, in mid-August, and again in mid-September. In severely infected plantings an additional spray may be required in late September or early October, especially if wet weather prevails during this period. Conversely, in lightly infected plantings, the initial, late July spray may be omitted if dry weather prevails at this time. Studies show September to be the key month in which maximum spore release and infection occur.

When application of chemical disease control is economically feasible, as in the case of Christmas trees or forest nursery stock, the pest manager must understand the life cycle of the disease to be controlled. For many diseases, only one short window of control may be available in a calendar year, or the control spray may have to be applied preventively – before any signsor symptoms of disease are present. Chemical control measures must be applied when infection is most likely to occur or it will be a waste of time, effort and money. Undersanding the life cicle of disease organism enables to make a proper and timely management decisions.

It is known that the occurrence and development of parasites in special area is largely affected by meteorological factors (temperature, humidity, precipitation, etc.). In addition, to undrestand the disease it is important to monitoring phenological phases through which the plant passes during the growing season, especially the "critical stage" of development during which the plant is vulnerable to attack parasites. In addition to these and many other factors (sources and amounts of inoculum, plant resistance, cultural practices applied) affect the emergence and spread of harmful organisms. Study of all these factors, as well as using appropriate forecasting model, it is possible to predict the occurrence of pathogens in a specific location and signal the optimal timing of pesticide applications, ie. promptly notify the producers on the implementation of these measures.

Thus, obtaining a high-quality information in a short period of time, and appropriate professional advice about the time of application, the required dose and choice of products, farmers can save time, reduce losses and increase production yield and quality of its products.

Forecast of harmful organisms is its contribution to the rational use of pesticides and is increasingly gaining in importance. As crop production affected the emergence of different groups of harmful organisms (fungi, bacteria, insects, weeds), which differ in their nature, biology and way of causing damage to the area and share forecasts and develops in accordance with the area to which it relates.

Forecast of harmful organisms is a complex process, the quality of the work requires continuous and thorough implementation of all necessary procedures. A very important role during this process belongs to the monitoring of meteorological conditions in the crop or plantation, necessary for the emergence and growth of harmful organisms. The rapid technological advances in this field of meteorology has enabled the introduction of more modern and easier monitoring devices required meteorological elements.

When it comes to diseases by fungi nature, among the first instruments that were used for these purposes were different thermohygrograph production, which recorded data on the length of wetting rate, relative humidity and air temperature. Today, the prognosis of the disease using automatic weather stations whose work supports various software: µMetos (PESSL instruments-Austria), WatchDog (Spectrum Technologies, Inc.-USA), BAHUS (Faculty of Agriculture - Novi Sad) and others. This way is much easier to monitor the conditions of infection in orchards and crops, and created the ability to view a large number of locations depending on the number of cells, which allows for easier monitoring procedure and prognosis of the disease in a large area, making forecasts gets much broader dimension. Thanks to all of the above, achieved significant progress in setting deadlines for the treatment plants and the rational use of pesticides.

The incubation method is the most versatile because it follows the development of parasites, hosts and climatic conditions. As such it is necessary organization of the plant protection service. They also predict the use of fungicides to protect pine seedlings from pathogen *L. seditiosum* at the end of the incubation period. In cases where conditions for realization of infection are more frequent and shorter, the treatment should comprise block use of systemic fungicides, while in cases of less frequent infections and longer incubation periods it is possible to use non-systemic fungicides whose persistence is shorter. By combining the application of fungicides from different chemical groups in this way we avoid the possibility of pathogen resistance to some active ingredients. Treatment with the appropriate fungicide, in terms which we were identified on the basis of calculation by methods of incubation, ensures reduction of the inoculation potential during the following year, which consequently scales down the infection of pine seedlings with this pathogen.

To facilitate the production of healthy and quality seedlings, it is necessary to use appropriate phytosanitary measures with the use of appropriate pesticides. I n Serbia there are still not in full use of legislation which includes all allowed products to protect seedlings

in nurseries. The certification of forest products is in progress and many are discharged from service, and for which no adequate and field tested replacement. So often in nursery production in Serbia in recent years using insufficiently tested means, or which have shown efficacy in other climates, and have a certificate in relation to our weather.

Therefore, this study should complement and demonstrate the practical use of the fungisides are effective in Serbia, and that without being toxic and the fungicides approved for use. In addition, given that the same is climate different area each year (weather conditions change from year to year, which is particularly acute in recent years, because there are large fluctuations and climate change), models are essential for the prognosis of the disease each year, which also gives the study. Model predictions of some infectious diseases occur in a specific time period each year, the timely preparation of appropriate means to protect effectively to prevent disease and rehabilitation centers in the beginning of infection, before the advent epiphytotia, which would result in a greater amount of healthy trees with a minimum expenditure of resources.

On the basis of previous positive experiences of colleagues scientists from other fields, we tried to apply their experience and to pathogens which will induce diseases in forest plantations. In the field of forestry is limited use of pesticides because of the introduction of FSC policies, generally there are no registered products for the prevention of pathogens in the field of forestry, techniques and the application is poorly developed. We wanted to give our contribution in the first implementations of forecasts from pathogens in foresty according to models that are already used in agriculture. Further research should lead to registration of fungicides for this purpose in the territory of the Republic of Serbia in line with the FSC and the forestry policy, and to study the possibility resistance of fungus to recommended active ingradients since the use of fungicides were for several years undocumented.

2. *Lophodermium* needle cast

2.1. Biology of the pathogen

Lophodermium is a genus of Ascomycete fungi that contains both needlecast pathogens and hostspecific endophytes of conifers [33, 34]. Although Minter *et al* [3] recognised at least four species of *Lophodermium* which can infect pine needles, only *L. seditiosum* Minter, Staley & Millar is considered to be pathogenic [4, 32].

The disease has previously been considered to be caused by *Lophodermium pinastri* (Schard. ex Hook) Chev. [48], and in older reports, i.e. Costonis [49], the disease has been attributed to this fungus. Two biotypes of *L. pinasri,* differing in their pathogenicity and morphology, were recognised on Scots pine both in the plantation and in the nursery in Scotland [50]. Form A produced apothecia and black diaphragms on completely brown needles in the litter. In contrast, form B produced larger apothecia without diaphragms on the brown part of one-year-old needles whilst they were still attached to the tree [50]. Later Minter *et al* [3] showed that there are at least four *Lophodermium* species which can infect pine needles, and

described Millar and Watson's form B as a new species L. *seditiosum* Minter, Staley & Millar. Although Kurkela [32] did not test the pathogenicity of *L. seditiosum,* which was the only *Lophodermium* species isolated from newly browned needles, he concluded that this fungus was the cause of needle cast epidemic of Scots pine in 1975 in Finland. Later inoculations made on Scots pine seedlings confirmed the pathogenicity of *L. seditiosum* [30, 4].

First symptoms of the disease may be visible on pines in September – October as small yellow to brown spots on needles [12, 30, 4]. Later, 5 months after inoculation, needles turned brown and died, but the death and shedding of needles did not kill the plants although the new shoots were visibly weakened [4, 31, 32, 56]. Owing to the loss of healthy foliage, tree growth and quality were lower in outplanted infected trees than in healthy ones [51]. *L. seditiosum* was found to be active in seedlings 1 year after planting out [51]. In Finland diseased seedlings have occasionally been planted in the forest because in early spring, just after snow melt, diseased seedlings may still be green. The weakened seedlings have not tolerated the planting stress and have died [44]. *L. seditiosum* has been shown to infect green primary and secondary needles and only occasionally 2- or 3-year-old needles [52].

Ascocarps of *L. seditiosum* mature on fallen needles [35] and begin to release ascospores in late summer and peak between September and October in Europe [36, 37] and North America [38]. *L. seditiosum* preferentially infects green primary and secondary first-year needles [39] via ascospores [40, 41, 39]. The occurrence of apothecia was found to be highest on younger primary needles and on 1-year-old secondary needles [41]. According to Kurkela [32], the abundance of *L. seditiosum* must vary considerably from year to year in Finland. The persistent populations of the fungus seem to be very low and ascospores may arrive from Central Europe or from Estonia where needlecast has proved to be more persistent [57]. Severe epidemics occur only when the weather conditions are suitable for the pathogen [32].

Germinating ascospores form germ tubes ending in melanized, appressorium-like structures [30, 53] that penetrate the cuticle and epidermis [42]. The yellow margin of the lesion is caused by starch-free cells in the mesophyll and these cells were externally shown to form the yellow margin of the lesion [30]. Furthermore, the amount of chlorophyll and carotenoid pigments in infected needles is 1.2–3.8 and 1.3–2.4 times lower than in healthy ones, respectively [43]. Seedlings infected with *L. seditiosum* may be undetected as they can appear healthy when planted in the early spring. Minter and Millar [54] compared reports of the ascospore dispersal period, and concluded that different workers have trapped spores of different *Lophodermium* species. The dispersal of ascospores of *L. seditiosum* was been shown to start in June and reach its peak between September and October in Sweden [55], Estonia [36], Yugoslavia [37], Germany [10] and the USA [38]. High precipitation during late summer and fall creates conditions favourable for infection [32, 30].

Typically, infected seedlings do not survive planting stress [44] and so there is a need to identify latent infections in material stored below 0° C prior to planting. Stenström and Ihrmark [45] have developed species-specific PCR primers which detect also latent

infections of *L. seditiosum*. In Finland, needlecast caused by *M. laricis* has mainly been a problem in Siberian larch during their second growing season [46]. *Lophodermium* needlecast was among the first diseases to be controlled with fungicides in Finnish nurseries [47]. Although severe epidemics only occur under particular conditions [32], the potential economic loss is so great that annual application of fungicides is standard in southern and central Finland [44, 46]. Larch needlecast can be reduced by producing only 1-year-old seedlings - by meaning of Martinsson study [12].

The study of Martinsson [12] described that development of the fungus is very irregular and depends on environmental factors. The life cycle of the pathogen the commonest in central Europe has been described by Rack [10]. In damp weather during late summer and autumn ascospores spread from the needles on the ground. The spores germinate on the surface of living needles. From the beginning of August small brown infection spots can be observed. When the temperature increases in April-May, a rapid change occurs. Within a few days a whole stand of pine can change its colour from green to reddish-brown. Depending on the strength of the wind, the needles then fall off fairly rapidly. As a rule, severely affected seedlings stand completely devoid of needles for some time, until the new shoots have had time to develop new needles. The conidial stage of the fungus is developed first and can sometimes be observed before the needles are shed. After the needles have fallen, the apothecia of the fungus develop during late summer and autumn. They occur as dark elliptic spots on the surface of the needle. Later apothecia develop from the tissue of the needle into gatherings, slitting longitudinally in damp weather and discharging the spores. The fungus can also have a longer life cycle lasting for several years [8, 9, 10, 11]. The filiform ascospores have a length of 100-160 *p*, a width of 2-2.5 *p* and are enclosed by a mucilaginous envelope. A variety which is slightly shorter can occur on cones [13]. The vegetatively formed conidia are smaller and rod-shaped, and, according to the same source, about 7 x0.7 *p*. These conidial spores are unable to germinate and their significance to the fungus is unknown [14]. When the ascospores germinate on the surface of the needle, at least three different types of spores can be distinguished with regard to the cell division of the germ hyphae, the number of nuclei and growth [15]. There are relatively few stomata on one infection spot which are not penetrated by germ hyphae. On a double needle 200 individual infection spots can exist [10]. The anatomy of the fungus inside the needle has been described in detail by Jones [14]. After the fungus has penetrated the endodermis of the needle, the hyphae grow intracellularly. Since the stomata of the needle have been destroyed by the fungus, the hyphae, after having penetrated the endodermis, cause an uncontrollable transpiration of water through the hyphae of the fungus from the conducting tissue of the needle to its surface [92]. This initiates a reaction in the pine seedling, which leads to the shedding of the whole needle. The ripe apothecium is elliptic when viewed towards the surface of the needle, and has a length of 1-2 mm and a width of about half that size. It splits longitudinally in damp weather and closes again when the humidity level drops. According to Rack [10] each apothecium discharges about 2000 spores.

The fungus has a parasitic and a saprophytic stage. The parasitic stage constitutes the part of the life cycle in which the fungus lives on and in the live needle. The saprophytic stage

constitutes the other part of the life cycle. The development of the apothecia is most rapid in those needles that have been shed in June-August. The development is accelerated by high humidity. The optimum temperature for an apothecia formation seems to be 13 to 14°C, which is lower than the optimum temperature for the vegetative growth of the mycelium, which is about 18°C [10, 16]. During its saprophytic stage the fungus is highly dependent on the environmental factors influencing the moisture on the ground level. This is considered a contributory cause to the fact that the damage is especially frequent in dense plantations in grasscovered habitats and in nurseries [8]. There are also examples of stands considerably exposed to the wind being heavily attacked by *Lophodermium* [17]. In such cases, however, it seems to be the parasitic stage which is favourably affected. The uncontrolled transpiration in the tree is of decisive importance. *Lophodermium* needle cast seems able to exist on all types of forest soil and in nurseries. The physiological investigations performed have mostly been made *in vitro.* The fungus can be cultivated on artificial media. Growth is stimulated by the addition of pine needle extract [18]. So far nobody has been able to make fructifications with germinable spores develop on artificial media [19]. The pH-value of the needle tissue as well as its osmotic pressure have not proved to affect the development of the fungus [20]. In different isolated cultures the optimum pH-value for vegetative growth in *Lophodermium* cultures can vary between 4 and 6. The fungus, however, will grow within the pH-interval of 3-9 [21]. Fries and Stephan [22, 21] investigated the vitamin requirements of the fungus and found that in most *Lophodermium* strains the vegetative growth was favoured by biotin, thiamin and inositol. By nature the fungus is able to hydrolyze starch [21].

2.2. The diseased pine

High precipitation during late summer and autumn creates favourable conditions for infection [4, 32]. Due to the less severe climate, southern populations of *L. seditiosum* are more stable and can supply infective propagules to northern latitudes [36, 32]. Climatic factors such as lower temperatures during autumn and early snow cover may explain the lower risk of needlecast in the northern part of Finland [32].

Also, in his study Martinsson [12] finds that pine seedlings with secondary needles usually survive single attacks by the pathogen. Even if all the needles have been affected and fall off in spring, the seedling is usually able to develop a new shoot with healthy needles out of the apical bud by means of the stored nutrients in the stem, branches and roots [8]. Seedlings with primary needles only, however, have less chance of surviving the attack. These small seedlings have less nutrients stored and the primary needles do not fall off as easily as do the secondary needles; thus, the fungus grows into the shoot as well. In seedlings with secondary needles the fungus does not usually have time to reach the dwarf shoot, since the needle is shed before that. This needle shedding is a defence mechanism initiated by the pathogen. The infection starts the processes that cause the needle to fall off [23, 24]. The growth of a pine seedling depends upon the photosynthesis of the green biomass. If a large number of the needles formed during or before the preceding year are lost, root as well as stem and shoot growth is affected both directly and indirectly. The earlier during the year

the needles are lost the more serious will be the negative influence [10]. The nitrogen and carbohydrate supply in the seedling are built up in the previous year's needles during the spring. These nutrient reserves are largest immediatly before the buds open [25], i.e. usually during the season in which the pine seedling can be deprived of its needles to a greater or lesser degree by *Lophodermiurn.* The number of needles on the growing shoot is predestined in the bud, which is formed as early as the year before the disease breaks out. The elongation of the shoot, however, is dependent upon the available nutrient supply and assimilated material formed in the older needles [26, 25]. If older needles are missing completely or partially, this implies a considerable loss of nutrient supply. A shorter shoot grows out, and the amount of nutrients in the roots, stem and branches is reduced. The seedling has regulating mechanisms which endeavour to adjust the proportion of the dry weight in shoots and roots to a specific value; however, this value is dependent on environmental factors [27]. If a seedling is deprived of some of its needles, thereby receiving a reduced supply of assimilated material, an unbalance arises temporarily between shoot and root. Relatively speaking, the growth of the root will suffer more from the shortage of carbohydrate than will the growth of the shoot. Due to reduced water and nutrient absorption in the root, growth of the shoot and bud is impaired. If the attack does not cease, the seedling will finally die of nutrient deficiency [28].The upper parts of a pine will be attacked less than the lower parts. The reasons may be that the infection spreads from the ground and that the microclimate higher up prevents fungus growth [10, 29]. When the pine has reached a certain height, the upper parts usually escape attack completely. A young pine stand at a height of two metres has therefore usually passed the limit below which the trees are susceptible to severe attack by *Lophoderrniurn* needle cast. Thus, the growth ability of the pine and, consequently, its provenance and site quality class, indirectly affect the resistance of pine to *Lophoderrniurn*.

2.3. Economical significance

When chemical disease control application is economically feasible, as in the case of Christmas trees or forest nursery stock, it is essential that the pest manager understand the life cycle of the disease to be controlled. For many diseases, only one short window of control may be available in a calendar year, or the control spray may have to be applied preventively before any signs or symptoms of disease are present. Chemical control measures must be applied to the plant when infection is most likely to occur or it will be a waste of time, effort, and money. By understanding the life cycle of the disease organism, you will be able to make proper and timely management decisions.

2.4. The efficacy of current management strategies

Disease surveys are important and are the first step in application of control measures. Detection, appraisal, and control surveys are made for early recognition of disease; for information on scope of attack, extent of damage, possibilities for control, estimates of costs, and delimitation of control areas; and for assessing the effectiveness of control programs.

The major objective of disease management is to prevent or minimize losses while preserving tree quality. Absolute disease control is rarely achieved or even attempted. More often, management efforts are directed toward preventing disease or reducing it to the status of a tolerable nuisance. In most instances, forest disease management requires preventive methods over a long period of time and considers the stand as a whole rather than specific diseased individuals. Christmas tree disease management, on the other hand, is more likely to consider the value of each tree. Management measures must be economically feasible expenditures must not exceed the expected benefits. Direct control of disease in the forest is limited by many factors, including:

- The vast areas involved.
- The inaccessibility of many stands.
- The long life cycle of trees.
- The relatively low per acre or per individual tree values

Thus, spraying, dusting, or other direct control procedures commonly employed with high-value crops such as Christmas trees, forest nursery crops, and valuable seed orchards are rarely applicable in the forest. Occasionally, however, disease epidemics of introduced forest pests warrant drastic and costly direct control measures to meet the emergency.

3. Other *Lophodermium* species that seem to be involved in the disease complex

Different periods of mass infection during a year, then different types of ascospore germination, penetration of germ tubes directly through the cuticle or through the stomata, significant anatomic-morphological differences of fruiting bodies (in particular apothecia) on primary and secondary needles, as well as numerous other ecological, biological, physiological and pathological characteristics point to the existence of a number of *Lophodermium* species which differ significantly [50, 42, 40, 58, 59].

Lazarev [6] had noted that *Lophodermium* species, as the primary pathogens, have a significant role in the succession and inter-relations with other pathogens on the needles. This is especially the case in Scots pine, which is much more susceptible to the attack of *Lophodermium* species than Austrian pine. By all means, the succession and connexion of other fungi is affected by weather factors. He concluded that the older primary needles of Scots pine and Austrian pine are infested by *Lophodermium pinastri*, and the younger ones by *L. seditiosum*. Based on the symptoms and the obtained isolates from region in Serbia, it can be concluded that Scots pine seedlings (primary needles) are more susceptible to the attack of *Lophodermium* species than Austrian pine. The same conclusion also refers to secondary needles - primary pathogens on Scots pine secondary needles are *Lophodermium seditiosum, Lophodermella sulcigena* and *Lophodermium pinastri* (on older needles), and on Austrian pine *Dothistroma pini, Sphaeropsis sapinea* and *Lophodermium pinastri*. The secondary pathogens, which occur massively on the needles diseased by primary pathogens, are the species in the genera *Cyclaneusma (C. minor, C. niveus)* and *Cytospora friesii* [60, 61, 62].

Lophodermium Chev. includes 145 species, mostly from pine hosts, and there is some degree of host specificity [63, 64]. Morphological characteristics of this genus include a single longitudinal slit opening of the apothecia, and the fusiform shape of the ascospores [65]. As it is known today, the *Lophodermium* species complex on *P. sylvestris* in Scotland includes two endophytes and one pathogen. The two endophytes differ in their ecology. *L. pinastri* ascocarps are found on naturally shed needles, while *L. conigenum* fruits on prematurely killed needles [54]. The pathogen *L. seditiosum* causes needlecast disease which is particularly a problem on young *P. sylvestris* [4].

4. The history of fungicide usages against pine nidle cast

Several factors must be considered before a chemical control procedure is followed. First, the type of *Lophodermium* must be determined. If only the older needles of the tree are affected, no control may be needed or desired. The disease on the most recently formed needles is the most destructive and usually requires fungicide applications. Second, the degree of desired control must be determined. For a high degree of control, monthly sprays throughout the year may be necessary; however, fairly good control has been achieved by monthly sprays during the late summer and fall (August to October). The fungicide label will indicate whether an additional spreader sticker is needed to give good coverage on the waxy needles [66].

Chemical control of *L. seditiosum* is necessary and very important in the protection of pine seedlings. It is possible to use a systemic and non-systemic fungicide from all chemical groups registered for that purpose (Table 1). In investigations of Cordell [67] we can see that two fungicides chlorothalonil and maneb are registered for control of *Lophodermium* needle cast. A surfactant is needed for maneb.

During the 1990s, there were serious outbreaks of the pathogen *Lophodermium seditiosum* on pine seedlings in Swedish forest nurseries, even though the seedlings had been treated with the fungicide propiconazole [68]. The experiment was carried out to evaluate two other fungicides, fluazinam and azoxystrobin, as possible alternatives to propiconazole. In the tests, which were all carried out in the same forest nursery, seedlings were treated with either propiconazole, fluazinam or azoxystrobin, and the proportion of needles with ascocarps of *L. seditiosum* and the number of ascocarps per needle were recorded over the following 2 yrs. Seedlings treated with azoxystrobin already appeared healthier than control seedlings in September of the first year, and by November all azoxystrobin-treated seedlings had fewer ascocarps per needle compared with control seedlings. In autumn of the second year, there were no ascocarps on seedlings treated with fluazinam or azoxystrobin, whereas seedlings treated with propiconazole had similar numbers of ascocarps to non-treated control seedlings [68]. The similar results were and in investigations of Ostry [69] with benomyl, maneb and chlortalonil.

Chemical controls have been successful in managing *Lophodermium* needle cast disease in nurseries and Christmas tree plantations [70, 71, 38]. However, outbreaks of this disease continue to occur periodically when control measures are not applied and where climatic

conditions are conductive to disease development and pathogen spread. Recent outbreaks of *Lophodermium* needle cast were reported in the southern United States in 1986 [72] and in Michigan in 1988 [73]. In Europe, genetic differences and correlations were found for height growth of Scotch pine and severity of *Lophodermium* needle cast [12, 74], and young Scotch pine protection from *Lophodermium* infection by three fungicide applications were taller than untreated control [75]. The impact of this needle cast disease on the growth of nursery seedlings has not been documented in the Unated States. This informations is critical to advising nursery managers on how to control this pathogen.

Nicholls [76, 51] suggested to apply a registered, preventive fungicide such as chlorothalonil or maneb, once every 2 to 3 weeks during the major infection period from late July through October. Apply more frequently if wet weather persists. Do not grow seedlings next to red or Scotch pine windbreaks that can serve as a *Lophodermium* inoculum source. Plant only *Lophodermium*-free stock. If you suspect infection, have seedlings examined by a pest specialist.

The result of experiments on the influence of 12 fungicides such as Carbendazim, Cholrtalonil, Dithiocarbamates, Metham-sodium, Thiophanate-methyl, Bactericide (Tuzet), Benomyl etc. on conidia germination of *Lophodermium pinastri* (Schrad.) Cher. showed that different fungicides had distinct control effect. The fungicides screening trial demonstrated that the contorl effect of Metham-sodium, Carbendazim, Chlortalonil, Bactericide (Tuzet) on *L. pinastri* were better. The experiment of control of *L. pinastri* were carried out in the field and in the forest. The results showed spraying 45% Metham-sodium Solution could reach an efficacy of 81.2% [77].

During disease susceptible periods, apply sprays at two- to three-week intervals. See below for fungicides and application timing: Bordeaux mixture, chlorothalonil, dithiocarbamates (ferbam, or mancozeb) - Mid-July through October. [78].

Two fungicides: chlorothalonil and dithiocarbamate (maneb) are registered for control of *Lophodermium* needle cast. A surfactant is needed for maneb. During rain, do not apply fungicide as it tends to wash off. Spray applications are best during periods of low air movement during the still, early morning hours, for example-because these periods allow more uniform coverage. Chlortalonil is registered and effective against this disease. Use according to label directions. Nursery stock should be sprayed four times at 2-week intervals, beginning about August 1. For larger trees (2.5- 5.5m), two or tee applications of eiter chemical are suggested at 3-week intervals. Begin the treatment when temperatures remain above 26^0C, typically about mid- July, and continue until early September. Two applications should be adequate for lightly infected trees [79].

In Serbia, as well as protective measures against these diseases are given mainly preventative, such as raising nurseries away from infested pine trees from which can carry spores of these fungi. In the critical period for infection, mainly used Dithiocarbamates (Cineb a S-65 and Ortocid-50) in a concentration of 0.3%, which provides almost complete

protection against infection of new needles parasites. Number of spraying is reduced to four or five (depending on the quantity and frequency of rainfall). Usualy, the first spraying for *Lophodermium* controling is done around mid-June, and later as needed, ie. when rain washed out with needle tool. This means that the number of required spraying depends on the year. As a rule, should strive to do all the needles fall to be enough fungicides. It is recommended that the fungicidal soup add wetting agents, it is then easier to fine droplets of wax coating on the needles. Every annual regular spraying of pine seedlings in the nursery should be a rule in all the nurseries where the disease is observed, even if to a lesser extent, because once the disease is established, there will be wet in the first year to major damage, and then it was too late. In any case, the infected seedlings may not be transplanted, because it will be developed the disease and after transplanting, and lead to deterioration of the transplanted material. Accordingly, the spray is indispensable, because only it can ensure a sufficient quantity of healthy seedlings for afforestation.

Timing of fungicide applications may vary with geographic location and species of *Lophodermium*. In the Lake States and the Northeast, apply four sprays (August 1, August 15, September 1, and September 15) just before and during the period when spores are released. Where infection is severe and prolonged rainy weather is expected, spraying again on October 1 may be necessary. In the Pacific Northwest, where mild, moist conditions are expected most of the year, experience has shown that from 9 to 12 sprays are most effective. Apply year-round at approximately 1-month intervals except when beds are covered by snow [67].

When practical, diseased needles which have fallen and have piled up on branches or under trees should be raked up and destroyed. This will reduce the number of spores in the vicinity of the tree, and should help reduce the amount of future infection. Also, fungicide applications are more effective in preventing disease when the number of spores has been significantly reduced. When an affected tree is in an area where there are no other pines nearby, it is possible to keep the disease under control without fungicide application by thorough removal of dead needles from the tree and ground. The removed, dead needles should be destroyed by burning, depositing in the garbage, or in some other suitable way. Do *not* throw them on a compost pile or use them for mulching.

Application of chemicals to plants in order to prevent or inhibit disease development is a fundamental means of managing diseases caused by fungi. Knowledge of the effectiveness of particular compounds is important for achieving effective disease control. Equally important is an understanding of the underlying physiological mode of action of plant disease management materials.

Why is it important to know the physiological mode of action of fungicides ? It is important for resistance management and preservation of fungicide effectiveness. This meanes to incorporate fungicides with different modes of action into a disease management program as an alternation or as a mixture, to prevent the resistance of these pathogens on this fungicides.

FRAC CODE	MODE OF ACTION	CHEMICAL FAMILY(GROUP)	ACTIVE INGREDIENTS
11	Respiration	methoxyacrylates	Azoxsystrobin
M	Multi-site contact activity	inorganic	Basic Copper Sulfate
1	Mitosis and cell division	benyimidazoles	benomyl
	Multi-site contactactivity	phthalimides	Captan
	Multi-site contact activity	chloronitriles (phthalonitriles)	Chlorthalonil
	Multi-site contact activity Mitosis and cell division	chloronitriles (phthalonitriles) thiophanates	Chlorthalonil – Thiophanate Methyl
M	Multi-site contact activity	inorganic	Copper Hidroxide
M	Multi-site contact activity	Inorganic dithiocarbamates	Copper Hidroxide+Mancozeb
M	Multi-site contact activity	inorganic	Copper Hidroxide+ Copper Oxychloride
M	Multi-site contact activity	inorganic	Copper Salts of Fatty and Rosin Acids
	Multi-site contact activity	dithiocarbamates and relatives	Ferbam
29	Respiration	2, 6-dinitro-anilines	Fluazinam
	NADH cytochrome c reductase in lipid peroxidation (proposed)	dicarboximides	Iprodione
	Multi-site contact activity	dithiocarbamates	Mancozeb, zineb, ziram
	Multi-site contact activity, Sterol synethesis	Dithiocarbamates triazoles	Mancozeb+Myclobutanil
28	Cell membrane permeability		propamokarb-hidrohlorid
3	Sterol synethesis	triazoles	Propiconazole
1	Mitosis and cell division	thiophanates	Thiophanate-methyl
3	Sterol synethesis	triazoles	Triadimefon

Table 1. Fungicides used in the protection of *Lophodermium* spp. - FRAC list

However, the risk of economic losses with both bareroot and container Scots pine seedlings during an epidemic is so high that routine control with fungicides is considered to be necessary every year [44]. A lower occurrence or absence of the fungus, together with climatic factors such as lower temperatures during autumn and earlier snow cover, may explain the lower risk of needlecast [32, 56]. In general the fungicide spraying times are

consistent with the sporulation period of *L. seditiosum* [54]. In Finland spraying with fungicides such as maneb and chlorothalonil [38, 51] should be carried out at 2-week intervals, from June to October [44, 12].

5. Biological examination of efficacy of some fungicides in serbia

5.1. Material and methods

5.1.1. Experimental conditions

Selection of crop and cultivar, test organisms - This study was established in a forest tree nursery "Barje" in Pirot, Serbia. The research was carried out on the beds of three -year-old seedlings of red pine (*Pinus sylvestris*). The container bed was seeded to a density of 35 seedlings per 0.3 m. Test organism: *Lophodermium seditiosum*, Miller. Visual estimation of test organism was made based on the results of professor Lazarev [6] and professor Karadzic [7] for the locality of Serbia. The intensity of disease was assessed by the method of PP1/100 (2) EPPO: Guideline for the efficacy evaluation of fungicides – *Lophodermium seditiosum* [81]. Trial conditions - Only field trials were conducted. Trials were carried out in nurseries with 3-year-old seedlings. Homogeneous seedling lots were selected for the trials from seedling beds in nurseries where the inoculum potential of the disease was high and uniformly distributed. Design and lay-out of the trial - The trials were set in accordance with methods PP 1/152 (2) (EPPO, 1997) [80], and the treatment plan was made according to a fully randomized block design. The experiment was conducted in four repetitions. Plot size: 2 m2 for seedlings.

5.1.2. Application of treatments

Test products are: a) Blue bordo (i.e. cooper (II) sulphate pentachloride and calcium hydroxide 200g/kg) used in the concentration of 0.5%, b) Captan WP 50 used in the concentration of 0.3%, c) Previcur 607 Sl used in doses of 75 ml/20 L H20. Mode of application - Applications was done with good forestry practice. Type of application and equipment - Regarding the method of application and amount of water per unit surface, the fungicides were applied using the backstroke sprayer "Solo"; with a consumption of 1000 L/ha of water. Time and frequency of application - Applications of the product were administered so as to coincide with the major spore release and infection periods, i.e. late summer and early autumn. There were 2 applications in total, administered on 21st October and 03rd November 2010. Data on chemicals used against other pests - There were no treatment with other chemicals which have to be used for other pests.

5.1.3. Mode of assessment, recording and measurements

Type, time and frequency of assessment - The appearance and development of flushing and dispersal of pine needles was followed by the initial appearance and development of the

disease on the control variation, as well as through accomplishment of a clear difference between the control and other variations on which fungicides were applied. A single assessment was made after overwintering in the spring, on 21st February 2011. Each seedling was scored for percentage of needles affected. The estimation of pine needles with secondary infection was conducted by scale of values which was used to record the results of each needle, as follows: 0=healthy, 1=mild symptoms, 2= medium infection, less than 50% of the needles affected, 3=severe infection, over 50% of the needles affected. The intensity of disease was assessed by the method of EPPO: Guideline for the efficacy evaluation of fungicides - *Lophodermium seditiosum*, EPPO Bulletin 17, 389-394 (1987), No. 100[16]. Direct effects on the crop - The crop was examined for presence or absence of phytotoxic effects. Phytotoxicity was estimated according to instructions of PP methods (1/135 (2) [82].

5.1.4. Statistical data

Data processing was performed using standard statistical methods (intensity of infection according to Towsend- Heuberger [83], the efficiency according to Abbott [84], analysis of variance according to Duncan test [85] and methods PP/181 (2) [86]. The differences of the disease intensity were evaluated by the analysis of variance and LSD-test.

5.1.5. Incubation methods

According Sisakovic [87] for forecasting of pathogen are used incubation methods : a) The method according to Müller, b) The method according to Shatski and c) The method according to Mrezanin and Lipickaja . All of the described methods are for determining the duration of the incubation period and timing of treatment.

5.1.5.1. The method according to Müller – Method 1

On the basis of Müller's incubation curve, given with the data in Table 2, it can be inferred that the shortest duration of incubation period at the end of which is ascospores capable to erupt ascocarpes. The duration of individual incubations is determined computationally by means of a formula:

$$C = A / B - B$$

C = number of days until the end of the incubation
A = sum of daily incubations starting from the day of onset of the infection until the time it ends
B = number of days elapsed from the day of onset of the infection until the end of the incubation (days with mean daily temperatures of 12^0C and below are excluded).

5.1.5.2. The method according to Shatski – Method 2

The second method is based on following the incubation, using the percentage of incubation progress for relevant sums of mean daily temperatures for each day separately.

Temperature										
T°C	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
12	13.0	12.8	12.5	12.3	12.0	11.8	11.6	11.5	11.2	11.1
13	10.9	10.7	10.5	10.4	10.2	10.1	10.0	9.9	9.7	9.5
14	9.4	9.3	9.2	9.0	8.9	8.8	8.7	8.7	8.4	8.3
15	8.2	8.1	8.0	7.9	7.8	7.6	7.5	7.4	7.3	7.2
16	7.1	7.1	7.0	6.9	6.8	6.7	6.6	6.5	6.5	6.4
17	6.3	6.2	6.1	6.1	6.0	5.9	5.8	5.8	5.7	5.6
18	5.6	5.5	5.4	5.4	5.3	5.3	5.2	5.1	5.1	5.0
19	5.0	4.9	4.9	4.8	4.8	4.7	4.7	4.6	4.6	4.5
20	4.5	4.4	4.3	4.3	4.3	4.2	4.2	4.2	4.2	4.2
21	4.1	4.1	4.1	4.1	4.1	4.0	4.0	4.0	4.0	4.0
22	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
23	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
24	4.0	4.1	4.1	4.1	4.1	4.1	4.2	4.2	4.2	4.2
25	4.3	4.3	4.3	4.4	4.4	4.5	4.5	4.5	4.6	4.6
26	4.7	4.7	4.8	4.8	4.9	5.0	5.0	5.0	5.1	5.2
27	5.3	5.3	5.4	5.5	5.6	5.6	5.7	5.7	5.9	6.0
28	6.1	6.2	6.3	6.4	6.6	6.7	6.9	7.0	7.2	7.3

Table 2. Values of Müller curve for incubation period expressed in days of incubation at specified mean daily air temperatures

The percentage is calculated according to the pattern by Sisakovic [87], where the temperature of 8°C is taken as the lower threshold of the parasite development. The percentages are added up and the incubation is over once the sum reaches 100%. Compared to the previous one, this method has certain advantages as it starts from a lower temperature threshold of development of 8°C. The pattern comprises the following elements:

$$I = \sum(t-8)/60.\ 100$$

I = duration of the incubation period in %
Z = sum of effective temperatures (t-8) per days
t = mean daily temperature in ^{0}C
60 = sum of temperatures (t-8) at the end of the incubation

The incubation period is over once the value I reaches 100%.

Under the optimal conditions for the development of the pathogen (23-24^0 C), the daily incubation is achieved with 25% and the incubation period lasts for 4 days.

The timing of treatments according to this method is similar to the one applied in the previous method, towards the very end of the incubation or once 50% of the incubation period has elapsed, depending on the possibility for administering the treatment.

5.1.5.3. The method according to Mrezanin and Lipickaja – Method 3

The third method, Mrezanin and Lipickaja, is based on the sum of effective temperatures that amounts to 61^{0}C. According to these authors, forecast of the occurrence and development of the pathogen, as well as duration of the incubation period, starts from 7.9^{0}C (8^{0}C).

The sum of effective temperatures for individual mid-day temperatures is determined according to the formula

$$I = \sum (t - 8)$$

I = duration of the incubation in days
Z = sum of the effective temperatures (t-8)
8 = minimum temperatures for growth and development of the mycelia
t = mean daily temperature
t-8 = effective temperature that affects the development of the mycelia

The basis is reduction of mid-day temperatures by 8. If the mid-day temperature is 15.3 (15.3 – 8 = 7.3), the effective temperature is 7.3. Once the sum of effective temperatures reaches 61, the incubation period is over.

The timing of spray applications is determined in a similar fashion as in the previous methods, towards the end of the incubation 40-50^{0}C or upon expiration of one-half of the incubation period (30-35^{0}C), if treatment cannot be completed in a single day. The advantage of this method lies in the simplicity of the calculations. It may be applied in cases of considerable temperature fluctuations, which are frequent during primary infections.

All of the described methods for determining the duration of the incubation period and timing of treatment are satisfactory and may be applied in practice.

Air humidity also impacts the duration of the incubation. If the air is saturated with moisture at the temperature of 14^{0}C, the incubation lasts for 6 days. At the same temperature, if the humidity is 80 and 90%, the incubation lasts for 10 days, whereas at the humidity <60%, Josifovic [88] according to Schad, the mycelium in the tissue develops very slowly. This is an indication of another factor that needs to be taken into account when calculating the duration of the incubation.

The duration of the incubation may also depend on the variety of the host plant, which according to Josifovic [88] may cause the incubation to be longer or shorter by 1-2 days.

As stated above, the incubation method forecasts the application of fungicides for the purposes of protecting pine seedlings from the pathogen *L. seditiosum* towards the end of the incubation period. It takes into account the local climate, phenology of plant development and biology of the pathogen development.

6. Results

The Table 3. presents the results of intensity of infection by *L. seditiosum* on seedlings of red pine (%) in Pirot, Serbia. Based on the variance analysis of the randomized block design, it

was determined that the difference between the medians was statistically significant at the probability of 95%, since F0>F0.05. Moreover, a statistically significant difference was found between mid treatments at the probability of 99%, since F0>F0.01. There is no statistically significant difference between mid treatments of other variances and all untreated treatments, and the differences are incidental.

By means of a multiple comparison procedure [85], two homogenous groups were identified with statistically significant differences at 99%.

As stated above, the incubation method forecasts the application of fungicides for the purposes of protecting pine seedlings from the pathogen *L. seditiosum* towards the end of the incubation period. It takes into account the local climate, phenology of plant development and biology of the pathogen development.

No	*Fungicide*	Conc. (%)	Infection of *L.seditiosum* (%)	*Efficacy* (%)	*Standard* (%)
1.	Blue bordo	0.5%	1.61 a	90.87	99.83
2.	Captan WP 50	0.3%	1.58 a	91.03	100.00
3.	Previcur 607 Sl	0.3%	1.53 a	91.35	100.36
4.	Untreated	-	17.64 b	0.00	0.00
	lsd 005		5.32		

Table 3. Biological efficacy on *L. seditiosum* on seedlings of red pine in Serbia

The level of consumption of fungicides is the result of spreading pathogen in forest production. However, often the use of pesticides in crop production are excessive and inadequate, and the costs arise because of this is unnecessary. The most important negative consequence of excessive use of pesticides are their environmental aspect, which is reflected in an increased content of residues in food and human health.

Forecasts of harmful organisms and its contribution to the rational use of pesticides are even more important. Forecast of harmful organisms is a complex process, the quality of the work requires continuous implementation of all necessary procedures. A very important role during this process belongs to the monitoring of meteorological conditions in the crop or plantation, necessary for the emergence and growth of harmful organisms. The rapid technological advances in this field of meteorology has enabled the introduction of more modern and easier monitoring devices for required meteorological elements.

In order to make an accurate determination of the incubation period according to the described methods, we followed mean daily temperatures [°C] and relative air humidity [%] in the period 1st August 2010 through 31st March 2011, as presented in Picture 1, in which the following cases may be distinguished: mean daily temperature above 12°C and relative humidity above 60%; mean daily temperature below 12°C and relative humidity above 60%; mean daily temperature above 12°C and relative humidity below 60%; mean daily temperature below 12°C and relative humidity below 60%

The most favourable conditions for the duration of the incubation are those presented under item 1 in Figure 1, i.e. the conditions with mean daily temperature above 12°C and relative humidity above 60%.

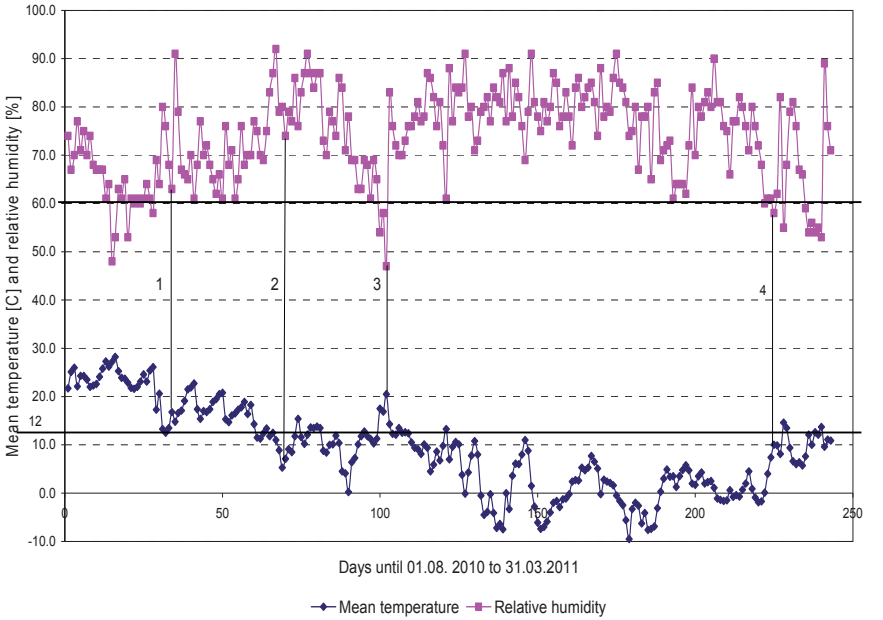

Figure 1. Mean daily temperature and relative humidity in the period 01 August 2010 through 31 March 2011

Figure 2 presents the number of consecutive days which meet both conditions and have mean daily temperatures above 12°C as well as relative humidity above 60%, and the number of consecutive days which only meet the condition to have mean daily temperatures above 12°C. The period during which the conditions were observed was 1st August 2010 through 31st March 2011. Picture 2 shows that the most favourable conditions lasted over the first 60 days from the beginning of measuring for observation of the set conditions.

Figure 3 presents the number of days required for achieving the duration of the incubation period – METHOD 1. For example, by observing the 17th number in the group of days we may see that the duration of the incubation period I = 60 (the blue column) while the required number of days is 11 (dark red column). In the beginning of measuring, up to the 14th group of days, average duration of the incubation period I is approximately 55 (blue columns) while the number of required days is about 4 (dark red columns).

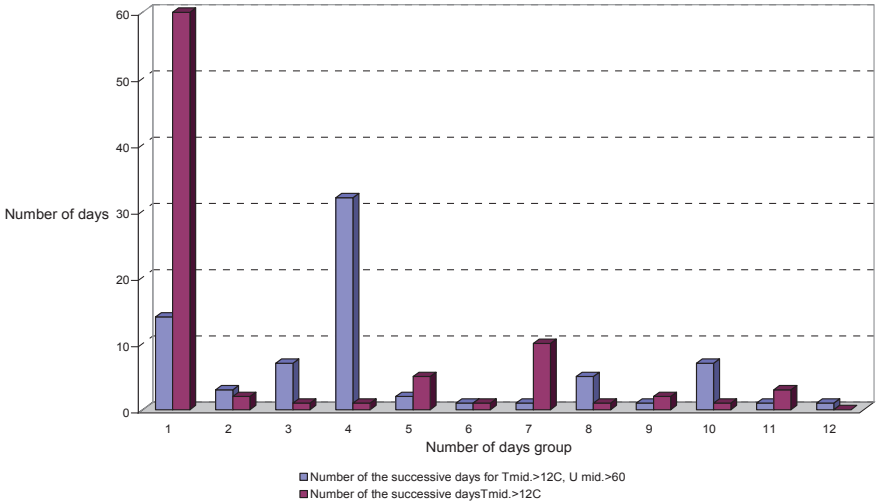

Figure 2. The number of consecutive days that provide the conditions for the formation of infection

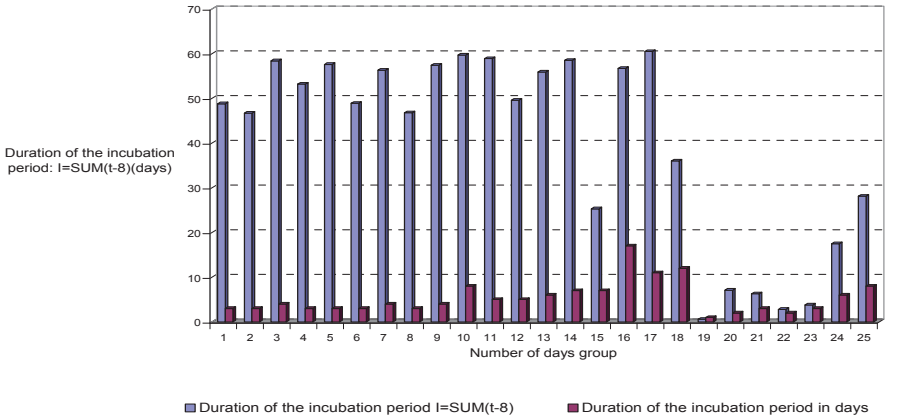

Figure 3. Duration of the incubation period I=SUM (t-8) and number of days

Figure 4 presents the days required for achieving the duration of the incubation period I given in percentages [%] - Method 1. It may be clearly seen that there is one case in which the incubation is completed (I = 100%), which is the 17th measuring (number of the group of days) where the duration of the incubation period I = 100%, (dark red column) while the required number of days is 11 (blue column).

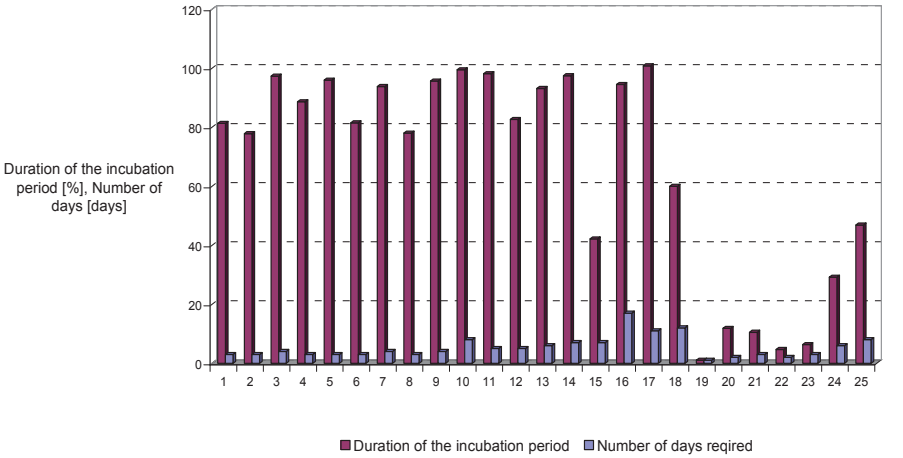

Figure 4. Duration of the incubation period I=SUM (t-8)/60*100 in [%] and needed number of days

Figure 5 shows the number of days until the end of the incubation for the period 1st August 2010 through 28th August 2010 (the most favourable conditions for achieving the incubation period), Method 2. It is clear that the average value of the number of days until the end of the incubation is 10 days.

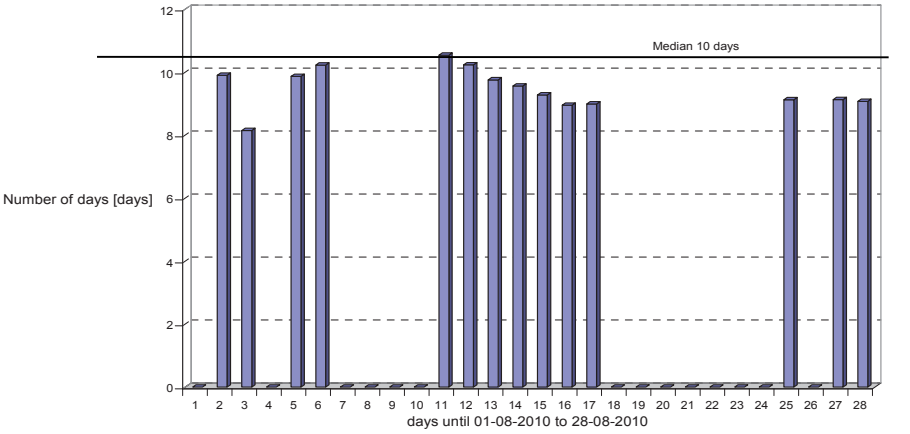

Figure 5. Number of days until the end of incubation, C=A/B-B

Softvrer for data calculation for Method1 and Method 2 is written in MATLAB. Thanks to this program it was possible to do the calculation for the measured data and Method 1 and Method 2, and the results were used in Microsoft Excel for graphical display of their comparative analysis.

A comparison of Method 1 and Method 2 leads to conclusion that Method 1 is "more accurate" with the average forecast of the period of duration of the incubation about 4 days (green line on Figure 6.), while in Method 2 this period is about 10 days (blu line on Figure 6). The relative air humidity for all measurements was taken to be equal to or above 60.

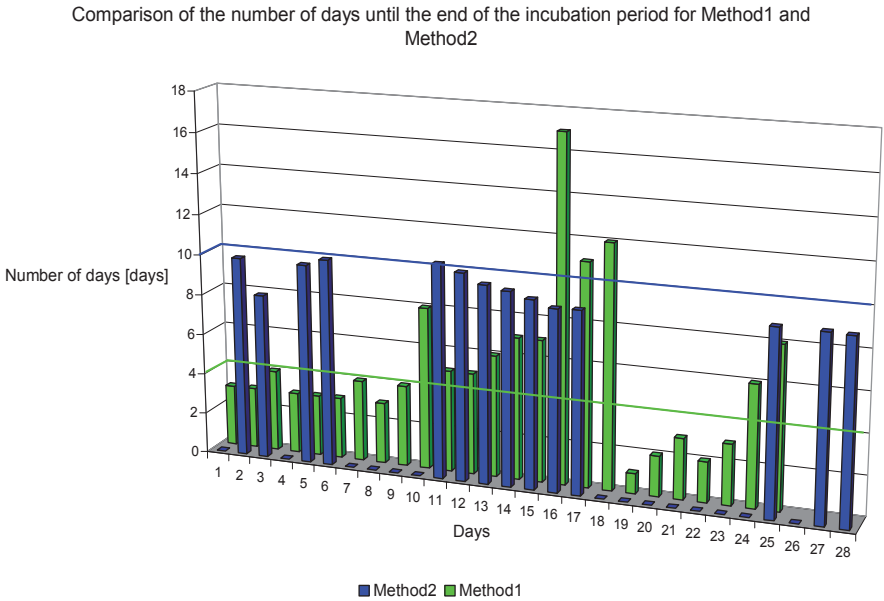

Figure 6. Comparsion of Method 1 and Method 2

7. Conclusion

Species from the genus *Lophodermium* Chevall. is the most dangerous and the most harmful pathogenic fungus in nurseries and young crops, which causes redness and dispersal of pine needles.

Forecast of harmful organisms is a complex process, the quality of the work requires continuous and thorough implementation of all necessary procedures. A very important role during this process belongs to the monitoring of meteorological conditions in the crop or plantation, necessary for the emergence and growth of harmful organisms. The rapid technological advances in this field of meteorology has enabled the introduction of more modern and easier monitoring devices required meteorological elements.

In order to use pesticides to rationalize and optimize, the proposed prognostic models were predict the development of this disease. In this way the forecasting models allow producers the timely and successful intervention in order to prevent damage of the causal disease.

Preventive as weel as cultural and chemical controls have been developed to minimase damage.

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Section 2

Exploring Natural Products

Chapter 9

Natural Products from Plants and Fungi as Fungicides

Marina D. Soković, Jasmina M. Glamočlija and Ana D. Ćirić

Additional information is available at the end of the chapter

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1. Introduction

In early fifties of the twentieth century the agrochemical industry provided agriculture with a vast array of chemicals for crop protection, including fungicides. Random synthesis, biological screening and empirical optimization yielded many effective compounds (Cremlyn, 1991). Whereas it is generally acknowledged that the use of pesticides has large benefits to farmers, the present use of pesticides in agriculture also causes negative environmental (and health-related) effects to society. For example, during and after application of pesticides a substantial amount of it could end up in soil, ground- and surface water or air. These negative effects demands for an effective policy. Such policies have been initiated, both at the level of the individual Member States of the European Union and at the level of the European Union itself (Oppenheimer & Donnelly, 1997). Their selectivity between target organisms and plants is mainly based on differences in uptake. The more recently developed protective chemicals are more potent in terms of dose required to control the pest or disease, and in distinguishing between target and non-target organisms. They usually have a specific mode of action. Since selective compounds are specific site inhibitors in the metabolism of target organisms, the risk of developing resistance is high. This has occurred for a number of fungal plant pathogens (Delp, 1988). Although pathogenic microorganisms are mainly controlled chemically, the use of synthetic compounds is limited due to several undesirable aspects, which include carcinogenicity, teratogenicity, acute toxicity and the requirement of an extended degradation period with consequent development of environmental pollution problems. The new awareness of modern consumers about these problems has created a "green" consumer profile that demands the absence of synthetic chemicals in food production and preservation together with extended shelf life of the majority of food products. Fungal infections remain a therapeutic problem in many fields despite the availability of a number of treatments. Such diseases in humans have markedly increased during the past ten years, especially in immunocompromised

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patients. Consequently, up to 10% of hospital acquired systemic infections are caused by fungi. Altogether this forces the scientific community, agro–industry and pharmaceutical companies to search for natural compounds that will satisfy consumer requirements (Harvey, 2008). Furthermore, there is growing concern about chemicals for protection because of their undesirable side effects in humans, other target organisms and their behavior and fate in the environment (Jespers, 1994).

The total number of all known natural products is around one million, including both bioactive and inactive compounds, plants metabolites 600000, fungal metabolites 8600, microbial metabolites recognized until now is around 50000. It is an obvious question, where is the border in the diversity of natural products? The general needs of the human society are continuously increasing. We need every new compounds which may be useful for the human society. More food, new drugs, and other goods are highly necessary for the benefit of humankind. The only question is the existence of sufficient natural and technical resources to fulfill these demands. Fortunately, in the area of the research of bioactive microbial products it seems that the ever expanding scientific and technical possibilities are increasing together with the continuously widening needs of the human therapy, veterinary and agriculture. The problem really is not whether we would be able to discover further new useful microbial compounds, but rather how can we optimize and quickly and effectively apply the chances derived from the new discoveries. How can we pick up and use effectively the proverbial needle found in the haystack (Berdy, 2005). However, screening of more than a million substances in the last decade has resulted in the introduction of only a very limited number of compounds with novel modes of action and resistance. This explains the renewed interest of the chemical industry in natural compounds with a variety of unique characteristics, waiting to be exploited. Natural products derived from plants and fungi have traditionally been used in ethnomedicine. Throughout the development of both Western and Eastern civilizations, whole plants, fungi, their parts, derived compounds and extracts have functioned as sources of food and medicine, symbolic articles in religious and social ceremonies, and remedies to modify behavior. Plant and fungal extracts and compounds containing physiologically active biochemicals have immense potential for producing new agents of great benefit to mankind. In this context, systematic screening of secondary metabolites of folk herbs and fungi may result in the discovery of novel and effective antimicrobial compounds (Hussain et al., 2011). Recently, interest has been growing in natural products due to their availability, fewer side effects and less toxicity as well as better biodegradability when compared to other available antimicrobial agents and preservatives. Thus, plants and mushroom may offer great potential and hope. Consequently, natural products are attracting the attention of scientists because they are cheaper, safer, eco-friendly and within the reach of the current medical community. This paper gives an overview on the activity of plant and fungi derived extracts as well as their constituents against a wide variety of microfungi, methodology and potential uses (Figure 1.).

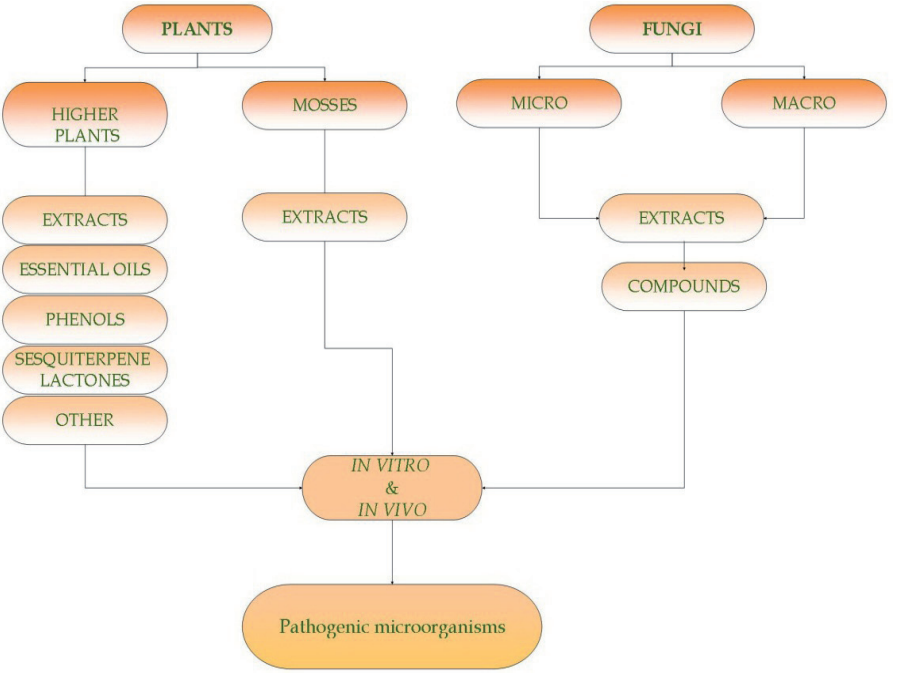

Figure 1. Our approach for testing of antifungal activity of natural products from fungi and plants.

2. Methodology

In order to test antifungal activities of natural products derived from plants and fungi few conventional and non-conventional methods were applied. Method is selected depending on the characteristics of extracts and compounds tested. A various number of plant, animal and human fungal pathogens were used. Growth cultures are conducted under optimal physical conditions for individual species. The growth of fungi was assessed visually or instrumentally. Two replicates were done for each compounds and the experiment was repeated two times.

Agar diffusion method is suitable for testing of antifungal activity of hydrophilic compounds which easily could be dispread trough to the agar medium. The compounds investigated were mixed with 0.01% Tween 20 surfactant and dissolved in molten MA medium. The fungal species were cultured for 7 days on Malt agar medium. Micromycetes were inoculated in the centre of Petri dishes and incubated for 21 days at 25° C. Mycelial growth was observed every 7 days and compared with the control. The commercial fungicide was used as a positive control (Ishii, 1995). The minimum inhibitory concentration (MIC) of compounds was determined when it achieved a complete stop in the growth of mycelium.

Microdilution method is suitable for testing small quantities of extracts, fractions or components, simultaneously in many different concentrations. In order to investigate the antifungal activity of the compounds, a modified microdilution technique was used (Hanel & Raether, 1988; Daouk et al., 1995). The fungal spores were washed from the surface of agar plates and adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μl per well. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (1 mg/ml) and added in broth Malt medium with inoculum. The microplates were incubated at Rotary shaker (160 rpm) for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μl of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 μl of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, commercial fungicides were used as positive controls.

Microatmosphere test, a slightly modified agar disc diffusion method, is perfectly suitable for the estimation of essential oil activity in vapor phase (Zollo et al., 1998). The assay was performed using mushroom pathogenic fungi. Petri dishes were filled with malt agar (MA), and then seeded with a 7 day-old mycelial culture of the tested fungi. The Petri dishes were then inverted and the determined amount of essential oils impregnated on sterile filter paper discs (4 mm) attached to the inverted lid (1 disc per lid). The Petri dishes were wrapped with parafilm along the rim, inverted and incubated for 21 days at 25 °C in an incubator. The results are presented as the diameter of the microorganism growth inhibition zone, or as the essential oil minimal inhibitory quantity (MIQ), which inhibits the total growth of microorganism. Commercial fungicides were tested as a control.

Bioautography is widely used for the examination of extracts. When the solvent evaporates, the broth and microorganisms are applied on the chromatographer paper or plates, and after the incubation time, growth is scrutinized. No growth is observed on the active spot components. Simultaneously, the components of the extract are eluted and identified. Different volumes of the mycelium extracts and pure compounds were dissolved in appropriate solutions. Ten micro liters of each sample were applied on TLC plates and sprayed either with freshly prepared fungal suspensions in nutrient broth (TSB). The plates were incubated for 18 h at 37°C and then sprayed with aqueous sol. 3% of p-iodonitrotetrazolium violet and stored for another 3 h. After this period plates were sprayed with 70% EtOH to stop fungal growth and were incubated for 36 h at 27 °C. White inhibition zones on a pinkish background were indicative of antimicrobial activity of tested extracts or compounds. The widths of these zones (mm) are the measure of efficiency and presented as minimal inhibitory concentration (MIC) (Pacher et al., 2001). As positive controls commercial fungicides have been used.

3. Antifungal activity against plant pathogens

3.1. Fungal extracts and metabolites

The fungi constitute a very large group of organisms that are found everywhere and are of great importance to life on earth and to human society. This is mainly due to the many interactions among fungi and other organisms. Most fungi produce a wide variety of secondary metabolites with biological activity. A wealth of literature exist on the discovery and potential use of mycotics in agriculture (Berdy, 1980; Guterson, 1990). However, their exploitation in agriculture remained limited, because widespread application in crops might simultaneously select for resistance to these mycotics in human pathogens (Isono, 1990). The concept that substances derived from one living organism may affect another organsm is old. Some of the secondary metabolites that occur in fungi are fairly widespread, but many are confined to a few species. Hence, screening of further fungi species usually leads to the discovery of new bioactive secondary metabolites. The broad diversity of the fungi, as well as their easy acquisition makes them especially interesting for natural products screening program. Among fungal species, the various microscopic (filamentous) fungi (ascomycetes, fungi imperfecti, *etc.*) are the most frequent producers with about 6400 produced compounds. From the most common ascomycetes, namely from *Aspergillus*, *Penicillium* and *Fusarium* species 950, 900 and 350 compounds have been isolated, respectively. Besides them several other filamentous and endophytic species (*Trichoderma*, *Phoma*, *Alternaria*, *Acremonium* and *Stachybotrys*), are also good producers, each produces several hundreds of bioactive compounds. From higher fungal species – basidiomycetes or mushrooms – exemplified by *Ganoderma*, *Lactarius* or *Agaricus* species, altogether about 2000 active compounds have been derived. From yeasts, only 140 and from Myxomycetes (slime moulds) species 60 bioactive metabolites have been isolated. The chemically relatively simple fungal compounds, over the antibiotic activities frequently exhibit diverse biological effects, mainly phytotoxic and pharmacological activities. We should not forget, the great practical and historical importance of beta-lactams (penicillins, cephalosporins), the cyclosporin, and various statins (mevinolin, compactin, lovastatin, pravastatin, atrovastatin), which are all fungus derived compounds. Recently it is unquestionable that the interest to all types of fungal species, but mainly to endophytic and the so called marine fungi as possible sources of new bioactive compounds is highly increasing. The expansion of the very quick new screening methods led to the appearance of the increasing number of "unidentified" fungus as bioactive metabolite producers (over 250 new metabolites in the last two years), especially in the patent literature. It indicates the high speed of isolation, and identification/patenting process of new fungal products, and the long time need for taxonomical identification of new fungal species (Berdy, 2005). Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by differet modes of action from existing agents and, consequently, lack cross-resistance to chemicals currently used. Kurobane et al., (1981) reported that *Penicillium brefeldianum* produces fulvic acid which possesses antiviral, antifungal, antioxidant and antibiotic activities. Maskey et al., (2003) isolated two active substances,

8-O-methylaverufin and 1,8-O-dimethylaverantin as new antifungal agents from *Penicillium chrysogenum*. Nam et al., (2000) found that 8-O-methylsclererotiorinamine isolated from *Penicillium multicolor* showed antimicrobial activity. Funiculosin, found in *Penicillium funiculosum* possesses antibiotic activity (Ando et al., 1969), as well as substance SQ 30,957, a new antibiotic produced by *P. funiculosum* (Singh et al., 1986). We recently started an investigation on the antifungal activity of fungal extracts and metabolites from both micro- and macrofungi. The extracts of 17 microfungi (*Alternaria alternata, Cladosporium cladosporioides, C. fulvum, Fusarium sporotrichioides, F. trincintum, Paecilomyces variotii, Penicillium ochrochloron, P. funiculosum, Phoma magdonaldii, Phomopsis helianthi, Stachybotrys chartarum, Trichoderma viride,* and five dermatomycetes, *Epidermophyton floccosum, Microsporum canis, Trichophyton mentagrophytes, T. rubrum* and *T. tonsurans* were tested against the yeast *Candida albicans* using the bioautographic assay test on TLC plates (Rančić, 2004).

While herbs are rather commonly used in the Western hemisphere, medicinal use of mushrooms, which has a long tradition in Asian countries, has also slightly increased in Europe during the last few decades. Although there has been extensive research on properties of medicinal mushrooms, their true potential is yet to be revealed. A number of compounds possessing significant antimicrobial activity have been isolated from polypore fungi. They provide a rich variety of active secondary metabolites and polysaccharides. Medicinal mushrooms such as *Agaricus brasiliensis, Coprinus comatus, Coriolus versicolor, Ganoderma lucidum, Lentinula edodes, Phellinus linteus*, and many others have traditionally been used as health foods or supplements for the prevention and cure of a range of diseases, including atherosclerosis, cancer, chronic hepatitis, and diabetes. The preventive and therapeutic effects of these mushrooms and their components have been well documented in mouse and rat model systems and in cancer cell lines. This has led to a considerable amount of knowledge about the effects of mushroom extracts and of their modes of action. It is generally accepted that mushroom extracts contain a variety of components, such as polysaccharides (*i.e.* glucans), small proteins, lectins and polyphenols, each of which may have its own biological or medicinal effect. The most common immunomodulatory action of mushroom are attributed to β-(1→3)-(1→6)-glucans, which have been studied in some detail (Smiderle et al., 2010). Vaz et al., (2011) described and compared the chemical constituents (phenol compounds, macronutrients, sugars, fatty acids, tocopherols and ascorbic acid) of four wild edible mushrooms widely appreciated in gastronomy: *Armillaria mellea, Calocybe gambosa, Clitocybe odora, Coprinus comatus*. Polysaccharides have emerged as an important class of bioactive substances, and many medicinal and therapeutic properties are attributed to them (Alquini & Carbonero, 2004). *Trametes versicolor, Laetiporus sulphureus* and *Ganoderma lucidum* are just some of the known mushrooms with this potential. This alone has made them suitable candidates for critically needed new antibiotics and antimycotics (Zjawiony, 2004). *Laetiporus sulphureus* is a wood-rotting basidiomycete, growing on several tree species and producing shelf-shaped fruit bodies with a bright yellow fleshy margin. This recognizable pigmentation along with the fruit body form is

responsible for the trivial name under which this fungus is known, and that is sulfur shelf (Weber et al., 2003). Even though it is recognized as a source of active compounds, and is widely used as a food among human, reports on the antimicrobial activity of *L. sulphureus* extracts are scarce (Turkoglu et al., 2007; Zjawiony, 2004). The potential barrier to everyday use of medicinal mushrooms as therapy is the manner in which the mushroom is consumed. Most research on fungi as potential antimicrobial agents is based on ethanol and methanol extracts of the fungal fruit body (Barros et al., 2007; Turkoglu et al., 2007). Consumption of products on this basis is of no practical use. To test the antifungal activity of extracts and metabolites of macrofungi we chose the wood-rotting basidiomycete, *L. sulphureus*, also named chicken of the woods. It is known for its nutritional value. An aqueous extract obtained from *L. sulphureus* was investigated for antimicrobial properties using a microdilution assay *in vitro* against seven fungi (four *Aspergillus,* two *Penicillium* species and *Trichoderma viride*). This extract showed strong activity against the tested microorganisms in a dose dependent manner (Šiljegović et al., 2011a). The presence and growth of microfungi in food may cause spoilage and result in reduction in quality and quantity. The presence of toxigenic fungi in foods stored for long periods of time is a potential hazard to human and animal health. Consumption of tomato products has been associated with a lower risk of developing digestive tract and prostate cancer. Therefore, preservation of tomato paste seems to be of great importance, both for the food industry, and for human well-being. We have used a methanol extract of *L. sulphureus* as an *in vivo* inhibitor of *Aspergillus flavus* growth in tomato paste. The results indicated complete inhibition of *A. flavus* growth in tomato paste for 15 days. An inhibition rate of 99.83% was achieved with 0.15 mg/ml of extract. Complete fungicide activity (100%) and no spore survival in the tomato product was recorded using 0.25 mg/ml of *L. sulphureus* extract in tomato medium. Since *L. sulphureus* is widely consumed as an edible macrofungus, its use as a natural preservative in tomato products can be considered as safe (Stojković et al., 2011a).

3.2. Plant extracts and metabolites

With increasing acceptance of traditional plants as an alternative form of health care the search for active compounds in plants becomes very important. Medicinal and aromatic plants have been employed for many centuries and they are mentioned in folklore from ancient times. After the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobials become virtually nonexistent to be rediscovered, as well as other alternative forms of medical treatments in the late 1990s (Cowan, 1999). There are several approaches to choosing sources of natural products for the discovery of potential antifungal compounds. One of approach is to investigate whole extracts of potential antifungal plants. Other approaches are to obtain biological material, which has not previously been studied for fractionation and testing, or some other sources. One strategy is to use ethnobotanical and/or chemical ecology clues to select which plants to sample (Duke et al., 2000). Here at first we will discuss the antifungal activities of plants extracts and after that some secondary metabolites derived from plants.

3.2.1. Plant extracts

There are many reports concerning the antifungal activity of plant extracts, but we will mention only a few. Ushiki et al., (1996) found that root extracts from twelve medicinal plants displayed antimicrobial activity against certain pathogens of soil-borne plant diseases. Among these plants, *Geranium pratense* (Bigroot geranium) strongly inhibited the growth of *Streptomyces scabies* which causes common scab of potato. It was shown that geranin, isolated from Bigroot geranium roots possessed a 1.25% higher antimicrobial effect than streptomycin (Ushiki et al., 1997). Previous studies indicated that certain crops and vegetables contain antimicrobial substances in their roots, and that these substances directly suppress growth of the pathogen and development of the disease (Clarke, 1966; Masaoka et al., 1993; Naqvi & Chauhan 1980; Yoshihara et al., 1988). In a program to screen extracts from medicinal plants for fungicidal activity, it was found that aqueous extracts of *Reynoutria suchalinensis* (Polygonaceae) showed favorable protecting control of powdery mildew (Herger et al., 1988).

An ethanol extract of *Phlomis fruticosa* (Jerusalem sage) (Labiatae) tested by diffusion method inhibited *Aspergillus niger, Penicillium ochrochloron, Trichoderma viride, Fusarium tricinctum* and *Phomopsis helianthi.* Moreover, this extract had fungicidal activity against *Cladosporium cladosporioides* and *Aspergillus ochraceus* at a very low concentration (10-20 μg/ml), (Ristić et al., 2000). Further investigation showed that when this extract was hydrolyzed with HCL and β-glucosidases, which remove sugars from flavonoids, it possessed greater antifungal activities than the original ethanol extract. Lower antifungal activity of the whole ethanol extract may be due to the presence of some aglycones, unstable flavonoid glycosides (Soković et al., 2000). It is generally known that flavonoid glycosides show lower activity against the microorganisms than aglycones (Raoha et al., 2000). Strong antifungal activity of a dealcoholized extract of leaves of *Cassia tora* was obtained against *Aspergillus niger* (Mukherje et al., 1996). Sato et al., (2000) analyzed 29 plants extracts against *Arthrinium sacchari* and *Chaetonium funicola.* The ethanol extracts of fifteen plants showed antifungal activity, but *Acer nikoense* (Nikko maple), *Glycyrrhiza glabra* and *Thea sinensis* (Tsa) were the most effective plants in very low amounts. Whole, fresh involucral bracts of cardoon, *Cynara cardunculus* L. (Compositae), were extracted with EtOH and an aqueous suspension of this extract was partitioned successively with $CHCl_3$, EtOAc and n-BuOH, leaving a residual water extract. Each extract was evaluated for antifungal properties. Antimicrobial activity was estimated using a microdilution technique against *Aspergillus niger, A. ochraceus, A. flavus, Penicillium ochrochloron, P. funiculosum, Trichoderma viride, Fusarium tricinctum* and *Alternaria alternata*. All cardoon extracts were found to possess antifungal activity comparable with standard mycotics (Kukić et al., 2008). Antifungal assays of branched centaury *Centaurium pulchellum* (Gentianaceae) extracts and secoiridoid glycosides isolated from this extracts have been studied as potent bioactive compounds against five fungal species. Methanol extracts from both aerial parts and roots exhibited excellent antifungal (0.1-2 mg/ml) activity. Pure secoiridoid glycosides isolated from these extracts demonstrated very strong antifungal (0.001-0.1 mg/ml) activity (Šiler et al., 2010). The antifungal activity of methanol extracts of three different Labiatae species (Catmint),

Nepeta rtanjensis, N. sibirica and *N. nervosa* (grown *in vitro*) against eight fungal species, was evaluated. All tested extracts showed significant antifungal activity, with that from, *N. rtanjensis* being the strongest (Nestorović et al., 2010).

Genuine mosses constitute a large group of nonvascular higher plants, consisting of about 14 000 species. Generally, bryophytes are not damaged by microorganisms, insects, snails, slugs, and other small mammals. Up to date, over several hundred new compounds have been isolated from bryophytes and their structures elucidated (Veljić et al., 2009). In spite of a number of secondary metabolites identified from various mosses, the chemical profiles of most species are insufficiently known or even unknown. Secondary metabolites from mosses, identified so far, include terpenoids, flavonoids and bibenzyls, and also derivatives of fatty acids. Mosses rich in flavonoids has been found to possess strong antimicrobial activity (Veljić et al., 2009). An ethanol extract of bryophyte, *Bryum argenteum* (silver moss), showed antifungal activity against two fungi (*A. niger* and *P. ochrochloron*) (Sabovljević et al., 2006). Our investigations also demonstrated that methanol extracts of selected genuine mosses (*Pleurozium schreberi*, *Palustriella commutata*, *Homalothecium philippeanum*, *Anomodon attenuatus, Rhytidium rugosum*, *Hylocomium splendens*, *Dicranum scoparium* and *Leucobryum glaucum*) possess antimicrobial activity when tested by the microdilution method (Veljić et al., 2008). When the antifungal activity of methanol extracts of the mosses *Fontinalis antipyretica* var. *antipyretica*, *Hypnum cupressiforme* and *Ctenidium molluscum* were analyzed, that of the first species showed strongest activity against the following micromycetes: *Trichoderma viride*, *Penicillium funiculosum*, *P. ochrochloron*, *Aspergillus fumigatus, A. flavus* and *A. niger* (Veljić et al., 2009). The antifungal activity of extracts of three bryophyte species, two mosses (*Atrichum undulatum, Physcomitrella patens*) and a liverwort (*Marchantia polymorpha* ssp. *ruderalis*), grown under natural conditions and in axenic culture, was evaluated by the microdilution method against five fungal species. Each bryophyte extracts was active against all fungi tested. In general, extracts made from material grown in laboratory (*in vitro*) conditions express stronger antifungal activity than those made from material from under natural conditions. Some of the fungi tested reacted similarly to both extracts (Sabovljević et al., 2011). The antimicrobial activity of a dimethyl sulfoxide extract of the moss *Rhodobryum ontariense* was evaluated by microdilution method against *Aspergillus versicolor*, *A. fumigatus*, *Penicillium funiculosum*, *P. ochrochloron* and *Trichoderma viride*. The extract was active against all the fungi tested but to varying degrees. This finding implies that *R. ontariense* could be considered as a promising material for natural antifungal products (Pejin et al., 2012 in press).

3.2.2. Plant secondary metabolites

Plants produce a diverse array of secondary metabolites, many of which have antifungal activity. Some of these compounds exist in healthy plants in biologically active forms. Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Compounds

belonging to the latter category are still regarded as constitutive because they are immediately derived from preexisting constituents (Mansfield, 1983). A large number of plant compounds have been reported to have antifungal activity. Well known examples include; flavonoids (Ćirić et al., 2011; Karioti et al., 2011; Weidenbörner & Jha 1997), lactones (Djeddi et al., 2007; Janaćković et al., 2002; Skaltsa et al., 2000a, 2000b; Vajs et al., 1999, 2004), proteins (Giudici et al., 2000), sulfur compounds (Ilić et al., 2012), cyanogenic glycosides and glucosinolates (Osbourn, 1996) and essential oils (Daouk et al., 1995; Džamić et al., 2010; Garg & Siddiqui, 1992; Glamočlija et al., 2006a, 2006b, 2009; Marinković et al., 2002; Mishra & Dubey, 1994; Müller-Riedau et al., 1995; Rančić et al., 2005; Shimoni et al, 1993; Soković, 2001, 2002, 2008a, 2008b, 2009a, 2009b, 2009c; Stojković et al., 2011b, 2011b; Thompson, 1989).

3.2.2.1. Essential oils

Essential oils from aromatic and medicinal plants have been known since antiquity to possess biological activity and constitute one of the most investigated groups of secondary metabolites. With growing interest in their use in the pharmaceutical and agrochemical industries, systematic examination of oils for these properties has become increasingly important. Over the last hundred years antimicrobial properties of common spice oils have been demonstrated (Bullerman et al., 1977) and many studies have been made on antifungal activities of essential oils (Daouk et al., 1995; Garg & Siddiqui, 1992; Glamočlija, 2006b, 2009; Kalemba & Kunicka, 2003; Mishra & Dubey, 1994; Müller-Riedau et al., 1995; Shimoni et al, 1993; Soković, 2001; Thompson, 1989). Thus, Maruzzela & Balter (1959) found that, out of 119 spice oils tested, 100 essential oils possessed an antagonistic effect to at least one of twelve phytopathogenic fungi and 50 of these compounds showed wide spectrum activity against all fungi tested. The essential oil of *Origanum majorana* exerted considerable inhibitory powers against *Aspergillus flavus, A. niger, A. ochraceus, A. parasitcus* and *Trichoderma viride* (Deans & Svoboda, 1990). A comparative study of the antifungal activity of essential oils extracted from thyme, rosemary, eucalyptus and mugwort was carried out by a group of investigators against 39 mold strains. The essential oil of thyme was found to be the most effective (Conner & Beuchat, 1984). Essential oils of allspice and cloves totally inhibited *Trichoderma viride, Alternaria alternata, Fusarium oxysporum, Mucor circinelloides, Rhizopus stolonifer, Cladosporium cladosporioides, Aspergillus versicolor* and *Penicillium citrinum* in a concentration of 2% (Schmitz et al., 1993). Essential oils from other plants such as Wormwood *Artemisia afra,* Lavender tree, *Heteropyxis natalensis* and Sweet gale *Myrica gale,* were found to have strong inhibitory effects against a broad spectrum of fungal species. The essential oil of *Origanum syriacum* showed very strong antifungal activity against *Penicillium, Aspergillus* and *Fusarium* species (Daouk et al., 1995 and references cited therein). The essential oil of Soldier's herb *Piper angustifolium* was very effective effect against *Aspergillus niger* and *A. flavus* (Trillini et al., 1996). The antifungal activities of the essential oils from Lemon mint *Monarda citriodora* and Tea tree *Melaleuca alternifolia* were evaluated *in vitro* on fifteen common post-harvested pathogens of a variety of crops. Both essential oils exhibited a high level of antifungal activity, by direct contact and in the vapor phase. Oil from Lemon mint was generally more active than that from Tea tree, particularly against rapidly growing fungal species (Bishop & Thornton, 1997). Baratta et al., (1998) examined essential oil from

eight commercial plants (*Cinnamomum zeylanicu* (Cinnamon), *Cananga odorata* (Ylang ylang), *Ocimum basilicum* (Sweet basil), *Citrus limon* (Lemon), *Cymbopogon citratus* (Lemon grass), *Baswellia thurifera (*Boswellia), *Majorana hortensis* (Marjoram) and *Rosmarinus officinalis* (Rosemary) and showed that all the oils tested were able to inhibit the growth of the common spoilage fungus, *A. niger*, even at a concentration of 1 µl/ml broth, with the exception of lemon and rosemary oils which exhibited inhibitory effects on higher concentrations. Essential oils extracted from different parts of some angiosperms (Cedarwood *Cedrus deodara* and Ajwain *Trachysremum ammi*) were analyzed for fungitoxicity against the mycelial growth of *Aspergillus niger* and *Curvularia ovoides,* two fungi found in *Vigna mungo.* Since the essential oils from both plants exhibited fungitoxic properties, it may be possible to use them to control various fungi and exploit them as fungicides (Singh & Tripathi, 1999). The antifungal activities of four essential oils from spice (sage, thyme, oregano and savory) were analyzed against *Fusarium oxysporum, Macrophomina phaseoli, Botrytis cinerea, Rhizoctonia solani, Alternaria solani* and *Aspergillus parasiticus.* Earlier results showed weak activity for sage, while thyme, oregano and savory were active against all moulds tested (Ozcan & Boyraz, 2000). Different essential oils have antifungal activity against a wide range of fungi. Thus, considering the importance of these oils, 75 different essential oils were tested against *A. niger*. All the oils possessed antifungal activity (Pawer & Tacker, 2006). We examined a variety of essential oils from several plant families (Compositae, Labiatae, Lauraceae, Apiaceae, Cupressaceae, Poaceae, Illiaceae, Myrtaceae, Verbenaceae). Essential oils of Wild marjoram *Origanum onites,* Thyme-leaved savory *Satureja thymbra,* Greek sage *Salvia fruticosa* and *S. pomifera* subsp. *calycina* plants growing wild in Greece and their components; carvacrol, camphor, and 1,8-cineole, were assayed for antifungal activity against thirteen fungal species. The oils inhibited all fungi investigated. The highest and broadest activity was shown by oils containing the carvacrol (Wild marjoram and Thyme-leaved savory, while Greek sage was the least effective (Soković et al., 2002). The antifungal activity of essential oils from three *Micromeria* species: *M. dalmatica*, *M. albanica* and *M. thymifolia* was investigated against seven fungal species. The oils from all three *Micromeria*: (*M. dalmatica*-0.2-0.4 µl/ml, *M. thymifolia*-0.4-2 µl/ml) and particularly *M. albanica* (0.2-0.4 µl/ml) showed strong antifungal effects against all fungi tested: *Aspergillus niger*, *A. ochraceus, Penicillium ochrochloron, Cladosporium cladosporioides, Fusarium tricinctum, Phomopsis helianthi* and *Trichoderma viride* (Marinković et al., 2002). The essential oil of *Foeniculum vulgare* was tested for antifungal activity against nine different plant pathogenic fungi. This oil was most effective against *C. cladopsorioides* and *P. helianthi* (0.8-4.0 µl/ml), more effective than bifonazole (Mimica-Dukić et al., 2003). The antifungal activity of oils from eight *Stachys* species were tested against fungal pathogens (*A. niger* and *Penicillium ochrochloron*). The greatest activity was obtained for *S. scardica* due to the high content of sesquiterpene hydrocarbons (69.3%) in this oil (Skaltsa et al., 2003). Essential oil from *Juniperus excelsa* (Cupressaceae) was evaluated for antifungal activity against twelve micromycetes from the following genera (*Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium, Phomopsis* and *Trichoderma*). This essential oil showed moderate-high antifungal characteristics with MIC of 8.0–40.0 µl/ml and MFCs of 10.0–50.0 µl/ml. The essential oil was the most effective against the phytopathogenic species *Phomopsis helianthi* while *Trichoderma*

viride was the most resistant species treated with this essential oil. This essential oil exhibited higher antifungal activity than the commercial fungicide bifonazole (Soković et al., 2004). The antifungal activity of essential oils from different plant species of Lauracaea family (*Aniba rosaeodora* Rosewood, *Laurus nobilis* Bay tree, *Sassafras albidum* Sassafras and *Cinnamomum zeylanicum* Cinnamon) was investigated against seventeen micromycetes. In order to determine fungistatic and fungicidal concentrations (MIC and MFC) both diffusion and microdilution tests were employed. Essential oil from cinnamon was the most effective as an antifungal agent, followed by rosewood and sassafras oils. Laurel oil possessed the lowest antifungal activity (Simić et al., 2004). The antimicrobial activities of essential oils isolated from Sweet cicely *Myrrhis odorata*, St. John's wort *Hypericum perforatum* and *Helichrysum arenarium* were determinated by microdilution test. The greatest antifungal activity was observed for sweet cicely oil, while *H. arenarium* showed the lowest antifungal potential. Minimal inhibitory and fungicidal concentrations were 0.5-60 μg/ml. The oil of sweet cicely showed had higher activity than commercial product and was very effective against all fungi tested (Rančić et al., 2005).

Essential oils and their components from three spices of *Salvia* (Labiatae) were tested for antifungal activity in our laboratory. The antifungal activity of the corolla, calyx and leaf oils of *Salvia brachyodon* were analyzed. Antifungal activities of the essential oils were determined by the microdilution method against *Aspergillus niger*. The oil from the calyx possessed the strongest antifungal activity probably due to synergistic activity of all the components present. This oil contains high concentrations of sesquiterpenes and diterpenes, which were not found in the other samples investigated (Soković et al., 2005). Another species of *Salvia* genus, Clary sage (*Salvia sclarea*) was also tested as an antifungal agent. A concentration of 25 μl/ml showed fungicidal activity against *Aspergillus*, *Penicillium*, *Trichoderma viride* and *Fusarium* species. For *Mucor mucedo* the MFC was 15 μl/ml. Fungistatic and fungicidal activities of the oil against *Cladosporium cladosporioides* was recorded at concentrations of 2.5 μl/ml and 5 μl/ml. The most sensitive micromycetes were *Cladosporium fulvum*, *Alternaria alternata*, *Phomopsis helianthi*, and *Phoma macdonaldii*, where a concentration of 2.5 μl/ml was lethal (Džamić et al., 2008). The essential oil of Sardinian sage *S. desoleana* and its main components were investigated for antifungal activity against eleven micromycetes by macro- and micro-dilution methods. The essential oil and components investigated were diluted in ethanol and Tween 80 in both methods. We analyzed whole essential oil from Sardinian sage and its main components; linalyl acetate, 1,8-cineole and linalool, which together represent 52.71% of the total oil. Whole essential oil of Sardinian sage and linalool possessed strong antifungal activity, while 1,8-cineole exhibited only moderate potential. Linalyl acetate had the lowest antifungal potential. Values for MIC were lower with microdilution method (Soković et al., 2009a). The antifungal activity of *Nepeta rtanjensis* essential oil on mycelia growth has been determined by the diffusion method. It acted most efficiently against *Alternaria* species (0.6 μl/ml). *Bipolaris spicifera* and *Cladosporium cladosporoioides* had MIC values of 1.0 μl/ml, whereas *Trichoderma viride* with an MIC value of 1.6 μl/ml against this essential oil (Ljaljević Grbić et al., 2007). Antimicrobial activity from Citronella *Cymbopogon winterianus* (Poaceae) and Karawya *Carum carvi*

(Apiaceae) essential oils was investigated against seventeen fungal species. Oil of Karawya at concentration of 0.25–2.5 μl/ml stopped the growth of all tested micromycetes except for *T. viride*. For this resistant fungus, the concentration of Karawya oil had to be increased to 10 μl/ml. The essential oil from Citronella was less effective but still showed stronger antifungal activity than the commercial drug bifonazole. The MIC and MFC values of this oil were 1–20 μl/ml (Simić et al., 2008).

Essential oils isolated from the aerial parts during the flowering and vegetative phases, roots and seeds of the plant *Portenschlagiella ramosissima* were tested for antimicrobial activity against micromycetes (*Aspergillus flavus, A. niger, A. versicolor, Penicillium ochrachloron, P. funiculosum* and *Trichoderma viride*). All of the oils tested showed activity. The most effective was that isolated from the aerial parts of the plant during flowering, followed by the oil from the vegetative phase, seeds and roots. The most resistant fungal species were *Aspergillus flavus* and *A. versicolor* (Soković et al., 2008b). Essential oils of Star anise *Illicium verum* (Illiaceae) and Clavos *Eugenia caryophyllata* (Myrtaceae) were investigated as a potential antifungal agents. Star Anise oil exhibited fungicidal characteristics with MIC and MFC values of 2.5–25 μl/ml. Clavos oil showed strong antifungal activity at 0.1–2.5 μl/ml. The most resistant fungi were *Trichoderma viride*, *Penicillium* and *Aspergillus* species. The antimicrobial activity of star anise is mainly due to anethole while eugenol is responsible for antifungal effect of cloves oil. The authors raised the possibility that interactive effects of other compounds present in smaller quantities may also contribute. Thuse, both oils, but especially clove, showed powerful antifungal activity (Džamić et al., 2009). The antifungal activity from essential oils of wild carrot *Daucus carota* L. (Apiaceae) collected in Serbia were tested. The antifungal activity of oils from the ripe fruits, unripe fruits, flowers, root, leaves, and stem, were examined against eight fungal strains (*Fusarium sporotrichoides, Fulvia fulvum, Trichoderma viride, Penicillium ochrochloron, P. funiculosum, Aspergillus ochraceus, A. flavus* and *A.fumigatus*) by the microdilution technique. The essential oil of unripe fruits manifested the strongest antifungal potential followed by oils from ripe fruits, roots, stems, leaves and flowers. These oils were more efficient than the commercial drug bifonazole and much more active than ketoconazole. The most prominent biological activity was exhibited by the essential oils from ripe and unripe fruits of wild carrot oil (Soković et al., 2009c). The chemical composition and effectiveness of the essential oil obtained from *Echinophora spinosa* (Apiaceae) was tested on different fungi. The most resistant fungal species were *Penicillium ochrochloron* and *P. funiculosum* while *Trichoderma viride* was the most sensitive. This essential oil tested showed higher antifungal potency against *T. viride* than the commercial drugs bifonazole and ketoconazole (Glamočlija et al., 2011a). The essential oil of *Lippia alba* Bushy lippia (Verbenaceae) is reported as an antifungal agent against human pathogenic microorganisms but few articles concern its use for green mould control. We determined the antifungal activity of Bushy lippia essential oil against green molds (*Aspergillus ochraceus, A. niger, A. versicolor. A. fumigatus, Penicillium ochrochloron, P. funiculosum* and *Trichoderma viride*) as an alternative to synthetic fungicides. Microdilution assays evaluated the essential oil MIC and MFC. Bushy lippia essential oil has MIC of 0.3-1.25 mg/ml and MFC of 0.6-1.25 mg/ml. Bushy lippia essential oil is classificated as citral type and the results indicate that it

is a potential alternative to synthetic fungicides (Glamočlija et al., 2011b). The chemical composition and antimicrobial activities of the essential oils isolated from Pink savory *Satureja thymbra* and Black thyme *Thymbra spicata* (Labiatae) were compared. The oil of Black thyme possessed higher antifungal potential than Pink savory *S. thymbra* oil. *A. versicolor* and *A. fumigatus* were the most sensitive species, while *P. ochrochloron* was most resistant to these oils. Both oils showed much greater antifungal activity than a commercial antifungal agent (Marković et al., 2011). The essential oil of *Seseli montanum* subsp. *tommasinii* was tested for antifungal activity on four fungal species (*Aspergillus ochraceus*, *A. fumigatus*, *Penicillium ochrochloron* and *Trichoderma viride*). It showed moderate activity against all the tested fungi, but activity against *A. fumigatus* and *T. viride* was stronger than that of bifonazole. In the case of *A. fumigatus*, which is a very common and invasive pathogen, this is important due to the rising problem of fungal resistance to antifungal agents (Šiljegović et al., 2011b). We have studied the antimicrobial activity of *Seseli* species, examining essential oils obtained from the aerial parts of *S. anuum* (Milosavljević et al., 2007), *S. globiferum* fruits (Stojković et al., 2008a), *S. globiferum* aerial parts (Janaćković et al., 2011) and flowers of *S. rigidum* (Stojković et al., 2009). Differences in their activity were found. The essential oil from aerial parts of *S. anuum* showed activity against twelve fungi in the range of 12.5 to 50 μl/ml. That from fruits of *S. globiferum* had the strongest antifungal activity with the MICs and MFCs in the range of 0.5-50 μl/ml. Oil from the aerial part of *S. globiferum* showed significant activity against micromycetes (2.5-10 μl/ml). The essential oil from the aerial parts of *S. montanum* subsp. *tommasinii* was more active than that from the aerial parts of *S. anuum*. In the case of *P. ochrochloron* and *T. viride* this activity was twice that of *S. anuum* oil. On the other hand, the essential oil from *S. rigidum* had greater antifungal activity than that of *S. montanum* subsp. *tommasinii* against *A. fumigatus* and *P. ochrochloron* (Šiljegović et al., 2011b). The results of our investigation of the antifungal activities of essential oils from sixteen aromatic and medical plants and their components (Bitter orange *Citrus aurantium*, Lemon *C. limon*, *Hyssopus officinalis*, Lavender *Lavandula angustifolia*, Wild lavender *L. stoechas*, Chamomile *Matricaria chamomilla*, Melissa *Melissa officinalis*, Peppermint *Mentha piperita*, Spearmint *M. spicata*, Sweet basil, Rusmary *Rosmarinus officinalis*, Sage *Salvia officinalis*, Sardinian sage *S. desoleana*, Clary *S. sclarea*, Thyme *Thymus vulgaris* and *T. tosevii*) by the microdilution method showed that *Thymus* essential oils were the most effective in tests *in vitro*, while those from *Citrus* species and *S. officinalis* showed the lowest antifungal activities (Soković, 2001, 2009b). The same essential oils plus Oregano *Origanum vulgare* oil were assayed for inhibitory activity against major pathogens of the button mushroom, *Agaricus bisporus*, i.e. the fungi *Verticillium fungicola* and *Trichoderma harzianum*. The highest and broadest activity was shown by oregano and thyme oils with very low active concentrations (0.05-5.0 μg/ml) (Soković & van Griensven, 2006a). All the essential oils mentioned previously were also tested by microatmospheric method *in vitro* against 5 different isolates of *Mycogone perniciosa* (causal agent of wet bubble disease) from *Agaricus bisporus*. Essential oils which contained phenol components (thymol and carvacrol), (oregano, Greek oregano *O. heracleoticum*, Pink savory *S. thymbra*, thyme and *T. tosevii* with MIC values of 0.001 to 0.7 μl/disc and MFC of 0.1 to 1.0 μl/disc) showed significantly

stronger antifungal potential than those with high alcohol contents (Spearmint and Peppermint with MIC 1.0 μl/disc and MFC 2.5 μl/disc), and those oils with high keton contents (*S. pomifera* with MIC 1.0 μl/disc and MFC 15.0 μl/disc and *H. officinalis* with MIC 5.0 μl/disc and MFC 25.0 μl/disc), and especially than oils with monoterpenic hydrocarbons as dominant components (Lemon with MIC 4.0 μl/disc and MFC 5.0 μl/disc Bitter orange with MIC 0.7 μl/disc and MFC 1.0 μl/disc and Lavender MIC 1.5 μl/disc and MFC 2.5 μl/disc). The fungicide prochloraz showed much lower antifungal potential than all the oils tested, with MIC 5.0 μl/disc and MFC 50.0 μl/disc (Glamočlija, 2006a, 2006b, 2009). Also, there is only limited information in the literature on the antifungal activity of essential oils *in vivo.* The experiments *in vivo* are in relation with several problems of application of essential oils. Those related to the volatility of the oils and their poor solubility in water must be resolved before trials are performed *in vivo*. The persistence of a volatile oil on the treated plant and consequently its protection against pathogens is of short duration. The solubility problem means that organic diluents may have to be used, with the risk of environmental pollution and even phytoxicity. In an attempt to overcome these problems, some experimental formulations of essential oil and camphor, the most active components of Sage against *Botrytis cinerea* were prepared using as excipient a polymeric matrix obtained by the graft polymerization of acyclic monomers on gelled starch. Besides being absolutely harmless to plants, these polymers showed the capacity to form aqueous dispersions of the oil that were homogenous and adhered well to the surface of the leaves in some preliminary tests. Once the water had evaporated, the dispersions left a solid film which could be reduced to a hydrogel on the laminae of the leaves, from which the oil was gradually released in concentration related to the humidity of the microenvironment. This guarantees an extended duration of effective concentrations of the oil on treated plants (Moretti et al., 1998). The same researchers analyzed the effects of essential oil from Sage and camphor on tomato plants infected with *Botrytis cinerea*. A significant reduction of infection was found, but they not able to eliminate entirely the appearance of spots on the leaves. However, this encouraged further research in this field, with other essential oils which possessed greater antifungal activitiy *in vitro.* The effects of eleven plants essential oils for protecting maize kernel against *Aspergillus flavus* were studied. The optimal doses for maize protection, the influence of combinations of oils, residual effects and toxicity of the essential oils to maize plants were determinated. The principal constituents of eight essential oils were tested for their ability to protect maize kernels. Essential oils of Cinnamon, Peppermint, Sweet basil, Oregano, *Telaxys ambrosioides, Syzygium aromaticum* and thyme totality inhibited fungal development on maize kernel. Thymol and methoxycinnamaldexyde significantly reduced maize grain contamination. No phytotoxic effect on germination and corn growth was detected with any of these oils (Montes-Belmont & Carvajal, 1998). Reddy et al., (1998) showed *in vivo* that essential oils of thyme exhibited antifungal activity against *Botrytis cinerea* and *Rhizopus stolonifer,* two common pathogens of *Fragaria anamassa.*

Among a variety of oils tested *in vitro* against the pathogenic fungi *Mycogone perniciosa,* Oregano oil was singled out as the best. Although disease caused by *M. perniciosa* is routinely controlled with different fungicides, it remains a constant threat. In order to find

alternative preventive methods we evaluated the antifungal activity of Oregano oil *in vivo* in a mushroom growing unit. To treat experimentally induced mushroom disease in the growing house we tested the antifungal activity of oregano oil when applied in casing soil. The most favorable results were achieved with 2% of oregano oil and simultaneous application of the spores suspension when oregano oil completely inhibited the growth of *M. perniciosa*. As essential oils are largely nontoxic and easily biodegradable we advise on disinfection of commercial casing soil with 2 % oregano oil before applying the casing to the compost (Glamočlija, 2006a, 2007). The chemical composition and antimicrobial activity of essential oils of *Vitex agnus-castus* L. and their main constituents *in vitro* and *in vivo* showed that these oils could be used equally *in vitro* and *in vivo* (Figure 2.). The oils from all plant parts possessed great antifungal potential against eight fungal plant pathogens. Using the same technique 1,8-cineole and α-pinene (dominant compounds) showed very high antifungal potency as well. As 1,8-cineole was the predominant constituent of the oils, we tested it *in vivo*. Randomly chosen apples were treated with 1,8-cineol solution and infected with *Aspergillus niger* in order to provoke Aspergillus rot in apples. As apple fruits were treated with an acceptable amount of 1,8-cineole, we suggest it as a potent agent for preventing apple rot caused by *A. niger* and its application as a bioactive compound to control *A. niger* infection during apple storage (Stojković et al., 2011b).

Figure 2. Treatment of Aspergillus rot in apples with 1,8-cineol

3.2.2.1.1. Essential oils components

We will also discuss here the antifungal activities of essential oil components. Eugenol has exhibited very strong antifungal activity against *Absida glauca, Aspergillus nidulans, A. niger, Colletotrichum capsici, Fusarium monoliformae, Pestolotia psidi* and *Rhizopus nadssus*, while caryophyllene inhibited *A. glauca*. Cineole showed favorable activity against *Fusarium monoliformae* and *Alternaria alternata* and good to moderate activity against the remaining fungi tested. The chillie crop in India suffers from rips tot and die Back diseases caused by *C. capsici*. The growth of this fungus has very successfully been inhibited by cumaldehyde and eugenol. Cineole, cumalaldehyde and eugenol also very satisfactorily inhibited growth of *F. monoliformae, P. psidi* and *R. nadssus. F. monoliformae* causes localized "Softrot" of apical tissue, head blight, scab and root rot of wheat and other cereals, beke disease of rice and twisted top of sugarcane. These compounds could be used for inhibition of *A. alternata*, which attacks crucifers such as mustard, cauliflower, knol-knol and radish. Cumalaldehyde may also find application as an inhibitory agent against the growth of *P. expansum* which

causes infection if it gains a foothold in injured tissues. It also causes soft rot in apple fruit (Garg & Siddiqui, 1992). The essential oil of *Foeniculum vulgare* and components (anethole, fenhone and camphor) were screened for antifungal activity against nine plant pathogens. Anethole possessed the greatest activity (1.3-2.8 μl/ml), then fenchone (3.7-6.0 μl/ml), while camphor had the lowest antifungal effect (2.8-9.7 μl/ml) (Mimica-Dukić et al., 2003).

Essential oil components (β-caryophyllene, β-caryophyllene oxide, α-pinene, cadinene, linalool) from eight *Stachys* species were analyzed for antifungal activity. Linalool exhibited the greatest activity (0.03 mg/ml), while the lowest effect was observed for β-caryophyllene (0.3 mg/ml) (Skaltsa et al., 2003). We investigated the antifungal activity of limonene against fourteen fungal species using micro- and diffusion tests. Limonene showed antimicrobial activity against all fungi tested, in concentrations of 8.0-13.0 μl/ml in the diffusion and 6.0-10.0 μl/ml in the microdilution method. Differences in MICs obtained by these two methods could be attributed to low solubility of limonene in the agar medium used in the diffusion method. Limonene showed stronger antifungal potential than bifonazole, especially in the microdilution assay when the component was dissolved in Tween (Rančić et al., 2003). Phenol compounds were the most active among the components investigated. Limonene and linalyl acetate were less effective against micromycetes. All essential oils and components were investigated against twelve fungi: *Aspergillus niger, A. ochraceus, A. versicolor, A. flavus, A. terreus, Alternaria alternata, Penicillium ochrochloron, P. funiculosum, Cladosporium cladosporioides, Trichoderma viride*, *Fusarium tricinctum* and *Phomopsis helianthi* (Soković, 2001, 2009b). The components linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol and menthol were assayed for inhibitory activity against three major pathogens of the button mushroom, *Agaricus bisporus*, the fungi *Verticillium fungicola* and *Trichoderma harzianum*. The highest and broadest activity was shown by carvacrol and thymol with very low MIC and MFC values (0.02-1.5 μl/ml), while linalyl acetate and limonene possessed the lowest activity (MIC/MFC 5.0-11.0 μl/ml) (Soković & van Griensven, 2006a). The essential oil of *Critmum maritimum* and its components (α-pinene and limonene) possessed antifungal activity against the mycopathogen *M. perniciosa* tested by the microatmospheric method. MIC for α-pinene was 5 μl/disc, and MFC 10 μl/disc, while limonene showed higher antifungal activity with MIC 1 μl/disc, and MFC 5 μl/disc (Glamočlija et al., 2009) (Table 1.).

It can be seen that growth of the tested fungi responded diversely to the essential oils and their components, which indicates that different components may have different modes of action or that the metabolism of some fungi is able to overcome the effect of the oil or adapt to it. Terepenic compounds inhibit electron transport, proton translocation, phosphorylation steps and other enzyme-dependent reactions or act on the cell membrane. Their antifungal activity will depend on the chemical composition of the cell wall and on the structure of the terpenoid molecules. Terpenic hydrocarbons are water insoluble and revealed poor activity, while among the water soluble compounds vanilin, piperonal and camphor, were not remarkably active, whereas the non-aromatic ester borneol acetate showed antiseptic effects. Aliphatic alcohols, such as linalool or citronellol and ketones like pipertone or carvone exhibited antifungal properties. Phenol compounds

showed very strong antifungal activity in spite of their relative low capacity to dissolve in water (Knobloch et al., 1988). The most active terpenoids were found among phenols, followed by aldehydes and ketones, alcohols and hydrocarbons. Thymol and carvacrol were the most effective compounds which causing total inhibition of oxidative phosphorylation. The ability of terpenoids to inhibit the reactions described above arises both from lipophilic properties, which enables them to dissolve in the cytoplasmic membrane, and from their functional groups, which interfere with enzyme structure (Griffin et al., 1999; Knobloch et al., 1988; Shelef, 1983; Soković, 2001). Studies of antimicrobial activity of essential oils and their components showed that, terpene acetates and hydrocarbons tended to be relatively inactive, regardless of their structural type, and that this inactivity appears to be closely related to their limited hydrogen bonding capacity and water solubility. Ketones, aldehydes and alcohols showed activity but with differing specificity that was not always defined by the functional group present but was associated with hydrogen-bonding parameters in all cases (Griffin et al., 1999). Our results concerning the antifungal activity of many essential oils and their components indicate different efficacy. Also, the modes of action of essential oils differ among fungal species. The strong antifungal activity of some oils (*Mentha* species, Thyme, Oregano) can be explained by the high percentage by their high percentage of active components such as menthol, thymol, carvacrol. For the remaining oils, no significant correlation between antifungal activity and relative amounts of the major components has been found. This suggests that the components present in large proportions are not necessarily responsible for a great share of the total activity. Different antifungal activity exhibited by an oil, compared with those of it major components, can be explained by either synergistic effect of diverse components in the oil and/or by the presence of other components that may be active even in small concentrations (Soković & van Griensven, 2006a). To examine the problem of a lack of unified criteria in greater depth, we can look particularly at studies of the antimicrobial activity of essential oils. Janssen et al., (1987) reviewed the characteristics of complex mixtures as well as the techniques used to evaluate them and concluded that many results are difficult to compare as the test methods differed so widely. They proposed that in future the strain number of the tested microorganism, the composition of the essential oil and the conditions under which it was obtained be included as an integral part of the report.

Recently, Kalemba & Kunicka (2003) reviewed the classical methods commonly used to evaluate the antibacterial and antifungal activities of essential oils, including the agar diffusion method (paper disc and well), the dilution method (agar and liquid broth) and turbidimetric and impedimetric monitoring of microorganism growth in the presence of these oils. Besides drawing conclusions about factors that influence the antimicrobial activity of essential oils *in vitro* and their mechanisms of action, they included an overview of the susceptibility of human and foodborne fungi towards different essential oils and their constituents. The most relevant ones, which included oils from Thyme, Oregao, Mint, Cinnamon, Sage and Clove, have antimicrobial properties. Other criteria were the study of plants used as preservatives, as well as examination of the use of spices

as antimicrobial agents. The cited criteria seem sufficient to justify the studies, but Ríos & Recio, (2005 and references cited therein) believe that research should be focused on achieving definitive knowledge about the plant and its properties. With increasing acceptance that the chemical diversity of natural products is well suited to provide core scaffolds for future antimicrobial agents, there will be more developments in the use of novel natural products and chemical libraries based on natural products (Harvey, 2008). The methodology employed is another point that needs to be considered in depth. For non-polar extracts, the use of diffusion techniques is probably inadequate, although many reports employing these techniques have been published. Solid dilution techniques are suitable for studying plant extracts or nonpolar compounds. Only when the amount of sample available is small is the use a diffusion techniques possibly more appropriate (Rios & Recio, 2005).

Our results showed that the MICs for essential oils and their components are generally higher, in disc-diffusion assays and with diffusion methods than with the microdilution method. Poor activity can be explained by low water solubility of the oil and its components, which limits diffusion through the agar medium in the disc diffusion and agar diffusion methods. Only more water-soluble compounds, such as 1,8-cineole, diffuse into the agar. The hydrocarbon components either remain on the surface of the medium or evaporate. This could be the reason for better results obtained using the microdilution method. Also, essential oil and their components showed greater antifungal activity when diluted in Tween 80. Both MICs and MFCs were lower in the microdilution than in the diffusion method, especially when Tween 80 was employed. Non-ionic emulsifiers, such as Tween 20 or 80, are relatively inactive when tested alone and have been widely reported as useful emulsifying agents (Soković, 2001; Soković et al., 2002; Soković & van Griensven, 2006a). We observed that some oils and compounds acted not only as fungicidal agents but also inhibited sporulation of different fungi (Glamočlija et al., 2006a, 2006b; 2007; Soković & van Griensven, 2006a). Treatment was not only effective in solution or by contact, but even in a vapor treatment their were very effective enabling fungal growth to be inhibited by a smaller amount of essential oil while also acting as a potent inhibitor of sporulation. Vapor concentration and the duration of exposure are important. The gaseous contact activity was demonstrated primarily by the maximum vapor concentration at an early stage of incubation. Maintaining a high vapor concentration for long periods of time appeared to be unnecessary. Essential oil vapors might serve to control proliferation of moulds that are now treated with other sanitizing agents. Oils and their components have high vapor pressures and are relatively volatile. Solutions and emulsions used in the form of sprays with or without a carrier therefore represent the preferred form in which these agents should be applied to large areas of casing soil surface with minimal effort. Also evaporation by heating could be considered. An additional advantage of the volatile of essential oils is that no or only little residue will be left on the product after treatment. Our own experience leads us to propose the use of the microdilution method, carried out in microtiter trays, which involves a low

workloads for a larger number of replicates and the use of small volumes of test substance and growth medium (Soković 2001, Soković et al., 2006a, 2009b). On other hand, for investigation of some plants and fungal extracts and separation of fractions and highly pure compounds bioautographic methods on TLC plates were recommended. This qualitative techniques will only give an idea of the presence or absence of substances with antimicrobial activity in very small amounts. The method was useful for screening plants and fungi for antimicrobial activity and for the bioassay-guided isolation of natural antimicrobial compounds. Bioautography allows easy localization of activity even a matrix as complex as that derived from natural products. Comparison chromatograms developed under identical conditions and visualized using suitable chromogen reagents can provide useful information about the nature of active compounds (Figure 3.) (Rančić et al., 2006, Ćirić, 2010).

Considerable changes in legislation have been made and there are increasing consumer trends for more natural alternatives to chemical fungicides (Brul & Coote, 1999). The use of essential oils is particularly advisable because herbs and spices are common plant additives. Among the natural antimicrobials that were tested in our laboratory the essential oils of Oregano and Thyme, as well as their components, carvacrol and thymol were the most promising. Addition of various plant derived antimicrobials in combination should improve both the spectrum of activity and the extent of inhibition due to synergistic effects. Thus, combination of these compounds might have even higher potential. The use of essential oils is limited, and possible reasons for this may be their strong smell and taste when used at effective doses (Skandamis & Nychas, 2000). Although the majority of essential oils are classified as Generally Recognized As Safe (GRAS) (Kabara, 1991), their use in foods as preservatives is often limited due to flavor considerations, as effective antimicrobial doses may exceed organoleptically acceptable levels. Therefore, there is an increasing demand for accurate knowledge of the minimum inhibitory (effective) concentrations (MIC) of essential oils to enable a balance between sensory acceptability and antimicrobial efficacy (Lambert et al., 2001).

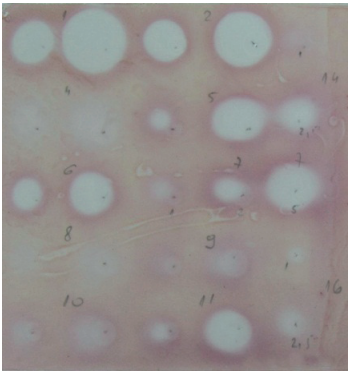

Figure 3. Antifungal activity of sesquiterpene lactones using a bioautographic method on TLC plate

Group of compounds	Species	Methods	Activity	References
Fungal extracts	*Cladosporium fulvum* *Fusarium sporotrichioides* *Penicillium ochrochloron* *P. funiculosum* *Phomopsis helianthi* *Stachybotrys chartarum Trichoderma viride* *Microsporum canis* *Trichophyton rubrum* *T. mentagrophytes* *T. tontonsurans*	bioautographic assay on TLC plates bioautographic	50-100μg/ml 50-100 μg/ml 50-100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 50-100 μg/ml	Rančić et al., 2006
	Laetiporus sulphureus	microdilution	0.30 mg/ml	Šiljegović et al., 2011a
plant extracts	*Phlomis fruticosa*	diffusion	10-20 μg /ml	Ristić et al., 2000
	Cynara cardunculus	microdilution	1.0–1.5 mg/ml	Kukić et al., 2008
	Centaurium pulchellum	microdilution	0.1-2.0 mg/ml	Šiler et al., 2010
	Nepeta rtanjensis, N.sibirica, N. nervosa	microdilution	25-100 μg/ml	Nestorović et al., 2010
mosses	*Bryium argenteum*	microdilution	0.29-0.52 μg/ml	Sabovljević et al., 2006
	Pleurozium schreberi *Palustriella commutata* *Homalothecium philippeanum* *Anomodon attenuatus* *Rhytidium rugosum* *Hylocomium splendens* *Dicranum scoparium* *Leucobryum glaucum*	microdilution diffusion	0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 0.5-10 mg/ml 0.5-10 mg/ml 0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 0.5-10 mg/ml	Veljić et al., 2008
	Fontinalis antipyretica var. *antipyretica* *Hypnum cupressiforme Ctenidium molluscum*	microdilution	2.5-5 mg/ml 5 mg/ml 5 mg/ml	Veljić et al., 2009
	Atrichum undulatum, Physcomitrella patens, Marchantia polymorpha ssp. *ruderalis*	microdilution	0.1-2 mg/ml 0.1-2 mg/ml 0.25-1 mg/ml	Sabovljević et al., 2011
	Rhodobryum ontariense	microdilution	0.25-1 mg/ml	Pejin et al., 2012
essential oils	*Origanum onites, Satureja thymbra, Salvia fruticosa, S. pomifera* subsp. *calycina*	microdilution	0.05-25 μl/ml	Soković et al., 2002
	Micromeria dalmatica, M. albanica, M. thymifolia	diffusion	0.2-2.0 μl/ml	Marinković et al., 2002
	Foeniculum vulgare	microdilution	0.8-4.0 μl/ml	Mimica-Dukić et al., 2003
	Stachys sp.	microdilution	0.01-1.0 mg/ml	Skaltsa et al., 2003
	Aniba rosaeodora *Laurus nobilis* *Sassafras albidum* *Cinnamomum zeylanicum*	diffusion microdilution	0.5-7.5 μl/ml 1.0-20 μl/ml 10-40 μl/ml 10-50 μl/ml 5-15 μl/ml 5-30 μl/ml 0.1-1 μl/ml 0.1-2.5 μl/ml	Simić et al., 2004
	Juniperus excelsa	microdilution	8–50 μl/ml	Soković et al., 2004
	Myrrhis odorata *Hypericum perforatum* *Helichrysum arenarium*	microdilution	0.5-2.5 μg/ml 15-30 μg/ml 10-60μg/ml	Rančić et al., 2005
	Salvia brachyodon	microdilution	15-40 μl/ml	Soković et al., 2005
	Salvia sclarea	microdilution	2.5-25 μl/ml	Dzamić et al., 2008
	Salvia desoleana	diffusion microdilution	1.5-6 μl/ml 1.5-6 μl/ml	Soković et al., 2009a
	Nepeta rtanjensis	diffusion	0.6-1.8 μl/ml	Ljaljević Grbić et al., 2007
	Cymbopogon winterianus *Carum carvi*	microdilution	0.5-20 μl/ml 0.25-20 μl/ml	Simić et al., 2008
	Portenschlagiella ramosissima	microdilution	50-200 μl/ml	Soković et al., 2008b
	Eugenia caryophyllata *Illicium verum*	microdilution	0.1-2.5 μl/ml 2.5–25 μl/ml	Dzamić et al., 2009
	Daucus carota	microdilution	2-150 μl/ml	Soković et al., 2009c

Group of compounds	Species	Methods	Activity	References
	Echinophora spinosa	microdilution	0.0625-1 mg/ml	Glamočlija et al., 2011a
	Lippia alba	microdilution	0.3-1.25 mg/ml	Glamočlija et al., 2011b
	Satureja thymbra *Thymbra spicata*	microdilution	1.25-5 μg/ml 0.3-2.5 μg/ml	Marković et al., 2011
	Seseli anuum aerial	diffusion	12.5-50 μl/ml	Milosavljević et al., 2007
	Seseli globiferum fruits	microdilution	0.5-50 μl/ml	Stojković et al., 2008a
	Seseli globiferum aerial	microdilution	2.5-10 μl/ml	Janaćković et al., 2011
	Seseli rigidum flowers	microdilution	10-50 μl/ml	Stojković et al., 2009
	Seseli montanum subsp. *tommasinii*	microdilution	25-100 μl/ml	Šiljegović et al., 2011b
	Matricaria chamommilla, *Mentha piperita, M. spicata* *Lavandula angustifolia* *Ocimum basilicum,* *Origanum vulgare, Salvia officinalis,Citrus limon, C. aurantium, Thymus vulgaris*	microatmosphere diffusion microdilution microatmosphere	0.5-15 μl/ml 0.5-35 μl/ml 0.125-20 μl/ml 0.001-25 μl/disc	Soković & van Griensven, 2006[a] Glamočlija, 2006a; 2006b, 2009
	Vitex agnus-castus	microdilution	44.5-267 μg/ml	Stojković et al., 2011
components of essential oils	carvacrol, camphor 1,8-cineole	microdilution	0.1-0.5 μg/ml 3.0-10 μg/ml 4.0-15 μg/ml	Soković et al., 2002
	anethole fenhone camphor	microdilution	1.3-2.8 μl/ml 3.7-6.3 μl/ml 2.8-9.7 μl/ml	Mimica-Dukić et al., 2003
	β-caryophillene, β-caryophillene oxide, α-pinene, cadinene, linalool	microdilution	0.03-0.3 mg/ml	Skaltsa et al., 2003
	limonene	diffusion microdilution bioautographic	8-13 μl/ml 6-10 μl/ml 5 μl/ml	Rančić et al., 2003
	linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol, menthol	diffusion microdilution	0.05-13 μl/ml 0.02-11 μl/ml	Soković & van Griensven, 2006a
	linalyl acetate 1,8-cineole linalool	diffusion microdilution	7.0-11.5 μl/ml 7.5-11 μl/ml 2-8 μl/ml 3-8 μl/ml 2-7 μl/ml 2-7 μl/ml	Soković et al., 2009a
	α-pinene limonene	microatmospheric	5-10 μl/disc 1-5 μl/disc	Glamočlija, 2009
	1,8-cineole α-pinene	microdilution	3.5-7 μg/ml 4-8 μg/ml	Stojković et al., 2011
Flavonoids	*Quercus ilex*	microdilution	0.056-2.95 μmol/ml	Karioti et al., 2011
	Centaurea spruneri	microdilution	0.694-1.3 μmol/ml	Ćirić, et al., 2011
Sesquiterpene lactones	*Centaurea nicolai*	diffusion	0.8-25 μg/ml	Vajs et al., 1999
	Centaurea achaia, C. thessala, C. attica	microdilution	0.03-4 μg/ml	Skaltsa et al., 2000a;b
	Centaurea pullata	microdilution	0.0001-0.0007 μmol/ml	Djeddi et al., 2007, 2008
	Anthemis melanolepis	microdilution	25 μg/ml	Saroglou et al., 2010
sulphur products	*Allium sativum*	microdilution	0.001-0.03 mg/ml	Ilić et al., 2012

Table 1. An overview of antifungal activities of natural products from fungi and plants

3.2.2.1.2. Morphophysiological changes in fungi due to inhibition activity by essential oils

Dematiceous fungi are characterized by the presence of the dark brown pigment – melanin within their cell wall structure. Melanins are negatively charged, hydrophobic biopolymers of high molecular weights. They are typically brown or black and formed by the oxidative polymerization of phenol or indolic compounds in organisms from all biological kingdoms, including fungi. Fungal melanins are usually found in the cell walls of spores, sclerotia,

mycelia or fruiting bodies. They enable fungi to survive adverse environmental conditions by protecting them from oxygen free radicals, UV radiation and wall-degrading enzymes produced by antagonist microbes (Butler et al., 2001).

Many human pathogenic fungi contain melanin within their cell wall structure (e.g. *Aspergillus fumigatus, A. nidulans, A. niger, Alternaria alternata, Cladosporium carionii, Cryptococcus neoformans, Exophiala jeanselmei, Fonsecaea compacta, F. pedrosoi, Hendersonula toruloidii, Histoplasma capsulatum, Paracoccidioides brasiliensis, Penicillium marneffei, Phaeoannellomyces wernickii, Phialophora richardsiae, P. verrucosu, Sporothrix schenckii, Wangiella dermatitidis*). For several of these fungi, melanin has been described as a virulence factor due to its ability to reduce a pathogen's susceptibility for killing by host antimicrobial mechanisms and by influencing the host immune response. Due to the protective role of fungal melanin, dematiceous fungi are extremely difficult to treat with antifungal drugs (Nosanchuk & Casadevall, 2006). Plant secondary metabolites could be a suitable alternative for the treatment of fungal infections in the light of increasing fungal resistance to commercial antifungal agents (Vivek et al., 2009). Essential oils are known to cause morphophysiological changes in fungi through a lack of sporulation, depigmentation and aberrant development of conidiophores (e.g. Sharma & Tripathi, 2008; Moreira et al., 2010). We tested several essential oils for this purpose. Using the diffusion method it was observed that essential oils of *Hyssopus officinalis*, Thyme, *T. tosevii*, Spearmint and Peppermint induced changes in some morphophysiological characteristics of the fungi *Trichoderma viride*, *Penicillium ochrochloron* and *Aspergillus niger*. Depigmentation of the colonies of *A. niger* was noted. Untreated control colonies were black, blue, green and the mycelium was well developed, while the colonies treated with oils were white with sparse mycelium. In the treated *A. niger, P. ochrochloron* and *T. viride* cultures, mycelium is rare, the conidiogenic apparatus was atypical, the vesicles deformed, phialides were abnormal and other variations included lack of sporulation, visible loss of pigmentation and aberrant development of conidiophores (Soković, 2001). *Nepeta rtanjensis* an essential oil showed the ability to interfere with all stages in the reproduction cycle of the human pathogenic fungus *Bipolaris spicifera*: conidia germination, mycelial growth and intensity of sporulation which is demonstrated with radial mycelial growth inhibition. Thus, inhibition of conidia germination and low conidia production were demonstrated in the treated samples. The most significant documented orphophysiological changes in *B. spicifera* included demelanization (bleaching) and an aberrant conidial apparatus (Ljaljević Grbić et al., 2011).

3.2.2.2. Flavonoids

Flavonoids are another group of secondary metabolites with great antifungal potential. Besides other biological activities, have been shown to be active against microorganisms. At least in some cases their presence might serve as a chemical barrier to invading microorganisms. Since they are natural compounds and possess highly specific antimicrobial activity, flavonoids may be an alternative to conventional fungicides in the control of plant diseases caused by fungi. Twenty-five flavonoids were examined for their effect on the mycelial growth of a crop pathogen, *Verticillim albo-atrum*. The minimum

inhibitory concentrations (MIC) for the two most active compounds, flavone and flavanone. Other flavonoids inhibited hyphal growth and some compounds were ineffective at the highest concentration used. Active compounds did not share a common pattern of substitutions. The unsubstituted flavonoids were stronger growth inhibitors and, in most cases, increasing the number of substittions (hydroxylation, methoxylation and glycosylation) resulted in the loss of antifungal activity (Picman et al., 1995). The fungicidal activity of two isoflavones, one isoflavanone and seven isoflavans was tested against *Aspergillus repens*, *A. amstelodami*, *A. chevalieri*, *A. flavus* and *A. petrakii* (Weidenbörner et al., 1989). While the isoflavones showed low activity, the two isoflavans were highly inhibitory (Weidenbörner & Jha, 1997). Two naturally occurring isoflavones, genistein and biochanin A, and their dihydroderivates (isoflavanones) as well as nine perhydrogenated isoflavones (isoflavans) were tested for their effects against *Rhizoctonia solani* and *Sclerotium rolfsii* (Weidenbörner et al., 1990). All the isoflavonoids of the biochanin A series showed high antifungal activity. Genistein isoflavan and the other isoflavans with two hydroxyl groups and one methoxy group were fungitoxic, while isoflavans with two or three methoxy groups were almost inactive (Weidenbörner & Jha, 1997), although earlier results demonstrated that isoflavans, generally possess higher activity than the corresponding isoflavones and isoflavanones.

Since the individual unsubstituted flavonoids showed strong antifungal activity, various mixtures have been tested against fungi occurring on grain to enhance the fungicidal potential of each substances by anticipating synergy. In general a combination of flavones and flavanones in different proportions was most effective. However, it was interesting that a mixture containing flavonoid molecules with one methoxy group and several hydroxy groups in general exhibited higher activity than a mixture containing only hydroxylated flavonoids. It becomes obvious that combinations of several suitable flavonoids (depending on the number, kind and location of the substitutents) may result in even greater increase in antifungal potential. Consequently, lower active concentrations may make flavonoids more attractive as natural protectants (Silva et al., 1998; Weidenbörner & Jha, 1997). Concerning the antifungal activity of flavonoid glycosides, it should be noted that no substantial effect could be detected (Weidenbörner & Jha, 1997). Krauze-Baranowska et al., (1999) found that cupressuflavone and 4'-O-methylcupressuflavone, isolated from leaves of *Cupressocyparis leylandrii*, possessed antifungal activity against *Alternaria alternata, Cladosporium oxysporum, Fusarium culmorum* and *F. avenaceum.* Matshumoto & Tahara, (2001) separated ampelosin, a flavonol, from *Salix sachalinensis* leaves and reported antifungal activity against *Cladosporium herbarum*.

The antifungal activities of many phenol compounds isolated from Holm Oak *Quercus ilex* leaves, belonging to the classes of flavonoids, proanthocyanidins, and phenol acids, have been examined against fourteen fungal species (Karioti et al., 2011). Two coumarins, scopoletin and isoscopoletin, two simple phenol acids, protocatechuic acid and isovanillic acid and one flavonoid, eriodictyol separated from the aerial parts of *Centaurea spruneri*, showed fungistatic activity at 0.259–2.38 μmol/ml and fungicidal at 0.69–2.6 μmol/ml against all fungi tested. The flavonoid, eriodictyol, possessed the greatest antifungal activity in the

range of 0.694–1.388 μmol/ml for MIC and MFC, while the activity of protocatechuic acid was lower (0.65–1.3 μmol/ml for MIC and 1.3–2.6 μmol/ml for MFC). The simple phenol acids, protocatechuic acid expressed the lowest antifungal activity (Ćirić et al., 2011). The inhibitory activity of flavonoids generally decreased with the increasing number of substitutions on the molecule, and the strongest inhibitors were unsubstituted compounds. The most active flavonoids, flavone and flavanone, have excellent potential as new natural antifungal agents (Picman et al., 1995).

3.2.2.3. Sesquiterpene lactones

Sesquiterpene lactones are natural products present in many families of plants, but mostly distributed in the family Compositae. They display a wide spectrum of biological activity, one of the most important of which is antifungal activity. The general mechanism of action is considered to be alkylation of biological nucleophiles such as cysteine (cys) and glutathione or sulfhydryl-containing systems, phosphofructoki-nase and glycogen synthetase by a,b-unsaturated carbonyl structures in a Michael-type addition (Koukoulitsa et al., 2005). This group of compounds was analyzed for potential antifungal activity, *in vitro* against *Aspergillus niger, A. ochraceus, Penicillium ochrochloron, Trichoderma viride, Fusarium tricinctum, Phomopsis helianthi* and *Cladosporium cladosporioides*. Guaianolides from *Centaurea nicolai* were found to be highly active (0.8-25 μg/ml) (Vajs et al., 1999). Sesquiterpene lactones isolated from *Centaurea achaia, C. thessala* and *C. attica* also exhibited excellent antifungal activity against *Aspergillus niger, A. ochraceus, A. versicolor, A. flavus, Penicillium ochrochloron, P. funiculosum, Trichoderma viride, Fusarium tricinctum, Phomopsis helianthi, Alternaria alternata* and *Cladosporium cladosporioides* with germacranolides providing the greatest antifungal activity (Skaltsa et al., 2000a; 2000b).

The fungicidal activities of 36 natural and synthetic sesquiterpene lactones with guaianolide, trans-germacranolide, cis-germacranolide, medampolide, and eudesmanolide carbon skeletons were evaluated against the phytopathogenic fungi *Colletotrichum acutatum, C. fragarie, C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea* and *Phomopsis* sp. by Wedge et al., (2000). Dehydrozaluzanin showed the highest antifungal activity due the presence of an α,β-unsaturated carbonyl group in the cyclopentananone ring. In addition to the previously isolated sesquiterpene lactones, 11,13-dihydrocnicin and 11,13-dihydro-19-desoxycnicin, the aerial parts of *Centaurea pullata* afforded three minor sesquiterpene lactones, namely, a new germacranolide, 8R-*O*-(4-acetoxy-5-hydroxyangeloyl)-11,13-dihydrocnicin, and two new eudesmanolides, 8R-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11,13-dihydrosonchucarpolide and 8R-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11,13-dihydro-4-*epi*-sonchucarpolide. The antimicrobial activity of all previously mentioned compounds and some newly isolated sesquiterpene lactones (a novel elemanolide with an α-methyl-γ-lactone moiety, 8α-O-(4-hydroxy-2-methylenebutanoyloxy)melitensine, in addition to other four known sesquiterpene lactones with the same ring, melitensine, 11β-dihydrosalonitenolide, 8α-hydroxy-11β-13-dixidroxy-4-epi-sonchucarpolide and 8α-hydroxy-11β-13-dihydroxy-onopordaldehyde) from *Centaurea pullata* was tested against eight fungal species, using a

microdilution method. All compounds evaluated showed greater antifungal activity than the positive controls used. Moreover, the pharmacokinetic profile of these compounds was investigated using computational methods and was in agreement with our *in vitro* data (Djeddi et al., 2007, 2008). Three linear sesquiterpene lactones, anthecotulide, hydroxyanthecotulide and acetoxyanthecotulide were isolated from the aerial parts of *Anthemis auriculata* together with five known flavonoids, taraxa-20(30)en-3â-ol and methyl vanillate. Comparing these results with previously published data (Konstantinopoulou et al., 2003) concerning the antimicrobial potential of sesquiterpene lactones of *A. altissima*, it was concluded that linear lactones are more active. This differentiation in antimicrobial activity could be explained in terms of solubility, as linear lactones are more lipophilic than the oxygenated eudesmanolides and germacranolides of *A. altissima* (Theodori et al., 2006). Nine sesquiterpene lactones, anthemin A, 1α-hydroxydeacetylirinol-4 α,5β-epoxide, anthemin C, tatridin A, 1-epi-tatridin B, anthemin B, 6-deacetyl-β-cyclopyrethrosin, elegalactone A and 1β,4 α,6trihydroxyeudesm-11-en-8 -12-olide were isolated from the aerial parts of *Anthemis melanolepis*. All sesquiterpene lactones showed an inhibitory effect against almost all fungi tested (Saroglou et al., 2010), which adds to evidence from previous studies concerning related compounds isolated from other *Anthemis* species (Konstantinopoulou et al., 2003).

The biological activity of sesquiterpene lactones is generally attributed to the alkylating property of the α-methylene-γ-lactone moiety. Moreover, the presence of other alkylating sites (epoxides and conjugated carbonyl groups) may enhance their biological activities. Lipophilicity seems to play an important role in antifungal activity. Since the chemical composition of fungal cells walls is highly lipophilic, they generally provide a strong barriers against penetration of hydrophobic compounds and transport of polar compounds through the outer lipid layer. According to Skaltsa et al., (2000a) an inverse relationship exists between polarity and antifungal activity for sesquiterpene lactones in general. Their polarity decreases in the order eudesmanolides > elemanolides > germacranolides. Some of the differences between the responses of *Verticillium albo-atrum* to flavonoids and sesquiterpene lactones suggest these two groups of plant metabolites have different modes of action. Thus, inhibition of mycelial growth by sesquiterpene lactones remained relatively constant during incubation times of 24, 48 and 72h. This suggests that these lactones affected the metabolism of the pathogen, slowing its growth, but that the fungus evidently did not produce specific enzymes to degrade the lactones. In contrast, flavonoids and especially flavanone, significantly reduced hyphal growth during the first 24h by up to 100% but growth was much less inhibited during the next 48h of incubation which indicated that the flavonoids were subject to degradation by enzymes produced by the pathogen (Picman et al., 1995).

3.2.2.4. Other compounds tested

Inhibition of certain thiol-containing enzymes in microorganisms by the rapid reaction of thiosulfinates with thiol groups was assumed to be the main mechanism involved in the antimicrobial effect of allicin. The mode of action of allicin on the fungal cell has not yet been elucidated but it is assumed to act on thiol enzymes as in other microorganisms. Other

requirements such as molecular accessibility and lipophilicity seem to play an important role for in their antifungal activity (Yamada & Azuma, 1997). Antifungal activities of allicin and related organo-sulfur products obtained by microwave-assisted transformation of allicin in ethanol were studied in our laboratory against eight fungi: *Aspergillus flavus, A. niger, A. fumigatus, Penicillium funiculosum, P. ochrochloron, Trichoderma viride, Candida albicans* and *C. kruzei.* The mixture of transformation products of allicin was analyzed using liquid chromatography-mass spectrometry (LC-MS) and consisted of ajoenes, vinyldithiins and diallyl disulfide. Allicin showed very powerful antifungal activity with minimum inhibitory concentration (MIC) at 0.001 to 0.008 mg/ml and MFC at 0.004 to 0.03 mg/ml. The transformation products of allicin also possessed very strong antifungal activity, but less than allicin (Ilić et al., 2012).

3.2.2.5. Synergistic effect of different natural compounds and fungicides

Fungi that are pathogenic to plants which produce antimicrobial compounds often have greater tolerance to these natural compounds *in vitro* than do nonpathogens of these plants, suggesting that resistance may be a prerequisite for infection. Nevertheless, many other factors will be required for fungal pathogenicity in addition to resistance to host antimicrobial compounds. Although *in vitro* tests of antifungal activity usually involve individual purified compounds, phytopathogenic fungi may often be exposed to more than one antifungal compound simultaneously during infection of plants. The combination of different compounds may be synergistic. The combined therapy is used with the aim of expanding the antimicrobial spectrum, minimizing toxicity, preventing the emergence of resistant mutants during therapy, and obtaining synergistic antimicrobial activity. Some steroidal glycoalkaloids have low antifungal activity when tested separately, but exhibit pronounced synergistic activity when mixed in plants (Osbourn, 1996). Synergism between ketoconazole and *Agastache rugosa* oil against *Blastichizomyces capitatus* was reported, as well as between *Pelargonium graveolens* oil and amphotericin B plus ketoconazole on strains of *Aspergillus* sp. (Silva & Fernades, 2010). Thus, studies on the interactions between natural products and antifungal drugs have also multiplied in recent years, indicating the importance of elucidating types of interactions, which can be favorable, such as in synergism, or harmful, as in antagonism. Our results showed that proanthocyanidins (procyanidin and prodelphinidin) isolated from Holm oak when combined with bifonazole and ketoconazole, increased the activity of both conventional fungicides (Karioti et al., 2011). Compounds tested showed higher activity than bifonazole and ketoconazole, against *A. fumigatus* and *A. niger*. Almost all compounds tested exhibited good ability to inhibit fungi, even much better than commercial antifungal agents used as reference drugs. Even more, compounds showed few times higher activity than bifonazole and especially than ketoconazole. Also, we tested the synergistic effect of mixture of several essential oils and found that some combinations could possess great antifungal potential. The following mixture of essential oils: Thyme, Peppermint, Sage, *Hyssopus officinalis,* Sweet basil and Lavander showed greater antifungal potential *in vitro* than each oil separately (Soković, 2001). Structurally unrelated compounds are also likely to have combined and possibly synergistic antifungal activity. Therefore, this study supports the potential use of a weak

antifungal products together with another compound to increase its activity. This type of finding could further boost the use of medicinal plants, extracts or natural products, either alone, combined or together with mycotics.

4. Antifungal activity against animal and human pathogens

Among animal and human pathogens, dermatomycetes are the main cause of dermatomycoses (infections of the hair, skin, and nails), superficial infections that are not life threatening but are chronic and cause considerable morbidity. The unpleasant side effects of therapy including nausea, abdominal pain and itching, and its toxicity, can limits its therapeutic use in many cases (Shin & Lim, 2004).

Despite the advancements of science and technology, surprisingly the development of novel and efficient antifungal drugs is still lagging behind due to the very fact that fungi are also eukaryotic and have mechanisms similar to human beings. Hence it becomes very difficult to develop an antifungal agent that is more specific in targeting the fungi alone without any damage to human beings. For successful treatment of the disease, proper diagnosis of the disease is always essential. The treatment is chosen based on the infection site, etiological agent and penetration ability of the drug. The penetration ability and retention in the site of infection of the agent determines its efficacy and frequency of utility. Since the dermatomycetes reside in the stratum corneum especially within the keratinocytes, the antifungal agents should have a good penetrating ability (Lakshmipathy & Kannabiran, 2010). Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of dermatomycosis. Essential oils play a great role in these investigations, the majority have good penetration possibilities the lipophilic properties of oil components might have also aided in the ability of the oil to penetrate the plasma membrane, strong antifungal activity and if they are used in active (MIC and MFC) concentration they are not harmful for animals and humans.

Previous studies *in vitro* and *in vivo* on investigations of the antifungal activity of essential oils from some medicinal and aromatic plants, *Origanum vulgare* subsp. *hirtum*, Spearmint, Lavander, and *Salvia fruticosa,* indicated that they could be employed as effective antifungal agents (Adam et al., 1998). Our selection of plants for evaluation in our study was based on traditional usage for treatment of infectious diseases (Sokmen, 1999). The *in vivo* evaluation of antifungal activity of several essential oils and their components was tested for the therapeutic potency against experimentally induced dermatomycoses in rats (2-month old male Wistar rats), using the most frequent dermatomycetes, *Trichophyton mentagrophytes, T. rubrum* and *T. tonsurans.* Essential oil of Lavander exhibited therapeutic activity after 13 days of treatment. The group of rats treated with Sweet basil oil were cured after 25 days of treatment. The shortest period of currency was observed at animals treated with Sage-12 days. The longest period of treatment was observed at rats treated with oils of Bitter orange and Lemon, 45 days. The main essential oil components were also used as a potential antifungal agents. Linalool showed antifungal activity after 32 days of treatment, while limonene needs 50 days for this activity. Rats treated with 1,8-cineole were cured after 40

days. Camphor exhibited therapeutic and antifungal activity after 14 days of treatment. The best antifungal activity was observed for menthol, which showed therapeutic potential after 10 days of treatment (Soković, 2001). We examined the antifungal activity of essential oil from *Mentha* x *piperita* and menthol. The oil completely cured the animals infected with *T. mentagrophytes* within 15 days, with *T. rubrum* within 30 days and with *T. tonsurans* within 29 days. Menthol possessed higher therapeutic and antifungal activities than the essential oil, as it cured the animals within 10 days. Also, menthol showed stronger activity than bifonazole (Soković et al., 2006b). The antifungal activity of essential oil from Lavander showed therapeutic and antifungal potential during the 13-day observation period and cured the animals completely (Soković et al., 2007). The essential oil of thyme and its main component thymol was also tested for therapeutic potency *in vivo.* This oil completely cured animals infected with *T. mentagrophytes* within 24 days, with *T. rubrum* within 37 days, and with *T. tonsurans* within 32 days of treatment. The animals treated with the commercial drug bifonazole were cured after 14–15 days of treatment. Moreover thymol possessed higher therapeutic and antifungal activity than essential oil and cured the animals within 14 days (Figure 4.) (Soković et al., 2008c).

In vitro susceptibility of the turpentine oil obtained from Cluster pine *Pinus pinaster* oleoresin was evaluated against three Sudanese clinical isolates of *Actinomadura madurae*, which is the main causative agent of actinomycetoma in man and animals. The minimum inhibitory concentrations (MICs) of the oil ranged from 100.3-124.8 μl/ml, and the minimum microbicidal concentrations (MMCs) were between 100.3 μl/ml and 150.0 μl/ml. The main component of oil, α-pinene, exhibited prominent bioactivity with MICs ranging between 3.3 and 5.0 μl/ml, while the MMC was 10.0 μl/ml against the same clinical isolates. Cluster pine turpentine oil and α-pinene might be useful agents in the treatment of mycetoma caused by *A. madurae* (Figure 4.) (Stojković et al., 2008b). From the above results it can be concluded, that all essential oils tested showed beneficial antifungal activity both *in vitro* and *in vivo.*

After reviewing of the results of the antifungal activity of essential oils and individual components *in vivo* experiment, knowing that the composition of essential oils and the proportion of the tested individual components may be, to some extent, explain the differences between their activities. Menthol, camphor and thymol showed better antifungal activity than essential oils tested individually. Since the individual essential oils showed lower antifungal activity than the tested components, it is evident that the active principles can be explained by individual components. Although it is possible that interactions between the constituents of essential oils block the active principles of individual components when the treatment is the total essential oil. Added to that are antagonistic effect (Davidson & Parish, 1989) which does not mean that it can be completely neglected the role of individual components of essential oils on the expression of antifungal potential. Menthol, camphor and thymol, which showed the best antifungal activity *in vivo* among all tested components, are the dominant components of the essential oils of Spearmint and Thyme, and therefore can be justified by the high antifungal potential of these oils *in vivo*. The essential oil of Sweet basil, which is known for the beneficial activity of the skin, healing wounds, etc. is used to treat fungal infections showed good antifungal activity, but only

better than lemon and orange. Dominant component of this oils was linalool, which proved to be good, but the tested components as one of the weaker fungicides, in front of limonene. Similarly, Lemon and Bitter orange oil showed the lowest antifungal potential, as well as among individual components of limonene, which is present in these oils with a high proportion, which is certainly influenced the decrease in the efficiency of oil. Essential oils of *S. officinalis* and *L. angustifolia* have proved to be most effective in the treatment of experimental induced dermatomycoses. If we compare the results obtained during investigation of antifungal activity of essential oils *in vitro*, and this generated *in vivo*, it is obvious that the essential oil of Sage and Lavander reacted with lower potential *in vitro*. *In vivo* experiments, these oils, in contrast, have proved to be most effective. Obviously, they have better therapeutic activity than other essential oils. In addition, it is known that Sage, Lavender and above all, always been used to treat various skin diseases and cosmetic products for skin care (Bremnes, 1994). Lavender essential oil possessed as the dominant components linalool, linalyl acetate, limonene, cineole and camphor. Good efficacy of essential oil it can be explained by interactions of individual components, but given the importance of some of the components, especially interactions linalyl acetate and linalool (Lis-Balchin et al., 1998). These essential oils and components could represent possible alternatives for the treatment of animals and humans infected by dermatomycetes. However, there are still only limited data available on the antifungal activity of essential oils towards human fungal pathogens *in vivo* (Soković, 2001).

Extracts of seventeen microfungi (Alternaria alternata, Cladosporium cladosporioides, *C. fulvum*, Fusarium sporotrichioides, F. trincintum, Paecilomyces variotii, *Penicillium ochrochloron*, *P. funiculosum*, *P*homa magdonaldii, Phomopsis helianthi, *Stachybotrys chartarum*, *Trichoderma viride*, and five dermatomycetes, *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes*, *T. rubrum and T. tonsurans* were tested against the yeast *Candida albicans* using a bioautographic assay on TLC plates. The extracts weer active against *C. albicans* at concrentations of 50-100 μg/mL. The extact of *P. ochrochloron* was most active. Further bioguided chemical analysis of *P. ochrochloron* afforded two components with antimicrobial activity identified as (−)2,3,4-trihydroxybutanamid and (−)-erythritol. (−)-Erythritol showed moderate antifungal activity, while (−)2,3,4-trihydroxybutanamide was highly active against the fungi tested (Rančić et al., 2006). The antifungal activity of limonene was tested against *Candida albicans* and five dermatomycetes. The antifungal potential of limonene was evaluated against *C. albicans* using a bioautographic method on TLC plates. It showed better potential than bifonazole. When the activity of limonene towards five dermatomycetes was determined by the micro- and diffusion methods, it was more effective against these human and animal pathogens than bifonazole (Rančić et al., 2003). The antifungal activity of garlic bulb powder, allicin and the lozenge with 15% of garlic powder was tested using broth microdilution method against *C. albicans*. The tested garlic powder, as well the lozenge, have shown activity with MIC 1.25–7.50 mg/ml. The major compound, allicin, was highly active at a very low concentration with MIC 0.4 μg/ml. Those concentrations are lower then concentrations of commercially available fungicides (Kundaković et al., 2011). These antifungal agents in development offer

extended half-lives, possibly reduced drug interaction profiles and good tolerance. In addition to activity against animal and human pathogens, they have a broad spectrum of activity including activity against resistant and emerging fungal species. According to these results microfungi may be the source of new biologically active substances, so special attention should be given to research on the biological activity of fungal metabolites and their application.

5. Susceptibility of fungal species to tested compounds

In our investigation, the extracts of different plants exhibited inhibitory effect on the growth of micromycetes. Among the tested extracts one of *Phlomis fruticosa* showed the best activity (10-20 μg/ml) (Table 1.). All investigated mosses extracts have been proved to be active against all fungi tested, where the ethanol extracts of silver moss showed the best potential (0.29-0.52 μg/ml) (Table 1.). The results of the tested essential oils are summarized in Table 1. All tested essential oils exhibited antifungal activity ranging from 0.1-1250 μl/ml using different methods. Among all oils analyzed the essential oil of cinnamon was the most effective as an antifungal agent in concentration 0.1-1 μl/ml using microdilution method. Also, the components of essential oils such as carvacrol, thymol, menthol, showed very strong antifungal activity (0.02-300 μl/ml), where carvacol showed the best potential (0.1-0.5 μg/ml). Among all flavonoides, sesquiterpene lactones and other compounds analyzed in our investigation (Table 1), lactones from *Centaurea pullata* showed the best antifungal activity (0.0001-0.0007 μmol/ml). Thus, comparing the activity of our investigation (extracts, essential oils and components, pure compounds) we can conclude that the sesquiterpene lactones was the most effective as an antifungal agent, followed by essential oils and their components.

The human and food-borne pathogens are most frequently chosen for testing essential oil antimicrobial activity. Many laboratories deal with plant pathogens but fewer with animal pathogens. The essential oils which are the most tested compounds in our investigation of antifungal activity showed different effect on plant and human pathogens species. In our earlier investigation (Soković, 2001) essential oils in general exhibited higher antifungal activity against plant pathogen species (*Phomopsis helianthi, Cladospoirium cladosporioides, Alternaria alternata, Fusarium* species) than human (*Trichophyton* species, *Microsporum cannis, Epidermophyton flocosum*). The essential oils tested possessed different range of minimal fungicidal concentration where human pathogens were more resistant than plant pathogens: *Mentha spicata* 1-2 μl/ml for plant pathogens (pps) and 2 μl/ml for human pathogens (hps), *M. piiperita* 1.5-2.5 μl/ml pps and 2.5 μl/ml for hps, Thyme 0.125-0.25 μl/ml pps and 0.25 μl/ml for hps., *Salvia* species for pps 2-15 μl/ml and 3-20 μl/ml for hps, *Lavandula* species 0.5-9 μl/ml for pps and 0.5-10 μl/ml hps, *Citrus aurantium* 10 μl/ml for pps and 7-15 μl/ml for hps. Some oils had the same antifungal ability against both, pps and hps: *Melissa officinalis* (5-6 μl/ml), *Rosmarinus officinalis* (6-8 μl/ml), *Citrus lemon* (7-10 μl/ml), while *Matricaria chamommilla* exhibited better antifungal capacity against hps (6-9 μl/ml) than for pps (7-9 μl/ml) and Sweet basil (4-5 μl/ml for pps) and 3-5 μl/ml for hps. These results are confirmed

by our investigation of antifungal activity with some other essential oils that we tested latter (Soković et al., 2002; Ristić et al., 2004; Simić et al., 2008; Džamić et al., 2009). Other researchers also obtained the similar results and confirmed that dermatomycetes are the most resistant, although numerous essential oils demonstrate high effectiveness against them. Among 22 samples of essential oils from 11 species of *Cinnamomum*, the oil of *C. suvabenium* was the most active against *Microsporum canis*, *Trichophyton mentagrophytes* and *T. rubrum* as well as some candidiasis (*C. albicans* and *C. glabrata*), (Kalemba & Kunicka, 2003). The results indicate that different essential oils have different efficacy. Also, the modes of action of essential oils are not the same against different fungal species. The mode of action of antimicrobial agents also depends on the type of fungal species and is mainly related to their cell wall structure and the outer membrane arrangement (Villar et al., 1986).

6. A novel approach to solve the problem

Fungal disease is responsible for significant losses of global crop production every year, and thus has a major impact on the world's agricultural productivity. Numerous strategies have been developed in attempts to minimize the losses caused by plant pathogens. Traditional approaches are based on the avoidance of sources of infection, vector management, modification of cultural practices, the use of resistant varieties obtained through conventional breeding, cross protection and chemical control. While these methods have been successful in some cases, indeed there is a need for new approaches. Furthermore some fungicides are being withdrawn from the market because of their undesirable effects on the environment. Whereas this information is interesting with regards to economic aspects, such as the share of fungicides costs in the output or in the variable costs, the information is less useful with regards to environmental aspects: i.e., the amounts spent on fungicides do say little about the types and quantities used. Over recent decade, producers have used synthetic fungicides as the main tool to control this problem. It has been estimated that over 23 million kg of these synthetic fungicides are used annually worldwide and it is generally accepted that production and marketing of plants would be not possible without their use (Martinez-Romero et al., 2008). New strategies for disease control are therefore urgently required. The development of novel control strategies for plant diseases is particularly important for pathogens that are difficult to control using existing methods.

More recently the scientific community has turned its attention to secondary metabolites from actinobacteria and its exploitation for various purposes which include therapeutic, environmental and industrial applications. With developing microbial resistance and need for safe and cost-effective antifungal drugs, screening of some other source, i.e. micro- and macro-fungi, mosses, for potential bioactive secondary metabolites becomes necessary. Particularly desirable is the discovery of novel prototype therapeutic agents representing new chemical classes, that operate by different modes of action compared to existing agents. We were developed suitable approves during the last several years in order to find new solutions in the aim to discover new antifungal drugs either by testing already existing medical compounds, compounds from natural sources such as plants, micro- and

macrofungi, or by combination of chemical compounds with natural one. Researchers also strive to elucidate the underlying biology of fungal microorganism both *in vitro* and *in vivo*.

The majority of our results are focused on investigation of antifungal activity of natural products isolated from plants, especially essential oils. We used conventional research methods for testing of antifungal activity and introduced some modification for the corresponding class of compounds and microorganisms in which we operate. For example, for testing of compounds in small quantity, we recommend using a microdilution method, and microathmosphere method for testing of volatile compounds. Bioautogaphic method on TLC plates is suitable for testing of plant and fungi extracts and fractions. Considering the fact that the fungi (micro and macro) may be the source of new biologically active substances, special attention in our laboratory is given to the research of biological activities of fungal metabolites and their application in protection and treatment of diseases caused by fungi and environmental protection. The broad diversity of the fungi, as well as their easy acquisition make them especially interesting for natural products screening program. The fungi possess high capacity of bio-synthesizing various metabolites possessing different structural and pharmacological characteristics. Many medicinal and therapeutic properties are attributed to the presence of active substances in fungi. Some such compounds are investigated because their known triggering mechanisms important for fungi, while other compounds are tested blindly for their antifungal properties.

Finally, research should be kept up in order to uncover as much potentially interesting data as possible, including toxicity against animal or human cells, mechanisms of action, effects *in vivo*, positive and negative interactions with common fungicides and so forth. Currently, these studies have produced a compounds suitable for the clinical trial stage. In summary, it is our belief that the study of plant, fungi and other natural sources as antimicrobial agents is necessary but the use of a appropriate method for investigation is essential. Finally, our results could be suggested for further clinical tests and for getting new information and possible application. To this should be given high priority.

7. Conclusion and future trends

Natural product-based fungicides are generally considered safer than synthetic herbicides, because of their relatively short environmental half-life and they are not harmful. The recent resurgence of interest in natural sources of bioactive compounds may, in part, be attributed to improved methods and instrumentation that has greatly reduced the time and effort required in natural product discovery programs. This interest is also associated with several other factors, including the realization that nature has already selected for very specific biological activities, that many natural compounds have yet to be discovered, and that the biological activities of relatively few of the known natural products have been characterized. All of the described and possible secondary metabolites have some kinds of inherent activity but in many cases these activities have not yet been discovered. Only the methods to detect their possible, perhaps until now unknown type of activity, has to be developed. There is no reason to suppose that the majority of the natural products including fungal metabolites should not exhibit some kind of biological function.

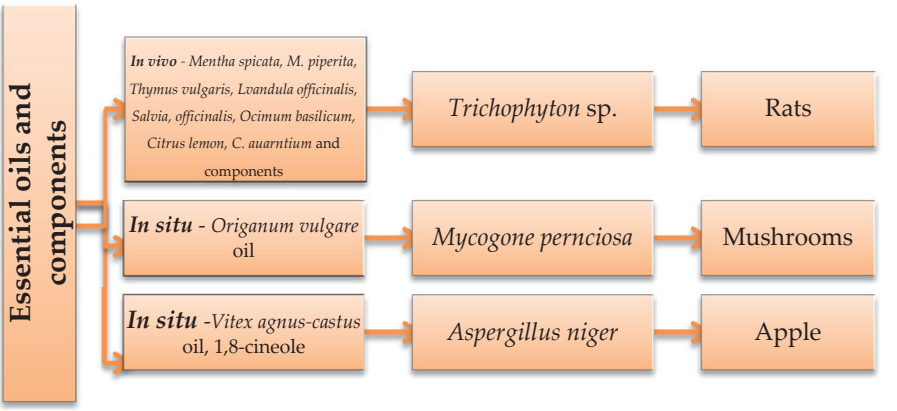

Figure 4. *In situ* and *in vivo* testing of antifungal activity of essential oils and components against pathogenic microfungi

Hundreds of presently known bioactive metabolites originally was discovered as "inactive", natural product and their activity was only discovered later, investigating them with new more specific methods, or reisolated them (sometimes from different species). In our days, in fact, there would not be any reason to talk about "bioactive" or "inactive" secondary metabolites and treat them separately. Moreover, the study of natural products may lead to the discovery of novel target sites, and/or new classes of chemistry that can be developed for pathogen management. Structural diversity has been, and still remains, an invaluable source of lead compounds in developing novel products. A recent study on complementary synthetic and natural products confirmed that the later generally have higher molecular weights than the former. Such diversity may be useful to the synthetic chemist in developing new classes of fungicides. One indirect and important benefit of the chemical composition and structural characteristics of natural products (the absence of "unnatural" ring structures and the low content of heavy atoms) is that most of them are rapidly degraded in the natural environment to benign products. In addition to their structural features, natural products tend to have different target sites from conventional fungicides.

Our results contribute to the development of safe, effective, and inexpensive formulations and processes to reduce the presence of pathogens. The antifungal compounds identified by us as the most active against major pathogens are candidates for future studies of synergism, compatibility and activity in different systems. Isolation and identification of natural active components may include a multitude of different extractions, chemical modifications, and increase knowledge of their mechanisms of action. As essential part of obtaining natural fungicides is the development of bioassays. The development of fungal resistance to synthetic drugs poses a serious long-term trait to plant, animal and human

health and environmental requirements. This could also possess as significant financial issues. The advantage of natural products compared to synthetic is not only in their non toxic characteristics but also in low costs. Growing of medicinal and aromatic plants and fungi is well established and in most cases economically justified. Identification and isolation of active components from plants and fungi is also good elaborated. Natural products with antifungal activity usually operate in very small concentrations, especially essential oils, and the for further application small amount are needed. All together makes them relative cheap and available as antifungal agents.

The future of fungicide management will probably be significantly influenced by research on natural products. Modern instrumentation has simplified the isolation and identification of lead compounds from which fungicides will be derived. The reviewed studies clearly demonstrate that natural products from plants and fungi present great potential for medical procedures and for the food, cosmetic, agricultural and pharmaceutical industries.

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Chapter 10

Natural Products from Plants as Potential Source Agents for Controlling *Fusarium*

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Additional information is available at the end of the chapter

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1. Introduction

At present, due to the fast expansion of the world's population, agricultural practices have been focused on increasing food production. Crop protection from competing weeds, insects and diseases has been necessary to obtain higher yields in agriculture. However, as a consequence of pest infection, a decrease in production performance has happened manifested in low food quality [1]. In order to solve this situation, synthetic chemical products have been widely used for controlling infectious diseases. Nonetheless environmental pollution due to their slow biodegradation, phytotoxicity, carcinogenicity and toxic waste in agricultural products is an important drawback [1, 2]. Agriculture production is currently trending to use eco-friendly methods for controlling diseases and pest infection [3, 4].

The growing demand of pesticide-free agricultural products has led to the search of novel, affordable, and less toxic strategies for pest control. Amongst those strategies biological agents, mineral salts and vegetable products have gained interest in the industry. Natural products are an important source of novel active chemical agents that could delay or inhibit pathogen growth and / or toxin production [5, 6]. Generally speaking, the plant derivatives (essential oils, extracts, fractions and compounds) are generally considered as non – phytotoxic and potentially effective for controlling pathogenic fungi in plants, [7]. Some of these natural subtances have showed antifungal, fungistatic or fungicidal activities which allow protected crops to have an extended shelf life by preventing enzymatic or metabolic processes of microorganisms. These fermentative or degradative microbiological processes can result not only in changes in odor taste, color and texture but also can cause potential harm to the consumer [8].

Natural compounds can be useful for crop and food protection. The use of botanicals for the management of the phytopathogens is gaining ground. Plant extracts and essential oils may

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have an important role to play in the preservation of foodstuffs against fungi. Recent literature has shown that biological activity of many plant-extracts, essential oils and their individual components is related with the inhibition of the growth of various fungi.From the 1970s there has been an increased interest on the study of the defensive mechanisms of plants for protecting against pathogenic agents or adverse environmental conditions. Phytochemical research has led to the isolation of active constituents synthesized by plants as a response to biotic or abiotic stresses, evidencing that these substances have insecticidal, fungicidal, bactericidal or herbicidal action. Throughout their evolution, plants have developed several defense mechanisms to prevent infections due to pathogens; also, plants synthesize a large number of secondary metabolites to protect themselves against biotic and abiotic stresses and for the maintenance of structure and vital functions. These are reasons to consider plants as an important source of new biopesticides [9, 10].

Fungi are a major cause of plant diseases and are responsible for significant economic losses to the food industry. These pathogens can cause local or systemic symptoms on their hosts. The most common symptoms are die-back (extensive necrosis of twigs), root-rot (disintegration or decay of the root system), leaf-spots (localized lesions on leaves consisting of death and collapsed cells), damping-off (rapid death of young seedlings), blight (general and extremely rapid browning death of leaves, branches, twigs, and floral organs), anthracnose (necrotic and sunken ulcer-like lesions of the stem, leaf, fruit, or flower), canker (localized necrotic lesion), basal stem rot (disintegration of the lower part of the stem), soft rots and dry rots (maceration and disintegration of fruits, roots, bulbs, tubers, and fleshy leaves) and decline (plants growing poorly, small and yellowish or red leaves) [11].

Species of the genus *Fusarium* are examples of phytopathogenic and toxine-producing fungi that have been reported to be widespread throughout the world, which can cause health problems associated with cell toxicity, cancer and adverse effects on growth and development of animals and humans [12, 13]. The genus *Fusarium* is a soilborne, necrotrophic, plant pathogenic fungus with many species causing serious harm to the plants. *Fusarium* infections are responsible for destroying crops and dramatically reducing production yields [9, 11]. *Fusarium* species have the ability to synthesize toxic mycotoxins, such as zearalenone, fusarins, fumonisins or trichothecenes which are detrimental to the consumer's health [14, 15, 16]. Some of these toxins, such as enniatin and fusaric acid are phytotoxins, whereas others, such as the mycotoxins, trichothecins and fumonisins, are toxic to animals [11].

Fusarium species are typically found in plants prior to harvest attacking cereals and often forming mycotoxins in the kernels. *F. oxysporum* causes primarily vascular wilts on many crops, whereas numerous species, especially *F. solani*, cause root and stem rots and rots of seeds that are accompanied by the production of mycotoxins. Moreover, *Fusarium* species causing disease in immunocompromised human population have been reported [11, 17]. The species *F. avenaceum, F. culmorum, F. graminearum, F. poae, F. semitectum, F. tricinctum and F. sporotrichioides* are found in cereals; *F. nygamai, F. verticilloides* and *F. subglutinans* in corn; *F. thapsinum* and *F. chlamydosporum* in sorghum, while *F. nygamaiand* and *F. fujikuroi* are found in rice. In legumes *F. chlamydosporum* and *F. tumidum* are typically encountered. *F.*

solani usually attack potatoes. The species *F. acuminatum, F. equiseti, F. oxysporum, F. proliferatum, F. solani* and *F. sambucinum* can attack a variety of substrates including fruits, vegetables and ornamental plants [18].

This chapter consists of a literature survey of the antifungal potential against *Fusarium* of substances obtained from different plant species. The chapter is organized as follows: in section 2 the main control methods against *Fusarium* spp are explained; in section 3 the main antifungal assays employed to evaluate the fungicide potential of different substances are described; in section 4 the results of antifungal activity of some plant natural products with potential for controlling *Fusarium* are presented and the potential role of these substances for sustainable plant disease management is discussed.

2. Control methods

The methods used to combat, control and prevent diseases in many crops by different strains of fungi, usually are divided into three groups according to the origin of the substance that makes the control. Currently, it is common the integral use of all kinds of control methods, practice that is known as integrated pest management. All methods have advantages and disadvantages, but the choice of a specific one for a particular crop depends on the state of the disease. In the following, a description of the chemical, biological and physical methods for controlling *Fusarium* is presented.

2.1. Chemical methods

Since the appearance in Europe of the fungus that caused the aggressive downy mildew disease of grape in the late 1870´s, many researchers have focused their efforts on the search for chemical entities that could control the diseases caused by fungal pathogens [11]. In particular, chemical fungicides have been used widely to control diseases caused by *Fusarium*, but all have a problem: high toxicity and accumulation of the active substance. In the following, the kinds of chemical compounds more commonly used for the treatment of diseases caused by *Fusarium* are presented.

2.1.1. Halogenated hydrocarbons

Halogenated hydrocarbons are used as soil fumigants because of its fast spread. These compounds are used before the sowing of seeds as a sterilization system. The compounds most commonly used are methyl bromide **1** and trichloronitromethane **2**, known as Chloropicrin, that were successfully employed in the treatment of root rot caused by different types of *Fusarium* species in crops of strawberry (*Fragaria vesca*), raspberry (*Rubus idaeus*), chile (*Capsicum annuum*), onions (*Allium cepa*), snuff (*Nicotiana tabacum*) [19]. Recent studies at University of Chile showed the combined use of the methyl bromide and chloropicrin in the successful control of vascular wilt in tomato plants by *Fusarium oxysporum* f. sp. *lycopersici* and of stem and root rot caused by *Fusarium solani* [20] at doses between 70 g/m^2 and 100 g/m^2.

However, the Enviromental Protection Agency classifies chloropicrin as a highly toxic and non-selective fungicide. The trichloronitro methane decomposes in the presence of light and heat and produces toxic gases like hidrogen chloride and nitrogen oxides. These compounds cause eye and skin irritation and adverse effects on the nervous system. Humans exposed for a long time to decomposition vapors of chloropicrin suffer severe headaches and pulmonary edema. Chloropicrin has a low accumulation in water due to its high volatility [21].

In the other hand, the Vienna Agreement of 1985 and the 1987 Montreal Protocol amended in London and Nairobi, classified the methyl bromide as a substance that ends up the ozone layer. Ozone (O_3) is a molecule consisting of three oxygen atoms, formed naturaly in the upper layers of the atmosphere by the sun's energy; ozone is a very unstable molecule, the solar radiation decomposes the ozone into molecular oxygen and atomic oxygen, which react to form O_3 again. The ozone's concentration in the atmosphere depends on a dynamic equilibrium between the rate at which forms and the speed with which destroys. When methyl bromide reaches the ozone layer, sunlight decomposes the halogenated hydrocarbon generating bromine radical. The bromine radical reacts with an oxygen atom of the ozone molecule, inducing a radical reaction that destroys ozone molecules quickly. For the serious environmental consequences generated by the destruction of the ozone layer many countries have outright the use of methyl bromide as a pesticide; however the countries that permit the use of this substance as a pesticide should be implement environmental care measures. Exposure to methyl bromide causes headache, vomiting, skin irritation and damage to the central nervous system [22].

2.1.2. EBDC's

EBDC's (Ethylenebisdithiocarbamates) are a group of non-systemic (surface acting) fungicides. EBDC active ingredients approved for their use are mancozeb, maneb, zineb and zineb ethylene thiuram disulphide adduct (metiram) [23]. The exact mechanism of action of EBDCs on fungi is not known. It is supposed that they act as fungicides when they are metabolised to an isothiocyanate radical (containing nitrogen-carbon-sulphur atoms) which inactivates the sulphydryl (sulphur-hydrogen) groups in amino acids (building blocks for proteins) contained within individual fungal pathogen cells [23]. Ethylenebisdithiocarbamates has been used for many years to control different diseases caused by *Fusarium* species in various crops like potato, guava and tomato.

EBDCs have relatively low acute toxicity. They are categorised by the World Health Organization (WHO) as Class III unlikely to present an acute hazard in normal use. However, some studies of toxicity in mice of EBDC's and some of their degradation products (like ethylenethiourea (ETU)) show that the principal target organ upon repeated exposure to all of the EBDCs is the thyroid [24]. For example, EBDCs and ETU altered thyroid hormone levels and/or weights in rats at the lowest dose after three months of dietary feeding. Other organs affected by ETU are liver at higher doses and pituitary gland: prolonged dietary feeding of ETU produces thyroid and pituitary tumors in rats and mice,

and liver tumors in mice [23]. ETU is considered an industrial contaminant of the EBDCs' industries.

2.1.3. Neonicotinoids

Chemical structures of neonicotinoids are obtained by synthetic methods from nicotine, an alkaloid derived from ornithine and obtained naturally from *Nicotiana tabacum* used since the mid-sixteenth century as a pesticide in multiple crops. Different synthetic series of neonicotinoids has been obtained. Imidacloprid **3** is an example of a type of neonicotinoid that has been used as a contact fungicide systemic in the treatment of vascular wilt caused by *F. oxysporum* and *F. moniliforme* [25, 26]. According to WHO imidacloprid is classified as moderate hazard or class II, the LD_{50} corresponds to 450 mg/kg. According to the EPA, neonicotinoid insecticides are classified as low toxicity to mammals [27]. However, countries like Germany and France banned its use because there are evidences that it causes collapse of bee colonies [28].

2.1.4. Benzimidazoles

The benzimidazoles are organic compounds resulting from the fusion of an aromatic ring and an imidazole ring, widely known for its effective use as dewormers of mammals and some of its derivatives are recognized as important antifungal substances. Benzimidazoles interfere with cell division and intracellular transport mechanisms of pathogenic fungi. The active substance with antifungal activity of more widespread use with chemical structure derived from the benzimidazole is known as benomyl **4**. It is a systemic foliar fungicide selectively toxic to microorganisms and invertebrates. Benomyl is used to treat vascular wilt of various crops (as tomato and carnation) caused by different special forms of *F. oxysporum*, and to treat potato dry rot caused by *F. graminearum* and *F. sambicinum*. Benomyl inhibits *F. oxysporum* growth in a percentage close to 60% [29]; and *F. graminearum* and *F. sambicinum* growth in around 90% [25].

However, benzimadozoles show low mobility in soil and do not volatilize, therefore, they produce high accumulation. Their agrochemical registration was canceled in the United States and the European Union. Since 1982 the use of benomyl has been restricted in Sweden and New Zealand for the birth of children with malformation whose mothers were exposed to this pesticide. In Latin America it has been registered the use of benzimidazoles; however, since 2006 in Brazil is no longer authorized to use fungicide whose active ingredient is benomyl. According to the World Health Organization, benomyl is a fungicide that it is safe for mammals the LD_{50} is greater than 10000 mg/kg. Other international institutions such as the EPA and the Academy of Sciences of the United States of America classified the benomyl as teratogenic substance, and one of the twelve chemicals responsible for cancer in the USA [30].

2.1.5. Phenyl pyrroles

Phenyl pyrroles are contact systemic fungicide used to control fungal phytopathogens, formulated mainly for *Botrytis* control in blueberries, tomatoes and grapes crops, and also to

control the sour rot complex formed by species of *Aspergillus*, *Alternaria*, *Rhizopus* and *Penicillium* in grape growing. They act by interfering the life cycle of the fungus, mainly in the processes of conidia germination, germ tube development, penetration and development of mycelium in the tissues of the host plant [31]. There are reports of the use of fludioxonil **5** in the control of several species of *Fusarium* which cause rot and common scab of potato [32]. Studies in Canada about potato crops in field conditions show the inhibition of about 70% and 90% of *F. solani* var. *coeruleum* and *F. sambusinum* growth, respectively [32]. Fungicides containing the active substance fludioxonil are classified by WHO as pesticides that do not present acute hazard in normal use, with LD_{50} greater than 5000 mg/kg [33]. Syngenta home manufacturer of a large number of fungicides with fludioxonil specified in the product data sheets that are moderately toxic to fish and should avoid contact with aquatic environments [31].

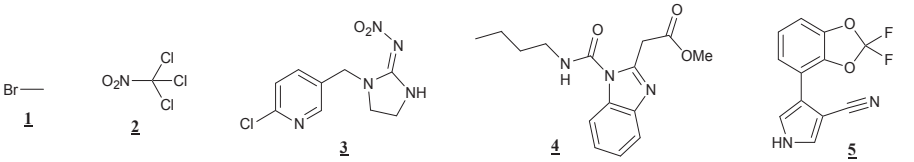


Figure 1.

2.2. Biological methods

Biological control is defined as the use of living organism to eliminate or control other. These control methods become an option that reduces the risks to health and to the ecosystem, and in many cases produce effects comparable to synthetic chemical pesticides. Biological controllers alternatively can be used in combination with synthetic pesticides to reduce substantially the amount of chemical product applied. In the case of diseases caused by *Fusarium* species, the biological control methods most commonly used involve the use of antagonistic organisms, particularly fungi and bacteria. Below, it is presented the antagonistic organisms more commonly used for *Fusarium* control.

2.2.1. Trichoderma spp.

Trichoderma species are a beneficial fungus that occurs naturally in all soils. In particular, *T. harzianum* is the most used specie for biological control of *Fusarium*, which acts as antagonist. In many markets worldwide, several products that contain *T. harzianum* are available. The fungus is applied to seeds or to plants in the crops, then it colonizes the roots and forms a kind of protective glove. *Trichoderma* spp. and the roots form a symbiosis: the fungus feeds and lives with the exudates produced by the roots and the fungus gives to the roots protection. The protection process consists of any of the following three ways [34]:

- The first one is given when the antagonist fungus consumes the root exudate of the host. As the exudate is the chemical signal that alerts the fungal pathogen to attack the plant, the infection does not happen.

- When any fungal pathogen gets to cross the protective glove, it is destroyed and used as food by *Trichoderma* spp.
- The third type of protection is by exclusion, considering that *Trichoderma* occupies all the space near to the roots, constituting a physical barrier and excluding from this area any fungal pathogen.

The previous considerations are very important because the biological control is a preventive but not curative methodology, therefore, when damage appears in plants must first be applied the chemical fungicide and seven days after *Trichoderma* should be applied. For use this antagonist fungi is necessary a pH between 4 and 8 and a temperature between 48 and 95 °F [34].

The Panamerican Agricultural School in Honduras has recorded, produced and developed a commercial fungicide based on *T. harzianum* spores known as Trichozam®. It is used in the treatment of root and stem rot caused by *Fusarium* species particularly in the follow crops: lettuce (*Lactuca sativa*), sweet chile (*Capsicum frutense*), tomato (*Licopersicum esculentum*), snuff (*N. tabacum*), potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*); in all cases the recommended dose is 240 g/ha [34]. In addition to *T. harzianum*, *T. lingnorum* strain has been used and processed in commercial products for control of phytopathogens of the genus *Fusarium* [35]. Together with the facts presented above, there is experimental evidence of control of *Fusarium* with *Trichoderma* spp. in cultures of Papaya in Mexico (*Carica papaya*) [36], bean in Colombia (*Phaseolus vulgaris*) [37] employing the antagonist fungus alone or in combination with other antagonists. It is important to make clear that the antagonic fungi are not toxic for mammals.

2.2.2. Antagonistic bacteria

Free-living bacteria or associations that inhabit the rhizosphere can control natural inhabitants of soil as phytopathogenic fungi, like *Fusarium* species. The mechanisms of action of these organisms are not clear; however, taking into account experimental evidence, some authors suggest the mechanism is related to mycelial growth inhibition and stimulation of plant defense-related enzymes [38]. Microorganisms that have been most studied belong to the genera *Azospirillum*, *Azobacter*, *Klebsiella*, *Pseudomonas* and *Bacillus*. Fungicides based in a combination of some of these bacteria have been used successfully in treatment of alfalfa wilt caused by *Fusarium* spp. [39]. Moreover, these organisms have the ability of produce growth promoting substances, since they belong to a group of organisms called plant growth stimulators. These substances stimulate the germination of seeds and accelerate plants growth, especially in the early stages, induce root initiation and increase the formation of roots and root hairs. The main substances that are produced are stimulating hormones like auxins, gibberellins and cytokines [39].

2.3. Physical methods

In addition to chemical and biological control, there are physical methods, which normally are used as prevention methods and always should be used in combination with other

methods of control. In the case of *Fusarium* control has been used three physical control methods:

1. Rotate crops: this method consist in planting of successive different crops in one field, following a defined order. In contrast, monoculture planting is repeated the same species in the same field year after year. The crop rotation is a practice that has positive effects on the crops, raise the production due to: Reducing the incidence of pest and diseases, to stop their cycle's life. Provides a better nutrients soil profile. Allows balancing the production of waste and when the crop is contaminated with a pathogen, the crop rotation provides a partial reduction of pathogen inoculums [37]. In the specific case of affected crops by *Fusarium* (like tomato and potato), the crop rotation is very common, the idea is to rotate the crops commonly attacked by *Fusarium* by other crops that are not attacked by the same pathogen.
2. Planting bed: this method is commonly used in many countries of Latin America to control *F. oxysporum* f. sp. *dianthi*. This technique involves the use of different planting substrates, which form a high bed that isolates the seed from the ground, natural habitat of the pathogens of *Fusarium* genus [40].
3. Solarization: according to the FAO, soil solarization is a term that refers to soil disinfestation by heat generated from solar energy captured. Soil solarization is a hydrothermal process that takes place in moist soil which is covered by a plastic film and exposed to sunlight during the warmer months [40]**.** The efficiency of soil solarization to control soil pests depends on the relationship between exposure time and temperature. This method is based on the fact that many pathogens are mesophiles in which a threshold temperature of 37 ^{0}C is critical and the accumulation of the effects of heat at that temperature or higher is lethal. It is important to note that there are thermophilic and thermotolerant organisms that can survive and even thrive at this temperature.This method has the advantage that it is not dangerous for farmers and does not transmit toxic waste to the consumers, being easy to educate farmers about their use. However, some disadvantages of this method are the lack of sufficient irrigation water and the survival of the pathogen in the deeper soil layers. For *Fusarium* control, experimental evidence shows that the use of the solarization combined with chemicals fungicides gives good results. For example, when solarization was used combined with fumigation with methyl bromide (at lower doses than those normally used) was observed the mycelial growth inhibition in carnation crops affected by *F. oxysporum* f. sp. *dianthi* [41].

It is clear that chemical control methods are most effective for the treatment of diseases caused by *Fusarium* species, however, exist many problems by toxicity of these substances in the short, medium and long term. It is also clear that the integrated management of different methods for controlling *Fusarium* pathogens has shown good results, however, there is still much to do in the search for methods of biological and physical control for gradually decrease the use of synthetic pesticides. In this way, plants are an important option, even more if one considers that from ancient times have been used in an empirical way in the maintenance of different crops by many civilizations.

3. Bioassays for antifungal activity evaluation

Several methods for testing antifungal susceptibility are currently used. So far, key areas for the application of antifungal bioassays include control of crop pathogens in phytopathology and human pathogenic fungi in antimycotic chemotherapy. Fungicidal testing includes either *in vitro* methods, such as minimum fungicidal testing methods or animal models [42, 43].

The available methods for detecting activity are not equally sensitive or not based upon the same principle; therefore results will be profoundly influenced by the method. The choice of assay constitutes the first arising difficulty when working with fungi. One of the most inherent problems is that the single methodologies do not really produce comparable results. The standardization of antifungal susceptibility testing methods is crucial for the evaluation and development of antifungal drugs and agrochemicals, because the successful use of a fungicide usually also requires the dissemination of its correct application procedure [42, 44].

The ability of a compound to kill a pathogen as opposed to simply inhibiting its growth is an apparently desirable quality, particularly in the setting of decreased immunity. Although several studies have characterized the fungicidal activity of antifungal agents, there is no standardized method for doing so [43].

Below is a description of the main features of methods to evaluate antifungal activity. The most used assays to detect antifungal substances are bioautography, disk diffusion, agar dilution and dilution tests. These antifungal test methods have been classified into three main groups: dilution, diffusion and bioautographic methods [44, 45].

3.1. Dilution methods

Dilutions assays, especially those that are carried out in microwell plates, are one of the most useful and efficient methodologies to evaluate antifungal activity of different substances [45, 46, 47].

In the dilution methods, the compounds are mixed with an appropriate medium that has been previously inoculated with the fungal strain. The assay can be carried out in liquid as well as in solid media. The results of these assays can be measured in many ways; being the minimal inhibitory concentration (MIC) and half effective concentration (EC_{50}) the most common forms of reporting results. Minimal inhibitory concentration (MIC) is defined as the lowest concentration capable to inhibit any fungal growth. Half effective concentration is defined as the the median concentration that causes 50 % of maximal response in a given system.

In liquid or broth-dilution methods, turbidity and redox-indicators are most commonly used. Turbidity can be estimated visually or achieved more accurately by measuring the optical density at 405 nm. However, test samples that are not entirely soluble may interfere with turbidity readings, emphasizing the need for a negative control or sterility control. The

liquid-dilution method also determines whether a compound or extract has a fungicidal or static action at a particular concentration. The serial dilution test yielded the best reproducible results on the MIC and was recommended as general standard methodology for testing natural products. The microdilution method is more sensitive and allows detecting the MIC more exactly [45].

3.2. Agar diffusion or disk diffusion methods

This technique is one of the most widely employed for antifungal activity screening, due to its simplicity and low cost. It is primarily used to determine if a compound or a compound mixture (like crude extracts, fractions and essential oils) possesses any activity. This assay is based on the use of disks containing solutions of the substances to be evaluated. The tested substance, at a known concentration, is in contact with an inoculated medium, and the diameter of the clear inhibition zone around the reservoir (inhibition diameter) is measured at the end of the incubation period. The results of this assay also can be reported as Minimal Inhibitory Quantity (MIQ) which is deffined as the minimal quantity of substance that causes some detectable inhibition of fungal growth. One of the major shortcomings of these methodologies is that, as for all diffusion assays, the concentration of the compound or compound mixture tested is unknown [42, 44].

The possibility to test up to six extracts per plate against a single microrganism and the use of small sample volumes are specific advantages of diffusion assays [45]. The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar. The antimicrobial potency of different samples may not always be compared, mainly because their differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Additionally, size of inhibition zones might be influenced by volatilization of antimicrobial active test material. Due to the absolute values of inhibition zones have only relative importance, the agar diffusion method is appropriate as pre-test only and should not be used for compounds of high lipophilicity, such as volatile sesquiterpenes [48]. Furthermore, agar-diffusion methods are difficult to run on high-capacity screening plataforms.

The composition of the medium could influence the activity of the tested substances. The agar diffusion assay is limited to substances with considerable water solubility. Growth media and compound doses employed in this test system vary much and hamper the interpretation of results. On the other hand, the disk diffusion method was used as a laboratory routine to perform a susceptible test for licensed drugs. There has been much research interest in agar-based antifungal susceptibility via disk diffusion method due to their relative ease and the lack of need for specialized equipment [49].

The inhibition zones are usually distorted as this application procedure does not guarantee the test compound to be evenly distributed across the disk. However, if the solvent has not been removed properly and causes inhibition effects by itself, the zones are highly concentric to the disk. The peculiarity of this phenomenon facilitates the experienced researcher to become aware of the deficiency in his work [42].

Conventionally, diameters of inhibition zones are presented to document the observed antifungal activity. In interpreting these diameters, should be considered that variable diffusion properties of the test compound may affect the outcome, especially if results from this assay are used to compare MIC values of different compounds. There exist modifications of this method, such as the agar well diffusion, including the hole-plate (diffusion of the aqueous test compound solution into the agar medium from a vertical hole in the agar layer) and the cylinder method (stainless-steel or ceramic cylinders placed on top of the agar medium) [50]. These two modifications have their merits when the test compound shows good solubility in aqueous solvents. However, as the majority of active compounds are better soluble in organic solvents, the addition of a specific portion of organic solvent to obtain an aqueous suspension of the test compound is required. This modification has the evident advantage that pure organic solvent can be used for the stock solution, which gets completely lost during the preparation of the disks after efficient drying [42].

3.3. Bioautographic method

This technique was introduced by Homans and Fuchs (1970) and is preferably carried out on thin-layer plates (TLC), but is also applicable on polyacrylamide gels [51, 52]. The bioautography can be done in three ways: (a) direct bioautography, where the microorganism grows directly on the thin-layer-chromatographic plate (TLC); (b) contact bioautography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (c) agar-overlay bioautography, where a seeded agar medium is applied directly onto the TLC plate. TLC has an enormous potential for separating mixtures of low-molecular weight compounds, reason that bioautography allows localizing substances with antimicrobial activity of an extract on the chromatogram; it supports a quick search for new antimicrobial agents through bioassay-guided isolation [44].

Autobiography on TLC plates facilitates the evaluation of a wide range of filamentous fungi to antifungal testing. Preference is given to those fungi that are characterized by pigmented hyphae, spores or conidia; if contrast is poor, it can be enhanced by treatments with iodine vapor [42]. The fungus is applied to the plates in a suspension, that usually consists of malt extract broth or glucose medium with mineral salts added [51]. However, the nutrient medium composition may have to be adjusted to the specific requirements of each test fungus.

Wedge and Nagle published the application of 2D-TLC as efficient approach to obtain improved separation of compounds with a concomitant gain in sensitivity of the assay [53]. Diffusion assays are generally less suitable to assess the quality of the antifungal activity in comparison to positive controls, despite their quick and versatile application.

Apart from the advantages of rapidly detecting active compounds in mixtures and high sensitivity, the depicted bioautography also points to a potential disadvantage of this diffusion assay. Its applicability is limited to microorganisms that easily grow on TLC plates. The diffusion effects may significantly hamper a comparison of activities between

different compounds with differing chemical properties. Another factor that may also affect results is the stability of the compound on the TLC plate as the duration of the assay may last for several days and exact quantization of the amounts of the compound that survived on the TLC plate are rarely performed due to the amount of effort required. This qualitative technique is not directly applicable in current high capacity screening designs and does not give data of values for Minimal Inhibitory Concentration (MIC). When pure compounds are evaluated at diferentt quantities in this assay, the results of its activity can be reported as Minimal Inhibitory Quantity (MIQ) which is deffined as the minimal quantity of substance that causes some detectable inhibition of fungal growth [54, 55].

3.4. Other methods

Flow cytometry (FC) has been described as an excellent tool for studying the susceptibility of different microorganisms, including fungi [48, 49]. The main advantages of FC are: 1) it yields higher susceptibility and precise results and 2) FC assays combine the speed of cell-by-cell analysis of very large populations with the independence from long incubation times, resulting in faster tests. However, there are still determinant disadvantages such as: the extremely high cost of the FC equipment, besides that, and in spite of the evolutions made in recent times regarding the user-equipment interface, the techniques still require an experience and skilled operator in order to obtain optimal results [56].

4. Plant natural products as potential agents for controlling *Fusarium*

Natural plant products have been used since the fifteenth century by different communities to control different pests. Today, interest in botanical pesticides has come back, reason why many phytochemical investigations have been focused on finding new products with pesticidal properties. It is important to note that many antifungal activity assays are used to determine the fungicidal potential of a substance, therefore, the results of antifungal activity forextracts, fractions, essential oils and pure compounds are reported in many ways, making difficult to compare results, in order to establish which substances are most promising to control fungi. The following sections present a review of studies of antifungal activity of different products from plants evaluated against different *Fusarium* species, carried out from 2000 to 2012, taking into account the studies that used dilution, diffusion and bioautography assays as methods for determine the antifungal activity.

4.1. Crude extracts and fractions

This section presents a review of the main results of antifungal activity of plant extracts and fractions that have been carried out in recent years. Some crude extracts from species of families Asteraceae, Rubiaceae, Rosaceae, Rutaceae, among other have excellent activities *in vitro* and *in vivo* against plant fungal pathogens of genus *Fusarium*. The reports of antifungal activity of extracts and fractions of plants against *Fusarium* species have been carried out mainly against *F. oxysporum*, *F. verticillioides*, *F. dimerum*, *F. proliferatum* and *F. solani*, using different assays.

The diffusion method is the most employed technique in the screening to the antifungal activity in extracts or fractions, because it is a fast - low cost assay and allows an approach to the presence of active compounds. Table 1 summarizes the main results of antifungal activity against species of *Fusarium* genus of different extracts and fractions obtained from some plant species of different families. It is shown the results of the antufungal activity as inhibition zones or minimum inhibitory quantity MIQ.

The antifungal activity of extracts of four plants from Lake Manzalah in Egypt was tested *in vitro* against *F. oxysporum*. Extracts were obtained from dried leaves employing different solvents (methanol, ethanol, water and chloroform). The antifungal activity was measured using disk diffusion method. All aqueous extracts showed the highest inhibitory activity against *F. oxysporum* with inhibition zones between 38 and 48 mm; therefore, polar compounds are responsible for the antifungal activity [57].

In a study of the antifungal activity against *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *dianthi*, 100 extracts and fractions obtained from Colombian species belonging to the families Lauraceae, Rutaceae, Piperaceae and Myristicaceae were tested by disk diffusion method using quantities of 500 μg of extract or fraction [58, 59]. The results of this study, expressed as MIQ, porved that the ethanolic extract and chloroform fraction from *Compsoneura capitellata* wood (Myristicaceae), ethanolic extract of the bark of *Zanthoxylum monophylum* (Rutaceae), chloroform fraction from alkaloid extract of *Z. quinduense* bark

Specie	Part of plant	Sample	Measured variable	*Fusarium* species	Results (Concentration)	Reference
Ceratophyllaceae						
Ceratophylum demersum	Leaves	Aqueous	Inhibition diameter (ID)	*F. oxysporum*	45.0 mm (300 mg/mL)	57
		Chloroform			23.0 mm (300 mg/mL)	
		Ethanolic			24.0 mm (300 mg/mL)	
		Methanolic			11.0 mm (300 mg/mL)	
Lauraceae						
Ocotea callophyla	Leaves	Ethanol	Minimum inhibitory quantity (MIQ)	*F. oxysporum* f. sp. *dianthi*	250 μg	58
Ocotea macrophylla	Steam	Alcaloids fracction	MIQ	*F. oxysporum* f. sp. *lycopersici*	500 μg	59
		Chloroform			500 μg	
Moraceae						
Maclura tictoria	Leaves	Ethanolic	MIQ	*F. oxysporum* f. sp. *lycopersici*	500 μg	59
Myristicacea						
Compsoneura capitellata.	Leaves	Ethanolic	MIQ	*F. oxysporum* f. sp. *dianthi*	100 μg	58 y 59
		Petroleum ether			100 μg	
		Chloroform		*F. oxysporum* f. sp. *lycopersici*	500 μg	
		iso-propil acetate			500 μg	
	Wood	Ethanolic		*F. oxysporum* f. sp. *lycopersici*	250 μg	
		Hexane			250 μg	

		Chloroform		*F. oxysporum* f. sp. *dianthi*	100 μg	
		iso-propil acetate		*F. oxysporum* f. sp. *lycopersici*	100 μg	
Piperaceae						
Piper eriopodon	Fruits	Ethanolic	MIQ	*F. oxysporum* f. sp. *dianthi*	250 μg	58
P. holtoni	Leaves				250 μg	
P. hispidum	Roots				250 μg	
P. aduncum	Leaves				250 μg	
P. bogotense	Leaves				100 μg	
P. bogotense	Fruits				250 μg	
P. artanthe	Aerial part				100 μg	
P. artanthe	Wood				250 μg	
P. arboreum	Aerial part				100 μg	
Pontederiaceae						
Eichhornia crassipes	Leaves	Aqueous	ID	*F. oxysporum*	44.0 mm (300 mg/mL)	57
		Ethanolic			12.0 mm (300 mg/mL)	
		Methanolic			14.0 mm (300 mg/mL)	
Potamogetonaceae						
Potamogeton crispus	Leaves	Aqueous	ID	*F. oxysporum*	48.0 mm (300 mg/mL)	57
		Chloroform			11.0 mm (300 mg/mL)	
		Ethanol			16.0 mm (300 mg/mL)	
		Methanol			21.0 mm (300 mg/mL)	
Potamogeton pectinatus	Leaves	Aqueous	ID	*F. oxysporum*	38.0 mm (300 mg/mL)	57
		Chloroform			28.0 mm (300 mg/mL)	
		Methanol			26.0 mm (300 mg/mL)	
Rosaceae						
Rubus ulmifolius	Shoots	Metanolic	ID	*F.dimerum*	10.7 mm (35 mg/mL)	61
				F. solani	24.3 mm (35 mg/mL)	
				F. sp	11.7 mm (35 mg/mL)	
		Fenols fraction		*F. solani*	18.0 mm 35 mg/mL)	
Rutaceae						
Zanthoxylum quinduense	Leaves	Ethanolic	MIQ	*F. oxysporum* f. sp. *lycopersici*	500 μg	59
		Alkaloids – $CHCl_3$			250 μg	
	Wood	Ethanolic			500 μg	
		Ethyl acetate			100 μg	
		Acetone			500 μg	
Z. monophyllum	Bark	Ethanolic	MIQ	*F. oxysporum* f. sp. *lycopersici*	250 μg	58 y 59
				F. oxysporum f. sp. *dianthi*	250 μg	
Z. rhoifolium	Bark	Ethanolic	MIQ	*F. oxysporum* f. sp. *lycopersici*	500 μg	59
Esenbeckia runyonii	Wood	Ethanolic	MIQ	*F. oxysporum* f. sp. *dianthi*	250 μg	58
Rubiaceae						
Uncaria guianensis	Leaves	Ethanolic	MIQ	*F. oxysporum* f. sp. *lycopersici*	500 μg	59

Table 1. Results of diffusion method (inhibition grown or minimum inhibitory quantity) of plant extracts and fractions against *Fusarium* species

(Rutaceae) and ethyl acetate fraction from *Z. quinduense* wood were substances that showed the highest antifungal activity against *F. oxysporum* f. sp. *lycopersici* [59]. The extracts that showed the higher antifungal activity against *F. oxysporum* f. sp. *dianthi* are Leaves of *C. capitellata* (Myristicaceae), petroleum ether fraction from *C. capitellata* leafs, chloroform fraction from *C. capitellata* wood, *Piper bogotense* leaves and aerial parts of *P. artanthe* and *P. arboreum* [58].

Plants species of *Rubus* genus are known to have antimicrobial properties mainly due to their high content in phenolic compounds [60]. At 2008 it was investigated the micropropagation of *R. ulmifolius* and it was evaluated the *in vitro* antifungal activity of shoots against different species of genus *Fusarium*. The methanolic extract was fractionated by column chromatography on sephadex LH-20 and it was obtained fractions enriched in phenols. The antimycotic activity of the crude metanolic extract and fractions were tested using the disk diffusion method. The methanol extract showed low activity against *F. dimerum* and *Fusarium* sp. with inhibition zones of 10.7 and 11.7 mm at 25 μl, equivalent to 100 mg of dried plant material. The crude extract showed good activity against *F. solani* (24.3 mm) at 25 μl, equivalent to 100 mg of dried plant material. The fractions with higher polarity, rich in tannins, showed moderate inhibition against *F. solani* with IZ of 12.5 and 19.0 mm at 25 μl (35 g/L) [61].

The dilution method is used to determine more specifically the activity of an extract or fraction. In this technic, minimum inhibitory concentrations MIC are determined by agar-dilution method or microdilution method. The results of the antifungal activity using dilution methods are summarize in Table 2.

Specie	Part of plant	Sample	Measured variable	*Fusarium* species	Results (Concentration)	Reference
Achariáceas						
Xylotheca kraussiana	Leaves	Acetone	Minimum inhibitory concentration MIC	*F. oxysporum*	0.63 mg/mL	62
		Methanol			0.32 mg/mL	
		Hexane			0.32 mg/mL	
		Dichloromethane			0.32 mg/mL	
Asteraceae						
Baccharis glutinosa	Aerial part	Methanolic	% inhibition	*F. verticiloides*	67 % (8,4 mg/mL)	64
Flourensia microphylla	Leaves	Ethanolic	% Inhibition	*F. oxysporum*	85 % (100 μl/l)	65
F. cernua					80 % (100 μl/l)	
F. retinophylla					90 % (100 μl/l)	
Anacardeaeceae						
Harpephyllum caffrum	Leaves	Acetone	MIC	*F. oxysporum*	0.08 mg/mL	62
		Methanol			0.63 mg/mL	
		Hexane			0.32 mg/mL	
		Dichloromethane			0.16 mg/mL	
Caparaceae						
Capparis decidua	Fruits	Ethanolic	% Inhibition	*F. oxysporum*	70 % (50 mg/mL)	66
Caesalpinaceae						
Peltophorum pterocarpum	Leaves	Aqueous	% Inhibition	*F. equiseti*	74 % (3,33 mg/mL)	67
				F. graminearum	69 % (3,33 mg/mL)	
				F. proliferatum	60 % (3,33 mg/mL)	
				F. semitectum	60 % (3,33 mg/mL)	

Combretaceae						
Bucida buceras	Leaves	Acetone	MIC	*F. oxysporum*	0.02 mg/mL	62
		Methanol			0.63 mg/mL	
		Hexane			0.32 mg/mL	
		Dichloromethane			0.04 mg/mL	
Euforbiaceae						
Emblica officinalis Gaertn	Leaves	Aqueous	% Inhibition	*F. equiseti*	75 % (3,33 mg/mL)	67
				F. graminearum	71 % (3,33 mg/mL)	
				F. lateritium	64 % (3,33 mg/mL)	
				F. moniliforme	69 %(3,33 mg/mL)	
				F. oxysporum	79 % (3,33 mg/mL)	
				F. proliferatum	89 % (3,33 mg/mL)	
				F. semitectum	80 % (3,33 mg/mL)	
				F. solani	76 % (3,33 mg/mL)	
Myrtaceae						
Eucalyptus globulis Labill.	Leaves	Aqueous	% Inhibition	*F. equiseti*	62 % (3,33 mg/mL)	67
				F. graminearum	69 % (3,33 mg/mL)	
				F. lateritium	76 % (3,33 mg/mL)	
				F. moniliforme	66 % (3,33 mg/mL)	
				F. proliferatum	81 % (3,33 mg/mL)	
Prosopis juliflora Swartz	Leaves	Aqueous	% Inhibition	*F. equiseti*	76 % (3,33 mg/mL)	67
				F. graminearum	72 % (3,33 mg/mL)	
				F. lateritium	70 % (3,33 mg/mL)	
				F. moniliforme	90 % (3,33 mg/mL)	
				F. oxysporum	80 % (3,33 mg/mL)	
				F. proliferatum	91 % (3,33 mg/mL)	
				F. semitectum	81 % (3,33 mg/mL)	
				F. solani	80 % (3,33 mg/mL)	
Olinaceae						
Olinia ventosa	Leaves	Acetone	% Inhibition	*F. oxysporum*	0.08 mg/mL	62
		Methanol			0.63 mg/mL	
		Hexane			0.32 mg/mL	
		Dichloromethane			0.16 mg/mL	
Rubiaceae						
Breonadia salicina	Leaves	Acetone	MIC	*F. oxysporum*	0.32 mg/mL	62
		Methanol			0.08 mg/mL	
		Hexane			0.16 mg/mL	
		Dichloromethane			0.16 mg/mL	
Mimusops elengi L.	Leaves	Aqueous	% Inhibition	*F. equiseti*	86 % (3,33 mg/mL)	67
				F. graminearum	70 % (3,33 mg/mL)	
				F. lateritium	78 % (3,33 mg/mL)	
				F. moniliforme	79 % (3,33 mg/mL)	
				F. oxysporum	86 % (3,33 mg/mL)	
				F. proliferatum	93 % (3,33 mg/mL)	
				F. semitectum	84 % (3,33 mg/mL)	
				F. solani	80 % (3,33 mg/mL)	
Verbenaceae						
Lantana cámara	Stems	Ethanolic	% Inhibition	*F. oxysporum*	60 % (5,0 mg/mL)	66
	Leaves				70 % (5,0 mg/mL)	
	flowers				80 % (5,0 mg/mL)	

Table 2. Results of dilution method (percentage of inhibition or minimum inhibitory concentration) of plant extracts and fractions against *Fusarium* species

The antifungal activity of acetone, methanol, hexane and dichloromethane leaf extracts of six African plants, *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana* were evaluated against *F. oxysporum*. The microdilution assay was used to determine the minimum inhibitory concentration (MIC) values for each plant extracts. All plant extracts were active against the phytopathogenic fungi. *B. buceras* had the best antifungal activity against *F. oxysporum*, with minimum inhibitory concentration (MIC) of 0.02 g/L. The number of active compounds in the plant extracts was determined using bioautography method, this compounds was separated with CEF had similar Rf values of 0.70, 0.85 and 0.95 in acetone, hexane, DCM and methanol extracts, respectively [62].

The genus *Baccharis* belonging to the Asteraceae family is mainly distributed in central and South America and it is characterized by the presence of phenolic compounds with significant activity against different pathogens [63]. The antifungal activity of *B. glutinosa* was evaluated against *F. verticillioides*. Sample of aerial part was extracted with 70% methanol and sequentially partitioned with hexane, ethyl acetate and n-butanol. The crude and partitioned extracts were evaluated in their capacity to inhibit the radial growth of fungi. The results showed that the partitioned ethyl acetate extract exhibited the highest antifungal activity. The ethyl acetate extract inhibited completely the growth of *F. verticillioides* at 0.8 g/L (Table 2) [64]. Some other Mexican endemic species of the Asteraceae family presented fungicidal activity against *F. oxysporum.* The antifungal activity of ethanol extracts from *Flourensia microphylla*, *F. cernua*, and *F. retinophylla* were tested against micelial growth Inhibition of *Fusarium oxysporum*. The three *Flourensia* species showed growth inhibition of *F. oxysporum* at 10μl l^{-1} and *F. microphylla* had the highest inhibition at this concentration. *F. cernua* and *F. microphylla* had the highest efficiency [65].

Aqueous extracts of 46 plants belonging to 32 different families were tested for antifungal activity against eight species of *Fusarium* genus (*F. equiseti*, *F. moniliforme*, *F. semitectum*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. solani* and *F. lateritium*). Table 2 shows that only 12 plants exhibit significant antifungal activity (inhibition percentages greater than 60%). The antifungal activity of aqueous extracts varied among the test pathogens and it was compared with that of the synthetic fungicides. *F. proliferatum* was the strain that showed the highest susceptibility for the aqueous extracts [67].

4.2. Essential oils

Amongst alternatives for natural biological control, are found essential oils and their components, which have showed therapeutic activities and toxicity facing fungi, bacteria and insects. These substances could be an alternative to inhibit pathogen fungi growth such as Fusarium species. They have as advantages specificity, evaporation (avoiding residues) and biodegradability; and furthermore they are considered non aggressive from the standpoint of health. Although their action mechanisms are not totally clear, it has been reported that chemical components present in essential oils produces the following effects:

protein denaturalization in the cell membrane, precipitation of cell proteins and enzymatic inhibition, provoking the loss of amino acids [68, 69, 70]. Thus, each component in an essential oil has its own contribution upon the whole biological activity. Amongst those reported compounds that showed antimicrobial properties are found thymol, carvacrol, geranial, citronellal, geraniol, linalool, citronellol and lavandulol [6].

Despite the advantages that have essential oils for control of fungal pathogens, their use as commercial products is still incipient, due to its high cost-benefit ratio due to the low extraction yields of essential oils. Another reason is the low development of efficient formulations to maintain its effective concentration for long periods of time, due to that essential oils: they are very complex mixtures, they have high evaporation rate and they degrade quickly even at room temperature. At present they has proposed the use of waxes that are widely used in the food industry for incorporate the essential oils. It has also been proposed to prepare emulsions with controlled release of essential oils, as part of the solution to counteract some of the above mentioned disadvantages [71].

The following will be a review of essential oils that could be considered as an alternative for plant disease control produced by Fusarium genus fungi. Among the plants that are important for its antifungal activity are found the plants of the families Annonaceae, Apiaceae, Asclepidaceae, Asteraceae, Caryphyllacaeae, Cupresaceae, Chenopodiaceae, Geraniaceae, Lamiaceae, Lauraceae, Myrtaceae, Piperaceae, Poaceae, Rosaceae, Rutaceae, Solanaceae Unmbeliferacea, Verbenaceae and Zingiberaceae. Lamiaceae family is the one with the largest number of antifungal activity reports, with studies of species belonging to genera *Mentha*, *Ocimum, Origanum* and *Thymus.*

Also was observed that *Fusarium* species that have been object of the highest number of studies are: *F. oxysporum, F. solani* and *F. moniliforme*. The results of antifungal activity evaluation for essential oils are reported as follows: inhibition diameter, inhibition percentage, minimal inhibitory concentration (MIC); minimal fungicidal concentration (MFC); concentration producing 50% of inhibition (IC50); half effective concentration (EC50). However, during the review were found other reported variables such as: antifungal index, biomass production inhibition percentage and conidium inhibition percentage; but they will not be considered for not being the variables most common to report the results of antifungal activity.

The information from the review are shown on Tables 3 and 4, according to the type of assay used, such as previously was described in section 3. In the tables is possible to see that the assay most commonly used for evaluate the antifungal activity of essential oils was the dilution method (Table 3).

Melaleuca alternifolia (Myrtaceae) and *Salvia lanigera* (Lamiaceae) were the species with the best results of antifungal activity in dilution assay with MIC values of 0.23 μg/mg and 0.63 L/mL, respectively [2, 91]. While for the diffusion test, the best results were for essential oils of *Piper betle* (Piperaceae) and *Lippia berlandieri* (Verbenaceae) with MIC values of 0.20 μL/mL and 0.50 μL/mL respectively [112, 114].

Specie	Plant part	Isolation Method	*Fungi*	Variable	Result	Reference
Annonaceae						
Artabotrys odoratissimus	Leaves	Hydrodistillation	*F. oxysporum*	Percentaje of growth inhibition	100% (500 µL/L)	72
Monodora myristica	Seeds		*F. moniliforme*		66% (500 µg/mL)	73
Apiaceae						
Carum carvi L.	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	92% (1000 µg/mL)	74
Citrus carvi L	No report		*F. verticillioides*		88% (1 µg/mL)	75
			F. oxysporum		94% (1 µg/mL)	
Cuminum cyminum	Comercial		*F. solani*	MFC	72 µg/mL	6
			F. oxysporum		202 µg/mL	
			F. verticillioides		96 µg/mL	
			F. paoe		75 µg/mL	
			F. equiseti		99 µg/mL	
Foeniculum vulgare L.			*F. solani*		77 µg/mL	
			F. oxysporum		151 µg/mL	
			F. verticillioides		73 µg/mL	
			F. paoe		69 µg/mL	
			F. equiseti		63 µg/mL	
	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	83% (1000µg/mL)	74
Heraclum persicum	Comercial		*F. solani*	MFC	70 µg/mL	6
			F. verticillioides		952 µg/mL	
			F. equiseti		795 µg/mL	
Pimpinella anisum L.	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	100% (500µg/mL)	74
Pituranthos tortuosis	Aerial part	Hydrodistillation	*F. graminearum*	MIC	3,6 µg/mL	76
Asteraceae						
Achillea fragrantissima	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	89% (1000µg/mL)	74
Achillea millefalium			*F. moniliforme*		67% (1000µg/mL)	
Bidens pilosa Linn. var. Radiata	Leaves		*F. solani*		78% (400 µg/disco)	77
			F. oxysporum		89% (400 µg/disco)	
	Flowers		*F. solani*		98% (400 µg/disco)	
			F. oxysporum		95% (400 µg/disco)	
Calendula ofricinalis L.			*F. moniliforme*		76% (1000µg/mL)	74
Matricaria chamomilla L.	Whole plant		*F. moniliforme*		76% (1000µg/mL)	
Caryphyllaceae						
Silene armeria L	Flowers	Hydrodistillation	*F. oxysporum*	MIC	500 µg/mL	7
			F. solani		125 µg/mL	
Cupresaceae						
Juniperus excelsa	Berries	Hydrodistillation	*F. tricinctum*	MIC	10 µg/mL	78
Metasequoia glyptostroboides Miki ex Hu	Floral cones		*F. solani*		500 µL/mL	79
			F. oxysporum		500 µl/mL	
Chenopodiaceae						
Chenopodium ambrosioides	Leaves	Hydrodistillation	*F. oxysporum*	Percentaje of growth inhibition	100% (100 µg/mL)	80

Specie	Plant part	Isolation Method	*Fungi*	Variable	Result	Reference
Geraniaceae						
Pelargonum roseum L.	No report		*F. oxysporum*	Percentaje of growth inhibition	86% (1 µg/mL)	75
			F. verticillioides		74% (1 µg/mL)	
Lamiaceae						
Hyptis suavelons	Leaves	Hydrodistillation	F. oxysporum sp. gladioli	Percentaje of growth inhibition	100% (0,6 µL/mL)	81
Lavandula angustifolia Mill	Leaves	Hydrodistillation	*F. solani var. Coeruleum*	EC50	520 µg/mL	82
	No report		*F. verticillioides*	Percentaje of growth inhibition	69% (1 µg/mL)	75
Mentha arvensis	Aerial part	Hydrodistillation	*F. oxysporum*		100% (3.12 µg/mL)	83
Mentha longifolia ssp. longifolia	Leaves		*F. acuminatum*	MIC	63 µg/mL	84
			F.oxysporum		63 µg/mL	
			F. tabacinum		31 µg/mL	
Mentha pulegium			*F. solani var. Coeruleum*	EC50	400 µg/mL	82
Mentha piperita	Commercial		*F. oxysporum* sp. gladioli	Percentaje of growth inhibition	83% (300 µg/mL)	70
Mentha viridis	No report	Steam distillation	*F. moniliforme*		77% (1000 µg/mL)	74
Nepeta cataria L	No report		*F. oxysporum*		98% (1 µg/mL)	75
			F. verticillioides		92% (1 µg/mL)	
Ocimum basilicum L	No report	Steam distillation	*F. moniliforme*		67% (1000 µg/mL)	74
	No report		*F. oxysporum*		75% (1 µg/mL)	75
			F. verticillioides		78% (1 µg/mL)	
Ocimum basilicum Yatta	Leaves	Hydrodistillation	*F. verticillioides*		100% (5 µL/mL)	85
	Flowers		*F. verticillioides*		100% (5 µL/mL)	
Ocimum basilicum Sagana	Leaves		*F. verticillioides*		100% (5 µL/mL)	
	Flowers		*F. verticillioides*		100% (5 µL/mL)	
Origanum glandulosum	Aerial part		*F. oxysporum*	MIC	56 µg/mL	86
		SFME	*F. oxysporum*		57 µg/mL	
Ocimum gratissimum Sagana	Leaves	Hydrodistillation	*F. verticillioides*	Percentaje of growth inhibition	75% (5 µL/mL)	80
Ocimum gratissimum Yatta.	Flowers		*F. verticillioides*		75% (5 µL/mL)	
	Leaves		*F. verticillioides*		75% (5 µL/mL)	
Ocimum gratissimum	Leaves		*F. moniliforme*		100% (500 µL/mL)	87
Origanum dictamnus	Leaves		*F. solani var. Coeruleum*	EC50	76 µg/mL	82
Origanum majorana	Leaves		*F. solani var. Coeruleum*		120 µg/mL	
Origanum vulgare	Leaves		*F. solani var. Coeruleum*		50 µg/mL	
Origanum vulgare ssp. vulgare	Aerial parts		*F. acuminatum*	MIC	63 µg/mL	88
			F. oxysporum		63µg/mL	
			F. tabacinum		31 µg/mL	
Orthosiphon stamineus Benth	Leaves		*F. solani*		500 µg/mL	89
	Stems				500 µg/mL	
Plectranthus cylindraceus	Aerial part	Steam distillation	*F. oxysporum*	Percentaje of growth	100% (125 µg/mL)	90

Specie	Plant part	Isolation Method	*Fungi*	Variable	Result	Reference
				inhibition		
Rosmarinus officinalis	Leaves	Hydrodistillation	*F. solani var. Coeruleum*	EC50	668 μg/mL	82
Salvia lanigera	Aerial part		*F.oxysporum*	MIC	100 μL/mL	91
Salvia officinalis			*F. moniliforme*		0.63 μL/mL	92
Salvia sclarea L	No report		*F. verticillioides*	Percentaje of growth inhibition	65% (1 μg/mL)	75
Teucrium marum subsp. marum	Aerial part	Steam distillation	*F. oxysporum Sch*	MIC	450 μg/mL	93
Thymus capitatus	Leaves	Hydrodistillation	*F. solani var. Coeruleum*	EC50	71 μg/mL	82
Thymus spathulifolius	Whole plant		*F. acuminatum*	MIC	125 μg/mL	94
			F. tabacinum		125 μg/mL	
			F. oxysporum		63 μg/mL	
Thymus vulgaris	Whole plant		*F. moniliforme*	Percentaje of growth inhibition	100% (600 μg/mL)	73
	Commercial		*F. sp*		100% (200 μg/mL)	70, 95
			F. oxysporum sp. gladioli		100% (100 μg/mL)	
Thymus vulgaris L	No report		*F. oxysporum*		98% (1 μg/mL)	75
			F. verticillioides		98% (1 μg/mL)	
	Whole plant	Steam distillation	*F. moniliforme*		100% (250 μg/mL)	84
	No report	Hydrodistillation	*F. oxysporum*		100 % (0.7 μL/mL)	96
Zataria multiflora	Herb	Commercial	*F. solani*	MFC	153 μg/mL	6
			F. oxysporum		184 μg/mL	
			F. verticillioides		99 μg/mL	
			F. paoe		145 μg/mL	
			F. equiseti		99 μg/mL	
Lauraceae						
Cinnamomum zeylanicum	Commercial		*F. sp*	Percentaje of growth inhibition	68% (200 μg/mL)	95
			F. oxysporum sp. gladioli		100% (100 μg/mL)	
	Bark	Hydrodistillation	*F. solani*	MIC	150 μg/mL	97
	Leaves	Commercial	*F. proliferatum*		50 μgLmL	98
	Bark		*F. proliferatum*		50 μL/mL	
Cinnamomum zeylanicum L.	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	100% (1000 μg/mL)	74
Laurus novocanariensis	Leaves	Hydrodistillation	*F. oxysporum*	EC50	280 μg/ml	99
			F. moniliforme		440 μg/ml	
			F. solani		410 μg/ml	
Myrtaceae						
Leptospermum scoparium	Leaves	No report	*F. circinatum*	Percentaje of growth inhibition	62% (26 μg/ml de air)	100
Melaleuca alternifolia	Leaves and flowers	Comercial	*F. culmorum*	MIC	0.23 μg/mg	2
			F. graminearum		0.12 μg/mg	
Pimenta dioica L. Merr	No report		*F. oxysporum*	Percentaje of growth inhibition	100% (1μg/mL)	75
			F. verticillioides		100% (1 μg/mL)	

Specie	Plant part	Isolation Method	*Fungi*	Variable	Result	Reference
Syzygium aromaticum	Clove	Comercial	*F. proliferatum*	MIC	50 µL/mL	98
Poaceae						
Cymbopogon citratus	Leaves	Hydrodistillation	*F. moniliforme*	Percentaje of growth inhibition	100% (800 µg/mL)	73
Cymbopogon flexuosos	Aerial parts		*F. oxysporum*		100% (3.12 µg/mL)	83
	Leaves			MIC	1,1 µL/mL	101
Cymbopogon nardus L.	No report			Percentaje of growth inhibition	86% (1 µg/mL)	75
			F. verticillioides		76% (1 µg/mL)	
Rosaceae						
Agrimonia eupatoria	Whole plant	Steam distillation	*F. moniliforme*	Percentaje of growth inhibition	75% (1000 µg/mL)	74
Rutaceae						
Citrus reticulata Blanco	Peel of ripe fruits	Hydrodistillation	*F. oxysporum*	MIC	200 µL/mL	102
Solanaceae						
Cestrum nocturmum L.	Flowers	Hydrodistillation	*F. solani*	MIC	250 µg/mL	103
Umbeliferaceae						
Trachyspermum ammi Lin	Seeds	Hydrodistillation	*F. oxysporum f.sp. lycopersici,*	MIC	240 µg/mL	104
			F. oxysporum f.sp. cubense		240 µg/mL	
			F. oxysporum f.sp. capsici		240 µg/mL	
Verbeneaceae						
Vitex agnus-castus	Unripe fruits	Hydrodistillation	*F. tricinctum*	MIC	178µg/mL	8
	Ripe fruits				89 µg/mL	
	Leaves				178µg/mL	
Zingiberaceae						
Zingiber cassumunar Roxb	No report		*F. verticillioides*	Percentaje of growth inhibition	67% (1 µg/mL)	75
Zingiber officinale	Rhizomes	Hydrodistillation	*F. moniliforme*		100% (1000 µg/mL)	73

Table 3. Essential oils with antifungal activity against *Fusarium* species evaluated by the diluttion assays.

Specie	Plant part	Isolation Method	*Fungi*	*Variable*	*Result*	Reference
Asclepidaceae						
Periploca laevigata	Root	Hydrodistillation	*F. oxysporum*	Diameter zone inhibition	48 mm (50 µg/mL)	105
			F. solani		55 mm (50 µg/mL)	
Asteraceae						
Achillea gypsicola	Whole plant	Hydrodistillation	*F. aveneceum*	Percentaje of growth inhibition	60% (1000 µg/mL)	106
			F. culmorum		82% (1000 µg/mL)	
			F. oxysporum		69% (1000 µg/mL)	
			F. sambucinum		79% (1000 µg/mL)	
			F. solani		66% (1000 µg/mL)	
Haplopappus baylahuen	Leaves		*F. oxysporum*		76% (100 µg/mL)	107
Tanacetum aucheranum	Aerial parts		*F. acuminatum*		63% (1.5 µL/mL)	108

			F. chamydosporum		67% (1.5 μL/mL)	
			F. culmorum		67% (1.5 μL/mL)	
			F. equiseti		66% (1.5 μL/mL)	
			F. graminearum		73% (1.5 μL/mL)	
			F. nivale		83% (1.5 μL/mL)	
			F. proliferatum		64% (1.5 μL/mL)	
			F. sambucinum		78% (1.5 μL/mL)	
			F. semitectum		84%(1.5 μL/mL)	
Tanacetum chiliophyllum var. Chiliophyllum			*F. acuminatum*		78% (1.5 μL/mL)	
			F. chamydosporum		75% (1.5 μL/mL)	
			F. culmorum		78% (1.5 μL/mL)	
			F. equiseti		67% (1.5 μL/mL)	
			F. graminearum		81% (1.5 μL/mL)	
			F. nivale		83% (1.5 μL/mL)	
			F. oxysporum		71% (1.5 μL/mL)	
			F. proliferatum		75% (1.5 μL/mL)	
			F. sambucinum		84% (1.5 μL/mL)	
			F. scirpi		63% (1.5 μL/mL)	
			F. semitectum		86% (1.5 μL/mL)	
			F. tabacinum		68% (1.5 μL/mL)	
			F. verticillioides		68% (1.5 μL/mL)	
Lamiaceae						
Origanum acutidens Hans	Aerial parts	Hydrodistillation	*F.culmorum*		81% (25 mg/disc)	1
			F. equiseti		70% (25 mg/disc)	
			F. nivale		86% (25 mg/disc)	
			F. oxysporum		82% (25 mg/disc)	
			F. sambucinum		89% (25 mg/disc)	
			F. semitectum		87% (25 mg/disc)	
			F. solani		79% (25 mg/disc)	
Origanum vulgare	Inflorescenses		*F. avenaceum*	Diameter zone inhibition	20 mm (500 μg/mL)	109
	Leaves				22 mm (500 μg/mL)	
Origanum onites	Leaves and flowers		*F. semitectum*	MFC	1.8 μg/mL	110
			F. oxysporum		7.0 μg/mL	
Thymus algeriensis Boiss. Et Reut.	Aerial parts		*F. solani*	MIC	1 μL/mL	111
Piperaceae						
Piper betle L. var magahi	Leaves	Hydrodistillation	*F. oxysporum*	MIC	0.50 μL/mL	112
Rutaceae						
Zanthoxylum monophyllum	Fruits	Steam distillation	*F. oxysporum*	EC50	0.140 μL/mL air	113
Zanthoxylum fagara					0.183 μL/mL air	
Zanthoxylum rhoifolium					0.245 μL/mL air	
Verbenaceae						
Lippia berlandieri Shauer	Leaves and flowers	Steam distillation	*F. oxysporum lycopersici*	Percentaje of growth inhibition	100% (0.20 μL/mL)	114

Table 4. Essential oils with antifungal activity against *Fusarium* species evaluated by the diffusion assays.

For the Myrtaceae family, the best results were obtained for *M. alternifolia* oil, with values lower than 1% w/w, which were attributed to the presence of terpinen-4ol [2]. Another species from the same family having a high activity is *Syzygium aromaticum*; which at 100 mg/L of oil inhibits 100% of growth of *F. oxysporum* sp. *gladioli* and at 50 μL/mL of oil inhibits totally the growth of *F. proliferatum* [70, 98]. Other oil that showed the best results in the dilution test was the obtained from *Salvia officinalis*, which presented a MIC and MLC of 0.63 μg/mL, which indicates a fungicide effect [92]. In this same genus, the oil of *S. lanigera* presented a moderated effectiveness at 100 mg/L. In this two studies, activity is attributed to the presence of phenolic compounds (Thymol and carvacrol) [91].

Piper betle (Piperaceae) is another species with very good results, with a value MIC of 0.50 mg/L, where once more, the activity is attributed to phenolic compounds. Due to OH presence, it is able to form hydrogen bonds with the active spot enzymes and increases the activity via enzyme denaturalization [112].

The species *Citrus carvi*, *Foeniculum vulgare*, *Pimpinella anisum* and *Piruranthos tortuous* of Apiaceae family, presented a strong inhibitory power against the growth of Fusarium species. The essential oil *P. tortuous* is one of the oils showing the lowest MIC with a value of 3,6 mg/L. In regard to these results, the authors of this paper mentioned that the activity is related with the high monoterpenoids contents in this oil. These substances have the capacity of altering the morphology of hyphae and aggregates, reducing the diameter each time they interact with cell membranes of pathogen agents [76].

Rutaceae family also presented good results in the diffusion test, being *Zanthoxylum* genus the most representative. The species *Z. monophyllum, Z. fagara* and *Z. rhoifolium* were evaluated against F. oxysporum, and it was found that essential oils from the fruits of these species show similar or higher results compared to the positive controls used. *Z. monophyllum* was the most active, followed by *Z. fagara* and *Z. rhoifolium*. These results are attributable to the presence of some compounds that are in these fruits essential oils in low concentrations such as (E)-caryophyllene, T-muurolol, and α-cadinol, compounds that have been previously reported as antifungal substances by other researchers [113].

As is observed in Tables 3 and 4, Asteraceae and Lamiaceae families have the highest number of reports of antifungal activity against Fusarium species. For the Asteraceae family, two genera (*Achillea* and *Tanacetum*) showed moderate results, against to a large number of *Fusariu*m species. Both studies showed a correlation of results with a high monoterpenoids content such as: camphor, 1,8-cineole, piperitone, borneol and α-terpineol [106, 108].

In the Lamiaceae family there were the highest inhibition percentages with small amounts of oils from *Hyptis suaveolens L, Lavandula angustifolia Mill, Mentha arvensis, Nepeta cataria L, Ocimum basilicum, Ocimum gratissimum, Origanum majorana L, Salvia scalrea* and *Thymus vulgaris*. An example of the use of essential oils from species of this family as an alternative to control *F. oxysporum*, is the integration of the oil from *H. suavelons* species in hot water and ultraviolet radiation, as a treatment which gives excellent results with a high reduction of pathogen population in artificially inoculated in gladiolus corms [81]. From the same

family, the potential of *M. arvensis* as antifungal, is observed taking into account that when using a concentration of 3.12 mg/L of this oil, there would be a 100% inhibition in *F. oxysporum* growth, this is an effect attributed to its high menthol contents (71.50%) [83]. In this family, the *Ocimum* genus is one of the most studied, and an example of this is the report by Dambolena, where all oils assessed came from leaves and flowers, had inhibitory effects upon the growth of *F. verticillioides*. Authors report that inhibition degree depends widely upon composition and concentration of components of each of those oils. Oils with a high eugenol contents showed the best results. These results are consistent to other prior works that have shown that *O. basilicum* oil has antifungal and antibacterial activities due to its oxygenated monoterpenoids high contents, following the rules that antifungal activity is related with content to phenols > alcohols> aldehydes> ketones> esters> hydrocarbons contents [85].

The review confirmed the importance of establish the criteria for obtain the essential oils and how are affect the results of th antifungal activity when the oils are obtained from different forms and from plants cultivated in different conditions. Also it is important establish the criteria for evaluate the antifungal activity. The most important factors are: trial type, time of vegetal matter collection, parts of the plant used and maturity status, and extraction methods to obtained essential oils [5], [8], [87]. An example of this is found in the study of *Ocimum onites*, where it was established that there are differences in antifungal activities of the oils, when are used fresh or dried fruits, being the most active of fresh fruits oil [110]. Additionaly this species is the one showing the lowest MFC values with 1.8 y 6.0 μg/mL facing *F. semitectum* and *F. oxysporum* species respectively.

4.3. Pure compounds

Several reviews have discussed the activity of isolated natural products against a wide range of fungi including human pathogenic species such as *Candida albicans* or *Aspergillus* species, or plant pathogens such as *Colletotrichum* and *Cladosporium* species [115, 116, 117]. However it is difficult to find a comprehensive review of natural products specifically active against *Fusarium* plant pathogens. Here we describe some of these natural agents that according to their activity against *Fusarium* have the potential to be used either directly as pure constituents, or they can serve as starting point for generating more potent and selective antifungals.

4.3.1. Alkaloids

Plant alkaloids are biologically active entities that often confer chemical protection against pathogen infections or herbivory [118]. Different types of nitrogen-containing substances have been found to display antimicrobial [119, 120], antifungal, antiparasitary [121] and antiviral [122] effects. Chinese traditional phytomedicine is a vast source of bioactive scaffolds, and berberine **6** is one interesting representative, originally isolated from *Coptis chinensis* rhizome [123]. It is a yellow, bitter, and a very active agent against bacteria, fungi, protozoa, trypanosome and mammalian cells from higher organisms. The biological action is thought to be pleiotropic, inhibiting mainly protein synthesis but also intercalating into

DNA strands [124]. Berberine has an spore germination half inhibitory concentration ($SPIC_{50}$) of 599 mg/L against *F. oxysporum,* being also active against other fungi such as *Botrytis cinerea*, *Alternaria solani* and *Monilinia fructicola* [125]. Dehydrocorydalmine and oxyberberine, two berberine structurally-related alkaloids were found to be less active against *Fusarium* species with $SPIC_{50}$ close to 1000 mg/L. Nonetheless two other interesting alkaloids sharing some similarity to berberine, are corydalmine **7** and isocorypalmine **8** isolated from *Corydalis chaerophylla* which displayed an $SPIC_{50}$ close to 300 and 800 mg/L respectively against *F. nudum* [126]. Certainly isoquinolines are attractive chemotypes with antifungal activity and studies of their mechanism of action and structure-activity relationships can boost the development novel *Fusarium* inhibitors.

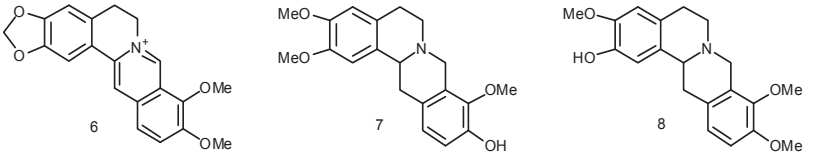


Figure 2.

Another type of interesting alkaloid which has attracted the attention of researchers because of their pronounced biological activity specifically against lower organisms is the class of pyrrolizidines [127]. Against a species of *Fusarium*, a notable antifungal activity was observed for floridinine **9** obtained from *Heliotropium floridum*, showing an inhibitory concentration of 500 mg/L against *F. oxysporum* [128]. Europine **10** isolated from *H. bovei* showed an $SPIC_{50}$ of 740 mg/L against *F. moniliforme*, while 7-acetyleuropine was inactive [129]. It is interesting to see that a related alkaloid, lycopsamine isolated from *H. megalanthum* was inactive against *Fusarium* while being active against other phytopathogenic fungi [130]. From the plant *Senecio jacobaea*, retrorsine and retrorsine-*N*-oxide were isolated and showed to reduce the growth of *F. oxysporum* but were not able to arrest their development [131]. The pyrrolizidine alkaloids are thought to be involved in crucial ecological relations, for example, these alkaloids can be sequestered by some butterflies offering them with chemical protection against depredators [132]. It can also be hypothesized that the insects also utilize them for preventing microbial infection.

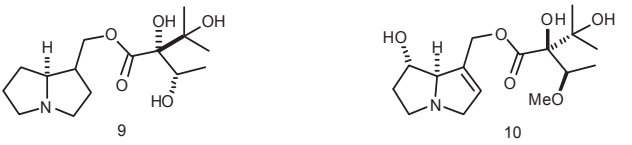
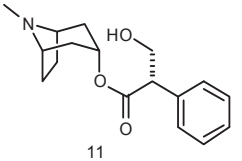


Figure 3.

Tropane alkaloids are interesting phytochemicals with a wide array of biological activity typically present in species of the Solanaceae family. Hyoscyamine **11** isolated from traditionally medicinal plant *Hyoscyamus muticus* was show to inhibit several species of pathogenic fungi including *F. dimerum*, *F. nivale* and *F. oxysporum* in an autobiography assay

[133]. *Ent*-norsecurinine **12** isolated from *Phyllantus amarus* is a bioactive scaffold for antifungal drug development, as it is very active against a wide range of phytopathogenic fungi [134]. Specifically, against *F. nudum* infecting *Cajanus cajan*, the alkaloid displayed an $SPIC_{50}$ of around 250 mg/L. The *allo*- form of securinine was found to be less active with an $SPIC_{50}$ close to 450 mg/L [135, 136].



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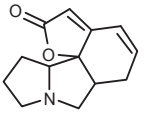

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Figure 4.

Steroidal alkaloids have also been found to display activity against *Fusarium* species. *Solanum* glycoalkaloids solasonine **13** and solamargine were able to slow down the growth of three different species of *Fusarium* [137]. Tomatidine, the aglycone of α-tomatine **14**, was found to be less active against *F. subglutinans*, than the parent glycoside [138]. Tomatidine inhibited almost 90% of the growth of the phytopathogenic fungi at a concentration of 0.30 mM. However the strain *F. oxysporun f. sp. lycopersicum* was found to produce an extracellular hydrolase named tomatinase which is able to hydrolyze α-tomatine rendering it inactive [139].

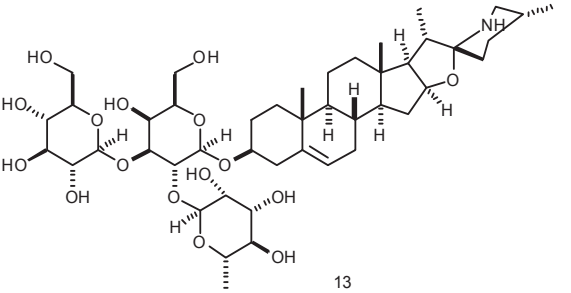

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Figure 5.

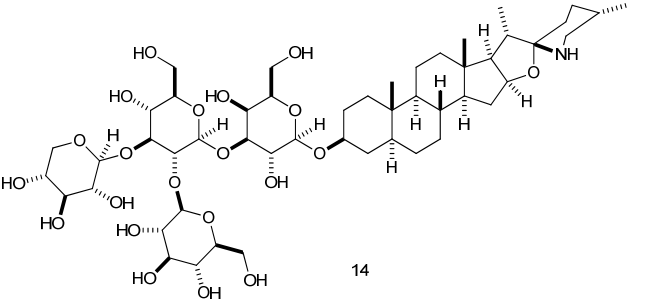

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Figure 6.

Another type of steroidal alkaloids which have also been found to be active against *Fusarium* is the ceveratrum alkaloids. The seeds of *Schoenocaulon officinale*, commonly known as sabadilla powder have been used traditionally as insecticide in South America [140]. This plant possesses cevadine **15** and veratridine, which are active against *F. graminearum* [141] however being less effective against *F. oxysporun.* Venenatine **16** is an indole alkaloid isolated from the bark of *Alstonia venenata* which displayed antifungal activity against a wide array of phytopathogenic fungi [142]. Against the strain *F. udun* a $SPIC_{50}$ close to 400 mg/L was found. This alkaloid was particularly active against the fungi *Ustilago cynodontis*. In another study a closely related alkaloid, Δ^3-alstovenine was isolated and shown to be inactive against *F. udun* even at 1000 mg/L while sustaining a marked inhibition against *Helminthosporium maydis* and *Erysiphe pisi* [143]. Fistulosin **17**, an oxyndole alkaloid, was isolated from roots of Welsh onion (*Allium fistulosum* L.). This compound exhibited antifungal activities against diferent phytopathogenic filamentous fungi, especially varieties of *Fusarium oxysporum* at 1.62 ± 6.5 g/L of MICs. Fistulosin inhibited protein synthesis and had a slight inhibitory efect on DNA synthesis, but no inhibitory effect on RNA synthesis [144].

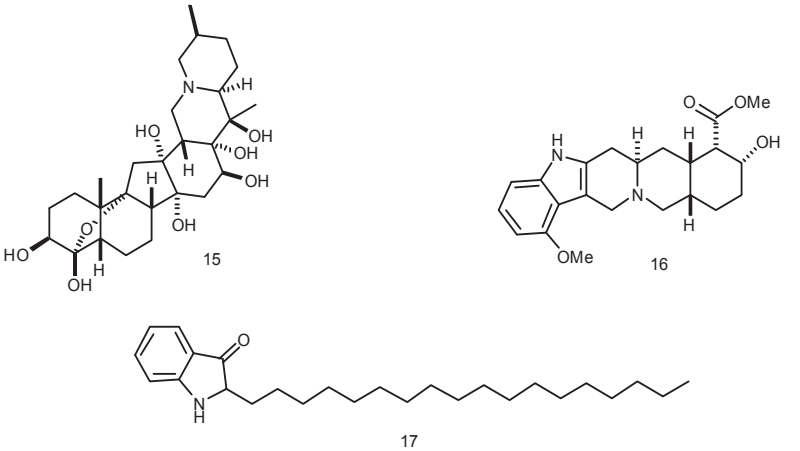

Figure 7.

The antifungal activity of four aporphine alkaloids isolated from *Ocotea macrophylla* was evaluated using the disk diffusion method against *F. oxysporum* f. sp. *lycopersici*. The inhibitory activity against the growth of the fungi was moderate at 5 g/L for (*S*)-3-methoxynordomesticine **18**, while the other alkaloids were inactive, suggesting that the presence of electron withdrawing substituents on the nitrogen atom decrease the antifungal activity [145]. The antifungal activity against the same phytopathogenic strain was evaluated by direct bioautography in a TLC bioassay [42,146] for the compounds isolated from *Z. monophyllum* and *Z. quinduense*. The minimum growth inhibitory amount was determined for each compound taking into consideration a growth inhibition with less than 100 μg. Among the evaluated compounds three benzophenanthridine alkaloids (norchelerytrine **19**, 6-

acetonyl-dihydrochelerythrine **20** and chelerythrine **21**), a berberine alkaloid (jathrorrhizine **22**) and a quinolone alkaloid (thalifoline **23**) were found to display inhibitory properties [147-149].

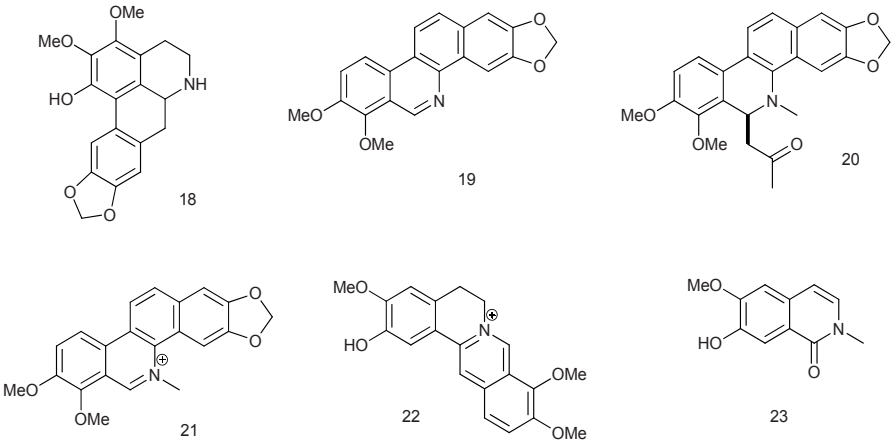

Figure 8.

4.3.2. Phenolic compounds

A comprehensive study from 1998, evaluated several flavones on the growth inhibition of five different species of *Fusarium*: *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium poae*, *Fusarium avenaceum* and *Fusarium nivale* [150]. Unsubstituted flavone showed the highest growth inhibition among all flavones [151], preventing 70% of the growth in comparison with the control against all the *Fusarium* strains tested except *F. culmorum*. Interestingly the combinations of oxygenated substituted flavones with unsubstituted flavone or flavanone were more active than the pure constituents [150]. This synergistic effect was not observed for others flavonoids, indicating the specific substitution on the flavonoid skeleton was responsible for this effect.

Oxygenated flavonoids isolated from the stems and leaves of *Ficus sarmentosa* also showed inhibition of the mycelium growth *of F. graminearum* [152]. Luteoline **24** was able to inhibit half the growth of the fungi at a concentration of 56 mg/L. The reduction of luteoline to a flavanone, the substitution on C-3 or the methoxylation of phenolic in C-3′ produces as result a significant loss in activity. Other flavonoids and phenols have also shown to inhibit the growth of *F. culmorum*, notably taxifolin **25** [153].

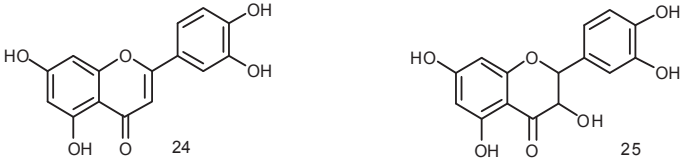

Figure 9.

Glycosylated flavonoids trifoline **26** and hyperoside **27** have been isolated from the medicinally important species *Camptotheca acuminata*, source of useful metabolites for cancer chemotherapy such as camptothecin [154]. These two galactosides inhibited half of the growth of *Fusarium avenaceum* at a concentration of 75 mg/L with a complete inhibition at 125 mg/L for trifolin and more than 150 mg/L for hyperoside. They showed also inhibition against other fungal pathogens such as *Pestalotia guepinii* and *Drechslera* sp. In general it is well accepted that flavonoids alter the growth of *Fusarium*, some flavonoids can induce sporulation in *F. solani* [155] and they are therefore thought to be involved in host-pathogen regulatory relations. There is also specificity in the activity of flavonoids, some fungi being more sensitive to certain flavonoids than others, and also synergistic effects have been observed [156].

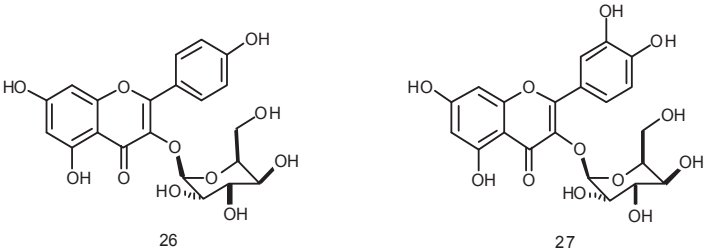

Figure 10.

Non-flavonoid phenols have also attracted the attention of researchers, as other biosynthetic pathways have evolved extremely potent phenols either from shikimate or acetate precursors or by mixed routes. In this class we can find simple phenols, mono and poly-prenylated phenols, lignans, coumarins, stilbenes and others. For example rotenone **28** and related metabolites known as rotenoids, are well established insecticides and piscicides [157]. Specifically rotenone had the highest anti-fungal activity from the rotenoids isolated from *Pachyrhizus erosus* against *F. oxysporum,* significantly inhibiting its growth at 250 mg/L [158]. Some lignans have also demonstrated activity against *Fusarium* pathogens. (-)-Taxiresinol **29** obtained from the medicinally important *Taxus baccata*, source of the anticancer agent paclitaxel, displayed 60% inhibition of the growth of *F. solani* at 200 mg/L [159]. Aryltetraline lignans isolated from the same plant were found to be less active as antifungals however they displayed higher cytotoxicity towards cancer cell lines.

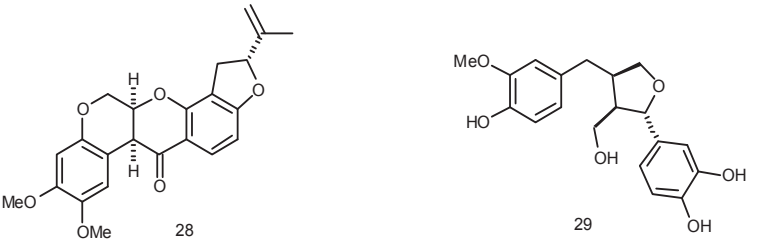

Figure 11.

Three napthoquinones isolated from *Moneses uniflora* showed interesting antifungal activity against *F. tricuictum.* Chimaphylin **30**, 8-chlorochimaphylin **31** and 3-hydroxychimaphylin **32** showed complete inhibition of the fungi at a concentration between 12.5 and 25 mg/L, which is remarkable for such relatively simple chemical structures [160].

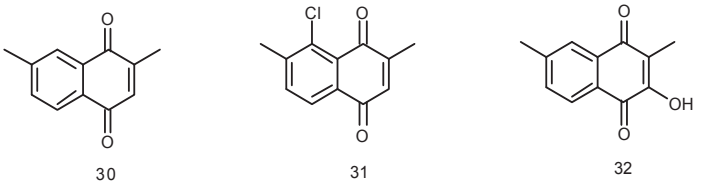

Figure 12.

Surangin B **33** is a coumarin isolated from *Mammea lonfigolia* that showed significant antifungal activity against many different pathogenic species with very low inhibitory concentrations [161]. Surangin B reduced to half the level of spore germination at 2.3 μM. It was found that the mechanism of action of this interesting coumarin was through inhibition of electron transport chain in the mitochondrion. Another coumarin with antifungal activity is osthol **34**, isolated from species of the genera *Angelica* and *Cnidium. F. graminearum* is sensitive to this coumarin at a concentration from 25 to 100 mg/L, with an IC_{50} around 57 mg/L [162]. Osthol was shown to reduce considerably intracellular glucose levels which were detrimental to fungal development. In a research carried out in Colombia, the activity of phenolic derivatives obtained from different plant species has been reported against *F. oxysporum* f. sp. *dianthi*. Direct bioautography in a TLC bioassay showed that the minimum amount required for the inhibition of fungal growth was 1 μg for cumanensic acid **35** obtained from *Piper cf. cumanense* [163], 5 μg for evofolin-C **36** isolated from *Z. quinduense* [147], and 2 μg for uvangoletin **37** and chrysin **38** obtained from *P. septuplinervium* [164].

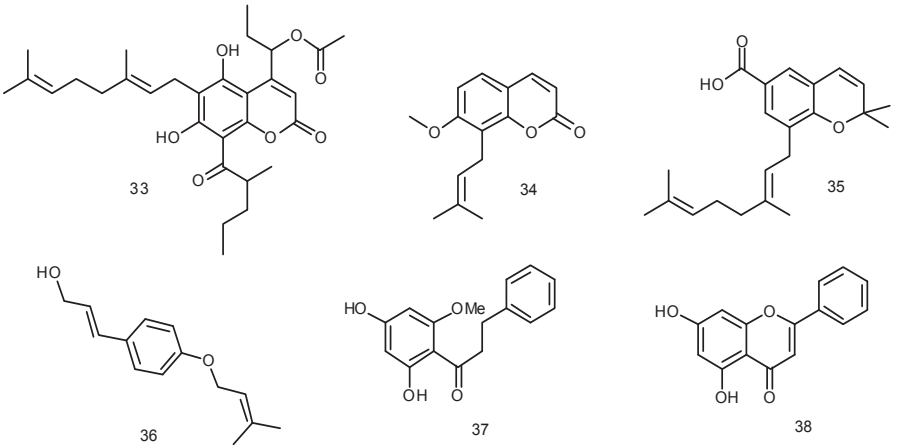

Figure 13.

4.3.3. Terpenes

The antifungal activity of essential oils is well documented [165], however less is known about the effects of isolated compounds from the oils. A study from 2005 reported the inhibitory activity of several isolated essential oil components using six different phytopathogenic fungi, including *F. oxysporum*. Specifically against this pathogen, chlorothymol **39**, thymol **40**, carvacrol **41** and carveol **42** inhibited half the growth of the fungi with an IC_{50} less than 30 mg/L [166]. Other monoterpenoids such as geraniol, citronellol, eugenol, and vanillin were of moderate potency (around 100-200 mg/L for IC_{50}), while benzyl alcohol, camphor, carvone, menthol, cinammaldehyde, borneol, cineol were found to be less active.

On another study of antifungal potential of compounds usually encountered in essential oils, aromatic aldehydes such as benzaldehyde **43** and salicylaldehyde **44** showed a remarkable inhibition of *F. sambucinum* [167]. Their minimum inhibitory concentration (MIC) was found to be 40 mg/L and 4 mg/L respectively for several strains of this pathogen when tested in a vapour phase assay. Interestingly cinammaldehyde was confirmed to be less active against *Fusarium* species, with an MIC value superior to 400 mg/L. Moreover when the compounds were tested in dissolution in the media, cinammaldehyde **45** and thymol were able to inhibit completely the growth of the pathogenic fungi at 0.1 and 1% concentrations [167].

In a recent study of antifungal metabolites isolated from ethnomedicinal *Wardburgia ugandensis*, several sesquiterpenoids were found to inhibit the growth of different species of *Fusarium* [168]. Polygodial **46**, warburganal **47** and mukaadial **48** were potent inhibitors of the growth of pathogenic fungi showing MIC values below 25 mg/L against *F. solani* and 100 mg/L against *F. oxysporum*. Interestingly lactonic related natural products were much less active, suggesting that free aldehyde groups are essential for the antifungal effect, and this observation is supported by the fact that aromatic aldehydes are also highly active antifungals.

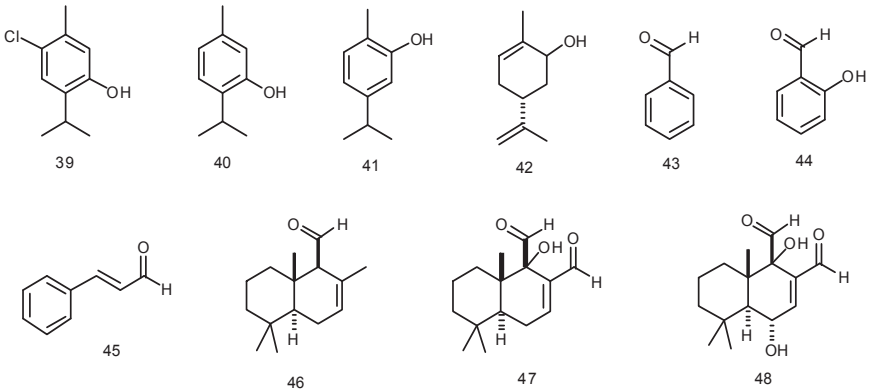

Figure 14.

Clerodane diterpenoids isolated from seeds of *Polyalthia longifolia* have shown moderate antifungal activity towards *Fusarium sp* [169]. 16α-hydroxy-cleroda-3,13(14)-*Z*-diene-15,16-olide **49** and 16-oxo-cleroda-3,13(14)-*E*-diene-15-oic acid **50**. These diterpenoids were slightly more active against bacteria than against fungi, displaying MIC values of 100 mg/L and 50 mg/L respectively against *Fusarium sp*.

Costunolide and parthenolide are active sesquiterpene lactones typically found in *Magnolia* species. Parthenolide **51** was found to inhibit the growth of *F. culmorium* at 50 mg/L, while not inhibiting *F. oxysporum* [170]. In contrast costunolide was found inactive to both *Fusarium* species even at the highest concentration tested of 1000 mg/L. 1,10-epoxyparthenolide **52** was found to be slightly less active than parthenolide with MIC value of 230 mg/L against *F. culmorium*, being also inactive against *F. oxysporum*.

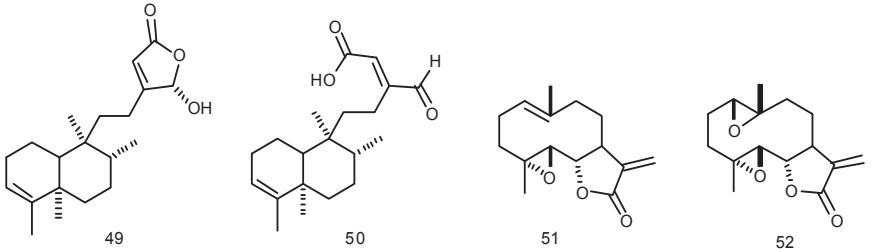

Figure 15.

5. Conclusions

The control of diseases caused by microorganisms such as bacteria and fungi in commercially important crops continues to be a large problem worlwide. Although different methods of control exists (chemical, physical, biological and combinations of these), these are still insufficient or have been found ineffective. Reports related to resistance mechanisms developed by the plagues are increasing; in many cases, the emergence of resistant strains is associated with a badly use of control products (under- or overdose). Especially, synthetic chemical control methods are associated with higher pollution and residues in crops, and in many cases poor selectivity of the pesticides causes alterations of biological balance of ecosystems. These observations have justified the growing research in the field of natural products in order to find effective and safe control methods for plagues that affect different products of economic importance.

To evaluate the antifungal power of a substance different methods have been developed. For these methods for antifungal activity evaluation, it should indicate that they are complementary methods, because some provide qualitative information and others shows quantitative data. The criterion for the selection of a test depends mainly on the characteristics of the sample to prove, principally purity and solubility. The bioautography method is inexpensive, very useful for screening large numbers of samples (especially crude

extracts). Although results are not entirely quantitative, it can give information about how many and which substances in a mixture showed antifungal activity. Dilution methods are employed for quantitative analysis and require little amount of sample, therefore, are suitable for evaluating compounds which do not present problems of solubility.

As a result of the intense research activity, a large number of species with potential for controlling phytopathogenic organisms have been identified. According to this review, approximately 150 plants species belonging to 30 plant families and about 50 compounds have promisory antifungal activity. These substances should be postulated as interesting agents for the control of *Fusarium* species.

The antifungal bioactivity was observed in crude extracts, fractions of varying polarity and essential oils. Studies have identified the individual compounds responsible for the activity. These results demonstrate that not a single type of extract, fraction or compound is responsible for antifungal activity, but sometimes they work in a synergistic fashion and therefore there is the possibility of using the whole extract instead of the pure compounds, which will be a cheaper and more feasible strategy in rural settings with low income.

The numerous reports of antifungal activity of natural products, contrast with the poor number of publications in relation to the mechanisms of antifungal activity. Therefore, the challenge is not only finding potential species to pest control, but determining the biochemical mechanisms that these products target. Understanding these mechanisms is important to developing products with higher selectivity. In the investigations in this field, it is necessary to involve computational methodologies and structure-activity relationships (SAR) that may lead to the identification of highly effective natural products, synthetic derivatives or structural analogues.

Although there have been found extracts, essential oils and pure compounds with antifungal activity comparable to that shown by current commercial products, these have not been converted into commercial products because there is still no large-scale production. To achieve production levels that are marketable missing including the following steps: a) studies of propagation and domestication of wild species that have shown activity, b) studies of agronomic crop species; c) study methods of harvesting and processing of plant material for obtaining the extracts; d) conducting formulation studies for extracts, oils and compounds.

The development of these activities will complement the work done so far as prospecting phase and lead to obtaining a standardized and reliable product that can be used by farmers for the partial or total replacement of the traditional synthetic products. In addition to not only provide a reliable product from the point of view of activity, it must be competitive in price, so search should focus on crude extracts or fractions, because with these substances is possible avoid separation process which could raise production costs.

We should take advantage of the fact that different types of substances and structures show antifungal activity, and if the mechanisms of action are varied, the commercial products based on these substances will be possible to avoid development of resistance by the

pathogen. Thus, the window of permanence of the products on the market will be higher. Although it is unrealistic to develop a unique product to control Fusarium, it is plausible to think that naturalproducts used in combination with other methods could have a dramatic impact achieving higher level of protection and therefore greater productivity with fewer environmental problems.

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Section 3

Fungicide-Fungus Interactions

Chapter 11

Detection of Fungicide Resistance

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Additional information is available at the end of the chapter

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1. Introduction

Fungicide resistance is a form of selection that describes a fungus's ability to survive and reproduce in the presence of a fungicide[1]. Practical resistance (field resistance) results when the prevalence of fungicide resistant isolates reaches a critical threshold where disease control is no longer observed. The primary factors that select for fungicide resistance in an organism are:

i. the biology of the pathogen,
ii. the mechanism(s) of action of the fungicide,
iii. the rate and frequency of fungicide application.

It is important to note that most fungi show a broad range of sensitivities to the spectrum of different fungicides. For example, most fungicides for the control of Chromista (e.g., mefenoxam, fluopicolide, dimethomorph) do not control true fungi; some fungicides are specific for ascomycete (e.g., thiophanate-methyl) or basidiomycete fungi (flutolanil), and species within the same genus of fungi may respond differently to certain fungicides (Leroux et al. 2012). Additionally, different genotypes exist within a spectrum of sensitive to resistant (Albertini et al. 1999). The relationship between the frequency of fungicide application and resistance has been established (King and Griffin, 1985; Suzuki et al. 2010), and the role of rate or dose in this process is deserving of further study (Genet et al. 2006; van den Bosche et al. 2011).

In 2006, the world market consumed approximately 520 million pounds of fungicide (Grube et al. 2011), a number that is expected to increase as the consumption of other pesticides declines (Troy, 2011). The discovery, development, and registration of a new pesticide comes at a cost (not including the capital costs or production). In 2006, this price was

[1] Due to issues of length, brevity and complexity, this review will only deal with fungicides that are for the control of true fungi, and not fungicides for the management of Chromista.

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estimated to exceed $180 million (Whitford et al. 2006). With fewer fungicides available (particularly with new modes of action) and increasing consumption, risk of fungicide resistance is even greater, and failures will more profoundly impact cropping systems. All of these factors increase the need for rapid detection of fungicide resistance. Historically, the detection of fungicide resistance in the field has been difficult because disease control failures can be caused by factors other than resistance, including improper fungicide selection, improper timing, reduction of recommended rate, and erroneous sprayer calibration (Latin, 2011).

Rarely, are molecular proofs of fungicide resistance ever fulfilled with plant pathogenic fungi, although many of the genes involved in fungicide resistance are studied in model systems to elucidate mechanisms (Zhang et al. 2002). Usually, resistant individuals are detected using bioassays (sensitivity tests or poison plate assays) and those resistant isolates may be correlated with molecular markers, many of which are based upon previous studies (Koernraadt et al. 1992; Albertini et al. 1999; Schnabel and Jones, 1991; Lesniak et al. 2011). In some instances, segregation analysis between crosses of sensitive and resistance phenotypes is often used to strengthen this correlation (Faretra and Pollastro 1993; Orth et al. 1995; Dyer et al., 2000); however, the ultimate mechanism of resistance is often lacking. Ultimately, the success of molecular detection is only useful if the expected genetic profile that is being screened correlates to the phenotype of resistance. Laboratory tests are required to determine the level of resistance in numerous suspect isolates of the pathogen before an assessment of the status of the orchard, plantation, vineyard or field can be determined. Traditionally, this meant direct-plating of single-spore isolates or mycelial plugs on medium amended with various concentrations of fungicides under specified growth conditions, and then determining inhibition of growth and/or spore germination (Koller et al. 1997). Many fungi, including the economically important downy mildews, powdery mildews, and rust, cannot be cultured. Other fungi, like *Venturia inaequalis,* grow slowly in culture, taking at least four weeks to obtain results, which, when achieved, are no longer useful for in-season disease management recommendations (Chapman et al. 2011, and others). Unfortunately, the phenotypic comparison of fungicide resistance can be inconsistent due to media choice (Cox et al. 2009; Rampersad 2011), choice of active ingredient (technical grade versus field fungicide), degradation of material, stability of resistance (Zhu et al. 2012; Cox et al. 2007), and genetic background of isolates. One method of circumventing this inconsistency is through the direct testing of genotype, which has resulted in an emerging paradigm of nucleic acid-based detection systems for the rapid identification of fungicide resistance.

Molecular methods have the potential to provide a more rapid and reliable assessment of fungicide resistance, and there are many examples of the successful use of these methods in applied plant pathology (for a review, see Vincelli and Tisserat, 2008). Genetic testing of potentially resistant isolates can be performed directly from disease lesions, obviating the need for isolation, and subsequent growth of the fungus *in vitro* (Quello et al. 2009; others). Furthermore, molecular methods characterize genotype, not phenotypic expression under laboratory conditions. This is particularly important in the case of recalcitrant fungal

pathogens, like *V. inaequalis*or *Mycosphaerella fijiensis* and obligate pathogens such as *Blumeria* and *Plasmopara*. In the case of obligate pathogens in particular, genotype can be readily identified long before phenotype can be determined due to the slow growth of the organism.

As a paradigm, nucleic acid-based detection systems offer rapid (within hours) and sensitive (to picograms) methods to detect the presence of alleles known to confer resistance. First used by Koenraadt et al. (1992b) to detect benomyl resistance in *V. inaequalis,* a variety of sensitive and sophisticated nucleic acid-based detection systems have since been developed and promoted to identify resistance.

However, it is impossible to review the developing paradigm and not question how we implement these detection systems to provide the end-user, in this instance, the farmer, with the information necessary to make the appropriate management decision. The objective of this review is to briefly discuss the current fundamental approaches of nucleic acid based systems, and the currently known targets for the molecular detection of fungicide resistance. The paper concludes with the limitation of these techniques, the impact detection has had on the management of fungicide resistance, and future directions.

2. Nucleic acid based detection techniques

The molecular detection of fungicide resistance can be boiled down into three fundamental techniques: Hybridization, amplification, and sequencing. It is important to stress though, that most amplification technologies used today are also partly based on hybridization technology, and that all sequencing technology is based upon amplification. This article is not designed to review the preponderance of available techniques; for that information, the reader is encouraged to review the current literature, as new application technologies are produced every year. Instead, this article focuses on certain paradigms that have developed between laboratories for the detection of fungicide resistance, and to provide both structure and context as to where we are currently positioned.

3. Hybridization

Hybridization is one of the oldest molecular techniques: Sample DNA is denatured into single strands and allowed to anneal with a single-stranded probe labeled with some type of signal (radioactive isotopes, antibodies, enzymes or chemiluminescent compounds) to permit detection. Target DNA is bound to solid support (historically nitrocellulose then nylon, although today numerous substrates are available, including magnetic beads and polystyrene microspheres). Direct hybridization is the simplest assay for single nucleotide discrimination. For 15 to 20-base oligonucleotides, the approximate melting temperature for hybridization of a perfectly matched template compared to one with a single base mismatch can differ by several degrees (Ikuta et al. 1987)—a fact that can be exploited to create a variety of multiplex detection techniques, and discriminate within samples or between samples to a single nucleotide polymorphism.

4. Amplification

Briefly, polymerase chain reaction (PCR) is the basis for all amplification-type reactions and involves the heating of the sample DNA for denaturing, followed by the annealing of the small, oligonucleotides that serve as primers for DNA polymerase, followed by the extension of the primers by a thermostable, DNA polymerase. Numerous books and laboratory manuals exist as references for PCR operation and optimization (Sambrook et al, 1989). PCR based detection of fungicide resistance depends upon the ability of the reaction to selectively amplify specific regions of DNA, and usually require several post-PCR steps, including agarose gel electrophoresis for either confirmation of amplicon presence or size, or restriction enzyme analysis (Lesniak et al. 2011; Fontaine et al. 2009; Quello et al. 2009; and reviewed by Ma and Michailides 2005). The development of new fluorescent techniques (LAMP, etc) has led to novel assay formats that greatly simplify the protocols used for the detection of specific nucleic acid sequences (Nurmi et al 2000) and allow for the detection of a specific PCR product in a homogeneous solution without the need to open the amplification tubes after PCR or gel electrophoresis (Tomlinson et al. 2012). In these techniques and their variations, PCR products are monitored as they are generated during the course of the reaction via one of two ways: By fluorescent or chemiluminescent dyes that bind to double-stranded DNA in a nonspecific fashion, or by fluorescence-labeled probes that bind to specific sequences. As a result, PCR amplification, amplicon detection and analysis are all achieved in a single reaction (Figure 1). If this is not enough detection power, multiple, sequence-specific probes with unique fluorescent reporters can be added to the reaction, allowing for additional, and simultaneous, determination of multiple products. Techniques such as this are ideally suited for the detection of fungicide resistance, particularly if multiple alleles are involved. Furthermore, the results can be read in real time as the PCR product accumulates or at the end of the thermal cycling protocol directly from the amplification wells. Although many scientists believe that the choice between real time or "standard" PCR (and gel electrophoresis) depends on whether a quantitative or qualitative assay is desired (Nurmi et al. 2000), the reality is that equipment expense and laboratory expertise limits most "applied" labs, resulting in a preponderance of scientifically dazzling techniques that may be used in a human clinical setting, or a plant pathology laboratory focused on basic science, but are rarely, if ever, subsequently tested using isolates from a field failure.

5. Hybrid technologies

Technologies such as Luminex Xmap powerfully combine hybridization and PCR to create a technique that is capable of discriminating and reporting up to 500 different reactions in a single reaction vessel in just a few seconds per sample (Dunbar 2005; Luminex 2011). The approximate melting temperature for 15 to 20-base oligonucleotides can differ by several degrees compared to a "perfectly matched" hybridization (Ikuta et al. 1987). By exploiting and combining this discriminatory hybridization temperature with PCR and microarrays systems and digital imaging, the user can screen up to several hundred thousands of DNA probes (either PCR products or synthetic oligonucleotides) per square centimeter of a solid matrix. Multiple readings per beadset provide built-in internal controls (Luminex 2011). For

medically relevant human pathogens, whole genome arrays have been developed; it is hardly a stretch to imagine development of arrays for a few dozen alleles for important genes, or even genes important for fungicide resistance. Although financially out of reach for most labs, few could envision the sequencing of entire genomes for a few thousand dollars as we do today, and it is hopefully only a matter of time before microarrays to detect fungicide resistance in agriculturally important crops are widely available. Today, and certainly for the next few years, both cost and accessibility also remain obstacles to the development of such arrays. Within reason and immediate reach is a 96-well format that provides fast, simple, and highly reproducible analyses of up to 96 PCR products—which still translates into an assortment of alleles for a variety of fungicide resistance genes.

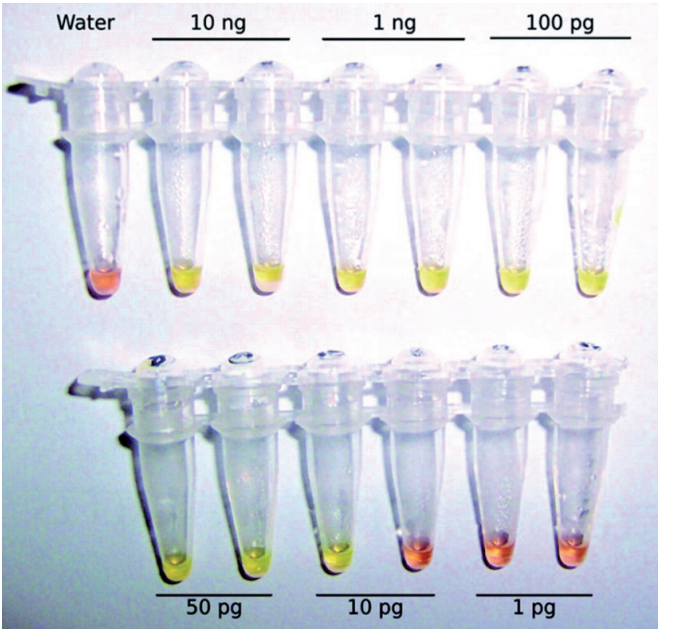

Figure 1. LAMP technologies have been used to detect the presence of invasive species, like *Phytophthora ramorum*. The presence of LAMP product in positive reaction mixtures causes a color change from orange to yellow. In time, this technology could be used to detect some type of fungicide resistance in the field. Photo from Tomlinson et al. 2007.

For most, smaller laboratories, a more realistic approach concerns the use of polymerase chain reaction coupled with cleaved amplified polymorphic sequences (PCR-CAPS). Restriction-fragment length polymorphisms due to small nucleotide polymorphisms (SNP) that co-segregate or are caused by fungicide resistance create or abolish restriction sites in PCR products, and can be exploited for detection of fungicide resistance through the careful selection of locus-specific oligonucleotide primers (Banno et al. 2008; Lesemann et al. 2007, Quello et al. 2009, Fontaine et al. 2009; others).

Assuming that a SNP is present and can be used, oligonucleotide primers with unique sequences are used to amplify a defined locus, followed by the use of a restriction enzyme that can discriminate between resistant and sensitive isolates (Figure 2). An alternative to PCR-CAPS is allele specific (AS-PCR), in which the mutation that confers resistance is used to design primers that specifically amplify the mutated allele, but not the wild-type one (Fontaine et al. 2009, Lesniak et al. 2011, others). This approach is more sensitive, and does not require a RFLP to detect resistance, but also does not detect any heteroplasmy or heterozygosity.

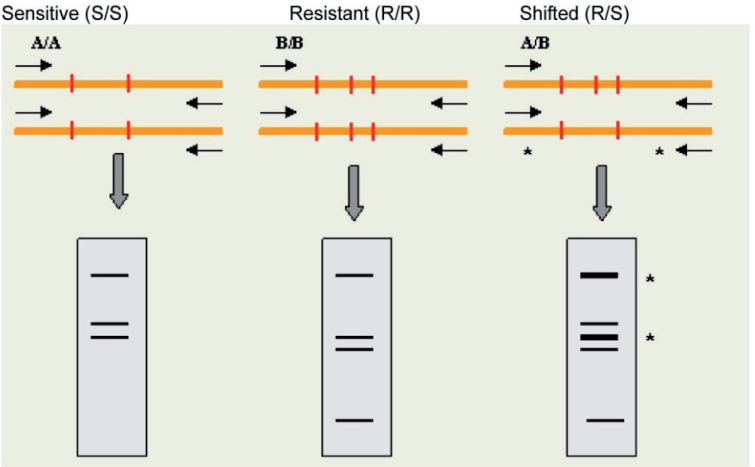

Figure 2. Cleaved Amplified Polymorphic Sequences (CAPS) polymorphisms result from single nucleotide polymorphisms (SNPS), insertions or deletions (INDELS) that create or destroy restriction enzyme recognition sites in polymerase chain reaction (PCR) amplicons. For example, three isolates have different level of fungicide resistance to azoxystrobin: Sensitive S and Resistant R, and shifted (moderately resistant) from the isolate with mitochondrial heteroplasmy R/S. The amplified fragments from S and R contain two and three RE recognition sites, respectively, which is sufficient discrimination in the case of fungi, which are mostly haploid. (Sierotzki et al. 2000; Avenot and Michailides, 2010). This can also result in the identification of heteroplasmy in resistance (as seen in Fontaine et al., 2009) with restriction patterns that resemble those found in heterozygous diploids, or even incomplete digestions. When fractionated by agarose or acrylamide gel electrophoresis, the PCR products digested by the RE will give readily distinguishable patterns. Image from http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechCAPS.shtml

6. DNA sequencing

DNA sequencing, as performed for the last 30 years, has been done via the Sanger method and is the most commonly used sequencing technique available. Genome-sequencing efforts have resulted in technological advances in DNA sequencing and led to the improvement of longer sequencing length, done faster and with less expense. It has also permitted the rapid sequencing of isolates (in part or into total) that did not conform to expected genotype:

phenotype relationships (Lesniak et al. 2011; Quello et al. 2010;Leroux et al. 1999, and many others).

Metzker (2009) provides an excellent review of 'next generation sequencing' technology, although the rapid pace of technology marches on. One of the newer sequencing technologies that have immediate application to the detection of fungicide resistance is pyrosequencing. DNA pyrosequencing, a method of sequencing by synthesis, was first introduced in 1996, and is faster and less expensive than traditional Sanger (dideoxy sequencing) DNA sequencing methodologies. Unlike the Sanger method, DNA pyrosequencing utilize a cascade of enzymatic reactions that yield detectable light proportional to incorporated nucleotides. As a result, pyrosequencing yields relatively short read lengths and limited amounts of sequence data per pathogen or microbe. However, with careful target selection and primer placement, DNA pyrosequencing has been used for genotyping, SNP detection, and identification of microbes (Petrosino et al. 2009). Most importantly, pyrosequencing has been used to detect point mutations in antimicrobial resistance genes as a means of molecular resistance testing, including antifungal resistance in clinically important fungi (Wiederhold et al. 2008).

Pyrosequencing offers comparable accuracy to conventional DNA sequencing via the Sanger method, but provides greater opportunity for large sample numbers to be processed in parallel. The reaction is performed in real-time, obviating the need for electrophoresis, labeled nucleotides and primers. This is a technique that can be multiplexed, which economically enables rapid and accurate screening of a large number of samples, however, the prohibitive cost of equipment [$200,000-$1million per machine (Metzker 2009)], and technical expertise limits the use of this technology to research universities and industry.

Finally, a review of the variety of molecular techniques used for diagnostic applications (and the constant development of 'new' techniques) demonstrates that no universal technique exists which is optimal for detection of nucleic acids. The choice of a particular technique is also dependent on the information required, the targets under consideration, and obviously, cost. Regardless of any given or "popular" technique, new techniques continue to be developed which involve new approaches to amplification, hybridization, formats, imaging, and labels.

7. Detection of fungicide resistance

Regardless of the techniques used, the primary targets of fungicide activity (and thus fungicide resistance) have remained remarkably constant, and include mitosis and cell division; sterol biosynthesis; respiration; nucleic acid synthesis; and signal transduction. The widespread use of fungicides has also resulted in resistance in non-target genes, namely drug transporters, which are included in this review due to the role they play in fungicide resistance. The mechanisms of fungicide resistance that have been identified to date, include: i) a mutation in the target site of the antimicrobial agent that reduces the binding of the fungicide; ii) overproduction of the gene that is the target of the fungicide; (iii) reduced uptake of the antimicrobial agent, and (iv) active efflux of the fungicide. Other mechanisms

that may have roles in fungicide resistance (based upon host-pathogen interactions, or basic fungal and other microbe biology) include, the possible presence of an enzyme that inactivates the antimicrobial agent (e.g., pisatin-pisatin demethylase) or even mutations that result in the posttranscriptional or posttranslational modification of the target enzyme or other regulatory factor (Mann and Jenson, 2003), resulting in reduced binding of the antimicrobial. Resistance may also be caused by unrecognized mechanisms—a problem for scientists trying to understand mefenoxam, phosphorous acid, or dodine resistance, to name a few of the fungicides where resistance is known, but the primary mechanism of resistance has not yet been identified.

7.1. Mitosis and cell division: Beta-tubulin assembly inhibitors

Introduced in the 1960s, benzimidazoles were the first penetrant fungicides. Compared to their predecessors, the carbamates, dithiocarbarmates and pthalimides, they were a revolutionary change in fungicides, in that they were noncontact, and extremely effective at low rates. First introduced as benomyl (other MBC fungicides include thiabendazole, and thiophanate-methyl, to name a few), once inside the plant it is metabolized to form methyl benidimidazole carbamate (MBC), which inhibits fungal mitosis via binding to tubulin, the subunit of microtubules essential to forming the mitotic spindle (Ma and Michailides, 2005).

As one of the first noncontact fungicides, it is not surprising that it was one of the first instances of resistance reported, to powdery mildew in greenhouse cucumbers (Schroeder et al. 1969). Since that time, resistance to the benzimidazole class of fungicides has been detected in many fungal species (Ma and Michelides, 2005). Resistance is correlated with point mutations in the β -tubulin gene, with different mutations resulting in altered amino acid changes at the benzimidazole-binding site. These various mutations at different codon sites also result in different levels of resistance: In *V. inaequalis*, mutations at codon 198 resulted in medium resistance, at codon 200 it resulted in very high resistance (Koenraadt et al. 1992a), and at 240 it resulted in low resistance (Quello et al. 2010). In *Monilinia*, only low and high resistance to benomyl and thiophanate-methyl has been observed for field isolates of *M. fructicola* (Ma and Michelides, 2005). Sequence analysis of the β-tubulin gene showed that a single base pair mutation at codon 6 was responsible for the low resistance level to benzimidazoles in all the LR isolates of *M. fructicola* examined. Curiously, different substitutions at the same codon resulted in different degrees of resistance in the cereal eyespot fungus, *Tapesia yallundae* (Pseudocercosporella herpotrichoides): codon changes from Glu to Ala, Gly, Lys, and Gln at position 198 had 50% effective concentration (EC50) values to carbendazim ranging from 0.5 to more than 25 m g/ml in some isolates (Albertini et al., 1999), one of the first instances that demonstrates that differences in genetic background may play a role in expression of specific fungicide resistance genes.

PCR-CAPS was used to determine thiophanate-methyl resistance *Helminthosporium solani* (Cunha and Rizzo, 2005). In *V. inaequalis*, a screen using this approach resulted in the identification of previously unidentified alleles conferring resistance in this fungus, and would have under-reported MBC resistance in 31% of the isolates (Quello et al. 2009) if PCR

alone was used for detection. Although the authors continue that a PCR-RFLP based assay may be the best option to screen for fungicide resistance from late-season scab lesions because *V. inaequalis* cannot be reliably cultured due to the application of protectant fungicides for other diseases and competing leaf microflora later in the season, it still leaves open the possibility of new alleles for resistance not being identified, and resistance being under-reported. At this point in time, most apple growers have abandoned thiophanate-methyl for the control of apple scab in the field, although it continues to be used for other diseases. However, these studies are a cautionary tale regarding the sole use of a PCR-based detection to identify fungicide resistance, particularly when no in vitro screening is performed in parallel. This is particularly important as Kawchuk et al. (2002) found that mechanisms other than point mutations in the β -tubulin gene play a role in resistance on *Gibberella pulicaris*; work in the human pathogen, *Candida albicans*, identified the role of a multi-drug resistant transporter (MDR) as responsible for benomyl resistance (Ben-Yaacov et al. 1994). Later work by Sanglard et al. (1995), identified the BEN^R gene that confers resistance to β -tubulin as an ATP-binding cassette (ABC) transporter (Sanglard et al. 1999).

Detection of specific types of benzimidazole resistance also identifies any negative cross-resistance with diethofencarb (Leroux et al. 1999). The phenylcarbamate diethofencarb was introduced in 1984, and has a similar mode of action to the MBC class of fungicides. In fact, single base pair mutations in codons 198 and 200 result in a readily detectable, negative cross-resistance to diethofencarb (Faretra and Pollastro, 1991; Yarden and Katan, 1993). Negative cross-resistance (NCR) occurs when a novel allele that confers resistance to one toxic chemical results in hyper-sensitivity to another. This mutation therefore results in efficacy to benzimidazole-resistant isolates that possess that allele, but not wild-type isolates. In *B. cinerea*, high levels to resistance carbendazim and thiabendazole, conferred hypersensitivity to diethofencarb, even more so than the benzimidazole sensitive type (Leroux et al. 1999). Furthermore, this negative cross-resistance involved other N-phenylcarbamates and other herbicides that target microtubule assembly (Leroux and Gredt, 1989). Negative cross-resistance was also observed between benzimidazoles and several aromatic hydrocarbon fungicides (e.g., dicloran, OPP (o-phenylphenol)); the phenomenon was first described in cereal eyespot fungus, *T. yallundae*, as well (Leroux and Gredt, 1989), but is more likely due to multi-drug resistance (MDR) mutations than a single structural gene.

8. Signal transduction: Dicarboximides and phenylpyrroles

The dicarboximides are composed of three major products: Iprodione, vinclozolin, and procymidone. The introduction of this class of fungicides coincided with the failure of the benzimidazoles in control of Botrytis in grape. As with benzimidizoles, resistance developed rapidly, due to a combination of concurrent applications of this class without rotation or tank-mixing with other products, coupled with a limited understanding of the process of fungicide resistance. This class of fungicides was primarily used for the control *Botrytis, Alternaria, Sclerotinia* diseases, although they are effective on other pathogens like

Rhizoctonia spp., and *Fusarium* spp., as well. Not surprisingly, field resistance was first observed in *B. cinerea* (Pommer and Lorenz, 1982), *M. fructicola* (Ritchie, 1983), and *Sclerotinia* spp. (Detweiler et al., 1983).

The molecular mechanisms of dicarboximide resistance involve two separate signal transduction pathways: The two-component histidine kinase and mitogen-activated protein (MAP) kinase cascades. Both MAP kinase and two-component histidine kinase are involved with regulating a diversity of cellular responses including differentiation, cell division, gene expression, heat shock and osmotic response. The first molecular mechanism of dicarboximide resistance was identified in *U. maydis* (Orth et al. 1994), and is one of the few instances where the gene was found to be sufficient for conferring resistance to a wild-type isolate via transformation (Orth et al. 1995). The gene, termed *adr1*, is a 1,218 bp open reading frame with homology to serine/threonine protein kinases that was later identified as the major cAMP dependent protein kinase; However, later studies by Ramesh et al. (2001), found that the *adr1*-encoded enzyme was not the direct target of vinclozolin inhibition but that mutants with a defect in the regulatory subunit of cAMP-dependent protein kinase (*ubc1*) exhibited resistance to vinclozolin and the aromatic hydrocarbon, chloroneb. Mutants with a defect in the *ubc1* gene also display interesting changes in morphology, including a reduction in multiple budding in the presence of the fungicides and osmotic sensitivity, suggesting a connection between fungicide mode of action and morphogenesis and glycerol accumulation (which would also be related to osmotic, turgor regulation and osmotic shock), which may explain why this mutation has never been reported from field isolates with fungicide resistance phenotypes (Ramesh et al. 2001).

Studies by Leroux et al. (1999) in *B. cinerea* in French vineyards found strains resistant to multi-site fungicides, and multiple combinations of fungicide resistant phenotypes in vineyards in France: two types of benzimidazole (e.g. carbendazim, thiabendazole)-resistant strains were detected, with negative cross-resistance towards the n-phenylcarbamates (diethofencarb, diphenylamine and dicloran) found only in one type of resistant strain, as previously stated above. However, this study also found that most dicarboximide (e.g. iprodione, procymidone, vinclozolin)-resistant strains were also weakly resistant to aromatic hydrocarbon fungicides (e.g. chloroneb, dicloran, tolclofos-methyl) but remained sensitive to phenylpyrroles (e.g. fenpiclonil, fludioxonil). However, in some other dicarboximide-resistant strains, resistance was observed either as being restricted to dicarboximides or as extending weakly to phenylpyrroles. Dicarboximides, phenylpyrroles and the aromatic hydrocarbon fungicides (e.g. chloroneb (PCNB), dicloran, quintozene, tolclofos-methyl) are not chemically related, but obvious structural similarities can be observed (Fig. 3), and in laboratory studies, mutants of *B. fuckeliana* and several other fungi, have a positive cross-resistance between them (Leroux et al., 1992; Faretra and Pollastro, 1993).

Similar observations regarding the complexity of resistance profiles were observed in *B. cinerea* in the laboratory (Oshima et al. 2002). Dicarboximide- resistant laboratory mutants selected for high resistance to dicarboximides, aromatic hydrocarbons, and phenylpyrroles, were hypersensitive to osmotic stress, and were rarely obtained from the field (Leroux et al.,

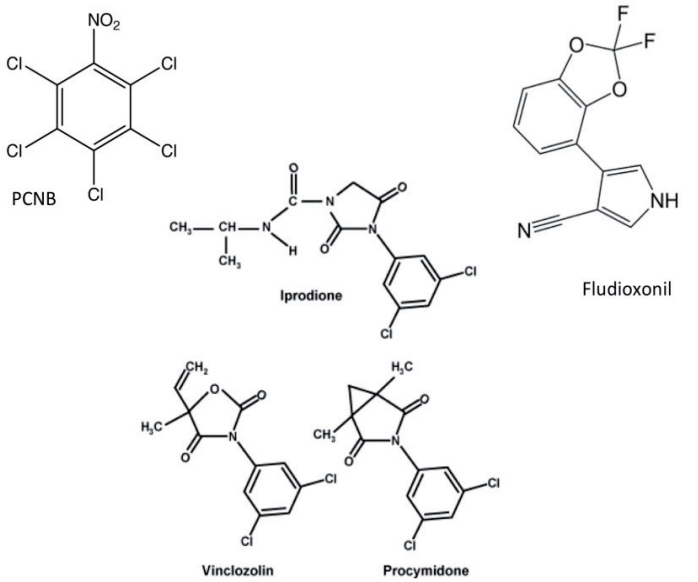

Figure 3. Comparison of members of the aromatic hydrocarbons (PCNB), phenylpyrrole fungicide fludioxonil and three dicarboximides, iprodione, vinclozolin and procymidone. All five structures share low molecular weight and aromatic hydrocarbon moieties that may explain why some cross resistance between these distinct classes of fungicides is observed.

1992; Faretra and Pollastro, 1993; Lyr, 1995). Instead, only moderately resistant, osmotically-stable strains were recovered from the field, and these field isolates of *B. cinerea* showed cross-resistance to aromatic hydrocarbons but not to phenylpyrroles (Oshima et al. 2002). In fact, phenylpyrrole- resistant mutants from *B. cinerea* that were first isolated and characterized in the laboratory were due to mutations at a different locus (*Daf1*—later identified as *BcOS1*) locus responsible for dicarboximide resistance (Faretra and Pollastro, 1993) and were only later found in the field, and were only described as "weakly resistant"(Leroux et al. 1999). These early studies found that mutations of the *Daf1* gene are highly variable, with at least five classes of alleles identified and associated with differing levels of resistance (sensitive (S), low resistance (LR), moderate resistance (MR) or highly-resistant (HR)) to dicarboximides and phenylpyrroles, respectively: S/S, LR/S, HR/LR, HR/MR, HR/HR (Faretra and Pollastro, 1993). Field resistance was due to a single amino acid substitution, from Ile to Ser, which occurred at codon 365 in *BcOS1* gene (Oshima et al. 2002), and at two other amino acid substitutions at the same position, 365 Ile to Asn or Arg, in field-resistant isolates. Work done by Oshima et al. (2006) used PCR-CAPS, exploiting a *Taq*1 restriction site that results in the mutant allele that confers resistance. Approximately 41 percent of the isolates had the type I mutation that could be detected by PCR-CAPS. However, other types of dicarboximide resistance were detected and sequence analysis of these mutants classified them into type II isolates that have three amino acid substitutions

within *BcOS1*p (V368F, Q369H, and T447S) or type III isolates that have two amino acid substitutions within *BcOS1*p (Q369P and N373S) (Oshima et al. 2006).

Many fungi accumulate glycerol to increase osmotic pressure to contend with osmotic stress, or for pathogenicity. Studies done by Fujimura et al., (2000) in *Neurospora crassa* with osmotic-sensitive (os) mutants with *os-1*, *os-2*, *os-4* and *os-5* mutations (similar to the previously described resistances in *B. cinerea*) showed cross-resistance to dicarboximides (iprodione and vinclozolin), but also the aromatic hydrocarbons (PCNB). All of the os mutants except for some os-1 mutant alleles were resistant to the phenylpyrrole fungicide, fludioxonil (Ochiai et al. 2002). Previous work by Grindle (1982) found other osmotic sensitive mutants (cut) that were not resistant to these fungicides, suggesting that osmotic sensitivity alone was not the mechanism. However, the original report by Grindle (1982) on dicarboximide resistance in *N. crassa* shows that multiple alleles that segregate independently were involved with this os-1 resistance, suggesting that other genes may still be involved in resistance (See section on MDR for further information). Work by Zhang et al. (2002), identified a mitogen activated protein (MAP) kinase (HOG1) that was resistant to phenylpyrrole, and were caused by a frame shift from tryptophan to a stop codon or nonsense point mutations, allowing for a screenable genotype. However, field resistance of phenylpyrrole has not been reported to date, and the complex phenotype involved in this type of resistance makes it biologically interesting, but hardly defining in the diagnosis of fungicide resistance.

8.1. Sterol biosynthesis inhibitors

This group of fungicides exploits one of, if not the most important mode of action for both antifungals in medicine and fungicides in agriculture. The sterol biosynthesis inhibitor (SBI) fungicides inhibit a precursor of ergosterol that is essential for the development of the fungal membrane (Brent, 1995). These fungicides can be classified based upon their target sites in sterol biosynthesis, with inhibitors of squalene epoxidase (e.g. naftifine, terbinafine, tolnaftate) primarily used for mammalian mycoses. The remaining classes are used for agricultural purposes. 'Amines' or 'morpholines' (e.g. fenpropidine, piperalin, spiroxamine, tridemorph) act as inhibitors of sterol Δ14-reductase or Δ8->Δ7-isomerase, and target the products from the *Erg24* and *Erg2* genes; hydroxyanilides, represented by fenhexamid, act on the 3-keto reductase, C4- demethylation encoded by *Erg27* and is specifically used for the control of *Botrytis;* inhibitors of sterol 14-alpha-demethylase (e.g. bitertanol, triazoles, imidazoles, in addition to imazalil, prochloraz, pyrifenox, triadimenol) are referred to as demethylase inhibitors (DMIs) and primarily target *Erg 11/CYP51*(FRAC 2012), and are effective against a wide variety of phytopathogens.

DMIs were introduced for plant disease management in the 1970s, with resistance and reduced efficacy reported soon after (Brent, 1995). Due to both agricultural and medical importance, the molecular mechanisms of SBI resistance have been highly studied and include (i) mutations in 14-alpha-demethylase (CYP51) structural gene (Delye et al. 1997; 1998; Canas-Gutierrez et al. 2009, and others) and ERG27 gene (Albertini and Leroux, 2004;

Fraaije et al. 2007); (ii) overexpression of the CYP51 gene, leading to increased production of the target enzyme (Cools et al. 2012; Luo et al. 2008; Ma et al. 2006; Schnabel and Jones, 2001; Hamamoto et al. 2000), and (iii) overexpression of the ATP-binding cassette (ABC) transporters (Zwiers et al. 2002; Hamamoto et al. 2000; Nakaune et al. 1998) which will be addressed later in the chapter. Several studies, at least in the laboratory, have demonstrated that multiple mechanisms contribute to the variation in azole susceptibility (Stergiopoulis et al. 2003; Zwiers et al. 2002), and genetic analysis of progeny from a cross between *M. graminicola* isolates with differing sensitivities to DMI fungicides revealed a continuous distribution of resistance, leading the researchers to conclude on the polygenic nature of inheritance (Stergiopoulis et al. 2003), leaving open the possibility that other mechanisms may also be at work.

Mutations in the 14-alpha-demethylase (*ERG11/CYP51*) structural gene that lead to a decreased affinity of the target protein to DMI fungicides have been found in powdery mildews (Delye et al. 1998; Delye et al. 1997); two *Mycosphaerella* pathogens—*M. graminicola* (Leroux et al. 2007; Cools et al. 2006) and the black Sigatoka fungus, *M. fijiensis*, (Gutierrez-Canas et al. 2009); and the cereal leaf spot pathogens--*Tapesia* spp. (Albertini et al. 2003), although work by Wood et al. (2001) did not find a correlation between resistance and the presence of known mutations in the structural gene. In *M. graminola*, reduced sensitivity to DMIs could be correlated with an alteration in the CYP51 structural gene at codons 459–461 (Cools et al. 2005; Leroux et al. 2007). In the barley powdery mildew, *Blumeria graminis*, amino acid substitutions in CYP51 at Y136F and K147Q, were detected and a very high level of resistance was associated with the allele containing K147Q mutation. Sequence analysis of the CYP51 gene from the progeny of a cross between DMI-sensitive and resistant isolates demonstrated co-segregation between the mutant alleles and resistance. Consistent with other studies, the authors found that genetic analysis of resistance to the triadimenol indicates that mutation of the *CYP51* gene is not the only mechanism of resistance operating in *B. graminis*: Two moderately resistant isolates had no mutations in the *CYP51* gene, and had identical sequences to that of the sensitive isolate, suggesting that resistance in at least these two isolates must be due to a mutation in an entirely different gene (Wyand and Brown 2005). The identification of isolates such as these should serve as adequate warning that molecular detection of mutations in the structural gene may result in an under-reporting of actual incidence of resistance in the field. Finally, mutations in the structural gene of *ERG27* in *B. cinerea* were detected and correlated with mutations (F412I and R496H) that would lead to a decreased affinity of fungicide to the target protein, but it was regarded as low resistance or moderate resistance (Albertini and Leroux, 2004; Leroux et al. 2007).

A second mechanism of DMI resistance involves overexpression of the *CYP51* gene. Overexpression of CYP51 has been associated with DMI resistance in *P. digitatum* (Hamamoto et al. 2000), *V. inaequalis* (Schnabel and Jones, 2001), *M. fructicola* (Luo et al. 2008), *B. jaapi*i (Ma et al. 2006), C. *beticola* (Bolton et al. 2012) and *M. graminicola* (Cools et al. 2012). In all but *C. beticola*, overexpression of CYP51 has been associated with insertions in the upstream promoter: A tandemly repeated 126 bp fragment was found in the promoter of *P. digitatum*; Ma et al. (2006) identified a variably-sized retrotransposon-like element in *B.*

jaapi; Schnabel and Jones (2001) reported on a 533-bp fragment found in some (but not all) DMI-resistant isolates of *V. inaequalis*; and Luo et al. (2008) found a 65 bp 'Mona' element in the promoter of *CYP51* in *M. fructicola* that was strongly linked to the DMI resistance phenotype. However, subsequent work by Villani and Cox (2011), found that the 'Mona' element was present in a range of sensitivities, and not always present in resistant isolates, suggesting it is just one of many possible mechanisms of resistance. Overexpression of *ERG27* has not yet been identified as having a role in resistance to the hydroxyanilide class (FRAC 17) of fungicides.

A final mechanism of DMI resistance relies upon "drug" transporters, trans-membrane proteins located in the plasma membranes that utilize ATP to translocate compounds, including toxins, out of the cell, preventing the accumulation of these products to toxic levels, as has been shown for *B. cinerea* (Leroux and Walker 2011; Leroux et al. 2002), *M. graminicola* (Cools et al. 2007) *Penicillium digitatum* (Nakaune et al. 1998), and possibly *V. inaequalis* (Koller and Wilcox, 2001). This will be discussed in a later section. Some moderate resistance to DMI fungicides in *B. cinerea* (termed Ani R2 and Ani R3 by Leroux et al., 1999) was previously described (Stehmann and DeWaard, 1995; Del Sorbo et al. 1997). Later studies found isolates with this similar phenotype demonstrated pronounced increases in expression level of the ABC transporter genes (Kretschmer et al. 2009) and not the structural genes themselves, creating greater complexity and less certainty in molecular detection of fungicide resistance. As a result, DMI resistance in numerous species has been identified, but only 10 species have had the molecular mechanism of resistance studied in depth.

Although fungicide resistance baselines and thresholds have been previously established in many agricultural systems, the primary stumbling block to the rapid identification of DMI fungicide resistance for most fungi is the nature of its quantitative resistance (Koller et al. 1991; Schnabel and Jones, 2001; Ishii, 2009). Unlike qualitative resistance that results from a single gene mutation (e.g., *TUB1R* conferring resistance to benzimidazoles), DMI resistance is much more complex. Due to the diversity of mechanisms of DMI resistance in different pathogens (and even the variation in response by pathogens to different DMIs), it is unlikely that any one approach, with the exception of phenotypic analysis, will provide the screening necessary to detect fungicide resistance in a diversity of pathosystems, although the possibility exists that the monogenic DMI resistance observed in powdery mildew pathogens may be amenable to this approach. In V. *inaequalis*, examples of DMI resistance to date are associated with an insertion in the promoter of the CYP51 target gene that has been correlated with overexpression of CYP51. The authors of this work (Schnabel and Jones, 2001) noted that the correlation was strong, but not absolute. Subsequent work to utilize this knowledge to develop PCR based screening of resistant isolates can detect the insertion, but not predict the degree of resistance that results (Villani, Cox and Beckerman, in preparation and Fig 4.), preventing the rapid detection of resistance in the field. Ultimately, *in vitro* screening of radial growth in the presence of fungicide (a 4-week process after a single-spore isolate in pure culture is established) is needed to determine the degree of resistance in the isolate, and at least in *V. inaequalis*, it does not seem to be currently possible to rapidly detect fungicide resistance in the field, and certainly not to a degree that thresholds could be

identified to warn when a fungicide failure will occur. This situation is quite different than the detection of fungicides that impact respiration.

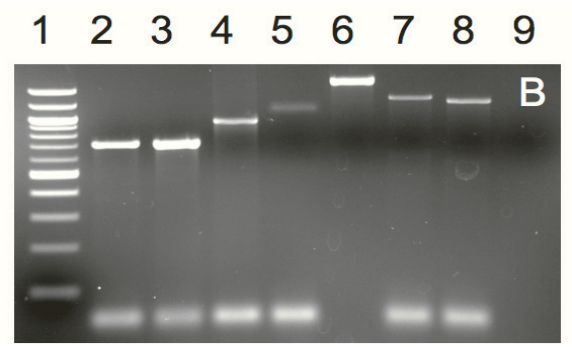

Figure 4. Amplicon size is not related to overexpression of *CYP51* in *V. inaequalis*. Primers flanking the promoter region of *CYP51* were used to examine the relationship between the 533 bp insertion, size of promoter insertion and resistance to myclobutanil. Many of the highly resistant isolates did not yield an amplicon, and those that did did not have the expected 533 bp insertion. From Villani, Cox, and Beckerman, unpublished.

8.2. The respiration inhibitors

Respiration is an obvious target for fungicides. The oldest synthetic fungicides still used today include dithiocarbamates (e.g., mancozeb, thiram, ziram) or pthalimide derivatives (e.g., captan), and prevent plant disease by the inhibition of spore germination, and subsequent germ tube elongation. To date, there have been no credible reports of resistance to these older fungicides. Furthermore, older synthetic multi-site fungicides are effective against a wide range of plant-pathogenic fungi, and Chromista. These multisite inhibitors are thio-reactant with those enzymes involved in respiration (Lyr, 1977); Other inhibitors of respiration work at complex II at the succinate dehydrogenase gene, complex III, the cytochrome bc1 (ubiquinol oxidase) at Qo site, or cytochrome bc1 (ubiquinol oxidase) at Qo site; or complex V, where fungicides like fluazinam actually work by uncoupling electron transport and ADP phosphorylation of oxidative phosphorylation (FRAC, 2009). As such, this target is essential to aerobic eukaryotes, and present targets for disruption. The most recently developed group of respiration inhibitors targets mitochondrial complex III (the cytochrome bc1 complex), and targets the outer quinol-oxidizing pocket (Qo site), and complex II at the succinate dehydrogenase gene, which are the areas of most recent and active interest due to their recent release, the nature of fungicide resistance, and potential ease of detection.

8.3. QoI—The quinone outside inhibitors

Fungicides that inhibit the cytochrome bc1 enzyme complex (complex III) at the Qo site (Qo inhibitors, QoIs) were first introduced to the market in 1996. QoI resistance has now been

reported in more than 20 pathogens, including the Chromista pathogens, downy mildew of grape and cucurbit, and the fungi that cause apple scab, wheat powdery mildew and Septoria leaf blotch, to name but a few. Resistance has been reported in all major continents: Asia, Australia, Europe, North America, and South America (FRAC 2006; Wilson and Wicks, 2011). The majority of the reports associate the point mutation that results in a change from glutamate to alanine at codon 143 (G143A), followed by the resistance that results from a transition from phenylalanine to leucine at position 129 (F129L) (Pasche et al. 2005; Sierotzki et al. 2007); the least commonly observed mutation involves a change from glycine to arginine at position 137 (G137R) (Sierotzki et al. 2007), and of course, unknown causes (Lesniak et al. 2011). These easily identified mutations allow for the rapid detection for the potential of QoI-resistance in a variety of fungi and Chromista. Furthermore, the degree of resistance is much greater in the G143A allele (referred to as high resistance), and has consistently resulted in a significant reduction in disease control, as compared to resistance associated by F129L and G137R which results in more moderate or partial resistance, and some degree of control at highest rates of fungicide.

A more recent study by Lesniak et al. (2011), found ninety-eight percent of QoI-resistant/shifted isolates screened in Michigan were associated with G143A, consistent with other phytopathogenic fungi resistant to this class of fungicides (Ishii et al., 2009; Ishii et al. 2007). Sequencing the entire *CYTb* gene of resistant isolates that did not test positive for the G143A transition did not reveal other mutations in the structural gene, and suggests other mechanisms of resistance (Lesniak et al. 2011). Regardless of mutation present, functional analysis of the *CYTb* gene is confounded by the complexity of fungal mitochondrial genetics. A fungal cell may contain >250 mitochondria and an equal copy number of mitochondrial DNA (mtDNA). A single point mutation in a single copy of a mitochondrial gene could be detected, but would not be sufficient to confer resistance, unless multiple copies of the allele were present. However, over time, and under increasing selection pressure, the allele would become fixed, and the mitochondria population within the cell would shift from sensitive to increasing numbers bearing the resistant allele, shifting resistance and becoming homeoplasmic over time. Thus, early detection of this shift could preserve long-term fungicide efficacy by short-term use of alternative fungicides until the allele shifts back to the previous susceptible homeoplasmy. Early detection with quantitative PCR (qPCR) could theoretically enable the assessment of either gene expression or copy number that is required for QoI resistance to develop the field. However, to date, this work has not been attempted with any field populations. Preliminary data suggests that the populations in New York, Michigan and Indiana are not yet fixed (heteroplasmy). Laboratory analysis of QoI resistant mutants found that serial passage of trifloxystrobin-resistant isolates in vitro has resulted in a loss of resistance In *V. inaequalis*,after only two rounds of propagation allowed a significant majority of mitochondria containing the wild-type cytochrome b sequence to re-appear; This was not observed when the fungus was under continued selection (Zheng et al. 2000) . The conversion of heteroplasmic isolates back to wild type in the absence of QoI fungicides would imply that a G143A mutation is associated with a fitness penalty, although Chapman et al. (2011) found no evidence of this. Early detection of the resistance allele, and prophylactic switching to non-QoI fungicides may return the

population of a sensitive level in a few years, and with proper monitoring, allow the use of QoI fungicides for scab control again. Similar findings on *M. graminicola* also showed conversion of mutated *CytB* gene back to wild-type in *M. graminicola* (Fraaije et al. 2002).

With QoI resistance, allele specific PCR (Lesniak et al. 2011 and many others) and restriction enzyme digest of the amplicon (Leroux et al. 2010 and many others) are both used to detect the presence of the allele. Work by Fontaine et al. (2009), found that the QoI-resistant allele could not be detected after *Fnu*4HI digestion for R : S ratios equal to or below 1:9 (w:w). When compared to the allele specific (AS-PCR) PCR, the mutant allele was amplified at the lowest ratio tested, much more sensitive than the PCR-CAPS technique (Fontaine et al. 2009). However, work by Chapman et al. (2011), suggests an over prediction of resistance, if the mutation is used as a sole criterion for identification of resistance. Similar finding occurred in work done by Lesemann et al. (2007), on apple powdery mildew, with PCR of cleaved amplified polymorphic sequences (CAPS) analyses suggest that the proportion of mitochondria carrying the G143A exchange determines the degree of strobilurin resistance. Lesemann et al. (2007) also found a high variability in the cytB gene of *P.leucotricha* and discusses the role that mitochondrial heteroplasmy may play in conferring a selective advantage under changing conditions, as does Avila-Adame et al. (2003). Many papers on QoI-resistance in phytopathogens fail to discuss mitochondrial heteroplasmy (Patel et al. 2011). This is important as for *P. leucotricha* (Lesemann et al. 2007) and *V. inaequalis* in the laboratory (Zheng et al.2000) and in the field (Lesniak et al. 2011), clearly demonstrated that all isolates of the apple powdery mildew and scab pathogens tested were heteroplasmic for the G143A mutation. Lastly, a study by Miguez et al. (2003), examined the role of alternative oxidase in reducing the sensitivity of *M. graminicola* to the QoI azoxystrobin. Although the level of resistance was lower than the more commonly identified, G143A, sufficient resistance was incurred despite the absence of this mutation, suggesting yet another mechanisms of resistance.

8.4. SDHI

Succinate dehydrogenase inhibitor (SDHI) fungicides include “first generation” SDHIs, like flutolanil, carboxin, followed by later (and improved) “second generation” compounds, including boscalid, and even “third generation” products like penthiopyrad, and fluopyram. This class of compounds targets and binds to the ubiquinone-binding site (Q-site) of the mitochondrial complex II, specifically the succinate dehydrogenase (SDH) complex in the respiratory chain also referred to as complex II or succinate:ubiquinone oxidoreductase (SQR), inhibiting fungal respiration by blocking electron transport [Kuhn, 1984; reviewed by Avenot and Michailides (2010), including a recent list of resistant organisms and corresponding mutations]. There is no evidence of cross-resistance with other similarly acting chemical classes such as QoI fungicides (which also affect energy production and electron transport) due to their unique mode of action and target site. The primary target of the SDHI fungicides is the SDH complex that consists of four subunits: a flavoprotein (Fp) subunit (SdhA), an iron-sulfur protein (Ip) subunit (SdhB), and two membrane-anchored protein subunits (SdhC and SdhD). Mutations conferring resistance have been found in the SDHB (Avenot et al 2008) and SDHC (Ito et al. 2004) and SDHD subunits (Avenot et al. 2009).

The first widely used SDHI fungicide, carboxin, was used in the late 1960s, and its applications were limited due to its primary activity against basidiomycete pathogens, namely rusts, *Rhizoctonia* spp., and corn smut (*Ustilago maydis*), and limited activity against other pathogens (Sisler 1988). In contrast to old SDHIs, newer active ingredients of SDHIs comprise compounds such as boscalid, penthiopyrad or fluopyram, and are characterized by a broad spectrum of fungal activity on various crops (Stammler et al., 2007; Stammler et al., 2006) particularly *Botrytis* and *Alternaria* species. Carboxin is less efficient than boscalid in controlling the wild-type sensitive isolates (Avenot et al. 2008). Unlike carboxin, boscalid prevented *B. cinerea* spore germination completely at high concentrations, and inhibited germ-tube elongation at low concentrations. This phenomenon was particularly noticeable if biological tests were conducted in media containing succinate rather than glucose as the carbon source (Lyr, 1977). This is an important point when performing in vitro screens of fungicide resistance phenotypes.

Field and laboratory mutants resistant to carboxin and boscalid have been reported in a variety of fungi and cropping systems (For a recent review see Avenot and Michailides 2010). Carboxin and other SDHI fungicide resistances are described as monogenic, and have been identified in both field and laboratory. Sequence analysis of the gene encoding the target protein, the succinate dehydrogenase enzyme (SDHB), revealed that single or double point mutations in the highly conserved regions of gene were associated with resistance. Different levels of resistance are associated with mutations in different alleles: In *B. cinerea*, mutations resulted in P225L or P225F transitions that confer high resistance, and in a histidine to tyrosine replacement at position 272 (H272Y) or Arg (H272R) (Avenot et al. 2008), although Angelini et al. (2010) found these two mutations resulted in a lower level of resistance. In *A. alternata*, sequence analysis of the SDHB gene from sensitive and resistant isolates identified a H277Y and H277R transitions, as well. (Avenot et al. 2008). It is important to note that in some boscalid-resistant strains of *A. alternata*, there were no identifiable mutations in the AaSdhB gene as compared to the wild type, suggesting that mutation(s) in other loci are involved in the boscalid resistance phenotype. This is not surprising since the boscalid mode of action involves at least two other genes of the Sdh complex (SdhC or SdhD) (Avenot et al. 2009).

Despite the elucidation of the molecular mechanisms of resistance, mycelial growth assay in liquid medium in microtiter plates were developed to monitor fungicide resistance, in *A. alternata* (Avenot and Michailides 2007) and a variety of other fungi, eventually resulting in the development of rapid in vitro monitoring procedures with a single discriminatory dose of boscalid (Avenot and Michailides, 2010), despite the eventual elucidation of many of the molecular mechanisms of resistance.

8.5. MDR-efflux pumps and resistance

All living organisms, eukaryote and prokaryotic, are exposed to both food and toxins in their natural environments. To quote Pao (1998), "Transport systems allow the uptake of essential nutrients and ions, excretion of end products of metabolism and deleterious

substances, and communication between cells and the environment." Some of these deleterious substances may be antibiotics produced by bacteria and actinomycetes, antifungals [e.g., strobilurin A produced by *Strobiluris tenecellus*, killer yeast strains, plant defense compounds (phytoalexins, alkaloids, small molecular weight peptides)], and heavy metals. There are two protein families that are key players in this type of transport process: The ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters. ABC transporters are able to bind and hydrolyze nucleotide triphosphates (mainly ATP) due to a conserved cytosolic, nucleotide-binding fold (NBF or ATP-binding domain) and use this energy to transport solutes across cell membranes (Higgins, 1992). MFS transporters work as a "secondary" active transport system that does not require ATP for functionality, and are capable only of transporting small solutes in response to chemiosmotic ion gradients (Pao 1998). These two families of transporter proteins can mediate a quantitative multidrug resistance (MDR) to multiple classes of fungicides, however, the resistance levels conferred against individual fungicides are greatly reduced as compared to fungicide resistance conferred by target site mutations (de Waard et al. 2006; Mernke et al. 2011).

In the human pathogen *C. albicans*, the role of MDR transporters and resistance is fairly straightforward: The ABC transporter, CDR1, plays a major role in resistance (in a majority of isolates) to fluconazole and miconazole due to upregulation of the gene (Sanglard et al., 1995) resulting in increased efflux of the antifungal and its decreased accumulation in the cell, thereby reducing inhibition of the ERG11/CYP51 gene in most isolates resistant to fluconazole. In addition to its role in resistance to azoles, CDR1 also confers resistance to other sterol biosynthesis inhibitors, including allylamines and morpholines, in addition to several other drugs. A separate transporter, referred to BENR, was highly overexpressed and conferred resistance to azoles and benomyl (Sanglard et al., 1999; Sanglard et al., 1995).

Detection of this type of fungicide resistance has proven to be more difficult, or at least less published, in plant pathogens. Numerous reports exist about multiple fungicide resistances in plant pathogens (Leroux et al. 1997, Nakaune et al. 1998; Leroux et al. 1999; Kretchmer et al. 2011; Chapman et al. 2011). It is important to stress that not all of these multiple fungicide resistances are due to MDR, but in fact, are due to other mechanisms (see the section on Dicarboximides for elucidation of one such mechanism) including segregation of separate, multiple resistances (Chapman et al. 2011). One of the first studies (Leroux et al. 1999) that screened multiple fungicide resistances in *B. cinerea* found resistance to anilopyrimidines (AP, e.g. cyprodinil, mepanipyrim, pyrimethanil) and identified two distinct resistance phenotypes: The most AP-resistant isolates were resistant to only anilinopyrimidines, suggesting action on the hitherto unidentified, single target gene (Hilber and Hilber-Bodmer 1998) and a second phenotype that included resistance to distinctly different classes of fungicides including dicarboximides, phenylpyrroles, sterol biosynthesis inhibitors (e.g. tolfanate, prochloraz, tebuconazole) and the hydroxyanilide derivative, fenhexamid, consistent with an MDR phenotype.

The role of MDR in fungicide resistance was further strengthened by work by Kretschmer et al. (2011) who found that fungicide resistant field isolates from France and Germany

exhibited three distinct MDR phenotypes of increased fungicide efflux activity and overexpression of efflux transporter genes. In this study, MDR1 strains were found to possess mutations in the transcription factor, *Mrr1*, that controls the ABC transporter gene AtrB; the MDR2 strains possessed insertions of a retrotransposon-derived sequence in the promoter region of the major facilitator superfamily (MFS) transporter gene mfsM2 (more thoroughly described by Mernke et al. 2011), and the MDR3 strains which showed the highest levels and broadest spectrum of resistance against most fungicides tested, and was identified as recombinants carrying both MDR1-specific mutations in mrr1 and MDR2-specific mutations in mfsM2 (Kretschmer et al. 2011). All MDR strains showed strong constitutive overexpression of either one (MDR1, MDR2) or two (MDR3) drug efflux transporter genes. MDR1 and MDR3-described isolates had an increased efflux for fludioxonil whereas MDR2 strains did not, while bitertanol efflux was observed for all MDR phenotypes, although MDR1 possessed a less resistant phenotype. Prior to this, Hayashi et al. (2001) found in laboratory isolates, overexpression of the ABC Transporter Gene *BcatrD* involved in resistance to two azoles, in addition to the dicarboximide fungicide iprodione, the benzimidazole fungicide carbendazim, and the antibiotic cycloheximide.

In *M. graminicola*, laboratory isolates were selected with decreased azole susceptibilities and cross-resistance to chemically unrelated of low molecular weight compounds (Zwiers et al. 2002). Later studies found field isolates showed differences in both basal and induced levels of ABC transporter gene transcript, although no correlation between increased expression and azole sensitivity was evident (Stergiopoulos et al., 2003); Cools et al. (2007) were unable to establish a direct relationship between over-expression of the ABC transporters and decreased azole sensitivity, and later studies, using cDNA microarrays to profile the transcriptional response of *M. graminicola* to epoxiconazole, and compared the expression profiles of an azole-sensitive and less sensitive *M. gramincola* isolates did not find a relationship. They found upregulation of ten genes that provided different constitutive expression profiles between the two strains, including drug transporters, a cell surface glycoprotein, stress response protein rds1, and an unknown gene encoding a homologue of the antibiotic response protein in addition to differential expression between components of the sterol biosynthesis pathway between sensitive and less sensitive isolates, and components of the mitochondrial respiratory chain. (Cools et al. 2007). Thus, at least in *M. graminicola*, studies have not demonstrated a relationship between expression (or overexpression) of ABC transporter genes directly (Stergiopoulos et al. 2003) or by microarray (Cools et al. 2007), despite its demonstrated role in field resistance in *B. cinerea*.

Although the role of MDR genes in fungicide resistance is clear in some pathogens, rapid detection of fungicide resistance due to MDR is not. Some of these difficulties reside in the unclear and multiple mechanisms of DMI and other fungicide resistances discussed above, others in the role and regulation of MDR. To date, most differences identified in fungicide resistance where MDR is implicated have been found to be due to overexpression of ABC transporter genes. Most important to stress is the lack of correlation between expression level specific ABC transporter gene with fungicide resistance, suggesting that multiple transporters

may be involved (at least in DMI resistance) or that other mechanisms in addition to upstream transcription factors, have not yet been identified (Cools et al. 2007; Leroux et al. 2011).

9. Conclusions

Fungicide resistance can be conferred by a variety of mechanisms, and plant pathologists need to recognize that multiple genes may be necessary for this resistance, and that single genes, although easily scored or detected by molecular means, may not be sufficient for determination of resistance. Central to these studies is the recognition of the strengths and the limitations of molecular detection, and the importance of phenotypic versus genotypic resistance. This requires that we recognize that the phenotype of fungicide resistance consists (in many instances) of "major" genes, "minor" genes, the interactions between these genes (epistasis) and the interactions between genes and the environment, and that many of the known mechanisms conferring quantitative fungicide resistance utilize alternative metabolic pathways, exclusion or efflux of fungicides, and hitherto unknown mechanisms (Brent and Holleman 1998; Leroux et al. 1999; Lesniak et al. 2011).

The detection of single gene targets is only the beginning, and understanding the mechanism of resistance does not provide a blueprint as to how to manage the crop in the absence of the fungicide lost. Previous studies labored under an assumption that "a timely detection of resistance levels in populations of phytopathogenic fungi in a field would help growers make proper decisions on resistance management programs to control plant diseases" (Ma and Micheilides 2005). It certainly doesn't hurt, but few instances are available where this knowledge has been translated for use to growers and resulted in in-season changes of management beyond cessation of use of the fungicide in question. To date, most resistance management consists of abandoning the fungicide that has failed, to use others (Chapman et al. (2011); Lesniak et al. (2011), Avenot et al., others); Chapman et al. (2011) is one of the few instances where fungicide resistance detection was used many years later to confirm that a fungicide could be used with some degree of successful certainty. This was determined by mycelial growth assay as no molecular detection methods currently exist to screen for dodine resistance.

Fungicide sensitivity testing using mycelial growth or germination inhibition (in petri dishes or microtitre plates) lacks the excitement and appeal of the many different molecular approaches to detect fungicide resistance. It is certainly not as fast as any molecular test, although microtiter-based assays combined with Alamar blue (AB), or resazurin provide quantifiable and early detection within days, for fast growing pathogens like *Monilinia* (Cox et al. 2009) or *Verticillium* (Rampersad 2011). AB is an oxidation-reduction indicator dye used to detect microbial respiration. In the presence of actively growing cells, the resazurin indicator is changed from an oxidized, nonfluorescent blue form to a reduced, fluorescent pink form. Inhibition of growth maintains an oxidized environment, leaving the indicator blue. Results can be easily discerned with the naked eye due to the colorimetric nature of the test, or more rapid and sensitive measurements can be taken with spectroscopy equipment. In these studies, the AB assay provided a rapid and reproducible method of testing

fungicide efficacy and provide an option of deriving quantitative data in the form of degree of resistance versus the qualitative data associated with molecular detection. Use of AB in fungicide resistance requires aseptic techniques that are essential for any microbial assay (Cox et al. 2009). This prerequisite proved to be insurmountable for use of this technique for fungicide resistance screening in *V. inaequalis*, and possibly other fungi. Finally, the calculated the cost of 96 reactions, excluding labor and plate reader, was found to be under $4 per isolate for AB assays and over $15 per isolate for mycelial growth assays (Cox et al. 2009) making this affordable for multiple labs and diagnostic clinics to screen for fungicide resistance as a service. Despite the lack of intellectual appeal, there are other significant advantages of fungicide resistance phenotype screening, chief amongst which is the low cost, accuracy and reliability of the screening for the fungicide resistant phenotype over the genotype, because, unless all known genes and alleles are screened are known and screened, the risk of under-reporting actual resistances exists (Quello et al. 2010), and is rarely discussed.

This is not to say that molecular detection of fungicide resistance is without merit. One of, if not the most critical challenges facing applied plant pathologists is the need for the early detection of fungicide resistance in a population. Molecular methods can be used to powerfully detect and monitor the emergence of resistance in those instances where detection is possible, to a degree that phenotypic assays cannot achieve. Unfortunately, there are few studies where this has been done, and most research ends with a proof of concept, and not the implementation of the developed technique to better manage fungicide resistance. Currently, there are few studies monitoring sensitivity and early detection through the use of sentinel plants coupled with molecular detection in those instances where the mechanism can be detected, which could provide powerful information in an early warning system to anticipate the emergence of fungicide resistance in a population and prevent its occurrence. One problem with this approach is that most models for the detection of fungicide resistance emergence should assume an initial resistance mutation frequency from anywhere from as high as 10^{-4} (Zwiers et al. 2002) to as low as 10^{-8} (Zheng et al. 2000) based upon laboratory studies.

The developing paradigm that has emerged has been the generation of mutants in the laboratory, followed by their isolation from the field in some instances (but not all), followed by phenotypic screen, and identification of mechanism (Albertini et al. 1999 to Zwiers 2002, and others.) Genotypic screens have been developed, but are rarely evaluated for efficacy and accuracy over time, let alone predictive ability or utility. Furthermore, these developments have still not helped the farmer, nor provided him or her with the information necessary to make the appropriate in-season management decision. The question that remains is if we should be willing to shift the paradigm and use the best technique to adequately address the problem of fungicide failure in the field. As we enter a post-genome period, the technology is available for the detection of even single nucleotide polymorphisms. Whether it is available to rapidly detect fungicide resistance in plant pathogens in a way that is useful for growers remains to be seen.

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Chapter 12

Hormesis: Biphasic Dose-Responses to Fungicides in Plant Pathogens and Their Potential Threat to Agriculture

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Additional information is available at the end of the chapter

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1. Introduction

Hormesis is a toxicological concept characterized by low-dose stimulation and high-dose inhibition (1, 2, 3, 4, 5). Extensive examinations of scientific literature by Calabrese and his collaborators reported that hormetic dose-responses are common across biological systems and stressors (4, 6, 7, 8). Scientific literature provides evidence that hormesis can be caused by multiple stimuli (9), such as chemicals (4, 10, 11), radiation (12; 29), heat (13), stress (14), and even exercise (15). Dose-response curves displaying hormesis are characterized by a biphasic behavior (Fig.1). The hormetic zone includes a range of subinhibitory doses that are stimulant, with a peak at the maximum stimulation dose (MSD), and ends at the no observable adverse level (NOAEL), that typically precedes the inhibitory doses (Fig. 1). Our interest in hormesis pertains to the effects of fungicides at subinhibitory doses on fungal and oomycete growth and pathogenicity. Thereafter, for the purposes of this book, we will focus on chemical hormesis alone. Some of the most familiar examples of biphasic dose-responses include vitamins, alcohol, essential minerals, and many drugs (16, 17, 18, 19, 20). Hormesis has been measured using diverse endpoints in multiple biological systems (8). One of the most common endpoints used in hormesis research is growth, but several others have been studied including CO_2 production (1; 21), longevity (14), other metabolic processes, and cellular functions (22). Southam and Ehrlich (2) coined the term hormesis to describe the biphasic dose-response because its Greek etymological root *horm-* means "to exite" (18). Several reports of subinhibitory stimulation of fungi and oomycetes due to exposure to fungicides are available in the mycological and phytopathological literature, but little attention has been paid to fungicide hormesis in spite of its potential detrimental effects to crop productivity.

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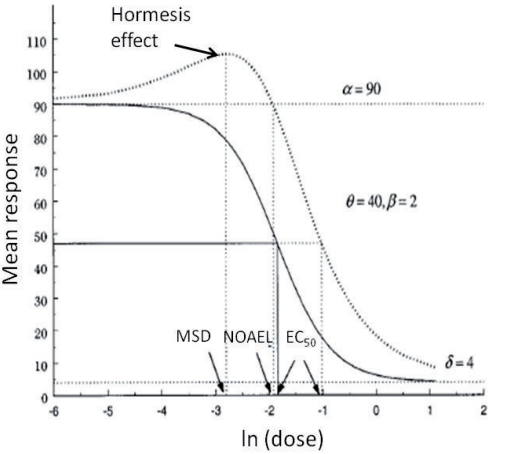

Figure 1. Dose-response curves comparing the traditional threshold model (solid) and the Brain-Cousens model (dotted) that includes hormesis in the dose-response concept. The Brain-Cousens model permits to estimate the no-observable adverse effect level (NOAEL), the maximum stimulation dose (MSD), and the half maximal effective concentration (EC_{50}). Modified from Shabenberg *et al.* (23).

2. Historic background

The first references to dose-responses have been attributed to Paracelsus, who allegedly said "all things are poison and nothing is without poison, only the dose permits something not to be poisonous" (24). However, the first scientific reports of chemical stimulation at low doses and inhibition at high concentrations go back to the 1800s. In 1854, Virchow (22) reported that low concentrations of sodium hydroxide increased the frequency and intensity of beating of ciliae of human tracheal ciliated epithelia; while at higher concentrations or longer exposures the same compound paralyzed ciliae and caused cell death. In 1865, Reveil (25) reported that sodium hypochlorite stimulated seed germination at low concentrations (0.1% solution) but it was phytotoxic at high concentrations. According to Calabrese and Baldwin, in their review of the historical foundations of chemical hormesis as a biological hypothesis (5), two researchers are considered as the founders of modern hormesis, Rudolf Arndt and Hugo Schulz. In 1887, Schulz (1) observed experimentally that several chemicals caused low-dose stimulation and high-dose stimulation on yeast fermentation. Because Schulz's views supported those of Arndt, a homeopathic physician, soon they were merged in to what was known as the "Arndt-Schulz law". The law stated that "for every substance, small doses stimulate, moderate doses inhibit and large doses kill" and that their findings could be generalized to all organisms and all toxic agents. Mainly because of the difficulty at the time to demonstrate the universality of this law without offering explanation of its biological causes, and probably also because at the time it was conceptually associated to homeopathy, over time it fell out of use. However, a few years later Ferdinand Hueppe

(1896), a distinguished bacteriologist, made similar observations in bacteria and described the phenomenon in his books and scientific publications (26). Hueppe recognized the validity of Schulz's scientific research but stated certain limitations and exceptions to the Arndt-Schulz law. Soon the notion that "substances which inhibit biological processes at sublethal doses may be expected to stimulate them at lower levels" became known as the "Hueppe rule" and was broadly adopted in international literature (3, 5).

Years later, in 1929, Branham (21) confirmed Schulz's observations using a series of different chemicals and an improved apparatus to detect CO_2 production, and demonstrated that very small doses of inhibitor compounds had an apparent stimulatory effect on carbon dioxide production by yeasts. One of her experiments assessed the effect of adding crystals of 1,2,5,6-dibenzanthracene to yeast suspensions, finding that at a concentration of 9×10^{-4} molar yeast proliferation increased. The term hormesis was coined by Southam and Ehrlich in their 1943 report of growth stimulation of a wood-decaying fungus (*Fomes officinalis*) in culture by extracts of western red-cedar heartwood at low doses, while higher doses were inhibitory (2).

Research focused on chemical subinhibitory dose-responses continued through the early decades of the 1900s, but scientific attention faded away as the science of Toxicology became established and few hormesis studies were published until the 1980s (3, 27, 28). Toxicology is the study of the adverse effects of chemical, physical, or biological agents on biological systems, their prevention and amelioration. Hence, by definition, Toxicology deals with the negative effects of such agents at doses above the no observed adverse effect level (NOAEL), and essentially ignores the effects of subinhibitory doses. Stebbing (3) reexamined the hormesis concept and provided the first update on the validity of this concept based on the abundance of scientific reports of data with biphasic distributions. Stebbing reported his conclusions after searching the scientific literature for an explanation to his own observations of growth stimulation of the colonial hydroid *Campanularia flexuosa* (3) by exposure to sublethal concentrations of various metals and organometallic compounds; and raised the question whether growth hormesis by diverse toxic substances in multiple biological systems had a common explanation. Although examples of stimulation by subinhibitory levels of chemicals and radiation are numerous in the toxicological literature, interest on hormesis has slowly increased again in the last few decades. Several studies on radiation hormesis followed Stebbing's paper (29, 30, 31, 32), with limited initial impact initially, but the interest on the subject gathered momentum by the end of the decade (33, 34). The few chemical hormesis reports that followed Stebbing's paper had much better acceptance (35, 36, 37). Hormesis once again, although still viewed as a polemic concept by some toxicologists, attracted the attention of the scientific community on the basis of its relevance to risk assessment and optimization of research resources (38). In the late 80s Dr. Edward Calabrese, currently the Director of the Department of Public Health at University of Massachusetts School of Public Health and Health Sciences, became deeply interested in hormesis and began a campaign to create awareness of the biological significance of this phenomenon.

Calabrese first report related to hormesis described stimulation of *Mentha piperita* grown, in soils and *in vitro*, by the growth retardant phosfon (2,4 dichlororobenzyl tributyl phosphonium chloride) at concentrations of $1.26x10^{-5}$ M to $7.77x10^{-4}$ M and $6.30x10^{-7}$ M to $3.78x10^{-5}$ M respectively, while higher concentrations inhibited plant growth (39); nonetheless, no reference to hormesis was made, but wondered about the nature of the stimulation. Eleven years later, Calabrese and collaborators examined the occurrence of chemically induced hormesis in biological and toxicological systems by looking for evidence of low-dose stimulation in literature (37). They found multiple historical and contemporary publications reporting biphasic dose-responses to chemical stressors in plants, fungi, and animals. During the decade that followed Calabrese and Baldwin published four articles reviewing possible examples of chemical hormesis in previously published studies, and discussing the importance of expanding the reference dose concept to incorporate the effect of subinhibitory doses (4, 40, 41, 42). Products of their work were the description of the first quantitatively-based methodology to evaluate chemical hormesis and the development of a chemical hormesis database. These publications served as catalysts for what Calabrese and Baldwin referred to as "the dose-response revolution". The prolific research inspired by these papers has produced hundreds of publications, providing well supported evidence of hormetic effects of a broad spectrum of stressors, including radiation, heat, caloric intake, and even exercise, on plants, fungi, bacteria, protozoa, and animals, including humans (6, 10, 12, 43, 44, 45, 46, 47).

3. Hormesis as a general phenomenon

Based on their extensive literature review, Calabrese and Baldwin stated that hormetic responses often, but not always, display the following characteristics: i) Stimulation zone of the dose-response could be found within a 10-fold range; ii) Stimulatory responses were 30-60% greater than the controls; and iii) the NOAEL three to six-fold greater than the MSD (4). Using these criteria they identified hundreds of toxicological studies that potentially displayed hormetic responses (48). In recent years researchers from many different disciplines have been inspired by Dr. Calabrese's work and have studied hormesis in their biological systems of interest. There are numerous recent reports of biphasic dose-responses in plants, animals, as well as eukaryotic and prokaryotic microorganisms in the scientific literature. A few examples of studies reporting chemical hormesis are presented below.

Velini *et al.* (49) examined the effect of the herbicide Glyphosate on target and non-target plants. The growth of glyphosate sensitive soybean was inhibited when applied at concentrations between 72–720 g AE ha^{-1}; however, low doses (1.8 – 18 g AE ha^{-1}) of herbicide induced significant increases in shoot and total dry weight (up to 28% and 22%, respectively). Similar growth enhancements were observed in maize, *Eucalyptus grandis* Hill ex Maiden, *Pinus caribea* L. and *Commelina benghalensis* L. by 1.8 – 36 g AE ha^{-1}. Barceló and Poschenrieder (10) reported their observations of rapid root growth in corn plants sensitive to soil aluminum (Al). Hormesis was observed in plants exposed to subinhibitory Al levels or as a transient effect after brief exposure to potentially toxic concentrations. A study by Migliore *et al.* (50) of the effects on plants of fluoroquinolone antibiotics administered to

cattle and excreted in their feces, which are later used as field manure, demonstrated that enrofloxacin was toxic to *Cucumis sativurs* L., *Lactuca sativa* L., *Phaseolus vulgaris* L., and *Raphanus sativus* L. seedlings at concentrations equal or above 5000 μg l^{-1}, while growth hormesis was often observed at concentrations 50-100 μg l^{-1} in the four plant species.

The better documented example of hormesis in animals is lifespan increase as a result of restricted caloric intake in diet (24). While high calorie diets have been associated with increased risk of several age-related diseases in animal systems (cardiovascular disease, type 2 diabetes, stroke, among others), dietary energy restriction (i.e. controlled caloric restriction or intermittent fasting) has been reported to have anti-oxidative effects, increasing the cells' tolerance to several types of stresses. For example, restricted calorie diets protected rodents against several types of cancers (51); furthermore, alternate day calorie restricted diet in humans seems to improve inflammatory symptoms in asthmatic patients (52). A now classic example of chemical hormesis are vitamins in human diet, since small amounts of them are necessary and beneficial, but large amounts are toxic and can cause hypervitaminosis, tissue mineralization, and chemical imbalances (45). Other examples of hormesis in animal systems include inhibition of N-diethylnitrosamine (DEN)- initiated pre-neoplastic lesions by phenobarbital at low-doses, while higher doses promote activity (53), and survival and fertility enhancement in *Podisus distinctus* Stål (Heteroptera: Pentatomidae) due to exposure to sublethal doses of the pyrethroid insecticide permethrin (54), among others.

There are abundant examples of hormesis in prokaryotic and eukaryotic microbial systems. As related in the historical review, the first reports of biphasic dose-responses were on bacteria and fungi (1, 2, 26, 21) and several more studies have been published in more recent years. Hotchkiss (55) found that $TiCl_2$, $MgCl_2$ and, NaCl had hormetic effects on the growth of *Escherichia coli* in culture. Low doses of penicillin produced doubled the growth of *Staphylococcus* (No. 6571 N.C.T.C.) in culture compared to the non-treated control (56). Linares *et al.* (57) demonstrated that three antibiotics (tobramycin, tetracycline, and norfloxacin) trigger the expression of determinants that influence the virulence of *Pseudomonas aeruginosa* at subinhibitory concentrations. Wang *et al.* (58) and Gong *et al.* (59) reported increased production of mycrocystin by *Microcystis aeruginosa* (cyanobacteria, Cyanophyta) in non-linear responses to nonylphenol and arsenic pollution. Hong et al (60) reported growth stimulation in *Selenastrum capricornutum* (Chlorophyta, Selenastraceae) due to exposure to subinhibitory doses of the algicide ethyl 2-methyl acetoacetate at high initial algal densities. Many of the early studies on hormesis were done on the effects of multiple chemicals on yeast metabolism (2, 21). Yeasts continue to be used as models for the study of hormesis, particularly related to cancer (61), ageing (43, 62), and UV radiation hormesis research (63).

4. Evidence of chemical hormesis in phytopathological literature

Our review of mycological and phytopathological literature found several studies of fungicide effects on fungi and oomycetes with results that reflect potential hormetic responses. We present some interesting examples below, while an exhaustive literature

review will be reported elsewhere. Southam and Ehrlich (2) observed that extracts of western red-cedar heartwood were stimulatory at low doses on the growth of a wood-decaying fungus (*Fomes officinalis*) in culture, while higher doses were inhibitory. This was the first scientific study that demonstrated stimulation by a mycotoxic compound on a plant pathogenic fungus, and the authors coined the term hormesis to describe their observations. Later studies of hormesis in plant pathogens demonstrated a positive effect of trichothecin, a compound produced by *Trichothecium roseum,* on the growth of *Fusarium oxysporum* (64, 65) while assessing the production of trichothecin in different soil types. Although not further references to hormesis were made in the phytopathological literature until recently (66), several reports of stimulation by exposure to fungicide are available. Baraldi *et al.* (67) reported higher percentages of germination in seven out of 41 thiabendazole (TBZ) resistant isolates of *Penicillium expansum* from pear when grown TBZ-amended media than without the fungicide. The authors suggested that germination stimulation and fitness advantage in certain TBZ-resistant *P. expansum* isolates could be due to the ability of this isolates to metabolize the fungicide as a nutrient compound, but further study was recommended to understand the nature of the stimulation. Audenaert *et al.* (68) observed increased production of the mycotoxin deoxynivalenol (DON) by *Fusarium graminearum*, *in vitro* and *in planta*, when exposed to sub-lethal doses of the triazole fungicide prothioconazole, and proposed that mycotoxin production was stimulated by H_2O_2 production triggered by the fungicide at low concentrations. While studying the effects of different fungicides on the infection of *Sphagnum* by fungi, Landry *et al.* (69) observed significantly increased radial growth of *Lyophyllum palustre* (Peck) *in vitro* when media was amended with the fungicide propamocarb compared to the non-amended control.

Similar references can be found on oomycetes literature. Fenn and Coffey (70) observed that 69 μg/ml of phosphorous acid (H_3PO_3) was stimulatory on the growth of *Pythium ultimum in vitro* and that 138 μg /ml was also stimulatory on the growth of *Pythium myriotylum*. In a study comparing the sensitivities of various oomycetes to the fungicides mefenoxam (a metalaxyl enantiomer, FRAC code: 4) and hymexazole (FRAC code: 32), Kato *et al.* (71) observed a range of responses to these fungicides among the different groups that could be used to classify them by taxa as reflected by DNA analysis. Significant findings included the differential fungicide sensitivity of the plant pathogens *Pythium* and *Phytophthora*. Although fungicide sensitivity responses varied within the two genera, general trends suggested that *Pythium* species were more sensitive to hymexazol than to mefenoxam, while the opposite was true for *Phytophthora* species, with a few exceptions. The reported sensitivity response curve for *Phytopthora undulata* reflected slight stimulation at the lowest hymexazol doses. Radial growth stimulation was observed in three out of four metalaxyl-resistant *Phytophthora infestans* isolates when grown on cleared lima bean agar medium amended with 20μl/ml (72). Since one of the isolates grew more in the presence of metalaxyl only when nutrients were limited, it was hypothesized that under certain circumstances metalaxyl could be beneficial to metalaxyl-resistant *P. infestans* strains. Moorman and Kim (73) reported for the first time strains with dual resistance to mefenoxam and propamocarb in *Pythium aphanidermatum*, *P. irregulare,* and *P. ultimum* and described radial growth

stimulation in some strains of the three species by propamocarb at a concentration of 1μg/ml, and of *P. aphanidermatum* by 1,000 μg/ml propamocarb.

5. Fungicides hormesis and its impact on fungal plant pathogens

Recent research on chemical hormesis on fungal pathogens has focused on the effects of subinhibitory doses of fungicides on radial growth and pathogenicity of fungi and oomycetes (66, 74). Garzon *et al.* (66) examined the effects of subinhibitory doses of mefenoxam on the radial growth and pathogenicity of a mefenoxam and propamocarb-resistant isolate of *P. aphanidermatum*. In this study we found modest radial growth stimulation, with an average of 10% increase over the control, and very significant increase of pathogenicity, with an increase of 61% severity of damping-off of seedlings in geranium. Flores and Garzon (74) reported standardized laboratory and statistical protocols for detection of chemical hormesis using radial growth *in vitro* as endpoint. Using the reported methods hormetic responses were detected in *Pythium aphanidermatum*, *Rhizoctonia solani* and *R. zeae* exposed to ethanol (Fig. 2), and on *P. aphanidermatum* exposed to subinhibitory doses of the fungicides propamocarb and cyazofamid (Fig. 3).

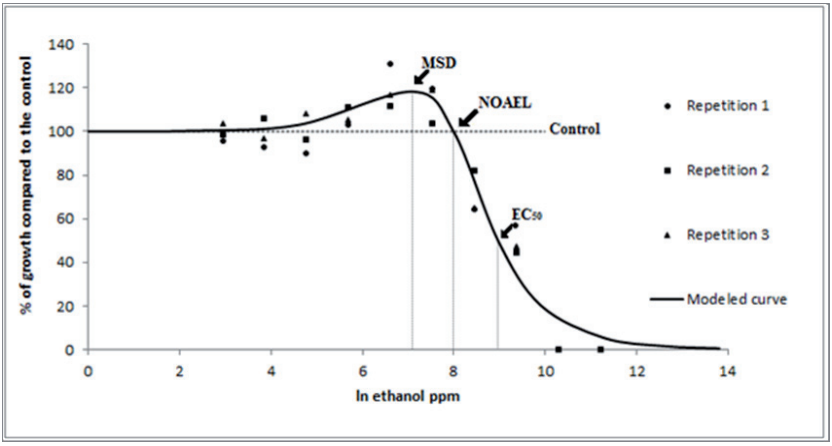

Figure 2. Modeled curve of the radial growth *in vitro* of *P. aphanidermatum* in response to subinhibitory doses of ethanol (Flores and Garzon *in press*). Radial growth is expressed as percentages relative to a non-amended control, and concentrations as natural logarithm of ppm. Figure reproduced with permission of Dose-Response Journal.

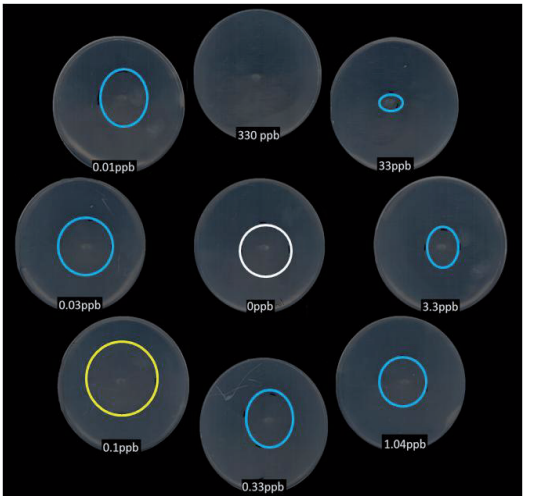

Figure 3. Radial growth of *Pythium aphanidermatum* on corn meal agar amended with cyazofamid. Significant hormetic stimulation was observed at 0.1 ppb cyazofamid, with 17% radial growth increase (yellow) over the control (white).

6. The biological basis of hormesis

Hormesis can result from overcompensation after a disruption of homeostasis by stressors, by direct stimulation, or as a response to an adaptating dose followed by a larger dose (3, 75, 76). The research by Branham (21) on the effect of 16 chemicals on CO2 production by Baker's yeast, provided clear evidence, in 12 of the chemicals, of an initial mild inhibition followed by significant stimulation. These results supported the hypothesis of stimulation due to overcompensation, being most evident for formaldehyde, phenol, iodine, and metaphen. Other examples of over-compensatory responses include ethanol stimulation of locomotion in mice (77), increased serotonin levels in rat neurons after treatment with below toxic doses of 5,6-dihydroxytryptamine (78); and growth stimulation in peppermint following an initial decrease after treatment with phosfon, a plant growth regulator (39), among others. Stebbing (79) provided an "improved" explanation for hormesis due to overcompensation by describing a model using two overlapping curves, an *effect* curve and a *response* curve, relative to a particular endpoint and the compensatory mechanisms involved, respectively. Under this model, below a threshold an stressor may be undetectable by an organism, and after reaching that threshold compensatory mechanisms would be triggered by a range of doses (represented by the *response* curve), however no effects would be visible, due to neutralization by compensation responses; effects would be evident only after the capacity to neutralize inhibition is exceeded and may follow a linear pattern. In systems where compensation responses completely neutralize inhibition, the alfa curve is present (threshold dose-response) with no evident low-dose effects, while in those where compensation responses exceed inhibition the beta curve is observed (hormetic curve).

Overcorrection of inhibition may have an adaptation role with impact on fitness. This hypothesis was supported by Stebbing's observations that hydroid colonies pre-exposed to a subinhibitory dose of copper (10 $\mu g.l^{-1}$) had increase tolerance across the range (0 – 50 $\mu g.l^{-1}$), when compared with non-pre-exposed colonies. Calabrese suggested direct stimulatory response as another possible cause of hormesis (8).

The underlying mechanisms that generate hormetic responses have not yet been fully understood. Conolly and Lutz (80) hypothesized that hormetic responses may occur due the superimposition of two monotonic dose-responses, one that takes effect at low doses and other that overtakes at higher doses undermining the first one. They demonstrated by computational modeling that four different cellular models could generate biphasic dose-responses: i) Membrane receptor subtypes with opposite downstream effect; ii) Androgen receptor mediated gene expression; iii) Induction of DNA repair and "co-repair" of background DNA damage; and, iv) Modulation of the cell cycle and effect on rate of mutation (80). Subsequent studies have found empirical evidence of hormesis attributable to the presence of antagonistic membrane receptors (81) or to the induction of DNA repair (82). Bae *et al.* (83) suggested that hormesis may arise because of the heterogenic susceptibilities of different tissues to the same stimulus; such difference can result in the expression of a U shaped dose-response curve. The observations that drove this conclusion were made from the response of different cell types normally present in human blood vessels to the presence of small doses of arsenic and a reactive oxygen species generator (menadione). Allender (84) and Allender *et al.* (85) provided indirect evidence of the influence of calcium influx to the cell on hormetic responses related to plant growth. The diversity of the models that may show a hormetic response suggests that the mechanisms acting may not be the same for different systems. Experimental evidence suggests that multiple metabolic processes may be involved in hormetic responses, some acting during the stimulation phase and others, probably different, acting during the inhibition phase of the beta curve.

7. Studying hormesis

Detection of hormesis is often challenging due to the multiple factors that can affect metabolic responses of the target organisms. For example, when studying fungicide hormesis in oomycetes using radial growth as endpoint it is fundamental to standardize every experimental factor involved; in addition to growing media type and concentration, fungicide treatments, and incubation temperature, other factors are also relevant, including light, growing media depth, inoculum age and developmental stage, mixing time when preparing fungicide dilutions, using fungicide stock solutions prepared on the same day of the experiments, etc. Variation in any of these parameters can influence mycelial growth significantly, hence introducing experimental variation that could affect the reproducibility of results (66).

When trying to prove the existence of hormesis there are some requirements that the experimental design should fulfill: i) The NOAEL should be determined; ii) doses below the NOAEL need to be tested with five equally spaced doses providing enough data to detect

hormesis; and iii) the separation between doses should generally be smaller than one order of magnitude since the hormetic zone is usually within a ten-fold range (42). To test for hormesis researchers must compare the effect of small doses with the response of the non-treated control. Therefore, there should always be background incidence in the control, without background incidence there is no way to detect a stimulus (80). Evaluation of data is very important when proving hormesis. Crump suggests the criteria for evaluating hormesis as follows: strength of evidence, soundness of data, consistency and biological plausibility (86). Statistical analyses should be performed in order to differentiate a small stimulus from background occurrence.

Different methods have been used throughout the years for the detection and estimation of hormesis including parametric, non-parametric, and model-based approaches. The hormetic zone of a dose-response curve follows a non-monotonic relationship between two variables, similar to what is known as umbrella alternatives. Umbrella alternatives are important in many fields of science; a classical example is the ability of learning as a function of age in humans (87). As we grow older our ability to learn new things reaches a peak and later declines. Tests for umbrella alternatives can be used to detect if a dose-response curve follows a non-monotonic trend compared to a monotonic one where hormesis would not be present. The firsts to describe a test for umbrella alternatives were Mack and Wolfe (87) who used a non-parametric method where the distribution of the data is not assumed a-priori. In the Mack and Wolfe test (87) the maximum stimulation detected experimentally is compared to the response at all the other doses using Mann-Whitney counts, a test statistic is calculated and compared with simulated critical values to determine if the dose-response is biphasic. Buning and Kossler (88) demonstrated that the Mack and Wolfe (87) test with Mann-Whitney counts is appropriate for testing data with symmetric and medium-up to long tailed distributions but they suggested the use of different two-sample statistics, i.e. Hogg *et al.* (89) and Gastwirth (90), for asymmetric and short-tailed distributions respectively.

For the detection of hormesis there are also parametric analyses which assume a normal distribution of the data and can have more statistical power than non-parametric analyses if such assumptions are correct. Among the parametrical tests we can highlight the one proposed by Buning and Kossler (88), a modification for umbrella alternatives of the test by Barlow *et al.* (91) for monotonic alternatives. And the method by Bailer and Oris (92) that employs generalized linear models for the detection of hormesis. There are also parametric models that can be used to detect the hormetic response. A parametric model is an equation with a finite number of parameters that describe the relationship between two variables, in the case of hormesis it describes a byphasic relationship between dose and response. If the data fits the model within a confidence limit, usually of 95%, it is assumed that hormesis is present. There are some models that can be modified to better fit the hormetic response, including the quadratic function (93), Gompertz function (94, 95) and logistic function (96). Deng *et al.* (97) summarized some of these model-based approaches and developed a method to estimate the magnitude of the hormetic response. When testing for chemical hormesis the Brain and Cousens model (96), based on the logistic function, is the most

commonly used. Further modifications of the Brain and Cousens model have been made by Van Ewijk and Hoekstra (98), and Schabenberger *et al.* (23) in order to allow estimation of different parameters that are relevant for toxicology such as EC_{50}, NOAEL and maximum stimulation dose (MSD). According to Cedergreen *et al.* (99), a deficiency of the Brain and Cousens model is that if the slope at the EC_{50} (β) is smaller than 1 then the model will not yield a curve; therefore they proposed a model that circumvents this drawback. Cedergreen *et al.* (99) provide a robust detection method but it does not estimate the EC_{50}, NOAEL or MSD. In general, a combination of methods may be used to detect hormesis, determine its magnitude and estimate the EC_{50}, NOAEL and MSD, depending on the type of data that is being analyzed and on the behavior of the dose-response curve.

When testing for the stimulation at low doses of chemicals on fungal plant pathogens, Flores and Garzon (74) used a model-based approach. The Brain and Cousens model was appropriate for this case where most of the dose-response datasets analyzed yielded a β higher than 1. Because of the relevance of EC_{50}, NOAEL, and MSD for disease management, the modified model by Schabenberger *et al.* (23) was used. Flores and Garzon (74) tested 9 to 11 doses of different chemicals including pesticides and disinfectants on *Pythium aphanidermatum* and *Rhizoctonia zeae* with at least five doses below the NOAEL. Datasets were analyzed using a non-linear modeling procedure (PROC-NLIN, SAS 9.2, SAS Institute, Cary, NC) and EC_{50}, NOAEL, and MSD were estimated as described by Schabenberger *et al.* (23). The procedure yielded dose-response curves that properly described the behavior of the data and showed their biphasic nature (Fig 4).

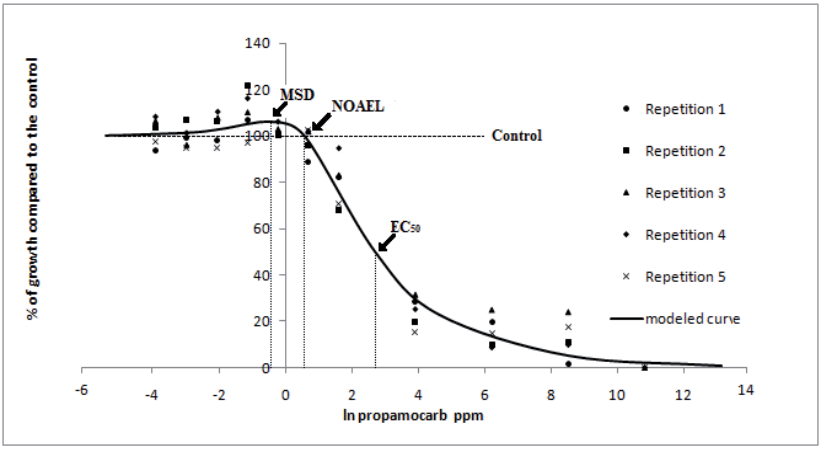

Figure 4. Observed values and modeled curve of the radial growth *in vitro* of *P. aphanidermatum* in response to low doses of propamocarb. Radial growth is expressed as percentages relative to a non-amended control, and concentrations as natural logarithm of ppm. Each data point in the figure represents the mean value of five replicates. Arrows indicate the natural logarithm of the EC_{50} (14.91 ppm), NOAEL (1.77 ppm) and MSD (0.64 ppm). The slope of the curve at the EC_{50} (β) was 1.37 and the rate of increase at low doses (γ) was 35.17. Low-dose stimulation was observed (Flores and Garzon, [74]). Figure reproduction authorized by Dose-Response.

8. Why is chemical hormesis relevant for crop management?

Multiple chemicals with distinct modes of action are available for management of fungal and oomycete diseases. Although integrated disease management is practiced extensively, the productivity of many agricultural systems relies strongly on chemical control. The limited access to registered products for certain agricultural environments, such as greenhouses, as well as inappropriate use has led to the emergence of fungicide resistant strains in multiple species (100, 101, 102, 103, 104, 105, 106). Currently, the effects of subinhibitory doses of fungicides on fungal plant pathogens are unknown. The evidence gathered from literature indicates that stimulation of fungi and oomycetes by sub-inhibitory doses of fungicides has been observed in ascomycetes (64, 65, 67, 68), basidiomycetes (2, 69, 74), as well as in oomycetes (66, 70, 71, 72, 73, 74). Several fitness factors could be affected for the benefit of pathogens, including mycelial growth, spore germination, toxin production and pathogenicity (66, 67, 68, 74). Exposure to subinhibitory doses can occur accidentally in agricultural fields, orchards, nurseries and greenhouses, under diverse circumstances, such as inappropriate fungicide application, low-dose applications to reduce costs, presence of fungicide resistant strains, etc. Thereafter, the possibility of fungal pathogen stimulation due to fungicide hormesis in actual agricultural scenarios is real. The potential effects of fungicide hormesis are highly detrimental, since it could result in larger crop losses, reduced seed and crop quality, higher mycotoxin levels in grain, and wasteful use of fungicides.

In spite of the potential detrimental effects of fungicide hormesis on fungal plant pathogens to agricultural productivity, a complete lack of awareness has meant the exclusion of this important concept from the design of disease chemical management strategies. In some fungicide-pathogen systems, the value of the EC_{50} can be different when hormesis is included in the analysis, hence it is important to consider this concept to avoid bias in EC_{50} calculations. Awareness of the risk taken by growers by the inappropriate use of reduced-dose fungicides (reduced-dose fungicides can be used in combination with two or more other formulations, with different active ingredients [107]) and careless chemical application will help to promote the use of best-management practices and responsible use of fungicides.

More research is needed to understand the processes involved in fungicide hormesis, the prevalence of hormesis in fungi and oomycete species and populations, fungicide class risks, whether mixtures can prevent stimulation, etc. Hormesis is not a new concept but its use in plant pathology is recent, and its application to disease management may open new opportunities to improve plan health and crop productivity.

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