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Target-site mutations and Multidrug resistance of *Botrytis cinerea* and its control through potential biocontrol agents

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I hereby declare that the work submitted is mine and that where I have made use of another's work, I have attributed the source(s) according to the Regulations set in the Student's Handbook.

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Abstract

This dissertation was written as part of the MSc in Sustainable Agriculture and Business at the International Hellenic University.

Over the last decades, fungicide resistance has become an even greater threat to the agricultural world. It is vital to understand in depth the resistance mechanisms and the effect of potential biocontrol agents. This literature-based dissertation focuses on creating a data pool, mainly over the last decade, concerning different traits of fungicides used against the plant pathogen *Botrytis cinerea*, while also analyzing Multidrug resistance (MDR) characteristics and potential microbes and extracts with anti-Botrytis effect.

In this study, every class of botryticides is analyzed regarding the mode of action, resistance conferring mutations and resistance frequency. Particular importance is given to a couple of classes, anilinopyrimidines and phenylpyrroles, for which certain aspects, like their specific molecular target protein and resistance-conferring mutations are yet to be defined. Moreover, some insight is provided on the emergence of Multidrug resistance, being a phenomenon new to the agricultural worlds, with MDR1h phenotype displaying unexpectedly higher frequencies, significantly wider distribution and greater resistance levels compared to the other Multidrug resistant phenotypes. Finally, certain newly published potential biocontrol agents are summarized, in particular their effect and results. Bacteria in the genus *Bacillus* and certain fungal species like *Aureobasidium sp.* and *Candida sp*. seem to exhibit the greatest effect in inhibiting the growth *of B. cinerea* and thus the grey mould disease.

Concerning Target-site resistance, it is a common phenomenon in *Botrytis* because the principal method for controlling this pathogen is chemical control. However, the repeated use of fungicides harbours many dangers and threats, one of them being fungicide resistance development. In addition, *Botrytis cinerea* biology renders it a high risk

pathogen concerning resistance development. Target-site alterations in some gene targets grant resistance to different fungicide classes. Regarding *B. cinerea*, there have been found mutations, granting resistance to almost every class of botryticides currently in use.

Apart from target-site resistance, Multidrug resistance has also been recently discovered in *B. cinerea*. MDR is a known phenomenon in medicine, since it occurs in cancer cells and human pathogenic *Candida spp*., making their treatment way more demanding. It involves mutations in certain genes, leading to overexpression of drug efflux pumps of the cell, granting simultaneous resistance against many different-mode-of-action drugs.

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1 Contents

1. Introduction

1.1 Pathogen agent, symptoms, and host range

Botrytis cinerea, with the teleomorphic stage *Botryotinia fuckeliana* (de Bary 1866) is a necrotrophic plant pathogen that belongs in the family Sclerotiniaceae, class Leotiomycetes and division Ascomycota. Its host range is notably wide, including more than 220 plant species (Jarvis et al. 1977). More recent studies showed that *B. cinerea* has been found on 586 plant genera, representing more than 1300 plant species (Elad et al. 2015). Infections by this pathogen usually manifest themselves in the overground plant organs (leaves, fruit, stems, etc.). However, infections can occur in seeds and propagation material (Elad et al. 2004) while during the bloom stage the fungus can cause latent infections (Williamson 1994). Worldwide, it causes annual crop damages which result in $10 billion to $100 billion financial losses. It possesses a wide enzymatic and structural arsenal which enables the pathogen to elude a broad range of plant defense compounds. Moreover, concerning the molecular approach, *B. cinerea* is a model organism, being one of the most explicitly studied plant pathogen (Boddy 2016).

Botrytis cinerea is the pathogen cause of grey mould disease. The pathogen has a wide range of hosts and can cause significant damage to many crops. Great losses have been recorded in vegetable crops in the field and even more in greenhouses and tunnels since the conditions there favor the pathogen's development.

Table 1: Most significant plant hosts of *Botrytis cinerea* (Data taken from study: Elad et al. 2015)

Genus	Species	Family	Disease
Vitis	*V. vinifera*	*Vitaceae*	Grey mould
Solanum	*S. lycopersicum*	*Solanaceae*	Grey mould, ghost spots
Fragaria	*F. ananassa*	*Rosaceae*	Grey mould, dry crown
Actinidia	*A. deliciosa*	*Actinidiaceae*	Grey mould, fruit rot

Certain crops are devastated by grey mould disease (Table 1). In heated tomato greenhouses infections are restricted to the stem area (Elad et al. 1995). Furthermore, grey mould is one of the major diseases of strawberry crops, both in the field and in specific plastic tunnels, where it causes both pre and post-harvest rot (Boff et al. 2001). *B. cinerea* is also a great threat to vineyards because it can infect grapes and cause drastic yield reduction and wine quality reduction, especially in red wines (Bulit and Dubos 1988).

1.2 Pathogen Biology

B. cinerea is a necrotrophic fungus which after infection, it kills cells, causes host tissue death, and grows on them by sporulating or by constructing mycelia structures of long-

term survival. In this way, the pathogen can survive in different environments with various forms like mycelia, microconidia, macroconidia, chlamydospores, sclerotia, apothecia, and ascospores (Jarvis 1980). These structures are formed in living or dead tissues and constitute the pathogen's inoculums.

The fungus forms sclerotia which vary in shape and size, depending on the fungal strain and environmental conditions. Sclerotia constitute the most important survival structure of the pathogen since they can survive harsh and unfavorable conditions and then germinate to mycelia, conidiophores with conidia or apothecia (Coley – Smith 1980).

Another survival organ is chlamydospores. The pathogen can effectively pass through unfavorable conditions by forming chlamydospores in aged or infected hyphae, which in turn germinate to mycelia hyphae or conidia (Holz et al. 2004).

In contrast to the other reproductive formations, conidia are generally short-lived. Conidia are asexual spores that germinate during their contact with plant surface and form certain structures like the appressorium and the penetration peg, which help the pathogen to breach the cuticle of the plant host. Physical pressure alone is not likely to be sufficient at granting access to the plant interior because the appressorium is tied with the germ tube, with the absence of a septum, and thus not enough turgor can be generated. To make up for that, the pathogen possesses enzymes and production systems of H2O2. Once the cuticle is breached, the penetration peg invades an epidermal cell and grows in the pectin-rich cell wall (Boddy 2016)

Conidia lifespan is short. Their survival depends on temperature, available humidity, microbial action, and solar exposure. Nevertheless, the main environmental factor that affects conidia survival is ultraviolet solar radiation. Moreover, the production of microconidia, an alternative form of reproduction, has also been observed during harsh conditions (Jarvis 1980).

The fungus *B. cinerea* shows great variability in all levels, corporal, metabolic, and genetic (Munoz et al. 2002). This happens mainly due to the phenomena of heterokaryosis and

aneuploidy and much less to sexual reproduction, which occurs only rarely in nature (Jarvis 1980). The mating-type which controls *B. fuckeliana* reproduction is heterothallic with two gene alleles MAT 1-1 and MAT 1-2. However, some strains are heterokaryotic regarding their mating type containing both alleles. This phenomenon makes them self-fertile (Faretra and Pollastro 1993). Faretra et al. (1988) confirmed the importance of heterokaryosis by reporting that in single-spore isolates from homothallic strains, there appeared both homothallic and heterothallic isolates. The transmission of resistance to the offspring also proves the significance of heterokaryosis since it was discovered that some pathogen isolates do not transfer their resistance to their descendants or that resistance is lost in some asexual ones. This means that maternal isolates are heterokaryons between resistant and sensitive genotypes. The reproduction of the pathogen is intriguing and resistance inheritance is tightly attached to it (Farretra and Pollastro 1993; Pollastro 1996).

1.3 Epidemiology

Botrytis cinerea inoculum is always present in the environment and its production and dispersion are procedures that happen constantly and are directly connected to the environmental conditions (Jarvis 1980). The pathogen can overwinter as mycelia in living or dead tissue and sclerotia in plant debris (StrØmeng et al. 2009). Through these forms, conidiophores with conidia will emerge in early spring, marking the primary inoculum. The major factor which affects conidia production and dynamics is the humidity of the attaches surface and environmental temperature (Jarvis 1980).

Airborne conidia constitute by far the most significant infecting unit of the pathogen, though every part of the fungus can serve as a dispersion unit. They are dispersed by wind, rain, and insects. Wind velocity and inoculum position (high or low) play an important part in dispersion (Fitt et al. 1985). After conidia dispersion, their adhesion to the host surface follows, leading to their germination. The fungus' entrance occurs with the aid of

appressorium which is notably different from other plant pathogens (Mendgen et al. 1996).

The most important factors affecting conidia germination are the high relative humidity, the water film presence in the host tissue, and the appropriate temperature (10-20oC) (Mendgen et al. 1996). Natural openings and wounds can facilitate pathogen entrance. However, it is known that cellular membrane disruption can also occur enzymatically with enzymes like chitinases, proteases, pectinolytic enzymes, etc. (Cotoras and Silva 2005). Another phenomenon that is connected to the epidemiology of the fungus is its ability to remain in the latent situation inside host tissue for a significant period from cultivating season to harvest period, causing latent infections (Elad 2004)

1.4 Pathogen Control

Control of the pathogen is still a major issue and it includes a combination of different methods, chemical, biological, and cultivating practices. Chemical control is until now the main control method of this fungus. Fungicide application with a different mode of action is made by spraying the above-ground parts of the plant. However, botryticides application can occur in seeds, bulbs, and harvested fruit. However, consumers are skeptical about the safety of these products. In addition to that, resistance, which was developed after the intensive use of chemicals, has made chemical control problematic.

Cultivating practices and sanitary measures can significantly contribute to disease limitation through prevention. These measures aim to create an unfavorable environment for the pathogen. For instance, lack or overflow of certain nutrients (calcium, potassium) can directly affect the vulnerability of the tissues, thus facilitating pathogen infections. Another example, common in grapes, is that plant and foliage density is connected to increased fungal infections (Steel et al. 2001). Cultivating techniques like pruning and leaf removal near the fruit zone can lead to increased aeration and lighting preventing the development of favorable conditions for the pathogen. In greenhouse crops, heat and ventilation are used to prevent the accumulation of humidity (Morgan et al. 1984). In

general, the main target of these measures is the reduction of the relative humidity since it is the main factor harboring pathogen infection.

Finally, during the latest years, new measures and techniques are more and more frequently applied. Such is biological control (Köhl et al. 2020). Some advantages of this method is the absence of resistance development and the easy acceptance from the consumer public (Elad and Stewart 2004).

1.5 Resistance Development

B. cinerea is considered a high-risk pathogen concerning resistance development mainly due to its significant genetic variability, intense sporulation, a great number of hosts, and excessive applications of botryticides used in each cultivating season (Myresiotis et al. 2007)

1.5.1 Resistance mechanisms

Chemical control is the most common and efficient weapon to combat plant pathogenic fungi. For more than 7 decades, the use of synthetic fungicides has been intensified. As a result, the selection of resistant mutants has emerged (Maia et al. 2020). These resistant strains preexist in nature but their frequency depends on the selection pressure forced by the repeated applications of fungicides. That which determines the survival of these strains is their fitness compared to the sensitive wild type ones (Ma and Michailides 2005). One of the major problems that farmers face worldwide is the evolution of fungicide resistance, especially where high resistance factors have been observed and the frequencies of mutant phenotypes are high. As a consequence, this phenomenon could dramatically lower the efficacy of the active ingredients in use, which could increase chemical control cost and the unveiling of lurking dangers concerning the environment and non-target organisms because of repeated treatments (Brentand and Hollomon 2007).

Several resistance mechanisms have been detected in plant pathogenic fungi. The most common resistance mechanism in plant pathogenic fungi is the alterations in the target

site proteins. These altered proteins provide at the same time both functionality and reduced susceptibility to fungicides. Point mutations, insertion, or deletion mutations can occur in the target gene which could result in target site changes in the protein. Nevertheless, there are also other resistance mechanisms identified at lower frequencies. They include: (i) target site overproduction which results from mutations leading to the overexpression of the target gene, (ii) activation of an alternative pathway compensating for inhibition of the major pathway, (iii) greater fungicide detoxification or lower levels of profungicide activation, and (iv) decreases in fungicide influx or increases in fungicide efflux, resulting in lower fungicide content within the cell (Brent and Hollomon 2007). Moreover, target-site resistance has been recorded against all site-specific fungicides and often leads to high levels of resistance against different fungicides that share the same mode of action (cross-resistance). The first three mechanisms often lead to specific resistance, i.e., resistance concerning only one molecule or a class of fungicides. The last mechanism generally involves the constant overexpression of membrane drug efflux proteins mainly ABC (ATP binding cassette transporters) and MFS (Major facilitator superfamily). Because of their relatively low substrate specificity, both types of transporters can lead to multidrug resistance (MDR) to various unrelated classes of fungicides. MDR is an important phenomenon in human pathogenic microbes and cancer cells and is beginning to emerge in plant pathogenic fungi, even though resistance levels against individual fungicides are generally much lower than those achieved by target site mutations.

In this project, the classes of drugs used against *B. cinerea* are analyzed with emphasis on classes for which certain aspects have not yet been deciphered, like mode of action and resistance mechanisms. Also, the search also focused on the effect of certain fungicides on *Botrytis cinerea* resistant populations after their discontinued use. Moreover, the types of Multidrug resistance are summarized. This type of resistance is known to other scientific fields like medicine, but it is new to the agricultural world. Finally, a brief collection of potential published biocontrol agents of *B. cinerea* is presented.

1.6 Aim of Study

The aim of this study is to gather and assemble recent bibliographic data concerning fungicide resistance in *Botrytis cinerea* and its biological control, with emphasis on published articles of the last decade. Its goal is to aid future surveys regarding i) the fungicide resistance in *B. cinerea*, more particularly mode of action, resistance-conferring mutations and resistance frequencies of each class of available botryticides, ii) the development of Multidrug resistance and its possible hazardous consequences in agriculture since it is a newly discovered phenomenon in *Botrytis cinerea* and vigilance is required, iii) the alternative control method of this disease, biological control, and present some of the latest published biocontrol agents.

2. Fungicide Classes

In this section, the major botryticides classes will be analyzed, along with their mode of action, chemical structure and function of target protein, resistance-conferring mutations, and resistance frequencies.

2.1 Multisite inhibitors

Multisite inhibitors are some of the oldest fungicides still in use. They include dithiocarbamates (e.g., mancozeb, thiram) or trihalomethylthio derivatives (phthalimides) (e.g., captan, folpet) (Fig. 1). They act by blocking certain thiol-containing enzymes and thus interfering with the process of respiration (Lyr 1977). Although their first use dates decades ago, they play only a minor role in plant protection nowadays, compared to more active synthetic compounds. Because of their non-specific mode of action, they pose only low resistance risk. However, reduced sensitivity of *B. cinerea* to these multisite inhibitors has been observed in a few cases, probably because they shared the same mode of detoxification (Barak and Edgington (1984).

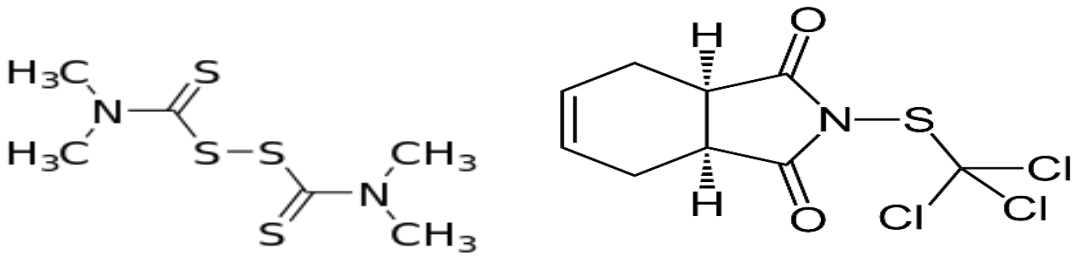


Figure 1: Chemical structure of thiram (left) and captan (right)

Table 2: Studies concerning different fungicide classes, resistance mutations, and results.

Fungicides used in each study	Target / Mode of action	Mutations involved	Effects and results	Host species	Reference
Benzimidazoles (carbenazim)	β tubulin	E198A/K/V F200Y	Fitness assessment and application value of carbendazim	Cucumber, Tomato, Pepper, Kidney bean	He et al. 2020
Benzimidazoles Diethofencarb	β tubulin	E198A/K/V F200Y	Resistance monitoring	Strawberry	Liu et al. 2019
Benzimidazoles	β tubulin	E198A/K/V F200Y	Mutation frequencies and LAMP method development	Tomato, Strawberry	Ya Bing Duan et al. 2018
Dicarboximides (iprodione)	Osmosensing histidine kinase, MAPK cascade	I365	Resistance mechanisms and evaluation of resistance adoption	Tomato	Maqsood et al. 2020

Dicarboximides, Benzimidazoles	Osmosensing histidine kinase, MAPK cascade,β tubulin	-	Resistance monitoring	Wine grape	Bertetti et al. 2018
Phenylpyrrole (Fludioxonil)	Inhibition of HOG cascade of MAPK pathway	-	Fitness evaluation and resistance mechanisms	Tomato	Zhou et al. 2019
Phenylpyrrole (Fludioxonil)	Histidine kinase BOS1	I365N/S	Resistance detection and mechanisms	Strawberry	Gong et al. 2019
Dicarboximide (iprodione) Anilinopyrimidine (cyprodinil)	Osmosensing histidine kinase, MAPK cascade	I365N/S, Q369P, N373S	Resistance monitoring	Pistachio, Grape, Pomegranate	Avenot et al. 2018
QoI (pyraclostrobin)	Qo site in cytochrome b	-	Effect on membrane integrity and fitness	Tomato	Xiong et al. 2020
QoI (azoxystrobin)	Qo site in cytochrome b	G143A, Bcbi 143/144 intron	On- site evaluation of resistance development	-	Hu et al. 2017
QoI (trifloxystrobin, pyraclostrobin)	Qo site in cytochrome b	G143A	First report of QoI resistance	Strawberry	Trkulja et al. 2016
Hydroxianilide (Fenhexamid)	3-ketoreductase	-	Resistance monitoring and Botrytis group S identification	Strawberry	Yin et al. 2015
Hydroxianilide (Fenhexamid)	3-ketoreductase	F412I/L	Selective system for genetic modification	Hydrangea	Cohrs et al. 2017

Hydroxianilide (Fenhexamid)	3-ketoreductase	V365A,E368D,A378T	Reistance monitoring, patterns and fitness evaluation	Strawberry	Zhou et al. 2017
Hydroxianilide (Fenhexamid)	3-ketoreductase	F412S/I, V309M, L400F	Reistance frequency and fitness assessement/ Mutations characterization	Pistachio, Grapes	Avenot et al. 2020
SDHIs	Succinate dehydrogenase	H272R/Y, P225F, N230I	Efficacy and activity of a novel SDHI fungicide	Vegetables	He et al. 2020
SDHIs	Succinate dehydrogenase	G85A, I93V, M158V, V168I (SdhC)	Mutations in SdhC subunit concerning resistance and fitness	Apple, Cherry, Blueberry, Pear, Strawberry	Amiri et al. 2019
SDHIs	Succinate dehydrogenase	H272R/Y/L, P225H/F/L, N230I	HRM analysis as a detection tool for sdhB mutations	Strawberry, Stone fruit rootstocks	Samaras et al. 2016
SDHIs	Succinate dehydrogenase	H272R	LAMP method for mutation detection	Tomato, Strawberry	Fan et al. 2018
SDHIs	Succinate dehydrogenase	H272R/Y, N230I	SDHI Resistance monitoring	Strawberry	Fernández-Ortuño et al. 2017

2.2 Benzimidazoles

Benzimidazoles were the next class of fungicides to follow. They were introduced during the late 1960s. The benzimidazoles are renowned for their broad-spectrum activity against a wide range of pathogens, including most ascomycetes, and some basidiomycetes (Leadbeater 2014). Certain molecular examples of this class are carbendazim, thiophanate-methyl, and benomyl (Fig. 2). They are the first systemic fungicides with protective and therapeutic action (Leroux 2004). Because of these aspects, these fungicides proved excellent at controlling certain plant diseases which in turn, however, made the farmers use them frequently and sometimes exclusively. Under such conditions, resistance development was swift. In cases where benomyl was used as a mixture along with other unrelated drugs from the beginning, resistance was delayed (Delp 1980).

Their mode of action is interference with the process of mitosis. They bind to the main protein of microtubules, tubulin, which inhibits microtubule assembly. Resistance to these fungicides has been constantly recorded many times and is correlated with point mutations in the *β*-tubulin gene (Davidse and Ishii 1995). Single mutations at amino acid positions 198 (E198A, E198K, E198V, E198G or E198L) and 200 (F200Y) can confer resistance of different levels (Leroux et al. 2000; Zhang et al. 2010; Ya Bing Duan et al. 2018).

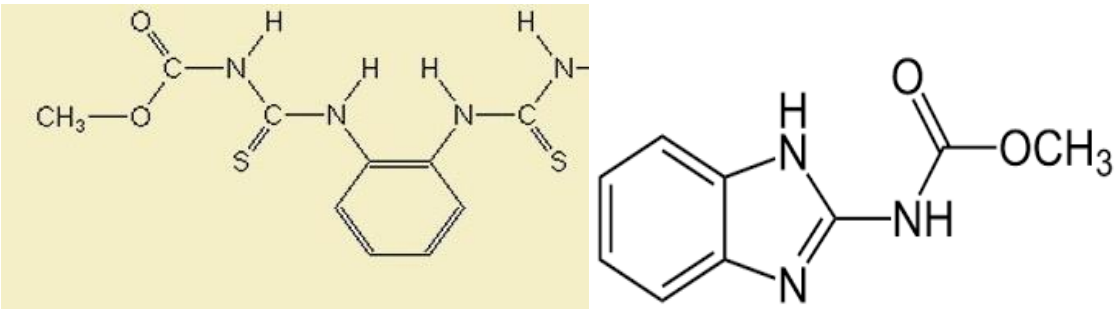


Figure 2: Chemical structure of thiophanate-methyl (left) and carbendazim (right)

Two types of benzimidazole-resistants phenotypes exist in nature, Ben HR (high resistance to benzimidazole fungicides) and Ben MR (moderately resistant to benzimidazoles). The most common mutant strain, E198A, is highly resistant to carbendazim and sensitive to diethofencarb. This negative cross-resistance pattern led to the introduction of a mixture of carbendazim (or thiophanate-methyl) and diethofencarb. However, strains with the F200Y or E198K mutations emerged which are resistant to both drugs. Strains with the F200Y mutation are moderately resistant to benzimidazoles, while the E198K mutants, like the E198A mutants, are highly resistant to benzimidazoles (Yarden and Katan 1993) (Leroux et al. 2002). During the last years, the use of benzimidazoles has significantly decreased. However, despite the absence of selection pressure, resistant populations still dominate the fields with frequencies over 80%. This underlines the competitive advantage and the adequate fitness level of the resistant mutants (Liu et al. 2019). On some occasions, resistance frequencies reach up to 95% even with the absence of benzimidazole usage. However, there has been observed a difference in the composition of the resistant populations. Isolates possessing E198V and E198K mutations have diminished while it seems that E198A mutation seems to be the most dominant now (He et al. 2020).

2.3 Dicarboximides

Dicarboximides with the major compound iprodione, came to replace benzimidazoles in the 1970s (Fig. 3). Their mode of action was not fully understood for many years, although it was observed early on that resistance to dicarboximides is often coupled to hypersensitivity to hyperosmotic stress (Beever 1983). It is now established that dicarboximides interfere with osmoregulation mediated by an osmosensing histidine kinase and a downstream MAP kinase cascade. However, their particular mode of action remains to be revealed (Filinger et al. 2012). *B. cinerea* field strains with low, medium, and high resistance levels to dicarboximides contain one or more mutations in the *BcOS1* gene encoding the osmosensing histidine kinase (Oshima et al. 2006). The predominant mutation was located at codon I365S/N. Furthermore, Shinpei Banno et al. (2008) revealed two other types of resistance groups. Group A type II carries mutations that yield three amino acid substitutions (V368F, Q369H, plus T447S), and the other group type III

carries mutations causing two amino acid substitutions (Q369P plus N373S) in the *BcOS1* gene (Avenot et al 2018). These three types of dicarboximide-resistant isolates possess almost equal levels of resistance and pathogenicity (Oshima et al. 2006; Maqsood et al. 2020).

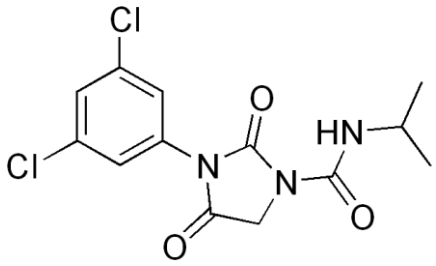


Figure 3: Chemical structure of iprodione

Dicarboximide resistant isolates rarely were observed in nature in the years immediately after the introduction of dicarboximide fungicides in the mid-1970s; however, by the early 1980s, resistant isolates were common on several crops (Locke and Fletcher 1988). Because of their excessive use and lack of other different fungicides, efficacy loss and even total control failure have been recorded (Katan 1982; Lorenz 1988). Dicarboximides are no longer registered for use anymore in several countries, because of resistance problems and their relatively low activity compared to the newer anti-Botrytis fungicides. In contrast to benzimidazoles, monitoring has revealed a decrease in the frequency of dicarboximide resistant strains in the field, following their discontinuation of fungicide treatments. This could be explained by the increased fitness cost of the mutants (Gouot 1988; Pak et al. 1990; Leroux 1995; Pommer and Lorenz 1995). This is said to occur during the saprophytic stage of Botrytis rather than during its parasitic phase (Raposo et al. 2000).

Concerning the frequencies of resistant phenotypes to benzimidazoles and dicarboximides, a recent study by Bertetti et al. (2016) confirmed the same pattern. All the sampled vineyards were benzimidazoles and dicarboximide free for more than 20 years. Despite this fact, *B. cinerea*-resistant strains to benzimidazoles still displayed a frequency

of 48%. In general, benzimidazole resistant strains are quite stable and they may yet remain in a pathogen population, even in the absence of application with benzimidazole fungicides (Gullino et al. 2000; Banno et al. 2008; Bertetti et al. 2016). In contrast, there were not identified any dicarboximide-resistant strains in 2018. This fact is a confirmation of the previously reported pattern on vineyards in Piedmont and other countries as well (Bertetti et al. 2016; Avenot et al. 2018; Baggio et al. 2018). In addition, these data confirm the fact that benzimidazole-resistant strains possess much greater stability than the dicarboximide-resistant ones and that reduction in the application rates of dicarboximide fungicides leads to a significant decrease of dicarboximide resistance in the fields (Gullino et al. 2012).

2.4 Quinone outside inhibitors (QoIs)

Quinone outside inhibitors (QoIs), (mainly strobilurins), were developed from the natural products of β-methoxyacrylate acid, including strobilurin A and oudemansin A (Kraiczy et al. 1996). The most common strobilurins used in agricultural practice are pyraclostrobin and azoxystrobin (Barlett et al. 2002). QoIs effectively bind to the Qo site in cytochrome b. Cytochrome b is a component of the cytochrome bc1 complex in the inner membrane of mitochondria. Following the binding of a QoI to cytochrome b, electron transfer between cytochrome b and c1 is prevented and as a result, mitochondrial respiration is inhibited (Hu et al. 2017; Xiong et al. 2020). However, crops usually receive QoIs applications for control of diseases other than grey mould, like downy and powdery mildew (Hahn 2004). This phenomenon increases the risk of resistance development (Trkulja et al. 2016).

The most common resistance-conferring mutation is located in the cytochrome b gene (*cytb*) resulting in an amino acid substitution of alanine to glycine at codon 143 (G143A). Although this is the most common and highest resistance level conferring mutation, there have been found other mutations as well, like F129L and G137R, which result in moderate levels of resistance (Kim et al. 2003; Pasche et al. 2005; Sierotzki et al. 2007; Fernandez et al. 2008). Nevertheless, regarding QoI resistance, the mutation must not coexist with an intron, located next to the codon for G143. The presence of this intron is shown to

interfere with the proper slicing and the production of the mature mRNA, meaning that isolates carrying both the mutation and the intron, would not survive (Grasso et al. 2006; Samuel et al. 2011; Hahn 2014).

Concerning the frequency of resistance isolates, a study in strawberry population in Greece showed that the percentages were high; in particular resistance, frequencies were as high as 45% and 52% in years 2008 and 2009 respectively (Fig. 4) (Samuel et al. 2011). Ishii et al. 2008 recorded resistance frequencies to even 100% in 2008 from strawberry populations.

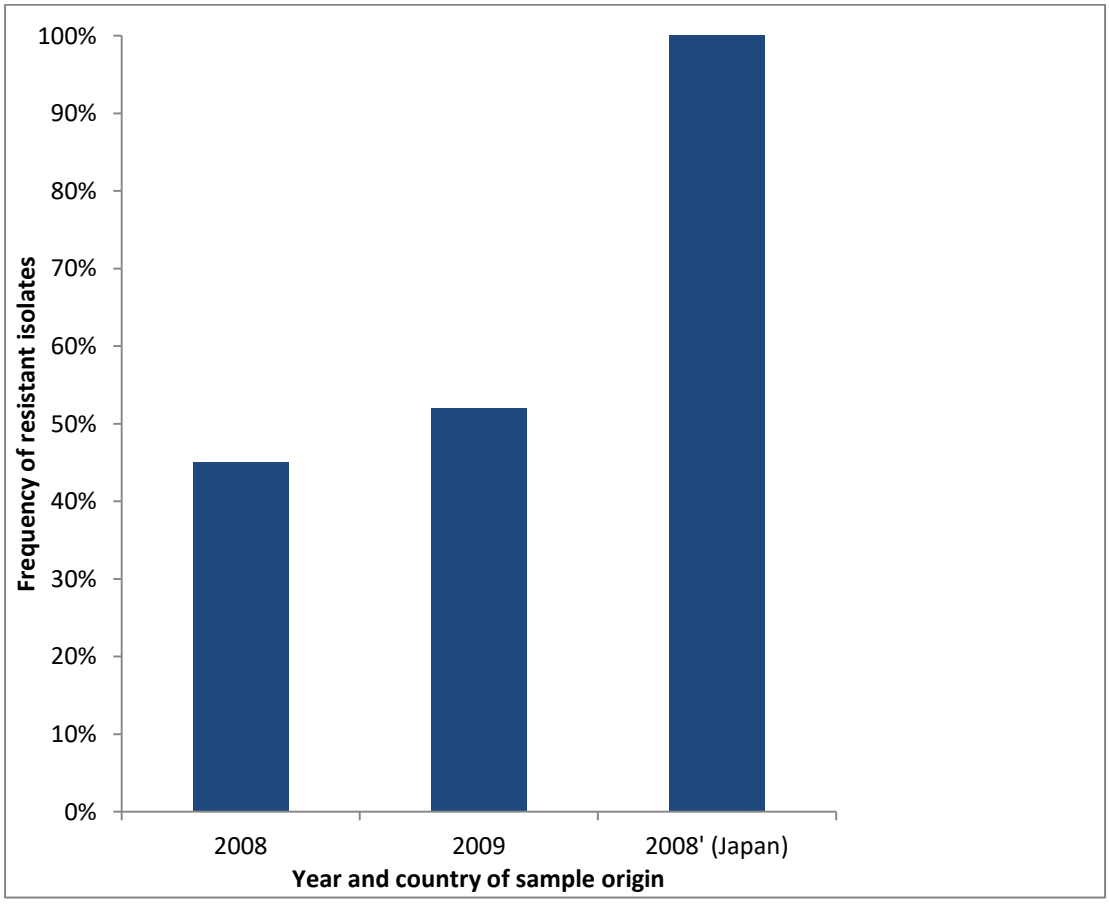


Figure 4: Quinone outside inhibitor resistance frequencies from strawberry fields in Greece and Japan (we used data from Samuel et al. 2011 and Ishii et al. 2008)

While the mutated gene responsible for this resistance could become unstable over time due to the dynamics of the mitochondria, it is clear that sole and excessive use of QoI fungicides, targeting the control of *Botrytis* or/and other pathogens, could create serious resistance development and disease control inefficacy (Zhend et al. 2000; Fountaine et al. 2007; Ishii et al. 2008).

2.5 Hydroxianilides

The major representative of this class is fenhexamid (Fig. 5). It is widely used because of its specific mode of action and high efficiency at controlling grey mould. It belongs to the SBI (sterol biosynthesis inhibitors) and it blocks the 3-ketoreductase enzyme, whose role is the catalysis of C-4 demethylation during the process of ergosterol biosynthesis (Debieu et al. 2001). The corresponding gene is *erg27*. Fenhexamid application results in inhibition of the germ tube elongation and mycelia growth of the fungus (Rosslenbroich et al. 1998; Rosslenbroich and Stuebler 2000; Myresiotis et al. 2007). Concerning resistance development, it is classified as low to medium risk. However, because of its widespread use and the specificity of its mode of action, resistance emergence was sure to follow. Resistant isolates were soon found in many countries like France, Japan, USA, and Germany (Forster et al. 2007; Fillinger et al. 2008; Mercier et al. 2009; Saito et al. 2011, 2014; Weber 2011; Moorman et al. 2012; Grabke et al. 2013).

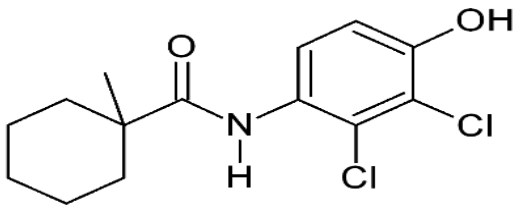


Figure 5: Chemical structure of fenhexamid

Genetic polymorphism and mutations in the *erg27* gene can lead to a weaker affinity of fenhexamid to its target 3-ketoreductase and thus confer resistance to this botryticide (Debieu et al. 2013; Yin et al. 2016).

The resistant isolates of Botrytis have been categorized into 4 groups depending on their resistance levels. Among them, HydR1 is found only in *B.pseudocinerea* isolates which are naturally resistant to fenhexamid. The other three phenotypes are found in *B. cinerea* isolates and are classified as HydR2, HydR3, and MDR2 (Leroux et al. 2002).

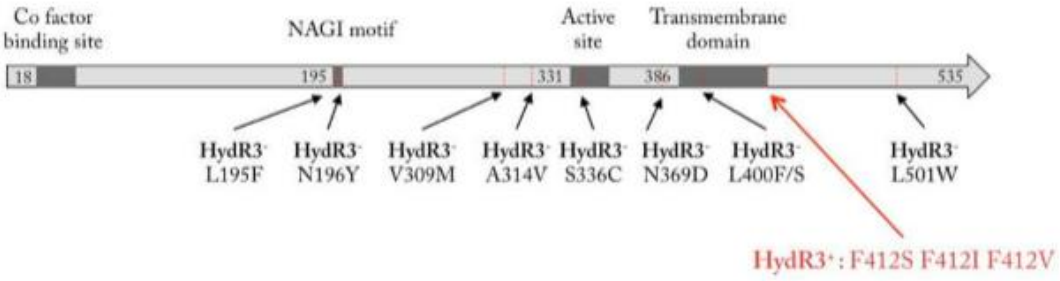
HydR1 and HydR2 phenotypes transfuse moderate to high resistance levels almost exclusively to mycelial growth. In contrast, the other phenotypes show an equal resistance level to sporulation and germ tube elongation (Debieu et al. 2011). HydR3 phenotype generates the highest resistance level and is the one responsible for resistance during germ tube elongation (Leroux et al. 2002). This category is divided into two subcategories HydR3- and HydR3+ with low and high resistance respectively (Fillinger et al. 2008). The first HydR3 isolates were detected in 202 in Germany and two years later in French vineyards.

The resistance mechanism of fenhexamid is composite since each phenotype is correlated with a different mechanism. HydR1and HydR2 phenotype isolates show increased detoxification of hydroxianilides due to the blockage of cytochrome P450 monoxygenase (Suty et al. 1999; Leroux et al. 2002).

Sequencing of *erg27* gene, which codes the target protein, 3-ketoreductase, showed that point mutations are connected with fenhexamid resistance in HydR3 isolates. However, erg27 is characterized by great polymorphism, and thus not every point mutation confers resistance. Concerning HydR3- phenotype, many point mutations have been recorded like F26S,N93V, D146N, V192L, L195F,N196T,I211V, I215L, M218T, I232M,V234A, I235V, P238S, P250S,D261G, S264T, P269L, A285T, ΔP298, V309M, A314V, S336C,Q354K,N369D,

L400F/S, Y408S,A461S,R496T, ΔP238. In HydR3+ fewer mutations have been identified with the most common ones being the replacements of phenylalanine by serine, isoleucine, or valine in codon 412 (F412S/I/V) and the more rare T496R, G170R, and A210G (Fig. 6) (Albertini and Leroux 2004; Fillinger et al. 2008; Esterio et al. 2011; Amiri and Perez 2014).

Isolates carrying the aforementioned mutations differentiate not only regarding their resistance levels but also other adaptability traits like mycelial growth, sporulation, and sclerotia production (Fillinger et al. 2008; Esterio et al. 2011; Amiri and Perez 2014). Several studies have suggested that these mutations carry also a fitness cost. Ziogas et al. (2003), conducted a research with 6 laboratory mutant isolates with different resistance levels. Isolates possessing the highest resistance levels to fenhexamid were burdened by decreased sporulation capacity, sclerotia production, and pathogenicity. Another study revealed that isolates with laboratory resistance to fenhexamid showed reduced mycelia



growth (DeGuido et al. 2007). Moreover, Saito et al. (2010) found that resistant mutants completely lost their ability to infect cucumber cotyledons. Billard et al. (2012) examined *in vitro* isolates carrying the F412S/I/V mutations. Certain traits like conidia and sclerotia production, mycelia growth, and pathogenicity were measured. The results revealed that pathogenicity was not affected in contrast to the other attributes which were decreased.

Figure 6: Protein structure and function in 3-ketoreductase *erg27* (Billiard et al. 2011)

In the fields, isolates with high resistance to fenhexamid are not particularly usual suggesting their fitness cost is often high enough to hinder their emergence. Low to

moderate resistance patterns are much more frequent but still fungicide applications can control Botrytis in some extent. However, it is vital to take into consideration that highly resistant isolates with little to no fitness cost have also been described and thus control strategies must be improved (Avenot et al. 2020).

2.6 SDHIs (Succinate Dehydrogenase Inhibitors)

Fungicides belonging to the group of SDHIs are systemic and were discovered around 1960 while their entrance to the market was delayed for 10 years approximately. Their main use was against Bacidiomycetes usually as seed coating (Kulka and von Schmeling 1995). However, a new generation of SDHIs, with boscalid as the main representative, introduced in the market in 2003, broadened their range to also include Ascomycetes (Glattli et al. 2011). SDHI fungicides consist of 10 different chemical groups and 20 different molecules in total (Table 3). Out of the most recent and usually used molecules are boscalid and fluopyram from Pyridine-carboxamides and Pyridinyl-ethyl-benzamides group respectively.

SDHI fungicides inhibit mitochondrial respiration by blocking the function of mitochondrial respiratory complex II that consists of a flavoprotein (*SdhA*), an iron-sulfur protein (*SdhB*), and two membrane-anchored proteins (*SdhC* and *SdhD*) (Fig. 8) (Hägerhäll 1997). An important feature of this group of fungicides is the absence of cross-resistance with other different chemical groups making them a useful choice for resistance management and disease control optimization (Zhang et al. 2007; Avenot et al. 2008; Veloukas and Karaoglanidis 2012).

Table 3: Succinate dehydrogenase inhibitors chemical groups and common names (Fungicide Resistance Action Committee; www.frac.info)

Target site of action	Group name	Chemical group	Common name
		Phenyl-benzamides	Benodanil Flutolanil Mepronil
		phenyl-oxo-ethyl thiophene amide	Isofetamid
		Pyridinyl-ethyl-	Fluopyram

Complex II; succinate-dehydro-genase	**SDHI** (**S**uccinate **dehy**drogenase **i**nhibitors)	benzamide	
		Furan-carboxamides	Fenfuram
		Oxathiin-carboxamides	Carboxin Oxycarboxin
		Thiazole-carboxamides	Thifluzamide
		Pyrazole-carboxamides	Benzovindiflupyr Bixafen Fluxapyroxad Furametpyr Isopyrazam Penflufen Penthiopyrad Sedaxane
		Pyridine-carboxamides	Boscalid
		N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides	pydiflumetofen
		pyrazine-carboxamides	pyraziflumid

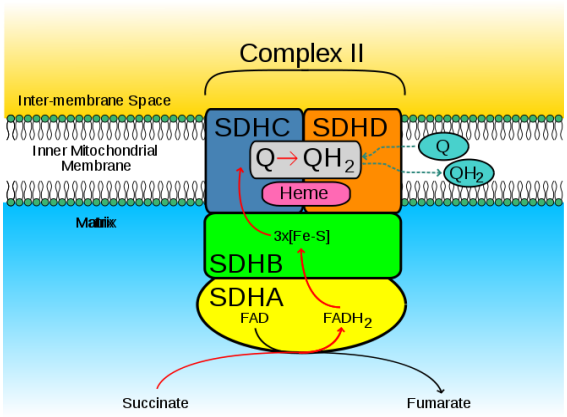


Figure 8: Structure of complex II of the respiration chain A, B, C, D (the four subunits) (Horsefield et al. 2006)

Concerning resistance, SDHIs are classified as medium to high resistance risk mainly due to the specificity of their mode of action and their worldwide widespread use. Resistance to several molecules of this class was reported shortly after their registration and was attributed to certain mutations in one of *SdhA, SdhB, and SdhC* subunits (Georgopoulos et

al. 1972; Gunatilleke et al. 1975; Broomfield and Hargreaves 1992; Matsson et al. 1998; Skinner et al. 1998; Ito et al. 2004; Avenot et al. 2008).

As far as *B.cinerea* is conserved, the most common resistance-conferring mutations are H272R and H272Y, due to the replacement of histidine by arginine or tyrosine respectively, at codon 272 of the *sdhB* locus (Leroux et al. 2010; Veloukas et al. 2011; Yin et al. 2011; Fernández-Ortuño et al. 2012). Another mutation in the same codon has been identified, namely H272L, however its frequency rates are considerably lower (Leroux et al. 2010). Apart from replacements at position 272, the following mutated alleles granting resistance to SDHI in *sdhB* have also been detected, namely P225F, P225T, P225L, P225H, and N230I (Leroux et al. 2010; Veloukas et al. 2011, 2013; Amiri et al. 2014; Esterio et al. 2015). In addition to mutations in the *sdhB* subunit, there have also been recorded mutations in the *sdhD* subunit, one of them being H132R (Leroux et al. 2010).

All of the aforementioned mutations yield different levels of resistance and follow different same -class cross-resistance patterns. The two most common mutations H272R/Y confer resistance to boscalid but are sensitive at the same time to other SDHIs, like fluopyram. Nevertheless, other mutations like the ones in P225 confer high resistance to both boscalid and fluopyram (Laleve et al. 2013; Veloukas et al. 2013; Samaras et al. 2016).

SDHIs have attracted a lot of attention, especially during the last years because of their efficiency, broad range, and use. Novel fungicides like pydiflumetofen show promise concerning future resistance management. He et al. (2020) conducted research on the specific drug and reported that it is both efficient against Botrytis and the most common resistant mutants, possessing one of the mutations H272R/Y, P225F, N230I show low to moderate resistance to pydiflumetofen. However, its mode of action is still very specific and caution must be taken when it comes to disease control implementation.

In addition, more recent studies have revealed that mutations in *sdhC* subunit may also confer resistance to some SDHI fungicides. Amiri et al. (2019) report that there exist two different genotypes of *Botrytis cinerea*, namely C+ and C-, due to the presence of 4 simultaneous mutations in the SdhC. C+ isolates show increased sensitivity to some SDHIs

like boscalid, while decreases sensitivity has been recorded to fungicides like fluopyram. It is still unclear how exactly these isolates have been selected in the fields, though monitoring and further research is crucial concerning future resistance development.

Following up, two newer classes of botryticides will be analyzed, Anilinopyrimidines and Phenylpyrroles. These fungicides are often coupled because of Switch, a product containing a mixture of cyprodinil and fludioxonil, and used extensively. Moreover, not much is known for these two botryticides classes, concerning their mechanism, their target protein, or even their resistance-conferring mutations.

2.7 Phenylpyrroles

Phenylpyrroles are derived from the antifungal antibiotic pyrrolnitrin, a tryptophan derivate produced by Pseudomonas species. Synthetic analogs of pyrrolnitrin have been produced and two of them, fludioxonil and fenpiclonil are used as a seed treatment (Leroux 2007). Fludioxonil is the major representative of this group and because of its good light stability, it was introduced as a foliar fungicide during the -mid-1990s. (Rosslenbroich and Stuebler 2000). A mixture containing fludioxonil and the AP cyprodinil is often used and is regarded as the most effective fungicide against *Botrytis* (Hahn 2014). Similar to the dicarboximides, fludioxonil interferes with the *BcOS1*- and MAP kinase-dependent osmoregulation pathway (Vignutelli et al. 2002). More recent studies have shown that field resistant mutants are hypersensitive to osmotic stress, possess mutations in *Bos1,* and express abnormally *Bchog1*. It is speculated that this is the result of inactivation of the HOG-MAPK pathway (Ren et al. 2016; Gong et al. 2018)

In sensitive cells, fludioxonil hyperactivates the pathway, which leads to a hyperosmolarity response, followed by glycerol accumulation and growth inhibition (Kojima et al. 2004).

Mutants of *Botrytis* that show high resistance to both phenylpyrrnoles and dicarboximides and are at the same time sensitive to osmotic stress, can be easily produced in the lab. However, such strains are pretty unusual in the fields. Strains moderately resistant to

dicarboximides but not to phenylpyrroles have been revealed by monitoring. They bear slight or no osmotic sensitivity. It seems that resistance in the fields is restricted to either dicarboximides (Leroux et al. 1999) or phenylpyrroles (Vignutelli et al. 2002). The absence of strains resistant to both dicarboximides and phenylpyrroles in the field undermines a reduced fitness level probably due to the increased osmotic sensitivity. Nevertheless, strains that are express low to moderate resistance to fludioxonil have been recorded in the fields, probably due to a drug efflux mechanism (Hahn 2014).

2.8 Anilinopyrimidines

Another major group of botryticides is Anilinopyrimidines (AP). They were firstly introduced in the market around the mid-1990s with three representative molecules pyrimethanil, cyprodinil, and mepanipyrim (Neumann et al. 1992; Heye et al. 1994; Maeno et al. 1990) (Fig. 9). Generally, their most common use involves treatments for the control of grey mould in a wide range of crops, from fruits, vegetables, ornamentals to control of apple scab disease (Müller et al. 1998). However, their use is not restricted against *Botrytis.*

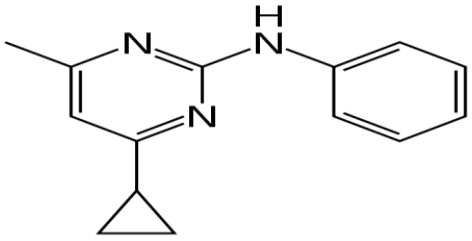


Figure 9: Chemical structure of cyprodinil

Class-specific cross-resistance has often been observed in APs (Leroux et al. 1999). Although the class is of medium resistance development risk by the Fungicide Resistance Action Committee (FRAC), adequate performance is still observed in the fields. However, it wasn't long before resistance rapidly emerged in trial sites. To combat this emergence, and to protect the efficacy of APs, mixtures of anilinopyrimidines and other active ingredients are used. Most common is the mixture of cyprodinil and fludioxonil for

sustained and effective control of grey mould (Forster and Staub 1996; Hilber and Hilber-Bodmer 1998; Latorre et al. 2002)

AP fungicides are still classified as potential inhibitors of methionine biosynthesis due to early studies on their mode of action that displayed reversal of growth inhibition when sulfur-containing amino acids, and in particular methionine or its upstream metabolite homocysteine, were added to culture media containing the fungicide (Leroux 1994; Masner et al. 1994; Leroux et al. 1995).

The lack of reversal by cystathionine, a metabolite one step before homocysteine, proposed that the potential mode of action could be methionine biosynthesis inhibition through the inhibition of the enzyme cystathionine β-lyase (Masner et al. 1994; Fritz et al. 1997). However, cystathionine β-lyase presence was not inhibited even at high doses of APs, as some enzymatic studies on *B.cinerea* revealed (Sierotzki et al. 2001; Fritz et al. 2003). In addition, more studies highlighted a new important feature of APs concerning their mode of action. It was revealed that AP fungicides prevent the secretion of fungal hydrolytic enzymes such as laccases, lipases, proteases, sugar modifying (invertase), and cell wall degrading enzymes (cutinases, pectinases, and cellulases). This in turn suggested that different levels of sensitivity to AP fungicides on different growth media could be caused by differential requirements for extracellular enzymes necessary for the mobilization of nutrients (Miura et al. 1994; Milling and Richardson 1995). Since several studies could not uncover the exact mode of action, it was deemed essential that further studies be conducted to decipher the molecular target site of this class. Nevertheless, Mosbach et al. (2017) identified 9 different mitochondrial proteins that confer resistance to AP fungicides suggesting that the molecular target of this class is a mitochondrial one. While there is no direct correlation between these genes and methionine biosynthesis, there is a suggestion that the methionine reversal in *B.cinerea* is the indirect effect caused by the intracellular concentration of the amino acid. More precisely, they identified and validated AP conferring mutations in 9 different genes most of which came from the mutant collection. However, only 2 genes, *Bcpos* and *Bcmdl* were recorded in the field. This indicated that the fitness cost of mutations in the other genes was too high.

Nevertheless, every gene product of the abovementioned is correlated to mitochondrial function. Yeast alternate *Pos5* is a mitochondrial NADH kinase, whose role is the production of the mitochondrial pool of NADPH from mitochondrial NADH and ATP (Outten and Culotta 2003; Bieganowski et al. 2006; Miyagi et al. 2009). On the other hand, yeast *Mdl1* is responsible for a mitochondrial inner membrane ABC transporter, putatively involved in the export of peptides derived from degradation of proteins, while physically interacting with F1FO ATP synthase (Young et al. 2001; Galluhn and Langer 2004; Hofacker et al. 2007).

Concerning field isolates, the most frequent mutations are L412F, L412V, and G408V in *Bcpos5* and E407K in *Bcmdl1*, but all possible resistant mechanisms are still not yet identified, and thus further research is required (Mosbach et al. 2017).

In general, as far as AP fungicide resistance is concerned, there exist three different phenotypes AniR1, AniR2, and AniR3. AniR1 phenotype is characterized by the highest resistance and the greatest sensitivity shift (up to 250-fold).Apart from its high resistance, this phenotype is also specific to AP fungicides and is also the phenotype that is correlated with the efficacy of solo applications of anilinopyrimidines (Forster and Staub 1996; Leroux et al. 1999). The other two phenotypes, AniR2 and AniR3, display sensitivity shifts below 20-fold and are mediated by the overexpression of drug efflux transporters and have recently been re-classified as multidrug-resistant (MDR). It is then speculated that by focusing on AniR1 phenotypes, the mode of action of APs could be unraveled. (Leroux et al. 1999; Leroux et al. 2002)

Concerning the frequency of resistant phenotypes regarding AP and phenylpyrrole fungicides, several studies have been conducted throughout the years. Strawberry fields in particular are burdened with many fungicide applications and so resistance is a common phenomenon. Scalliet et al. (2015) monitored *Botrytis* resistant populations concerning the use of Switch (a mixture of cyprodinil and fludioxonil). Regarding CDL resistance, the frequency varied from 30 to 62 % throughout the years of the monitoring (Fig. 10). For FDL, resistant frequencies were lower, around 30% (Fig. 11). However, most of these

isolates were of intermediate resistance, suggesting the very low frequency or absence of specific target site mutations conferring resistance in the fields. Most isolates expressed MDR phenotypes thus providing only a medium level of resistance to FDL (Scalliet et al. 2015).

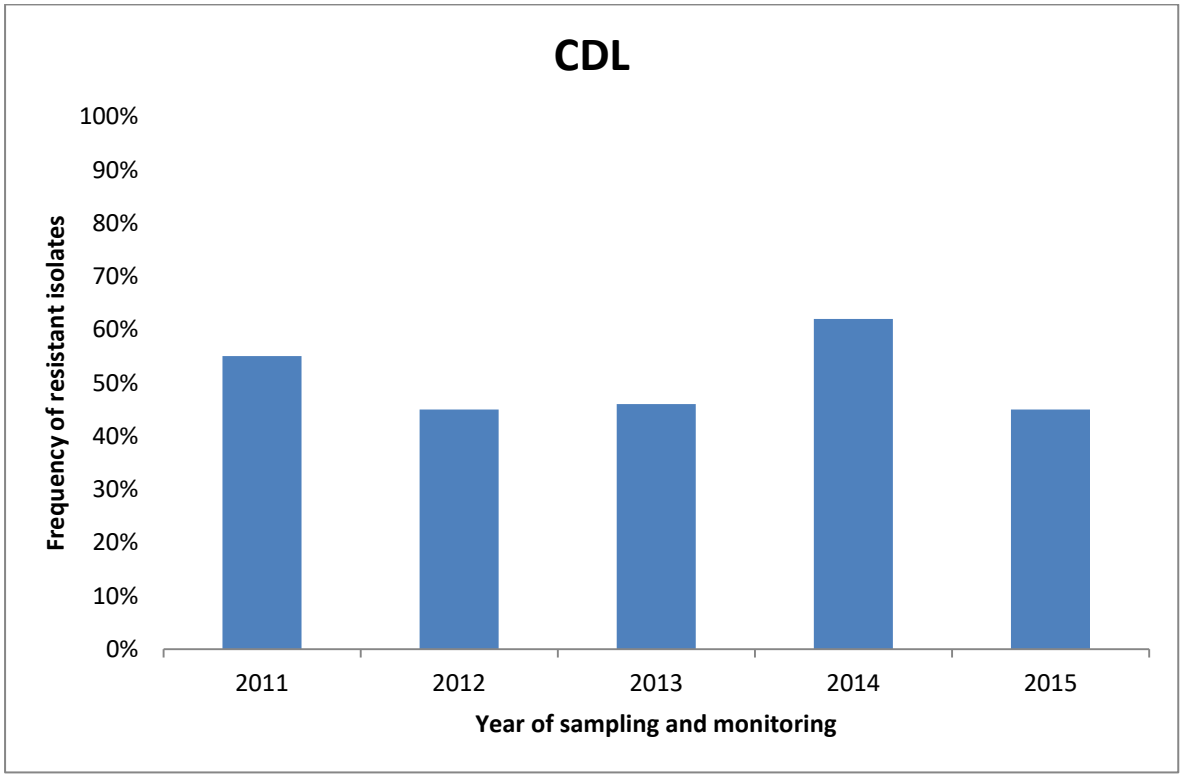


Figure 10: Frequency of cyprodinil resistant isolates (we used data from Scalliet et al. 2015)

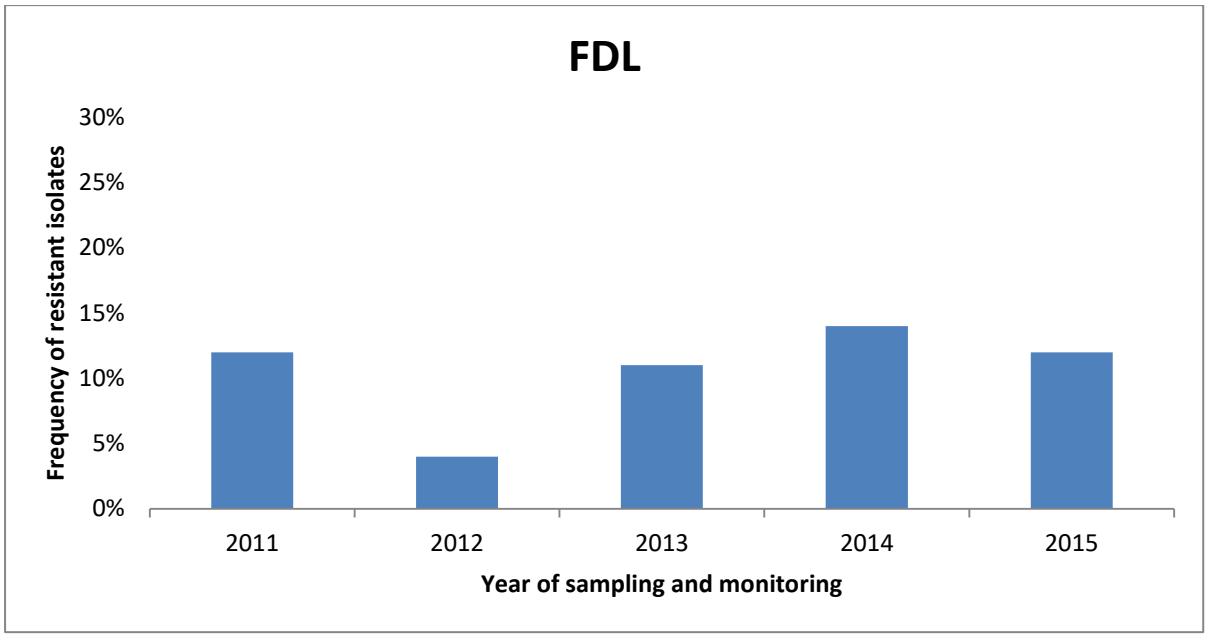


Figure 11: Frequency of fludioxonil resistant isolates (we used data from Scalliet et al. 2015)

Another study concerning resistance profiles of *Botrytis* was conducted in greenhouse strawberries in Lebanon during 2016 and 2017. The frequencies of the resistant isolates, regarding APs, were significantly higher, reaching almost 90% in sites where four sprays were applied, whereas frequencies of PPs resistance were about 33% (Fig. 12). (Habib et al. 2020)

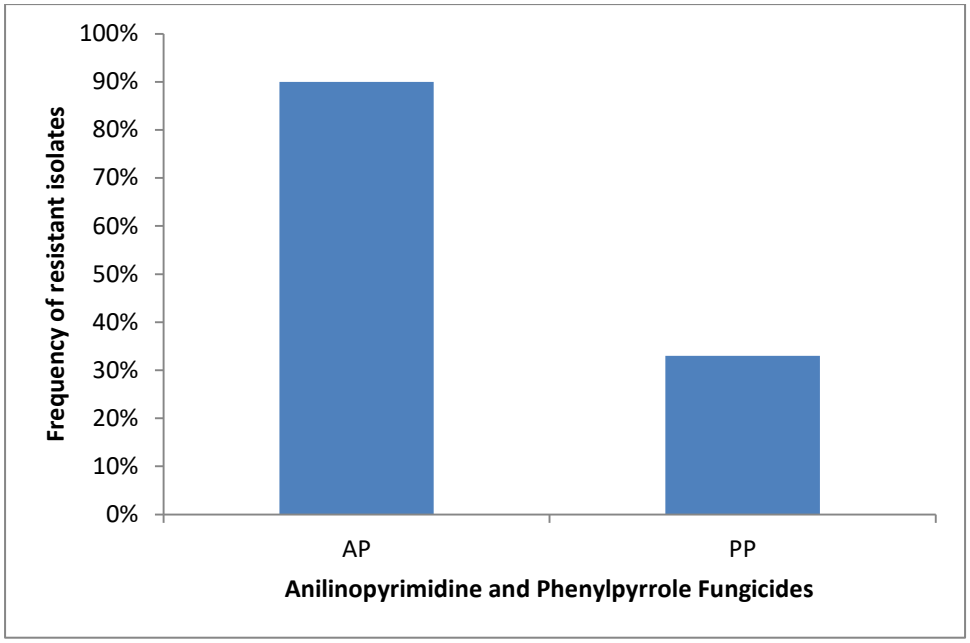


Figure 12: Frequency of isolates expressing AP and PP resistance (we used data from Habib et al. 2020)

In general, it can be observed that apart from their efficacy in controlling grey mould, phenylpyrroles are much more difficult to obtain resistance to. The resistance frequencies of PPs are significantly lower thus highlighting a potential resistance technique. It can be speculated that in areas with high resistance development pressure, products containing phenylpyrroles could help milden the problem.

2.9 Multiple Resistance

Fields that are heavily treated with fungicides against *Botrytis*, like strawberry fields, experience another phenomenon called multiple resistance. Isolates may accumulate target site mutations, over time, which confer resistance to many different classes of fungicides. As a result, isolates could be simultaneously resistant to more than one botryticides, or even to every available botryticide in use (Rupp et al. 2017). Disease

management faces a real threat in such circumstances (Leroch et al. 2011; 2013; Weber 2011). Frequencies of such *Botrytis* strains, according to some studies, have become more and more intense, especially in crops burdened with many fungicide applications. There are certain hypotheses why this happens, from a stepwise accumulation over time to immigration from other fields and even introduction to the fields from contaminated with MR strains nursery plants (Hu et al. 2016; Mernke et al. 2011). The fitness of these MR strains remains a controversial issue (Fernández-Ortuño et al. 2015; Chen et al. 2016). However, they can likely complete adequately in the field, since they are abundant on many occasions. Control strategies fall a bit off since MR strains are immune to almost every fungicide currently in use (Weber et al. 2015). Finally, it is believed that products that contain fungicide mix could deteriorate this problem, especially those that contain QoI compounds (Weber 2011).

Concerning the frequency rates, resistance monitoring in strawberry fields conducted by Habib et al. (2020), showed that multiple resistance to four and five fungicides was most common with the percentage fluctuating around 30%, resistance to six fungicides reached 19 % while there were some isolates which possessed resistance to every fungicide tested.

3. Multi-Drug Resistance (MDR)

Apart from target site mutations which usually confer high levels of resistance, there also exist other mechanisms responsible for resistance development. Overexpression of efflux transporters, which exhibit low substrate specificity, can lead to the secretion of many harmful compounds into the extracellular space and thus to detoxification of the cell. Such compounds can be of plant defense origin (phytoalexins), fungicides, antibiotics, microbial toxins, or self-produced harmful metabolites (Andrade et al. 2000; Hayashi et al. 2002; Samaras et al. 2020). The concentration of these substances intracellularly is therefore decreased and their toxic effect fades (de Waard et al. 2006). Due to their low substrate specificity, the enhanced activity of such transporters results in simultaneous resistance to

structurally unrelated and chemically different active ingredients known as Multidrug Resistance. MDR is thoroughly studied in human tumor cells, bacteria, and human pathogenic fungi like *Candida spp*. which greatly threaten cancer and immunocompromised patients (Holmes et al. 2016; Rahman et al. 2017; Wu et al. 2014). The two greater families of efflux transporters are ABC transporters (ATP-Binding Cassette superfamily transporters) and MFS-transporters (Major Facilitator Superfamily transporters) (de Waard et al. 2006; Gulshan and MoyeRowley 2007). Concerning plant pathogenic fungi, the evidence and the importance of MDR strains in the field are not yet clearly determined, nevertheless, it does have an impact regarding fungicide resistance and management.

Table 4: Table representing studies concerning MDR phenotypes and their transporter functions

Effect/Results of each study	Resistance Phenotype	Mutations involved	Gene/Protein involved	Reference
ABC Transporter function and architecture	-	-	ABC Transporters	Rees et al. 2009
MFS Transporter function in pathogenicity of Botrytis	-	-	MFS Transporters	Vela-Corcía et al. 2019
Detection of a new MDR1h phenotype and a novel clade Botrytis Group S	MDR1, MDR1h	ΔL497	*AtrB, Mrr1*	Leroch et al. 2012

Transcriptome analysis of MDR strains	MDR	-	ABC, MFS related genes	Samaras et al. 2020
Multiple and Multidrug Resistance	MDR1, MDR1h	-	*β-tubulin, cytb, bos1, erg27, sdhB*, and *mrr1* genes, and the mfsM2 promoter regions	Fernández-Ortuño et al. 2015
Two promoter rearrangements confer MDR2	MDR2	Insertion in Mfs promoter region	MfsM2	Mrenke et al. 2011
Molecular Basis of MDR	MDR1	V575M in mrr1	*atrB, mfs*	Kretschmer et al. 2009
Activity of modulators of membrane transporters against MDR strains	MDR1, MDR2	-	*BcatrB, BcmfsM2*	Leroux et al. 2013

3.1 ABC Transporters

The membrane transport proteins of this class use the energy of ATP hydrolysis to transfer solutes across lipid membranes. They serve many purposes some of which include nutrient absorption, protein secretion, xenobiotic compound resistance, and pathogenesis (Higgins 1992). They derive from ancient organisms and can be considered as effective devices that can translocate solutes, from ions to macromolecules, against the concentration gradient (Schneider et al. 1998). A typical structure of an ABC transporter

consists of four main parts: two transmembrane domains (TMDs) that are attached to the membrane bilayer and two nucleotide-binding domains (NBDs) which are situated in the cytoplasm (Fig. 14) (Wilkens 2015). While ABC transporters possess a highly conserved motif at the sequence level, TMDs' sequences, and structures vary greatly thus depicting the diversity of the potential translocated substrates. Transporters of the ABC superfamily are divided in importers and exporters. In eukaryotic cells they mainly serve as exporters (Rees et al. 2009).

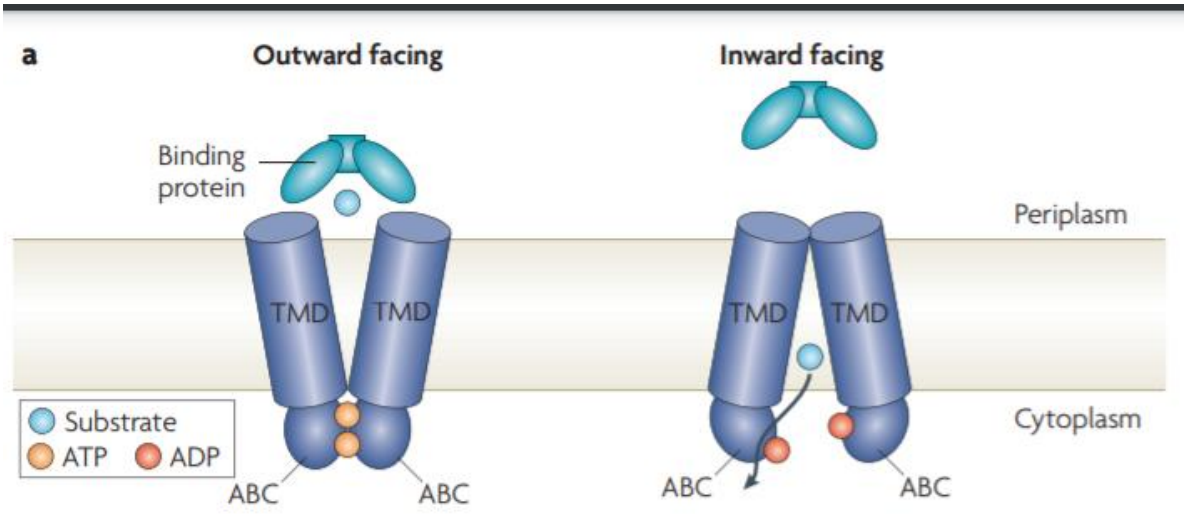


Figure 4: Structure of a typical ABC transporter (Rees et al. 2009)

A series of consecutive steps forms the catalytic cycle of ABC transporters. It starts with the direct binding of the substrate to the TMDs, followed by the binding of ATP molecules to the NBDs. Afterward, ATP hydrolysis to phosphate and ADP occurs and the transport substrate is released. The NBD dissociate and set the complex to its former state (Wilkens 2015).

3.2 MFS Transporters

In contrast to ABC, MFS transporters are categorized as secondary metabolites but are also involved in exporting different compounds of different sizes (carbohydrates, drugs, ions, etc) (Dos Santos et al. 2014). They can act as uniporters, symporters, or antiporters (Pao et al. 1998). Their length varies from 400 to 600 amino acids. In this superfamily, the energy needed to support the drug efflux mechanism derives from the electrochemical gradient generated across the cell wall due to ion and proton movement (Rahman et al. 2017; Vela-Corcia et al. 2019).

MFS proteins consist of a conserved 12 transmembrane (TM) α-helix fold (Reddy et al. 2012) that is made of two 6-TM helix bundles that are connected by a pseudo-two-fold axis of symmetry (Fig. 15). Both domains are presumed to be of equal functional importance for the transport mechanism, due to the ligand binding to the central TM cavity at the interface between the two domains (Pazdernik et al. 1997)

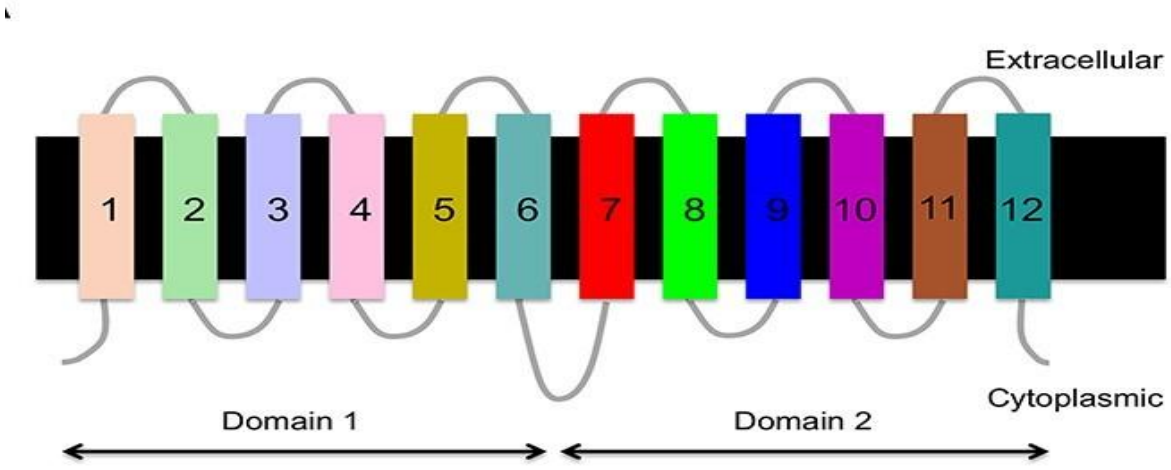


Figure 5: Structure of a typical MFS transporter (Lee J. et al. 2016)

3.3 MDR1

From the mid-1990s, strains of *B. cinerea* with no specific fungicide resistance have been detected. These strains show partial resistance to cyprodinil and fludioxonil, chemically and structurally different fungicides, and were originally called AniR2 but have been renamed to MDR1 due to their similarity to efflux multidrug resistance phenotypes of cancer cells and human pathogens (Alekshun 2007; Morschhauser 2010). MDR1 phenotype is attributed to overexpression and constitutive activation of the ABC transporter *BcatrB* which is in turn associated with gain of function mutations in the transcription factor *Mrr1*. There have been detected many point mutations in the mrr1 gene, which can lead to MDR1 phenotype. MDR1 has been observed in both vineyards and strawberry fields and may be a result of the extended use of Switch, a mixture containing cyprodinil and fludioxonil (Hahn 2014; Leroch et al. 2012).

3.3.1 MDR1h

More recently, a new stronger variant of the MDR1 phenotype called MDR1h was identified. MDR1h phenotype confers more than two-fold higher resistance to cyprodinil and fludioxonil but has been mostly detected in strawberry fields. A 3-bp deletion mutation (ΔL497) in *mrr1* is responsible for the emergence of MDR1h, though MDR1h strains may accumulate more point mutations in the same gene. Furthermore, MDR1h strains are more frequent to express multiple resistance, by accumulating many target site mutations in different genes (Leroch et al. 2012). In addition, according to Leroch et al. (2012), MDR1h strains belong to a novel clade called *Botrytis group S*, closely related to *B. cinerea* and *B. fabae*.

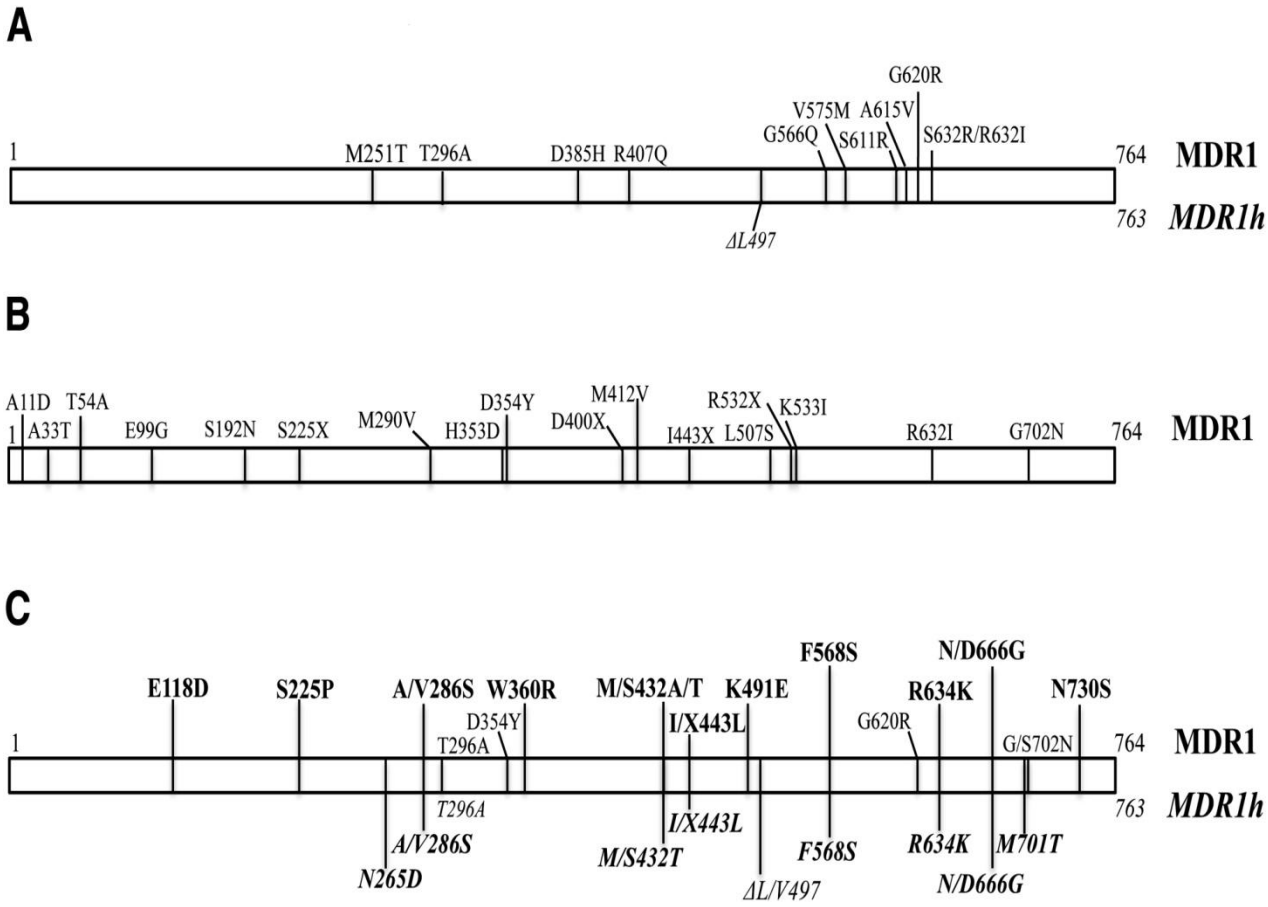


Figure 6: MDR1- and MDR1h-related deletions and mutations in the transcription factor *mrr1*. (A. Kretschmer et al. 2009; Leroch et al. 2013. B. Li et al. 2014. C. Fernández-Ortuño et al. 2015)

Concerning the frequencies of MDR1h isolates, they were found to be widely distributed in German strawberry fields, with frequencies reaching up to 60% (Fig. 17) suggesting that resistance management in such circumstances can become very problematic.

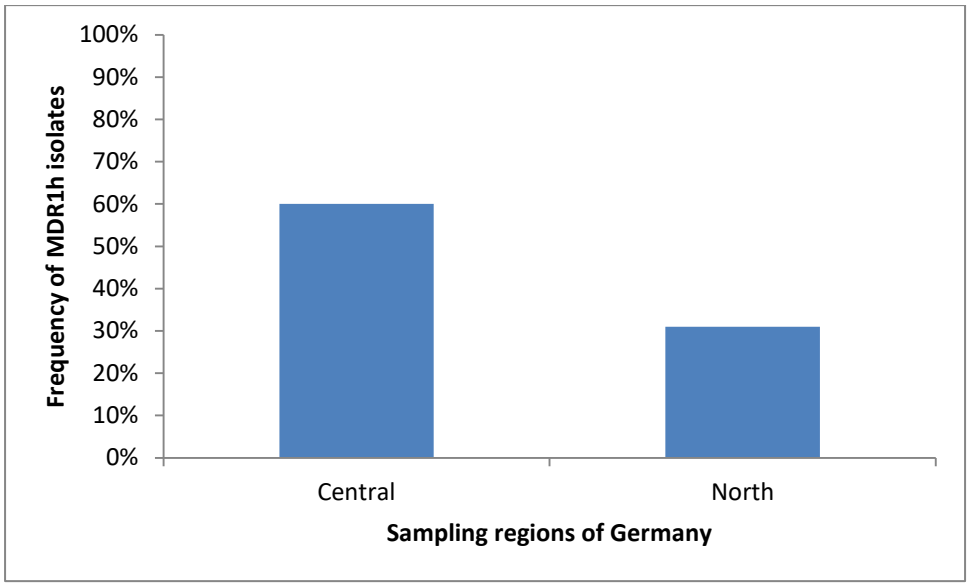


Figure 7: MDR1h frequency in north and central Germany (Data taken from following study: (Leroch et al. 2012)

3.4 MDR2

MDR2 is another multidrug resistance phenotype that is associated with the constitutive activation and overexpression of the MFS transporter superfamily. MDR2 (previously named AniR3) strains show partial resistance to fenhexamid, cyprodinil, and iprodione (Chapeland et al. 1999). The *mfsM2* gene, which encodes a transporter of the MFS superfamily, is constantly upregulated. The overexpression of this gene is associated with a unique rearrangement of its promoter induced by the insertion of a retrotransposon–derived sequence. More precisely, the promoter contained a 1326-bp insertion in conjunction with a 678-bp deletion (Kretschmer et al. 2009; Leroch et al. 2012). There have been detected two types of MDR2 phenotypes, type A and type B, both of which convert the *mfsM2* promoter to a strong, constitutive one, thus conferring fungicide resistance. Type B *mfsM2* promoter is accompanied by a 1011-bp insertion and a 76-bp deletion, but at different sites compared to type A (Fig. 18). MDR2 strains are widely spread in French and German vineyards but are absent from strawberry fields (Mernke et al. 2011).

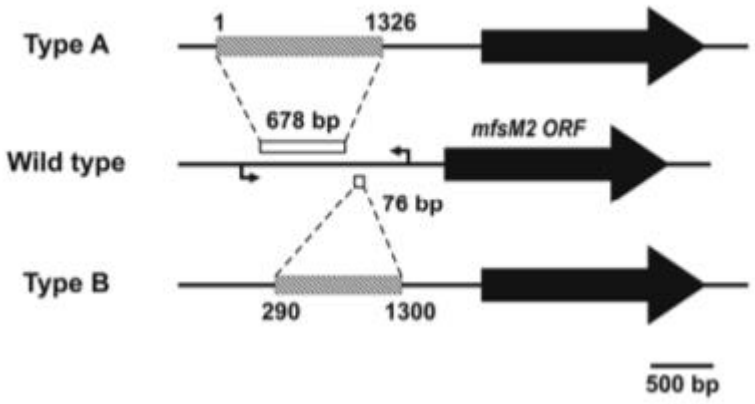


Figure 8: The two types of promoter rearrangements leading to MDR2 phenotype (Mernke et al. 2011)

Interestingly, *atrB* and *mfsM2* overexpression are adequate for MDR1 and MDR2 phenotypes respectively, since knock-out mutants of MDR1 and MDR2 strains lacking the respective genes lost their MDR traits (Mernke et al. 2011).

3.5 MDR3

MDR3 phenotype is another type of multidrug resistance. MDR3 strains are known to be genetic recombinants of MDR1 and MDR2 strains. As a result, they are generally resistant to most fungicides used against *Botrytis*, since they combine the resistance spectra of the other two MDR types. Concerning their genotype, these strains are characterized by the simultaneous mutations in the *mrr1* and the rearrangements of the *mfsM2* promoter. Just as MDR2 strains, MDR3 strains are solely distributed to vineyards (Leroch et al. 2012; Leroux et al. 2013).

4. Alternative Control Methods

The extensive use of fungicides in an irrational way and the rapid development of resistance, along with the increased cost of chemical control and the sensitization of consumers concerning the safety and the upshot of agro-pesticides in both man and

environment, have led to an uprising need of new alternative control methods of plant pathogens.

4.1 Biological Control

It has been discovered that many different biological agents, living organisms, and substances or components of natural origin could be used against plant diseases in the frame of biological control. Concerning the plant pathogen *Botrytis cinerea*, several biological products have been detected (Nicot et al. 2016). They can be classified into three categories: a) botanical extracts like *Melaleuca alternifolia, Reynoutria sachalinensis*, b) living organisms like bacteria (*Bacillus, Pseudomonas*), actinomycetes (*Streotimyces*), yeasts (*Aureobasidium, Candida*) and fungi (*Trichoderma, Gliocladium, Clonostachys, Ulocladium*) c) organic acids (Fillinger and Walker 2016).

Each biocontrol agent presents a different mode of action against pathogens. Microorganisms like the bacterium *Pseudomonas spp*. have been found to alter the properties of plant surface thus inhibiting the attachment and growth of *Botrytis cinerea* (Elad and Stewart 2007). Moreover, the ability of some biocontrol agents (*Candida pulcherrima*) to adhere to the pathogenic fungus can inhibit mycelia hyphae growth or affect the dispersion of conidia, thus effectively reducing the amount of inoculum (Elad and Stewart 2007).

Another mechanism is competition for nutrients and niche between pathogen and biocontrol agents like algae, bacteria, and fungi (Elad and Stewart 2007). This mode of action finds great efficacy against *B. cinerea* because antagonistic microbes (*Bacillus spp*., *Pseudomonas sp*.) possess the ability to colonize plant surface much faster, thus inhibiting the establishment of the pathogenic fungus. Moreover, biocontrol agents have been found to colonize wounds which are located at the epidermis of fruits and are regarded as a possible way to combat post-harvest infections (Haidar et al. 2016).

Inhibiting compounds secreted by biocontrol agents can also function as antibiosis against *B. cinerea* infections. *Trichoderma spp*. and *Gliocladium spp*. have been reported in many

studies for their action through antibiotic substances exudation (Elad and Stewart 2007). The suppressive effect of *Bacillus subtilis* and *Bacillus pumillus* against *B. cinerea* has been attributed to the mechanism of antibiosis, while the reduction of conidia germination of *Botrytis fabae* derives from the secretion of substances of *Penicillium chrysogenum* (Elad and Stewart 2007). Metabolites like enzymes (cutinases, glucanases, cellulases, proteases) of bacterial or algae origin can interfere with the development of pathogens. According to Essghaier et al. (2009) many bacterial species like *Bacillus subtilis, B. licheniformis, B. pumilis, Halomonas elongate, Staphylococus sp*. have been found to inhibit the growth of *B. cinerea* through exudation of lytic enzymes (Haidar et al. 2016).

Finally, induced systemic resistance (ISR) can be a result of many microbes, effectively dealing with pathogens (Paulitza and Matta 1999). Microorganisms that induce this kind of mechanism can be non-pathogenic, saprophytic, or even non-pathogenic strains of the potential pathogen (Elad and Stewart 2007). Many studies have been conducted on the mode of action of *Trichoderma spp*. It has been clear that it promotes the stimulation of several genes that are connected to the salicylic and jasmonic acid pathways, activating the plant defence system (Hermosa et al. 2012). However, the activation of these genes is affected by the host age, the tissue type, and the application way (Nicot et al. 2016). ISR has been proved to be activated by many microbes, some of which are *Micromonospora, Saccharothrix algeriensis,* and *Pseudomonas fluorescens* (Haidar et al. 2016).

Table 5: Biocontrol agents and their effects against *B. cinerea*

Biocontrol Agent examined in each study	Origin of Biocontrol Agent	Host species used in each study	Results	Reference
Fusarium oxysporum, *Aureobasidium pullulans,*	Fungi Fungi Fungi	Tomato leaf	Disease reduction: 44.8% for bacteria	Jürgen Köhl et al. 2020

Sporobolomyces roseus, *Chryseobacterium sp.*	Bacteria		53.1% for fungi	
Issatchenkia terricola	Yeast	Table grape berries	Inhibition percentage 80%	Vargas et al. 2012
Meyerozyma guilliermondii, *Candida membranifaciens,* *Bacillus sp.,* *Ralstonia sp.*	Yeast Yeast Bacteria Bacteria	Grape berries and leaves	Inhibition percentage 45% for yeasts 50-55% for bacteria	Kazem Kasf et al. 2018
Bacillus amyloliquefaciens, *Bacillus subtilis,* *Bacillus licheniformis*	Bacteria	Strawberry, tomato, grape fruits	Disease reduction ranging from 70-86%	Chen et al. 2018
Oxalate Degrading Bacteria (ODB)	Bacteria	Spinach, strawberries	Disease suppression effect	Lee et al. 2020
B. subtillis PHYS77, *B. subtillis PHYS78,* *Pseudomonas fluorescence*	Bacteria	Onion plants	Inhibition percentage of 62,60 45% respectively	Abo-Elyousr, K.A.M et al. 2020
Rodotrula sp., *Aureobasidium sp.,* *Cryptococcus sp.*	Yeast Fungi Fungi	Table grapes	Potential biocontrol agents	Carmichael et al. 2019
Burkholderia sp., *Paraburkholderia sp*	Bacteria	Maize rhizosphere	Disease suppression	Esmaeel et al. 2019

Jürgen Köhl et al. (2020) conducted an experiment bioassay on tomato leaf infection by *B. cinerea*. They excised leaflets from tomato plants, sprayed them with the potential

antagonists (water for positive control), and then each leaflet was inoculated with a suspension of *B. cinerea* spores. The lesion diameter was measured reaching up to 5.06mm for positive controls. The application of some antagonists reduced lesion development by up to 2.30mm with the most efficient isolates being *Fusarium oxysporum* HTS519 and *Aureobasidium pullulans* HTS551.

In another study, yeast isolates with potential biocontrol abilities against grey mould were examined for their antagonistic effect. The most promising ones were used in vivo on grape berries, then, the pathogen was applied 1 and 24 hours after the application of the yeasts. Some yeast isolates reduced the amount of disease incidence by up to 80%. Sequencing analysis later revealed that these isolates corresponded to *Issatchenkia terricola* (Vargas et al. 2012).

Kazem Kasf et al. (2018) isolated yeasts and bacteria from the epiphytic flora of grapes and leaves. The isolates were then screened concerning their potential antagonistic action against *B. cinerea*. The most effective ones were further tested on dual cultures and in vivo. Both fungi and bacteria showed inhibition percentages around 50%. The most noteworthy were then sequenced and identified as *Meyerozyma guilliermondii, Candida membranifaciens* regarding fungi and *Bacillus sp*. and *Ralstonia sp*. for bacteria.

Apart from testing bacteria per se, Chen et al. (2018) also examined their culture filtrates and extracts against *Botrytis*. They artificially infected strawberry, grape, and tomato fruits with both the bacteria and the pathogenic fungus. The different bacteria, *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis* reduced the disease development by up to 86% with the different organisms ranging among the three plant hosts. Abo-Elyousr, K.A.M et al. (2020) also examined the antagonistic potential of some strains of *Bacillus subtilis*, on onion plants, which in turn showed inhibition of grey mould disease by up to 62%.

Identification of potential fungi and yeasts also occurred by Carmichael et al. (2019). They showed that every development stage of grapes with a low concentration of *B. cinerea* had abundant populations of *Rhodotorula, Aureobasidium,* and *Cryptococcus*. Finally,

another study focused on some PGPR strains showed that strains of *Burkholderia* and *Parabulkholderia* can not only promote plant growth but also help plants resist pathogen attacks (Esmaeel et al. 2019).

Conclusions

To sum up, concerning *Botrytis cinerea*, resistance development is a crucial issue that besets farmers all around the globe. Resistance has been observed in every class of botryticides currently in use. However, it is vital to highlight that the mode of action, and thus the correlated resistance, of a specific class, anilinopyrimidines, has yet to be identified. Combining recent studies, it seems that the target of AP fungicides is a mitochondrial one, in contrast to what has been speculated, up to recently. Future studies should be conducted on this matter to enable easier and better monitoring procedures and resistance control. In addition to anilinopyrimidines, phenylpyrrole fungicides also require more deep understanding. While they show a surprisingly low resistance frequency in the field, resistance to this class should be analyzed and studied, to prevent the future loss of this specific mode of action, due to resistance prevalence. Except for target site-resistance-conferring mutations, multidrug resistance phenotypes of *B. cinerea* have been also somewhat recently identified. Up to now, their impact on the agricultural world remains obscure, nevertheless, it is proved that this type of resistance is responsible for the simultaneous moderate resistance to more than one different active ingredient. A deeper understanding of this issue could provide insight into this phenomenon, because a combination of multidrug resistance phenotype with target-site mutations could lead to simultaneous resistance to many botryticides, rendering many active ingredients inefficient. Biological control, on the other hand, is another, much promising control method. Resistance emergence is absent in biological control so it should be deployed in pathogen control schemes. During the last years, more and more studies are conducted on *B. cinerea* biological control, and many biocontrol agents have been discovered and

evaluated. Studies on this matter must continue, to enable more efficient and safe control of this plant pathogen.

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