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# Resistance to Botryticides

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## 1. Introduction

Plant pathogenic fungi and oomycetes as causal of plant diseases are responsible for economical looses in agricultural production worldwide. Therefore, their chemical control by products named fungicides is needed and justified especially when disease tend to became an epidemic. Without fungicides both yield and quality would be severely reduced by the ravages of fungi. The improved performance, specificity and environmental safety of the modern fungicides led to become their ever more widely used. But, as great Renaissance man Leonardo da Vinci said: "*The nature never breaks her own laws*", the fungi constantly found the new ways to adapt to conditions that human creates and keep existing and living. Fungus develops insensibility to chemical compound aimed to their suppression under constant pressure of often and continuous use of fungicide with specific mechanisms of action. This ability is nothing else than natural phenomenon or evolution. Today this phenomenon is less mysterious than three decades ago when first arise although some new challenges have spring up. Phenomenon of insensibility of fungus to the chemical compound used for controlling it is named resistance. With the increased use and specificity of the product comes a greater risk that resistance will developed because certain members of the target fungal population will not be affected by the product and therefore fungus cannot be controlled adequately any more. That is, they are genetically resistant to it. Although some plant diseases may be managed through the alteration of cultural practices, many diseases are only managed acceptably with the application of fungicides. One of them is grey mould of wine grape caused by ascomycete fungus *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). Even today the only effective control of *B. cinerea* remains application of fungicides specifically named botryticides. In the past *B. cinerea* has proved to be very prone to resistance development which makes it difficult to control. Those drown attention of scientists and catalyse studies of resistance phenomenon in *B. cinerea*. Furthermore, resistance phenomenon intensified the genetic studies of this fungus because it was assumed that limited understanding of the genetic structure of *B. cinerea* populations is reflecting in difficulties in managing the disease. Despite of gained knowledge about *B. cinerea* resistance and managing solutions the resistance is still an ever present threat with new cases arising and some old problems still continuing. A new segment of the topic becomes issue of multiple drug resistance (MDR). MDR phenomenon is common in human pathogens but it has been rarely described before in field strains of plant pathogenic fungi. Gaining knowledge about MDR revealed existence and involvement of some different mechanisms for resistance development. Fungicide resistance mechanisms

can relate to qualitative factors such as absence or presence of a sensitive target site. Beside this, qualitative factors like uptake, transport, storage and metabolism also need to be considered. The MDR phenomenon of *B. cinerea* was firstly recorded in 1998. Since than, more data of MDR monitoring were obtained indicating that *B. cinerea* MDR types in combination with other *B. cinerea* resistant types could represent a significant threat for future chemical control of *B. cinerea*.

## 2. Bunch rot of grapes: High standard disease of grapevine

Bunch rot of grapes is one of the grapevine diseases of great economical importance because it leads to substantial losses in yield and lowering in quality. Vineyard ecosystem is often difficult to manipulate both the crop and its environment. Also, it is a stage where *B. cinerea* can express its dual nature in causing the destructive bunch rot and, under certain conditions, the non-destructive noble rot, which is not paralleled in plant pathology. Noble rot yields vines of a special quality that are high economical. In the continental climate the bunch rot disease can inflict damages up to 50 or 60 percent and under the Mediterranean climate 3 to 5 percent. The damages are continuing in vine making process. Rotting of grape berries caused by fungus is probably old as winegrowing and some descriptions date from time of Roman Plinius the Older (1. century). Even the genus name *Botrytis* is derived from Latin for "grapes like ashes" by Micheli who erected the genus in 1729. Name of disease, grey mould, actually describes the grey coating spread over the bunch especially beacon before vintage when the most damage is already done. The coating is somatic filamentous body or sporulating mycelia of fungus *B. cinerea*. In grapevine *B. cinerea* causes massive losses of yield and quality of grape berries for vine production especially during cool and wet climatic conditions. This fungus is able to act as saprophyte, necrotroph as well as pathogen. In vineyards *B. cinerea* is present as part of the environmental micro-bionta and predominantly being saprophytic it colonize wounds or senescing tissue. From an economic point of view, only while acting as true pathogen infecting flowers and grape berries are of importance in terms of lowering quantity and quality of yield. Although there are numerous scientific contributions that continue to be published, there are still gaps in our knowledge about the etiology and epidemiology of bunch rot disease in vineyards. Disease starts with infections in flowering and even earlier. Establishment of *B. cinerea* on moribund and injured tissues normally allows pathogen to infect health tissues. Source of inoculum which will initiate further cycle of the disease are sclerotia and mycelium formed in the outer layers of the dead bark of shoots, cane or on plant debris of various origin. The sclerotia may be directly infective as sources of conidia yet some sclerotia are not conidia-bearing but form reproductive body apothecia. The ascospores produced from apothecia can also initiate primary infections although sexual stage is not considered as significant for epidemiology of grey mould yet Anotnin de Bary described easily found apothecia on dead vine leaves in late 1866. Sclerotia are rare in the regions with warm dry summers and therefore it is unlikely apothecia will be found either. Sporulation on sclerotia is repeated and this extend period of conidial production and infection. Rain and splashing water under natural conditions dislodge conidia from germinating sclerotia and conidia are dispersing in air currents, in splashing water droplets and by insects. The "fruit fly" *Drosopilla melanogaster* is considered as plurimodal vector of *B. cinerea*. The concentration of conidia in the air is increasing as the grapevines maturing. The mycelium spread through outer layers of the dead bark of shoots and the bark of invade cane is bleached to almost white colour. *Botrytis*

mycelium sometimes invades the nodes and buds on lower parts of the shoots especially if they had bad wood maturation in the autumn. Buds with dormant mycelia will be finally killed and this will reduce bud burst on the basal parts of the fruit canes in the next spring. Sclerotia and mycelium can also exist on various plants surrounding vineyard and from there conidia disperse in air currents are imported to vineyard. Sclerotia and conidia can be developed on pruned cans left *in situ* or on mummified berries. Abundance of described carry over inoculum in the beginning of new vegetation season at pre-flowering stage is quantitatively related and therefore important for flower infections. Infections are favoured by wet period, at least 12 hours duration, and temperature between 15 and 20 °C. Primary infections of grapes occurs at bloom time or at the end of it when *B. cinerea* starts it's life cycle as biotroph infecting flowers through the stigma and style and then into the stylar end to the ovary. Infected flowers are symptomless and only microscopic examination will reveal necrosis of stamens and growth of the pathogen on the style and stigma. These flower infections are invariably followed by a period of latency when fungus remains in a quiescent phase in receptacle area. Flower infection is believed to be an important stage in the epidemiology of *B. cinerea* in grapes. Furthermore, early infections of the generative organs can destroy flower bunches. Infected flowers, also could become potent inocula within developing bunches for berry rot. Because of the abundance of necrotic floral debris in the vineyards, the end of flowering represents an important epidemiological stage for *B. cinerea.* The floral debris provides an excellent nutrient source for the conidia. Floral debris bearing mycelium are dispersed in wind and rain (Jarvis, 1980) onto leaves and berries. After infection at bloom time following symptomless latent phase, generally until berries begin to ripen. Latency could be explained by the ability of the young berries to synthesize stilbenes until veraison (Pezet & Pont, 1992), maintaining the fungus in the receptacle area from where it can spread into the berry during ripening. During the development of berries until veraison, when the berries begin to soften, the berries are resistant to infection. The ripening process corresponds to a senescence process with a degradation of the berry tissues, especially activity allowing disease expression to occur. During this phase, the whole defence mechanisms controlling the pathogen loose their activity, allowing disease expression to occur. Grapevine tissues defend themselves against fungal attack by the accumulation of phytoalexins, like stilbenes, mostly in the green berries but stilbenes appears to be inactive during ripening (Pezet & Pont, 1992; Bais et al., 2000). After veraison the berries become increasingly susceptible to infection. At lower sugar content, less than 13° Brix, the so-called sour rot affects berries and leads very often to a complete loss of attached grapes. Sour rot is favourable with frequent rainfalls. At higher sugar content, attached berries can be processed normally but these forces growers to an earlier harvesting or to picking of moulded grapes. Infections of berries occur at temperature interval between 20 and 25 °C and are accomplished by conidia. Germlings that developed from conidia enter grape berries through different pathways, namely through stigmata, pedicels, natural openings and wounds, or by direct penetration of the cuticle (Coertze *et al*., 2001; Holz *et al*., 2003). Conidia are deposited on berry surphace by air, rain or insects. The most prominent symptom of the disease is found on the berries in the ripening period when the disease reaches its highest stage and lasts up to the end of harvest, being marked by softening and decay of grape berries. Infected berries are dark coloured and show the typical greyish, hairy mycelium all over their surface. Especially sporulating mycelium can be seen to grow along cracks or splits on the berries because tufts of condiophores with conida are protrude from stoma and peristomal cracks on the skin of the berry. The *B. cinerea* can also infect

young leaves and relatively older leaves. Leaf infections occur occasionally during long rainy periods with continuous leaf wetness over 48 hours and temperature between 15 and 20 °C. Heavy leaves infections are not very common because only long duration of leaf wetness allow mycelium to spread in the mesophyll. Therefore, leaves infections normally take place during rain spring. For the same reason in spring also young shoots can be infected from attached tendrils or small wounds. The quantitative relation between incidence of *B. cinerea* at critical stage in the growth of grapevines; pre-flowering (carry over), flowering and harvest was described by Nair et al. (1995). According to their observation the 50% incidence of *B. cinerea* monitored on grapevine tissues carried over from previous season during pre-flowering can predict 29% primary infections of flowers in the new season.

### 2.1 Managing grey mould

Although some prognostic models are developed based on etiology and epidemiology of grey mould disease the severity of the grey mould disease in vineyards cannot be easily predicted so therefore control based on prognosis may not be satisfactory. Effective control of grey mould in vineyard is usually based on preventive repeated fungicide applications during the season. Already the Romans used sulphur to control this disease. For the same purpose sulphur and potassium were recommended in 18th century. During the late 1950s fungicides were introduced in viticulture and until 1968 in many countries for *Botrytis* control were used: sulphamides (dichlofluanid), pthalimides (captan, captafol, folpet) and dithiocarbamate (thiram). At this point of time the efficacy of fungicidal treatments for *Botrytis* control ranged between 20 and 50 percent. All this fungicides were multi-site inhibitors, affecting many target sites in fungal cell and therefore acting as general enzyme inhibitors. In 1960s, first fungicides appeared which act primarily at one target site therefore referred to as single-site or site-specific and they more efficiently control pathogen. Today, several families of synthetic site-specific botryticides are available. They can be classified according to their biochemical modes of action into five categories: 1) anti-microtubule toxicants (benzimidazoles); 2) compounds affecting osmoregulation (dicarboximides, fludioxonil); 3) inhibitors of methionine biosynthesis (anilinopyrimidines) and 4) sterol biosynthesis inhibitors (fenhexamid); 5) fungicides affecting fungal respiration (fluazinam, boscalid and multi-site inhibitors). The era of sigle-site or site specific fungicides begun in late 1960s with introduction of benzimidazoles (benomyl, thiophanate-methyl, carbendazim) that improved *Botrytis* control (Dekker, 1977; Georgopoulos, 1979; Beever & O'Flaherty, 1985). Only a few years later the new group of dicarboximides become available and they shadowed all previously used ingredients. Dicarboximides were introduced into the market between 1975 and 1977 primarily for the control of *B. cinerea* in grapes (Beetz & Löcher, 1979). Due to good efficacy they were popularly named botryticides and it seemed that the problem of protection against *Botrytis* had been successfully solved. Dicarboximides or cyclic imides (e.g. chlozolinate, iprodione, procymidone, vinclozolin) are characterized by the presence of a 3,5-dichlorophenyl group. The activity of dicarboximides fungicides was first reported in the early 1970's with the three key commercial products being introduced within three years; iprodione in 1974 (Lacroix et al., 1974), vinclozolin in 1975 (Pommer & Mangold 1975) while procymidone was registered a year later (Hisada et al., 1976). They are typically protectant fungicides and although some claims to systemicity have been made (Hisada et al., 1976) they are best regarded as protectant materials. In the mid-1990s a novel

family of botryticides was arose, the anilinopyrimidines, with three representative ingredients: pyrimethanil, cyprodinil and mepanipyrim. Mepanipyrim and pyrimethanil exhibit a high activity against *B. cinerea,* while cyprodinil came in combination with fludioxonil (phenylpyrroles) in protection of grapes. Pyrimethanil and cyprodinil were introduced in French vineyards in 1994 (Leroux & Gredt, 1995) and in Switzerland they were registered since 1995 (Hilber & Hilber-Bodmer, 1998). In Italy cyprodinil was registered in 1997 (Liguori & Bassi, 1998.). Mepanipyrim was in 1995 registered in Switzerland, Japan and Israel (Muramatsu et al., 1996). Mixture of cyprodinil and fludioxonil was firstly introduced in Switzerland in 1995 (Zobrist & Hess, 1996). In Croatia pyrimethanil was acknowledged in 1997 under the commercial name Mythos and cyprodinil came as a mixture with fludioxonil named Switch while mepanipyrim was not registered at all (Topolovec-Pintarić & Cvjetković, 2003). Although anilinopyirimidines showed to be highly effective against *B. cinerea,* a high risk of resistance build up was already evident in the laboratory investigations at preregistration phase (Birchmore & Forster, 1996). In spite of that they have been registered in most European winegrowing countries since 1994 but with recommendations for restricted use: once per season when anilinopyirimidines are applied alone and a maximum of two applications per season is proposed for the mixture cyprodinil + fludioxonil (phenylpyrrol) (Fabreges & Birchmore, 1998; Leroux, 1995). Shortly after introduction of anilinopyrimidines in 1995 fludioxonil (phenylphyroles) start to be used in vineyards against *B. cinerea*. Fludioxonil is synthetic analouge of antibiotic pyrrolnitril (phenylphyrol), an antibiotic compound produced by a number of *Pseudomonas* spp. and is thought to play a role in biocontrol by these bacteria. Fludioxonil belong to class of fungicides affecting osmoregulation and is inhibitor of both spore germination and hyphal growth. In 1999 fluazinam (phenylpyridinamine) was introduced in French vineyards although in Japan has been used since 1990 against grey mould in various crops. Fluazinam belongs to group of fungicides that affecting fungal respiration so, it shows multi-site activity probably related to uncoupling of mitochondrial oxidative phosphorilation. It is highly toxic to spores and mycelia. Any shift of *B. cinerea* toward fluazinam in vineyards has still not revealed. In 1999, firstly in Switzerland, a botryticide with novel botryticidial action was registered, the fenhexamid (Baroffio et al., 2003). Early investigations on the fenhexamid mode of action suggested that it has different mechanism from than of all other botryticides (Rosslenbroich & Stuebler, 2000). Fenhexamid is a 1,4.hydroxyanilide with a high preventive activity against *B. cinerea*. It is easily degraded and therefore presents a favourable toxicological profile and environmental behaviour (Rosslenbroich et al., 1998; Rosslenbroich & Stuebler, 2000). It is characterized by a long duration action. Due to its lipophilic character it shows rapid uptake into the plant cuticle and within the upper tissue layer limited but significant locosystemic redistribution occurs (Haenssler & Pontzen, 1999) and as a result the rain fastness of fenhexamid is very pronounced. Fenhexamid suppresses the germination of spores only at relatively high concentrations but it is highly effective in inhibiting subsequent stages of infections. After the initiation of spore germination the fenhexamid inhibit the germ-tube elongations, germ-tubes collapse and die before they are able to penetrate plant surface. Also, treated hyphae frequently show a characteristic leakage of cytoplasm or cell wall associated material at the hyphal tip area (Haenssler & Pontzen, 1999; Debieu et al., 2001). It is sterol biosynthesis inhibitor and blocks the C4-demethylations (Rosslenbroich & Stuebler, 2000). The lastly released botryticide for use in grapevines, in 2004, is novel ingredient boscalid (syn. nicobifen). Boscalid from carboxamide group is systemic and is the only representative of

new generation of fungal respiration inhibitors. It act as inhibitor of fungal respiration morover it is new generation of succinate dehydrogenase inhibitors (SDHIs) which inhibit respirations by blocking the ubiquinone-binding site of mitochondrial complex II. In the future, arrivial of new anilide is expected, still described under code SC-0858.

## 3. Resistance to botryticides

In *B. cinerea* the resistance phenomenon, as in other plant pathogenic fungi, becomes apparent with the site-specific fungicides. Site-specific or single-site fungicides act primarily at single target under responsibility of single major gene. Thus, just a single gene mutation can cause the target site to alter (monogenic resistance), so as to become much less vulnerable to the fungicide (Brent, 1995). Therefore, within few years of intensive use of such fungicide, in populations of polycyclic pathogen with high propagation rate, can be found a high frequency of resistant mutants. The most common mechanism of fungicide resistance is based on alternations in the fungicide target protein. The resistance to multi-site fungicides, which effect many target sites in fungal cell, has been rarely reported. Multi-site fungicides have been considered as low-risk fungicide from the resistance point of view because they interfere with numerous metabolic steps and cause alternation of cellular structures.

### 3.1 Retrospective of botryticide resistance

As it was mentioned earlier, the oldest multi-site fungicides used in vineyards against grey mould, were thiram (dithiocarbamate), captan, folpet (chloromethylmercaptan derivates) chlorotalonil (phthalonitrile) and dichlofluanid (phenylsulphamide). This ingredients react with thiol, SH and amino group inducing formation of thiophosgene and hydrogen disulphide. They block several thiol-containing enzymes involved in respiratory processes during spore germination and this multi-site action is believed to prevent the development of resistance (Leroux et al., 2002). Therefore, they have been considered low-risk fungicide from the resistance point of view. But, in the 1980's strains resistant to dichlofluanid and to the chemically related tolylfluanid, chlorthalonil and even to phthalimides, captan and folpet, have occasionally been reported (Malatrakis, 1989; Rewal et al., 1991; Pollastro et al., 1996). Moreover, cross-resistance among captan, thiram, chlorothalonil and related fungicides were identified (Barak & Edington, 1984). Resistance to dichlofluanid is determined by two major genes, named *Dic1* and *Dic2*, probably involved in a detoxifying mechanism and in glutathione regulation (Pollastro et al., 1996; Leroux et al., 2002). The mutation of this genes lead to the formations of two sensitive phenotypes Dic1S and Dic2S, two phenotypes with low level resistance Dic1LR and Dic2LR and one high leveled resistant phenotype Dic1HR. In practice only a few cases of control failure due to dichlofluanid-resistant strains were noted. Although these ingredients are not at risk from resistance development and are still registered their practical use is restricted because they are weak botryticides and their residues can cause problems in vine making process (delay fermentation). First site-specific fungicide used in vineyards since the late 1960's was benzimidazole carbendazim or MBC. But, in the early 1970s, only a few years after commercialization loss of disease control due to resistance was reported in many crops especially in vineyards (Leroux et al., 1998). First report of surprisingly enhanced attacks of *B. cinerea*, rather then suppressed, after benzimidazole treatments was in Germany

(Ehrenhardt et al., 1973; Triphati & Schlosser, 1982; Bolton, 1976) but the outbreak of tolerant strains occurred simultaneously in many winegrowing countries in temperate climate. In Switzerland after only two years of use, in 1973, a complete loss of control by benzimidazole was observed and they were withdrawn (Schuepp & Küng, 1981). In Southern Europe where *B. cinerea* pressure is much lower, resistance appeared more slowly. In Mediterranean climate e.g. Italy satisfactory control was reported until 1977 (Bisiach et al., 1978). In Croatia benzimidazoles was used in protection of vineyards shortly from 1971 to 1974. Primarily they were redrawn from use in vineyards because of toxicological reason (negative residues in must and wine). A decrease in efficacy was in Hungary firstly observed in 1981 and it was confirmed by Kaptas & Dula (1984). In 1987 of special interest become mixture of carbendazime and dietophencarb owing to negatively correlated cross-resistance, allowing destruction of benzimidazole-resistant strains by dietophencarb. Soon, negatively correlated cross-resistance become positive as between 1988 and 1989 an overall increase of resistance from 4 to 22% to both components was detected. An explanation of the quick outcome of benzimidazole-resistance was the local existence of naturally resistant strains in the field population of *B. cinerea* before benzimidazole was introduced and their application acted as selected factor eliminating sensitive strains (Schuepp & Lauber, 1978). Benzimidazole carbendazim (MBC) does not affect spore germination but inhibit germ-tube elongation and mycelial growth at low concentrations. These anti-fungal impacts came from MBC binding to tubulin, which is the main protein in microtubules. Microtubules, one type of cytoskeleton filament, regulate organelle position and movement within the cell. Microtubules consist of long, hollow cylinders of repeating dimers of α- and ß-tubulin. MBC binding to tubulin leads to inhibition of the microtubule assembly (Leroux et al., 2002). Alterations in the binding sites on the ß-tubulin protein are related to benzimidazole-resistance (Leroux & Clearjeau, 1985). Approximately 10 mutations conferring resistance to MBC have been identified in the ß-tubulin gene in laboratory studies with a wide range of different fungi. Benzimidazole-resistance in *B. cinerea* is conferred by polyallelic major gene named *Mbc1* by Faretra & Polastro (1991) with at least four classes of alleles responsible for sensitivity or different levels of resistance variously accompanied by hypersensitivity to *N*-phenylcarbamates (Faretra et al., 1989; Faretra & Pollastro, 1991, 1993a; Pollastro & Faretra, 1992; Yarden & Katan, 1993; Davidse & Ishii, 1995, De Guido et al., 2007). The presumed mutated locus encoded the structural gene for ß-tubulin and single base pair mutations occurred in codons 198 and 200. Two phenotypes exhibiting benzidimadozle-resistance were determined by Leroux et al (2002) in *B. cinerea* populations from French vineyards. Phenotype Ben R1 exhibit high resistance levels (greater then 250) to MBC is simultaneously more sensitive to phenylcarbamate dietophencarb then the wild type strains. The second phenotype Ben R2 was detected after introduction of the mixture carbendazime+ dietophencarb in 1987. Ben R2 is moderately resistant to MBC (levels 100-200) and insensitive to dietophencarb, just like strains sensitive to MBC. In both phenotypes resistance was conferred by alleles of the *Mbc1*: in Ben R1, at position 198 an alanine replaced a glutamate, whereas in Ben R2, at position 200 a tyrosine replaced a phenylalanine (Yarden & Katan, 1993). Resistance to the MBC is a type of 'qualitative' or 'single-step' resistance characterised by a sudden and marked loss of effectiveness, and by the presence of clear-cut sensitive and resistant pathogen populations with widely differing responses (Brent, 1995). Once developed, it tends to be stable, resistant strains have persisted after many years of non-use and sensitivity will usually not be restored by cessation of their use.

Due to stable resistance in vineyards and also for toxicological reason (unwanted toxic residues in vine) MBI were redrawn from use in protection of vineyards.

Benzimidazole carbendazim was followed by dicarboximides which has been available since 1976 (Lorenz & Eichhorn, 1978). Owing to MBI resistance they were welcomed and become recognized as botryticides due to their efficacy superior to formerly used fungicides for that purpose. For almost a decade it seemed that the protection of vineyards against *B. cinerea* had been successfully solved. The appearance of resistance to dicarboximides did not come as so obvious and sudden loss of efficacy that gave first indication of resistance in the case of MBI. Dicarboximides efficacy was diminishing with time and protection slowly become insufficient. Therefore, resistance to dicarboximides, appears to involve slower shifts toward insensitivity because of multiple-gene involvement. As resistance management strategies were poorly understood at that time this inevitably led to dicarboximides overuse and resistance development. In spite of resistance development no total loss of control occurred so dicarboximides use was continued. Moreover, there were no alternative botryticides at the time and as consequence, the proportion of resistant strains in *B. cinerea* population increased considerably. Resistance to dicarboximides *in vitro* was achieved in 1976 (Leroux et al., 1977). Practical dicarboximides-resistance was firstly detected in 1978 in Switzerland (Schüepp & Küng, 1978). The first appearance of resistance in a particular fungicide-pathogen combination in one region has almost always been accompanied, or soon followed, by parallel behaviour in other regions where the fungicide is applied at a similar intensity (Brent, 1995). Thereby, resistance was determined in 1979 in Germany (Holz, 1979) and in Italy (Gulino & Garibaldi, 1979) and in 1982 in France (Leroux & Basselat, 1984; Leroux & Clerjeau, 1985). In Hungary dicarboximides were registered in 1978 and decrease in sensitivity was observed in 1988 and confirmed in 1994 (Dula & Kaptas, 1994). In Slovenian vineyads dicarboximides-resistance was reported (Maček, 1981). In Croatia dicarboximides were introduced in protection of vineyards in 1979. A decrease of efficacy was observed at the end of '80-ties and resistance was proved in 1990 (Cvjetković et al., 1994). Since the beginning of the 1980s, practical resistance to dicarboximides has been related to the selection of moderately resistant strains, named ImiR1 (Leroux & Clerjeau, 1985). Initial studies on dicarboximides-resistance management were started in Germany (Löcher et al., 1985) and France (Leroux & Clerjeau, 1985). To delay the selection of resistant strains during the vegetative period the use of dixarboximides was soon restricted to only two treatments after veraison in Europe (Besselat, 1984; Locher et al., 1987). Unfortunatelly, their efficacy seemed to decrease with infection pressure and goes under 40% and most of the dicarboximides-resistant strains also exhibited high simultaneous resistance to benzimidazoles (Schlamp, 1988). Dicarboximides disturb the synthesis of the cell wall of hyphae by inducing accumulation of glycerol, which burst eventually. A lot of effort was made to investigate primary mode of dicarboximides action. In 1977 was suggested that the primary effect of vinclozolin and iprodion is on DNA production and that lipid metabolism is also affected (Leroux et al., 1977). Following studies showed that dicarboximides have little effect on respiration or the biosynthesis of sterols, nucleic acids, proteins or chitin (Pappas & Fisher, 1979). Edlich & Lyr (1987) described that dicarboximides inactive enzymes are involved in electron transport, causing the production of reactive oxygen products (like $O_2^-$ and $H_2O_2$) and initiate lipid peroxidation. Moreover, enhanced levels of catalase and superoxide dismutase recorded in some dicarboximides-resistant strains could be responsible for the detoxification of peroxy radicals although a conclusive correlation

between amounts of such enzymes and the levels of fungicide resistance has not been found when comparing many field strains and laboratory mutants of *B. cinerea* (Leroux et al., 2002; Edlich & Lyr, 1992). According to Edlich & Lyr (1992) the potential target site of dicarboximides might be a plasma-membrane-bound NADPH-dependent flavin enzyme, inhibition of which would initiate pathological oxidative processes. Therefore, components of glutathione system are targets of dicarboximides. Several findings suggest that they interfere with the osmotic signal transduction pathway consisting of histidine kinase and MAP kinase cascades. Therefore, their primary target sites could be protein kinases involved in the regulation of polyol biosynthesis (Leroux et al., 1999; Schumacher et al., 1997). Set up of target site dicarboximides affecting should enable confirmation of gene responsible for resistance. But, despite of many long-term investigations the mechanism of dicarboximides resistance is not elucidating yet. The most comprehensive data on the genetics of dicarboximides-resistance have been obtained from studies of F. Faretra whose work has clarified the sexual behaviour and matting system of *B. cinerea* and resulted in a reliable technique for obtaining ascospore progeny under laboratory conditions (Faretra & Antonaci, 1987). Resistance to dicarboximides is encoded by a single polyallelic major gene named *Daf1* (Faretra & Antonaci, 1987). Firstly, two alleles of *Daf1* have been recognized (Faretra & Pollastro, 1991): *Daf1* LR and *Daf1* HR responsible for low and high resistance to dicarboximides. Alleles *Daf1* HR also result in hypersensitivity to high osmotic pressure. In further studies conducted with field isolates and laboratory mutants general, was perceived that the resistance mechanism of field isolates differs from that of laboratory isolates. Dicarboximides resistant field isolates were designate as Imi R1 and laboratory mutants as Imi R4 (Leroux et al., 2002). Practical resistance to dicarboximides was only detected with Imi R1 strains (carrying *Daf1* LR alleles) and not with Imi R4 (carrying *Daf1* HR alleles) because of the absence of Imi R4 strains under field conditions. Most dicarboximides-resistant laboratory mutants (Imi R4) acquire high resistance to dicarboximides, but also to aromatic hydrocarbons (AHF) and pheylpyrolles and they are hypersensitive to osmotic stress. High-level dicarboximides-resistant strains of *B. cinerea* have seldom been obtained in the field whereas low- and moderate-level resistant strains (Imi R1) are normally associated with field isolates and are still capable of causing disease control failure. Furthermore, from the field only moderately resistant strains (Imi R1) without osmotic-sensitive phenotypes are recovered (wild type strains are tolerant to osmotic pressure). In addition, dicarboximides-resistant field isolates (Imi R1) show various levels of cross-resistance to aromatic hydrocarbons (AHF) (due to similarity of chemical structure because both have benzene ring in chemical structure) but not to phenylpyrolles (fludioxonil).

Fungicidal toxicity of phenylphyroles is reverted by piperonyl butoxide and α-tocopherol in *B. cinerea*. Different levels of dicarboximides-resistance variously accompanied by resistance to phenylpyrrole fungicides and reduced tolerance to high osmotic pressure point to polymorphism of *Daf1* and with time become evident that there are at least five classes of responsible alleles (Hilber et al., 1995; Faretra & Pollastro, 1991; Faretra & Pollastro, 1993a, 1993b; Vignutelli et al., 2002; Baroffio et al., 2003). Recent studies suggested that an amino acid substitution of serine for isoleucine in the second unit of tandem amino acid repeats on 86 codon of BcOS1p gene is responsible for dicarboximides resistance in the field (Oshima et al., 2002). Preliminary data show that all strains containing a mutation from isoleucine to serine are resistant to dicarboximides without exception. However, some isolates with isoleucine at codon 86 in the second unit are resistant to dicarboximides, suggesting the

possibility of other types of resistant strains in the field. Furthermore, Oshima et al. (2002) suggest that most of the mutations within the *BcOS1* gene affect virulence or fitness in *B. cinerea* under field conditions owing to well known fact of dicarboximides-resistant strains rapid decreases after discontinues applications of dicarboximides. According to Leroux (Leroux et al., 2002) dicarboximides-resistant field strains (Imi R1) contained a single base pair mutation at position 365 in a two-component histidine kinase gene, probably involved in the fungal osmoregulation. Dicarboximides-resistant laboratory strains (Imi R4) contained a single base pare mutation on 325 codon in gene also responasble for histidine kinase. In addition, both field strains Imi R1 and laboratory resistant strains Imi R4 showed resistance to the aromatic hydrocarbon fungicides (AHF) and especially to dicloran which is effective against grey mould on lettuces and on fruits during storage. Other *B. cinerea* isolates, Imi R2 and Imi R3, with different patterns of cross-resistance, were also detected in French vineyards (Leroux et al., 1999). Dicarboximides-resistant strains Imi R2 show cross-resistance to both phenylpyrroles and AHFs while Imi R3 are more resistant to dicarboximides then Imi R1 but are weakly resistant to pheylpyrroles. In some *B. cinerea* mutants, fungicide resistance was caused by a mutation in another gene, Daf2, which did not seem to be linked to the Daf1 gene (Faretra & Pollastro, 1993b). Although the primary target site of dicarboximides, phenylpyrroles and AHFs has not been clearly identified, these fungicides are the only commercial ones that seem so far to interfere with plant pathogens through the inhibition of a protein kinase (cit. Leroux et al., 2002). *B. cinerea* practical resistance to phenylpyrroles has not been demonstrated in the vineyards to date.

In the mid-1990s arise a novel family of botryticides, the anilinopyrimidines, with three representative ingredients: pyrimethanil, cyprodinil and mepanipyrim. Although anilinopyirimidines showed to be highly effective against *B. cinerea* a high risk of resistance was already evident in the first laboratory investigations (Birchmore & Forster, 1996) and therefore were put on the market with recommendations for restricted use. In the field pyrimethanil- and cyprodinil-resistant strains of *B. cinerea* were detected during preliminary testing in 1993 and 1994 in French (Leroux & Gredt, 1995) and Swiss vineyards (Forster & Staub, 1996). In Italy resistant strains were detected in 1996 even in vineyards where anilinopyrimidines have never been used before (Gullino & Garibaldi, 1979). Resistance to mepanipyrim was tested only in Japan and was not detected (Muramatsu & Miura, 1996). Organisation FRAC (Fungicide Resistance Action Committee at Global Crop Protection Federation (GCPF)) formed a new working group for anilinopyrimidine-resistance which in 1995 organised "ad hoc EPPO Workshop" in Switzerland and addressed to all winegrowing countries because of: "*… emergent and critical situation of B. cinerea resistance to anilinopyrimidines especially in vineyards...*" Even then was emphasize that efficacy of anilinopyrimidines can be saved and prolonged only with well organized monitoring and antiresistant strategy. Anilinopyrimidines exhibit some systemic translocation in plant tissues, and together with their image of pathogenesis inhibitors they possess protective activity and as it is said also some curative activity. Yet, in order to achieve satisfactory botryticidal effect it is recommended to use them preventively. They do not affect spore germination but germ tube elongation is inhibiting as well as mycelial growth at low concentrations. Under *in vitro* studies toxicity toward mycelial growth depends upon nutrition status of media and is greatly reduced on rich complex media. They posses ability to prevent fungal secretion of hydrolytic enzymes such as protease, cellulase, lipase or cutinase which play an important role in the infection and therefore they are considered as

inhibitors of pathogenesis (Miura et al., 1994; Milling & Richardson, 1995). In *B. cinerea* anilinopyrimidines prevent secretion of laccase and in grape treated with pyrimethanil reduce laccase activity can be observed (Dubos et al., 1996). Laccasa is phenol oxidase and causes oxidation of must so reduction of their quantity by pyrimethanil is welcome effect. The exact mechanism of action in the protein secretory pathway is not yet understood; it has been hypothesized that the target of anilinopyrimidines could be a step involving the Golgi complex or a later stage (Milling & Rhichardson, 1995; Miura et al., 1994). Anilinopyrimidines are particularly inhibitors of methionine biosynthesis. Enzymes which are involved in methionine biosynthesis are cystathionine γ-sinthase and cystathionine β-lyase. The late one might be their primary target site (Leroux et al., 2002). Biochemical studies showed that methionine and homocysteine (prior to methionine) were lower in mycelia after treatment by pyrimethanil while slight increase of precursor cystathionine was recored. However, recent enzymatic studies revealed only weak inhibition of cystathionine β-lyase by anilinopyrimidines (Leroux, 1994; Masner et al., 1994) and conclusive results with the isolated enzyme were not given. Moreover, the relevance of the inhibition of methionine biosynthesis in the fungus while it is invading plant tissue (that offers a rich source of methionine) has yet to be elucidated (Rosslenbroich & Stuebler, 2000). According to Rosslenbroich & Stuebler (2000) the inhibition of methionine biosynthesis and the secretion of hydrolytic enzymes may be associated with the mechanism of antifungal action of the anilinopyrimidines but might also be only a secondary effect. The discovery of *B. cinerea* strains that exhibit *in vitro* and *in vivo* resistance to anilinopyrimidines suggests that they do not interfere with pathogenesis alone. Based on long-term monitoring conducted since 1993 in French vineyards Leroux (Leroux et al., 1999) distinguished two groups of strains resistant to anilinopyrimidines: I) highly resistant strains with EC 50 greater than 0.5 mg $l^{-1}$ and II) less resistant strains with EC 50 lower than 0.4 mg $l^{-1}$ . All AniR strains were *transposa* types but according to their response to other fungicides they proposed following three anilinopyrimidine-resistant phenotypes: AniR1, AniR2 and AniR3. Practical resistance was observed only with AniR1 strains. Phenotype AniR1 was found in most European countries. It is moderately to highly resistant to anilinopyrimidines and *in vitro* response to new experimental anilide SC-0858 and other fungicidal groups is similar to the wild-type strains. Phenotypes AniR2 and AniR3 are weakly resistant to anilinopyrimidines and resistance was mainly recorded at the germ-tube elongation stage. Most important was founding that AniR2 and AniR3 were cross-resistant to chemically unrelated fungicides: dicarboximides, phenylpyrroles, several inhibitors of sterol biosynthesis, fenhexamide, tolnaftate,14 α-demethylation (DMIs), anilide SC-0858 and cyclohexamide (Leroux et al., 1999; Chapeland et al, 1999). AniR3 isolates were also resistant to azole fungicides. Genetic analysis showed that fungicide resistance in phenotype AniR1 is encoded by one major gene (Chapeland et al., 1999; Hilber & Hilber-Bodmer, 1998) but AniR2 and AniR3 are encoded by two different single major genes. Chapeland et al (1999) hypothesize that AniR2 and AniR3 posse's mechanism of resistance which consist of reduced accumulation of fungicides within mycelium and could be mediated by excretion of toxic molecules. Mechanism is correlated with increased mRNA levels of specific transport genes. Phenomenon of reduced accumulation of fungicides is known as "pleiotropic drug resistance" (PDR) or "multi drug resistance" (MDR) which is discussed later in 3.2. Therefore, as these phenotypes are multi-drug resistant (MDR) Chapeland et al. (1999) renamed AniR2 as MDR1 and determined them as anilniopyrimidine-resistant strains with considerable cross-resistance levels mainly

towards fludioxonil, cyprodinil and tolnaftate. Ani R3 become MDR2 which is characterized by increased resistance to fenhexamid, tolnaftate, cycloheximide, and cyprodinil. A third MDR phenotype, MDR3, was first detected in 2001. It is characterized by the highest levels and broadest spectrum of resistance against most fungicides tested (Kretschmer et al., 2009) contraty to MDR1 and MDR2 which have overlapping but distinct profiles. The frequency of MDR strains in the Champagne steadily increased until 2008, when the three MDR phenotypes together represented 55% of the total population. In contrast to the Champagne in German Wine Road region between 2006 and 2009, increasing MDR populations were also observed, but the MDR1 phenotype was clearly dominating (Kretschmer et al., 2009). In Croatia the occurence of resistant phenotype of *B. cinerea* to pyrimethanil was determined for the first time in 1999 after three years of intensive use of pyrimethanil (Topolovec-Pintarić & Cvjetković, 2002). The cross-resistant strains to cyprodinil were detected also (Topolovec-Pintarić & Cvjetković, 2003). Pyrimethanil- and cyprodinil-resistant strains were also detected in vineyards where anilinopyrimidines had never been used and this strains seemed to be "naturally" resistant and could be of AniR1 type. The growing number of resistant phenotype from the first to the last year of the 3-years trial lead to the conclusion of the appearance of so called "acquired resistance". The testing was conducted *in vitro* by germ tube assay so the resistance was determined in the germ-tube elongation stage. All anilinopyrimidine-resistant strains were *transposa* (Topolovec-Pintarić et al., 2004) so they belong to AniR2 or AniR3. Some of isolates showed cross-resistance to unrelated vinclozolin and fenhexamid (Topolovec-Pintarić, 2009). Described profile imply that this strains could belong to MDR2 type.

In 1999 botryticide with a high preventive activity against *B. cinerea,* fenhexamide, was introduced into vineyards (Baroffio et al., 2003). Owing to its novel mode of action, differing from all other botryticides it was presumed that resistance to fenhexamid will not occur easily. Analyses of unsaponifiable compounds conducted by Debieu et al. (2001) revealed that fenhexamid inhibited sterol biosynthesis in *B. cinerea*. The major sterol constitutes in *B. cinerea*, as well as in most filamentous fungi, is ergosterol. In the presence of fenhexamide ergosterol is reduced while its precursors 4α-methyl and 4-desmethyl 3-keto compounds are accumulated. This indicates that fenhexamid inhibits the 3-keto reductase, one of the four proteins of the enzymatic complex implicated in sterol C-4 demethylation process (Debieu et al, 2001). Thus, inhibition of 3-keto reductase leads to sterol C-4 demethylation inhibition and as a result the 4α-fecosterone and fecosterone are produced. Subsequent isomerization of 4α-fecosterone and fecosterone would give 4α-methylepisterone and episterone. Sterone accumulation is linked to growth inhibition and therefore is responsible for fenhexamide fungi toxicity. High risk of resistance was already evident in the first laboratory investigations. The baseline sensitivity of *B. cinerea* towards fenhexamide was recorded in 1992 and afterwards resistant strains of *B. cinerea* were detected in French and Swiss vineyards but so far loss of the fungicide's effectiveness has never been observed (Leroux et al., 1999; Suty et al., 1997; Baroffio et al., 2003). In France were high level fenhexamid-resistant strains collected even before use of fenhexamid. In Switzerland was obtained that fenhexamid-resistance can develop very rapidly, during 4 years from 0% up to 100% (Barofio et al., 2003). Knowledge on the risk of resistance to this fungicide is so far scant, although limiting the number of sprays per season is recommended (de Guido et al., 2007; Fungicide Resistance Action Committee [FRAC], 2006). It seems that fenhexamid resistance is not easily induced in *B. cinerea* because experiments towards selection of laboratory

mutants resistant to fenhexamid (either spontaneous or UV-induced) produced only few mutants often with aberrant morphology and colony growth. Hence, there are an association between fenhexamid-resistance and reduced fitness (de Guido et al., 2007). Genetic analysis indicated that the resistant phenotypes are encoded by single major gene(s) which is/are not linked with *Mbc1* and *Daf1* (de Guido et al., 2007). Fenhexamid resistance is caused by mutations in the erg27 gene encoded 3-keto reductase according to Fillinger et al. (2008). Alberini et al. (2002) described four phenotypes according to their responses towards fenhexamid: HydS, HydR1, HydR2 and HydR3. A HydS type is wild type sensitive to fenhexamid. HydR1 is naturally fenhexamid-resistant strains with negative cross-resistance to other SBIs (sterol biosynthesis inhibitors) such as prochloraz (14α-demethylase inhibitor or DMI) and higher sensitivity to fenpropidin (Δ14-reductase inhibitor). HydR2 and HydR3 are insensitive to fenhexamid and are similar in lower sensitivity to SBIs fungicides and microtubule inhibitors (carbendazim and dietofencarb). They are representatives of acquired resistance differing in response toward fenhexamid in the stage of germ-tube elongation; HydR2 is weakly resistant while HydR3 is highly resistant to fenhexamid. This suggests that distinct mutation in the same locus or in different loci is involved. Because of their high resistance level Hyd R3 strains have to be considered relative to risk of resistance occurrence. But, their poor overwintering capacities suggesting that they probably do not impact fenhexamid field's efficacy as laboratory investigations as well as field trials indicated (Ziogas et al., 2003; Suty et al, 1997; Kretschmer & Hahn, 2008; Topolovec-Pintarić, 2009; Korolev et al., 2011). For example, in long term trial conducted in vineyard by Suty et al. (1999) fenhexamid was used for 3-4 sprays per year and no reduce of effectiveness was observed although *B. cinerea* isolate less sensitive than normal did appear in the field. The Albertini et al. (2002) analyzed gene CYP51 and determined its DNA sequence. The gene CYP51 was highly polymorphic and this allowed distinction among HydR1 and non-HydR1 strains. At HydR1 CYP51 gene show two non-silent mutations: at position 15 expressed is phenylalanin instead of isoleucin and a serine instead of asparagine at position 105. Recently, Billard et al (2011) described that the major mechanism responsible for fenhexamid-resistance at Hyd R2 and Hyd R3 is fenhexamid detoxification by cytochrome P450 named cyp68.4.

Recently released botryticidal ingredient for use in grapevines was boscalid (carboxamide). Preliminary survey of *B. cinerea* populations from Champagne vineyards did not detect any strains moderately or highly resistant to boscalid and showed the absence of cross-resistance with benzimidazoles, phenylcarbamates and anilinopyrimidines (Leroux et al., 2010). The first resistant strains were found in 2006 in French and German vineyards and their number increased till 2008 (Lerox et al., 2010). Boscalid is the succinate dehydrogenase inhibitor and inhibits the fungal respiration by blocking the ubiquinone-binding site at mitochondrial complex II. Therefore, boscalid-resistance is caused by alterations in the respiratory succinate dehydrogenase (Avenot et al., 2008). The six phenotypes were characterized by Laleve et al (2011) according to their resistance pattern: CarR1-CarR4 with low to medium level of resistance and highly resistant CarR5 and CarR6. CarR1 and CarR2 are currently most frequent in France and Germany. For boscalid-resistance seemes to be responsible mutations in SDH proteins. For all except CarR2 phenotype, putative mutatuion occuring in the sdhB gene lead to a specific amino acid change in the *sdhB* gene. Strains of CarR2 phenotype may be distinguished in at least 3 sub-groups: I) point mutation in the *sdhB* gene, II) point mutation in the *sdhD* gene and III) no mutation in any of the four *sdh* genes. To

summarize, *B. cinerea* resistant phenotypes in correlation to fungicides *in vitro* effect towards germ-tube elongation and mycelial growth can be separated into three main categories. Phenotypes which exibit resistance at both stages are: I) Ben R1 and Ben R2 resistant to anti-microtubule toxicants; II) Imi R1 resistant to dicarboximides, III) Ani R1 resistant to anilinopyrimidines and IV) Hyd R3 resistant to fenhexamid. Phenotypes which expressed resistance mainly at stage of germ-tube elongation are only anilinopyrimidine-resistant Ani R2 and Ani R3 (also MDR phenotypes). Finaly, only phenotype Hyd R2 expressed fenhexamid-resistance at mycelial growth stage. Leroux et al (2002) stated that only phenotypes whose exibiting in vitro resistance at both development stages of *B. cinerea* will probably lead to practical resistance. This found not to be true for Hyd R3 phenotype as pratical resistance to fenhexamide has never been recoreded naimly to the rarity of Hyd R3 strains in vineyards. Furthermore, three main mechanisms of *B. cinerea* resistance to botryticides are indentified: reduced penetration of toxicants, increased detoxification or decreased conversion to toxic metabolites and reduced sensitivity of the target site.

### 3.2 Multi drug resistance phenomenon in *Botrytis cinerea*

Multiple drug resistance (MDR) phenomenon imply simultaneous reduced sensitivity to several different unrelated compounds. MDR is known as common in human pathogenic microbes and even cancer cells. In agricultural practice obvious cases of MDR in field strains of plant pathogenic fungi are restricted. The classic example of an MDR phenotype in *B. cinerea* is the cross-resistance to aromatic hydrocarbons, dicarboximides and other fungicides. The actual mechanism for this type of B. cinerea mutants has never been elucidated although many putative mechanisms of resistance were suggested. Recently, de Waard et al. (2006) suggested that drug transporters may have played a role in this case of resistance. But, the first expression of *B. cinerea* MDR phenomenon in the vineyard was detected between 1993 and 1997 from French vineyards located in Alsace, Armagnac, Bordeaux, Champagne and Loire Valley and described by Leroux et al. (1999). In some anilinopyrimidine-resistant phenotypes, named AniR2 and AniR3, they observed resistance extending to dicarboximides, phenylpyrroles, sterol biosynthesis inhibitors (e. g. tolfante, prochloraz, tebuconazole), and finally to hydroxyanilide derivate, fenhexamide. In Italy in 1996 anilinopyrimidine-resistant strains were detected even in vineyards where ingredient from this botryticidal group were never used before. Moreover, one of the isolates showed to be resistant simultaneously to fludioxonil, dixarboximides and benzimidazoles although it's virulence was very low. According to Chapeland et al. (1999) three MDR phenotypes can be distinguished in *B. cinerea*: 1) MDR1 strains show reduced sensitivities against fludioxonil and cyprodinil, 2) MDR2 strains are less sensitive to fenhexamid, cyprodinil and iprodione, 3) MDR3 strains are MDR1xMDR2 recombinants and thus show further reduced fungicide sensitivity. Until today MDR strains with additional boscalid resistance were never observed. Recently description of MDR phenothypes was reported in Germany by Leroch et al. (2011). They found most dominant to be MDR1 phenotype but interestingly, they detected MDR2 in low frequencies (2 %) in 2006 but until 2009 their number escalated (up to 26.7 %). Furthermore, MDR2 strains were carbendasim resistant also. Their hypothesis is that MDR2 strains have migrated eastward from Champagne to Germany based on the investigations conducted by Kretschmeir et al. (2009). MDR monitoring data from various investigations indicate that fungicide resistance patterns in *B. cinerea* are following current fungicide treatments. Development of MDR in human pathogens is explained as

consequence of over expression of drug efflux transport proteins. In *B. cinerea*, as in other plant pathogenic fungi this is also one of the various mechanisms that allow them to survive toxic compunds in their environment such as plant defense compounds, antibiotics and fungicides (De Waard et al., 2006). Efflux transporters are plasma membrane factors with low substrate specificity and depended of energy. Mutations leading to over expression of individual transporters can result in increased export of drug molecules back into their outer environment and thereby reduced sensitivity to drug. Thereby, they prevent accumulation of drug up to fungictoxic concentrations at their target sites inside fungal cells, preventing or reducing their toxic action. The major types of drug efflux proteins are ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters. The role of ABC and MFS transporters in efflux of natural and synthetic toxicants is today well known (De Waard et al., 2006). The genome of *B. cinerea* encodes more than 40 ABC-type, and more than 100 putative MFS-type efflux transporters although most of these transporters are not yet functionally characterized. Several ABC transporter genes have been cloned with variant basal transcript level (Vermeulen et al., 2001): I) undetectable *BcatrC*, *Bcatr*J, II) low level *BactrA*, *BcatrB*, *BcatrE, BcatrG, BcatrK,* and III) high level *BcatrF, BcatrH* and *BcatrI. BcatrB* and *BctrD* are a true multidrug transporters (De Waard et al., 2006; Hayashi et al., 2002). *BacrB* is a determinant for the anilinopyrimidines, dicarboximides, phenyllpyroles fludioxonil and fenpiclonil and to antibiotic phenazines. *BctrD* affects the sensitivity of *B. cinerea* to azole fungicide oxpoconazol, dicarboximides and benzimidazoles as well as to the antibiotic cyclohexamide. Therefore, it is possible that *BcatrB* and *BctrD* function in the MDR1 and MDR2 isolates. Also, *BcatrK* is a determinant for orgnophosphorus fungicide iprobenfos and antibiotic polyoxin. The ABC transporter AtrB1 has been shown to transport a variety of natural and synthetic drugs (Stefanato et al., 2009). Transcription of *atrB* is induced by various drugs, and requires the zinc cluster transcription factor Mrr1. Factor Mrr is present in nucleus but remain inactive in the absence of inducing drugs. Permanent activation of Mrr1 due to mutations in *mrr1*, resulting in constitutive overexpression of *atrB* and multidrug resistance (MDR) phenotypes. Kretschmer et al. (2009) identified that in MDR1 strains mutations in the transcription factor Mrr1 lead to over expression of the ABC transporters AtrB but in MDR2 strains a promoter rearrangement leads to over expression of the MFS transporters MfsM2.

## 4. Why is *Botytis cinerea* prone to resistance building?

*B. cinerea* is pathogen which can inflict an extreme number of plants without apparent specialisation which point to considerable variation. Already in 1922 Brierley nicely expressed that as follows: "*Botrytis cinerea is perhaps the commonest and best known fungus and has been the centre of mycological research since the time of de Bary. The species B. cinerea may be visualized as, at any one moment, a cluster of numerous races or strains morphologically congruent on the host plant but in vitro showing marked and constant cultural differences*". Genetic studies of nature and extent of genetic variability in *B. cinerea* were initiated to provide clues to the mechanisms and adaptability on different hosts. The resistance phenomenon intensified the genetic studies of *B. cinerea* assuming limited understanding of the genetic structure is reflecting in difficulties in managing the disease. Also, behavioral differences towards fungicides have provided additional markers for the wide genetic polymorphism encountered in *B. cinerea* (Giraud et al. 1999; Leroux et al. 1999). Genetic studies showed that this fungus has really great morphological variability and metabolic and genetic diversity

but the genetic basis for this variability is not elucidated. As the sexual stages (*Botyotinia fuckeliana*) are rarely observed in vineyards it was generally accepted that sexual recombination played no role in somatic and genetic diversity of *B. cinerea* and that therefore diversity must be due to heterokaryosis and aneuploidy. Hence, the high genetic diversity as well as the equal distribution of the two matting types in field populations indicates that sexual reproduction is a major reason for the heterogeneity of *B. cinerea* population (Choquer et al., 2007). There are also other nonexclusive hypotheses under investigation: occurrence of microviruses among wild populations can be one source of variability and other source can be differences in gene content and gene variability among *B. cinerea* strains. The genetic diversity of *B. cinerea* vineyard populations is so opulent that always seems to exceed the sampling size that can be handled in investigations. Recent genetic analysis showed that one of the causes of *B.cinerea* genotype variability could be transposable elements. Investigation on *B.cinerea* populations in Champagne vineyard showed the existence of two sibling sympatric species which can be distinguished according to the content of transposable elements (Giraud et al., 1999). Transposable elements are parts of DNA molecule, jumping genes which can change their position inside genomes, which means to change their position or locus on the chromosomes causing appearances like: gene inactivation, reactivation of pseudogenes, gene expression disorder, and also mutation type: deletion, insertion, inversion and translocation (Daboussi 1996; Daboussi & Capy, 2003). These sympatric species have been described as *Transposa* and *Vacuma* by Giraud et al. (1999). *Transposa* contains two transposable elements: gypsi-like retrotransposon Boty (Diolez et al. 1995) and transposon Flipper while *Vacuma* strains are devoid of both elements (Levis et al., 1997). *Vacuma* is showing higher diversity than *Transposa*. Newly population studies and phylogenetic analyses in France (Fournier et al., 2003) consider *B. cinerea* as a complex of species containing two genetically distinct populations, namely "Group I" (referred as *Botrytis pseudocinerea*) and "Group II" (*B. cinerea sensu stricto*) that were proposed to be phylogenetic species (Choquer et al., 2007). Groups exhibit difference in ecology and their resistance pattern to fungicides but it should be emphasize that they are unable to cross with each other. Group I is characterized by *vacuma* isolates sensitive to fenhexamide which are genetically isolated from others and therefore form a true phylogenetic species. Group II are all fenhexamid-resistant *vacuma* strains and *transposa*. Therefore, they are three main genetic types: Group I, Group II *vacuma* and Group II *transposa*. In the vineyards, as on various host plant, evident are changes in *B. cinerea* population structure and dynamics between genetic types during season. Group I play only a minor role in the epidemiology of bunch rot and is found only sporadically mostly at flowering on leaves and blossoms. Group II *vacuma* reached maximum on senescing floral caps and then decrease significantly until harvest but increase from autumn to flowering. This type of isolates are mostly isolated from over wintering sclerotia and in order with this it is hypothesized that *vacuma* isolates will express a more ruderal life-strategy with greater saprophytic capability (Furnier et al., 2003). Furthermore, it is regarded as a mix of different migrant populations from other host (Giraud et al., 1999). Group II *transposa* is the most virulent one and seems well-adapted to the grapes cause it is dominant at every phenological stage and isolated from over wintering canes more then other types. As it's showing a peak occurrence in rotted grape berries an epidemic of bunch rot is dominated by Group II *transposa* (Matinez et al., 2005). Group II *transposa* it seams to represent commonly occurring population of *B. cinerea* in European vineyards (Kretschmer & Hahn, 2008; Furnier et al., 2003; Topolovec-Pintarić et al., 2004).

According to Martinez et al. (2005) differences in the saprotrophic and pathogenic ability of the *vacuma* and *transposa* combined with a switch in resource availability from dead to living tissues is the most likely mechanism accounting for the success of *transposa* isolates and the decline of *vacuma* isolates during the course of an epidemic. The isolates that had only Boty and therefore they were named "Boty only" and were detected in France (Giraud et al., 1999) and in Chile (Muñoz et al., 2002). The "Boty only isolates" were frequently isolated on kiwifruit and in lower frequency on grapes and tomato. It is hypothesized that "Boty only isolates" may be result of crosses between *vacuma* and *transposa* and the transposon Boty may even be invading isolates of *vacuma* gropus (Muñoz et al., 2002). The isolates containing only Flipper have been found by Albertini et al. (2002). These isolates were origin from France vineyard and from UK strawberry fields and were resistant to fenhexamide. Considering fact that transposones influence genetic changes, especially expression of genes and gene mutations, it was hypothesized about possible relation to fungicide resistance. Some data about possible influence of transposones on the resistance to botryticides were obtained recently (Giraud et al, 1999). Group I is characterized by natural resistance to fenhexamide (Leroux et al., 2002; Suty et al., 1999; Martinez et al., 2005). Group II t*ransposa* strains are multiresistant and frequently resistant to vinclozolin and diethofencarb (Giraud et al., 1999; Martinez et al., 2005). Alberini et al. (2002) described four phenotypes according to their responses towards fenhexamid: HydS, HydR1, HydR2 and HydR3. A HydS type is wild type sensitive to fenhexamid and is either *vacuma* or *transposa*. HydR1 is fenhexamid-resistant with negative cross-resistance to other SBIs (sterol biosynthesis inhibitors) such as prochloraz (14α-demethylase inhibitor or DMI) and higher sensitivity to fenpropidin (Δ14-reductase inhibitor). Genetic analyses showed that they are mostly *vacuma* although few was Flipper only type. In comparison to other HydR types conidia of HydR1 are oversized and mycelial growth rate is higher. It should be emphasize that all HydR1 strains are compatible and mating obtains progeny but crosses between HydR1 and HydS failed to obtain progeny. Types HydR2 and HydR3 are of *transposa* type having smaller macroconidia (Giraud et al., 1999) and exhibit slower rates of mycelial extension when grown on highly nutritive agar media at different favorable temperatures (Martinez et al., 2005). They are similar in lower sensitivity to SBIs fungicides and microtubule inhibitors (carbendazim and dietofencarb). They differing in response toward fenhexamid in the stage of germ-tube elongation; HydR2 is weakly resistant while HydR3 is highly resistant to fenhexamid. The Albertini et al. (2002) analyzed gene CYP51 and determined its DNA sequence. The gene CYP51 was highly polymorphic and this allowed distinction among HydR1 and non-HydR1 strains rather then between *vacuma* and *transposa*. At HydR1 CYP51 gene show two non-silent mutations: at position 15 expressed is phenylalanin instead of isoleucin and a serine instead of asparagine at position 105. Absence of genetic exchange between HydR1 and non-HydR1 combined with morphological and somatic incompatibility suggest that these two groups are from distinct genetic entities and might even be non HydR two different species. As types in non HydR group are more variable it is probably composed of different subpopulations whose phylogenetic relationship is still not resolved. The *transposa* isolates resistant to fenhexamid, carbendazim and vinclozolin were detected in other investigations (Martinez et al., 2005; Giraud et al., 1999) and resistance appeared to be associated with an increased virulence. The *transposa* resistant profiles, according to Martinez et al. (2005) are most likely a consequence of population dynamics and are generated by the application scheme of fungicides. Therefore, *transposa* as predominant at veraison is exposed to greater selective

pressure of vinclozoline, resulting in a greater frequency of vinclozoline-resistant strains. Also, with speculations about ability of *transposa* isolates to develop fungicide resistance, as well as increased virulence, arises a question if this ability could be based on MDR (multi-drug resistance) systems. The MDR system allows efflux of various cytotoxic drugs such as plant defense compounds (e.g., phytoalexins) and botryticidal coumpound. The role of such mechanisms in the population dynamics of *B. cinerea* genetic types warrants further investigation. At the end, *B. cinerea* can be considered as actually a complex of several different entities (Albertini et al., 2002).

## 5. Conclusion and future prospect

In order to overcome the problem of *Botrytis* resistance there are continuous world efforts to develop new active ingredients. Monitoring methods were developed and again improving. Antiresistant strategies are applied. However, resistance to botryticides still pursuing to be economically significant problem in *B. cinerea* management as it will be in foreseeable future, mainly due to selection of MDR mutants with high levels of resistance. Ironically, selection of MDR mutant is favoured by some recommendation of antiresistant strategy. In most European vineyards is obey the recommendation to alternate the various groups of botryticides with restriction to one spray per year for each chemical group because they are single-site fungicides and to use fungicide mixtures. However, this antiresistant measure is impeded by the development of MDR phenotypes. This measure delay or prevent the evolution of mutant with target site modifications but presents a multi attack by fungicides which favours stepwise evolution of polygenic resistance related to ABC transporters. On the other hand, gained knowledge of MDR mechanisms could be used as new weapon against not only *B. cinerea* but other phytopathogenic fungi as well. Fungal mutants that lack drug transporters become hypersensitive and can be used as tester strains in the creating new fungicidal compounds. New antifungals perhaps can be compounds that inhibit ABC transporters. Analogues in human medicine exit as blockers or modulators of ABC transporters. Furthermore, these compounds can be synergist of existing fungicides or the ones that annul MDR in phytopathogenic fungus. Another line of new antifungals can be disease control compounds that even do not posses a direct fungitoxic activity and may be considered as inducers of plant defence mechanism. Such compounds can act as inhibitors of ABC transporters involved in fungal virulence which will lead to enhancement of plant defence compounds (phytoalexines, pathogenesis-related proteins) in fungal cells.

Yet after all, the basic for resistance prevention and management remains necessity of updating our knowledge about *B. cinerea,* although it is already abundad and comprehensive. For this goal the data about structure and dynamics of *B. cinerea* populations in commercial vineyards as well as the distribution of different types of fungicide resistance, including MDR types, should be obtained. More attentions should be given to today available alternatives to classical botryticides like biological control, or use of mineral salts and plant activators.

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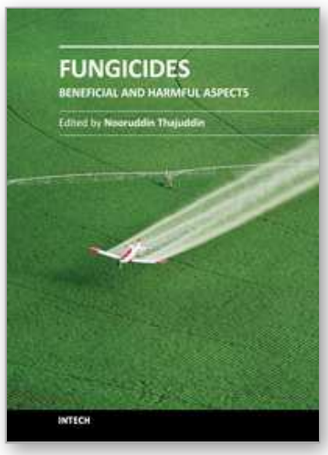


**Fungicides - Beneficial and Harmful Aspects**
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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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