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Fenhexamid Resistance in the *Botrytis* Species Complex, Responsible for Grey Mould Disease

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1. Introduction

1.1 Chemical control of grey mould in French vineyards

The three major fungal pests of grapevine, powdery and downy mildew and grey mould are mostly controlled through the application of fungicides. Some of those are particularly active against the grey mould agent *Botrytis cinerea*. The panel of fungicides authorized in France comprise since many years anilinopyrimidines, benzimidazoles, dithiocarbamates, dicarboximides, phenylpyrroles and pyridinamines. Lately, the panel has been completed by fenhexamid, a sterol biosynthesis inhibitor (SBI) and the pyridine boscalid, a succinate dehydrogenase inhibitor (SDHI). In addition, a biofungicide based on the *Bacillus subtilis* strain QST 713 (Serenade) has been authorized in 2010 against grey mould in vineyards. The compounds authorized against grey mould in French vineyards are listed in Table 1.

Since grey mould may infect grapevine from flowering until harvest, optimal protection needs to be obtained during this period. Nowadays up to three treatments per season are recommended in vineyards, corresponding to the stages A-C (A: flower cap falling – B: bunch closure – C: veraison). To reduce pesticide applications along with the general trend of reduction of chemical inputs in agriculture chemical treatments against grey mould are positioned according to epidemiological and meteorological parameters. Grey mould not only affects quantity of harvest but also the wine quality. Therefore, treatments also depend on the economic value of the wine. The number of treatments is variable between regions and years according to the factors cited above.

In those regions with regular applications of anti-*Botrytis* fungicides, especially in the Northern regions, resistant strains have been selected which can ultimately lead to treatment failure. In order to reduce the risk of specific resistance development each anti-*Botrytis* mode-of-action is limited to one application/season in France since the 90's, involving alternations of different chemical families to combat grey mould in the vineyards.

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Chemical families	Fungicides	Timing of application
Anilinopyrimidines	Pyrimethanil, Mepanipyrim, Cyprodinil	A, B or C
Benzimidazoles	Thiophanate-methyl	A or B
Carboxamides	Boscalid	A, B or C
Dithiocarbamates	Thiram	A
Hydroxyanilides	Fenhexamid	A, B or C
Dicarboximides	Iprodione	A, B or C
Phenylpyrroles	Fludioxonil	A or B
Pyridinamines	Fluazinam	A, B or C
Bacillus subtilis (biofungicide)	Serenade	up to 8 applications/year

Table 1. Active substances approved against grey mould of grapevine in France and recommended stages of applications (French National *Botrytis* Note - Vigne 2010). For further explanations, see text.

1.2 Fenhexamid: A single mode of action

Fenhexamid is a narrow spectrum fungicide (Rossenbloich and Stuebler, 2000) belonging to the chemical family of hydroxyanilides inhibiting sterol biosynthesis (SBI)(Debieu *et al.*, 2001). Bayer CropScience has introduced it to the French fungicide market more than 10 years ago as a specific anti-*Botrytis*. It is principally used in vineyards, but also on tomato and strawberry cultures up to two treatments per season (Couteux *&* Lejeune, 2003). Fenhexamid differs from other known SBIs (allylamines, azoles, imidazoles and morpholines/amines) by the enzymatic step that it inhibits. Sterols are essential lipid compounds found in all eukaryotes. They are principally localized to cytoplasmic or endo-membranes, although the latter are composed predominantly of phospholipids. Sterols participate in membrane permeability and rigidity, in the formation of lipid rafts and, eventually, in post-translational control of membrane proteins (Hac-Wydro *et al.*, 2007; Yeagle, 1990; Epand, 2008; Gilbert, 2010; Paila *&* Chattopadhyay, 2010). The major fungal sterol is ergosterol and specific to this kingdom. Therefore, many attempts to develop targets for antifungal treatments have been orientated towards the ergosterol biosynthesis pathway. Noticeably, all SBIs used against fungal diseases target enzymatic steps that are conserved among all kingdoms and not fungal specific.

The major class of SBIs used in agriculture represented by azoles, imidazoles and triazolinethions inhibits the 14α-demethylase enzyme therefore called DMIs (demethylation inhibitors), amines inhibit the Δ14-reductase and/or the Δ8➔Δ7-isomerase. The youngest SBI used against grey mould, fenhexamid, blocks the demethylation at C4 of sterols. This important process involves three different enzymes: Erg25 (C4-methyle oxidase), Erg26 (C3-dehydrogenase, C4-decarboxylase) and Erg27 (3-ketoreductase). The first two enzymes eliminate methyl groups at C4 leading to a keto group at C3. The Erg27 enzyme then reduces this keto group to a hydroxyl (Gachotte *et al.*, 1999) leading to a functional sterol.

This last step is the target of fenhexamid. Its mode-of-action has been uncovered in 2001, by the characterization of sterol composition of fenhexamid treated *B. cinerea* strains. Debieu and co-workers, observed an accumulation of compounds with a keto group at C3, suggesting the inhibition of the 3-ketoreductase step (Debieu *et al.,* 2001). Since this enzymatic step is conserved in all euascomycetes, one may expect fenhexamid to be a large spectrum fungicide as are the DMIs instead of its narrow spectrum (see paragraph 2.3).

1.3 Resistance to fenhexamid in the *Botrytis* species complex

The activity spectrum of a new fungicide is generally established by biological tests on diverse fungal species allowing distinguishing sensitive and tolerant species. While naturally resistant - or tolerant species - are insensitive to a given fungicide prior to its introduction and defining the fungicide's spectrum of activity, sensitive fungal species can evolve and select less susceptible - or resistant - isolates. Natural polymorphism or randomly occurring mutations may confer a certain level of resistance to some strains. Through the application of the corresponding fungicide, these isolates are preferentially selected and their proportion within the population will increase (selection pressure of the fungicide). The differentiation between natural and acquired resistance can sometimes lead to the identification of new fungal species; e.g. *Oculimacula yallundae* and *O. acuformis:* the second species is naturally resistant to triazole SBIs. Another example is *Rhizoctonia solani* which is subdivided into different anastomosis groups some of which are tolerant to pencycuron, an anti-microtubular agent (Campion *et al.,* 2005).

	Botrytis cinerea					
	EC50[a] HydS	**Resistance Factor**				***B. pseudocinerea* HydR1**
		HydR2	**HydR3$^-$**	**HydR3$^+$**	**MDR2**	
Germ tube	≈ **0.05**	≈ 2	≈ 75	> 350	≈ **10**	≈ 5 – 10
Mycelial growth	≈ **0.015**	≈ 50	≈ 20 - 30	> 350	≈ **10**	> 350

[a] concentration of fenhexamid (mg l^{-1}) inhibiting growth at 50%.

Table 2. Sensitivities of resistant phenotypes towards fenhexamid on germ tube elongation and mycelium growth stages. HydS= sensitive to fenhexamid; HydR= resistant to fenhexamid; MDR= multidrug resistant.

Also in the case of grey mould, it became clear that the species later on named *Botrytis pseudocinerea* a close relative of *B. cinerea,* was naturally resistant to fenhexamid at the stage of mycelial growth (Fournier *et al.,* 2005, Walker *et al.,* 2011). Later, resistant isolates were selected from the *B. cinerea sensu stricto* populations, initially sensitive to fenhexamid. Until now, four different fenhexamid resistance categories could be identified among grey mould populations (Table 2): HydR1, corresponding to the naturally resistant species *B. pseudocinerea* and the *B. cinerea* phenotypes MDR, HydR2 and HydR3 (Leroux *et al*, 2002).

For the phenotypes HydR1 (*B. pseudocinerea*) and HydR2 (*B. cinerea*), high to moderate resistance levels respectively, are observed nearly exclusively at the level of mycelial growth, whereas the other phenotypes display similar resistance levels at both developmental stages, germ tube elongation and mycelial growth. In addition, *B. pseudocinerea* (HydR1) presents increased sensitivities to other fungicides, such as DMIs, fenproprimorph, fenpropidine as well as to some SDHIs (Leroux *et al*., 2002). Only few HydR2 strains have been isolated and to our knowledge, they have not yet been found in French vineyard populations. On the opposite, HydR3 phenotypes are regularly found since 2003. This category has been subdivided into two sub-classes, HydR3$^-$ or HydR3$^+$ with moderate or high resistance levels respectively.

MDR2 (and MDR3) phenotypes display weak to moderate resistance levels (resistance factor <15) to various unlinked mode-of-actions including fenhexamid, SDHIs, dicroboximides, anilinopyrimidines etc. This **m**ulti**d**rug **r**esistance (MDR) is linked to the over expression of a membrane transporter leading to increased drug-efflux outside the fungal cell (Kretschmer *et al*., 2009). Anti-resistance strategies involving systematic alterations of different mode-of-actions seem to favor the selection of these MDR phenotypes instead of specific resistances, in particular in regions with more than one treatment per season such as the Champagne wine region.

2. Acquired resistance to fenhexamid in *Botrytis cinerea*

2.1 Target alterations cause reduced affinity of fenhexamid and are responsible for resistance

In agriculture, acquired resistance towards agrochemical products is frequently conferred by target site modifications leading to decreased inhibitor affinities. Target overproduction is rarely found. Pesticide detoxification is a mechanism particularly important for insecticide resistance involving diverse enzymes such as hydrolases, cytochrome P450 monooxygenases, glutathione-S-transferases or glycosyl transferases. In rare cases, resistance results from non-activation of pro-pesticides. Concerning antifungal compounds, the principal resistance mechanisms are target site modifications and increased efflux, in the agronomical field as well as in the medical field (Ma & Michaelides, 2005; Sanglard *et al*., 2009).

With respect to the *B. cinerea* HydR phenotypes, target site modifications were found in the HydR3 (+ and -) isolates, but not in HydR2 phenotypes (Albertini & Leroux, 2004; Fillinger *et al*., 2008). In *B. pseudocinerea* the fenhexamid target encoding gene, *erg27* shows a high degree of polymorphism compared to the sensitive *B. cinerea* allele. Its implication in fenhexamid resistance is described in paragraph 3.1.

Eight different amino acid replacements were found in *erg27* alleles from HydR3$^-$ field strains either as single or as double mutations (L195F, N196Y, V309M, A314V, S336C, N369D, L400F/S, et L501W). HydR3$^+$ field isolates display a single mutation of the phenylalanine at positions 412 (F412S, F412I or F412V) (Fig. 1; Fillinger *et al*., 2008).

Other *erg27* mutations have been found in fenhexamid resistant strains selected after chemical mutagenesis (G23S, C53R, T63I, K73E, H105Y, K159N, L195S, T273A, S310P, I397V, I411V, H423R, A452P, Q495R and C516R) (Saito *et al*., 2010). However, their involvement in resistance to fenhexamid remains to be shown.

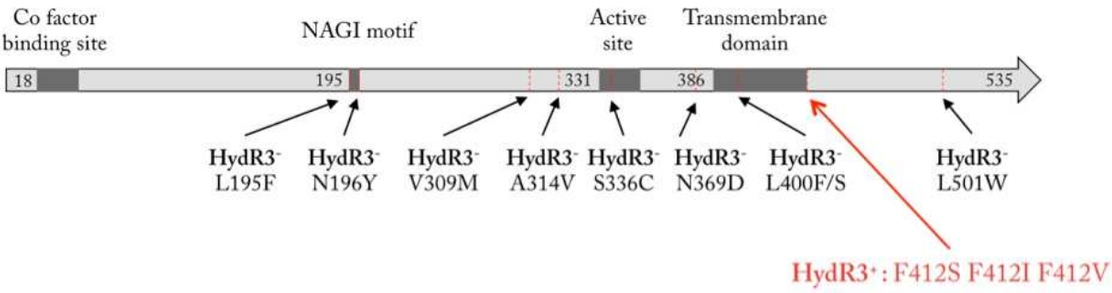

Fig. 1. **Protein structure and functional domains of the 3-ketoreductase Erg27 of *B. cinerea*.** The active site catalyzing the formation of a hydroxyl group on position C3 of sterols is located near the transmembrane domain allowing the anchorage of the enzyme to the endoplasmic reticulum. The position of the mutations detected in HydR3$^-$ resistant isolates are indicated by black arrows and concerning HydR3$^+$ isolates by a red one.

We analyzed the involvement of the natural HydR3 mutations in fenhexamid resistance by site directed mutagenesis. The *erg27* allele - encoding 3-ketoreductase – was replaced in a sensitive strain by the *erg27* allele of a HydR3 strain by homologous recombination. Two mutations identified from HydR3$^-$ strains (L195F et V309M) in addition to the three HydR3$^+$ alleles were studied. The fenhexamid sensitivity profiles of the generated mutants were similar to those from the field strains (Table 3). Indeed the transgenic *erg27L195F* or *erg27V309M* mutants display low to moderate resistance levels according to the developmental stage, whereas the transgenic HydR3$^+$ mutants (F412S, F412I, F412V) present high resistance levels at both growth stages, as do the highly resistant HydR3$^+$ field strains.

Fenhexamid susceptibilities	**EC50 Sensitive strain** (mg l^{-1})	Resistance level (RL)				
		HydR3$^-$ phenotype		HydR3$^+$ phenotype		
		Erg27::L195F	*Erg27::V309M*	*Erg27::F412S*	*Erg27::F412I*	*Erg27::F412V*
Germ tube	0.05	**MR**	**MR**	**HR**	**HR**	**HR**
Mycelial growth	0.015	**LR/MR**	**LR/MR**	**HR**	**HR**	**HR**

Table 3. **Sensitivity to fenhexamid of transgenic mutants**. LR=low resistance; MR=moderate resistance; HR=high resistance

In addition, the inhibition of 3-ketoreductase activity by fenhexamid is reduced in the artificial mutants (Billard *et al.*, unpublished), strongly suggesting a decrease in fenhexamid's affinity to its target 3-ketoreductase in HydR3 mutants. These results prove the direct correlation between the amino acid substitution and the specific resistance to fenhexamid in the *B. cinerea* HydR3$^+$ and HydR3$^-$ phenotypes. In addition they show target site modification as sole resistance mechanism in these isolates.

In contrast to HydR3 phenotypes, neither target site modification nor over expression seem to be responsible for fenhexamid resistance in HydR2 phenotypes. The only hint towards the potential resistance mechanism is an observed synergy between fenhexamid and DMIs suggesting fenhexamid metabolization involving a cytochrome P450 monooxygenase enzyme. Which mechanism precisely is at work in HydR2 strains needs yet to be identified.

We have modeled the *B. cinerea* Erg27 protein after alignment with the peptide sequences of various dehydrogenases/reductases whose crystal structures have been established. The homology-based model presented in Fig. 2 monitors the position of the HydR3 mutations relative to the substrate- and cofactor binding sites. None of the mutations are in the vicinity of these docking sites, although it cannot be excluded that the L195F and N369D replacements modify the Erg27 protein structure leading to different substrate- and co-factor affinities. Mutations of F412 however, potentially interfere with the helical structure of the transmembrane helix.

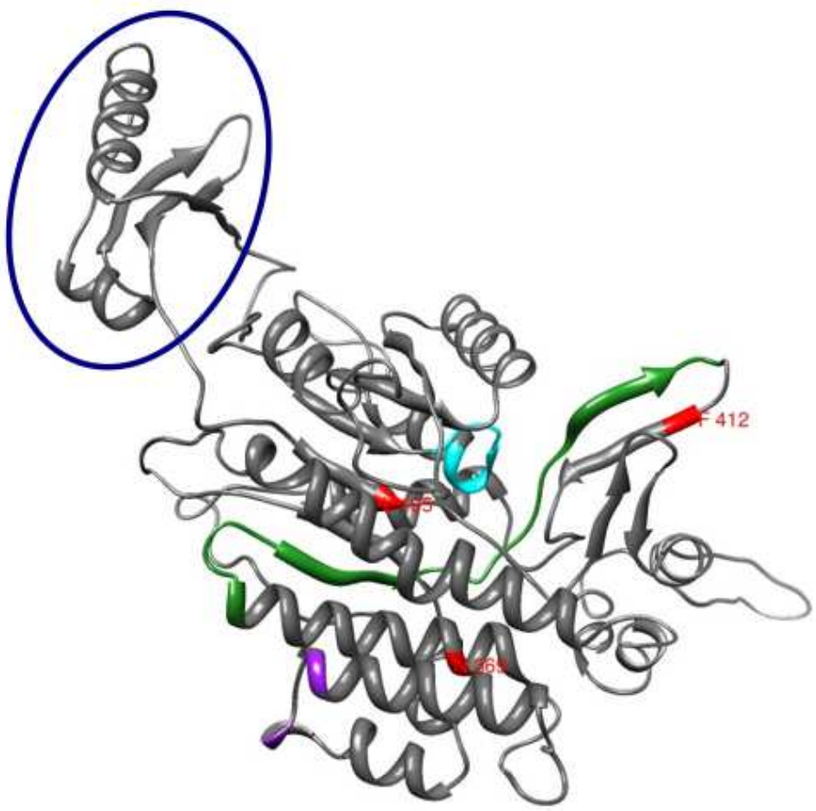

Fig. 2. Predicted 3D structure of the 3-ketoreductase (Erg27) of *Botrytis cinerea* based on the alignement with Salutaridine Reductase From *Papaver Somniferum* (Higashi *et al.,* 2011). The cofactor (NADHP) binding site and the active sites are highlighted in cyan and purple respectively. The green ribbon represents the transmembrane domain. The mutated residues responsible for specific fenhexamid resistance (HydR3) are highlighted in red. The blue circle designates the extension of *Botrytis* and *Sclerotinia* Erg27 proteins compared to other fungal orthologs.

2.2 Why fenhexamid has a narrow spectrum?

Fenhexamid is the sole SBI with a narrow antifungal spectrum. Its activity is restricted to close relatives of *B. cinerea*, e.g. *Botrytis* and *Monilinia spp.*, *Sclerotinia sclerotiorum* (Rosslenbroich & Stuebler, 2000). We therefore raised the question if 3-ketoreductase of ascomycetes naturally resistant to fenhexamid is insentitive to inhibition exerted by fenhexamid. The alignment of Erg27 to orthologous peptide sequences show that both 3-ketoreductase sequences, those from *B. cinerea* and *S. sclerotiorum*, harbor three additional fragments compared to the other fungal proteins, including one extension of 34 amino acids (Albertini *&* Leroux, 2004; Fillinger, unpublished; Fig. 2). In addition, we have shown by biochemical assays that 3-ketoredcutase activity of insensitive fungal species, is barely inhibited by fenhexamid, suggesting that fenhexamid has a strong affinity towards 3-ketoreductase of *Botrytis* related species only (Debieu *et al*., submitted). In how far the above mentioned protein extensions and/or specific residues of the *B. cinerea* Erg27 protein sequence are involved in this affinity remains to be established.

2.3 The HydR3^{+} resistance acquisition entails a cost in controlled conditions

The fitness corresponds to the capacity of one individual (strain) to survive among others under the same conditions. In the absence of selective pressure exerted by the application of fungicides, the relative fitness determines the persistence of a resistant isolate among a natural fungal population. Acquiring a fungicide resistance linked to a high fitness cost is a clear disadvantage for the fungal strain, as it is counter selected in absence of the corresponding fungicide from a mixed population with sensitive and resistant strains. In natural grey mould populations, this seems to be the case for the phenylpyrrol fungicides for which no (or only few) specifically resistant strains were detected up to now (Leroux *et al,* 2002; Moyano *et al*., 2004). The efficacy of such fungicides can be maintained over long periods. On the opposite, resistant mutations that are not associated to a fitness cost, may threaten the "life-time" of the corresponding fungicide. Indeed, such kind of resistances without associated fitness costs were obtained after few years only with the strobilurins used against cereal fungal pathogens or - on different crops and different pathogens - with the benzimidazoles. Resistance against either or both fungicides is generalized in many pests.

Concerning fenhexamid, the frequency of the HydR3^{+} strains of the greatest concern in practice, is in progression since 2003 and has reached now non-negligible levels, e.g. 27% in Champagne. This increase remains weak in regions despite annual applications and not comparable to the rapid generalization of strobilurin resistance. This evolution indicates that probably an effective anti-resistance strategy combined to a potential fitness cost linked to high levels of fenhexamid resistance limits the progression of HydR3^{+} frequencies among grey mould populations.

In order to evaluate the fitness cost linked to the HydR3^{+} phenotypes, we measured divers physiological parameters under controlled conditions. The general problem for fitness measurements in natural *B. cinerea* strains is the phenotypic variability between strains. Different growth parameters cannot be easily correlated to resistance due to different genetic backgrounds. We circumvented this problem by the construction of HydR3^{+} (F412S, F412I and F412V) mutants using site directed mutagenesis. The strains were isogenic except for the *erg27* allele providing the ideal material for fitness analyses. Particular attention was paid to temperature and nutritional factors in the measurement of the fitness parameters as well as the

quantification of developmental stages important for survival and dispersion (sclerotia and conidia respectively). Statistically significant decreases were observed in the mutants for mycelial growth – especially in extreme conditions (restricted nutrients at low temperatures), for conidia- and sclerotia-production – the last parameter especially at low temperatures, and for mycelial outgrowth from frozen sclerotia. Theses results obtained with isogenic strains, like those reported by Ziogas *et al* (2003) and Saito *et al.* (2010) for chemically resistant mutants of *B. cinerea*, indicate that the acquisition of high-level specific resistance to fenhexamid is associated with a decrease in fitness (Billard *et al.*, submitted). Our data show that *in vitro* pathogenicity is not affected (measured on detached bean leaves), whereas stages of the fungal life cycle during which there is no selection pressure seem to be the most affected.

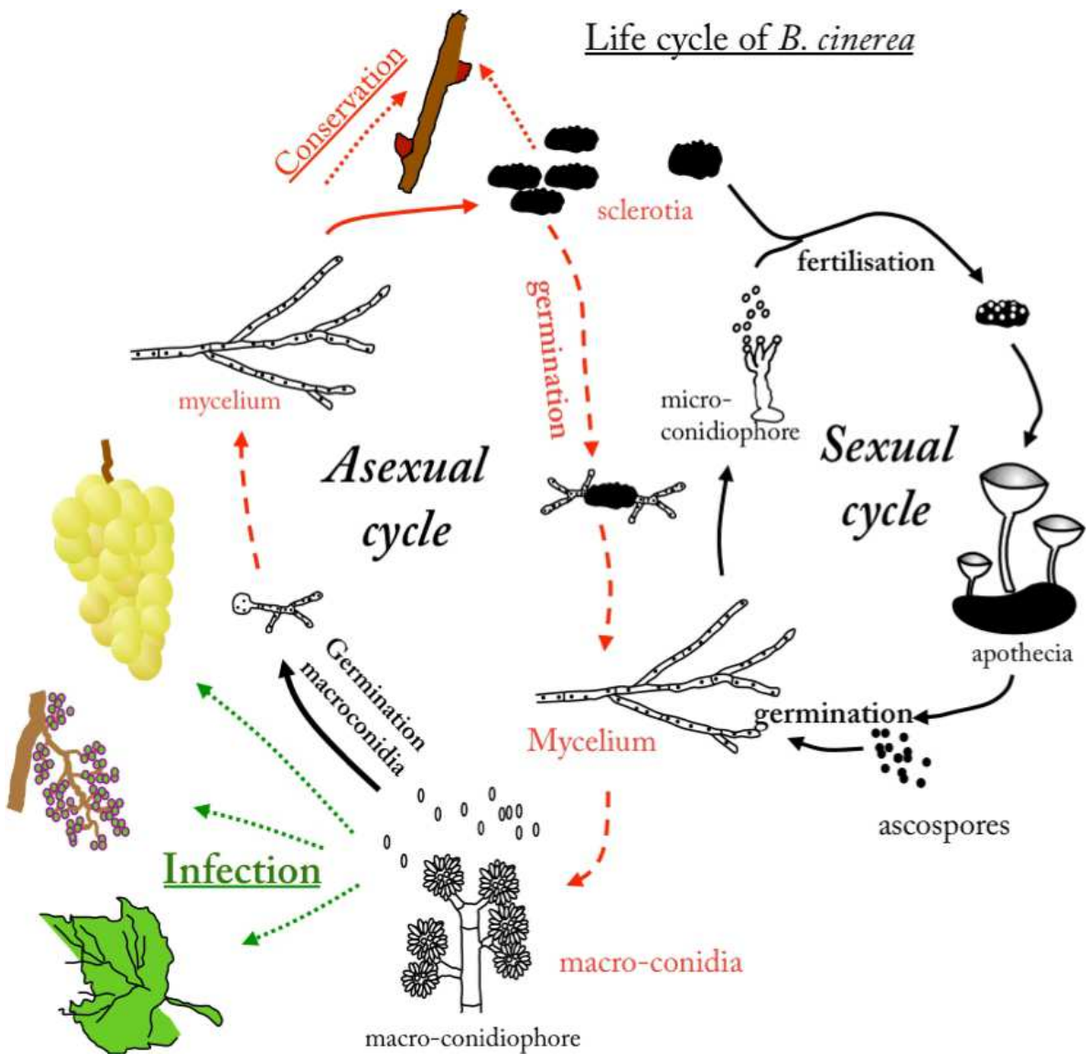

Fig. 3. **Life cycle of *Botrytis cinerea*.** In laboratory conditions, a fitness cost of the HydR3^{+} resistance was observed in isogenic mutants at different phases of the asexual cycle (in red) of *B. cinerea*. However, the pathogenicity of these mutants, measured on beans, remained unchanged from the parental strain sensitive to fenhexamid (green).

These findings for isogenic *erg27*^{F412} mutants suggest that the HydR3^{+} phenotype does not persist in the absence of the fungicide and that this phenotype has a moderate impact on the efficacy of fenhexamid treatments for controlling grey mould disease in vineyards. However long-term studies in vineyards are required to confirm these laboratory observations.

2.4 Fenhexamid monitoring in fields: A molecular approach

Monitoring of this resistance is a crucial area of research where our knowledge of the field distribution, evolution and impact of fungicide resistance depends on. In most cases, the degree of sensitivity of fungal populations to one or more fungicides is assessed by biological methods. These bioassays conducted, *in vitro* or *in vivo*, have been miniaturized (i.e. microtiter plate methods), but nonetheless consume considerable resources and remain time-consuming. When the molecular mechanisms of resistance are known (e.g. target mutation, target over expression, increased drug efflux), and particularly when the underlying DNA polymorphisms (single-nucleotide polymorphism (SNPs), deletions or insertions) have been defined, various molecular methods can be used to monitor antimicrobial resistance. The principal methods for quantifying resistance are based on real-time PCR (polymerase chain reaction) technology. Generally, studies have been made on isolated and purified strains using qualitative methods like RFLP, AS PCR or Real time PCR (Benzimidazole resistance in *Botrytis cinerea* Banno *et al*., 2008, *Venturia inequaelis* Koenraadt *et al*., 1992, *Rhyncosporium secalis* Wheller *et al*., 1995, *Helminthosporium solani* McKay *et al*., 1998; Strobilurines resistance on *Erisiphe graminis* Baümler *et al*., 2003, *Plasmopara viticola* Furuya *et al*., 2009, *Alternaria spp*. Ma *&* Michailides, 2003, *Mycosphaerella graminicola* Fraaije *et al*., 2007, *Mycosphaerella fijiensis* Sierotzki *et al*., 2000) and on the opposite some of them on populations, where resistance allele is quantify in gDNA pools by using Real time PCR technologies (Benzimidazole resistance in *Sclerotinia sclerotiorum* Chen *et al*., 2009 ; Strobilurins resistance on *Blumeria graminis* Fraaije *et al*., 2002 and *Pyrenophora teres* Kianianmomeni *et al*., 2007). However, one limitation of this method concerns the nonspecific amplification of alleles, which may affect precision. This limitation does not generally hinder detection of the polymorphism, but it may affect quantification capacity, particularly for mutated alleles with a low abundance.

Taking these results into account, we investigated the development of a new technique for quantifying, with a high precision, the three different *erg27* alleles from the HydR3^{+} phenotype. The underlying DNA polymorphism is the modification of the TTC codon encoding the F412 residue, which is converted into a TCC (serine), GTC (isoleucine) or ATC (valine) (Fillinger *et al*., 2008) codon. The best result was obtained with a non multiplexed method combining four allele-specific MGB Taqman® probes and four mismatched specific primers. This technique was named the *Allele Specific Probe and Primer Amplification Assay* (ASPPAA PCR), (Fig. 4; Billard *et al*., submitted).

The sensitivity of ASPPAA PCR is sufficiently good to quantify a SNP at a rate of 1% in a DNA pool. In the future, the multiplexing, in the same run, of the analysis of several polymorphisms at different genomic loci with probes picked up in different fluorophore channels is conceivable and would be expected to decrease the time required for monitoring, and its cost, significantly.

The principle disadvantage of these molecular quantification methods is that they do not allow to detect emerging resistances in contrast to biological methods. One alternative

molecular method to be considered is the HRM (high resolution melt curve) analysis (Pasay *et al.,* 2008*)*. HRM offers a fast and convenient method of assessing the presence of mutations without sequencing in a short (< 400 bp) defined genomic region. Using this tool, the identification of new mutations in the *erg27* gene is possible.

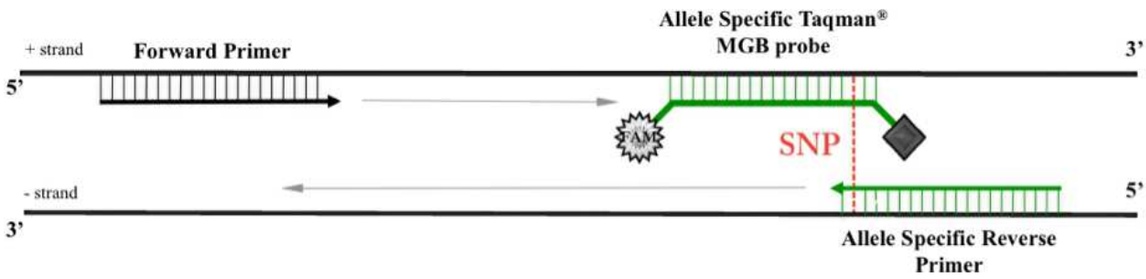

Fig. 4. **Principle of ASPPAA PCR for quantitative SNP detection.** The high robustness of this new allele quantification method using real-time PCR is caused by the specificity of amplification of the mutation by allele specific probes and primers present on both DNA strands.

3. Natural resistance to fenhexamid in *Botrytis pseudocinerea*

The genus *Botrytis* (Ascomycota) contains 22 highly specialised species and one hybrid. A multiple-gene gene genealogy study recently showed that this genus could be subdivided into two categories, one consisting of *Botrytis* species acting as pest on monocots, and the other containing *Botrytis* species acting as parasites on eudicots (Staats *et al.,* 2005). Within this second category, *Botrytis cinerea* has the widest host range, being able to infect more than 220 types of eudicot, including grapevine and many fruit and vegetable crops. However, *B. cinerea* has recently been shown to be a complex of two sibling species living in sympatry. *B. cinerea sensu stricto* is the predominant species (Fournier *et al.*, 2005). The other species, called *Botrytis pseudocinerea*, has been found at low frequency in French populations. This species is morphologically indistinguishable from *B. cinerea sensu* stricto, but Walker *et* al (2011) established several molecular markers to distinguish both species. In addition *B. pseudocinerea* has a different pattern of fungicide susceptibility (phenotype HydR1), displaying natural resistance to fenhexamid and hypersensitivity to fenpropidin and edifenphos (Leroux *et al*, 2002).

In contrast to the *B. cinerea* fenhexamid resistant phenotypes (HydR2 and HydR3), *B. pseudocinerea* was resistant to fenhexamid prior to its introduction, therefore displaying a natural resistance to fenhexamid.

3.1 Role of the natural target polymorphism in fenhexamid resistance

The genetic polymorphism of *B. pseudocinerea* compared to *B. cinerea* is also visible on the 3-ketoredcutase encoding gene *erg27*. Twelve codon modifications lead to amino acid replacements in the Erg27 protein (N93V, D146L, I211V, I215V, M218T, V234A, I235V, D261G, S264T, P269L, A285T and Q354K) (Albertini *&* Leroux, 2004). None of theses substitutions corresponds to any of the HydR3 mutations. In order to evaluate the impact of the *B; pseudocinerea erg27* allele in fenhexamid resistance we replaced the $erg27^{wt}$ allele in a

sensitive *B. cinerea* strain by that of a HydR1 "resistant" *B. pseudocinerea* strain. The gene replacement strategy is explained in Fig. 5.

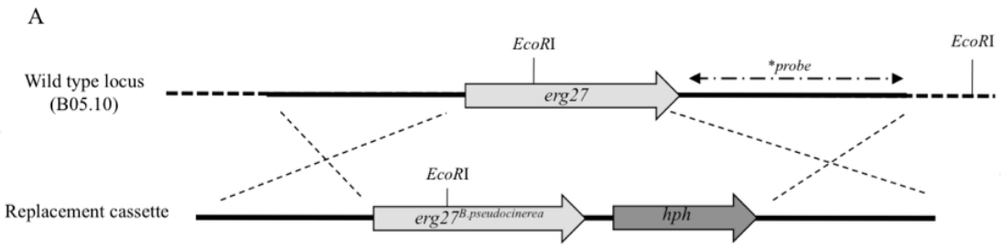

Fig. 5. Gene replacement strategy of the *erg27* allele by that of *B. pseudocinerea* in a sensitive strain of *B. cinerea*. The *hph* gene conferring resistance to hygromycine was used as transformation selection marker.

The transformants harboring the *B. pseudocinerea* allele instead of the *B. cinerea* allele showed a slightly increased resistance to fenhexamid (RF<10) compared to the parental *B. cinerea* strain (Billard *et al.*, unpublished). Those results indicate that the major part of fenhexamid resistance in *B. pseudocinerea* is conferred by a mechanism independent of the target.

3.2 Characterization of an unusual resistance mechanism towards fungicides: Detoxification

Indications about the second mechanism were obtained more than ten years ago by two studies. Suty and co-workers (1999) performed fenhexamid metabolization assays on *B. cinerea* and *B. pseudocinerea* and observed that naturally resistant species displayed a metabolization pattern different from *B. cinerea* suggesting fenhexamid degradation as possible resistance mechanism. These results were supported by the synergy between DMIs and fenhexamid observed only for *B. pseudocinerea* (Leroux *et al.,* 2002). Together these results suggested for *B. pseudocinera* an enzyme(s) similar to Cyp51 (the target of DMIs) to degrade fenhexamid and therefore conferring resistance to fenhexamid.

We have searched the *B. cinerea* genome database (Amselem *et al.,* 2011) (http://urgi.versailles.inra.fr/Species/Botrytis) for cytochrome P450 proteins similar to Cyp51 and compared their expression profiles between *B. cinerea* and *B. pseudocinerea*. The candidate gene showing the highest similarity to *cyp51* (19 % protein identity, 37 % protein similarity) showed increased expression in *B. pseudocinerea* (Billard *et al.*, unpublished). According to the nomenclature of P450s, after phylogenic analysis against all P450s of the ascomycete *Aspergillus nidulans* (Kelly *et al.*, 2009) the corresponding gene was named *cyp684*. We created *cyp684* deletion mutants in a *B. pseudocinerea* strain through a gene replacement strategy. The Δ*cyp684* mutants showed a 200 fold reduction in fenhexamid resistance and a simultaneous reduction in DMI-fenhexamid synergy (Table 4, Fig. 6). Although the *B. pseudocinerea* Δ*cyp684* mutants do not reach *B. cinerea* sensitivity levels, theses results show that the cytochrome P450 encoding gene *cyp684* is responsible for the biggest part of *B. pseudocinerea's* natural resistance to fenhexamid. Natural polymorphism between the *B. cinerea/B. pseudocinerea cyp684* orthologues may account for the different phenotypes. It remains to be shown if *cyp684* overexpression and/or differential enzyme parameters are involved.

In *B. pseudocinerea* however the *cyp684* deletion abolishes the hypersensitivity to the phosphothiolate edifenphos in addition to the loss of fenhexamid resistance (Table 6)

suggesting that Cyp684 could have a similar enzyme activity on fenhexamid as on edifenphos. Phosphorothiolates (e.g. edifenphos) are profungicides, nearly exclusively used against the rice-blast disease caused by *Magnaporthe oryzae*.

	B. cinerea	***B. pseudocinerea (RL)***	
		wt	***Δcyp684***
Fenhexamid (Hydroxyanilides)	S	HR	**MR**
Fenhexamid+DMI	Ind.	Syn.	Ind.
edifenphos (Phosphorothiolates)	S	HS	S

RL= resistance levels, S= sensitive, HS=hypersensitive, HR=highly resistant, MR=moderately resistant, Ind.= independent, Syn.= synergistic

Table 4. Sensitivity profiles to fenhexamid and edifenphos (phosphorothiolate) of *B. cinerea, B. pseudocinerea* and *Δcyp684* mutants of *B. pseudocinerea*.

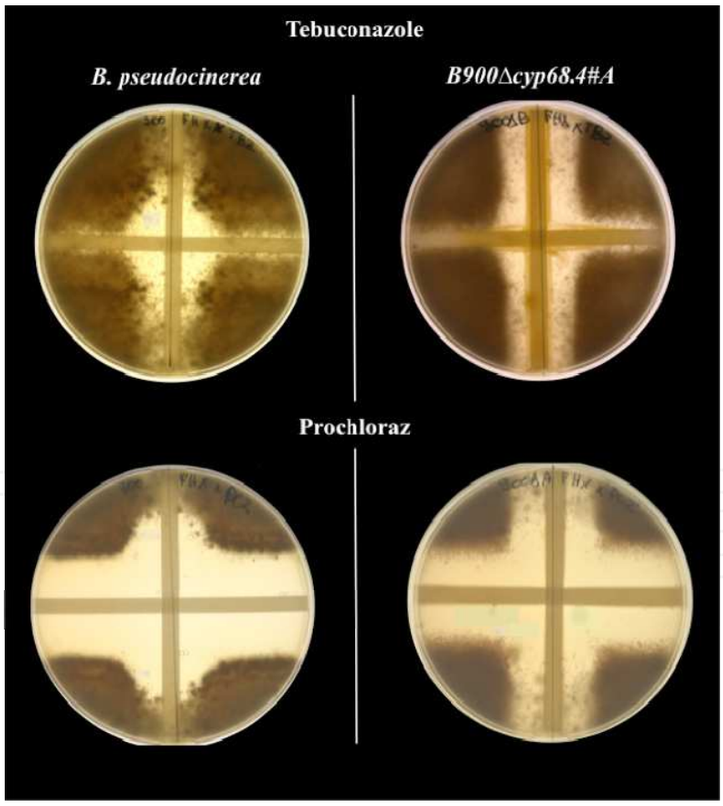

Fig. 6. Joint action between fenhexamid (vertical) and prochloraz or tebuconazole (horizontal strip) on the *B. pseudocinerea B900* strain and a B900Δ*cyp684* deletion mutant. The curves around paper crosses indicate strong synergism between chemicals (in the left panel). In the *B. pseudocinerea* Δ*cyp684* mutant growth inhibition is typical of independent actions (right panel).

These compounds need to be modified in order to become active. In the case of edifenphos, the active metabolite is produced by the cleavage of a phosphor-sulfur bridge (Uesugi, 2001). As for *B. pseudocinerea,* a strong synergy was observed between phosphorothiolates and DMIs. Moreover negative cross-resistance between phosphorothiolates and phosphoroamidates in *M. oryzae* allowed the identification of the biochemical reactions involved (Uesugi & Takenake, 1992 ; Fig. 7). The enzymes involved are yet unknown, but negative cross resistance between BPA and edifenphos on one hand, and the synergy between edifenphos and DMIs on the other, suggest the involvement of a cytochrome P450 accepting as a substrate edifenphos as well as BPA. The comparable situation observed in *B. pseudocinerea* is in favor of a comparable enzymatic reaction involved in the detoxification of fenhexamid. We are currently analyzing the metabolites produced by *B. pseudocinerea* from fenhexamid in order to unravel the reaction mechanism.

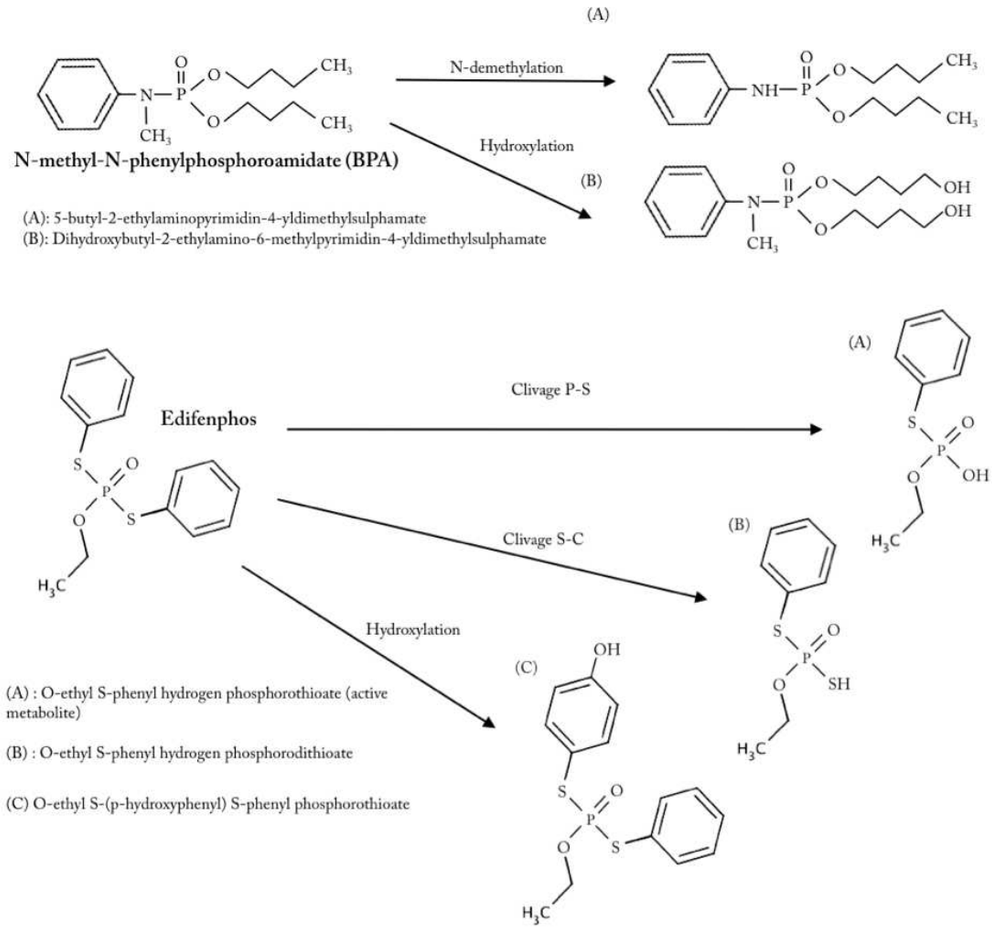

Fig. 7. Metabolic pathways of phosphorothiolates (edifenphos) and phosphoroamidates (BPA) in susceptible and resistant strains of *Magnaporthe oryzae.* (from Uesugi & Takenaka, 1992, modified).

4. Conclusions

We and others have identified and characterized various phenotypes displaying reduced sensitivity to fenhexamid among natural grey mould populations. In the case of the

phenotypes with low resistance at the spore germination stage (HydR1, HydR2, MDR2 et MDR3 phenotypes), Petit *et al.,* 2010 have shown these have only a limited impact on fenhexamid's efficacy in artificial inoculation studies and in vineyards studies. In the same line, monitoring started before the introduction of fenhexamid, show that the variations in frequencies of *B. pseudocinerea* – naturally resistant to fenhexamid – in grey mould populations, seem to be independent of selection by fenhexamid. *B. cinerea* and *B. pseudocinerea* are found in sympatry on the same hosts, but clearly differ in their phenology, demonstrating differences in ecological niche (Walker *et al.,* 2011): although present at low frequencies (0-15 %), *B. pseudocinerea* is predominantly found in spring populations and has only a reduced impact on grey mould epidemics.

Finally, the HydR3 phenotype comprises strains with moderate ($HydR3^-$) to high ($HydR3^+$) resistance levels at all growth stages. This resistance is conferred by target site modifications. $HydR3^+$ strains are the predominant strains resistant to fenhexamid in French vineyards and those of greatest concern in practice, because they threaten the sustainability of fenhexamid in combating grey mould. Frequencies of $HydR3^+$ strains are slowly but steadily increasing in French vineyard populations. Even if their mean frequencies stayed moderately low (e.g. 27 % in Champagne in 2010, Fig. 8), their proportion within same plots has strongly increased in the last years. Although one can observe over 50 % highly resistant strains at the end of the season after fenhexamid treatment, our multi-annual monitoring survey did not reveal sites with such important frequencies over several years. In addition, we are not aware of any loss-of-efficacy observed for fenhexamid due to the selection of resistant populations. Several hypotheses can be put forward to explain the situation: (1) the use of fenhexamid limited to one application per year (as for other botryticides), (2) the fitness cost of the $HydR3^+$ mutations and (3) the predominance of MDR strains at least in Champagne (55 % mean frequencies). Indeed, strains combining MDR (multiple drug resistance) and specific resistance to fenhexamid are rarely found.

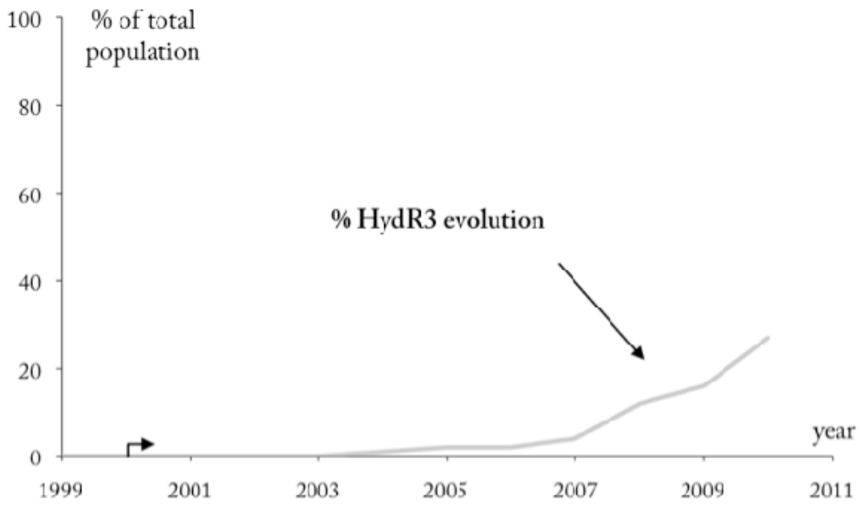

Fig. 8. Evolution of the HydR3 phenotype in Champagne since the introduction of fenhexamid (source : CIVC, AS Walker INRA).

We have developed a molecular tool allowing the rapid detection and quantification of the three major HydR3 alleles from a mixture of *B. cinerea* spores. (ASPPAA PCR). This tool will

make large scale field assays realistic to evaluate the percentage of HydR3$^+$ strains among populations subjected to different fungicide pressures in order to precise the best strategy for fenhexamid treatment and the best state for its application. Moreover, this method may monitor frequencies of HydR3$^+$ strains after stopping fenhexamid treatments and therefore allows to analyze the persistence of such resistance phenotype under real conditions.

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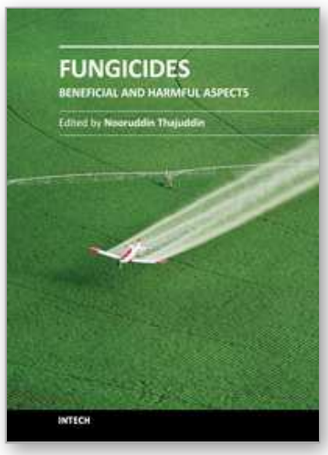

Fungicides - Beneficial and Harmful Aspects
Edited by Dr. Nooruddin Thajuddin

ISBN 978-953-307-451-1
Hard cover, 254 pages
Publisher InTech
Published online 16, December, 2011
Published in print edition December, 2011

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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A. Billard, S. Fillinger, P. Leroux, J. Bach, C. Lanen, H. Lachaise, R. Beffa and D. Debieu (2011). Fenhexamid Resistance in the Botrytis Species Complex, Responsible for Grey Mould Disease, Fungicides - Beneficial and Harmful Aspects, Dr. Nooruddin Thajuddin (Ed.), ISBN: 978-953-307-451-1, InTech, Available from: http://www.intechopen.com/books/fungicides-beneficial-and-harmful-aspects/fenhexamid-resistance-in-the-botrytis-species-complex-responsible-for-grey-mould-disease

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