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Review

# Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi

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ABSTRACT

Succinate dehydrogenase (Complex II or succinate-ubiquinone oxidoreductase) is the smallest complex in the respiratory chain and transfers the electrons derived from succinate directly to the ubiquinone pool. Succinate dehydrogenase inhibitor (SDHI) fungicides specifically inhibit fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II and play an important role in the integrated management programmes of many plant diseases. In contrast to first generation of SDHI fungicides (e.g. carboxin) exceptionally active against basidiomycetes, newer active ingredients in this class (e.g. boscalid, penthiopyrad, fluopyram) show a broad-spectrum activity against various fungal species. However, the consistent use of site-specific fungicides such as SDHIs can result in the selection of resistant fungal genotypes which may ultimately lead to a rapid decline of fungicide performance. This paper reviews previous and recent advances in understanding the molecular mechanisms and other factors controlling the evolution of resistance to SDHI fungicides. Furthermore, we provide recommendations on the future use of new developed molecules of this group as well as future research prospects.



## 1. Introduction

Over the years, industrial research programs have resulted in the development of valuable crop protection compounds with many desirable properties including specificity, systemicity, curative and eradicant action and high activity at low use rates (Russel, 2003). Succinate dehydrogenase inhibitor (SDHI) fungicides play an important role in plant protection against many phytopathogenic fungi. These molecules specifically bind to the ubiquinone-binding site (Q-site) of the mitochondrial complex II, thereby inhibiting fungal respiration. Due to their unique mode and site of action, they show no cross-resistance with other chemical classes such as strobilurins, benzimidazoles or anilinopyrimidines and therefore are excellent candidates for managing fungicide resistance development and optimizing diseases control (Avenot et al., 2008a; Leroux et al., 2003; Stammler et al., 2007a; Zhang et al., 2007). Early SDHI fungicides (Table 1) such as carboxin were first used in agriculture in the late 1960s and have a main activity against disease caused by basidiomycetes such as rusts and Rhizoctonia diseases whereas only a limited activity was reported against other pathogens (Motoba et al., 1988; Ulrich and Mathre, 1972; Yanase et al., 2007; Zhang et al., 2009). In contrast to old SDHIs, newer active ingredients of SDHIs (Table 1) comprise compounds such as boscalid, penthiopyrad or fluopyram, which are characterized by a broad spectrum of fungal activity on various crops (Stammler et al., 2007a; Yanase et al., 2007). Unfortunately, due to the specificity of their mode of action, the frequent and widespread use of these molecules can potentially cause selection of resistance among the pathogen population in the field (Dekker, 1995). Knowledge about the genetic factors influencing the dynamics of evolution of specific pathogens resistant populations is an important step to guarantee the sustainable use of SDHI fungicides. The mode of resistance at the molecular and genetic levels and the existence of mutants resistant to complex II inhibitors such as carboxin or flutolanil have been known for several years in several organisms. Recently, the release in the market of boscalid, first representative of newer SDHIs, improved diseases control, but shortly after its commercial use, resistance started to develop in field populations of a limited number of pathogens species. Here, we review prior and recent studies elucidating mechanisms and evolution of resistance to SDHI fungicides in phytopathogenic fungi. Furthermore, we provide

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**Table 1**
Examples of well documented and extensively used complex II inhibitors.

| Common name | Chemical name | Chemical group |
|---|---|---|
| Flutolanil | N-(3-Isopropoxy-phenyl)-2-trifluoromethyl-benzamide | Phenyl-benzamide |
| Atpenin A5 | (4S,5R)-5,6-Dichloro-1-(2,4-dihydroxy-5,6-dimethoxy-pyridin-3-yl)-2,4-dimethyl-hexan-1-one | 2-Pyridinol derivatives |
| Carboxin | 5,6-dihydro-2-methyl-1,4-oxathin-3-carboxanilide | Oxathiin carboxamides |
| Penthiopyrad | (*RS*)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide | Pyrazole carboxamides |
| Boscalid | 2-Chloro-N-(4′-chloro-biphenyl-2-yl)-nicotinamide | Pyridine carboxamides |
| Fluopyram | *N*-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-*o*-toluamide | Pyridinyl-ethyl benzamide |
| Dinitrophenol-17 | 2-(1-methyl-hexyl)-4,6-dinitrophenol | Dinitrophenol |

recommendations on future uses of new developed molecules of this group, as well as future research prospects.

## 2. Mode of action and molecular targets of SDHI fungicides

The target of SDHI fungicides is the succinate dehydrogenase (SDH) complex in the respiratory chain also referred to as complex II or succinate:ubiquinone oxidoreductase (SQR) (Hägerhäll, 1997; Kuhn, 1984). This enzyme complex couples the oxidation of succinate to fumarate in the mitochondrial matrix (or in the cytoplasmic membrane of bacteria) with the reduction of ubiquinone (UQ), a hydrophobic membrane bound electron carrier, to ubiquinol (UQH2) in the membrane during aerobic respiration (Horsefield et al., 2006). In addition to its function as a dehydrogenase in the respiratory system, complex II plays an important role in the tricarboxylic acid cycle. The mitochondrial SDH complex is composed of a membrane-peripheral domain and a membrane-anchor domain (Fig. 1). The peripheral domain, which consists of two hydrophilic subunits SDHA and SDHB, forms the soluble part of the complex and possesses the succinate dehydrogenase activity (oxidation of succinate to fumarate). SDHA is a flavoprotein (Fp) whose covalent FAD co-factor of the enzyme is part of the catalytic site, whereas SDHB is an iron-sulfur protein (Ip) containing three different iron-sulfur clusters [2Fe–2S], [4Fe–4S] and [3Fe–4S] for electron transfer between the FAD and the membrane quinone (Fig. 1) (Ackrell, 2000; Ōmura and Shiomi, 2007). The primary sequences of Fp and Ip are highly homologous among species and thus indicative of common ancestral genes (Ackrell, 2000). The integral membrane-anchor domain is composed of two hydrophobic membrane-spanning subunits SDHC or CybL and SDHD or CybS which form the large and small subunits of cytochrome b, each contributing three transmembrane K-helices (I, II, III, and IV, V, VI) (Ackrell, 2000; Ōmura and Shiomi, 2007). In addition to its protein components, complex II also contains a prosthetic heme b group complexed between SDHC and SDHD (Ackrell, 2000; Sun et al., 2005). This hydrophobic membrane domain shows greater diversity, with differing b-type heme content and little sequence homology. The membrane anchor subunits region contains specific binding sites for ubiquinone reduction and inhibitors and anchors the catalytic subunits (Fp and Ip) to the inner mitochondrial membrane, thus facilitating the transfer of electrons to ubiquinone (Ackrell et al., 1992; Hägerhäll and Hederstedt, 1996; Yankovskaya et al., 2003). In fact, insights about the location of the ubiquinone-binding sites have been revealed in several crystallographic and computational studies with the use of UQ-binding sites inhibitors (Table 1) (Horsefield et al., 2006; Huang et al., 2006; Sun et al., 2005; Yankovskaya et al., 2003). Analysis of complex II in mutants (as discussed in the next section) of several organisms resistant to SDH-inhibitors such as carboxin have also shed lights about the mechanism of inhibition and binding sites of SDH-inhibitors (Broomfield and Hargreaves, 1992; Keon et al., 1991). Accumulating results demonstrated that the UQ-binding site is formed by residues from subunits SDHB, SDHC and SDHD in close proximity to the [3Fe–4S] cluster and heme b, and is highly conserved between bacteria and eukaryotes (Horsefield et al., 2006; Sun et al., 2005; Yankovskaya et al., 2003). SDHI fungicides specifically interrupt fungal respiration by blocking the electron transport from the [3Fe–4S] cluster to ubiquinone at regions overlapping with the ubiquinone sites (Horsefield et al., 2006; Huang et al., 2006).

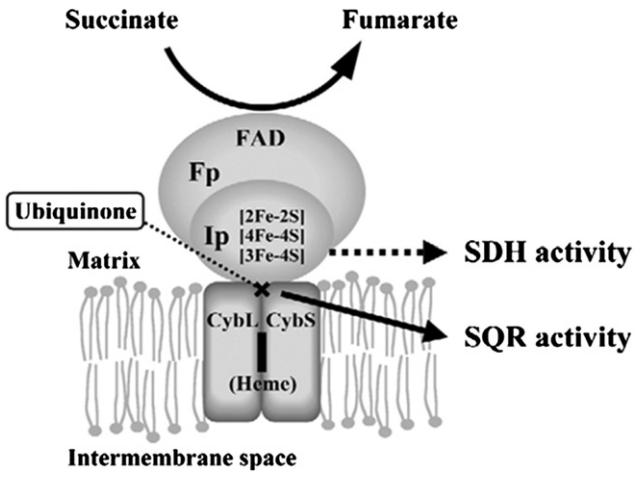


**Fig. 1.** Subunit structure and enzyme activities of complex II. (Adapted from Ōmura and Shiomi, 2007).

## 3. Molecular mechanisms of resistance to SDHI fungicides

### 3.1. Cases of mutants resistant to carboxin

Resistance to carboxin and structurally related fungicides was described as monogenic and the mutations in SDH genes mediating resistance have been characterized in carboxin resistant mutants of several organisms (Table 2) (Honda et al., 2000; Keon et al., 1991; Li et al., 2006; Matsson et al., 1998; Matsson and Hederstedt, 2001; Newcombe and Thomas, 2000; Shima et al., 2009; Skinner et al., 1998). In the maize smut pathogen *Ustilago maydis*, carboxin resistance was conferred by the replacement of a highly conserved histidine residue located at position 257 in the third cystein-rich cluster that binds the S3 center of the mitochondrial iron-sulfur subunit SDHB either by tyrosine or leucine (H257Y/L) (Broomfield and Hargreaves, 1992; Keon et al., 1991). The mutant gene has been successfully used as a dominant selectable marker gene for transformation of this species (Kinal et al., 1993). Structural analysis of the ubiquinone-binding site (Q-site) undertaken on *Escherichia coli* revealed two potential binding-positions; one potential Q-site was very close to the conserved histidine residue, suggesting a possible role in ubiquinone binding and reduction (Horsefield et al., 2006). Interestingly, the same study also revealed that carboxin docked in the same way as ubiquinone in close proximity to the conserved histidine. These results strongly suggested that the histidine residue, in addition to its crucial role in ubiquinone

**Table 2**
Mutations at the complex mitochondrial II genes causing resistance to SDHI in different organisms.

| Amino acid substitution in succinate dehydrogenase complex | | Organism | Mutant | | Reference |
|---|---|---|---|---|---|
| Subunit | Point mutation | | Phenotype | Type | |
| B | H252L | *Ustilago maydis* | Carboxin resistant (CbxR) | Lab | Keon et al., 1991; Broomfield and Hargreaves, 1992 |
| | H267Y | *Mycosphaerella graminicola* | CbxR | Lab | Skinner et al., 1998 |
| | H239L | *Pleurotus ostreatus* | CbxR | Lab | Honda et al., 2000 |
| | H228N | *Paracoccus denitrificans* | CbxR | Lab | Matsson and Hederstedt, 2001 |
| | H229L | *Xanthomonas campestris* | CbxR | Lab | Li et al., 2006 |
| | H277Y, R | *Alternaria alternata* | Boscalid resistant (BosR) | Field | Avenot et al., 2008b |
| | H278Y | *Corynespora cassiicola* | BosR | Field | Ishii et al., 2008 |
| | P225L, T, F; H272Y,R | *Botrytis cinerea* | BosR | Lab & Field | Stammler et al., 2007a |
| | H -> Y | *Didymella bryoniae* | BosR | Field | Stevenson et al., 2008 |
| | H -> Y | *Podosphaera xanthii* | BosR | Field | BASF 2007, Source FRAC |
| | H249Y, L, N | *Aspergillus oryzae* | CbxR | Lab | Shima et al., 2009 |
| C | N80K | *Coprinus cinereus* | CbxR | Lab | Ito et al., 2004 |
| | H134R | *Alternaria alternata* | BosR | Field | Avenot et al., 2009 |
| | S73P | *Corynespora cassiicola* | BosR | Field | Glaettli et al., 2009 |
| | T90I | *A.spergillus oryzae* | CbxR | Lab | Shima et al., 2009 |
| D | D89G | *Paraccocus denitrificans* | CbxR | Lab | Matsson et al., 1998 |
| | D123E, D133R | *Alternaria alternata* | BosR | Field | Avenot et al., 2009 |
| | S89P | *Corynespora cassiicola* | BosR | Field | Glaettli et al., 2009 |
| | D132R | *Sclerotinia sclerotiorum* | BosR | Field | Glaettli et al., 2009 |
| | D124E | *Aspergillus oryzae* | CbxR | Lab | Shima et al., 2009 |

binding, is essential for proper SDHIs binding, thus their inhibitory action. In *E. coli* succinate ubiquinone oxidoreductase (SQR), the conserved histidine residue in the UQ-binding pocket is located at position 207 (H207) and it is strictly conserved across species. In the ascomycete *Mycosphaerella graminicola,* alteration of the equivalent codon located at position 267 to either Tyr or Leu (H267Y; H267L) was also found to confer resistance to carboxin (Skinner et al., 1998). Recently, Li et al. (2006) observed that a His mutation at position 229 identical to that found in carboxin resistant mutants of *U. maydis* conferred resistance to carboxin in *Xanthomonas campestris*. An identical mutation has also been identified in homologs Ip genes of carboxin resistant mutants of *Paracoccus denitrificans* (Matsson and Hederstedt, 2001), *Pleurotus ostreatus* (Honda et al., 2000) and *Aspergillus oryzae* (Shima et al., 2009) (Table 2). In other species, carboxin resistance is also reportedly conferred by mutations in the genes SDHC and SDHD encoding the membrane-anchored subunits (Ito et al., 2004; Matsson et al., 1998). In *P. denitrificans*, an SDHD mutation conferring resistance to carboxin has been detected and corresponds to the replacement of Asp residue at position 89 to Gly (Matsson et al., 1998). Asp89 is invariant in succinate:ubiquinone reductases and is predicted to be located in a cytoplasmic loop connecting transmembrane segments number V and VI. The indicated invariant His residue in SDHB and the Asp residue in SDHD are both most likely involved, directly or indirectly, in the binding action of carboxin (Hägerhäll and Hederstedt, 1996; Matsson et al., 1998). In a mutant strain of the basidiomycete *Coprinus cinereus*, resistance to flutolanil was conferred by a single point mutation in the *SDHC* gene that results in the replacement of the amino acid Asn by Lys at position 80 (N80K) (Ito et al., 2004). This mutation also conferred cross-resistance against carboxin (Ito et al., 2004). More recently, mutants exhibiting resistance to the fungicide carboxin were isolated from *A. oryzae* and the mutations in the three gene loci, which encode succinate dehydrogenase (SDH) B, C, and D subunits, were identified to be independently responsible for the resistance (Shima et al., 2009). The mutated residue in the SDHC subunit (T90I) of *A. oryzae* (Shima et al., 2009) was distinct from the mutated residue that had been identified in the SdhC subunit in *C. cinereus,* whereas the mutated residue in the SDHD subunit (D124E) (Shima et al., 2009) of this fungus corresponds to the mutation identified in *P. denitrificans* (Matsson et al., 1998).

### 3.2. Cases of mutants resistant to boscalid

Field and laboratory mutants resistant to boscalid have been reported in a limited number of filamentous fungi (Table 2), including *Alternaria alternata* from pistachio (Avenot and Michailides, 2007; Avenot et al., 2008a), *Botrytis cinerea* from grapes, strawberry (Stammler et al., 2007a), apple (Kim and Xiao, 2009) and vegetable greenhouses (Zhang et al., 2007), *Corynespora cassiicola* isolates from cucumber greenhouses (Miyamoto et al., 2008, 2009), *Didymella bryoniae* which causes gummy stem blight of watermelons (Keinath et al., 2009; Stevenson et al., 2008), and *Podosphaera xanthii* on cucurbits (McGrath, 2008; Miazzi and McGrath, 2008). The molecular mechanisms of resistance have been studied and the sequencing of the orthologous of iron-sulfur gene of succinate dehydrogenase from the nuclear genome of resistant mutants have shown that the same mutation of the conserved histidine residue determining resistance to carboxin is also critical toward conferring resistance to boscalid in these ascomycetic fungi (Table 2). In *A. alternata,* isolates expressing high levels of resistance to boscalid were also cross resistant to carboxin (Avenot et al., 2008b). Polymorphism analysis of the *A. alternata AaSDHB* gene sequence between sensitive and resistant isolates showed that a conserved histidine residue at position 277 in the AaSDHB protein was mutated to either tyrosine (H277Y) or arginine (H277R) in some boscalid-resistant isolates (Avenot et al., 2008b). In *B. cinerea*, isolates from field sites carried mutations in the SDH B subunit at position 272 corresponding to the replacement of His by either Tyr (H272Y) or Arg (H272R) while at position 225, a Pro residue was replaced by Phe (P225F) or Leu (P225L) (Stammler et al., 2007a). In mutants generated in the laboratory the Pro residue at position 225 was replaced by either Leu (P225L) or Thr (P225T) (Stammler et al., 2007a). Mutations for the amino acid 225 have only been found in *B. cinerea* and have been not yet described for other insensitive organisms to SDHIs. P225L, P225F and H272Y cause significant losses in fungicide sensitivity, while in the case of H272R and P225T sensitivity losses were less pronounced.

Sensitivity studies with other SDHI fungicides (e.g. carboxin) showed cross-resistance (Stammler et al., 2007a). Molecular modeling studies showed that all mutations are positioned within or close to the ubiquinone-binding site and are expected to result in a decreased or loss in binding affinity for SDHI fungicides (Stammler et al., 2007a). A loss in binding affinity can be expected in the case of the mutations at H272, as its side-chain is located at the Q-site, and is directly involved in the binding of SDH-inhibitors via hydrogen-bonding (Horsefield et al., 2006). In *C. cassiicola,* partial nucleotide sequences of the Ip subunit were compared between boscalid-resistant and -sensitive isolates of *C. cassiicola* and a single point mutation from CAC to TAC leading to the substitution of His with Tyr at location 278 (H278Y) was found in two boscalid-resistant isolates (Ishii et al., 2008). The same histidine mutation was found in boscalid-resistant mutants of *P. xanthii* (Stammler, personal communication) and *D. bryoniae* (Stevenson et al., 2008). The Ip protein sequences were highly conserved across species (Avenot et al., 2008b; Stammler et al., 2007a) and the histidine residues at amino acid positions 277 and 272 in *A. alternata* (Avenot et al., 2008b) and *B. cinerea* (Stammler et al., 2007a) SDH B subunits, respectively, correspond to the histidine at position 257 in *U. maydis* (Keon et al., 1991), 267 in *M. graminicola* (Skinner et al., 1998), and 229 in *X. campestris* (Li et al., 2006). Interestingly, as it has been observed with carboxin, mutations were also found in the membrane-anchored subunits. In *A. alternata,* the polymorphism analysis of the *AaSDHB* gene sequence from 23 boscalid-resistant *A. alternata* isolates did not reveal any difference relative to the wild type (Avenot et al., 2008b). Subsequently, the *AaSDHC* and *AaSDHD* genes, encoding the *A. alternata* membrane-anchored proteins, were cloned and sequenced. As previously described (Baysal et al., 2001; Cecchini, 2003), the deduced amino acid sequences exhibited low similarities with SDHC and SDHD peptides from model organisms, namely *E. coli* and *Saccharomyces cereviviae*, but residues essential to form the ubiquinone-binding site or important in SQR assembly were particularly conserved (Avenot et al., 2009). Comparison of *AaSDHC* full sequence from sensitive and resistant isolates revealed that a highly conserved histidine residue (codon CAC in sensitive isolates) at position 134 was converted to arginine (codon CGC), in most resistant isolates (Avenot et al., 2009). In three other resistant isolates, two other mutations were detected at lower frequencies in the AaSDHD subunit. In one resistant isolate, a substitution of the highly conserved histidine residue by arginine was detected at position 133 (H133R), whereas an aspartate to glutamic acid substitution occurred at position 123 (D123E) (Avenot et al., 2009). A possible explanation for this low frequency is that the SDHD-mutations impose a selective disadvantage under field conditions for mutants having such genotypes. Additional tests revealed that the mycelial growth of these mutants was reduced when they were subjected to oxidative stress (Avenot et al., 2009). This latter result is in accordance with other studies demonstrating that mutations in SDH genes affecting the QP site in mutants of different organisms correlated with a hypersensitivity to oxidative stress (Baysal et al., 2000; Ishii et al., 1990, 1998; Szeto et al., 2007). The mutated aspartate residue D123 in *A. alternata* (Avenot et al., 2009) is conserved in the human SDHD homologous protein and is equivalent to Asp92 which was suggested to play a role in the protonation of ubiquinone upon reduction (Baysal et al., 2002; Yanase et al., 2002). Interestingly, the two histidine residues substituted in the boscalid-resistant mutants of *A. alternata*, namely His-C134 and His-D123, correspond to the residues His-C84 and His-D71 in the membrane anchor of the *E. coli* SQR. These residues are conserved throughout complex II and function as the axial ligands for heme b556, which is known to be essential for the assembly and structural stability of the enzyme (Maklashina et al., 2001; Vibat et al., 1998). However, based on the *E. coli* SQR structure, heme b556 does not seem to be involved in the direct electron transfer pathway to ubiquinone (Maklashina et al., 2001; Nakamura et al., 1996; Vibat et al., 1998). Nevertheless, the conservation of these histidine residues suggests a potential impact on the boscalid binding site and it is likely that alterations of these residues potentially change the structure of the fungicide molecular targets and therefore confer the resistant phenotypes. In contrast with the mutations in SDHC and SDHD subunits reported to affect the sensitivity to other SDHI fungicides in mutants of basidiomycetes species, the H134R and H133R mutations identified in AaSDHC and AaSDHD, respectively, appear unique, since they are located in the heme b ligands of the cytochrome II gene. This is apparently the first report of such mutations associated with resistance to SDHIs. Interestingly, the *A. alternata* SDHB mutants carried no other mutations in the AaSDHC and AaSDHD subunits and vice versa (Avenot et al., 2008b, 2009). Stammler et al. (2007a) also reported that field and laboratory mutants of *B. cinerea* isolated from grapes and strawberries with mutations within the homologous iron-sulfur gene had no mutations in the SDHC and SDHD subunits. In *C. cassiicola*, the histidine mutation was not detected in many other resistant isolates. Nucleotide sequences of the SDHC and SDHD subunits also revealed the presence of the point mutations S73P in SDHC and S89P in SDHD in boscalid-resistant isolates of *C. cassiicola* (Glaettli et al., 2009). Thus, each mutation found in one particular target-encoding gene can independently confer the resistance to boscalid. These findings are in agreement with results by Shima et al. (2009) that clearly demonstrated that independent mutations in the SDH B, C and D subunits confer resistance to carboxin in *A. oryzae* (Shima et al., 2009). Overall, it appeared clear that mutation of the conserved histidine residue occurred in many species of pathogens. This is in contrast with the SDHC and SDHD-mutations which seem to occur more variably. To what extent the described target site mutations occur in plant pathogens is currently not yet clear and the occurrence of additional residues in the SDHI fungicides binding and involved in resistance also cannot be ruled out. At least for *A. alternata* causing Alternaria late blight of pistachio among a sample of 38 boscalid-resistant isolates collected from 2005 to 2007, 39% had mutations in SDHB subunit, 52.6% in SDHC subunit, and 7.9% in SDHD subunit (Avenot et al., 2008b, 2009).

## 4. Methods of detection of boscalid-resistant mutants

### 4.1. Bioassays

Fungicide resistance monitoring programs are essential to evaluate fungicides effectiveness and to reveal early shifts in pathogens' sensitivity to a fungicide before resistance develops to the point of disease control failure (i.e., field resistance), thereby providing an opportunity to make changes in fungicide recommendations. Establishment of baseline sensitivity of a pathogen to a fungicide is the first step to detect fungicide resistance (Justum et al., 1998; Russel, 2004). Conventional bioassays have been used to assess baseline sensitivity profiles to boscalid and to subsequently detect resistant isolates of several important pathogens. For example, in some fungal species such as *B. cinerea* (Stammler and Speakman, 2006), *Sclerotinia sclerotiorum* (Stammler et al., 2007b), *Monilinia* spp. (Spiegel and Stammler, 2006), mycelial growth assay in liquid medium in microtiter plates have been developed as the method of choice to establish baseline sensitivity and to monitor fungicide resistance. In several other studies, conidial germination and mycelial growth assays on agar boscalid-amended media in Petri dishes have also been successfully used to test boscalid sensitivity of isolates of *A. alternata* (Avenot and Michailides, 2007; Avenot et al., 2008a,b), *A. mali* (Lu et al., 2004), *B. cinerea* (Zhang et al., 2007; Myresiotis et al., 2008), *C. cassiicola*

(Ishii and Nishimura, 2007), *Ascochyta rabiei* (Wise et al., 2008), *D. bryoniae* (Stevenson et al., 2008), *Rhizoctonia solani* (Zhang et al., 2009). Data obtained from these fungicide resistance testing have also resulted in the development of rapid *in vitro* monitoring procedures using one single discriminatory dose of boscalid and suitable for extensive monitoring studies. For examples, examination of the mycelial growth of *A. alternata* isolates on boscalid PDA-amended media showed that 10 μg/ml can be used as the cardinal concentration of boscalid for distinguishing resistant and sensitive isolates of *A. alternata* population (Avenot and Michailides, 2007); in *Monilinia* spp. 2.5 μg/ml was proposed as the discriminatory dose for identification of boscalid-resistance in *Monilinia* spp. (Spiegel and Stammler, 2006); in *D. bryoniae*, a boscalid concentration of 3.0 μg/ml was chosen as an appropriate discriminatory concentration for further sensitivity testing (Stevenson et al., 2008).

### 4.2. PCR-based assays with emphasis on A. alternata

The conventional methods for assessing fungicide resistance described above are extremely time and labor consuming. Where a clear phenotype-to-genotype relationship exists, DNA-based tests can be used to quickly and accurately detect fungicide resistant alleles within fungal populations both after and even before the introduction of the fungicide at risk. These techniques offer the opportunities to improve resistance risk assessment, optimize resistance management, and support new products (Brent and Hollomon, 1998). In the case of *A. alternata*, the presence of single nucleotide polymorphisms in *A. alternata* SDH genes have led to the development of molecular diagnostic methods for detecting boscalid resistant alleles in this pathogen. Recognition sites were identified, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific polymerase chain reaction (AS-PCR) methods were developed for identifying the boscalid resistant genotypes. Mutants carrying the alleles AaSDHB-H277R, AaSDHC-H134, AaSDHD-H133R and AaSDHD-D123E were successfully diagnosed by PCR-RFLP analyses using the restriction enzymes *Aci*I, *Aci*I, *Xcm*1, *Xmn*1, respectively (Avenot et al., 2008b, 2009). Molecular diagnosis of boscalid resistance in mutants carrying the *AaSDHB*-H277Y allele was performed by AS-PCR using relevant primers (Avenot et al., 2008b). Using these PCR assays, 180 isolates of *A. alternata* resistant to boscalid kept from a collection during the 2007 pistachio growing season were screened for the mutation AaSDHB-H277R/Y and AaSDHC-H134R. It was found that the genetic mutations in Ip gene were more prevalent in the tested resistant population with mutations H277Y and H277R in this gene accounting for 45% and 35% respectively. Only 5 isolates of remaining fraction of resistant isolates were analyzed for the presence of the AaSDHC-H134R mutation and all of them carried this mutation. Monitoring of the H134R mutation in isolates collected in 2006 indicated that this mutation was present at frequency of 53% whilst mutations in SDHD were much lower (7%). It is likely that the H134R mutation might be involved in resistance in the remaining group of isolates. Notably, the results from molecular diagnostic assays were in agreement with results obtained from conventional bioassays and proved the accuracy and reliability of these methods for routine monitoring studies of boscalid resistance, as well as for determining strategies for the use of new molecules.

## 5. Factors affecting the evolution of boscalid-resistant isolates

### 5.1. Boscalid usage patterns

The usage pattern of an active ingredient is a further factor with heavy usage increasing the selection pressure and competitive advantage for resistance. Intensive monitoring programs have been conducted to determine the distribution of boscalid resistance frequencies among isolates of several pathogens collected from fields in which unsatisfactory diseases control by the premix boscalid + pyraclostrobin were noticed. As it was suspected, the frequencies of boscalid-resistant isolates were significantly higher in fields where Pristine® (boscalid + pyraclostrobin) had been applied compared to those from boscalid-untreated fields. For example, 700 *A. alternata* single-spore isolates, collected from 42 commercial and untreated pistachio orchards during the 2005 and 2008 pistachio growing seasons were tested for sensitivity to boscalid. The analysis of the sensitivity data showed that the prevalence of resistant isolates was significantly higher in commercial pistachio orchards in which Pristine® (boscalid + pyraclostrobin) had been applied and in which Alternaria late blight proved difficult to be controlled. In contrast, percentage of resistant isolates from orchards with no history of boscalid exposure was low or equivalent to zero (Table 3) (Avenot and Michailides, 2008). Interestingly, high proportions of resistant phenotype were also recovered from pistachio plant tissues before any application of fungicides during the 2008 season (Avenot and Michailides, 2008). High frequencies of boscalid resistance were also reported in the Gummy stem blight pathogen *D. bryoniae* from watermelons in fields and experimental plots treated with boscalid (Stevenson et al., 2008; Stevenson, personal communication), in *B. cinerea* isolates collected all around Europe (Stammler et al., 2007a) and in *C. cassiicola* collected from cucumber greenhouses in Japan, which received regular sprays of boscalid (Miyamoto et al., 2009). Similarly, a recent monitoring study of the sensitivity of isolates of the grey mold pathogen originated from strawberry and kiwi fields in Greece, and

**Table 3**
Frequencies of boscalid-resistant isolates in Alternaria alternata populations collected from California pistachio orchards from 2005 to 2008.

| Orchard Location County | Year of isolation | History of pristine® sprays | Number of isolates tested | Frequency of boscalid-resistant isolates (%) |
|---|---|---|---|---|
| Orchard 4, (KAC) Fresno County | 2005 | None | 49 | 2.04 |
| Orchard 1, (SVO) Kern County | 2005 | Yes | 59 | 12.00 |
| Orchard 1, (SVO) Kern County | 2006 | Yes | 42 | 81.00 |
| Orchard 2, (SVO) Kern County | 2007 | Yes | 74 | 88.00 |
| Orchard 3, (SVO) Kern County | 2007 | Yes | 26 | 96.15 |
| Orchard 1 (NCL) Tulare County | 2007 | Yes | 27 | 89.00 |
| Orchard 2 (NCL) Tulare County | 2007 | Yes | 53 | 94.34 |
| Orchard 3 (ORD) Tulare County | 2007 | Yes | 88 | 94.32 |
| Orchards JV (Madera County) | 2008 | No | 19 | 15.80 |
| Orchards T8 Tulare County | 2008 | Yes | 106 | 75.50 |
| Orchard A1 Fresno County | 2008 | Yes | 9 | 56.00 |
| Orchard F1 Kings County | 2008 | Yes | 15 | 0.00 |
| Yolo County | 2008 | No | 30 | 0.00 |
| Orchards SQ1 Kern County | 2008 | No | 15 | 6.67 |
| Orchards M1 Madera County | 2008 | Yes | 38 | 18.42 |
| Orchards C1 Madera County | 2008 | Yes | 121 | 56.20 |

excessively treated with Signum® (boscalid + pyraclostrobin), showed that most of the *B. cinerea* isolates tested were resistant to boscalid ($EC_{50}$ > 100 ppm) (G. Karaoglanidis, personal communication). In *Monilinia fructicola*, the mean $EC_{50}$ values to boscalid of isolates collected from peach orchards with multiple years of boscalid exposure was significantly higher compared to the mean $EC_{50}$ value of the baseline population (Amiri et al., 2009). Overall, the sampled populations in each pathogen/crop system could be partitioned in two distinct groups of sensitive and resistant isolates. This distribution clearly indicated the occurrence of a disruptive selection type of resistance which is known to be one of the most important factors contributing to the rapid appearance and spread of fungicide resistant alleles (Milgroom, 1990). Undoubtedly, the reliance on boscalid in control programs since the activity of QoI (strobilurins) fungicides has declined (Ishii et al., 2007; Luo et al., 2007; Stevenson et al., 2004) has increased the selection pressure and competitive advantage for pathogens boscalid-resistant strains. Interestingly, in some cases (*A. alternata* on pistachio; *D. bryoniae* on watermelon) only sensitive isolates were found in some fields, even though they had been treated with Pristine® (boscalid + pyraclostrobin) (Avenot and Michailides, 2008; Stevenson et al., 2008). As suggested by Stevenson et al. (2008), while boscalid resistance has become common in some locations, in other areas the pathogens' populations remain predominantly sensitive. By 2006, due to the rapid increase of the frequency of recovery of *A. alternata* isolates with reduced sensitivity to boscalid across the pistachio producing areas of California, newly registered fungicides such as the anilinopyrimidine pyrimethanil (Scala), the premix formulation of cyprodinil (an anilinopyrimidine) and fludioxonil (a phenylpyrrole) (Switch), and the multisite inhibitor chlorothalonil were recommended for use in pistachio control programs in alternation with the premix boscalid + pyraclostrobin (Pristine®). A look at the schedule of fungicides used in the last 3 years in these orchards showed that growers have applied the premix boscalid + pyraclostrobin in alternation with pyrimethanil or the premix of cyprodinil + fludioxonil sprays. The introduction of these different chemistries provided alternative modes of action and potentially reduced the boscalid selection pressure on the *Alternaria* isolates in these orchards. Paradoxically, high level of resistant isolates was detected from a watermelon field that had no history of SDHI use (Stevenson et al., 2008) and the authors suggested that these isolates may have been introduced into the field as inoculum from outside source rather than arising from fungicide exposure in the field. Likewise, boscalid-resistant isolates of *A. alternata* were also detected at low proportions in some pistachio orchards with no history of boscalid use (Table 3). These isolates were also probably brought up from treated field, although resistant genotypes of fungi may be found in nature at very low frequency where no fungicidal selection pressure has been applied; such resistant alleles also can evolve in a population and spread rapidly, especially through airborne pathogen populations, and more particularly if selection pressure is maintained (Brent, 1995; Milgroom et al., 1989; Vendite and Ghini, 1999). In light of the monitoring data collected in different pathosystems, it appears that there is a high risk for boscalid-resistant pathogens to quickly develop and spread in the field overtime.

### 5.2. Fitness of boscalid resistant isolates

Fungicide resistance often is accompanied by fitness costs that may influence the risk of resistance build-up in the absence of selection pressure (Milgroom et al., 1989; Shaw, 1989). Thus, characterizing resistant isolates in terms of fitness is critical to predict the behavior of the entire population in the future and to implement disease control strategies. In the case of *A. alternata* on pistachio, measurements of predicted fitness components (Antonovics and Alexander, 1989; Dekker, 1981; Skylakakis, 1987) such as spore germination, hyphal growth, sporulation, or virulence on pistachio leaves of selected resistant strains have revealed no differences between boscalid-resistant and -sensitive isolates (Avenot and Michailides, 2007). These resistant isolates were later characterized in their AaSDHB and AaSDHC mutations (Avenot et al., 2008b, 2009). Experiments on the stability of the boscalid-resistant phenotype showed that the resistance level of resistant isolates did not decline following successive subculturing on fungicide-free PDA, indicating that the resistance to boscalid apparently is stable in the absence of selection pressure (Avenot and Michailides, 2007). In other pathogens, including *D. bryoniae* (Stevenson et al., 2008) and *C. cassiicola* (Miyamoto et al., 2009), no differences in the mycelial growth rates on boscalid-free media were observed between field boscalid-resistant and boscalid-sensitive isolates. Zhang et al. (2007) also showed no differences in the mycelial growth and spore germination rates of laboratory boscalid-resistant mutants and wild-type strains of *B. cinerea*. In *C. cassiicola*, boscalid-resistant and −sensitive isolates showed a similar degree of pathogenicity on cucumber (Miyamoto et al., 2009). On the other hand, as it was expected, application of boscalid confers a selective advantage for the resistant isolates to this fungicide. In *A. alternata*, a preventative application of a commercial formulation of boscalid (Endura™), at a concentration which is effective against naturally sensitive isolates, failed to control disease caused by the boscalid-resistant isolates in laboratory tests (Avenot and Michailides, 2007). As a result of Corynespora leaf spot inoculation tests which used potted cucumber plants, control failures of boscalid were observed against *C. cassiicola* boscalid-resistant isolates (Miyamoto et al., 2009). Amiri et al. (2009) also demonstrated that *M. fructicola* isolates with high $EC_{50}$ values toward boscalid were not fully controlled with the field rates of boscalid-containing fungicide. Similarly, in barley plants treated with carboxin, the level of infection caused by carboxin-sensitive isolates of *Ustilago nuda* was also significantly lower than that observed with the carboxin-resistant isolates (Newcombe and Thomas, 2000). Accordingly, measurements of some components of the predicted fitness seem to indicate an absence of fitness penalties in resistant isolates of several fungal species. In pistachio, the recovery of high proportions of *A. alternata* resistant phenotype from plant tissues before any application of fungicides during the 2008 season also seems to indicate that boscalid-resistant isolates of this pathogen may be well fit and overwinter in the field (Avenot and Michailides, 2008). However, in some cases, results regarding the values of predicted fitness parameters were rather inconsistent. In the case of *A. alternata*, analysis of SDH-mutants showed a reduction of their radial growth when subjected to oxidative stress, comparatively to the wild-type isolates (Avenot et al., 2009) and these isolates might also be affected in their fitness in the midst of such conditions in the field. Furthermore, the detection of *A. alternata* isolates carrying mutations in the AaSDHD subunit at a low frequency also seems to imply that the SDHD-mutations may possibly impose a selective disadvantage under field conditions for mutants having such genotypes (Avenot et al., 2009). Zhang et al. (2007) also showed that boscalid resistant mutants *of B. cinerea* had reduced sporulation *in vitro* and pathogenicity on aubergine, though these mutants were obtained after UV mutagenesis and might be different from field mutants. Likewise, *R. solani* mepronil-resistant mutants obtained through UV mutagenesis showed significant decreases in sclerotium production and pathogenicity (Zhang et al., 2009). Additional experiments measuring the total fitness values or “realized” fitness (Antonovics and Alexander, 1989; Dekker, 1981; Skylakakis, 1987) by competing two or more isolates (fungicide-sensitive and -resistant isolates) on the host

plant under natural or greenhouse conditions and comparing the changes in frequency of the isolates relative to the others after several generations of growth in absence and presence of the fungicide, can also be conducted to assess the competitive ability of resistant strains. In contrast with the predicted fitness experiments, the realized fitness approach generates information about the overall fitness. Therefore future inoculations experiments using mixtures of boscalid-resistant strains with either mutation in SDH B, C or D genes and -sensitive strains will provide a more realistic assessment about the fitness of pathogens boscalid-resistant isolates. It can be reasonably predicted considering the occurrence of a qualitative type of resistance in pathogens boscalid-resistant concomitantly with the absence of fitness costs that the frequency of isolation of the boscalid-resistant isolates would continually increase where resistance has not been detected, and this may ultimately lead to a rapid decline of boscalid-containing fungicides performance.

### 5.3. Cross-resistance relationships between boscalid, penthiopyrad and fluopyram

Penthiopyrad (pyrazole) (Dupont Co.) and fluopyram (benzamide) (Bayer CropScience Co.) are novel broad spectrum SDHI fungicides. Disease control results have been excellent, especially for ascomycete pathogens of horticultural crops (Fought et al., 2009; Yanase et al., 2007) and registrations of these fungicides are expected on several crops. Assuming a positive cross-resistance patterns for fungicides acting in mitochondrial complex II, the sensitivity profile of selected *A. alternata* boscalid SDHB, SDHC and SDHD-resistant mutants and wild-type isolates to penthiopyrad and fluopyram was determined by recording their relative mycelial growth in presence at 10 μg/ml penthiopyrad and fluopyram. All of the *A. alternata* isolates sensitive to boscalid were also sensitive to penthiopyrad and fluopyram (Table 4). As expected, *A. alternata* boscalid-resistant isolates carrying SDH-mutations were not affected by penthiopyrad (Table 4). Boscalid-resistant isolates of *D. bryoniae* collected from watermelon fields and carrying the histidine to tyrosine mutation in the Ip gene were also found to be resistant to penthiopyrad (Avenot et al., unpublished data). This correlation between the sensitivities of isolates to boscalid and to penthiopyrad seems to indicate that the SDH-mutations conferring resistance to boscalid are also associated with resistance to penthiopyrad. Consequently, applying penthiopyrad in crops where resistance to boscalid is established will lead to an increase of the selection pressure on resistant populations of the targeted pathogens hence in an increase of their frequencies. Interestingly and, in contrast to penthiopyrad, fluopyram *in vitro* strongly inhibited the mycelial growth of *A. alternata* SDH-mutants (Table 4), thus revealing the higher intrinsic activity of this molecule. These laboratory evaluations are consistent with the best performance observed for this product in control trials against Alternaria late blight (T. J. Michailides, personal communication). A close examination of the

**Table 4**
Comparison of the sensitivities of *Alternaria alternata* wild-type and boscalid-resistant mutants to penthiopyrad and fluopyram.

| Isolate | | Number | Inhibition of mycelial growth (%) at 10 ppm | | |
|---|---|---|---|---|---|
| Genotype | | | Boscalid | Penthiopyrad | Fluopyram |
| Locus | Mutation type | | | | |
| – | Wild-type | 8 | 87.23 ± 7.08 | 82.97 ± 4.10 | 89.73 ± 7.42 |
| AaSDHB | AaSDHB-H277Y | 8 | 30.70 ± 10.67 | 41.30 ± 7.43 | 90.53 ± 4.72 |
| AaSDHC | AaSDHC-H134R | 8 | 31.87 ± 5.99 | 11.27 ± 4.94 | 85.07 ± 4.67 |
| AaSDHD | AaSDHD-H133R | 2 | 47.46 ± 1.98 | 16.93 ± 3.06 | 88.70 ± 0.08 |

**Table 5**
Sensitivity ($EC_{50}$ values) of *Alternaria alternata* isolates to boscalid and fluopyram.

| Isolate | Genotype | Boscalid | Fluopyram |
|---|---|---|---|
| | | $EC_{50}$ | $EC_{50}$ |
| Aa16 | Wild-type | 0.5 | <0.25 |
| Aa29 | AaSDHB-H277Y | >50 | 0.25 |
| Aa111 | AaSDHB-H277Y | >50 | 0.25 |
| Aa122 | AaSDHC-H134R | >50 | 5 |

$EC_{50}$ values (Table 5) estimated for 4 *A. alternata* isolates showed that two boscalid-resistant *A. alternata* isolates Aa111 and Aa29 carrying SDHB- H277Y-mutation were not fluopyram-resistant (Table 5) whereas another *A. alternata* boscalid-resistant isolates (R122) carrying the H134R was clearly resistant to fluopyram (Table 5). However, this latter mutation seems to confer only a low level of resistance to fluopyram. It will be interesting to determine if other pathogens that have boscalid-resistance due to mutations similar to those reported in *A. alternata,* how they will behave to penthiopyrad and fluopyram fungicides. The discrepancy of cross-resistance pattern of fluopyram with compounds from the same cross-resistance group probably results from the higher intrinsic activity observed for this fungicide and this also suggests that the binding site of fluopyram in complex II may slightly differ from that of other SDHI fungicides and that additional unique mechanism of resistance to fluopyram may arise. Results demonstrating difference of activity within the complex II inhibitors have been obtained by Miyadera et al. (2003) which showed that atpenin A5, a specific Q-site inhibitor for complex II, was significantly more potent than the other complex II inhibitors, such as thenoyltrifluoroacetone (TTFA), and carboxin. These authors observed that the $IC_{50}$ value of atpenin A5 was 300-fold lower than that for carboxin and 1600-fold lower than that for TTFA. Earlier, we reported that mutations in SDH genes can evolve independently and at different proportions in boscalid resistant populations and the impacts of their presence on fluopyram efficacy in field conditions will need to be investigated. Monitoring the sensitivity to fluopyram in pathogens of specific crops will also be critical once this fungicide is introduced and widely used. Characterization of isolates, with only resistance to fluopyram, in their SDH genes will bring insight about potential amino acid residues that may specifically participate in the fluopyram binding site.

## 6. Conclusions

Effective prevention and resistance management strategies can best be achieved by understanding the factors relating to the origin, development, and spread of resistance. Although, fungicide resistance is a key factor in limiting the efficacy and lifetime of important useful fungicides, resistance may also be an important aid to our understanding, at molecular level, of the fungicidal mechanism of action of a particular class of chemicals (Steffens et al., 1996). Using the techniques of molecular genetics, single nucleotides polymorphisms associated with resistance to SDH-inhibitors have been identified in the SDHIs molecular targets genes in several fungal species. Novel SDHI fungicides have been developed, some of which seem to have differential cross-resistance pattern to pre-existing SDHI fungicides. The intrinsic activity of a fungicide is based on the specific affinity for and strength of binding to the target site (Yamaguchi and Fujimura, 2005). It is presumed that differences in activity between SDHI fungicides may result from differences in their binding affinity or interactions with amino acid residues in the succinate dehydrogenase subunits involved in the inhibitor binding sites. The characterization of the full spectrum of amino acid mutations in SDH genes conferring resistance to SDHI

fungicides in fungal pathogens will consolidate knowledge about the mechanisms of inhibition and resistance to these fungicides. This will ultimately result in the development of rapid and reliable molecular diagnostics to carefully monitor resistance to SDHI fungicides at large scale, and further help in the design of resistance management strategies and also alternative novel inhibitors of active ingredients targeted against the UQ-sites of specific fungal pathogens.

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