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First Detection of Boscalid-Resistant Strains of *Erysiphe necator* in French Vineyards: Biological and Molecular Characterization

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INTRODUCTION

Succinate dehydrogenase inhibitor (SDHI) fungicides target the ubiquinone-binding pocket of the enzyme which is structurally formed in the interface between SdhB, SdhC and SdhD subunits. Therefore, mutations within SdhB, SdhC and SdhD subunits can lead to amino acid substitutions conferring resistance to SDHIs. These mutations have also been found to confer resistance to boscalid in laboratory mutants and field isolates from different plant pathogenic fungi, including *Botrytis cinerea* (Lalève et *al.* 2014), *Alternaria alternata* (Avenot et *al.* 2008), *Alternaria solani* (Fairchild et *al.* 2012), and *Sclerotinia sclerotiorum* (Wang et *al.* 2015). In *Botrytis cinerea*, resistance to boscalid in both laboratory mutants and field isolates was associated to mutations in the *sdhB* gene. Substitution/replacement in the SdhB subunit of the conserved histidine (H272), proline (P225) and asparagine (N230) in *B. cinerea* led to different resistance levels to boscalid (Lalève et *al.* 2014). In addition, it has been reported that H272Y or H272R substitutions are frequent in boscalid-resistant field isolates of *B.cinerea* (Leroux et *al.* 2010).

During resistance monitoring of powdery mildew against the SDHI boscalid in French vineyards in 2015, several populations grew on leaf discs treated by discriminatory rates of boscalid (30 or 100 mg/L) (unpublished reports). Therefore, the aim of this study was to isolate some SDHI-resistant strains of *E. necator* from these populations. In this study, 31 singles-spore strains were isolated from three different regions in France. We performed biological tests on leaf disc treated with boscalid, fluopyram or fluxapyroxad at different concentrations. To gain further insights into potential molecular mechanisms of resistance to boscalid in *E. necator*, subunits SdhB, SdhC and SdhD from sensitive and resistant single-spore-isolates have been sequenced, and sequences were compared to characterize molecular mechanisms (point mutation associated with boscalid resistance). Only mutations in subunit SdhB were found in boscalid-resistant single-spore isolates and the most commonly identified mutations included SdhB-H242R and SdhB-H242Y.

MATERIAL AND METHODS

E. necator populations and culture conditions

Powdery mildew infected leaves and grapes were collected from vineyards of different French regions from May to September in 2014 and 2015. Populations were inoculated under sterile conditions onto decontaminated fungicide-free leaves from grape cultivar Cinsaut grown in a greenhouse. Leaves were placed onto water agar medium at the bottom of a disinfected Plexiglas settling tower and conidia were blown in at the top using sporulating leaves or grapes. Inoculated leaves were incubated for 12 days at 22°C at a 16:8h light dark photoperiod.

Sensitivity bioassays on field populations

Sensitivity tests were performed on leaf discs. Ten leaf discs were cut from 10 different leaves. These discs were placed in a Petri dish with the upper surface contacting a layer of filter paper impregnated with the fungicide (or water for control). After 24h, the discs were transferred into agar plates with adaxial surface facing up and dried in sterile conditions. To assess the sensitivity of mildew field populations to boscalid, three discriminatory concentrations were used: 15, 30 and 100 mg L^{-1}. Infected leaves with 12-14 days old powdery mildew cultures were used to inoculate the treated leaf discs. The plates were incubated as described above.

Boscalid-resistant single-spore isolates and cross-resistance assay

Leaf discs with colonies sporulating at 15, 30 or 100 mg boscalid/L were used to isolate resistant strains of *E. necator*. Single conidia were purified on leaf discs with tested concentration, and fresh subcultures of single spore strains were maintained on detached leaves of *V. vinifera* cv. Cinsault as described. To investigate cross-resistance to other SDHI fungicides, *in vitro* sensitivity of theses strains to boscalid, fluopyram or fluxapyroxad was measured. In order to determine the ED_{50}, isolates growing at discriminative concentrations were inoculated onto leaves treated with several different fungicide concentrations. Tests for each isolate were repeated twice per fungicide concentration.

Erysiphe necator SdhB, *SdhC* and *SdhD* gene sequencing and analysis

Publicly available genomic information for *E. necator* was scarce, reflecting the difficulty of working with an obligate parasite. However, sequences of SDH subunits from other pathogenic fungi, e.g. *B.cinerea*, are well documented (Leroux et *al.* 2010, Lalève et *al.* 2014, Walker et *al.* 2013). To identify similar putative sequences of SDH in *E. necator*, the published sequences of the *B. cinerea* BcSdhB, BcSdhC and BcSdhD proteins (National Center for Biotechnology Information [NCBI] accession numbers ACT83447, ACT83441 and ACT83437, respectively) were analyzed, using the BLASTp program against the NCBI's non-redundant database of proteins sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To amplify complete sequences of putative genes *SdhB*, *SdhC* and *SdhD* subunits in each *E. necator* single spore isolate, PCR primer sets were designed. *E. necator* mycelia of each single spore isolate collected from colonies growing on detached leaves approximately 14 days post inoculation

was used for DNA extraction and PCR amplification. Amplified products were then sequenced. To characterize *E. necator* SDH subunit genes and investigate point mutations in each resistant isolates, sequences of genes were compared with those of sensitive isolates, using the MAFFT sequence alignment program.

RESULTS AND DISCUSSION

Sensitivity of field isolates to boscalid, fluxapyroxad and fluopyram

Many field populations with low sensitivity to boscalid at different levels were observed in French vineyards in 2014 and 2015. Almost all populations growing on leaf discs impregnated with 30 and 100 mg boscalid L^{-1} were isolated in southern France. Single spores of these populations and of sensitive populations were used to generate single-spore isolates. According to their sensitivity to boscalid, one representative isolate of each population (i.e. a total of 9 isolates) showed ED_{50} values greater than 100 mg L^{-1} and 4 strains showed ED_{50} values lower than 1mg L^{-1}. Boscalid-resistant and -sensitive strains of *E. necator* were tested for their sensitivity to fluxapyroxad and fluopyram in order to evaluate cross-resistance between SDHI products used on grapes. Boscalid sensitive and resistant isolates were totally inhibited by 3 mg L^{-1} of fluxapyroxad and fluopyram in leaf-disc bioassays, with ED_{50} values between 0.13 and 0.9 mg L^{-1} for sensitive strains and 0.17 and 1.4 mg L^{-1} for resistant isolates respectively (Table 1).

Molecular analysis of field isolates *E. necator* SDH subunits EnSdhB, EnSdhC and EnSdhD.

H242R and H242Y substitution in EnSdhB subunit correlate with boscalid resistance

The sequence of the *B. cinerea* BcSdhB protein (accession number ACT83447) was used to analyze the SdhB subunit protein in *E. necator* by BLASTp. The conserved protein BcSdhB was highly similar (82% identity and e-value=8e-157) with a 271 amino acids putative succinate dehydrogenase iron sulfur protein (accession number KHJ35761.1) described in the *E. necator* genomic sequencing project (Jones et *al.* 2014). This protein shows similarities to the well-characterized mitochondrial iron sulfur protein of SDHs of *Blumeria graminis* (91%), *Alternaria alternata* (70%), *Saccharomyces cerevisae* (74%) and *Mycosphaerella graminicola* (85%). Subcellular localization prediction using the WoLF PSORT program (http://www.genscript.com/tools/wolf-psort) indicates that the putative EnSdhB contains an N-terminal mitochondrial targeting sequence, which is in accordance with the function of this protein. The EnSdhB amino acids sequence contains the three conserved cysteine-rich clusters associated with the iron-sulfur clusters [2Fe-2S], [4Fe-4S] and [3Fe-4S] for electron transfer between the FAD and the membrane quinone.

The complete *EnSdhB* nucleotide sequences of sensitive and resistant single spore-isolates were compared and revealed a nucleotide substitution of adenine in position 794 (codon CAT) with guanine (codon CGT) in almost all resistant isolates. Only the two isolates 15O90-1 and

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15O90-2 showed substitutions of cytosine at position 793 (codon CAT) with thymine (codon TAT). These substitutions correspond to the replacement of the conserved amino acid histidine at position H242 by arginine (H242R) or tyrosine (H242Y) respectively (figure 1).

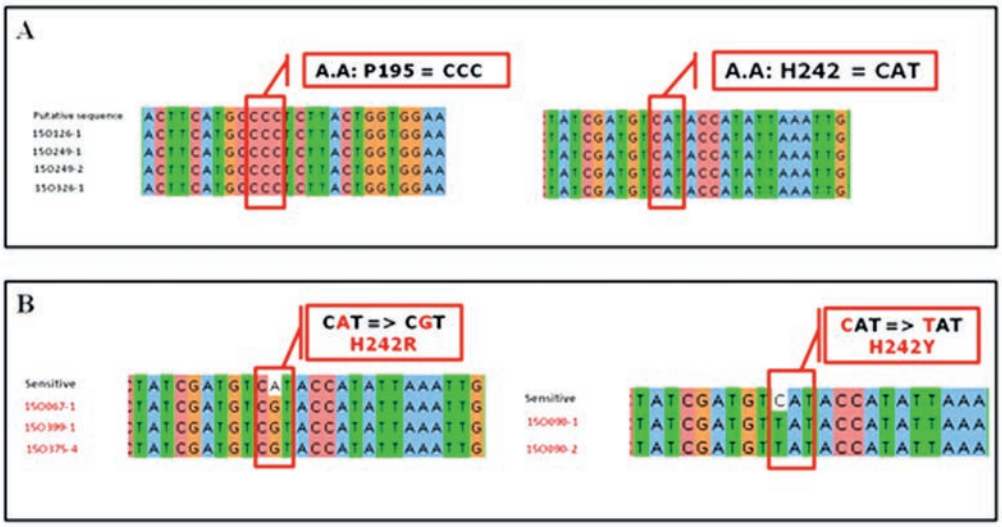

Figure 1: Sequence comparison of *EnSdhB* gene regions coding for conserved amino acid of sensitive (black) and resistant (red) *Erysiphe necator* isolates. **(A)** Comparison between putative *EnSdhB* gene and sequenced *EnSdhB* of sensitive isolates. **(B)** Comparison between *EnSdhB* sequences of sensitive and resistant isolates.

Comparison of the *SdhB* nucleotide sequences of *E. necator* single spore-isolates showed that H242R is the prevalent amino acid substitution associated with high level-resistance to boscalid (Table 1). No further change was observed in other conserved amino acids P195 or N200 in the EnSdhB subunit. It has been suggested that histidine and proline residues and other strictly conserved amino acids in SdhB and SdhC subunits mainly interact with a heteroatom of the core cycle carried by many SDHIs (Sierotzki & Scalliet 2013). These results show that in *E. necator* histidine at position 242 plays an important role in the mode of action of the SDHI boscalid. Furthermore, substitution of this amino acid by ariginine or tyrosine (H242R/Y) confers high resistance (RF > 100) to boscalid. The same amino acids substitutions were found in laboratory mutants or field isolates of different boscalid-resistant phytopathogenic fungi.

In *Botrytis cinerea*, H272Y and H272R were observed in boscalid-resistant strains isolated from vineyards (Leroux et *al.*, 2010). Other studies showed that inhibition of SDH activity in *B. cinerea* laboratory mutants carrying amino acid modifications in SdhB subunit (including SdhB P225T/L/F, N230I and H272Y/R/L) were mainly affected by SDHIs (Lalève et *al.* 2014). Boscalid-resistant field isolates carrying H277R/Y mutations have been detected in *A. alternata* isolated from pistachio (Avenot et *al.* 2008). Susceptibility to the SDHIs fluopyram

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or fluxapyroxad was assessed in four boscalid sensitive and nine resistants isolates. All *E. necator* carrying H242R/Y substitutions were sensitive to fluxapyroxad and fluopyram and no cross-resistance was observed. These results confirm that histidine at position 242 in the EnSdhB subunit slightly affects the interaction of fluopyram with SDH, as shown previously in *B. cinerea* mutants (Lalève et *al.* 2014).

Table 1: Response of *E. necator* field isolates to boscalid, fluxapyroxad and fluopyram

Strains	Regions	SdhB substitution	ED_{50} (mg L^{-1})		
			Boscalid	Fluxapyroxad	Fluopyram
15O249-2	Loire valley	H242	0,33	0,29	0,87
15O326-1	Cognac	H242	0,36	0,19	0,41
15O126-2	Languedoc	H242	0,29	0,16	0,19
15O126-5	Languedoc	H242	0,27	0,13	0,49
15O067-7	South East	H242R	> 100	0,159	0,68
15O067-8	South East	H242R	> 100	0,412	0,35
15O090-1	Languedoc	H242Y	> 100	0,351	0,19
15O090-2	Languedoc	H242Y	> 100	1,433	0,17
15O375-2	Champagne	H242R	> 100	0,171	1,41
15O375-4	Champagne	H242R	> 100	0,441	0,61
15O399-4	Languedoc	H242R	> 100	0,615	1,38
15O399-5	Languedoc	H242R	> 100	0,290	1,09
15O337-5	South East	H242R	> 100	0,343	0,41

CONCLUSIONS

This is the first detection and characterization of resistance to boscalid in field populations of *E. necator*. These resistant strains originated from monitoring 400 populations in the main wine growing areas in France in 2015. Strains isolated from population with loss of sensitivity to boscalid exhibit high level of resistance to this SDHI (ED_{50}>100 mg L^{-1}). However, these strains are still sensitive to fluopyram, another SDHI compound registered against grape powdery mildew, or fluxapyroxad, a candidate SDHI to be registered in France. This lack of cross resistance is confirmed for all the strains collected from the South East, Languedoc or Champagne areas.

Recent advances in *E. necator* genome sequencing facilitated molecular analysis of SDH subunits in sensitive and resistant isolate. Sequence comparison of sensitive and resistant subunits SdhB, SdhC and SdhD reveal the presence of single nucleotide mutation (codon

CAT) only in the SdhB subunit associated to boscalid resistance. The two different amino acid substitutions H242R and H242Y were observed in boscalid-resistant isolates, with H242R as the prevailing mutation. These mutations specifically confer resistance to boscalid, clearly explaining why to date no cross-resistance was observed between boscalid and fluxapyroxad or fluopyram in *E. necator* strains. Boscalid was the first compound registered in France against grape powdery mildew. This active ingredient was used in mixture with the QoI kresoxim-methyl. Recent introduction of fluopyram (also in mixture with a QoI) could modify the situation imposing another selection pressure on the populations of *E. necator*.

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