Characterization of Mutations in the Iron-Sulphur Subunit of Succinate Dehydrogenase Correlating with Boscalid Resistance in *Alternaria alternata* from California Pistachio

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ABSTRACT

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Thirty-eight isolates of *Alternaria alternata* from pistachio orchards with a history of Pristine (pyraclostrobin + boscalid) applications and displaying high levels of resistance to boscalid fungicide (mean EC₅₀ values >500 µg/ml) were identified following mycelial growth tests. A cross-resistance study revealed that the same isolates were also resistant to carboxin, a known inhibitor of succinate dehydrogenase (Sdh). To determine the genetic basis of boscalid resistance in *A. alternata* the entire iron sulphur gene (*AaSdhB*) was isolated from a fungicide-sensitive isolate. The deduced amino-acid sequence showed high similarity with iron sulphur proteins (Ip) from other organisms. Comparison of *AaSdhB* full sequences from sensitive and resistant isolates revealed that a highly

conserved histidine residue (codon CAC in sensitive isolates) was converted to either tyrosine (codon TAC, type I mutants) or arginine (codon CGC, type II mutants) at position 277. In other fungal species this residue is involved in carboxamide resistance. In this study, 10 and 5 mutants were of type I and type II respectively, while 23 other resistant isolates (type III mutants) had no mutation in the histidine codon. The point mutation detected in type I mutants was used to design a pair of allele-specific polymerase chain reaction (PCR) primers to facilitate rapid detection. A PCR-restriction fragment length polymorphism (RFLP) assay in which amplified gene fragments were digested with *AccI* was successfully employed for the diagnosis of type II mutants. The relevance of these modifications in *A. alternata* *AaSdhB* sequence in conferring boscalid resistance is discussed.

Additional keywords: complex mitochondrial II, fungicide resistance, PCR-based assays.

Alternaria late blight of pistachio caused by *Alternaria* spp. in the *alternata*, *tenuissima*, and *arborescens* species-groups is one of the most common fungal diseases of pistachio in California affecting both foliage and fruit (25). Control of the disease is a difficult task and requires a combination of management approaches and multiple fungicidal sprays. Quinone outside Inhibitors fungicides (QoIs) have become a key component of disease management strategies in California due to their excellent control efficacy and yield benefits. However, after only a few years of QoIs use on pistachio crops the development of resistant populations of *Alternaria* spp., caused by a mutation at position 143 (G143A) in the cytochrome b-target-encoding gene, has compromised their use (2,19,31).

As a strategy to manage the widespread resistance to QoIs in California pistachio orchards the new carboxamide fungicide, boscalid, has recently been introduced in a pre-packed mixture with the QoI pyraclostrobin. This mixture is used particularly against *Alternaria* late blight. Besides boscalid, the group of carboxamide fungicides also contains other compounds such as carboxin, flutolanil, benodanil, or furametpyr (6). These compounds are exceptionally active against Basidiomycete fungi and have been successfully used for many years in agriculture to

control bunt and smut diseases of cereals (13,23,30). Unlike most of these carboxamide fungicides, boscalid has an unusual fungicidal profile. It is highly effective against plant pathogens belonging to the Ascomycetes and the Deuteromycetes, such as *Botrytis cinerea*, and species of *Sclerotinia*, *Alternaria*, and *Monilinia*, as well as powdery mildews and other pathogens in fruit, vegetables, and vines (20,28,29).

Carboxamide fungicides are single-site inhibitors and their target is the succinate ubiquinone reductase or succinate dehydrogenase (Sdh) complex in the respiratory chain (3,17). This enzyme complex is a functional part of the tricarboxylic acid cycle and the mitochondrial electron transport chain and catalyzes both the oxidation of succinate to fumarate and the reduction of quinone. The Sdh complex consists of four subunits, a flavo-protein (Fp) (SdhA), an iron sulfur protein (Ip) (SdhB), and two membrane-anchored proteins (SdhC and SdhD). The Fp and Ip subunits form the soluble part of the complex and possess succinate dehydrogenase activity. The SdhC and SdhD subunits anchor Fp and Ip to the membrane and have quinone reduction activity (13,16).

The boscalid-containing product Pristine (BASF Corporation, Research Triangle Park, NC) was registered for use against *Alternaria* late blight of pistachio in 2003. However, after only 2 years of use, reductions in the efficacy of this product in the field have prompted concerns over development of resistance to boscalid, particularly in light of the widespread resistance to QoIs. Studies carried out to test the control efficacy of boscalid against the strobilurin-resistant strains of the pathogen and to determine the sensitivity profile of pathogenic isolates to boscalid, demonstrated

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the high efficacy of boscalid in controlling strobilurin-resistant strains. However, they also revealed the existence of *A. alternata* isolates expressing a high level of resistance to boscalid (1,2).

As was the case with resistance to Q₀Is, the rapid appearance of boscalid resistant populations of *A. alternata* in pistachio orchards when boscalid-containing fungicides are used to combat *Alternaria* late blight, indicates that it is imperative to monitor boscalid resistance in this fungus infecting pistachio. Elucidation of the molecular basis of this resistance will enable the development of quick methods for use in monitoring studies. Studies on the molecular mechanisms responsible for the acquisition of resistance to boscalid analog-fungicides in some resistant populations of bacteria and Basidiomycete and Ascomycete fungi have shown that mutations in conserved regions of the genes *SdhB*, *SdhC*, and *SdhD* encoding the boscalid targets result in reduced sensitivity (3,7,8,9,13,15,22,26,30). In fact, single amino acid substitutions found in the *SdhB* subunit within the third Cys-rich cluster that takes part in binding of iron-sulfur centers of *Ustilago maydis* (3,15), *Pleurotus ostreatus* (10), *Mycosphaerella graminicola* (26), and *Paracoccus denitrificans* (21) have been associated with increased resistance.

Resistant strains are frequently cross-resistant to structurally related chemicals or to chemicals with similar modes of action (5). For instance, cross-resistance has been demonstrated for flutolanil and carboxin both members of the carboxamide group (13). The fact that boscalid, carboxin, and flutolanil belong to the same chemical group infers that there is cross resistance to these compounds in *A. alternata* and that boscalid resistance in this fungus is governed by mutations in the target genes similar to those for carboxin and flutolanil resistance.

The objectives of the current study were to (i) establish the level of sensitivity to boscalid of *A. alternata* field isolates collected from 2006 to 2007 from pistachio orchards treated for at least three consecutive years with the boscalid-containing fungicide Pristine, (ii) investigate the cross resistance patterns between boscalid and carboxin in these isolates, (iii) elucidate the molecular mechanism responsible for boscalid resistance in *A. alternata* populations by isolating a putative iron sulfur gene and determining if mutations in this gene are responsible for boscalid resistance, and (iv) develop polymerase chain reaction (PCR)-based methods for the rapid identification of resistant mutants.

MATERIALS AND METHODS

Origin and collection of single spore isolates. All of the *A. alternata* isolates used in this study are listed in Table 1. They were isolated from pistachio leaves showing putative *Alternaria* infection and collected during the pistachio growing seasons from 2006 to 2007 from different pistachio orchards where boscalid had been used in mixture with pyraclostrobin (Pristine) for three or four consecutive years with two or three spray applications per season. Ten *A. alternata* boscalid-resistant and -sensitive isolates collected from an orchard located in Kern County that had been treated with boscalid (again in Pristine) for over 2 years, were also included in the study (1). Conidial isolates were obtained according to the procedure described by Pryor and Michailides (25). Single spore isolates were maintained on potato dextrose agar (PDA) at 4°C until use.

Media and fungicide sensitivity determination. Fungicides used in the study were pure (a.i., 98.4%) technical grade boscalid (BASF Corporation, Research Triangle Park, NC) and a commercial formulation of carboxin (Vitavax 34 FF, Chemtura Corp, Middlebury, CT). Technical grade boscalid was dissolved in acetone to provide stock solutions containing 10 and 50 mg a.i./ml. Autoclaved PDA was cooled to 50°C and boscalid was added to obtain concentrations of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 250, and 500 µg/ml. The concentration of acetone in all PDA media never exceeded 1 ml/liter. No fungicide was added

to the control medium. Tests for each isolate were repeated two times per fungicide concentration.

Mycelial plugs of each isolate were removed, with the aid of a 5-mm-diameter cork borer, from the colony margins of actively growing 72-h-old colonies on PDA and placed upside down on the center of 30 petri dishes containing the fungicide-amended or unamended media. Cultures were incubated at 24°C in the dark for 7 days. Radial growth of each isolate was measured (minus the diameter of inoculation plug) after 7 days by calculating the mean of two perpendicular colony diameters and expressed as a percentage of the mean diameter of the untreated control. The EC₅₀ value (effective concentration that reduces the mycelial growth by 50%) of each isolate was calculated by regressing the relative inhibition of growth against the log₁₀ fungicide concentration. Depending on their sensitivity to boscalid, isolates were graded into two classes. Isolates classified as sensitive (S) had EC₅₀ values ranging between 0 and 5 µg/ml, and highly resistant (HR) isolates had an EC₅₀ value higher than 100 µg/ml.

Determination of sensitivity to carboxin was carried out on *A. alternata* isolates that were either identified as boscalid-resistant or -sensitive. Measurements of sensitivity, in terms of EC₅₀ values, were based on inhibition of mycelial growth. For this purpose, autoclaved PDA was amended with 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 300, and 500 µg/ml a.i. carboxin. The sensitivity determination procedure was similar to that previously described for boscalid.

DNA manipulations. Genomic DNA was extracted from mycelium of *A. alternata* using the FastDNA kit (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions.

Primer set 1 (Table 2) was designed by aligning conserved sequences spanning the cysteine-rich clusters associated with the S1 and S3 iron-sulphur redox centers of the succinate dehydrogenase gene of *A. brassicicola* and *Stagonospora nodorum* (syn. *Phaeosphaeria nodorum*) (accession number SNOG_03351). This primer set was used to amplify the putative *SdhB* gene fragment from *A. alternata* genomic DNA from the boscalid-sensitive strain AaY16. PCR reactions were performed in a 50-µl volume containing 50 ng of DNA, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM of each dNTP (Promega, Madison, WI), 0.2 µM of each primer, and 1 unit of Pfu DNA polymerase (Stratagene, La Jolla, CA). PCR was carried out in a Mastercycler (Eppendorf, Hamburg) with an initial pre-heat for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 50 s, extension at 72°C for 1 min, and terminated with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 1.2% agarose gel in Tris-acetate (TAE) buffer. The PCR products from each isolate were purified from gel using the GeneClean Kit (MP Biomedicals, LLC., Solon, OH), cloned into a pGEM-T Easy vector (Promega, Madison, WI) and subsequently sequenced. The 5' and 3' regions of *A. alternata Sdh B* gene (*AaSdhB*) were obtained by PCR amplification using two sets of primer (Sets 2 and 3) designed from both the obtained fragment and conserved sequence of corresponding regions in *A. brassicicola* homologous gene (Table 2). PCR products were cloned and sequenced as described above.

The WoLF PSORT prediction program was used to predict the cellular localization of AaSdhB.

Allele-specific PCR (AS-PCR) and PCR-restriction fragment length polymorphism (RFLP) assays. In this study, the sequence polymorphism analysis of the *AaSdhB* gene revealed that some *A. alternata* boscalid resistant isolates had a single point mutation from C (CAC in sensitive isolates) to T (TAC in resistant isolates) causing a change of histidine to a tyrosine at codon position 277 (H277Y) of the AaSdhB. Based on this point mutation, a primer pair ADF1/ADR2 (primer set 5; Table 2) was designed to specifically prime the *AaSdhB* gene of resistant isolates having this mutation (type I mutants). The reverse primer

ADR2 was designed to match the putative point mutation T277 at the 3' end of the primer. A base mismatch located at the fourth base position from the 3' end was incorporated into the primer to enhance the specificity of the amplification (18,24). The primer pair was expected to generate a 250-bp PCR product from resistant isolates only. DNA extracted from the 10 type I mutants, and 3 boscalid-sensitive and 3 boscalid-resistant isolates, without the C to T mutation were used in the AS-PCR assay. PCR amplifications were performed in a 50- μ l volume containing 2 μ l of isolated genomic DNA, 0.2 μ M of each primer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 1 $\times$ Promega (Madison, WI) Taq polymerase buffer, and 2 U of Promega Taq polymerase. The PCR amplifications were performed using the following parameters: an initial preheat for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 65.5°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The amplicons of each isolate from the AS-PCR assay were analyzed by 1.5% agarose gels in 1 $\times$ Tris-acetate (TAE) buffer.

In the current study, an A-to-G mutation occurred at nucleotide 977 in the *AaSdhB* sequence in several other *A. alternata* mutants (type II mutants) creating an *AcI* restriction enzyme site. Primer, set 6 (Table 2), was designed to amplify mutation-containing frag-

ments that were subsequently digested with the enzyme *AcI* (New England BioLabs Inc., Beverly, MA) according to the manufacturer's instructions. Digestion products were then separated on a 2% agarose gel in 1 $\times$ TAE buffer.

RESULTS

Sensitivity of *A. alternata* isolates to boscalid and cross resistance to carboxin. Among the 46 isolates tested, 38 were highly resistant to boscalid with EC₅₀ values higher than 500 μ g/ml. Only eight isolates were found to be sensitive to boscalid with EC₅₀ values between 0.13 and 0.56 μ g/ml (Table 1). Notably, the sensitive isolates were able to grow slightly at some higher concentrations of boscalid but growth remained strongly inhibited compared to that observed for the resistant isolates at the same conditions.

All the 38 boscalid-resistant and the 8 boscalid-sensitive isolates detected were examined for cross-resistance to the carboxamide fungicide carboxin. The corresponding EC₅₀ values for carboxin of these isolates are shown in Table 1. The mean EC₅₀ values to carboxin for all wild-type boscalid-sensitive isolates was 15.53 μ g/ml ($\pm$ 2.80) and was higher than that obtained for the

TABLE 1. Characteristics and sensitivity to boscalid and carboxin of the *Alternaria alternata* isolates from pistachio orchards used in this study

Isolate	Location	Year of isolation	Number of years of boscalid exposure	EC ₅₀ boscalid (μ g/ml) ^a	EC ₅₀ carboxin (μ g/ml)
AaY16	Kern County, CA	2005	2	0.28	13.21
Aa4	Kern County, CA	2005	2	0.26	11.27
Aa127	Kern County, CA	2005	2	0.56	18.49
Aa20	Kern County, CA	2005	2	>500	77.37
Aa22	Kern County, CA	2005	2	>500	105.81
Aa29	Kern County, CA	2005	2	>500	54.41
Aa65	Kern County, CA	2005	2	>500	51.23
Aa111	Kern County, CA	2005	2	>500	43.58
Aa122	Kern County, CA	2005	2	>500	65.79
AaY1	Kern County, CA	2005	2	>500	38.06
Aa4y	Kern County, CA	2006	3	0.22	13.19
Aa35	Kern County, CA	2006	3	0.13	18.40
Aa6	Kern County, CA	2006	3	0.28	18.67
Aa61	Kern County, CA	2006	3	0.45	15.67
Aa62	Kern County, CA	2006	3	0.37	15.08
Aa2	Kern County, CA	2006	3	>500	55.67
Aa26	Kern County, CA	2006	3	>500	46.64
Aa51	Kern County, CA	2006	3	>500	74.67
Aa44	Kern County, CA	2006	3	>500	51.51
Aa41	Kern County, CA	2006	3	>500	54.03
Aa13	Kern County, CA	2006	3	>500	46.41
Aa48	Kern County, CA	2006	3	>500	109.10
Aa3	Kern County, CA	2006	3	>500	57.32
Aa68	Kern County, CA	2006	3	>500	57.82
Aa40	Kern County, CA	2006	3	>500	64.80
Aa10	Kern County, CA	2006	3	>500	98.53
Aa32	Kern County, CA	2006	3	>500	43.73
Aa54	Kern County, CA	2006	3	>500	68.71
Aa19	Kern County, CA	2006	3	>500	80.92
Aa94	Kern County, CA	2007	4	>500	62.17
Aa100	Kern County, CA	2007	4	>500	53.18
Aa105	Kern County, CA	2007	4	>500	63.67
Aa102	Kern County, CA	2007	4	>500	28.95
Aa103	Kern County, CA	2007	4	>500	41.97
Aa37	Kern County, CA	2006	3	>500	45.38
Aa45	Kern County, CA	2006	3	>500	53.51
Aa33	Kern County, CA	2006	3	>500	71.31
Aa58	Kern County, CA	2006	3	>500	80.29
Aa65n	Nichols, Tulare County, CA	2007	4	>500	34.52
Aa67n	Nichols, Tulare County, CA	2007	4	>500	57.68
Aa72n	Nichols, Tulare County, CA	2007	4	>500	99.81
Aa48o	Orandi, Tulare County, CA	2007	4	>500	40.44
Aa26o	Orandi, Tulare County, CA	2007	4	>500	41.17
Aa19o	Orandi, Tulare County, CA	2007	4	>500	52.02
Aa8o	Orandi, Tulare County, CA	2007	4	>500	136.26
Aa29ob	Orandi, Tulare County, CA	2007	4	>500	98.83

^a EC₅₀ values for each isolate are the mean from two experiments since the results did not differ statistically ($P > 0.05$) according to Fisher's least significant difference test.

same isolates in the presence of boscalid for which the mean EC₅₀ had a value of 0.32 ± 0.14 µg/ml. For all the boscalid-resistant mutants, the mean EC₅₀ value for carboxin was 63.35 (±23.70) µg/ml and was much lower compared to the mean EC₅₀ value (mean EC₅₀ > 500 µg/ml) obtained in the presence of boscalid. Furthermore, it could be noted that at higher concentrations of carboxin, the boscalid resistant-isolates were strongly inhibited. For instance, at 500 µg/ml of carboxin, the percentage of inhibition ranged from 75.00 to 90.74% (mean = 85.68%), whereas at the same concentration of boscalid, the same isolates were weakly inhibited (mean percentage of inhibition value = 17.94%).

Cloning of a succinate dehydrogenase iron sulfur gene from *A. alternata*. Primer pair, set 1, designed on the basis of fungal SdhB Ip from *A. brassicicola* and *S. nodorum* (Syn *Phaeosphaeria nodorum*) was successfully used to amplify a 602-bp fragment from the genomic DNA of *A. alternata* AaY16 a boscalid-sensitive strain. This fragment was cloned and sequenced. The resulting sequence was used to search the GenBank non-redundant database using the BLASTX algorithm. Significant matches were obtained with SdhB Ip orthologues from several filamentous fungi. This fragment contains the three conserved cysteine-rich clusters associated with the iron-sulphur centers S1, S2, and S3. The complete coding sequence (accession no. EU178851) was obtained by PCR amplification using primer set 2 and 3 designed both on the basis of the obtained DNA fragment and *A. brassicicola* SdhB DNA sequence.

The assembled DNA sequence contains an open reading frame (ORF) of 306 amino acids interrupted by three putative introns. *A. alternata* SdhB (AaSdhB) was highly similar to the well-characterized mitochondrial iron sulfur proteins of succinate dehydrogenase of *Saccharomyces cerevisiae* (72% identity), *Mycosphaerella graminicola* (82%), and *Ustilago maydis* (63%).

The three Cys-rich clusters of AaSdhB responsible for ligating the iron-sulphur centers (S1, S2, and S3) are highly conserved. These clusters were identified at amino-acid positions 125 to 144, 217 to 229, and 274 to 286, respectively (Fig. 1). Subcellular localization prediction using the WoLF PSORT program indicates that AaSdhB contains an N-terminal mitochondrial targeting sequence, which is in accordance with the function of this protein.

Sequence comparison of AaSdhB gene between boscalid-sensitive and boscalid-resistant *A. alternata* isolates. The complete nucleotide sequence of the AaSdhB gene was determined for seven boscalid-resistant isolates (Aa20, Aa22, Aa29, Aa111, AaY1, Aa122, and Aa65) and three boscalid-sensitive isolates (Aa4, Aa127, and AaY16). The Genbank accessions numbers of these sequences are EU178851 to EU178857. Comparison of the DNA sequences of sensitive and resistant isolates revealed that the C at position 996 in the boscalid-resistant isolates Aa20, Aa22, Aa29, and Aa111 was replaced by T resulting in H277Y substitution in the third iron-sulphur center S3. The nucleotide sequences of AaSdhB of the boscalid-resistant isolates (AaY1, Aa122, and Aa65) were identical to those of the wild-type sensitive isolates (Table 3).

To further clarify the relationship between the genotype (presence of the H277Y mutation) and the phenotype (resistance to boscalid) this region was sequenced for the remaining boscalid-resistant isolates identified in this study. A primer set 4 was designed to amplify the region containing the mutation from genomic DNA. PCR amplifications were carried out with high-fidelity *Pfu* DNA polymerase and individual 200-bp PCR prod-

TABLE 2. Primer sets used for amplification of the AaSdhB gene from *Alternaria alternata* genomic DNA

Primer set	5' primer	3' primer	Amplified DNA fragment ^a
1	TACGAGCTCGACCTCAACAAGAC	CTCGGCAACGCGGGGTTTCAGTC	+341 to +1049
2	GTGGCGTCGAAGGGCCGAAGAAGCCG	CAGCATCATGGGTCCGGTCTTGTGA	-33 to +349
3	TACTGGTGAACAGGAGGAGTA	CATACCACTCTAGGTGAAG	+856 to +1130
4	CGACGGACTCTACGAATGC	GCATGTCCTTGAGCAGTTGAG	+801 to +1020
5	AAGCTCGACGGACTCTACGAATGC	CTTGAGCAGTTGAGAATGGAGTA	+796 to +1013
6	AAGGAAGATCGCAAGAAGCTC	AATGGCTAGCGCAGGGTTCA	+781 to +1050
7	AAGCTCGACGGACTCTACGAATGC	CTTGAGCAGTTGAGAATGGAGTG	+796 to +1013

^a Fragment numbering corresponds to nucleotide positions in the AaSdhB gene, with the first nucleotide of the start codon considered as position +1.

Fig. 1. Comparison of the amino acid region, containing the three cysteine-rich clusters (shaded regions) of AaSdhB of *Alternaria alternata* with similar regions of SdhB subunits from other organisms. The highly conserved histidine residues within the third cysteine cluster are in bold type. Aa, *A. alternata*; Ab, *Alternaria brassicicola*; Sn, *Stagonopora nodorum* (Syn. *Phaeosphaeria nodorum*); Sc, *Saccharomyces cerevisiae*; and Um, *Ustilago maydis*.

ucts were purified and sequenced directly. Comparison of the partial sequences from amplified products of the *AaSdhB* alleles from boscalid-resistant isolates with that of the wild-type sensitive isolates revealed the presence of the mutation H277Y in six resistant isolates (Aa45, Aa65n, Aa26o, Aa48o, Aa67n, and Aa72n). In five other isolates (Aa33, Aa58, Aa19o, Aa8o, and Aa29bo) an A-to-G mutation occurred at position 997 affecting the conserved histidine residue and leading to an H277R substitution (Table 3). Among the strains analyzed, 23 fungicide-resistant isolates had no mutation in this region of *AaSdhB*, or in the remaining portion of the ORF (data not shown). The boscalid-resistant isolates described in this study were divided into three groups based on their sensitivity to boscalid and the presence/absence of mutation in *AaSdhB* (Table 3).

Molecular diagnosis of boscalid resistant isolates mutated in *AaSdhB*. At a high annealing temperature (65.5°C), the allele-specific primer pair ADF1 + ADR2 (primer set 5) amplified the expected 250-bp DNA fragment from the DNA extracted from the 10 boscalid-resistant type I mutants (Aa20, Aa29, Aa22, Aa45, Aa111, Aa65n, Aa48o, Aa67n, Aa26o, and Aa72n), but not from any of the eight sensitive *A. alternata* isolates and those of type II and III resistant mutants (Fig. 2). This process indicated that the allele-specific PCR primer pair ADF1 + ADR2 was specific only to boscalid-resistant type I mutants. A second pair of primers (primer set 7), with ADS R2 having its last base at the 3'-end matching the wild type nucleotide on which the putative point mutation was located in type I mutants and a base mismatch at the fourth base from the 3' end, was designed and successfully used to detect both the sensitive wild-type and the nontype I mutants alleles (data not shown).

Primer set 6, amplified the expected 300-bp DNA fragment for all boscalid-resistant and -sensitive isolates tested. The PCR products were then treated using a mutation specific enzyme *Acil*. The PCR products from type II resistant mutants carrying the mutated sequence CGC at position 997 were digested into 210- and 90-bp fragments, whereas products from sensitive isolates and other mutants remained undigested (Fig. 3).

DISCUSSION

The development of resistance to boscalid has been reported only in a small number of fungi, *A. alternata* from pistachio and *Botrytis cinerea* from grapes and strawberries (28). In contrast, resistance to structurally related chemicals, such as carboxin, is a pervasive phenomenon (3,10,13,15,22,26,30). As expected for compounds of the same chemical class, positive cross-resistance has been demonstrated in this group for flutolanil and carboxin (13). In the current study, field isolates of *A. alternata* were tested

for sensitivity to boscalid. Slight growth of wild type isolates was observed at high doses of boscalid. This could be attributed to the rich PDA medium used in the assay. It was previously shown that complete growth inhibition of sensitive isolates is sometimes not possible in boscalid petri dish assays using rich media (20,27,29). Despite this, the *Alternaria* isolates in this study were easily and clearly distinguished as sensitive and highly resistant to boscalid.

In this study, the response of *A. alternata* boscalid-resistant and -sensitive isolates in the presence of carboxin was also examined. Results showed that wild-type isolates were weakly affected by

Fig. 2. Specificity of allele-specific polymerase chain reaction (PCR) primer set 3 for detection of boscalid-resistant type I mutants of *Alternaria alternata*. Amplification products were obtained using genomic DNA from mutants type I Aa20, Aa29, Aa111, Aa26o, and Aa65n (lanes 4, 5, 7, 8, and 10, respectively), sensitive isolates Aa4 and Aa127 (lanes 1 and 2, respectively), and mutants type II Aa33 and Aa58 (lanes 3 and 6, respectively) and other mutant Aa65 (lane 9). M lane was loaded with a 100-bp DNA ladder (Invitrogen, Carlsbad, CA).

Fig. 3. Restriction fragment length polymorphism (RFLP) analysis of *Acil*-digested 300-bp polymerase chain reaction (PCR) products corresponding to the portion of the *AaSdhB* gene generated by the primer set 6: Amplification products were obtained using genomic DNA from *Alternaria alternata* mutants type II Aa33 (lane 2) and Aa8o (lane 8), sensitive isolates Aa4 (lane 3) and Aa127 (lane 4), mutants type I Aa20 (lane 5) and Aa29 (lane6), and mutants type III Aa65 (lane 7), and Aa40 (lane 9). M lane was loaded with a 100-bp DNA ladder (Invitrogen, Carlsbad, CA).

TABLE 3. Nucleotide mutations and deduced alterations in the iron sulphur *AaSdhB* from boscalid-resistant field isolates of *Alternaria alternata*

Isolate	Phenotype	Mutation at codon 277	Mutation type	Type of mutant
Aa22	Highly resistant (HR)	TAC	H to Y	I
Aa45	HR	TAC	H to Y	
Aa111	HR	TAC	H to Y	
Aa65n	HR	TAC	H to Y	
Aa48o	HR	TAC	H to Y	
Aa67n	HR	TAC	H to Y	
Aa26o	HR	TAC	H to Y	
Aa72n	HR	TAC	H to Y	
Aa20	HR	TAC	H to Y	
Aa29	HR	TAC	H to Y	
Aa33	HR	CGC	H to R	II
Aa58	HR	CGC	H to R	
Aa19o	HR	CGC	H to R	
Aa8o	HR	CGC	H to R	
Aa29bo	HR	CGC	H to R	
n ^a = 23	HR	none	?	III

^a Among the *A. alternata*-resistant mutants identified 23 had no mutation in the *AaSdhB* sequence.

carboxin alone. This lack of activity against *Alternaria* was not entirely unexpected as previous studies showed that the efficacy of carboxin towards complex II varies between organisms, with Ascomycetes being generally less sensitive to carboxin than Basidiomycetes (23,26). Moreover, the sensitivity of wild-type isolates to carboxin was lower compared to the sensitivity of the same isolates to boscalid. This finding showed that carboxin is less efficient than boscalid in controlling the wild-type sensitive isolates. However, carboxin expressed a strong fungicidal activity at high doses, inhibiting both highly boscalid-resistant and -sensitive isolates. These differences in efficacy between the two fungicides could result from differences in their intrinsic activity. Differences in levels of cross-resistance between fungicides due to differences in intrinsic activity have been reported for compounds that share same modes of action (4,12,14). Even so, data from this study clearly revealed that all carboxin-resistant isolates have a different pattern of boscalid resistance compared to the carboxin-sensitive isolates and displayed relative high levels of cross resistance to boscalid. Thus, even though *A. alternata* appears to have a higher basal resistance towards carboxin, the results clearly showed that the boscalid-resistant mutants exhibit positive cross-resistance to carboxin.

The site of action of both boscalid and carboxin is reportedly in the Sdh complex of the electron transport pathway and in light of the cross resistance results (Table 1) it is tempting to speculate that carboxin and boscalid may share similar resistance mechanisms. In the case of carboxin, biochemical and molecular studies of laboratory isolates showed that resistance is caused by point mutations in target genes that cause target-site modifications (3,10,13,15,22,26). In this study, to elucidate the molecular basis of boscalid-resistant phenotypes in *A. alternata* the iron sulphur subunit of the complex II was targeted for analysis. To initiate this work the gene encoding the *A. alternata* SdhB subunit was sequenced. Comparison of the predicted amino acid sequence of AaSdhB from *A. alternata* with the SdhB from other fungi showed that this region is particularly conserved between fungal proteins. Analysis of the AaSdhB protein structure indicates high conservation of three cysteine-rich clusters associated with iron-sulphur centers involved in electron transport. Sequence comparison of resistant isolates with those of the wild-type isolates showed that a single point mutation existed in fungicide-resistant *A. alternata* isolates. This mutation leads to a substitution of a highly conserved histidine residue, located in a region associated with the (3Fe-4 S) high-potential non-heme iron sulphur-redox (S3) center to either tyrosine (H277Y) or Arg (H277R). This finding is consistent with previously published reports in which a substitution of the conserved histidine residue was clearly demonstrated as the mechanism responsible for carboxin (Cbx) resistance in *U. maydis* (3,26). Structural analysis of the quinone-binding site (Q-site) undertaken on *E. 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 (11). Interestingly, the same study revealed that carboxin docked in the same way as ubiquinone in close proximity to the conserved histidine. These results strongly suggest that the histidine residue, in addition to its crucial role in ubiquinone binding, is essential for proper carboxamide (carboxin and boscalid) binding and thus their inhibitory action. The mutation responsible for the H277Y substitution in the *AaSdhB* gene is identical to that characterized in *M. graminicola* Cbx-resistant strains (26). More recently, Stammler et al. (28) reported that the same mutation was found within the iron sulphur gene homologue of boscalid-resistant field and laboratory mutants of *B. cinerea*. Furthermore, boscalid sensitivity losses were ascribed to this mutation. In the present study, results from sequencing resistant fungal strains were consolidated into PCR-based molecular diagnostic methods. Results indicating boscalid resistance in *A. alternata* strains using these molecular assays were in

general agreement with results obtained from conventional bioassays. Thus, and in the light of studies made in other fungal species, it is inferred that the two mutations H277Y and/or H277R are associated with high levels of resistance to boscalid. The introduction of these mutant alleles in the wild-type strain would constitute an ultimate proof that these mutations are responsible for the observed phenotypes.

In some boscalid-resistant strains there was no variation in the DNA sequence of the *AaSdhB* gene compared to the wild type. The absence of mutations in the *AaSdhB* gene in the remaining strains that showed high level of boscalid resistance raised the possibility that mutation(s) in other loci are involved in the acquisition of the boscalid resistant phenotype. This assertion is not surprising since the mechanism of action of boscalid involves at least two other genes of the Sdh complex. Indeed, previous studies have shown that mutations in *SdhC* or *SdhD* determine resistance to carboxin and flutolanil (13,22). As such, future work should attempt to isolate orthologues of these genes in *A. alternata* in order to further clarify the molecular basis of boscalid resistance. Moreover, another mutation has been detected in the SdhB sequence in *B. cinerea* (28) and it cannot be excluded that this particular genotype might also exist in the *Alternaria* populations.

The purpose of this study was not to dwell on the factors that could influence the development of boscalid resistance in *A. alternata* of pistachio. However, it was noted that even with the limited sampling of this study, there was variation in the frequency of the various resistance mutations. Further studies are needed to understand the behavior of mutants in pathogenicity assays in the presence or absence of a competitive wild-type population and in the presence or absence of fungicide treatments. Significantly, this study has produced molecular diagnostic methods that constitute reliable tests to identify boscalid-resistant isolates of *Alternaria* from pistachio and almond orchards. However, as mentioned above, more mutations than those characterized in this study are likely to contribute to boscalid resistance outbreaks. The characterization of *A. alternata* *SdhC* and *SdhD* genes is underway and will provide more robust molecular screening assays. This will ultimately lead to the development of efficient and sensitive molecular typing methods for the full spectrum of mutations causing boscalid resistance in *A. alternata* and will facilitate studies monitoring the relative frequencies of resistance phenotypes. These studies will provide information that is vital in the design of resistance management strategies.

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