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Research Paper

Molecular diversity of *Alternaria* spp. from leafy vegetable crops, and their sensitivity to azoxystrobin and boscalid

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Summary. Severe outbreaks of Alternaria leaf spot have occurred in Northern Italy on leafy vegetable and ornamental hosts. This disease is mainly controlled by two classes of respiration inhibitor fungicides, QoIs (including azoxystrobin) and SDHIs (including boscalid). Thirty-six *Alternaria* strains were isolated from five leafy vegetable crops, and subjected to molecular characterization. Multilocus phylogenetic analyses assigned most of the strains (86%) to *A. alternata*, while the rest were *A. arborescens* and other *Alternaria* spp. *In vitro* sensitivity assays showed that 3% of the strains were of intermediate resistance, and 11% of reduced sensitivity to azoxystrobin, while 8% of the strains were resistant to boscalid. Sequencing of cytochrome b in an intermediately resistant strain of *Alternaria* revealed the G143A mutation. This strain was also resistant to boscalid. None of the tested *Alternaria* strains had amino acid mutations associated with boscalid resistance coded by the *SdhB* and *SdhC* genes. This is the first report of azoxystrobin resistance in *A. alternata* in Italy, as well as the first record of resistance of *Alternaria* spp. found on leafy vegetables. As boscalid resistance was not associated with any frequently reported mutations, further investigations of the additional resistance mechanisms are necessary. These results demonstrate the need for well-organized chemical control of emerging Alternaria diseases, to prevent the increase of resistance to QoI and SDHI fungicide classes, and the possibility of double fungicide resistance in these pathogens.

Keywords. Alternaria leaf spot, fungicide resistance, QoIs, SDHIs, molecular characterization.

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INTRODUCTION

Alternaria species are becoming emerging threats in vegetable growing areas in Europe, as the consequence of the globalization of the trade of seeds and plants, climate changes and intensification of cultivation (Gilardi

et al., 2018). Alternaria leaf spot on leafy vegetables is caused by *Alternaria alternata* (Fr.: Fr.) Keissl. and other *Alternaria* spp., including *A. japonica* and *A. arborescens* (Gullino *et al*., 2014; Subbarao *et al*., 2017; Gilardi *et al*., 2018). Symptoms first appear as small leaf spots which expand to brown-black lesions encircled by yellow haloes on aging leaves. Progressive plant defoliation occurs at later stages with the occasional death of the plants. These pathogens overwinter on infected crop residues, seeds and weeds. Conidia are airborne and can be dispersed over great distances throughout the growing season. Transmission by seeds facilitates wide pathogen dissemination (Simmons *et al*., 2007), and this distribution occurs with different leafy vegetables (Gullino *et al*., 2014).

The presence of *Alternaria* spp. on new vegetable hosts has recently been reported in different countries, including Italy, Greece, Poland, South Africa, Algeria, Pakistan, China, and the United States of America (Farr and Rossman, 2019). Outbreaks of Alternaria leaf spot have increased in Italy, and they are mainly caused by small-conidium *Alternaria* spp. including *A. alternata* and *A. arborescens*. These species have been recorded for the first time on a few vegetable crops in Italy; *A. alternata* has been reported on sweet basil, cultivated and wild rocket, pepper, chili pepper, cabbage and spinach, and *A. arborescens* on sweet basil (Garibaldi *et al*., 2011; Gullino *et al.,* 2014; Woudenberg *et al*., 2015; Garibaldi *et al*., 2019a; Gilardi *et al*., 2019). In addition, *A. alternata* has been reported on ornamental hosts, including purple coneflower, pineapple sage, fruit-scented sage, peppermint, *Digitalis purpurea* and *Ceratostigma willmottianum* (Garibaldi *et al*., 2018a, 2018b, 2018c, 2019b, 2019c, 2019d).

Alternaria spp. are mostly controlled using fungicides. The fungicides registered in the European Union against *Alternaria* spp. are: 1) copper-based fungicides, 2) dithiocarbamates, 3) dicarboximides, 4) phenylpyrroles, 5) quinone outside inhibitors (QoIs), 6) succinate dehydrogenase inhibitors (SDHIs), 7) methyl benzimidazole carbamates, and 8) demethylation inhibitors (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN). The main fungicide groups used for management of Alternaria leaf spot are respiration inhibitors; QoIs (including azoxystrobin, pyraclostrobin and fluoxastrobin) and SDHIs (boscalid and fluopyram). Azoxystrobin and boscalid are widely used on leafy vegetables against a number of soil-borne and foliar pathogens, due to their broad activity spectra (Margot *et al*., 1998; Matheron and Porchas, 2004). Careful monitoring is important to determine change in sensitivity of *Alternaria* spp. to these fungicides. In Italy and other countries, the mixture pyraclostrobin + boscalid is frequently applied against different fungal diseases (e.g. *Botrytis cinerea* and *Sclerotinia sclerotiorum*).

The QoIs, which have a common single-site mode of action, inhibit mitochondrial respiration at the outer, quinone oxidizing pocket (Qo site) within the cytochrome bc1 enzyme complex. This causes impairment of the electron transfer chain, resulting in energy deficit and insufficient ATP production (Becker *et al*., 1981). The cytochrome b (*cyt b*) gene, one of the coding genes of the enzyme complex, is related to QoI resistance, which appeared soon after QoIs were introduced into plant protection markets. QoI resistance was first reported in *Blumeria graminis* f. sp. *tritici* and then on many plant pathogenic oomycetes and fungi, including *A. alternata* (Sierotzki *et al*., 2000; Ma *et al*., 2003). The cyt b amino acid substitution from glycine to alanine at position 143 (G143A) was mainly reported in QoI-resistant *A. alternata* (Ma *et al*., 2003), while *A. solani* showed other cyt b mutations (phenylalanine with leucine at position 129; F129L mutation) (Pasche *et al*., 2005).

SDHIs are another group of respiration inhibitors that act on the mitochondrial succinate dehydrogenase (Sdh) complex. The Sdh complex contains four subunits: flavoprotein (FP or SdhA), ironesulfur protein (IP or SdhB) and two integral membrane-anchor proteins (SdhC and SdhD) (Hägerhäll, 1997). Reduced sensitivity to SDHI fungicides has been related to several point mutations in four subunits of the Sdh complex. Boscalid resistance was reported for the first time in *A. alternata* in pistachio under field and laboratory conditions, followed by *A. solani* in potato and *A. alternata* in tomato (Avenot and Michailides, 2007; Wharton *et al*., 2012; Malandrakis *et al*., 2018). SdhB amino acid substitutions from histidine (H) to tyrosine (Y) or to arginine (R) at position 277 aa (H277Y), (H277R) in *A. alternata*, and the same substitution at amino acid position 278 (H278Y), (H278R) in *A. solani,* have been found in strains showing SDHI resistance (Avenot *et al*., 2008a; Mallik *et al*., 2014). SDHI resistance has also been associated with mutations in two other SDH subunits; H134R in SdhC, and H133R and D123E in SdhD (Avenot *et al*., 2009; Mallik *et al*., 2014).

The objective of the present study was to characterize *Alternaria* isolates from leaf spot-affected vegetable crops using molecular techniques, and to investigate their sensitivity to azoxystrobin and boscalid. For this purpose, 36 *Alternaria* isolates, obtained from symptomatic plants, were subjected to molecular characterization by four-loci phylogenetic analyses. The sensitivity of the identified *Alternaria* strains to azoxystrobin and

boscalid was also assessed using *in vitro* tests, and presence of the fungicide-associated mutations was investigated by characterizing the *cyt b* gene (related to azoxystrobin resistance) and the *SdhB* and S*dhC* genes (associated with boscalid resistance).

MATERIALS AND METHODS

Isolate collection

Thirty-six isolates were collected during 2013–2017 from leaf spot-affected tissues of cabbage, cauliflower, cultivated rocket, wild rocket and basil plants, grown in soil-less or conventional systems in Northern Italy (Table 1). On the basis of conidium observations, all the isolates belonged to the small-conidium *Alternaria* spp. Four additional strains, EGS 34015 (CBS 918.96), EGS 34016 (CBS 916.96) (E.G. Simmons, Mycological Services), CBS 124274, and CBS 124278 (CBS-KNAW Collection), were used as reference strains for *A. alternata* or *A. arborescens* (Table 1).

DNA extraction and PCR

The DNA of the 36 isolates was isolated using an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions, from 100 mg of mycelium grown on potato dextrose agar (PDA, Merck®) plates. Molecular identification was performed through amplification of the internal transcribed spacer (ITS; White *et al*., 1990) using primer ITS1/ITS4, endopolygalacturonase (*endoPG*; Andrew *et al*., 2009) using primer PG3/PG2b, β-tubulin (*tub2*; O' Donnell and Cigelnik 1997; Peever *et al*., 2004) using primer T2/β-tub2, and histone 3 (*H3*; Glass and Donaldson 1995) using primer H31a/H31b. The PCR products were purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and sequenced in both directions at the BMR Genomics Centre (Padua, Italy). Only the sequences of the studied and reference isolates with good quality scores (Phred scores greater than 30) were selected (Ewing *et al.,* 1998). These sequences were used for a successive contig assembly and sequence analyses. The sequences were deposited in GenBank under Accession Numbers: ITS (MH936379-MH936414), *endoPG* (MK140907-MK140935), *tub2* (MK044808-MK044820) and *H3* (MK239196-MK239231) (Table 1), with the exception of some isolates which had previously been sequenced in the *tub2* and *endoPG* region (Siciliano *et al*., 2017; 2018). The accession numbers of the reference isolates are also included in Table 1.

Sequence analyses

A sequence comparison with reference isolates of *Alternaria* spp. (Woudenberg *et al*., 2015; Siciliano *et al.,* 2018) available in the GenBank database was performed using the BLAST software package (www.ncbi.nlm.nih.gov). Phylogenetic analyses were based on Maximum Likelihood (ML) and Bayesian inference (BI). MEGA 7 software was used (Kumar *et al*., 2016) for the Maximum Likelihood analysis. A total of 1831 bp concatenated data sets were obtained with the ITS, *tub2*, *endoPG* and *H3* sequences. Findmodel was used to select the best-fit nucleotide model of each region (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) as follows: K80: Kimura 2-parameter for ITS, TrN: Tamura-Nei for *tub2*; GTR: General Time Reversible for *endoPG*, and TrN plus Gamma for *H3* and the concatenated tree. Maximum-likelihood trees were then constructed with bootstrap values obtained from 1,000 replications. The best-fit model of each dataset was determined for a Bayesian analysis (Huelsenbeck and Ronquist, 2001) using TOPALI v.2.5 (Milne *et al*., 2004): JC: Jukes and Cantor (ITS and *endoPG*), TrN plus Gamma (*tub2*), HKY85+I; Hasegawa, Kishino, and Yano, 1985 plus invariable sites (*H3*), and K81+I+G: Kimura, 1981 plus invariable sites plus Gamma (concatenated tree). The Bayesian analysis was performed discarding the first 25% of the sampled trees as burn-in phases, and the successive probabilities were estimated from the remaining trees (Ronquist *et al*., 2009).

In vitro *sensitivity testing of* Alternaria *spp. to azoxystrobin or boscalid*

The fungicides azoxystrobin (Ortiva®, 250 g L^{-1} a.i., Syngenta Italia S.p.A.) and boscalid (Cantus, 50% a.i., BASF Italia S.p.A.), each at concentrations of 0.1, 0.3, 1, 3, 10, 30, 100 or 300 mg L^{-1} of active ingredient, were used in Petri plate sensitivity assays. Salicylhydroxamic acid (SHAM, Sigma-Aldrich) was added to the medium at a final concentration of 100 mgL^{-1}, to prevent fungi from starting an alternative respiration process and to suppress resistance due to alternative oxidase (Kim *et al*., 2003; Pasce *et al*., 2004).

The effects of azoxystrobin and boscalid on the spore germination of different *Alternaria* isolates were evaluated on selective medium of corn meal agar (CMA, Sigma-Aldrich, 17 g L^{-1}) amended with streptomycin sulphate at 0.025 mg L^{-1} (AppliChem). Petri dishes containing CMA amended with antibiotic without fungicide, with or without SHAM, were used as experimental controls. Assay inoculum consisted of conidia gently scraped from a culture of each isolate grown on V8 medium

Table 1. *Alternaria* spp. isolates characterized by means of four molecular loci.

No.	Isolate	Host	Source	Location	ITS	*tub2*	*H3*	*endoPG*	Species
1	Cav 2/10	Cauliflower	Leaf	Italy	MH936379	*KT920427*	MK239196	MK140907	*A. alternata*
2	Cav 3/10	Cabbage	Leaf	Italy	MH936380	*KT920426*	MK239197	MK140908	*A. alternata*
3	Cav 4/10	Cauliflower	Leaf	Italy	MH936381	MK044808	MK239198	MK140909	*A. alternata*
4	Cav 5/10	Cabbage	Leaf	Italy	MH936382	*KT920423*	MK239199	MK140910	*A. alternata*
5	Cav 6/10	Cabbage	Leaf	Italy	MH936383	MK044809	MK239200	MK140911	*A. alternata*
6	Cav 7/10	Cabbage	Leaf	Italy	MH936384	*KT920425*	MK239201	MK140912	*A. alternata*
7	Cav 9/10	Cauliflower	Leaf	Italy	MH936385	MK044810	MK239202	MK140913	*A. alternata*
8	Cav 12/10	Cauliflower	Leaf	Italy	MH936386	*KT920424*	MK239203	MK140914	*A. alternata*
9	Cav 15/10	Cabbage	Leaf	Italy	MH936387	*KT920428*	MK239204	MK140915	*A. alternata*
10	Ruc 1/10	Cultivated rocket	Leaf	Italy	MH936388	*KJ909926*	MK239205	MK140916	*A. alternata*
11	Ruc 3/10	Wild Rocket	Leaf	Italy	MH936389	MK044811	MK239206	MK140917	*A. alternata*
12	Ruc 4/10	Wild Rocket	Leaf	Italy	MH936390	*KT920413*	MK239207	MK140918	*A. alternata*
13	Ruc 5/10	Wild Rocket	Leaf	Italy	MH936391	*KT920412*	MK239208	MK140919	*A. alternata*
14	Ruc 7/10	Wild Rocket	Leaf	Italy	MH936392	MK044812	MK239209	MK140920	*Alternaria* sp.
15	Ruc 8/10	Cultivated rocket	Leaf	Italy	MH936393	MK044813	MK239210	MK140921	*A. alternata*
16	Ruc 9/10	Cultivated rocket	Leaf	Italy	MH936394	*KT920411*	MK239211	MK140922	*A. alternata*
17	Ruc 10/10	Cultivated rocket	Leaf	Italy	MH936395	MK044814	MK239212	MK140923	*A. alternata*
18	Ruc 12/10	Cultivated rocket	Leaf	Italy	MH936396	*KT920417*	MK239213	MK140924	*A. alternata*
19	Ruc 13/10	Cultivated rocket	Leaf	Italy	MH936397	*KT920416*	MK239214	MK140925	*A. alternata*
20	Ruc PMP 4	Cultivated rocket	Seed	Italy	MH936399	KT920419	MK239216	MK140927	*Alternaria* sp.
21	Ruc PMP 8	Cultivated rocket	Seed	Italy	MH936398	KT920420	MK239215	MK140926	A. alternata
22	Ruc PMP 9	Cultivated rocket	Seed	Italy	MH936400	KT920418	MK239217	MK140928	*A. alternata*
23	Ruc PMP 12	Cultivated rocket	Seed	Italy	MH936401	KT920422	MK239218	MK140929	*A. alternata*
24	Ruc PMP 19	Cultivated rocket	Seed	Italy	MH936402	*KT920421*	MK239219	MK140930	*A. alternata*
25	Bas 1/10	Basil	Leaf	Italy	MH936403	*MF070269*	MK239220	*MF070304*	*A. alternata*
26	Bas 2/10	Basil	Leaf	Italy	MH936404	*MF070270*	MK239221	*MF070305*	*A. alternata*
27	Bas 4/10	Basil	Leaf	Italy	MH936405	MK044815	MK239222	MK140931	*A. alternata*
28	Bas 5/10	Basil	Leaf	Italy	MH936406	MK044816	MK239223	MK140932	*A. alternata*
29	Bas 6/10	Basil	Leaf	Italy	MH936407	*MF070271*	MK239224	*MF070306*	*A. alternata*
30	Bas G1	Basil	Seed	Italy	MH936408	*MF070272*	MK239225	*MF070307*	*A. arborescens*
31	Bas BIO 10	Basil	Seed	Italy	MH936409	MK044817	MK239226	MK140933	*Alternaria* sp.
32	Bas BIO 11	Basil	Seed	Italy	MH936410	MK044818	MK239227	MK140934	*Alternaria* sp.
33	Bas 4-1BA	Basil	Seed	Italy	MH936411	MK044819	MK239228	*MF070295*	*A. alternata*
34	Bas 18-1BA	Basil	Seed	Italy	MH936412	MK044820	MK239229	MK140935	*A. alternata*
35	Bas 23-1BA	Basil	Seed	Italy	MH936413	*MF070261*	MK239230	*MF070294*	*A. alternata*
36	Bas 27-1BA	Basil	Seed	Italy	MH936414	*MF070259*	MK239231	*MF070292*	*A. alternata*
37	EGS 34015	*Dianthus* sp.	-	The UK	*AF347032*	*MF070252*	-	*KP124026*	*A. alternata*
38	EGS 34016	Peanut	-	India	*AF347031*	*MF070244*	-	*JQ811978*	*A. alternata*
39	CBS124274	Cherry	Fruit	Denmark	*KP124413*	*MF070253*	-	*MF070287*	*A. arborescens*
40	CBS124278	Cherry	Fruit	Denmark	*KP124374*	*MF070256*	-	*MF070290*	*A. alternata*

GenBank accession numbers obtained from Woudenberg *et al.* (2015) and Siciliano *et al.* (2018) are shown in italics.

(100 mL Campbell's V8 juice, 1.5 g $CaCO_3$, 15 g of agar, 900 mL distilled water) using a sterile scalpel. Conidia were mixed into 4 mL of sterile distilled water containing 0.1% Tween-20 (VWR International), and adjusted to 10^4 conidia mL^{-1}. The conidium suspension of each isolate (100 µL) was spread on each fungicide-amended plate. The plates were then placed in the dark at 22±1°C for 4 to 6 h, and the germination of 100 conidia in each plate was assessed under a microscope. Germination of each conidium was defined as the presence of a germ tube at least half the length of the conidium. Plates were arranged in a completely randomized design with three treatment replicates per trial. The experiments were performed three times per each isolate.

Conidium germination for each fungicide concentration (Gf) was compared with the germination for the controls (Gc). The percent of germination inhibition (GI) was calculated as: % GI = (Gc – Gf / Gc) × 100. EC_{50} values (concentrations giving 50% inhibition) were calculated using the log/logit dose response relation of the GraphPadPrism® software (version 7.02; La Jolla, CA, USA). A log fungicide concentration versus normalized response-variable method was calculated as: Y = Bottom + (Top-Bottom) / {1 + 10 [($LogEC_{50}$-X) × HillSlope]}, where Y refers to the response (GI) and X indicates the fungicide concentration.

The *A. alternata* isolates were divided into four groups according to their sensitivity to azoxystrobin or boscalid. An isolate was considered sensitive (S) if EC_{50} was between 0 and 1 μg mL^{-1}, with reduced sensitivity (RS) if EC_{50} was between 1 and 15 μg mL^{-1}, intermediate-resistant (IR) for EC_{50} of between 15 and 100 μg mL^{-1}, and resistant (R) for EC_{50} > 100 μg mL^{-1} (Avenot *et al*., 2008b).

Cross-resistance relationships between the two classes of respiration inhibitor fungicides were assessed by regression analysis (regression coefficient r^2), where log EC_{50} values of the individual isolates were compared for boscalid × azoxystrobin pairs.

Molecular characterization of the cytochrome b gene

The portion of the *cytb* gene of eighteen *Alternaria* strains was amplified with the cytb2f (5'-CTA TGG ATC TTA CAG AGC AC-3') and DTRcytb2-INTr (5'-GTA TGT AAC CGT CTC CGT C-3') primers (Vega *et al*., 2012). The PCR cycling conditions included an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were purified and sequenced as described above. Using the distance-based matrix of the *cytb* gene, a Principal Coordinate Analysis (PCoA) was carried out by GenAlEx 6.502 software (Peakall and Smouse, 2012) to analyze the genetic structure among *Alternaria* subpopulations (sensitive or resistant to azoxystrobin).

Molecular characterization of the SdhB *and* SdhC *genes*

Amplification of the *SdhB* gene was performed with the SdhBF6 (5'-AAGGAAGATCGCAAGAAGCTC-3') and SdhBR6 (5'-AAT GGC TAG CGC AGG GTT CA-3') (Avenot *et al*., 2008a) primers, and the *SdhC* gene was amplified with the SdhC-(A-G)F1 (5'-CAC CTG GCC ATC TAC AAG C-3') and SdhC-(A-G)R1 (5'-TGG TTC TTG AAA CCA ATA CCG-3') primers (Avenot *et al*., 2009). The PCR conditions for both genes were as follows: an initial denaturing step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 50 s, an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were purified and sequenced as described above. A Principal Coordinate Analysis (PCoA) based on the concatenated *SdhB* and *SdhC* genes was performed to genetically distinguish between *Alternaria* subpopulations (sensitive or resistant to boscalid).

RESULTS

Molecular identification and phylogenetic analyses

During the 2013–2017 period, different leafy vegetable plants (2–5 months old) showed severe leaf spot symptoms in different areas of northern Italy. On the basis of morphological observations, the isolates obtained from infected plant tissues mainly belonged to small-conidium *Alternaria* species. Thirty-six *Alternaria* isolates were collected and subjected to molecular characterization. The ITS, *tub2, endoPG* and *H3* sequences of these isolates were compared with those available at NCBI, and all gave the greatest similarity with *A. alternata* and *A. arborescens* (98–100%). The only exception was the ruc PMP 4 isolate from cultivated rocket, which showed greatest similarity to *A. brassicicola* in ITS and the *H3* gene (respectively, 96 and 99%). Since the single gene sequence analyses were not conclusive in identifying *Alternaria* sp., four loci-phylogenetic analyses were carried out.

Phylogenetic analyses were performed on gene portions of 400–500 bp for ITS, *H3* and *endoPG,* and 700 bp for *tub2*. A concatenated tree, based on these genes, was used to study the genetic diversity of 36 tested *Alternaria* strains isolated from five vegetable hosts, together with four reference strains (Table 1). One main cluster, divided into two sub-clusters, was observed (Figure 1); the first sub-cluster grouped 31 strains together with two reference *A. alternata* strains (EGS 34015 and EGS 34016); the second sub-cluster grouped three strains, which gave the greatest similarity (99–100%) with *A. arborescens* in *tub2* and *endoPG* genes (ruc 7/10, bas BIO 10, and bas G1 strains). The reference *A. arborescens* strain CBS124274 was outside the main cluster. One minor cluster contained two *Alternaria* strains (ruc PMP 4 and bas BIO 11) that were not grouped together with the *A. alternata* or *A. arborescens* strains. No specific grouping of the strains was observed that was related to a plant host or source of

the isolation. Moreover, moderate intraspecies molecular diversity was observed for *A. alternata*, to which belonged a major number of identified strains with several phylogenetic subgroups from various hosts. The Bayesian consensus tree for four loci agreed with the tree topologies obtained from the ML analyses (Supplementary data, Figure 1). Furthermore, the phylogenetic analysis based only on the *endoPG* gene, as suggested by Woudenberg *et al.* (2015) for better separation of *A. alternata* and *A. arborescens*, again clustered the ruc 7/10, bas BIO 10 and bas G1 strains together with three reference *A. arborescens* and a few additional *A. alternata* strains (Figure 2).

Figure 1. Phylogenetic relationships of *Alternaria* spp. based on ITS, *tub2*, *endoPG* and *H3* sequences. The concatenated phylogenetic tree was obtained from a Maximum Likelihood analysis using a Tamura Nei model. The name and host affiliation are indicated for each strain. Reference isolates of *A. alternata* and *A. arborescens* (Woudenberg *et al.*, 2015) are shown in bold.

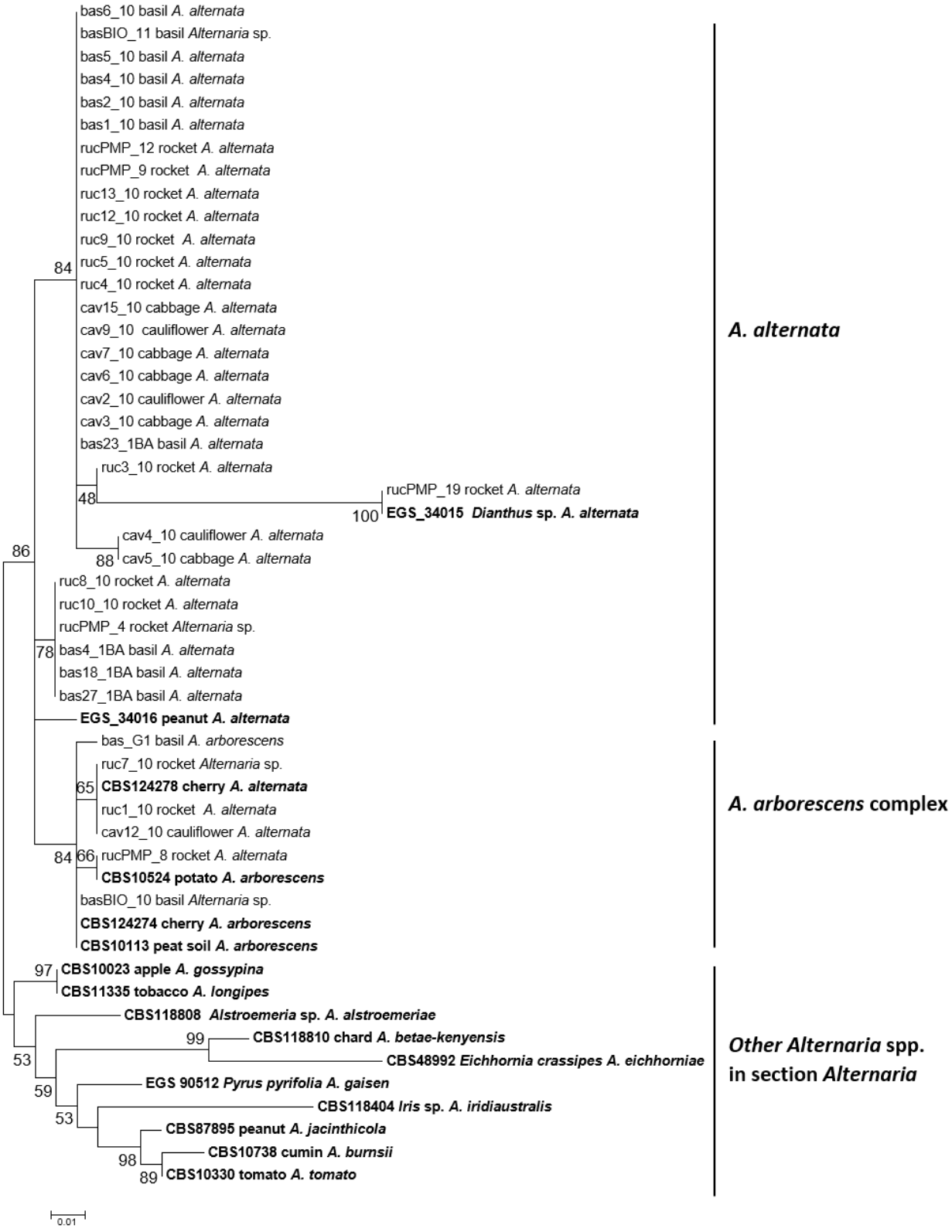

Figure 2. Phylogenetic relationships of the *Alternaria* species and the *A. arborescens* species complex within an *Alternaria* section based on *endoPG* sequences. The phylogenetic tree was obtained from a Maximum Likelihood analysis. The name, fungal species and host affiliation are indicated for each strain. Reference isolates (Woudenberg *et al*., 2015) are shown in bold.

Table 2. Sensitivity to azoxystrobin and boscalid of *Alternaria* spp. obtained from different hosts.

Isolate	Host	Species	Azoxystrobin EC_{50} (mg/L)			Boscalid EC_{50} (mg/L)			
			S	RS	IR	S	RS	IR	R
Cav 2/10	Cauliflower	*A. alternata*	0.01			0.06			
Cav 3/10	Cabbage	*A. alternata*	0.12				3.91		
Cav 4/10	Cauliflower	*A. alternata*	0.12					29.79	
Cav 5/10	Cabbage	*A. alternata*	0.04			0.54			
Cav 6/10	Cabbage	*A. alternata*	0.86				1.74		
Cav 7/10	Cabbage	*A. alternata*	0.06			0.34			
Cav 9/10	Cauliflower	*A. alternata*	0.86			0.14			
Cav12/10	Cauliflower	*A. alternata*	0.19			0.24			
Cav15/10	Cabbage	*A. alternata*	0.19				8.69		
Ruc 1/10	Cultivated rocket	*A. alternata*			30.42				134.4
Ruc 3/10	Wild Rocket	*A. alternata*	0.59				3.02		
Ruc 4/10	Wild Rocket	*A. alternata*	0.003				2.74		
Ruc 5/10	Wild Rocket	*A. alternata*	0.40			0.04			
Ruc 7/10	Wild Rocket	*Alternaria* sp.	0.13			0.04			
Ruc 8/10	Cultivated rocket	*A. alternata*	0.19			0.04			
Ruc 9/10	Cultivated rocket	*A. alternata*		2.42			6.48		
Ruc 10/10	Cultivated rocket	*A. alternata*		9.76			8.69		
Ruc 12/10	Cultivated rocket	*A. alternata*	0.20						153.2
Ruc 13/10	Cultivated rocket	*A. alternata*	0.43				1.04		
Ruc PMP 4	Cultivated rocket	*Alternaria* sp.		1.07			4.81		
Ruc PMP 8	Cultivated rocket	*A. alternata*	0.01			0.05			
Ruc PMP 9	Cultivated rocket	*A. alternata*	0.16			0.27			
Ruc PMP 12	Cultivated rocket	*A. alternata*	0.38				5.93		
Ruc PMP 19	Cultivated rocket	*A. alternata*	0.21				1.67		
Bas 1/10	Basil	*A. alternata*	0.11					38.85	
Bas 2/10	Basil	*A. alternata*	0.46				10.71		
Bas 4/10	Basil	*A. alternata*	0.61			0.46			
Bas 5/10	Basil	*A. alternata*	0.08				6.58		
Bas 6/10	Basil	*A. alternata*	0.02						102.8
Bas G1	Basil	*A. arborescens*	0.06				2.79		
Bas BIO 10	Basil	*Alternaria* sp.	0.02			0.20			
Bas BIO 11	Basil	*Alternaria* sp.		1.31			1.86		
Bas 4-1BA	Basil	*A. alternata*	0.007			0.11			
Bas 18-1BA	Basil	*A. alternata*	0.03			0.41			
Bas 23-1BA	Basil	*A. alternata*	0.10				1.44		
Bas 27-1BA	Basil	*A. alternata*	0.05			0.08			
Mean EC_{50}			0.22±0.02	3.64±0.44	30.42±2.93	0.23±0.02	4.51±0.38	34.32±2.31	130.13±15.67
EGS 34015*	*Dianthus* sp.	*A. alternata*	0.02				4.59		
EGS 34016*	Peanut	*A. alternata*	0.15			0.65			
CBS124274*	Cherry	*A. arborescens*	0.04			0.27			
CBS124278*	Cherry	*A. alternata*	0.05			0.11			

*Reference strains CBS.
S = sensitive isolates; RS= isolate with reduced sensitivity; IR = intermediate resistant isolates; R = resistant isolates.

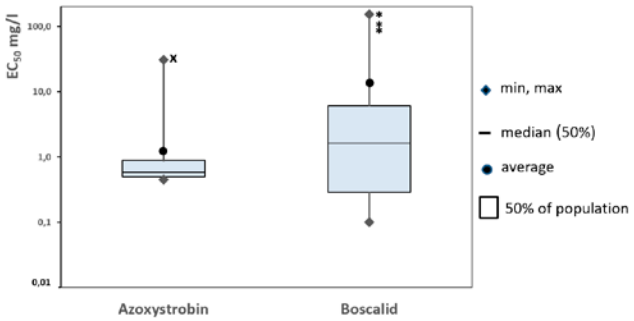

Figure 3. Sensitivity distribution (EC_{50}) of *Alternaria* spp. populations to azoxystrobin and boscalid. Reference isolates are included in the analyses. *Alternaria alternata* strain ruc 1/10 with intermediate resistance to azoxystrobin is indicated by a cross, and *A. alternata* strains (ruc 1/10, ruc 12/10, and bas 6/10) resistant to boscalid are shown by stars. The sensitivity distribution is plotted on a log EC_{50} scale.

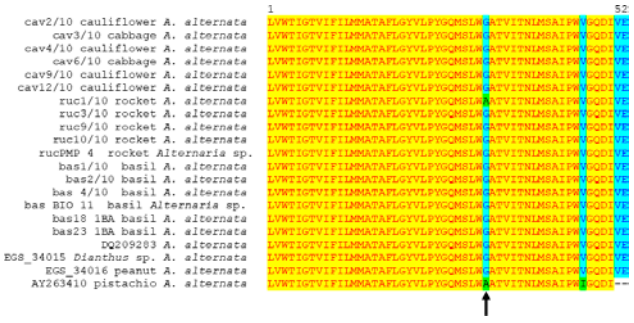

Figure 4. Amino acid alignment of the partial cytochrome b coding region. The eighteen studied and four reference *A. alternata* strains are shown (Ma *et al.*, 2003; Grasso *et al.*, 2006; Vega *et al.*, 2012). Amino acid position 143, where glycine (GGT) was substituted with alanine (GCT) in the ruc 1/10 strain, is indicated by the arrow.

Sensitivity of Alternaria *spp. to azoxystrobin or boscalid*

Thirty-six *Alternaria* isolates, originating from five vegetable hosts (and the four reference strains of *A. alternata* and *A. arborescens*), were evaluated in spore germination assays to establish their sensitivity to azoxystrobin (Table 2). Most of the isolates were sensitive to azoxystrobin, but four *Alternaria* isolates showed reduced sensitivity (mean EC_{50} = 3.64), and one isolate showed intermediate resistance (EC_{50} = 30.42). The four reduced sensitivity isolates had resistance factors of 17, and that for the intermediate resistance isolate was 138, compared to the sensitive isolates. The sensitivity range (between the most and the least sensitive isolate) was 122-fold, with a non-continuous sensitivity distribution of the isolates.

The *Alternaria* isolates were also tested for their sensitivity to boscalid (Table 2). Fifteen isolates (42%) were sensitive with a mean EC_{50} of 0.23. Sixteen isolates (44%) showed reduced sensitivity to boscalid (mean EC_{50} = 4.51), two isolates (6%) showed intermediate resistance (mean EC_{50} = 34.32), and three isolates (8%) were resistant (mean EC_{50} = 130.13). The sensitivity range was 14-fold, and this was narrower than the range for azoxystrobin, showing a more continuous sensitivity distribution of the isolates.

The box and whiskers plots showed high sensitivity variations in the 50% box of the population for sensitivity to azoxystrobin, and high maximum whiskers values for boscalid (Figure 3). The median line of the *Alternaria* spp. populations was less (EC_{50} < 1 mg L^{-1}) for azoxystrobin compared to that of boscalid. The sensitivity of the *A. alternata* isolate ruc 1/10 was outside the 50% box for azoxystrobin, and of the *A. alternata* isolates ruc 1/10, ruc 12/10, and bas 6/10 was outside the 50% box for boscalid, as well.

No cross-resistance was observed between azoxystrobin and boscalid (Supplementary data, Figure 2). A weak correlation (r^2 = 0.27) indicated that the isolates showing reduced sensitivity to both fungicides possessed a double resistance mechanism.

Molecular characterization of the cytb *gene*

Eighteen *Alternaria* strains (including the strain ruc 1/10 with intermediate resistance to azoxystrobin) were amplified in the *cyt b* region, in which the azoxystrobin resistance-associated mutation (G143A) was reported in *A. alternata* (Ma *et al.*, 2003). One intron was found, starting at position 164 aa (S164), after the T of the codon encoding for serine (TCA) in all of the sequenced strains (data not shown), as reported for other *A. alternata* strains (Vega *et al.*, 2012). Out of 18 strains, only the ruc 1/10 strain from cultivated rocket showed the cytb mutation at position 143 aa (glycine to alanine, G143A) (Figure 4). The rest of the cytb sequence was identical in all of the strains (either sensitive or with reduced sensitivity), with the exception of two nt polymorphisms in the exon region in the rocket ruc PMP 4 strain. The intron region was very similar to that of the group of *A. alternata* citrus strains reported by Vega *et al.* (2012), while it was different from those reported by Grasso *et al.* (2006).

It was not possible to distinguish between the sensitive *Alternaria* spp. subpopulation and the subpopulation with reduced sensitivity, based on the observed *cytb* nt polymorphisms and PCoA analyses (Supplementary data, Figure 4a). The only exceptions were the ruc 1/10 strain with intermediate resistance to azoxystrobin and

the cytb G143A mutation, and the ruc PMP 4 strain with reduced sensitivity to azoxystrobin. Both of these strains were distant from the rest of the analyzed strains.

Molecular characterization of the SdhB *and* SdhC *genes*

Eighteen strains (including boscalid-resistant strains and strains with reduced sensitivity) were sequenced in the portions of the *SdhB* and *SdhC* genes known to be related to boscalid resistance (Avenot *et al*., 2008a). Two synonymous mutations were found in the cauliflower cav 2/10 and rocket ruc 1/10 strains, while the basil bas BIO 10 strain showed only a few nt polymorphisms in both the *SdhB* and *SdhC* genes. However, no amino acid mutations, including the mutations related to boscalid resistance in *SdhB* (H277Y, H277R) and *SdhC* (H134R), were observed in either of the proteins (Supplementary data, Figure 3).

Identified *SdhB* and *SdhC* nt polymorphisms were not able to differentiate the subpopulations (sensitive, with reduced sensitivity, with intermediate resistance, or resistant) by PCoA analyses, and the resistant subpopulation was grouped together with sensitive subpopulations (Supplementary data, Figure 4b).

DISCUSSION

Alternaria sect. *Alternaria* comprises approx. 60 host-specific and small-conidium *Alternaria* species, which affect plants, animals and humans (Woudenberg *et al*., 2013). Recent genome and transcriptome studies of different *Alternaria* morphospecies have indicated that *Alternaria* sect. *Alternaria* contained only 11 phylogenetic species and one species complex (*A. arborescens* species complex), which are genetically very similar (97–98 % of the full-genome similarity; Woudenberg *et al*., 2015). Thirty-five morphospecies, which are indistinguishable according to multi-gene phylogeny, have also been synonymized as *A. alternata* (Woudenberg *et al*., 2015). Owing to the high genetic similarity, molecular characterization of the *Alternaria* sect. based on one locus is inconclusive, and is not sufficient to differentiate the small-conidium *Alternaria* species within this section. Thus, *A. alternata* cannot be differentiated from *A. arborescens* on the basis of only single markers, such as ITS, *tub2*, SSU, LSU or *gapdh* (Lawrence *et al*., 2013; Woudenberg *et al*., 2015). Multi-locus phylogenetic studies are widely used for the molecular characterization and better separation of *Alternaria* spp. (Woudenberg *et al*., 2015; Siciliano *et al*., 2018; Nishikawa and Nakashima, 2019). However, expanded multi-gene phylogenetics, in which the most diverse genes selected from the comparison of the whole-genome *Alternaria* sequences are considered, is not always sufficient to differentiate all of the *Alternaria* phylogenetic species in *Alternaria* sect. *Alternaria*. The markers such as *rpb2*, *tef1*, OPA10-2, *Alt a 1*, *endoPG*, KOG1058 and KOG1077 suggested by Woudenberg *et al*. (2015), and ATPase and cmdA used by Zhu and Xiao (2015), should permit differentiation of *A. alternata* and *A. arborescens*. This could be combined with morphological characteristics of conidium formation and culture, and a *TaqI* restriction site in the *endoPG* gene, as a specific marker for differentiation of *A. alternata* and *A. arborescens* (Andrew *et al*., 2009; Woudenberg *et al*., 2015; Ozkilinc and Sevinc, 2018).

Thirty-six *Alternaria* strains, isolated from leaf spot diseased plants of cauliflower, cabbage, cultivated rocket, wild rocket and basil, were characterized on a molecular basis in this study. Four commonly used markers for *Alternaria* sp. differentiation (ITS, *tub2*, *endoPG* and *H3)* were used for a multi-locus phylogenetic analysis. Some of these isolates had been characterized in a previous study, but not on the basis of all of these four loci (Siciliano *et al*., 2017; 2018). The present analysis showed that the majority of the strains were *A. alternata*. The ruc 7/10 and bas BIO 10 strains were grouped together with the bas G1 strain, which was identified by five other genes as *A. arborescens* in a study by Siciliano *et al*. (2018). These three strains also showed the TCGA sequence (*TaqI* restriction site), specific for *A. arborescens* (Ozkilinc and Sevinc, 2018). Based on morphological characteristics, the ruc 7/10, bas BIO 10 and bas G1 strains were also similar to each other, exhibiting ovate conidia (10.8 to 34.2 × 6.1 to 14.9 µm) and dark green-gray colonies (data not shown). In order to confirm the identification of the ruc 7/10 and bas BIO 10 strains as *A. arborescens*, it will be useful to include more molecular markers in future studies.

Two strains (ruc PMP 4 and bas BIO 11) were outside the main cluster that included the *A. alternata* and *A. arborescens* strains. These two strains need more profound molecular analyses in which other *Alternaria* sections should be included, since they were found to be more phylogenetically distant from all of the rest of the studied strains. Compared to the work of Siciliano *et al*. (2017), which was only based on the *tub2* gene, it was found that the strain ruc 1/10 from cultivated rocket was *A. alternata* instead of *A. japonica*. With respect to the work of Siciliano *et al*. (2018), which was based on seven genes, the basil strains were all confirmed as the same *Alternaria* spp.

Moderate molecular diversity with subgroup structuring to different plant hosts and isolation sources (seeds and leaves) was observed among the strains of *A. alternata*, which suggests non-recent introduction of the

pathogen into new areas and subsequent emergence of leaf spot diseases. More probably, a new disease outbreak is associated with the seed transmission of *A. alternata*, with globalization of the seed market and introduction of new agricultural practices (Rotem, 1994; Gullino *et al*., 2014; Mangwende *et al*., 2018). High percentage of seed contamination has been found for basil and rocket (respectively, 7% and 0.4% of non-disinfected seeds) (Gilardi *et al*., 2013a; 2015a). The pathogen can also be spread by airborne conidia (Simmons, 2007), that could explain the appearance of *A. alternata* on new ornamental hosts in Northern Italian areas close to the cultivation zones of leafy vegetables (Garibaldi *et al*., 2018a, 2018b, 2018c). Recent outbreaks of *A. alternata* on ornamental hosts should be investigated to determine if airborne inoculum came from leafy vegetable crops.

Emerging Alternaria leaf spot disease in Italy is predominantly controlled by two respiration inhibitor fungicide classes; QoIs using azoxystrobin, and SDHIs using boscalid. Based on the genetic diversity data obtained in the present study, indicating that introduction of the causative pathogen into Northern Italy has probably been non-recent, and its presence on other hosts previously treated with QoI and SDHI fungicides, the further objective of this study was to evaluate the sensitivity to azoxystrobin and boscalid of *Alternaria* strains isolated from vegetable hosts affected with this emerging disease. Soon after the first description of QoI resistance in the plant pathogen *B. graminis* f. sp. *tritici* (Sierotzki *et al*., 2000), QoI resistance also occurred in *A. alternata* on several vegetable and cereal crops, including pistachio, apple, citrus, potato and tomato, in different countries (Ma *et al*., 2003; FRAC, 2016; Duba *et al*., 2018). An amino acid change from glycine to alanine at 143 aa (G143A) has been reported in the majority of *A. alternata* strains resistant to azoxystrobin (Ma *et al*., 2003). In the present study, a low proportion (3%) of the azoxystrobin resistant *Alternaria* strains was found in the conidium germination assays. Only one *A. alternata* strain originating from cultivated rocket (ruc1/10 strain) was resistant to azoxystrobin. This is the first report in Italy of azoxystrobin resistance in *A. alternata* originating from leafy vegetable hosts. The reason why the G143A mutation was only found in the rocket ruc1/10 strain with intermediate resistance, and not in those strains with reduced sensitivity, could be related to the recent appearance of azoxystrobin resistance in leafy vegetable *A. alternata* strains, or to other mechanisms for this kind of resistance. The azoxystrobin resistance could be related to a recompense mechanism of the energy deficit caused by the fungicide, upstream of the NADH dehydrogenase in the respiratory chain, as has already been proposed for *Venturia inaequalis*. This is through modification of the alternative oxidase gene, or through a reduced accumulation of the bc1 inhibitor (Avila-Adame and Köller, 2002; Esser *et al*., 2014).

Alternaria strains were also tested to establish their sensitivity to SDHIs, represented by boscalid. The first descriptions of SDHI resistance in *A. alternata* were for isolates from pistachio in California, a few years after this fungicide was registered in the USA (Avenot and Michallides 2007; Avenot *et al*. 2008a, 2008b). This was followed by a report of boscalid resistance in *A. solani* from potato and *A. alternata* from peach in other USA states (Wharton *et al*., 2012; Yang *et al*., 2015). Four years later, boscalid resistance was found in *A. alternata* and *A. solani* populations from potato fields in Belgium (Landschoot *et al*., 2017). All of these reports associated resistance to boscalid with Sdh complex mutations, mainly with those in the *SdhB* or *SdhC* genes, or occasionally with *SdhD* mutation. However, in the present study, boscalid resistant strains isolated from leafy vegetable hosts did not show any aa mutation in SdhB or SdhC. This could mean that the boscalid resistance may be related to the *SdhD* gene or to some uncommon mutations in non-sequenced portions of the *ShB* and *SdhC* genes, which means further molecular studies are needed to verify these possibilities. It is also possible that the resistance in the evaluated *A. alternata* strains was governed by some other mechanism. There have been reports of SDHI resistance in other plant pathogens, such as *B. cinerea* from grapevine (Leroux *et al*., 2010), *Monilia fructicola* from peach, (Chen *et al*., 2013), *Pyrenophora teres* from barley (Wieczorek, *et al*., 2016) and *Zymoseptoria tritici* from wheat (Yamashita and Fraaije, 2018), which did not show any *SdhB* and *SdhC* mutations, or had some Sdh mutations present in both sensitive and resistant strains. The fungicide efflux membrane proteins, the ATP-binding cassette (ABC), and major facilitator superfamily (MFS) transporters (in *B. cinerea*), and nucleobase transporters (in *Aspergillus nidulans*), have been related to boscalid resistance and also to multi fungicide resistance (boscalid and other fungicides; Kretschmer *et al*., 2009; Leroux *et al*., 2010; Kalampokis *et al*., 2018).

In the present study, the ruc 1/10 strain with intermediate resistance to azoxystrobin was also resistant to boscalid, as has already reported for *A. alternata* (Avenot and Michailides, 2007; Avenot *et al*., 2008b; Landschoot *et al*., 2017). However, there was no cross-resistance between QoIs and SDHI in this isolate, suggesting the double resistance mechanism, and confirming the data of the previous study of *A. alternata* with double resistance (Malandrakis *et al.,* 2018).

Particular attention should be paid because QoI and SDHI fungicides can be formulated in combination products, and since multiple fungicide resistance is not associated with target gene alteration and provokes a wide spectrum of resistance (Leroux *et al*., 2010). Furthermore, the selection pressure exerted by both fungicide classes, used against other pathogens, such as *Peronospora belbahrii, Plectosphaerella cucumerina, B. cinerea* and *S. sclerotiorum* (Gilardi *et al*., 2013b; 2015b; Homa *et al*., 2014) should be considered for its effect on changes in sensitivity in *Alternaria* spp. originating from leafy vegetable hosts where pathogen populations already showed reduced sensitivity to both classes.

In conclusion, a small proportion of *Alternaria* strains identified from five vegetable crops were sensitive to azoxystrobin, while more than half of the strains showed reduced sensitivity or resistance to boscalid. These results are similar to recently reported resistance to pyraclostrobin and boscalid in *A. alternata* from tomato in Greece (Malandrakis *et al*., 2018). Resistance in the strains evaluated in the present study was not associated with commonly reported mutations, with the exception of one strain that was resistant to azoxystrobin. This aspect requires further investigation of the additional resistance mechanisms, with particular attention to fungicide efflux transporters. Adequate fungicide mixtures and rotations with chemicals with different modes of actions (particularly multi-site and eco-sustainable fungicides) may delay the development of single and double fungicide resistance in pathogen populations. This should be supported by improved management of Alternaria leaf spot disease on vegetable crops. This will include information on the sanitary status of seeds, host resistance of cultivars to these diseases, and appropriate choice of best agricultural practices.

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