Research Article

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Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species

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Abstract

BACKGROUND: *Colletotrichum* species cause anthracnose diseases on many plants and crops. A new generation of succinate dehydrogenase inhibitors (SDHIs) was developed recently. The inhibitory activity of the five SDHI fungicides against *Colletotrichum* species was determined in this study.

RESULTS: Isolates of *C. gloeosporioides*, *C. acutatum*, *C. cereale* and *C. orbiculare* were insensitive (naturally resistant) to boscalid, fluxapyroxad and fluopyram on YBA agar medium. In contrast, these isolates were relatively sensitive to penthiopyrad, except for *C. orbiculare*. Most interestingly, benzovindiflupyr showed highest inhibitory activity against all of these four species. Benzovindiflupyr was effective against *C. gloeosporioides* and *C. acutatum* on apple and peach fruit, as well as on cucumber plants inoculated with *C. orbiculare*. The *sdhB*, *sdhC* and *sdhD* genes encoding the subunits of fungicide-targeted succinate dehydrogenase were sequenced, but, despite high polymorphisms, no apparent resistance mutations were found in *Colletotrichum* species.

CONCLUSIONS: This is the first report on the activity of benzovindiflupyr against *Colletotrichum* species. The broad-spectrum efficacy of benzovindiflupyr within the *Colletotrichum* genus might be exploited when designing disease management strategies against various pathogens on a wide range of crops. Other mechanism(s) than fungicide target-site modification may be responsible for differential sensitivity of *Colletotrichum* species to SDHI fungicides.

Keywords: apple bitter rot; benzovindiflupyr; *Colletotrichum acutatum*; *Colletotrichum gloeosporioides*; cucumber anthracnose; fungicide resistance

1 INTRODUCTION

Colletotrichum species cause anthracnose diseases on many plants and crops and are among the 'top 10' fungal plant pathogens.[1] They cause important diseases, including apple bitter rot, grapevine ripe rot and anthracnose of strawberry, cucurbits, pear and other crops. Multiple species such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *C. acutatum* J. H. Simmonds and *C. orbiculare* (Berk. & Mont.) Arx are included in the genus, and these are also known to be species complexes.[2–5]

Although biofungicides such as Biotrust®, which contains *Talaromyces flavus* (Klocker) Stolk & Samson as the active ingredient, have been commercialised for anthracnose control,[6,7] and breeding programmes are focusing on anthracnose-resistant cultivars,[8] control of anthracnose diseases still relies largely on chemical fungicides. Fungicide resistance in plant pathogens is one of the most critical factors for chemical disease control. In *Colletotrichum* species, resistance has been reported to benzimidazole,[9,10] quinone outside inhibitor (QoI)[11–15] and sterol demethylation-inhibiting (DMI) fungicides,[16,17] often causing loss of efficacy of these fungicides.

Recently, a new generation of succinate dehydrogenase inhibitors (SDHIs) (mitochondrial complex II inhibitors) such as boscalid, penthiopyrad, fluopyram and fluxapyroxad was developed.[18] Benzovindiflupyr (Solatenol™) represents the latest release by Syngenta Crop Protection.[19,20] This fungicide controls rusts, many different leaf spots, apple scab, powdery mildew and Rhizoctonia, and will be available for use on wheat, corn, cucurbit and fruiting vegetables, grapevine, peanuts, pome fruit, potato and soybean in the United States (http://www.syngentacropprotection.com/news_releases/news.aspx?id=183015). A mixture of benzovindiflupyr with azoxystrobin has also been developed to combat Asian rust on soybean in Brazil.

The risk of resistance development to SDHI fungicides is regarded medium to high (Fungicide Resistance Action Committee, FRAC; http://www.frac.info/), and boscalid resistance has already been reported in *Alternaria alternata* (Fr.) Keissler on pistachio,[21] *Corynespora cassiicola* (Berkeley & Curtis) Wei on cucumber,[22] *Botrytis cinerea* Pers. on grapevine[23] and some other pathogen–crop relationships.

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The inhibitory activity of boscalid and fluopyram against *Colletotrichum* species has been shown to be low (www.papst.ch/files/Moon_Produkte_Spritzplaene.pdf). In spite of that, penthiopyrad was registered for the control of anthracnose on sweet cherry and ripe rot on grapevine in Japan, as well as soybean anthracnose in the United States. Therefore, the objective of this study was to compare the activity of benzovindiflupyr with other SDHI fungicides against various species of *Colletotrichum* causing anthracnose diseases on selected crops. The sequences of *sdhB*, *sdhC* and *sdhD* genes encoding the subunits of fungicide-targeted succinate dehydrogenases were analysed to help understanding the relationship between SDHI activity and the characteristics of fungicide-binding proteins. Brief results from these experiments have been reported earlier.[24,25]

2 MATERIALS AND METHODS

2.1 Fungal isolates and chemicals

Laboratory stock isolates of *Colletotrichum* species, including *C. gloeosporioides*, *C. acutatum*, *C. orbiculare*, *C. cereale* sensu lato Crouch, Clarke and Hillman and *C. truncatum* (Schwein.) Andrus & W. D. Moore obtained from various host plants, fruit trees, vegetables and turf, grown in Japan and the United States, were employed (Table 1). Five SDHI fungicides, boscalid (Endura™, 70% WDG, BASF), fluxapyroxad (Xemium®, 30% SC, supplied by BASF), penthiopyrad (DPX LEM17-090, 20% SC, gift from Du Pont), fluopyram (Luna®, 40.98% SC, from Bayer CropScience) and benzovindiflupyr (97% technical grade supplied by Syngenta Crop Protection), were used for sensitivity tests. The other commercial formulations of boscalid (Cantus®, 50% DF, BASF Japan) and penthiopyrad (Affet®, 20% SC, Mitsui Chemicals Agro, Japan) were used together with benzovindiflupyr for the *C. orbiculare* inoculation tests on cucumber plants.

2.2 Fungicide activity tests *in vitro*

For mycelial growth tests, isolates were cultured on potato dextrose agar (PDA) plates at 22 °C for 5 days in darkness to supply inoculum. Mycelial discs, 4 mm in diameter, were cut from actively growing colony margins and transferred onto YBA agar[22,26] plates containing SDHI fungicides at 0, 0.1, 1, 10 and 100 mg L^{-1} of active ingredient (AI). The commercial formulations of boscalid, penthiopyrad, fluxapyroxad and fluopyram were diluted with sterilised distilled water (DW) in a series and added to molten YBA agar after autoclaving. Benzovindiflupyr was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium. The final concentration of DMSO was 0.5% or less. After incubation at 22 °C for 3 days in the dark, the colony diameter of two replicates per treatment was measured, and EC_{50} values were calculated by regressing percentage growth inhibition against the log of fungicide concentration using GraphPad Prism® v.6.03 (GraphPad Software, Inc., La Jolla, CA).

For germination tests, fresh conidia were prepared following the method of Suzaki.[27] Briefly, agar pieces containing actively growing mycelium were taken from the colony of each isolate grown on PDA plates, homogenised aseptically in a 1.5 mL Eppendorf tube and spread on oat meal agar (OMA) plates, and the plates were incubated at 22 °C for 7 days under fluorescent light. Conidia formed on OMA plates were collected, filtered using cheesecloth and concentrated by centrifugation. After adjusting the conidial concentration to about 2×10^5 mL^{-1}, three drops of the conidial suspensions were separately placed on the surface of YBA agar plates supplemented with each fungicide. Twenty-four hours after incubation at 22 °C in the dark, 70% ethanol was added to cover fully the surface of all plates to stop fungal growth. Germination and germ tube growth of 50 conidia chosen arbitrarily were observed on three replicates per treatment under a microscope at 100× magnification. If the germ tube was longer than the length of the conidium, it was regarded to be germinated, and germ tube growth was scored as follows: −, no growth; +, 1–3 × length of conidium; ++, 3–10 × length of conidium; +++, >10 × length of conidium.

2.3 Inoculation tests

Conidia of the *C. gloeosporioides* isolate 5-2-1 and the *C. acutatum* isolate GC2-1 from apple and Japanese pear, respectively, and the two *C. gloeosporioides* isolates Cg_RR12-1 and Cg_EY12-2 and the *C. acutatum* isolate Ca_EY12-1 from peach were produced as described above. Conidia of the *C. orbiculare* isolates CL-2 and CL-10, the pathogen of cucumber anthracnose disease, formed on PDA plates, which included 0.5% yeast extract after incubation at 25 °C for 1 week in the dark and another week under fluorescent light.[28]

Apple fruit cvs 'Ginger Gold' and 'Golden Delicious' were purchased and used for inoculation. Fruit were washed with soap and water, dipped in 10% bleach for 10 min, rinsed with water and air dried. Conidial suspensions (20 µL) at a final concentration of about 1×10^5 conidia mL^{-1} were mixed at equal volume with each fungicide suspension or solution at a final concentration of 100 mg AI L^{-1} and injected into three equidistant points about 2 cm apart to a depth of 9.5 mm using a sterile 26G3/8 bevelled needle (Becton Dickinson & Co., Rutherford, NJ)[29] and incubated at 22 °C under a moist condition. Three fruit were used per treatment, and tests were conducted 3 times.

Commercial-grade peach fruit cv. 'Red Globe' were harvested from an experimental orchard at the Clemson University Musser Fruit Research Farm in Seneca, South Carolina, and kept in a cold room until use. They were surface disinfested as described above, air dried and sprayed with the suspension or solution of each fungicide at 100 mg AI L^{-1} using a plastic hand sprayer to run-off. Distilled water was used as a control. Conidial suspensions (10 µL, about 1×10^5 conidia mL^{-1}) were placed on three equidistant parts of fruit, and inoculated fruit were incubated at 22–25 °C under a moist condition. Five fruit were used per treatment, and tests were conducted 2 times.

For the inoculation of the cucumber anthracnose fungus, seedlings of cucumber cv. 'Shin Suyo Tsukemidori', supplied by Tohoku Seeds, Utsunomiya, Japan, were grown in plastic pots at 25 °C in a phytotron under natural light conditions, and only plants at the 2.5-leaf stage were used. All leaves of the plants were sprayed with suspensions of boscalid (334 mg AI L^{-1}) or penthiopyrad (100 mg AI L^{-1}) or solutions of benzovindiflupyr (100 mg AI L^{-1}), each containing 0.5% DMSO and 0.01% Tween 20, to run-off. Distilled water was used as the control, and three replicate plants served for each treatment. After incubation at 25 °C for 24 h in a phytotron, the treated plants were inoculated with conidial suspensions (about 1×10^5 conidia mL^{-1}) of two isolates of *C. orbiculare*. The inoculated plants were kept at 20 °C in a dew chamber for 24 h, followed by incubation at 25 °C in a phytotron. Disease suppression by SDHI fungicides was assessed 7 days after inoculation. Disease development on each leaf was recorded using the following scale: 0 = no visible symptoms, 0.1 = <1%, 0.5 = 1–5%, 1 = 6–10%, 2 = 11–20%, 3 = 21–30%,

Table 1. *Colletotrichum* isolates used for testing sensitivity to SDHI fungicides

Isolate	Year of isolation	Location	Species[a]	Host plant
Niitaka 3	2006	Chiba, Japan	*C. gloeosporioides*	Japanese pear
GC2-1	1998	Akita, Japan	*C. acutatum*	Japanese pear
5-2-1	2013	Akita, Japan	*C. gloeosporioides*	Apple
5-2-2	2013	Akita, Japan	*C. gloeosporioides*	Apple
Nagasaki 1	2012	Nagasaki, Japan	*C. gloeosporioides*	Grapevine
Nagasaki 2	2012	Nagasaki, Japan	*C. gloeosporioides*	Grapevine
AAU811-3	1999	Tochigi, Japan	*C. acutatum*	Strawberry
19002	2007	Nara, Japan	*C. gloeosporioides*	Strawberry
CL-2	2009	Saga, Japan	*C. orbiculare*	Cucumber
CL-10	2009	Saga, Japan	*C. orbiculare*	Cucumber
C-14	Unknown	Kanagawa, Japan	*C. orbiculare*	Cucumber
S0133[b]	2001	Yamaguchi, Japan	*C. cereale*	Orchard grass
Cg_RR12-1[c]	2012	SC, USA	*C. gloeosporioides* (= *C. fructicola*)	Peach
Cg_SE12-2	2012	SC, USA	*C. gloeosporioides*	Peach
Cg_EY12-2	2012	SC, USA	*C. gloeosporioides* (= *C. siamense*)	Peach
Cg_RR12-4	2012	SC, USA	*C. gloeosporioides*	Peach
Ca_EY12-1	2012	SC, USA	*C. acutatum*	Peach
CO4-35	2005	SC, USA	*C. acutatum*	Peach
Ct_RR13-1	2013	SC, USA	*C. truncatum*	Peach
Ct_RR13-2	2013	SC, USA	*C. truncatum*	Peach

[a] Identified by ITS1-5.8S-ITS2 sequencing.
[b] = MAFF306613.
[c] Refer to Hu *et al.*[45]

4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90% and 10 = >91% of leaf area diseased. Disease severity (DS) was calculated as

$$\frac{10A + 9B + 8C + 7D + 6E + 5F + 4G + 3H + 2I + 1J + 0.5K + 0.1L}{10M} \times 100$$

where *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K* and *L* are the number of leaves corresponding to the scales 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 and 0.1 respectively, and *M* is the total number of leaves assessed.[30]

2.4 Sequence analysis of *sdh* genes

The sequence of *sdhB*, *sdhC* and *sdhD* genes encoding the three subunits of the SDHI fungicide-targeted succinate dehydrogenase B, C and D were analysed by direct sequencing. Isolates of *Colletotrichum* species shown in Table 1 were cultured on PDA plates at 22 °C, and total DNA was extracted as described by Saitoh *et al.*,[31] with slight modifications. A small piece of agar medium with actively growing mycelium (about 1 cm^2 in size) was transferred in a 1.5 mL Eppendorf tube containing 500 µL of lysis buffer (200 mM of Tris-HCl, 50 mM of ethylenediaminetetraacetic acid (EDTA), 200 mM of NaCl, 1% *n*-lauroylsarcosine sodium salt, pH 8.0) and homogenised using a motor and an electric drill. The mixture was incubated at room temperature for 10 min, then centrifuged at 13 000 rpm for 5 min at 4 °C, and the supernatant (300 µL) was transferred to a fresh tube. After mixing with 750 µL of ethanol, the DNA was precipitated by centrifugation at 13 000 rpm for 2 min at 4 °C. The pellet was washed with 70% ethanol, air dried in a laminar floor bench and dissolved in 50 µL of Tris-EDTA (TE) buffer containing 10 mM of Tris-HCl and 1 mM of EDTA (pH 8.0).

To amplify the *sdhB* gene fragments from total DNA, the following PCR primers[32] were used: KES719 (forward), 5′-CTBCCNCACA CCTACGTCGTCAAGGAC-3′; KES729 (reverse), 5′-CTTCTTRATCTCVG CRATVGCC-3′. A quantity of 50 µL of PCR reaction mixtures contained 1 µL of total DNA, a set of forward and reverse primers (0.5 µM for each) and premixed Go Taq Green Master Mix (Promega, Madison, WI). PCR reactions were performed in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) programmed for 1 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C, 2 min at 72 °C, a final extension for 10 min at 72 °C and holding at 4 °C.

For amplification of the *sdhC* and *sdhD* subunit genes, the degenerate primer pairs CoCF: 5′-TGGGCGTGTCGGCCCT-3′ and CoCR: 5′-TACCACGCAAAGGCCAGGC-3′, as well as CSD2F: 5′-CGWCAAGA GTCGCCGCYTTC-3′ and CSDR: 5′-ACGTCGCTGGTCTCRAACT-3′, respectively, were designed on the basis of the sequences of the putative *sdhC* gene in *C. fioriniae* (Marcelino & Gouli) R. G. Shivas & Y. P. Tan (NCBI GenBank XM_007601458.1) and *sdhD* gene in *Colletotrichum* sp. (NCBI XM_007279924 and XM_007602242.1). PCR was performed in 50 µL volumes containing 2 µL of DNA, 25 µL of Go Taq Green Master Mix and primers at 0.3 µM each. PCR was carried out in a MyCycler Thermocycler (Bio-Rad Laboratories) with an initial preheat for 3 min at 95 °C, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and 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 on a 1.5% agarose gel in 40 mM Tris-acetate (pH 8.0) + 1 mM EDTA (TAE) buffer and stained with GelRed™ (Biotium, Hayward, CA). PCR products were cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the instructions supplied by the manufacturer. Sequencing was conducted at Clemson University Genomics Institute (CUGI, Clemson, SC). After sequencing, the nucleotide sequences were translated to amino acid sequences with NCBI/GenBank code.

Table 2. Sensitivity of *Colletotrichum* species isolates to SDHI fungicides (mycelial growth)

	EC_{50} (mg L^{-1}) of fungicide				
Species and isolate	Boscalid	Fluxapyroxad	Penthiopyrad	Fluopyram	Benzovindiflupyr
C. gloeosporioides					
Niitaka 3	>100	>100	2.6	>100	0.2
5-2-1	>100	>100	1.9	>100	<0.1
5-2-2	>100	>100	1.8	>100	<0.1
Nagasaki 1	>100	>100	0.8	>100	<0.1
Nagasaki 2	>100	>100	0.7	>100	<0.1
19002	>100	>100	1.6	>100	<0.1
Cg_RR12-1	>100	>100	1.2	>100	<0.1
Cg_SE12-2	>100	>100	1.1	>100	<0.1
Cg_EY12-2	>100	>100	2.6	>100	<0.1
Cg_RR12-4	>100	>100	1.1	>100	<0.1
C. acutatum					
GC2-1	>100	>100	0.3	>100	<0.1
AAU811-3	>100	>100	0.5	>100	<0.1
Ca_EY12-1	>100	>100	2.0	>100	<0.1
CO4-35	>100	>100	1.2	>100	<0.1
C. orbiculare					
CL-2	>100	>100	>100	>100	<0.1
CL-10	>100	>100	>100	>100	<0.1
C. cereale					
S0133[a]	>100	>100	1.5	>100	<0.1
C. truncatum					
Ct_RR13-1	>100	>100	4.4	>100	<0.1
Ct_RR13-2	>100	>100	5.3	>100	<0.1

[a] = MAFF306613.

Table 3. Sensitivity of *Colletotrichum* species isolates to SDHI fungicides (conidial germination)

	EC_{50} (μg mL^{-1}) of fungicide				
Species[a] and isolate	Boscalid	Fluxapyroxad	Penthiopyrad	Fluopyram	Benzovindiflupyr
C. g. 5-2-1	>100	>100	>100	>100	3.2
C. a. GC2-1	>100	>100	>100	>100	8.2
C. c. S0133[b]	>100	NT[c]	>100	NT	<0.1
C. o. CL-2	>100	>100	>100	10–100	<0.1

[a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*; *C. c.*, *C. cereale*; *C. o.*, *C. orbiculare*.
[b] = MAFF306613.
[c] NT, not tested.

2.5 Data analysis

For all the inoculation experiments, Brown–Forsythe tests (http://www.real-statistics.com/one-way-analysis-of-variance-anova/brown-forsythe-f-test/) were conducted to ensure the homogeneity between experiments. Disease suppression by each fungicide was compared within the isolate using the LSD test at $P = 0.05$. All statistical analyses were performed using JMP software v.10 (SAS Institute, Cary, NC).

3 RESULTS

3.1 Activity of SDHI fungicides against mycelial growth

Nineteen isolates of *Colletotrichum* species, including *C. gloeosporioides*, *C. acutatum*, *C. orbiculare*, *C. cereale* and *C. truncatum* (Table 1), were tested for their sensitivity to the five SDHI fungicides on YBA agar (Table 2). All isolates were insensitive (naturally resistant) to boscalid, fluxapyroxad and fluopyram (EC_{50} > 100 mg L^{-1}). In contrast, the EC_{50} values of penthiopyrad were relatively low and ranged from 0.3 to 5.3 mg L^{-1}, except for the *C. orbiculare* isolates from cucumber (EC_{50} > 100 mg L^{-1}). Interestingly, the new fungicide benzovindiflupyr was highly active against all isolates, including cucumber isolates, and EC_{50} values of this fungicide were typically lower than 0.1 mg L^{-1} (Table 2).

3.2 Activity of SDHI fungicides against conidial germination and germ tube growth

One isolate from each of *C. gloeosporioides*, *C. acutatum*, *C. cereale* and *C. orbiculare* was employed. None of the four SDHI fungicides boscalid, penthiopyrad, fluopyram and fluxapyroxad strongly

Table 4. Sensitivity of *Colletotrichum* species isolates to SDHI fungicides (conidial germ tube growth)

Fungicide	Concentration (μg mL^{-1})	Species[a] and isolate			
		C. g. 5-2-1	*C.a.* GC2-1	*C. c.* S0133[b]	*C. o.* CL-2
Boscalid	100	+++[c]	+++	+++	+++
Fluxapyroxad	100	+++	++	NT	++
Penthiopyrad	100	++	++	++	+++
Fluopyram	100	++	++	NT	+++
Benzovindiflupyr	1	+	+	−	−
Benzovindiflupyr	0.1	+	+	−	− − +
None		+++	+++	+++	+++

[a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*; *C. c.*, *C. cereale*; *C. o.*, *C. orbiculare*.
[b] = MAFF306613.
[c] −, no growth; +, 1–3 × length of conidium; ++, 3–10 × length of conidium; +++, >10 × length of conidium.

Table 5. Suppressive activity of SDHI fungicides against *Colletotrichum* species isolates on detached apple fruit

Species[a]	Isolate	Disease suppression (%)				
		Fungicide				
		Boscalid	Fluxapyroxad	Penthiopyrad	Fluopyram	Benzovindiflupyr
C. g.	5-2-1	−1.3 b[b]	2.0 b	11.9 b	3.8 b	40.0 a
C. a.	GC2-1	−43.2 b	−20.7 b	82.8 a	−14.9 b	60.6 a

[a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*.
[b] A Brown–Forsythe test was conducted to assess the equality of variance between experiments. Least significant difference (LSD) tests were conducted at $P = 0.05$. The same letters indicate no significant difference.

inhibited germination of conidia. EC_{50} values of these fungicides ranged between 10 and 100 mg L^{-1}. However, benzovindiflupyr exhibited very high inhibitory activity against the germination of *C. cereale* and *C. orbiculare* isolates (EC_{50} < 0.1 mg L^{-1}). High activity of benzovindiflupyr was also found for isolates of *C. gloeosporioides* and *C. acutatum*, with EC_{50} values of 3.2 and 8.2 mg L^{-1} respectively (Table 3).

Conidial germ tube growth of *C. gloeosporioides* and *C. acutatum* isolates was suppressed by benzovindiflupyr at 0.1 and 1 mg L^{-1}, but not by boscalid, fluxapyroxad, penthiopyrad and fluopyram even at 100 mg L^{-1} (Table 4). No germ tubes of *C. gloeosporioides* and *C. acutatum* isolates grew at 10 or 100 mg L^{-1} of benzovindiflupyr. For *C. cereale* and *C. orbiculare* isolates, benzovindiflupyr inhibited growth at 0.1 mg L^{-1}, but no or very little suppression of germ tube growth was recorded at 100 mg L^{-1} of the former four fungicides (Table 4).

3.3 Suppressive activity of SDHI fungicides against *Colletotrichum* isolates on detached apple and peach fruit

On apple fruit, treatment with the three SDHI fungicides boscalid, fluxapyroxad and fluopyram at 100 mg L^{-1} showed no suppressive activity against bitter rot caused by the isolates of *C. gloeosporioides* and *C. acutatum* (Table 5). Penthiopyrad was highly effective against the *C. acutatum* isolate at the same concentration (82.8% disease suppression) but not against the *C. gloeosporioides* isolate (11.9%). Benzovindiflupyr (100 mg L^{-1}) exhibited moderate levels (40.0 and 60.6%) of efficacy both against *C. gloeosporioides* and against *C. acutatum* isolates.

On peach fruit, none of the treatments with the four SDHI fungicides boscalid, fluxapyroxad, penthiopyrad and fluopyram at 100 mg L^{-1} showed efficacy on anthracnose disease caused by isolates of *C. fructicola* Prihastuti, L. Cai & K. D. Hyde, sp. nov., *C. siamense* Prihastuti, L. Cai & K. D. Hyde, sp. nov. and *C. acutatum* (Table 6). In contrast, benzovindiflupyr at the same concentration suppressed disease development of these three species (61.1, 77.9 and 68. 8%).

3.4 Suppressive activity of SDHI fungicides against *C. orbiculare* isolates on cucumber plants

Spray applications of benzovindiflupyr at 100 mg L^{-1} effectively suppressed anthracnose disease on intact cucumber plants inoculated with *C. orbiculare* (Fig. 1). However, the efficacy of boscalid at 334 mg L^{-1} and penthiopyrad at 100 mg L^{-1} was very poor. Disease suppression was 96.7, 20.0 and 21.6% on average, respectively, after treatment with benzovindiflupyr, boscalid and penthiopyrad (Table 7).

3.5 Nucleotide sequences of *sdh* subunit genes

Nucleotide sequences of *sdhB*, *sdhC* and *sdhD* genes, encoding fungicide-binding SDH subunit proteins, were analysed. When compared with NCBI GenBank XM_007276209.1 and XM_007596512.1, the *sdhB* sequence of *C. gloeosporioides* and *C. fioriniae* respectively, the nucleotide sequences of most isolates used in this study – *C. gloeosporioides* (*C. fructicola* and *C. siamense*), *C. acutatum*, *C. truncatum*, *C. orbiculare* and *C. cereale* – showed 90–100% identity. Only isolates of *C. orbiculare* and *C. cereale* showed 84–91% or 89% identity respectively. In *sdhB* genes from *Colletotrichum* species, no mutations known to be associated with SDHI resistance in other fungi were found.

The partial sequences of deduced amino acids of SDHB were compared with those of XM_007276209.1 (from *C. gloeosporioides*), XM_007596512.1 (from *C. fioriniae*) and ENH88418.1 (from

Table 6. Suppressive activity of SDHI fungicides against *Colletotrichum* species isolates on detached peach fruit

		Disease suppression (%)				
		Fungicide				
Species	Isolate	Boscalid	Fluxapyroxad	Penthiopyrad	Fluopyram	Benzovindiflupyr
C. fructicola	Cg_RR12-1	−5.5 bc[a]	−17.8 bc	15.7 ab	−42.7 c	61.1 a
C. siamense	Cg_EY12-2	−21.3 b	−15.9 b	4.3 b	0.0 b	77.9 a
C. acutatum	Ca_EY12-1	10.2 b	10.6 b	7.6 b	−2.0 b	68.8 a

[a] A Brown–Forsythe test was conducted to assess the equality of variance between experiments. Least significant difference (LSD) tests were conducted at $P = 0.05$. The same letters within rows indicate no significant difference.

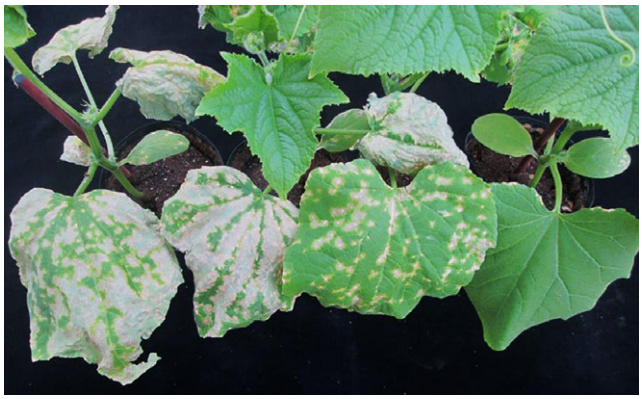

Figure 1. Suppressive activity of SDHI fungicides on cucumber anthracnose disease caused by *Colletotrichum orbiculare*. Left to right: distilled water, 334 mg L^{-1} of boscalid, 100 mg L^{-1} of penthiopyrad and 100 mg L^{-1} of benzovindiflupyr.

Table 7. Suppressive activity of SDHI fungicides against *C. orbiculare* on cucumber

	Disease suppression (%)		
	Fungicide		
Species	Boscalid	Penthiopyrad	Benzovindiflupyr
C. orbiculare	20.0 b[a]	21.6 b	96.7 a

[a] A Brown–Forsythe test was conducted to assess the equality of variance between experiments. Least significant difference (LSD) tests were conducted at $P = 0.05$. The same letters indicate no significant difference.

C. orbiculare). Amino acids P202, N207 and H249, homologous to P225, N230 and H272 in *B. cinerea*, were conserved in all isolates examined (Fig. 2), although their substitutions are reported to be associated with boscalid resistance.[33] When the amino acid sequences in the three datasets above were compared with those described as conserved residues in SDHB by Fraaije *et al.*,[34] a V113G variation was found both in *C. gloeosporioides* and *C. orbiculare* (Fig. 3). In *C. fioriniae*, T125S and V113G variations were detected (Fig. 3). However, these residues are not located in the binding site of SDHI fungicides. In *C. orbiculare* sequences from isolates naturally resistant to both boscalid and penthiopyrad but sensitive to benzovindiflupyr, five amino acid variations L11F, K16Q, P26S, Q36R and I50V were found (Fig. 3) compared with sequences from *C. gloeosporioides* and *C. fioriniae*.

The partial *sdhC* gene sequences revealed most nucleotide polymorphism in *Colletotrichum* isolates, including A46T/S, V48L, S49T, V50P, A51Q/D/E, N54L, E55S, I56L, R61H, H63S/N, I66V, T71G, T103S, V106I, V114E, X116S/A/T/V, A117Q, V120I/K, S121A, A122G, A123F, A124G, A126L, V128L/I, L130A and A132G (Fig. 4). The variations A51E, E55S, T103S, A122G and A132L were only found in *C. orbiculare* isolates CL-2 and/or CL-10. The same isolates possessed a M75L variation in the quinone-binding site of SDHI fungicides. However, there is no report describing this amino acid change as being involved in resistance to SDHI fungicides.

Partial *sdhD* gene fragments containing SDHI relevant sequences were obtained from one isolate of *C. gloeosporioides*, one isolate of *C. acutatum*, one isolate of *C. cereale* and two isolates of *C. orbiculare*, and were sequenced. Deduced amino acid variations S87T, I91V/L, T102A, T113A, I116V, A119S, L120M, I123V, T139I, R140K, T141H, L145S, G148A and T149S were identified (Fig. 5), but the alignment with NCBI submitted sequence XM_007279924 showed no mutations with known relevance to SDHI resistance in our *Colletotrichum* isolates. An intron was detected ahead of nucleotide position 201 in all isolates.

4 DISCUSSION

The release of 'new-generation' SDHI fungicides triggered a plethora of studies on efficacy and molecular mechanisms of resistance.[18,32–35] Resistance is mainly caused by mutations in the *sdh* subunit genes encoding the molecular target of SDHI fungicides. However, cross-resistance is not always uniform among these fungicides. For example, Ishii *et al.*[36] found a lack of cross-resistance to fluopyram in very highly and highly boscalid-resistant isolates of *Corynespora cassiicola* carrying H278Y and H278R variations in *sdhB*, respectively,[32] as well as highly boscalid-resistant isolates of *Podosphaera xanthii* (Castagne) U. Braun & N. Shishkoff carrying the H → Y (homologous to H272Y in *B. cinerea*) mutation in *sdhB*.[37] Similarly, fluopyram and isofetamid controlled boscalid-resistant isolates of *B. cinerea* that carried H272R/Y mutations in the *sdhB* gene.[38,39] As indicated by Scalliet *et al.*,[40] fluopyram does not have a hydrogen bond acceptor in its molecule, and thus its binding seems to be unaffected by the histidine to tyrosine substitution in the SDHB subunit. Nevertheless, the control efficacy of fluopyram was moderate towards H272L, N230I and P225F mutants of *B. cinerea*, suggesting that the sensitivity to SDHI fungicides varies greatly, depending on the point mutation in the *sdhB* subunit gene.[41]

In the present study, the insensitivity (natural resistance) of *Colletotrichum* species to boscalid, fluxapyroxad and fluopyram was confirmed by experiments conducted on fungicide-amended culture media and plants treated with fungicides prior to pathogen

Niitaka 3 (*C. g.*)[a]:	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
GC2-1 (*C. a.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRCHT	250
5-2-1 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
5-2-2 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Nagasaki 1 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Nagasaki 2 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
AAU811-3 (*C. a.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRCHT	250
19002 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
C-14 (*C. o.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
CL-10 (*C. o.*):	224	CPSYWWNSEENHRPAILLPSSRRLCDSRYWRKAERKANLDNSMSLYRCHT	250
0133[b] (*C. c.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDEKVAERKAALDNSMSLYRCHT	250
Cg_RR12-1 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Cg_SE12-2 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Cg_EY12-2 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Cg_RR12-4 (*C. g.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Ca_EY12-1 (*C. a.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRCHT	250
CO4-35 (*C. a.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRCHT	250
Ct_RR13-1 (*C. t.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHT	250
Ct_RR13-2 (*C. t.*):	224	CPSYWWNSEEYLGPAILLQSYRWLADSRD*RKEERKAALDNSMSLYRCHT	250

Figure 2. The partial sequences of deduced amino acids (positions 224 to 250) of SDHB in 19 isolates of *Colletotrichum* species. The names of isolates and species (in parentheses) are shown on the left. The conserved wild-type amino acids P202, N207 and H249, homologous to P225, N230 and H272 in *B. cinerea* and responsible for boscalid resistance,[33] are indicated in bold characters with underlines. [a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*; *C. o.*, *C. orbiculare*; *C. c.*, *C. cereale*; *C. t.*, *C. truncatum*. [b] = MAFF306613. * Not clear.

C. gloeosporioides (XM_007276209.1):	1	MAALRSSSRVLGTATKAAFRPIVTIPRRGLATPTDAPAVKEPKMKKFTIYRWNPDTPTEKPRMQEYTLDLNKTGPMMLDALIRIKNEIDPTLTFRRSCR	100
C. fioriniae (XM_007596512.1):	1	MASLRSSTRVLGSATKAAFRPAVTIPRRGLATPSDQVPATKEPKMKKFTIYRWNPDTPTEKPKMQEYTLDLNKTGPMMLDALIRIKNELDPTLTFRRSCR	100
C. orbiculare (ENH88418.1):	1	MAALRSSSRVFGSATQAAFRPAVTISRRGLATPSDRVPATKEPKMKKFTVYRWNPDTPTEKPRMQEYTLDLNKTGPMMLDALIRIKNELDPTLTFRRSCR	100

C. gloeosporioides (XM_007276209.1):	101	EGICGSCAMNINGQNTLACLCRIPTESASDVKVYPLPHTYVVKDLVPDLTQFYKQYRSIKPYLQRDTPAPDGKEYRQSVADRKKLSGLYECILCACCSTS	200
C. fioriniae (XM_007596512.1):	101	EGICGSCAMNINGQNTLACLCRIPSENASDVKVYPLPHTYVVKDLVPDLTQFYKQYRSIKPYLQRDTPAPDGKEYRQSVADRKKLSGLYECILCACCSTS	200
C. orbiculare (ENH88418.1):	101	EGICGSCAMNINGQNTLACLCRIPTESASDVKVYPLPHTYVVKDLVPDLTQFYKQYRSIKPYLQRDTPAPDGKEYRQSVADRKKLSGLYECILCACCSTS	200

C. gloeosporioides (XM_007276209.1):	201	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHTILNCTRACPKGLNPGKAIAEIKKAMAF	277
C. fioriniae (XM_007596512.1):	201	CPSYWWNSEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRCHTILNCTRACPKGLNPGKAIAEIKKQMAF	277
C. orbiculare (ENH88418.1):	201	CPSYWWNSEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRCHTILNCTRACPKGLNPGKAIAEIKKQMAF	277

Figure 3. The sequences of deduced amino acids of SDHB obtained from the NCBI GenBank database XM_007276209.1 (*Colletotrichum gloeosporioides*), XM_007596512.1 (*Colletotrichum fioriniae*) and ENH88418.1 (*Colletotrichum orbiculare*). The variations of amino acids are shown in bold characters with underlines. The shaded bold letters indicate the conserved wild-type amino acids P202, N207 and H249, homologous to P225, N230 and H272 in *Botrytis cinerea* and responsible for boscalid resistance.[33]

inoculation. This finding is meaningful, as boscalid is often applied in a mixture with a QoI fungicide such as pyraclostrobin in horticultural crops, either as prepacked formulations or in a tank mixture. This study confirms that the use of this combination of fungicides is risky for the control of *Colletotrichum* species in areas where QoI-resistant strains are widely distributed.

However, in contrast to the above observations, isolates of *C. gloeosporioides*, *C. acutatum*, *C. truncatum* and *C. cereale* were sensitive to penthiopyrad. This observation supports the fact that penthiopyrad is registered in Japan for the control of sweet cherry anthracnose, grapevine ripe rot and other diseases caused by *C. gloeosporioides*. Penthiopyrad is also registered on many crops, including apple, blueberry, stone fruit and vegetables, in the United States to control scab, powdery mildew, grey mould, brown rot and other diseases (http://www.dupont.ca/en/products-and-services/crop-protection/fruit-protection/products/fontelis.html). However, *C. orbiculare*, the pathogen of cucurbit anthracnose disease, was insensitive to penthiopyrad both on culture media and cucumber plants. Most interestingly, the new SDHI fungicide benzovindiflupyr exhibited high inhibitory activity against all isolates of the *Colletotrichum* species, including *C. orbiculare* used in this study. To our knowledge, this is the first report on the activity of benzovindiflupyr against *Colletotrichum* species.


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Niitaka 3 (C. g.)[a]:    41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
GC2-1 (C. a.):           41 SPVATTKLTPQDGLELLAKQRLSRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
5-2-1 (C. g.):           41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
5-2-2 (C. g.):           41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Nagasaki 1 (C. g.):      41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Nagasaki 2 (C. g.):      41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
19002 (C. g.):           41 *******VSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
CL-2 (C. o.):            41 SPVATSKLSVEDGNSILAKQRLSRPISPHLTIYKLEQTWFGASIWTRITGGGLSAAFYVY 100
CL-10 (C. o.):           41 SPVATSKLSVEDGNSILAKQRLSRPISPHLTIYKLEQTWFGASIWTRITGGGLSAAFYVY 100
0133[b] (C. c.):         41 SPVATSKLTPDDGLELLAKQRLNRPVSPHLGIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Cg_RR12-1 (C. g.):       41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Cg_SE12-2 (C. g.):       41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Cg_EY12-2 (C. g.):       41 SPVATAKVSVADGNEILAKQHLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Cg_RR12-4 (C. g.):       41 SPVATAKVSVADGNEILAKQRLHRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Ca_EY12-1 (C. a.):       41 ***ATTKLTPQDGLELLAKQRLSRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
CO4-35 (C. a.):          41 SPVATTKLTPQDGLELLAKQRLSRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Ct_RR13-1 (C. t.):       41 SPVATTKLTPQDGLELLAKQRLSRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100
Ct_RR13-2 (C. t.):       41 SPVATTKLTPQDGLELLAKQRLSRPISPHLTIYKMEQTWFGASIWTRITGGGLSAAFYVY 100

Niitaka 3 (C. g.)[a]:    101 FGTYLVAPLLGWHVEVASLVSAAAAAPIALKA 132
GC2-1 (C. a.):           101 FGTYLVAPLLGWHVESQSLIAAFGALPLAAKG 132
5-2-1 (C. g.):           101 FGTYLVAPLLGWHVEVASLVSAAAAAPVALKA 132
5-2-2 (C. g.):           101 FGTYLVAPLLGWHVEVASLVSAAAAAPVALKA 132
Nagasaki 1 (C. g.):      101 FGTYLVAPLLGWHVEAASLVSAAAAAPVALKA 132
Nagasaki 2 (C. g.):      101 FGTYLVAPLLGWHVEAASLVSAAAAAPVALKA 132
19002 (C. g.):           101 FGTYLVAPLLGWHVEVASLVSAAAAAPVALKA 132
CL-2 (C. o.):            101 FGSYLVAPLLGWHE**********          132
CL-10 (C. o.):           101 FGSYLVAPLLGWHVEAASLVAGFGALPVAAKL 132
0133[b] (C. c.):         101 FGTYLIAPLLGWHVETQSLVAAFGALPIAAKG 132

Cg_RR12-1 (C. g.):       101 FGTYLVAPLLGWHVEVASLVSAAAAAPIAL** 132
Cg_SE12-2 (C. g.):       101 FGTYLVAPLLGWHVEVASLVSAAAAAPIAL** 132
Cg_EY12-2 (C. g.):       101 FGTYLVAPLLGWHVEAASLVSAAAAAPVALKA 132
Cg_RR12-4 (C. g.):       101 FGTYLVAPLLGWHVEAASLVSAAAAAPVALKA 132
Ca_EY12-1 (C. a.):       101 FGTYLVAPLLGWHVESQSLIAAFGALPLAAKG 132
CO4-35 (C. a.):          101 FGTYLVAPLLGWHVESQSLIAAFGALPLAAKG 132
Ct_RR13-1 (C. t.):       101 FGTYLVAPLLGWHVESQSLIAAFGALPLAAKG 132
Ct_RR13-2 (C. t.):       101 FGTYLVAPLLGWHVESQSL********** 132
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Figure 4. The partial sequences of deduced amino acids of SDHC in *Colletotrichum* species isolates. The variations of amino acids are shown in bold characters with underlines. [a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*; *C. o.*, *C. orbiculare*; *C. c.*, *C. cereale*; *C. t.*, *C. truncatum*. [b] = MAFF306613. * Not clear.

Niitaka 3 (*C. g.*)[a]:	87	SFDRILAAGLVPITITPFAAGSLNPT**A**DAILC**S**LILIHSHTGFQNIIIDYVPTRTYPKLRKGTM	150
GC2-1 (*C. a.*):	87	SFDRILAAGLVPITI**A**PFAAGSLNPTTDA**V**LCA**M**ILIHSHTGFQNIIIDYVPT**KH**YPK**S**RK**A**T*	150
C-14 (*C. o.*):	87	*FDR**V**LAAGLVPITITPFAAGSLNPTTDAILCALIL**V**HSHTGFQNIIIDYVP**I**RTYPKLRKG**S**M	150
CL-10 (*C. o.*):	87	SFDR**V**LAAGLVPITITPFAAGSLNPTTDAILCALIL**V**HSHTGFQNIIIDYVP**I**RTYPKLRKG**	150
0133[b] (*C. c.*):	87	**T**FDR**L**LAAGLVPITI**A**PFAAGSLNPTTDAILCA**M**IL**V**HSHTGFQNIIIDYVPT**KH**YPK**S**RK**A**TM	150

Figure 5. The partial sequences of deduced amino acids of SDHD in *Colletotrichum* species. The variations of amino acids are shown in bold characters with underlines. [a] *C. g.*, *C. gloeosporioides*; *C. a.*, *C. acutatum*; *C. o.*, *C. orbiculare*; *C. c.*, *C. cereale*. [b] = MAFF306613. * Not clear.

Benzovindiflupyr applied at 100 mg AI L^{-1} suppressed the development of disease caused by *C. gloeosporioides* and *C. acutatum* on apple and peach fruit in inoculation tests. In addition, when benzovindiflupyr was sprayed at 100 mg AI L^{-1} prior to pathogen inoculation, this fungicide showed high control efficacy against anthracnose disease on cucumber. Although field trials still need to be done for confirmation, the broad-spectrum fungicide benzovindiflupyr seems to be promising and attractive either as a solo product or in combination with other fungicides for the control of diseases such as apple bitter rot, grapevine ripe rot and strawberry anthracnose caused by *Colletotrichum* species, in addition to other major diseases. As briefly reported recently,[25,42] boscalid-resistant isolates of *B. cinerea* showed high sensitivity to benzovindiflupyr in conidial germination tests conducted on YBA agar medium, irrespective of mutation types (H272R/Y and N230I) in their *sdhB* gene. Furthermore, all isolates of *A. alternata* resistant to boscalid showed sensitivity to benzovindiflupyr, irrespective of mutation types in the *sdh* subunit genes so far tested. Thus, the superior activity of benzovindiflupyr against *Colletotrichum* species compared with older SDHI fungicides might be exploited when designing disease management strategies in a wide range of crops.

As described by Sierotzki and Scalliet,[18] it is crucial to determine the genetic background to evaluate cross-resistance among SDHI fungicides. The sequences of *sdhB*, *sdhC* and *sdhD* genes of *Colletotrichum* species were investigated to improve our understanding of differential SDHI fungicide efficacy. No mutations previously reported to be associated with boscalid resistance were found, and thus it is still uncertain why *Colletotrichum* species are less sensitive to boscalid, fluxapyroxad and fluopyram but sensitive to benzovindiflupyr and/or penthiopyrad. The polymorphism in *sdhB*, *sdhC* and *sdhD* gene sequences found in our study resembled the polymorphism found in *A. alternata*. In *A. alternata* isolates resistant to boscalid, 21 mutations were identified in the genes encoding fungicide-targeted subunit proteins of SDH; however, no mutations were found in the highly conserved H277 in SDHB, H134 in SDHC and H133 in SDHD, typically observed in boscalid-resistant isolates.[43]

In laboratory-induced SDHI-resistant mutants of *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn, the causal agent of Septoria leaf blotch on wheat, 27 amino acid substitutions occurred at different positions in the three subunits SDHB, SDHC and SDHD, and it was indicated that resistance might be conditioned by sequence variation in multiple subunits of the enzyme.[40] Docking studies of SDHI fungicides in structural models, performed using wild-type and mutated SDH complexes,[34] may help understanding which residues are important for the binding of different SDHI fungicides and may show different binding for benzovindiflupyr. Moreover, sequence diversity in the large subunit of RNA polymerase I has been analysed recently using isolates of *Phytophthora infestans* (Mont.) de Bary, insensitive to the oomycete fungicide mefenoxam, and the association of one single nucleotide polymorphism in subunit RPA190 with insensitivity to this fungicide has been found.[44] The authors pointed out the possibility that insensitivity to mefenoxam might have arisen on multiple occasions in different pathogen genotypes, leading to independent SNPs encoding changes responsible for insensitivity. It might also be possible that other factors than target-site modification are involved in differential sensitivity of *Colletotrichum* species to SDHI fungicides. Further studies are required to confirm this possibility in the future.

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