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Sensitivity to fungicides in isolates of *Colletotrichum gloeosporioides* and *C. acutatum* species complexes and efficacy against anthracnose diseases

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ABSTRACT

Colletotrichum species cause diseases on many plants and are among the 'top 10' fungal plant pathogens. Species of the *C. gloeosporioides* and *C. acutatum* complexes are particularly important because they infect temperate fruit crops, but their control relies largely on chemical fungicides. In this study, differences in intrinsic fungicide sensitivity were determined *in vitro* using isolates of the *C. gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*) and the *C. acutatum* sp. complex (*C. fioriniae* and *C. nymphaeae*), which had never been exposed to fungicides. Mycelial growth of all isolates was sensitive to the QoI azoxystrobin, the SDHI benzovindiflupyr, and the new DMI fungicide mefentrifluconazole. The isolates of *C. nymphaeae* were highly sensitive to the phenylpyrrole fungicide fludioxonil. The isolates of *C. gloeosporioides* sp. complex were sensitive to the bis-guanidine fungicide iminoctadine-albesilate, whereas those of *C. acutatum* sp. complex were inherently insensitive. These results are valuable when sensitivity of field populations is monitored in resistance management. Although SDHI fungicides are largely not effective against diseases caused by *Colletotrichum* species, benzovindiflupyr controlled anthracnose disease of various crops such as kidney bean, garland chrysanthemum, and strawberry, caused by *C. lindemuthianum*, *C. chrysanthemi*, and *C. siamense*, respectively, demonstrating this fungicide to be unique among SDHIs and having a broad control spectrum against anthracnose. To help understanding the reason for differential activity of benzovindiflupyr and boscalid, *sdhB* gene sequences were analyzed but those of *C. lindemuthianum*, *C. chrysanthemi*, and *C. scovillei* revealed no known mutations reported to be responsible for SDHI resistance in other fungi, indicating that other mechanism(s) than target-site modification may be involved in differential sensitivity to benzovindiflupyr and boscalid, found in *Colletotrichum* species.

1. Introduction

Various fungal species of the genus *Colletotrichum* cause devastating diseases on large number of economically important crops and are among the 'top 10' fungal plant pathogens (Prusky et al., 2000; Wedge et al., 2007; Smith, 2008; Dean et al., 2012; Dowling et al., 2020). *C. gloeosporioides* and *C. acutatum* are particularly important because they infect temperate fruit crops causing apple bitter rot (Moreira et al., 2019), grapevine ripe rot (Nita and Bly, 2016), and anthracnose of strawberry (Peres et al., 2005; Smith, 2008), pear (Tashiro et al., 2019) and peach (Chen et al., 2016). Although resistance to diseases caused by *Colletotrichum* species is a high priority in modern crop plant breeding programs (Jacobs et al., 2019) and new resistant cultivars are grown locally in strawberry (Kitamura et al., 2015), control of diseases caused by *Colletotrichum* species still relies largely on the use of fungicides. Consequently, *Colletotrichum* species develop field resistance to fungicides such as benzimidazole (MBC) on pear (Tashiro et al., 2019), grapevine (Chung et al., 2006) and apple (Moreira et al., 2019), sterol demethylation inhibitor (DMI) on peach (Chen et al., 2016), and quinone outside inhibitor (QoI) fungicides on strawberry (Inada et al., 2008; Forcelini et al., 2016; Espinoza-Altamirano et al., 2017; Forcelini and Peres, 2018), peach and blueberry (Hu et al., 2015), grapevine (Kondo, 2011; Nita and Bly, 2016), apple (Moreira et al., 2019), pear (Tashiro et al., 2019), and mango (Takushi et al., 2014; Kongtragoul

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et al., 2020) making it difficult to control diseases caused by *Colletotrichum* species.

Both *C. gloeosporioides* and *C. acutatum* are species (sp.) complexes that each contains large numbers of species (Cannon et al., 2012). Little more than 10 years ago, *C. gloeosporioides* sp. complex comprised of 22 species plus one subspecies including *C. fructicola*, *C. siamense*, *C. tropicale* etc. (Damm et al., 2010; Weir et al., 2012). Similarly, *C. acutatum* sp. complex was defined as a collective of *C. acutatum* sensu stricto and 29 closely related species including *C. fioriniae*, *C. nymphaeae*, and others (Cannon et al., 2012). The number of species has now been revised to 38 and 36 for *C. gloeosporioides* and *C. acutatum* sp. complex, respectively (Marin-Felix et al., 2017; Zhang et al., 2020). Importantly, differences in fungicide sensitivity were reported among *Colletotrichum* species. It is well known that *C. acutatum* sp. complex is intrinsically insensitive (naturally resistant) to MBC fungicides (Adaskaveg and Hartin, 1997; Sato et al., 1998; Peres et al., 2004) and some reports indicated that in contrast to *C. acutatum* sensu lato, *C. gloeosporioides* sensu lato was sensitive to the bis-guanidine fungicide iminoctadine-acetate (Fukaya, 2002; Chung et al., 2006). Hu et al. (2015) found that *C. siamense* isolates with intronless cytochrome *b* genes were resistant to both the MBC fungicide thiophanate-methyl and the QoI fungicide azoxystrobin. Munir et al. (2016) reported that the isolates of *C. fioriniae* and *C. nymphaeae* in *C. acutatum* sp. complex were more tolerant to the QoI fungicide trifloxystrobin compared with those of *C. siamense* and *C. fructicola* in *C. gloeosporioides* sp. complex. Yokosawa et al. (2020) state that *C. fructicola* isolates from Japan were resistant to both azoxystrobin and the MBC fungicide benomyl. Importantly, intrinsic QoI sensitivity may differ between *Colletotrichum* species (Dowling et al., 2020). Sensitivity to DMI fungicides differed between isolates of *C. gloeosporioides* and *C. acutatum* sp. complexes (Chen et al., 2016). Furthermore, the isolates of *C. gloeosporioides* sp. complex were shown to be more tolerant to fludioxonil than those of *C. acutatum* sp. complex (Schnabel et al., 2021). For these reasons it is of benefit to know the *Colletotrichum* species involved when designing fungicide programs for diseases caused by *Colletotrichum* species (Dowling et al., 2020).

Most studies conduct efficacy tests on isolates collected from the field after fungicide treatment. To examine intrinsic fungicide sensitivity, it is necessary to use so-called baseline isolates which had never been exposed to selection pressure by fungicides. Fungicide Resistance Action Committee (FRAC) states that "For fungicide resistance research and management a 'baseline' can be defined as: A profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide" (https://www.frac.info/) as of August 2021.

The European Union (EU) recently increased regulation of DMI fungicides, specifically azoles and imidazoles, due to their suspected endocrine disrupting properties. However, the new isopropanol-triazole fungicide mefentrifluconazole, registered in the EU, USA, and other areas of the world (https://agriculture.basf.com/global/en/innovations-for-agriculture/innovation-for-fungicides/revysol.html, as of August 2021) revealed no embryofetal toxicity or teratogenicity (European Food Safety Authority (EFSA), 2018; Tesh et al., 2019) and was reported to possess high control efficacy against *Zymoseptoria tritici* (teleomorph: *Mycosphaerella graminicola*), the causal agent of *Septoria tritici* blotch of wheat (Jørgensen et al., 2020; Strobel et al., 2020). Mefentrifluconazole also revealed high efficacy against key fungal diseases of pome and stone fruit, grapevine, potato, and soybean (Heinecke et al., 2019).

A variety of new generations of succinate dehydrogenase inhibitor (SDHI) fungicides have been developed (Sierotzki and Scalliet, 2013; Stammler et al., 2015; Umetsu and Shirai, 2020) and sales growth of this fungicide class is evident by taking market share from QoI fungicides due to resistance issue (Phillips, 2020; Hagerty et al., 2021). In general, however, SDHI fungicides such as boscalid, fluxapyroxad and fluopyram are not effective intrinsically against diseases caused by *Colletotrichum* species with exception of penthiopyrad (Ishii et al., 2016; Liang et al., 2020). This inherent tolerance was not based on mutations in *sdhB*, *sdhC* and *sdhD* genes encoding the subunits of SDHI targets (Ishii et al., 2016). Penthiopyrad, however, inhibited the mycelial growth of *C. gloeosporioides* and *C. acutatum* sp. complexes, *C. cereale*, and *C. truncatum* isolates but lacked activity against *C. orbiculare*, the causal agent of cucurbit anthracnose. In contrast, benzovindiflupyr was found to inhibit spore germination and mycelial growth of all these *Colletotrichum* species *in vitro* and revealed high control efficacy *in planta* (Ishii et al., 2016). Benzovindiflupyr may therefore be effective against diseases caused by other *Colletotrichum* species of multiple crops than those already reported.

The objectives of this study were to (1) determine the sensitivity of mycelial growth to azoxystrobin, fludioxonil, mefentrifluconazole, benzovindiflupyr, and iminoctadine-albesilate in isolates of various *Colletotrichum* species, which had never been exposed to fungicides, (2) examine the inhibitory activity of benzovindiflupyr against mycelial growth in culture and its control efficacy against anthracnose disease of kidney bean, garland chrysanthemum, and strawberry caused by *C. lindemuthianum*, *C. chrysanthemi*, and *C. siamense*, respectively, and (3) sequence the *sdhB* genes of *Colletotrichum* isolates with differential sensitivity to boscalid and benzovindiflupyr.

2. Materials and methods

2.1. Fungal isolates and fungicides

Eighteen baseline isolates, 3 for each species of *C. gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*), *C. acutatum* sp. complex (*C. fioriniae* and *C. nymphaeae*), and *C. truncatum* (Table 1), isolated from various plants, were supplied by Genebank Project, National Agriculture and Food Research Organization (NARO), Japan. Baseline isolates to the best of our knowledge had never been exposed to selection pressure of the fungicides examined in this study. Two isolates for each of *C. lindemuthianum* from kidney bean, *C. graminicola* from corn and oat, and *C. dematium* from white mulberry and Japanese radish (Sato et al., 2005) from Genebank Project, 3 isolates of *C. chrysanthemi* from garland chrysanthemum (Misawa and Horita, 2017) and 3 isolates of *C. scovillei* from sweet pepper (Kanto et al., 2014) of the *C. acutatum* sp. complex were used (Table 1). These isolates were identified taxonomically in morphological and molecular studies based on sequences of *actin*, *tub2* (β-tubulin-2), *CHS-1* (chitin synthase 1), *GPDH* (glyceraldehyde-3-phosphate-dehydrogenase), *H3* (histon 3), and rDNA-ITS (internal transcribed spacer) regions (https://www.gene.affrc.go.jp/databases-micro_search_en.php, as of August 2021). The other 2 isolates of *C. siamense* derived from strawberry fruit grown in Florida, USA (Hu et al., 2015; Table 1).

Fungicides employed were formulations of azoxystrobin (Amistar® 20% flowable, Syngenta), fludioxonil (Savior® 20% flowable, Syngenta), mefentrifluconazole (Belanty® 34.9% flowable, BASF), benzovindiflupyr (Solatenol®10% emulsion, Syngenta), iminoctadine-albesilate (Bellkute® 30% flowable, Nippon Soda), boscalid (Cantus® 50% dry flowable SG, BASF), and penthiopyrad (Affet® 20% flowable, Mitsui Chemicals Agro). Mefentrifluconazole and benzovindiflupyr were supplied by BASF and Syngenta, respectively, and commercial formulations of the other fungicides were purchased.

2.2. Mycelial growth tests on culture medium

Isolates of various *Colletotrichum* species (Table 1) were cultured on potato dextrose agar (PDA) plates at 22 to 25 °C for 5 days in darkness. Mycelial discs, 4 mm in diameter, were cut from actively growing colony margins and transferred onto PDA plates containing each fungicide. YBA agar was used only for the SDHI fungicides benzovindiflupyr and boscalid as described previously (Ishii et al., 2016). Sensitivity to azoxystrobin was examined at 100 mg/L of active ingredient (AI) in the presence of 100 mg/L of salicylhydroxamic acid (SHAM) or 6 mM of *n*-

Table 1
List of *Colletotrichum* isolates used in this study.

Species	Isolate	Location	Source	Year of isolation	Reference
C. fructicola	MAFF 306735	Gunma, Japan	Common Hydrangea, Japanese Hydrangea	2008	Sato et al., 2013
C. fructicola	MAFF 410177	Iwate, Japan	Walnut	1950	Ito and Kobayashi, 1956
C. fructicola	MAFF 410178	Ibaraki, Japan	Japanese walnut	1950	Ito and Kobayashi, 1956
C. siamense	MAFF 239160	Kagoshima, Japan	Japanese spindle tree	2001	Sato et al., 2012
C. siamense	MAFF 239282	Kanagawa, Japan	Maidenhair tree, Ginkgo	2003	Sato et al., 2012
C. siamense	MAFF 242619	Ibaraki, Japan	Japanese mahonia	2010	Sato et al., 2013
C. tropicale	MAFF 239182	Kagoshima, Japan	Screw-pine	2001	Yumiki et al., 2008
C. tropicale	MAFF 239549	Okinawa. Japan	Litchi	2000	
C. tropicale	MAFF 240452	Okinawa. Japan	Chinese cricum	2007	Kageyama, 2010
C. fioriniae	MAFF 238654	Tokyo, Japan	*Castanopsis cuspidata* var. *sieboldii*	1996	Sato et al., 2013
C. fioriniae	MAFF 242591	Tokyo, Japan	Celery	1999	Sato et al., 2013
C. fioriniae	MAFF 306247	Chiba, Japan	Prairie gentian	1986	Sato et al., 2013
C. nymphaeae	MAFF 242590	Nagano, Japan	Celery	2009	Yamagishi et al., 2016
C. nymphaeae	MAFF 240036	Ibaraki, Japan	Japanese flowering cherry, Yoshino	2006	
C. nymphaeae	MAFF 242412	Chiba, Japan	Parsley	1987	
C. truncatum	MAFF 239896	Kagawa, Japan	Poinsettia	2004	Sato et al., 2015
C. truncatum	MAFF 305754	Tokyo, Japan	Soybean, Soya	1988	Sato et al., 2015
C. truncatum	MAFF 306552	Ibaraki, Japan	Buckwheat	1998	Sato et al., 2015
C. lindemuthianum	MAFF 305390	Tochigi, Japan	Kidney bean	1974	Sato et al., 2012
C. lindemuthianum	MAFF 510897	Tochigi, Japan	Kidney bean	Unknown	
C. graminicola	MAFF 244463	Hokkaido, Japan	Corn	2012	
C. graminicola	MAFF 305075	Saga, Japan	Oat	1966	Moriwaki et al., 2002
C. dematium	MAFF 236699	Kochi, Japan	White mulberry	1992	
C. dematium (*C. incanum*)	MAFF 238704	Miyazaki, Japan	Japanese radish	1998	Sato et al., 2015
C. siamense	FL98-139	Florida, USA	Strawberry	1998	Hu et al., 2015
C. siamense	FL99-21	Florida, USA	Strawberry	1999	Hu et al., 2015
C. chrysanthemi	GF1522	Gifu, Japan	Garland chrysanthemum	2014	
C. chrysanthemi	GF1523	Gifu, Japan	Garland chrysanthemum	2014	
C. chrysanthemi	GF1524	Gifu, Japan	Garland chrysanthemum	2014	
C. scovillei	GF1528	Gifu, Japan	Sweet pepper	2014	
C. scovillei	GF1529	Gifu, Japan	Sweet pepper	2014	
C. scovillei	GF1530	Gifu, Japan	Sweet pepper	2014	

propyl gallate (PG), an inhibitor of alternative oxidase (AOX, Ishii et al., 2009). Fludioxonil was added at 1, 10, and 100 mg/L AI and mefentrifluconazole was amended at 0.1, 0.3, 1, 3, and 10 mg/L AI. Benzovindiflupyr and boscalid were tested at 0.1, 1, 10, and 100 mg/L AI and iminoctadine albesilate was examined at 1, 10, 100, and 1000 mg/L AI. Un-amended medium was used as a control. After incubation at 22 to 25 °C in the dark for 3 days, the colony diameter (minus 4 mm for the disc) of two replicates per treatment was measured using a handheld digital caliper. For azoxystrobin, growth inhibition at 100 mg/L AI in the presence of an AOX inhibitor relative to that on untreated medium plates was calculated. For fludioxonil, growth at each fungicide concentration relative to that on untreated medium plates was calculated. The values of 50% effective concentration (EC_{50}) were calculated for mefentrifluconazole, benzovindiflupyr, boscalid, and iminoctadine albesilate by regressing percentage mycelial growth inhibition against the log of fungicide concentration using software kindly gifted by K. So, ZEN-NOH (Tokyo). Experiments were conducted two times.

2.3. Inoculation tests on host plants

To examine the spectrum of benzovindiflupyr efficacy against *Colletotrichum* species on various crops, test was conducted using kidney bean, garland chrysanthemum, and strawberry as follows:

(1) Two-leaf-stage of kidney bean (cv. Green mild, Tohoku Seed, Utsunomiya, Japan) and 4- to 6-leaf-stage of garland chrysanthemum (cv. Chuba Shungiku, Sakata Seed, Yokohama, Japan) plants in a pot were grown at 25 °C in a greenhouse under natural light conditions. After spraying the formulations of benzovindiflupyr at 200 mg/L AI and boscalid at 334 mg/L AI separately until run-off, the plants, 3 replicates for each treatment, were kept at the same greenhouse. Distilled water (DW) was used as a control. Because the isolates of *C. lindemuthianum* used did not produce conidia in this study, their mycelial discs, 4 mm in diameter, taken from actively growing colony margins were placed upside down on both sides of adaxial leaves of kidney bean, and 20 μL DW was dropped on top to avoid dryness 1 day after fungicide or DW treatment. The inoculated plants were transferred to a moist plastic box, kept at 25 °C for 2 days in the dark, and incubated at the greenhouse. Diameter of the lesion, developed on the leaves of kidney bean was measured 14 days after inoculation using a handheld digital caliper. Disease control (%) by fungicide treatment was evaluated as follows: (average length of lesions on DW-treated plants – average length of lesions on fungicide-treated plants) / (average length of lesions on DW-treated plants) × 100. Inoculation experiments were conducted twice independently.

For garland chrysanthemum, conidial suspensions (about 1×10^6 conidia/mL) of *C. chrysanthemi*, prepared from the colony on oatmeal agar plates after incubation at 25 °C for 2 weeks under fluorescent light, were spray inoculated until run-off 1 day after treatment with fungicide or DW. Penthiopyrad at 100 mg/L AI was included in the tests in addition of benzovindiflupyr at 200 mg/L AI and boscalid at 334 mg/L AI. The inoculated plants, 3 replicates for each treatment, were transferred to a moist plastic box and kept at 25 °C in the dark. The box was opened 3 days later and closed again on the following day. Seven to 8 days after inoculation, the percentage (%) of anthracnose incidence on garland chrysanthemum was calculated as follows: (number of diseased leaves / number of leaves inoculated) × 100. Disease control (%) by fungicide treatment was evaluated according to the formula: (mean incidence on DW-treated plants – mean incidence on fungicide-treated plants) / (mean incidence on DW-treated plants) × 100. Inoculation experiments were conducted twice independently.

(2) Transplants of strawberry, cvs. Earliglow and Camarosa, purchased from the commercial nursery Nourse Farm, Westborough, MA, USA, and Fresh-Pik Produce, Inc., Kenly, NC, USA,

respectively, were propagated in plastic pots with soil at a greenhouse (17.8 to 32.5 °C with the average of 23.6 °C) until use. Whole leaves and petioles of 4- to 5-leaf-stage of stolon-derived plants, 3 replicates for each treatment with one per pot, were sprayed with the formulations of benzovindiflupyr at 100 mg/L AI or DW, used as a control until run-off, and the plants kept at the same greenhouse. Conidial suspensions (about 1 to 1.5 × 10^6/mL) of the *C. siamense* isolates FL98-139 and FL99-21, suspended in decoction of healthy strawberry leaves (40 g/ L), effective to enhance conidial germination (Ishikawa, 2005), were spray-inoculated to petioles until run-off 1 day after preventative treatment. The inoculated plants were rapped with clean plastic bags individually, placed in a moist plastic box then kept at a growth room maintained from 18.4 to 29.5 °C with the average of 23.9 °C under fluorescent light. The bags were removed 2 days after inoculation and the plants transferred onto a bench at the same growth room until disease assessment 7 and 14 days after inoculation. Anthracnose symptoms were rated according to level of the disease development on petioles (Miller-Butler et al., 2018) as: 0 = no visible lesions, 1 ≤3 mm long petiole lesion, 2 = 3 to 10 mm long petiole lesion, 3 ≥10 to 20 mm long petiole lesion, 4 ≥20 mm long petiole lesion, 5 = leaf wilted, and 6 = dead. Disease severity (DS) was calculated as $(6A + 5B + 4C + 3D + 2E + 1F)/6G \times 100$ where *A*, *B*, *C, D, E and F* is the number of petioles corresponding to the scales 6, 5, 4, 3, 2, and 1, respectively, and *G* is the total number of petioles assessed. Disease control (%) by fungicide treatment was evaluated according to the formula: (mean DS on DW-treated plants – mean DS on benzovindiflupyr-treated plants) / (mean DS on DW-treated plants) × 100. The tests were conducted four times for cv. Earliglow and one time for Camarosa independently.

2.4. Sequence analysis of sdhB genes

Sequence of the *sdhB* genes encoding the subunit of the SDHI fungicide-targeted succinate dehydrogenase was analyzed by sequencing PCR-amplified products. The isolates of *C. lindemuthianum* (MAFF305390 and MAFF510897), *C. chrysanthemi* (GF1522 and GF1523), and *C. scovillei* (GF1528, GF1529, and GF1530), shown in Table 1, were cultured on PDA plates at 25 °C, and total DNA extracted as described by Saitoh et al. (2006) with slight modifications (Ishii et al., 2016). To amplify the *sdhB* gene fragments from total DNA, the primers SDHMF-1 (forward): 5′-TGYCCVTCBTACTGGTGGAA-3′ (Sakurai, 2007) and KES729 (reverse): 5′-CTTCTTRATCTCVGCRATVGCC-3′ (Miyamoto et al., 2010), synthesized by TSUKUBA ORIGO SERVICE (Ushiku, Japan), were used. 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, USA). PCR reactions were performed in a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany) or a PCR Thermal Cycler Dice Touch (Takara Bio, Kusatsu, Japan) programmed for 1 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 48 °C, 2 min at 72 °C, a final extension for 10 min at 72 °C and holding at 4 °C or 8 °C. PCR products were separated by electrophoresis on a 1.5% agarose gel in 89 mM Tris-borate (pH 8.2) + 2 mM EDTA (TBE) buffer or 40 mM Tris-acetate (pH 8.0) + 1 mM EDTA (TAE), stained with GelRed™ (Biotium, Hayward, CA, USA), and cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the instructions supplied by the manufacturer. Sequencing was conducted at Macrogen Japan Corp. (Kyoto, Japan) using the same primers employed for PCR. After sequencing in both directions, the nucleotide sequences were analyzed with the National Center for Biotechnology Information (NCBI)/GenBank database using basic local alignment search tools (BLAST).

2.5. Statistical analysis

The 95% confidence interval (95% CI = ±1.96 × SE) of mean values, calculated by Excel, was compared. When the 95% CI of the mean does not overlap, there is a statistical difference between treatments at $p = 0.05$ level.

3. Results

3.1. Fungicide sensitivity of baseline isolates

The isolates, 3 for each species, which had never been exposed to fungicides showed high sensitivity to the QoI fungicide azoxystrobin in the presence of the AOX inhibitors SHAM or PG. Mean mycelial growth inhibition of azoxystrobin at 100 mg/L, supplemented with 100 mg/L SHAM, ranged from 52.0% for the isolates of *C. truncatum* to 73.5% for those of *C. nymphaeae* but no difference was shown in azoxystrobin sensitivity between the isolates of *C. gloeosporioides* (*C. fructicola*, *C. siamense*, and *C. tropicale*) and *C. acutatum* (*C. fioriniae* and *C. nymphaeae*) sp. complexes (Fig. 1). Mycelial growth of all the isolates was completely inhibited at 100 mg/L azoxystrobin in the presence of 6 mM PG irrespective of species difference (Fig. 1). For the phenylpyrrole fungicide fludioxonil, the 3 isolates of *C. truncatum* and *C. nymphaeae* were highly sensitive, and null or only limited growth was observed at 1 mg/L (Fig. 2). In contrast, isolates of the other 4 species *C. fructicola*, *C. siamense*, *C. tropicale* and *C. fioriniae* grew even at 100 mg/L. However, among these species, the isolates of *C. fioriniae* were more sensitive than those of *C. fructicola* and *C. siamense*. Moreover, there was a statistical difference in fludioxonil sensitivity between the 2 species complexes, *C. gloeosporioides* (*C. fructicola* and *C. siamense*) and *C. acutatum* (*C. fioriniae* and *C. nymphaeae*) (Fig. 2).

The SDHI fungicide benzovindiflupyr revealed very high activity to inhibit mycelial growth with mean EC_{50} values less than 0.1 mg/L for all the isolates regardless of their species difference among *C. gloeosporioides* and *C. acutatum* sp. complexes, and *C. truncatum*. The growth was substantially reduced at 1 mg/L (Fig. 3) and completely stopped at 10 mg/L. The DMI fungicide mefentrifluconazole strongly inhibited mycelial growth of the isolates belonging to *C. gloeosporioides* and *C. acutatum* sp. complexes. The mean EC_{50} value of mefentrifluconazole was 1.25, 1.83, 1.92, 0.81, and 0.48 mg/L for the 3 isolates of *C. fructicola*, *C. siamense*, *C. tropicale*, *C. fioriniae*, and *C. nymphaeae*, respectively (Fig. 4). The isolates of *C. nymphaeae* were more sensitive than those of *C. siamense* and *C. tropicale* significantly. Furthermore, the isolates of *C. fioriniae* were more sensitive significantly than those of *C. tropicale*. *C. truncatum* isolates also showed high sensitivity to mefentrifluconazole except for the isolate MAFF 239896. For the bisguanidine fungicide iminoctadine-albesilate (Fig. 5), the isolates of *C. fioriniae* and *C. nymphaeae* were inherently resistant and thus it was not possible for these isolates to estimate an EC_{50} within the range of concentrations applied. In contrast, the isolates of *C. fructicola*, *C. tropicale*, and *C. truncatum* were sensitive with mean EC_{50} value of 0.00, 0.62, and 2.53 mg/L, respectively. The EC_{50} value for the two isolates of *C. siamense* was 3.60 and 0.00 mg/L, respectively, but the isolate MAFF 242619 was less-sensitive to iminoctadine-albesilate with EC_{50} value higher than 100 mg/L (Fig. 5).

3.2. Boscalid and benzovindiflupyr sensitivity of other Colletotrichum isolates in culture

The isolates of *C. lindemuthianum*, *C. graminicola*, *C. dematium*, *C. chrysanthemi*, and *C. scovillei* were all insensitive to boscalid with EC_{50} values higher than 100 mg/L. On the contrary, these isolates revealed high sensitivity to benzovindiflupyr. Mycelial growth was completely

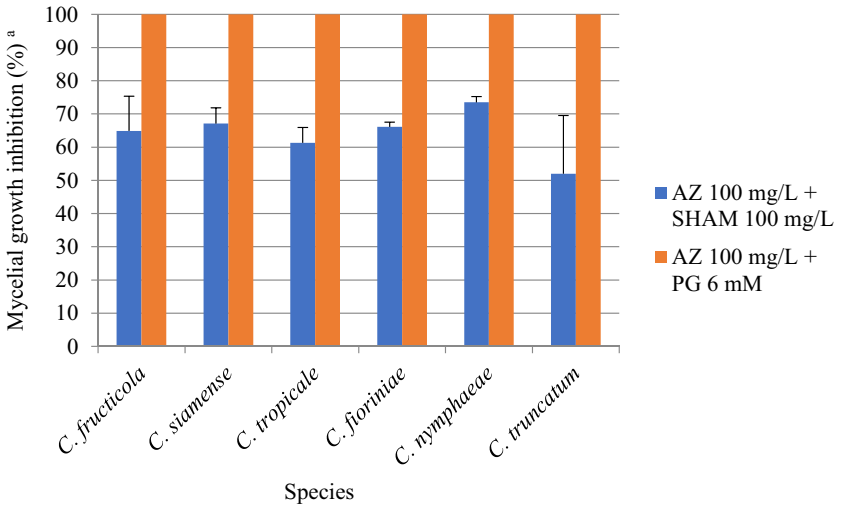

Fig. 1. Mycelial growth inhibition (%) against the isolates of *Colletotrichum gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*), *C. acutaum* sp. complex (*C. fiorinuae* and *C. nymphaeae*), and *C. truncatum*, 3 for each, which had never been exposed to fungicides, on potato dextrose agar plates amended with azoxystrobin (Amistar® 20% flowable) at 100 mg/L AI in the presence of 100 mg/L salicylhydroxamic acid (SHAM) or 6 mM *n*-propyl gallate (PG). [a]Mean values and their corresponding 95% CIs.

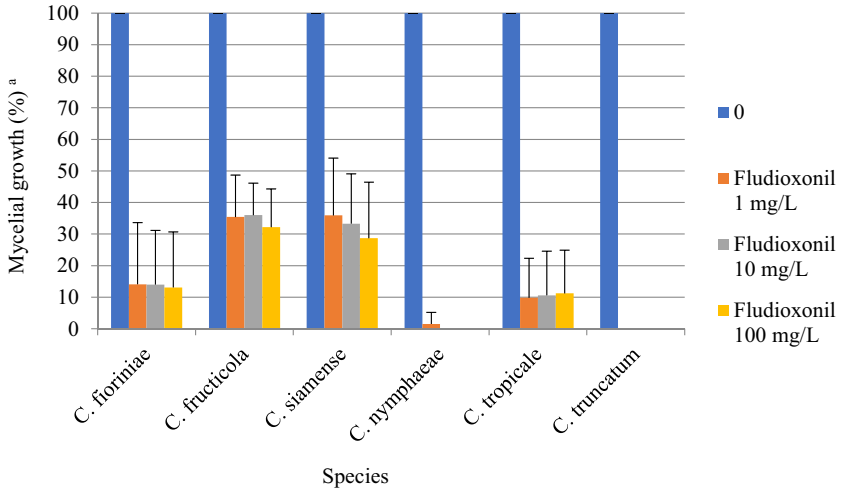

Fig. 2. Mycelial growth (%) of the isolates of *Colletotrichum gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*), *C. acutaum* sp. complex (*C. fioriniae* and *C. nymphaeae*), and *C. truncatum*, 3 for each, which had never been exposed to fungicides, on potato dextrose agar plates amended with fludioxonil (Savior® 20% flowable) at 0, 1, 10, and 100 mg/L AI. [a]Mean values and their corresponding 95% CIs.

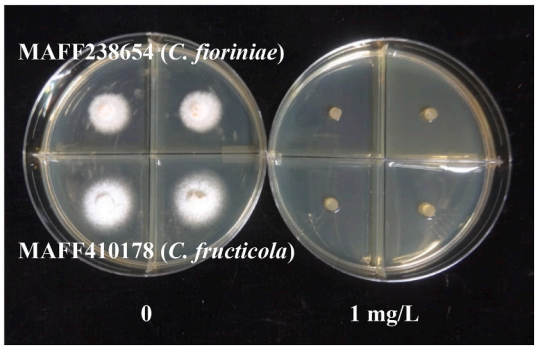

Fig. 3. Inhibition of mycelial growth of the isolate MAFF238654 (*Colletotrichum fioriniae*) and MAFF410178 (*C. fructicola*), which had never been exposed to fungicides, on YBA agar medium amended with 1 mg/L AI of benzovindiflupyr (Solatenol®10% emulsion). The photo was taken 3 days after incubation.

inhibited by benzovindiflupyr at 0.1 mg/L, the minimum concentration tested, for *C. lindemuthianum*, *C. graminicola*, and *C. dematium*, and the mean EC_{50} value for benzovindiflupyr was 0.069 mg/L ranging from 0.048 to 0.103 mg/L and 0.014 mg/L ranging from 0.001 to 0.039 mg/L for *C. chrysanthemi* and *C. scovillei*, respectively.

3.3. Efficacy of boscalid, penthiopyrad, and benzovindiflupyr against anthracnose in planta

The efficacy of boscalid against kidney bean anthracnose caused by *C. lindemuthianum* isolates was null at 334 mg/L whereas benzovindiflupyr controlled the disease by 85.2 ± 29.11% (mean and 95% CI) following preventative sprays at 200 mg/L (Fig. 6). On garland chrysanthemum, high incidence of anthracnose, 94.9 ± 10.09% and 86.7 ± 0.00%, was recorded for boscalid at 334 mg/L and penthiopyrad at 100 mg/L, respectively, under severe disease pressure as indicated by disease incidence of 86.0 ± 17.05% on DW-treated plants after inoculation with the *C. chrysanthemi* isolates. In contrast, disease incidence was 12.0 ±

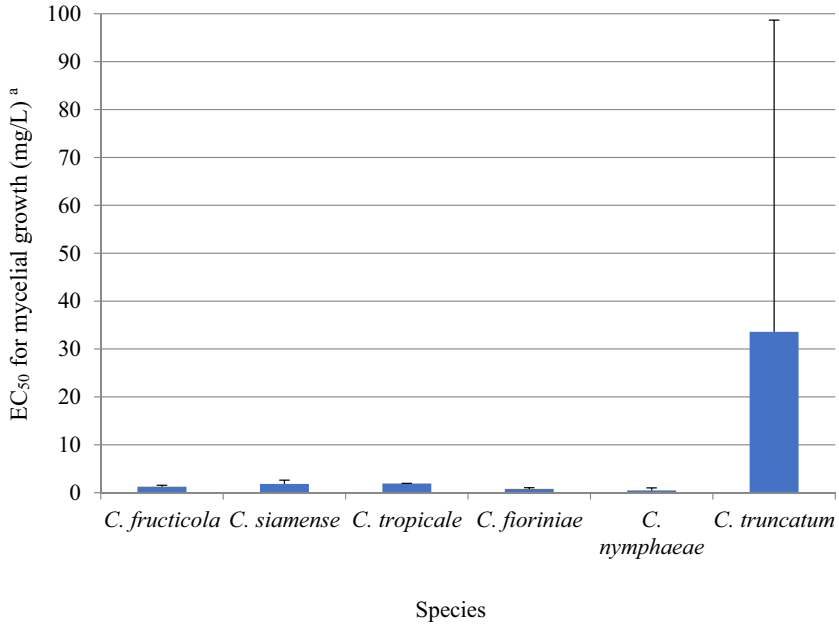

Fig. 4. 50% effective concentration (EC_{50}) AI for mefentrifluconazole (Belanty® 34.9% flowable) against mycelial growth of the isolates of *Colletotrichum gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*), *C. acutaum* sp. complex (*C. fioriniae* and *C. nymphaeae*), and *C. truncatum*, 3 for each, which had never been exposed to fungicides, on potato dextrose agar plates. [a]Mean values and their corresponding 95% CIs.

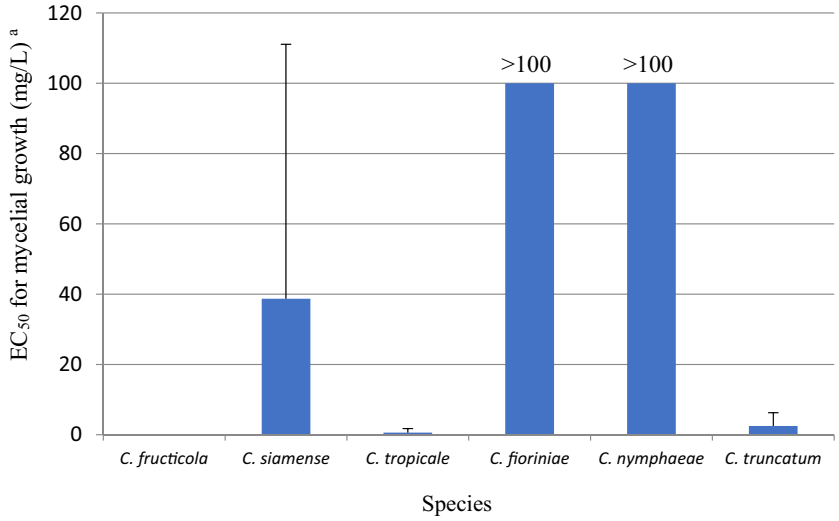

Fig. 5. 50% effective concentration (EC_{50}) AI for iminoctadine-albesilate (Bellkute® 30% flowable) against mycelial growth of the isolates of *Colletotrichum gloeosporioides* sp. complex (*C. fructicola*, *C. siamense*, and *C. tropicale*), *C. acutaum* sp. complex (*C. fioriniae* and *C. nymphaeae*), and *C. truncatum*, 3 for each, which had never been exposed to fungicides, on potato dextrose agar plates. [a]Mean values and their corresponding 95% CIs.

2.55% on plants treated with benzovindiflupyr at 200 mg/L (Fig. 7). These differences in anthracnose control were significant statistically.

For strawberry anthracnose caused by the *C. siamense* isolates, preventative sprays of benzovindiflupyr at 100 mg/L, one day before inoculation, exhibited 100% control efficacy in the experiments conducted four times using cv. Earliglow (Table 2). The mean DS on DW-treated plants of this cultivar was 25.6 ranging from 20.8 to 34.1 and 35.6 ranging from 29.8 to 46.2 at 7 days and 14 days after inoculation, respectively. When spraying benzovindiflupyr at 100 mg/L, one day before inoculation preventatively, 100% disease control was also achieved on cv. Camarosa at 7 days and 14 days after inoculation under lower disease pressure (Table 2).

3.4. Sequence analysis of sdhB genes

Nucleotide sequences of *sdhB* genes, encoding fungicide-binding SDH subunit proteins, were analyzed. When compared with NCBI GenBank XP_036585698.1, the *sdhB* sequence of *C. truncatum*, sequences of the 2 *C. lindemuthianum* isolates used in this study showed 98.0% and 100% identity. Sequences of the 2 *C. chrysanthemi* isolates and 3 *C. scovillei* isolates used in the present study were 100% identical to the sequence of *C. scovillei* (XP_035335452.1). Partial sequences of deduced amino acids of the sdhB were compared with those of TDZ13144.1 (from *C. spinosum*) and XP_035335452.1 (from *C. scovillei*). Amino acids P202, N207 and H249, homologous to the wild-type P225, N230 and H272 in

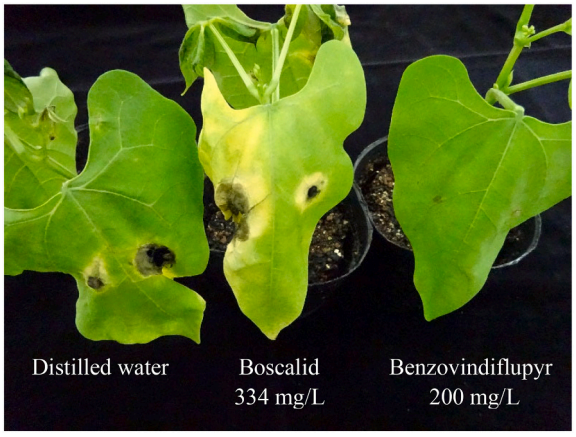

Fig. 6. Control efficacy of boscalid (Cantus® 50% dry flowable SG, 334 mg/L AI) and benzovindiflupyr (Solatenol®10% emulsion, 200 mg/L AI) against anthracnose disease caused by *Colletotrichum lindemuthianum* on kidney bean. The photo was taken 14 days after inoculation.

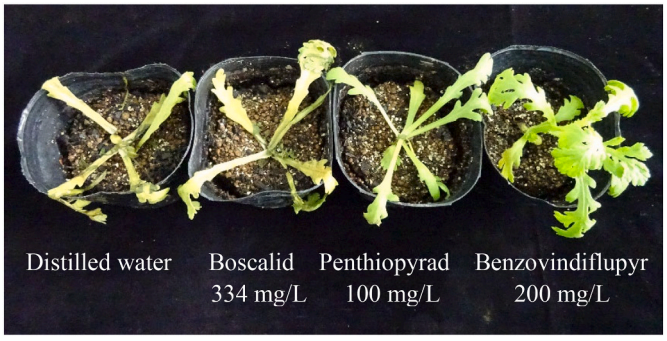

Fig. 7. Control efficacy of boscalid (Cantus® 50% dry flowable SG, 334 mg/L AI), penthiopyrad (Affet® 20% flowable, 100 mg/L AI), and benzovindiflupyr (Solatenol®10% emulsion, 200 mg /L AI) against anthracnose disease caused by *Colletotrichum chrysanthemi* on garland chrysanthemum. The photo was taken 7 days after inoculation.

Botrytis cinerea, were conserved in all the isolates compared and no mutations known to be associated with SDHI resistance in other fungi (Sierotzki and Scalliet, 2013; Stammler et al., 2015) were found (Fig. 8).

4. Discussion

In this study, mycelial growth of all the isolates of *C. gloeosporioides* and *C. acutatum* sp. complexes tested was completely inhibited by 100 mg/L azoxystrobin with 6 mM PG and judged as sensitive (Yokosawa et al., 2017). No difference was thus observed in the inherent sensitivity to QoI azoxystrobin for the isolates of these species, which had never been exposed to fungicides. In citrus, mean EC_{50} values of the QoI trifloxystrobin were as low as 0.14 μg/mL against *C. acutatum* baseline isolates (Gama et al., 2020). Yokosawa et al. (2017) suggested that the frequency of developing fungicide resistance is species-specific because isolates of *C. fructicola*, the dominant species causing apple bitter rot in Nagano Pref., Japan, often developed resistance to QoI fungicides, whereas isolates of other species such as *C. siamense* remained sensitive. It is possible that a higher number of resistant isolates remains in large populations of *C. fructicola* after fungicide selection (Yokosawa et al., 2017). *In vitro* azoxystrobin sensitivity of *C. siamense* and *C. fructicola* isolates showed resistance to the fungicide with EC_{50} values higher than 100 mg/L (Zhang et al., 2020). Furthermore, the mean EC_{50} value of azoxystrobin differed largely depending on the *Colletotrichum* species collected from apple: 55.7, 11.6, 0.14, and 0.06 μg/mL for *C. fructicola*, *C. siamense, C. fioriniae*, and *C. nymphaeae*, respectively (Kim et al., 2020). The latter studies, however, did not use AOX inhibitors and thus results may differ from ours. Again, the effect of AOX on QoI sensitivity may be species-specific. Mondal et al. (2005) described that inclusion of SHAM in the medium did not greatly affect azoxystrobin sensitivity of the mycelial growth of fungi including *C. acutatum*. For *C. gloeosporioides*, Inada et al. (2010) observed that both resistant and sensitive isolates grew over 50% at 100 mg/L of azoxystrobin on plates not containing SHAM in comparison to the growth on control plates clearly indicating usefulness of AOX inhibitors in QoI sensitivity tests. In the current study, growth of isolates belonging to the 5 species in *C. gloeosporioides* and *C. acutatum* sp. complexes was inhibited more than 50% by the treatment with 100 mg/L of azoxystrobin plus 100 mg/L

Table 2
Control efficacy of benzovindiflupyr at 100 mg/L AI against strawberry anthracnose in growth room experiments.

Experiment No.	Fungal species	Strawberry cultivar	Days after inoculation	Disease control (%) based on severity [a]	Disease severity on DW-treated plants
1	*C. siamense*	Earliglow	7	100	34.1 ± 37.42 [b]
			14	100	32.6 ± 26.05
2	*C. siamense*	Earliglow	7	100	20.8 ± 21.61
			14	100	46.2 ± 33.83
3	*C. siamense*	Earliglow	7	100	25.2 ± 12.90
			14	100	29.8 ± 22.90
4	*C. siamense*	Earliglow	7	100	22.1 ± 18.27
			14	100	33.7 ± 14.05
5	*C. siamense*	Camarosa	7	100	10.7 ± 3.59
			14	100	14.7 ± 4.75

[a] Disease severity (DS) was calculated as $(6A + 5B + 4C + 3D + 2E + 1F)/6G \times 100$ where *A*, *B*, *C*, *D*, *E* and *F* is the number of petioles corresponding to the scales 6, 5, 4, 3, 2, and 1, respectively, and *G* is the total number of petioles assessed. Disease control (%) by fungicide treatment was evaluated according to the formula: (mean DS on DW-treated plants – mean DS on benzovindiflupyr-treated plants) / (mean DS on DW-treated plants) × 100.
[b] Mean and 95% CI values.

MAFF305390 (*C. l.*) [a]:201 C**P**SYWW**N**SEEYLGPAILLQSYRWLADSRDERKAERKAALDNSMSLYRC**H**T 250
GF1522 (*C. c.*): 201 C**P**SXWW**N**SEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRC**H**T 250
GF1523 (*C. c.*): 201 C**P**SYWW**N**SEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRC**H**T 250
GF1528 (*C. s.*): 201 C**P**SYWW**N**SEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRC**H**T 250
GF1529 (*C. s.*): 201 C**P**SYWW**N**SEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRC**H**T 250
GF1530 (*C. s.*): 201 C**P**SYWW**N**SEEYLGPAILLQSYRWLADSRDQKKAERKAALDNSMSLYRC**H**T 250

Fig. 8. The partial sequences of deduced amino acids (positions 201 to 250) of sdhB in 6 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 *Botrytis cinerea* and responsible for boscalid resistance (Stammler et al., 2015), are indicated in bold characters with underlines. [a] *C. l.*, *Colletotrichum lindemuthianum*; *C. c.*, *C. chrysanthemi*; *C. s.*, *C. scovillei*.

SHAM. The inhibition was slightly lower for *C. truncatum* isolates but the difference was not significant statistically.

The baseline fludioxonil sensitivity was assessed for *C. acutatum* isolates previously (Gao et al., 2018). The EC_{50} values based on mycelial growth inhibition on PDA plates ranged from 0.011 to 0.080 μg/mL with an average of 0.031 μg/mL and resistance risk of *C. acutatum* to fludioxonil was considered low to medium. Recently, Schnabel et al. (2021) reported that the American isolates of *C. acutatum* sp. complex were sensitive but those of *C. gloeosporioides* sp. complex were inherently tolerant to fludioxonil in culture. This phenomenon may be important in practice because fludioxonil is sprayed to strawberry plants in a mixture with the anilinopyrimidine fungicide cyprodinil in the United States. Fludioxonil is also registered as a sole product for strawberry anthracnose control in Japan where *C. gloeosporioides* is predominant in the populations. In the present study, the isolates of *C. truncatum* and *C. nymphaeae* were highly sensitive to fludioxonil but those of the other four species were less sensitive. Particularly, the isolates of *C. fructicola* and *C. siamense* were less sensitive than those of *C. fioriniae*. There was a statistical difference in fludioxonil sensitivity between the two species complexes, *C. gloeosporioides* (*C. fructicola* and *C. siamense*) and *C. acutatum* (*C. fioriniae* and *C. nymphaeae*) except *C. tropicale* in the former sp. complex. Efficacy of fludioxonil needs to be confirmed in the field in future experiments.

The mode of action of iminoctadine-albesilate, the bis-guanidine fungicide, is thought to be disruption of membrane function and lipid biosynthesis by the Fungicide Resistance Action Committee (FRAC), the Ministry of Health, Labour and Welfare, Japan (https://www.mhlw.go.jp/content/11120000/000556143.pdf) as of August 2021 and others (Zhu et al., 2019). This protectant fungicide has broad spectrum of efficacy and is widely sprayed to control various diseases including anthracnose, powdery mildew, scab, brown rot, gray mold and many others in Japan. Iminoctadine-albesilate has also been shown to be effective against *Corynespora cassiicola*, the cause of Corynespora leaf spot on cucumber (Zhu et al., 2019) with relatively low risk of resistance. For this reason, it is frequently used in the field as solo product or a tank mixed with single-site inhibitors such as DMI, QoI, and SDHI fungicides. In this study, differential intrinsic sensitivity to iminoctadine-albesilate was confirmed between *C. gloeosporioides* and *C. acutatum* sp. complexes and *C. truncatum*. The 11 out of 12 isolates of *C. fructicola*, *C. siamense*, and *C. tropicale* in *C. gloeosporioides* sp. complex and *C. truncatum* were sensitive. In contrast, the 6 isolates of *C. fioriniae* and *C. nymphaeae*, belonging to *C. acutatum* sp. complex, were insensitive to iminoctadine-albesilate. These results were consistent with the early reports which indicated insensitivity (natural resistance) of *C. acutatum* sensu lato to the related fungicide iminoctadine-acetate (Fukaya, 2002; Chung et al., 2006).

To avoid pathogen resistance, multi-site fungicides are important because they possess a broad control spectrum and low risk for resistance development in general (Thind and Hollomon, 2018; Phillips, 2020; Reis et al., 2021). However, regulation of the conventional multi-site fungicides such as chlorothalonil and mancozeb has become a concern (Jones et al., 2020; Uppala et al., 2020; Government of Canada, 2020; Lynxee Consulting, 2021). Moreover, many of the existed DMI fungicides are now subjected to stricter regulation in the EU especially due to their suspected endocrine disrupting properties. Despite that, mefentrifluconazole, a new generation of DMI fungicide, possesses low risk to human and environment (Tesh et al., 2019). Mefentrifluconazole has high potential to control many pathogens including *Colletotrichum* species (Ishii et al., 2021; Li et al., 2021; Gao et al., 2021) and may become an alternative of existed DMIs in the future. Although cross-resistance was clearly proven between this fungicide and the other DMI fungicides in our previous tests (Ishii et al., 2021), *Colletotrichum* isolates are basically sensitive to mefentrifluconazole as shown in this study, suggesting its useful role in a chemical management program.

Benzovindiflupyr is a third generation of SDHI carboxamides (Guicherit et al., 2013; Bradley et al., 2020) with great potential due to its high activity (Gao et al., 2020; Ishii et al., 2020) and is expected to become Syngenta's best-selling fungicide (Dang and Liu, 2017). This fungicide was found to be effective against *C. gloeosporioides* and *C. acutatum* sp. complexes on peach fruit as well as on cucumber plants inoculated with *C. orbiculare* (Ishii et al., 2016). Chechi et al. (2019) reported that isolates of *C. fructicola* and *C. siamense* were sensitive to benzovindiflupyr. Subsequently, benzovindiflupyr was shown to be more effective than penthiopyrad on *Colletotrichum* species isolated from persimmon in Brazil (Carraro et al., 2020). In our current study, the activity of benzovindiflupyr was greater against *Colletotrichum* isolates than boscalid and penthiopyrad irrespective of the species. These included *C. lindemuthianum*, *C. graminicola*, *C. dematium*, *C. chrysanthemi*, and *C. scovillei*, which had never been tested before against this SDHI. Additionally, benzovindiflupyr revealed high protective activity against *C. siamense* isolates on young strawberry plants. It may suggest the usefulness of this fungicide for anthracnose control in nursery plants, the primary source of inoculum for field infections (Peres et al., 2005; Forcelini and Peres, 2018). Oliveira et al. (2020) determined baseline sensitivity of the *C. gloeosporioides* isolates *in vitro* using a spiral gradient dilution method and confirmed its superior inhibitory activity compared to penthiopyrad. It is worthy of notice that high control efficacy of benzovindiflupyr on strawberry anthracnose was demonstrated in field trials (Oliveira et al., 2020). Result from the present study is strengthening our previous hypothesis (Ishii et al., 2016) that the broad-spectrum efficacy of benzovindiflupyr within the *Colletotrichum* genus is helpful when designing control strategies against various pathogens on a wide range of crops. However, resistance to benzovindiflupyr has already been reported in other diseases such as Asian soybean rust, caused by *Phakopsora pachyrhizi* (Simões et al., 2018; Müller et al., 2021; Reis et al., 2021) and *Z. tritici* (Hagerty et al., 2021). Thus, resistance management is of upmost importance. Furthermore, possible eco-toxicity of benzovindiflupyr on non-targeted soil organisms such as earthworms (Yao et al., 2021) will need to be substantiated and considered.

In *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot in various plants, differential antifungal activity of SDHI fungicides was suggested to be mainly due to the affinity and binding characteristics of the fungicides to the SDH enzyme (Gao et al., 2020). Therefore, to investigate the superior activity of benzovindiflupyr to boscalid, sequences of the *sdhB* gene encoding the subunits of fungicide-targeted SDH were analyzed in this study using isolates of the 3 *Colletotrichum* species, *C. lindemuthianum*, *C. chrysanthemi*, and *C. scovillei*. No mutations known to be associated with SDHI resistance in other fungi (Stammler et al., 2015; Klappach and Stammler, 2019) were found. The results were consistent with those of our earlier study in which *C. gloeosporioides*, *C. acutatum*, *C. orbiculare*, *C. truncatum*, and *C. cereale* were investigated (Ishii et al., 2016). The docking study conducted by Gao et al. (2020) showed stronger binding affinity of benzovindiflupyr and fluxapyroxad to the SDH enzyme complex than boscalid and fluopyram in *S. sclerotiorum*. These results offer an explanation for the superior activity of benzovindiflupyr against *Colletotrichum* species but only partially because *Colletotrichum* species are insensitive (naturally resistant) to boscalid, fluopyram, and fluxapyroxad (Ishii et al., 2016). Other mechanism(s) leading to the superior activity of benzovindiflupyr against *Colletotrichum* species are yet to be uncovered. Non-target site resistance mechanisms such as involvement of drug efflux transporters (Yamashita and Fraaije, 2017) and detoxification (Sang et al., 2018) may apply (Hu and Chen, 2021).

5. Conclusions

The isolates of both *C. gloeosporioides* and *C. acutatum* sp. complexes, which had never been exposed to fungicides, revealed high sensitivity to azoxystrobin. This was in clear contrast to their differential sensitivity to the other class of fungicides, i.e., the isolates of *C. gloeosporioides* (*C. fructicola* and *C. siamense*) were less sensitive to fludioxonil than

those of *C. acutatum* (*C. fioriniae* and *C. nymphaeae*). High intrinsic difference was further shown for iminoctadine-albesilate. Isolates of *C. gloeosporioides* sp. complex were sensitive but those of *C. acutatum* sp. complex were naturally resistant. Mefentrifluconazole, a new generation of DMI fungicide, was effective against *Colletotrichum* isolates regardless of the species. Excellent activity against anthracnose was demonstrated for benzovindiflupyr when kidney bean, garland chrysanthemum, and strawberry plants were inoculated with the pathogen. Mechanisms of the insensitivity (natural resistance) to boscalid in *Colletotrichum* species and the high performance of benzovindiflupyr are yet to be explored.

Declaration of Competing Interest

None.

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