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Baseline Sensitivity of *Botryosphaeria* spp. Isolated from Apples to Pyraclostrobin in Korea

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The genus *Botryosphaeria* cause white rot disease on apple trees, and control of this pathogens were primary relied on the fungicide applications. To investigate the pyraclostrobin sensitivity of *Botryosphaeria* spp. in Korea, 329 isolates were collected from eight regions between 2005 and 2023. Phylogenetic analysis based on the concatenated sequences of internal transcribed spacer, *tef*1, and *tub*2 revealed *B. sinensis* (287 out of 329 isolates) and *B. kuwatsukai* (42 out of 329 isolates). EC_{50} values of isolates ranged from 0.01 to 34.16 µg/ml (average, 3.03 µg/ml). Mean EC_{50} values and frequency distributions were similar among isolate groups, indicating no significant differences in sensitivity. Twenty less-sensitive and 20 sensitive isolates were selected and their *cytochrome b* (*cyt b*) genes analyzed, revealing no mutations in codons 129, 137, and 143. Whole gene sequencing revealed three distinct *cyt b* gene structures among *Botryosphaeria* spp., and all strains, including those with different EC_{50} values and species, showed consistent amino acid sequences. Furthermore, control efficacy on pyraclostrobin-treated apple fruits indicated no significant differences between the five least sensitive and five most sensitive isolates. These results provide the baseline sensitivity of *Botryosphaeria* spp. to pyraclostrobin and highlight the structural characteristics of their *cyt b* gene. In conclusion, the assessment of *Botryosphaeria* isolates from various regions in Korea revealed no evidence of resistance to pyraclostrobin so far. However, the risk of resistance of *Botryosphaeria* populations still exists so it is assumed that continuous monitoring of risk assessment is necessary for *Botryosphaeria* in Korea.

Keywords **:** *Botryosphaeria* spp., *cytochrome b* gene, QoI fungicide

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Apple (*Malus* × *domestica* Borkch.) is one of the most economically important and widely cultivated fruit crops in the world (Food and Agriculture Organization of the United Nations, 2023). As of 2023, the apple cultivation area in Korea covered 33,789 hectares, accounting for 21.3% of the overall fruit cultivation area (Statistics Korea, 2023). However, various diseases affecting apple yield and quality are major threats in apple orchards (Fan et al., 2022). The genus *Botryosphaeria* is associated with stem canker, fruit rot, and decline in apples, adversely affecting yields in major producing regions around the world, including the United States, China, and Korea (Jurick et al., 2013; Lee et al., 2023b; Xu et al., 2015). This fungal pathogen is capable of infecting the branches and fruits of apple trees and causes

fruit decay during storage (Lee et al., 2023a; Marsberg et al., 2017). Currently, the application of fungicides remains the primary method of controlling white rot caused by genus *Botryosphaeria* on apples (Lee et al., 2023a; Wang et al., 2022). In Korea, various systemic or translaminar fungicides have been registered to control white rot disease on apples, including benzimidazole, sterol demethylation inhibitors, and quinone outside inhibitor (QoI) fungicides (Rural Development Administration, 2024). However, QoI fungicides, including kresoxim-methyl, azoxystrobin, and pyraclostrobin, possess a high risk for the emergence of resistance due to their single-site mode of action (Fungicide Resistance Action Committee, 2024). Indeed, although QoIs serve as broad-spectrum fungicides, various plant pathogens, including *Botrytis cinerea*, *Alternaria* sp., and *Colletotrichum acutatum*, have shown resistance with single amino acid substitutions, F129L and G143A, in the *cytochrome b* (*cyt b*) gene (Banno et al., 2009; Kim et al., 2019; Olaya et al., 2017). However, no new information has been reported on *Botryosphaeria* spp. resistance since a 2019 pyraclostrobin sensitivity assessment was performed in China (Fan et al., 2019), and there have also been no studies documenting sensitivity to QoI fungicides in Korea. Therefore, an evaluation of baseline sensitivity will provide foundational information for further resistance monitoring of *Botryosphaeria* populations in Korea (Russell, 2002). The current study assessed the sensitivity of *Botryosphaeria* spp. on pyraclostrobin-amended media and compared the EC_{50} distribution among isolate groups. Additionally, the whole *cyt b* gene was analyzed for the detection of amino acid substitutions associated with QoI resistance, and the effectiveness of fungicides on apple fruits was determined.

Materials and Methods

Fungal isolates. Including isolates from a previous study (Lim et al., 2024), mono-conidial isolates of 329 *Botryosphaeria* spp. were derived from infected apple trees and fruits collected from 2005 to 2023 in Korea (Supplementary Table 1). Sampling covered various apple trial sites in Gyeongbuk, Gyeongnam, Chungbuk, Chungnam, Jeonbuk, Jeonnam, Gangwon, and Gyeonggi provinces. All isolates were cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25°C and stored at −80°C in a 25% glycerol solution.

***In vitro* assay for the evaluation of *Botryosphaeria* spp. isolate sensitivity to pyraclostrobin.** *Botryosphaeria* strains were precultured on PDA media and incubated at 25°C for 5 days. To evaluate hyphal growth rate when challenged with pyraclostrobin, mycelial disks (4 mm in diameter) were cut from the edge of precultured fungal colonies and inoculated onto PDA media amended with pyraclostrobin WG (a.i. 20%) at 0.016, 0.08, 0.4, 2, or 10 μg/ml. All pyraclostrobin concentrations were tested in the presence of salicylhydroxamic acid at 100 μg/ml. The experiment was conducted in triplicate for each isolate. After incubation for 6 days at 25°C, the mean diameter minus the diameter of inoculation disk was measured at two perpendicular axes using a digital caliper (Mitutoyo, Kawasaki, Japan). The EC_{50} (effective concentration for 50% inhibition) values for each isolate were determined using the "log/logit dose response" parameter of the GraphPadPrism software (version 10.2.3, GraphPad Software, La Jolla, CA, USA) (Pugliese et al., 2018). Twenty isolates with the lowest EC_{50} values were designated as sensitive, and 20 isolates with the highest EC_{50} values were designated as less-sensitive for, and their sensitivity to three additional QoI fungicides, azoxystrobin WP (a.i. 10%), kresoxim-methyl WG (a.i. 50%), and trifloxystrobin WG (a.i. 50%), was evaluated.

PCR amplification, sequencing, and molecular phylogenetic analysis. The total genomic DNA was extracted from mycelia cultured on PDA using HiGene Genomic DNA prep kits (BIOFACT, Daejeon, Korea) according to the manufacturer's protocol. Molecular identification of the strains was conducted by phylogenetic analysis based on the concatenated nucleotide sequences of the internal transcribed spacer (ITS) regions and the translation elongation factor 1-alpha (*tef*1) and beta-tubulin (*tub*2) genes using the neighbor-joining method in the MEGA 11.0. program (Tamura et al., 2021). The ITS regions, *tef*1, and *tub*2 genes were amplified using the primer sets ITS1F/ITS4 (Gardes and Bruns, 1993; White et al., 1990), EF1-668F/EF1-1251R (Alves et al., 2008), and Bt2a/Bt2b (Glass and Donaldson, 1995). The amplified PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Macrogen Co. Ltd. (Daejeon, Korea).

PCR amplification and sequencing of the whole *cyt b* gene. Genomic DNA of the 40 less-sensitive and sensitive *Botryosphaeria* strains were used for the detection of F129L, G137R, and G143A mutations in the *cyt b* gene using the six different primers (Supplementary Table 2). In addition, the full-length *cyt b* gene of two less-sensitive and two sensitive strains was amplified using 24 primers for the detection of additional amino-acid substitutions and

analysis of the *cyt b* gene structure (Supplementary Table 2). All primer pairs were designed based on the mitochondrion sequence of *Botryosphaeria* spp. (accession nos. KY801668, MG593780, and MG593782). The amplification was conducted using an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min and 30 s and a final extension at 72°C for 10 min. Purification and sequencing were performed as described above. The sequence data were analyzed using MEGA 11.0 software and compared with reference strains (Tamura et al., 2021).

RNA extraction and RT-PCR. Total RNA from the four less-sensitive and sensitive *Botryosphaeria* strains was extracted using column-based RNeasy Mini Kits (Qiagen, Mississauga, ON, Canada) following the instructions of the manufacturer. After measuring concentrations using a NanoDrop 2000 (Thermo Fisher Scientific), the RNA was subjected to a reverse transcription-PCR (RT-PCR) using the One-Step RT-PCR kit (Qiagen, Hilden, Germany), as described by the manufacturers, along with four additional primers annealing to the exon sequences of the *cyt b* gene (Supplementary Table 2). The reverse transcription and PCR conditions included an initial 30-minute step at 55°C, and then initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. All products were directly purified and sequenced as described above. The nucleotide sequences and amino acid sequences of each strain were compared using MEGA 11.0 (Tamura et al., 2021).

***In vitro* assay for the evaluation of *Botryosphaeria* sensitivity to pyraclostrobin in apple fruits.** Apple fruits (cv. 'picnic') were sterilized with 70% ethanol for 1 min and washed using double-distilled water (ddH_2O). After drying to remove the remaining moisture, three consistent wounds were created using a sterile 4 mm-diameter cork borer. Subsequently, wounded fruits were dipped for 30 s in 500 ml of a 67 µg/ml (recommended concentration) pyraclostrobin solution or ddH_2O (control). After drying, the treated fruits were inoculated with 4 mm-diameter agar plugs taken from the five least sensitive and five most sensitive isolates. Subsequently, the inoculated fruits were incubated at 25°C in darkness for 7 days, and the lesion diameter was measured using a digital caliper. The control efficacy was calculated with the formula

$$\text{Control efficacy (\%)} = \left(1 - \frac{Lt}{L0}\right) \times 100$$

, where Lt represents the mean lesion diameter when treated with pyraclostrobin and $L0$ represents the mean lesion diameter of the control group. This experimental procedure was performed in triplicate.

Statistical analysis. The normality of the data for the individual control efficacy of each *Botryosphaeria* strain was assessed using the D'Agostino-Pearson normality test in the GraphPadPrism software. After calculating control efficacy parameters, the Mann-Whitney test was performed to evaluate the significance of differences between the less-sensitive and sensitive strains. The significance level was set at $\alpha = 0.05$.

Results and Discussion

Phylogenetic relationships based on multiple loci. A multilocus phylogenetic analysis was carried out based on the concatenated ITS, *tef*1, and *tub*2 sequences with *Botryosphaeria parvum* (ATCC 58191) as the outgroup taxon (Supplementary Fig. 1). The phylogenetic tree showed that 287 strains (approximately 87%) were identified as *B. sinensis,* a close relative of *B. dothidea* (Zhang et al., 2021), while the other 42 (approximately 13%) were clustered with *B. kuwatsukai*. Therefore, *B. sinensis* was the dominant species in Korean apple orchards, with some *B. kuwatsukai* also present in the white rot population, which is consistent with our previous study's results (Lim et al., 2024).

Sensitivity of *Botryosphaeria* spp. to pyraclostrobin. The sensitivity of the *Botryosphaeria* strains against pyraclostrobin WG covered a broad mycelial inhibition range at each concentration. The EC_{50} value of all 329 strains ranged from 0.01 to 34.16 µg/ml (average: 3.03 ± 3.62 µg/ml) (Table 1). Estimated EC_{50} values were < 2.5 µg/ml in 59.3% of the isolates, 2.5 to 9.5 µg/ml in 37.1% of isolates, and > 10.0 µg/ml in 3.6% of isolates (Fig. 1). The frequency distribution of the EC_{50} values of these *Botryosphaeria* isolates showed a unimodal curve with a long right-hand tail. The EC_{50} values of the less-sensitive group ranged from 5.49 µg/ml (strain 20-GC72) to 34.16 µg/ml (strain GwYwBD22-2-1). For the sensitive group, EC_{50} values ranged from 0.01 µg/ml (strain GsBhBD23-5-1) to 0.99 µg/ml (strain CcYsBD22-2-1) (Table 1).

Pyraclostrobin sensitivity was compared between various isolate populations using the EC_{50} values. When comparing sensitivity of those isolated from 2005 to 2020 to those isolated from 2021 to 2023, the mean EC_{50} value was 2.57 µg/ml in the former group and 3.17 µg/ml in the latter

Table 1. EC_{50} values against pyraclostrobin WG of various *Botryosphaeria* isolate groups

Grouping criterion	Groups	No. of isolates	EC_{50} value (μg/ml)		
			Minimum	Average	Maximum
Isolation year	2005-2020	75	0.10	2.57	24.97
	2021-2023	254	0.01	3.17	34.16
Tissue source	Apple trunk	145	0.05	2.22	24.97
	Apple fruit	184	0.01	3.68	34.16
Region	Gyeongbuk	110	0.01	2.29	21.89
	Gyeongnam	11	0.15	3.85	11.62
	Jeonbuk	48	0.10	2.79	9.88
	Jeonnam	4	1.88	2.30	2.97
	Chungbuk	52	0.01	3.34	24.97
	Chungnam	9	0.39	4.62	17.58
	Gangwon	52	0.10	3.26	34.16
	Gyeonggi	43	0.29	4.09	12.42
Species	*B. sinensis*	287	0.01	3.04	34.16
	B. kuwatsukai	42	0.09	3.27	17.58
Fungicide sensitivity	Less-sensitive	20	5.49	12.70	34.16
	Sensitive	20	0.01	0.44	0.99
Total		329	0.01	3.03	34.16

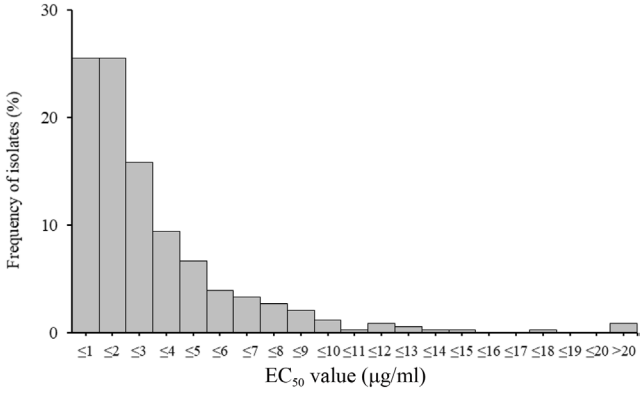

Fig. 1. Frequency distribution of the EC_{50} values of 329 *Botryosphaeria* spp. isolates against pyraclostrobin fungicide.

(Table 1), and 76% and 64% of the strains, respectively, were below the total mean EC_{50} value of 3.03 μg/ml (Fig. 2A). Comparing the sensitivity of the *B. sinensis* and *B. kuwatsukai* strains based on the phylogenetic analysis, mean EC_{50} values were similar, 3.04 μg/ml for *B. sinensis* and 3.27 μg/ml for *B. kuwatsukai* (Table 1). For both species, 67% and 65% of the isolates, respectively, presented EC_{50} values below 3.03 μg/ml (Fig. 2B). When comparing the EC_{50} values of isolates collected in the eight different regions in Korea, the lowest mean EC_{50} value was 2.29 μg/ml, in Gyeongbuk province, and the highest was 4.62 μg/ml, in Chungnam province (Table 1, Fig. 2C), and no significant differences in sensitivity among regions were detected (Fig. 2C). When comparing strains by isolation source, trunk and fruit, most strains (82 and 55%, respectively) were distributed below 3.03 μg/ml (Fig. 2D), with mean EC_{50} values of 2.22 μg/ml and 3.68 μg/ml, respectively, for the two groups (Table 1). All groups showed a similar EC_{50} value range and a unimodal curve with a positive skew, indicating no significant differences in EC_{50} distribution.

The sensitivity of the 40 less-sensitive and sensitive strains were evaluated against other QoI fungicides, including azoxystrobin WP (a.i. 10%), kresoxim-methyl WG (a.i. 50%), and trifloxystrobin WG (a.i. 50%), using the same methods as those for pyraclostrobin WG. Compared to the results for pyraclostrobin, all isolates showed relatively lower sensitivity to these fungicides. However, no significant differences in growth inhibition rates were observed between the less-sensitive and sensitive strains for all three fungicides at each concentration (Supplementary Fig. 2). The emergence of resistant strains in a pathogen population leads to a bimodal EC_{50} value distribution and differences in mean EC_{50} values when comparing isolate populations (Brent and Hollomon, 2007). For example, the EC_{50} value

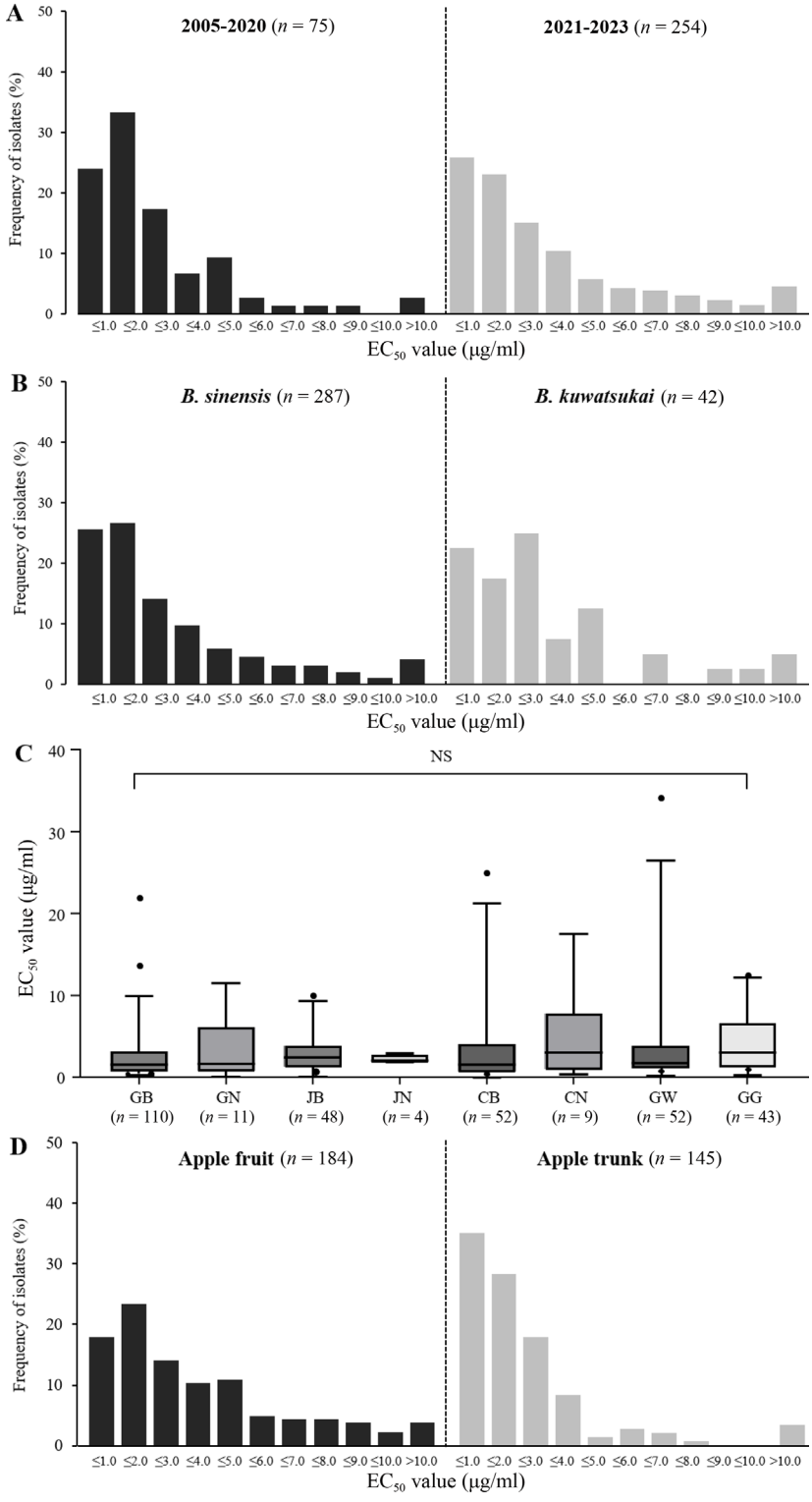

Fig. 2. EC_{50} values against pyraclostrobin among populations of *Botryosphaeria* isolates defined based on various criteria: (A) the EC_{50} frequency distributions of populations collected from 2005 to 2020 and 2021 to 2023; (B) the EC_{50} frequency distributions of *B. sinensis* and *B. kuwatsukai* populations; (C) a box and whisker plot of EC_{50} values by isolation region, including Gyeongbuk (GB), Gyeongnam (GN), Chungbuk (CB), Chungnam (CN), Jeonbuk (JB), Jeonnam (JN), Gwangwon (GW), and Gyeonggi (GG); and (D) the EC_{50} frequency distribution of different isolation sources. Black dots indicate EC_{50} values that were not assigned within the 2.5-97.5 percentile range (outliers), and "NS" indicates non-significant differences between groups.

distributions of *Macrophomina phaseolina* against fludioxonil changed from a unimodal distribution into a bimodal distribution between 2018 and 2020 (An et al., 2022). *Oculimacula acuformis* and *O. yallundae* showed changes in the distributions of their EC_{50} values against prochloraz and carbendazim between 1984 and 2000 (Parnell et al., 2008). Similarly, the sensitivity of *Zymoseptoria tritici* to pyraclostrobin differed significantly between isolation years, with the EC_{50} values in 2022 being 7 times higher than those in 2019 (Lavrukaitė et al., 2024). In the present study, *Botryosphaeria* populations did not show a bimodal distribution or significant differences in EC_{50} values among isolate populations based on any categorization criteria, indicating consistent pyraclostrobin sensitivity across the entire Korean population and no significant resistance development. Similarly, a previous study found that 97 *B. dothidea* isolates from China indicated low EC_{50} values (avg. 3.09 μg/ml) with a unimodal distribution, revealing that all isolates were sensitive to pyraclostrobin (Fan et al., 2019).

Comparison of the *cyt b* gene in less-sensitive and sensitive isolates. Sequencing of the partial *cyt b* gene from the 40 less-sensitive and sensitive isolates revealed a 1,700 bp sequence for *B. sinensis* using the BDcy687F/BDcy2467R primer pair and a 1,800 bp sequence for *B. kuwatsukai* using the BDcy687F/BDcy9347R primer pair. For *B. kuwatsukai* CcYsBD22-3-1, the primer pairs BDcy4986F/BDcy1542R and BDcy7586F/BDcy9347R were used to

	Strain name	EC_{50} (μg/ml)	129th																			137th								143rd	
Sensitive strains	*B. sinensis* GsBhBD23-5-1	0.01	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcGsBD23-3-1	0.01	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 21-CS29	0.05	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. kuwatsukai 22-MG32	0.09	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 20-HC78	0.10	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. kuwatsukai 21-MJ82	0.10	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. kuwatsukai CcJcBD23-3-1	0.11	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 20-GC72	0.15	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcCjBD23-1-1	0.16	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GsYjBD22-1-1	0.25	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GsDgBD23-1-1	0.31	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GwHcBD22-1-1	0.52	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcCjBD22-1-8	0.65	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GgIcBD22-2-1	0.82	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GwHwBD22-4-1	0.87	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcCjBD22-4-1	0.89	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GwCcBD22-1-1	0.90	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. kuwatsukai GsCsBD22-2-1	0.92	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. kuwatsukai GgPcBD22-2-1	0.92	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcYsBD22-2-1	0.99	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
Less-sensitive strains	*B. sinensis* 20-GC72	5.49	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 22-MJ42	5.49	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 20-YC55	6.31	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 22-CS7	6.34	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcYdBD 23-3-2	8.80	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GgIcBD 23-1-1	8.89	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GsBhBD 23-1-1	9.09	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GgPjBD 23-2-1	9.81	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis JlMjBD22-2-1	9.88	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GwJsBD22-2-1	10.99	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GgYnBD22-3-1	11.18	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GsGcBD22-2-1	11.62	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcCjBD22-2-8	12.07	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GgPjBD23-1-1	12.42	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcBeBD22-2-1	13.03	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcCjBD22-3-1	14.01	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis CcYsBD22-3-1	17.58	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GsUsBD22-2-1	21.89	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis 20-BY27	24.97	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	B. sinensis GwYwBD22-2-1	34.16	···	I	L	I	L	M	M	A	T	A	F	L	G	···	Y	V	L	P	Y	G	Q	M	S	L	W	G	···
	Resistance amino acid substitutions												A									R								A	

Fig. 3. Amino acid sequences of *cyt b* gene regions containing the primary mutation sites conferring quinone outside inhibitor resistance (F129L, G137R, and G143A; dark-gray boxes). These sequences were obtained from 20 less-sensitive and 20 sensitive *Botryosphaeria* strains. The resistance-conferring amino acid substitutions are indicated in red in the bottom row.

reveal partial *cyt b* gene sizes of 2,200 bp and 3,000 bp, respectively (Supplementary Table 2). The sequencing results covered the mutations at codons F129, G137, and G143, which are associated with resistance or reduced sensitivity to QoI fungicides. All 40 isolates had TTC (phenylalanine) in the 129th, GGT (glycine) in the 137th, and GGT (glycine) in the 143rd codon, which are all codons associated with sensitivity to QoI fungicides (Fig. 3). The primary mechanisms of fungicide resistance in phytopathogenic fungi are target gene mutations (He et al., 2021). Especially for QoI fungicides, single amino acid substitutions in the *cyt b* gene have been found in various plant pathogens, including *Alternaria alternata*, *Pyrenophora teres*, and *Colletotrichum acutatum* (Kim et al., 2019; Ma et al., 2003; Sierotzki et al., 2007). In most cases, resistance was induced by a single point mutation in the 143rd codon of the *cyt b* gene that led to the substitution of glycine by alanine (Banno et al., 2009). In some species, such as *Pyricularia grisea* and *Colletotrichum* spp., other amino acid substitutions (F129L and G137R) have also been associated with QoI resistance (Gisi et al., 2002; Shi et al., 2023). However, the 40 less-sensitive and sensitive isolates in this study showed no mutations at the 129th, 137th, or 143rd codon, indicating no resistant isolates based on these known QoI resistance mutation sites. These findings were consistent with the calculated EC_{50} values against pyraclostrobin, which indicated no significant resistance against pyraclostrobin among the *Botryosphaeria* isolates evaluated in this study (Table 1).

Structure of the *cyt b* gene in *Botryosphaeria sinensis* and *B. kuwatsukai*. To compare the *cyt b* exon/intron sequences and structures in *Botryosphaeria* spp., full-length gene sequences were determined through the primer walking strategy. The junctions between intron and exon sequences were identified by comparing their corresponding mRNA sequences to each other and to those of *Botryosphaeria* isolates from a previous study (Wang et al., 2021). All of the *cyt b* exon sequences from *B. sinensis* GsYjBD22-1-1 (EC_{50}: 0.25 μg/ml), *B. sinensis* GwYwBD22-2-1 (EC_{50}: 34.16 μg/ml), *B. kuwatsukai* CcYsBD22-3-1 (EC_{50}: 17.58 μg/ml), and *B. kuwatsukai* 22-MG32 (EC_{50}: 0.09 μg/ml) were 1,158 bp in length (Fig. 4A) and highly conserved (at least 98.5% identity) at the nucleotide level among all four isolates. Only 17 nucleotide substitutions were observed, all at different locations between *B. sinensis* and *B. kuwatsukai*, and these variations did not lead to any differences in amino acid sequences among the four isolates. Consequently, the primary mutations associated with QoI fungicide resistance (i.e., F129L, G137R, and G143A) were not observed in any isolates, nor were other mutations, including S34L, G37V, Y132C, D203V, and D230N (Fig. 4B and C), which are also known to potentially lead to reduced sensitivity or resis-

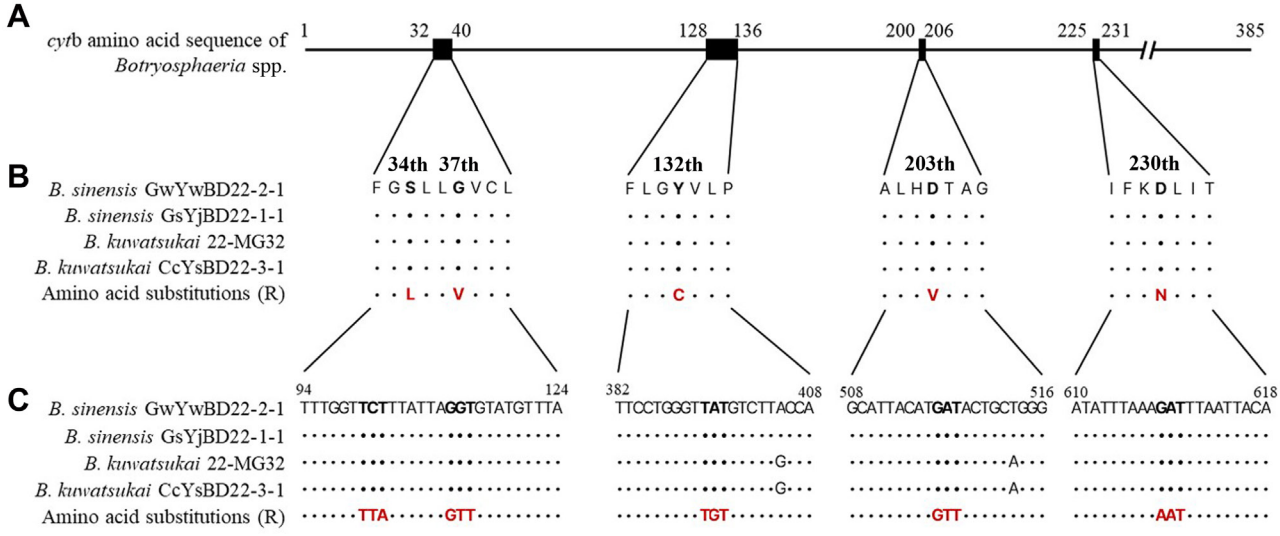

Fig. 4. Amino acid and nucleotide sequences of secondary mutation sites in the *cyt b* gene of *Botryosphaeria sinensis* and *B. kuwatsukai* isolates with different EC_{50} values. (A) A schematic diagram of the *cyt b* protein with boxes indicating regions containing secondary mutation sites associated with quinone outside inhibitor (QoI) resistance (i.e., sites other than the primary mutation sites F129L, G137R, and G143A). Amino acid (B) and nucleotide (C) sequences are shown within regions where secondary point mutations associated with QoI resistance have been reported in previous studies. Dots indicate amino acids/nucleotides that are identical to the corresponding residue in *B. sinensis* GwYwBD22-2-1 (top sequence). QoI resistance-conferring residues are indicated in red along the bottom row.

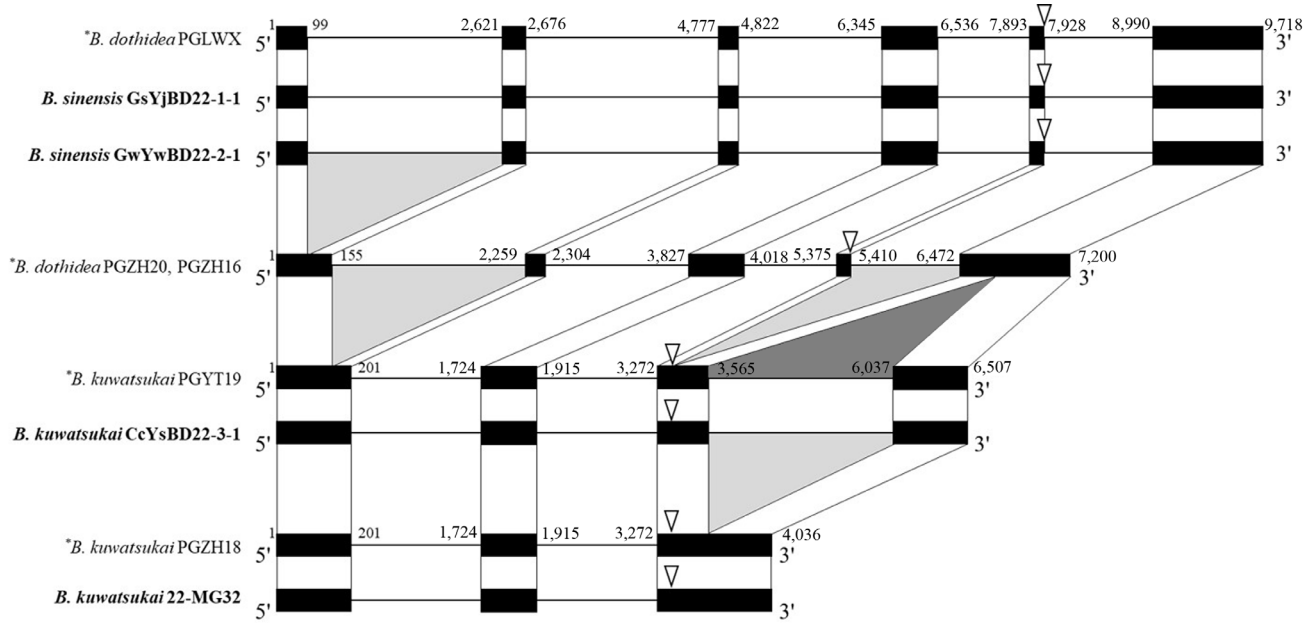

Fig. 5. Structures of the *cyt b* genes from the *Botryosphaeria sinensis* and *B. kuwatsukai* isolates from this study (bold) with different EC_{50} values along with those of available reference strains with consistent structures. Boxes denote exons, while lines signify introns. Numbers indicate the nucleotide positions at the beginning and end of exons. Lines connecting the exons of different isolates connect regions of homology. Light-gray and dark-gray areas indicate relative intron absence or presence between different isolates. The vertical open arrows show the location of the G143 codon. * indicates reference strains from previous study (Wang et al., 2021).

tance against QoIs (Fontaine et al., 2019; Fouché et al., 2022; Oliver et al., 2024; Zhou et al., 2015). However, the structures of *cyt b* gene differed in other ways among four *Botryosphaeria* isolates.

The *cyt b* gene sequences of the two *B. sinensis* isolates were consistent in their exon/intron organization but differed from those of both *B. kuwatsukai* isolates. In brief, the four isolates revealed three distinct *cyt b* structures (Fig. 5). Although exons of the *cyt b* genes were relatively similar in sequence and structure, vast differences in their lengths, approximately 9.7 kb for both *B. sinensis* strains and 6.5 and 4.0 kb for the *B. kuwatsukai* strains, respectively, was mainly the result of presence/absence of introns. *B. sinensis* GsYjBD22-1-1 and GwYwBD22-2-1 revealed *cyt b* genes sized at 9,718 bp, containing five introns of 2,521, 2,100, 1,522, 1,356, and 1,061 bp in length. In contrast, *B. kuwatsukai* CcYsBD22-3-1 had an additional 2,402 bp long intron between the 229th/230th codons, and the introns between the 33rd and 34th, 51st and 52nd, and 143rd and 144th codons in the *B. sinensis* isolates were absent, and *B. kuwatsukai* 22-MG32 had an additional missing intron between the 229th and 230th codons that was present in *B. kuwatsukai* CcYsBD22-3-1. This presence or absence of introns in the four selected isolates were consistent with structures seen in the reference strains, including *B. dothidea* PGLWX, *B. kuwatsukai* PGYT19, and *kuwatsukai* PGZH18 (Wang et al., 2021). Based on these similarities, *B. sinensis* GwYwBD22-2-1 and GsYjBD22-1-1 were estimated to be constructed with four group I introns and one group II intron, while *B. kuwatsukai* CcYsBD22-3-1 was estimated to have two group I introns and one group II intron and *B. kuwatsukai* 22-MG32 to have two group I introns only.

Mitochondrial *cyt b* genes in fungi can contain group I and II introns, which encode catalytic RNA sequences that self-splice and join exons during transcription (Tourasse and Kolstø, 2008). Group I introns in particular are primarily reported in the mitochondria of plant pathogenic fungi, and the number and locations of group I introns in the *cyt b* gene are highly conserved within closely related species (Grasso et al., 2006; Malbert et al., 2023). However, the presence or absence of specific group I introns have been reported even within the same species. It has been suggested that the absence or presence of a group I intron immediately after codon 143 is associated with the recognition of splice sites, thereby effecting the occurrence of the G143A mutation and the development of qualitative resistance to QoI fungicides (Rosenzweig et al., 2008; Sierotzki et al., 2007). To date, the presence of this group I intron has been associated with fewer G143A mutations in *Puccinia* spp.,

B. cinerea, *Alternaria solani*, and *Pyrenophora* spp. (Banno et al., 2009; Grasso et al., 2006; Jiang et al., 2009; Sierotzki et al., 2007). Notably, QoI fungicide resistance due to the G143A mutation has been observed in *B. cinerea* strains in which this intron was absent (Banno et al., 2009; Yin et al., 2012). Intriguingly, while exons 5 and 6 were separated by the 143/144 group I intron in *B. sinensis*, this intron was absent in both *B. kuwatsukai* strains (Fig. 5). This intron was observed in *B. dothidea* PGLWX, PGLB9, and SLBM-2-2, which have *cyt b* structures similar to those of *B. sinensis* GsYjBD22-1-1 and GwYwBD22-2-1 (Wang et al., 2021). Additionally, as this intron was absent in both *B. kuwatsukai* strains from the present study, as well as those from previous studies, the possibility of QoI resistance emergence due to the G143A mutation is thought to be higher in *B. kuwatsukai* than in *B. sinensis* and *B. dothidea*. Notably, the mean EC_{50} value of *B. kuwatsukai* strains increased from 2.34 to 3.40 μg/ml when comparing the 2005-2019 group and 2020-2023 group, while that of *B. sinensis* decreased from 3.13 to 2.98 μg/ml. In addition, the increase in the maximum EC_{50} values between the 2005-2019 and 2020-2023 groups for *B. sinensis* and *B. kuwatsukai* were approximately 2.9- and 5.5-fold, respectively (Supplementary Fig. 3). Therefore, further studies are necessary to monitor changes in the pyraclostrobin sensitivity of *B. kuwatsukai*. Additionally, some *B. cinerea* and *Monilinia fructicola* isolates have reported G143A mutations despite the presence of the intron immediately after G143, suggesting an association with not only presence/absence patterns but also the mobility of intron (Cinget and Bélanger, 2020; Leroux et al., 2010; Luo et al., 2010). Therefore, the possibility of QoI resistance still exists in *B. sinensis* and *B. dothidea*. Clearly, further evaluation of the association between *cyt b* structure and QoI resistance is essential, and continuous resistance monitoring should be conducted for *Botryosphaeria* spp.

Control efficacy of pyraclostrobin against *Botryosphaeria* spp. on apple fruits. The effectiveness of pyraclostrobin fungicides in controlling *Botryosphaeria* infection in apple fruits of five sensitive and five less-sensitive strains and the phenotypic differences between them were exam-

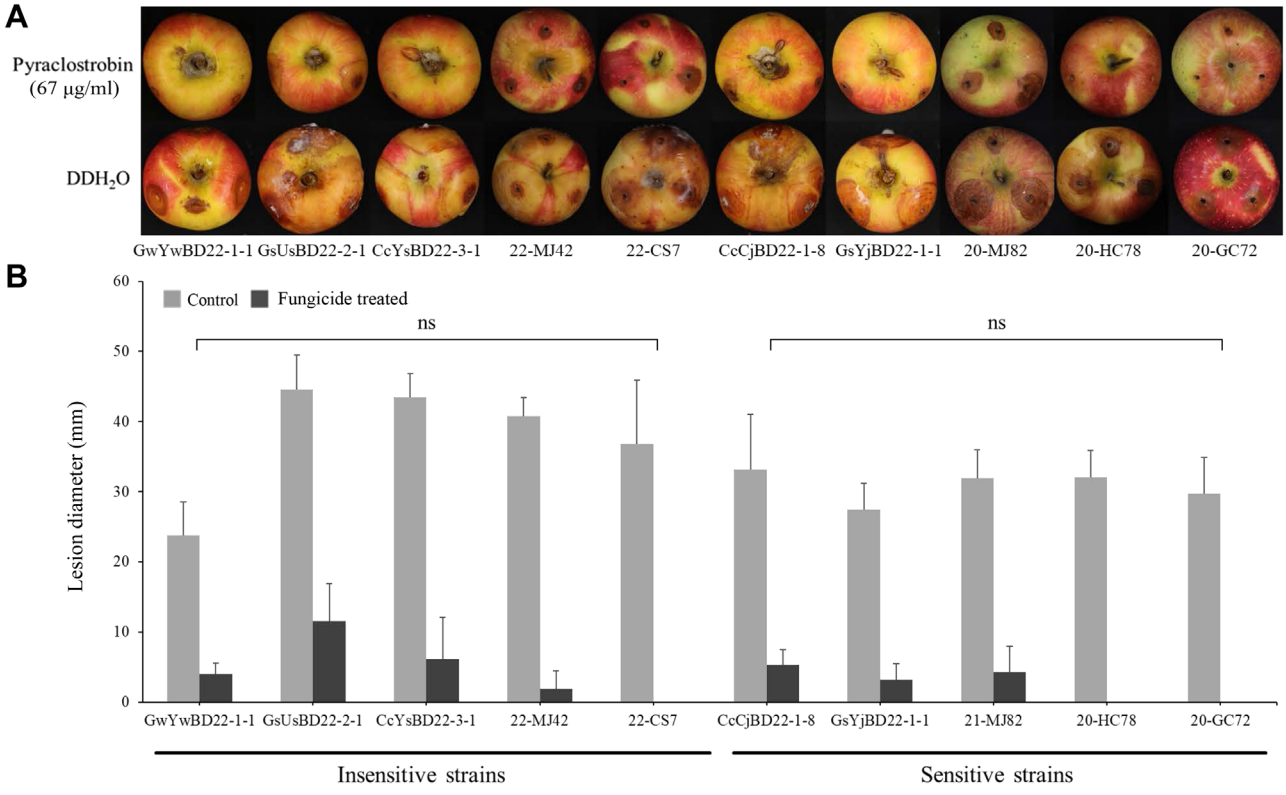

Fig. 6. Control efficacy of pyraclostrobin against less-sensitive and sensitive *Botryosphaeria* spp. strains in apples (cv. 'Picnic'). (A) Pictures of *Botryosphaeria* inoculated apples which were pretreated with pyraclostrobin or ddH_2O (control). (B) Mean lesion diameters (±standard errors) caused by *Botryosphaeria* strains on apple fruits treated with pyraclostrobin (dark gray) or ddH_2O (light gray). No statistical differences in control efficacy between the less-sensitive and sensitive strains were found using the by Mann-Whitney test ("ns" indicates non-significant differences between groups).

ined. When treated with the recommended concentration of pyraclostrobin (67 μg/ml), a fungicide typically used in orchards, effective disease control was observed in both sensitive and less-sensitive strains seven days after inoculation (Fig. 6A). The control efficacy (%) of the less-sensitive strains ranged from 74.1% to 100.0% (Avg. 89.9%), and that of the sensitive strains ranged from 84.1% to 100.0% (Avg. 91.8%) (Fig. 6B). Since the normality tests showed that the control efficacy of all isolate groups did not follow a normal distribution, a statistical analysis was performed using the Mann–Whitney test to investigate the differences between less-sensitive and sensitive isolates, and no significant difference was observed S (P = 0.3346). Fungicide-resistant strains exhibited lower control efficacy than sensitive strains when inoculated on fungicide-treated fruits or leaves. For instance, in a previous study, benzimidazole-resistant *B. cinerea* strains showed a control efficacy of approximately 10% or less, revealing lower inhibition rates compared to sensitive strains when inoculated on the fruits and leaves of strawberry (Kim et al., 2023). Additionally, prothioconazole-resistant *Leptosphaeria maculans* and *L. biglobosa* also revealed larger lesion diameters compared to sensitive strains in *in planta* assays (Sewell et al., 2017). In the present study, there was no significant difference in control efficacy between the less-sensitive and sensitive strains, again indicating that no significant fungicide resistance currently exists in Korea. In addition, the mean lesion diameters of control fruits were similar, averaging 30.9 mm and 37.8 mm for less-sensitive and sensitive strains, respectively (Fig. 6B), which is consistent with a previous study indicating that resistant *B. dothidea* isolates and sensitive isolates have similar virulence (Fan et al., 2019).

In this study, 329 *Botryosphaeria* spp. were found to be sensitive to pyraclostrobin WG, providing a reference for future evaluations of pyraclostrobin sensitivity among *Botryosphaeria* populations in Korea and allowing for the identification of potential shifts toward resistant strains. While apparently not currently present, the risk of fungicide resistance emergence in *Botryosphaeria* spp. still exist, and resistance to thiophanate-methyl, benomyl, and tebuconazole has already been reported. In addition, multiple studies have reported QoI resistance associated with non-target-site mechanisms, such as drug efflux transporters, alternative respiration, and intron deletions (Dorigan et al., 2023; Sun et al., 2024). Therefore, examining both target- and non-target site resistance mechanisms will be necessary for the regular long-term monitoring of changes in fungicide resistance, and the association between various *cyt b* gene types in *Botryosphaeria* and QoI fungicide resistance should be demonstrated.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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