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High Quinone Outside Inhibitor (QoI) Resistance Risk in *Colletotrichum* Species Causing Strawberry Crown Rot in Korea

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Anthracnose crown rot caused by *Colletotrichum* spp. is one of the most important diseases of strawberry (*Fragaria* × *ananassa*) in Korea. Single spore isolates of 72 *Colletotrichum* species collected in 2018, 2020, and 2023 from different locations in Chungbuk, Chungnam, Gyeongbuk, Gyeongnam, and Jeolla regions were characterized using a multi-locus sequence typing (MLST) of four genetic loci (*GAPDH, ACT, TUB2,* and *ITS*). Isolates were identified as *C. fructicola* (*n*=64) and *C. siamense* (*n*=8). Quinone outside inhibitors (QoIs) active ingredient pyraclostrobin is utilized to manage anthracnose crown rot in the field. Sensitivity to pyraclostrobin were assessed on potato dextrose agar using a mycelial growth inhibition assay at 20, 4, 0.8, 0.16, and 0.032 µg/ml fungicide concentrations. Our results demonstrated that all the 72 *Colletotrichum* spp. isolates were not inhibited by pyraclostrobin at all the tested fungicide concentrations, with EC_{50} ranging between 13 and >100 µg/ml. Sequences of the partial cytochrome b (*cyt b*) gene flanking the mutation site revealed the presence of the G143A mutation in all the isolates. No other site modifications were detected in the *cyt b* gene. Cross resistance assays with 10 selected isolates involving EC_{50} values of pyraclostrobin, mandestrobin, trifloxystrobin, and kresoxim-methyl showed positive correlations among these fungicides. These findings suggest a high risk of resistance development associated with using QoI fungicides to control the anthracnose crown rot of strawberries in Korea.

Keywords: *Colletotrichum* spp., *cyt b* gene, QoI fungicides, Strawberry anthracnose

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Introduction

Strawberry (*Fragaria* × *ananassa*) is a widely cultivated fruit in Korea, with an annual production valued at $1.029 billion in 2021 (Korea Statistical Information Service, 2021). Production is challenged by fungal diseases including crown rot induced by *Colletotrichum* spp. Anthracnose crown rot is characterized by black spots on leaves, reddish-brown necrosis of the crown, poor rooting, wilting of the plant, and eventual death (Nam et al., 2022). In the past, *C. gloeosporioides* and *C. acutatum* were reported to be associated with the anthracnose crown rot symptoms on strawberry (Kim et al., 1992; Nam et al., 2008), but the former was later reclassified as *C. fructicola* using a more advanced molecular tools (Nam et al., 2013). In recent years, *C. fructicola* and *C. siamense* have been more frequently isolated from anthracnose crown rot infected strawberries in South Korea, with incidence ranging between 10 to 30% (Nam et al., 2022). Chemical control through fungicide application offers one of the most effective management of this disease (Louws et al., 2024; Nam et

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al., 2014). Various fungicide treatments and spray regimes have been demonstrated to effectively reduce crown rot severity in strawberry (Kim et al., 2002; Nam et al., 2011).

Strobilurins, more properly known as quinone outside inhibitors (QoIs) that include fungicides such as pyraclostrobin, azoxystrobin, trifloxystrobin, mandestrobin, kresoxim-methyl, etc. were designed to inhibit the respiratory chain in fungal mitochondria by binding to the quinone outside site of cytochrome b involved in electron transport, thereby blocking the transfer of electrons from cyt b to cyt c1, that leads to disruption in the production of ATP (Bartlett et al., 2002; von Jagow and Link, 1986). Extensive and consistent use, in addition to the single site action of this fungicide group have resulted in the emergence of resistant isolates among fungal pathogens. Resistant isolates have been reported in many studies in some genera including *Colletotrichum* (Freeman et al., 1997; Vega and Dewdney, 2013). Furthermore, there have been several reports of variable fungicide sensitivity among fungal species belonging to different monophyletic groups, and between isolates of a species from different hosts (Chen et al., 2022; Kenny et al., 2012; Mondal et al., 2005; Moreira et al., 2019), and this variability poses challenges to effective management of the disease (Freeman et al., 1997; Vega and Dewdney, 2013).

The *in vitro* screening of these fungicides to determine their half maximal effective concentration (EC_{50}) allows tracking of the sensitivity of *Colletotrichum* species to the fungicides and helps to manage the risk of resistance, which results partly from application of ineffective concentrations of fungicides (Beckerman et al., 2015). Also, understanding the diversity of these species through continuous chraracterization of isolates is important for effective control since variations in fungicide sensitivity could exist among these species.

The aim of this study is therefore to characterize some isolates of *Colletotrichum* spp. collected in 2018, 2020, and 2024 from crown rot infected strawberries across Korea and evaluate their sensitivity to QoI fungicides.

Materials and Methods

DNA extraction, PCR and phylogenetic analysis. A total of 72 isolates obtained through single spore isolation from crown rot infected strawberries across some regions in Korea in 2018, 2020, and 2024 were used in this study (Table 1). The isolates were re-cultured on potato dextrose agar (PDA). Total genomic DNA of the isolates was extracted from a 5-day old culture using Exene™ Plant SV mini extraction kit (GeneAll Biotechnology, Seoul, Korea) following the manufacturer's instruction. Four genetic loci namely actin (*ACT*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), as well as the internal transcribed spacer (*ITS*) region were amplified using the primer pairs shown in Table 2. A 20 µl PCR mixture containing 12 µl sterile distilled water (SDW), 4 µl EzPCR 5X PCR master mix (Elpis Biotech., Daejon, Korea), 1 µl each of the forward and reverse primers and 2 µl gDNA was prepared for each PCR reaction. The PCR reaction was performed in a thermal cycler (Bio-Rad 100Tm). PCR conditions include intial denaturation at 95°C for 5 min, followed by 35 cycles involving denaturation at 95°C for 1 min, annealing at 52-61°C for 30 sec, extension at 72°C for 1 min, and then final extension at 72°C for 5 min. The PCR products were separated by electrophoresis in 1% agarose gel in Tris-Borate (TBE) buffer for 40 min at 100 volt and viewed using transilluminator. The amplicons were purified using the Expin™ SV PCR mini purification kit (GeneAll Biotechnology) and sent for sequencing at Macrogen (Daejeon, Korea). The forward and reverse

Table 1. Number of isolates, source and year of isolation of *Colletotrichum* spp. used in this study

Region	Farm location	Number of isolates	Year of isolation
Chungnam	Nonsan-si	22	2018
	Buyeo-gun	4	2018
	Cheonan-si	1	2018
	Hongseong-gun	1	2020
	Kongju-si	2	2020
Chungbuk	Cheongju-si	8	2024
	Jincheon	6	2024
Gyeongnam	Pohang-si	3	2018
	Hamyang-gun	1	2018
		1	2020
Gyeongbuk	Daegu	2	2018
	Gumi-si	1	2018
Jeonnam	Boseong-gun	1	2018
	Damyang-gun	1	2020
Jeonbuk	Wanju-gun	1	2018
		1	2020

Table 2. List of primers used in this study

Gene	Primer	Sequence	Reference
ACT	ACT512F	5'-ATGTGCAAGGCCGGTTTCGC-3′	Carbone and Kohn (1999)
	ACT783R	5'-TACGAGTCCTTCTGGCCCAT-3′	
GAPDH	GDF1	5'-GCCGTCAACGACCCCTTCATTGA-3′	Guerber et al. (2003)
	GDR1	5'-GGGTGGAGTCGTACTTGAGCATGT-3′	
TUB2	BTUB2FD	5'-GTBCACCTYCARACCGGYCARTG-3′	Woudenberg et al. (2009)
	BTUB4RD	5′-CCRGAYTGRCCRAARACRAAGTTGTC-3′	
ITS	ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3′	White et al. (1990)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3′	
CYT b	R-CytbF	5′-GRCAAATGTCWTTATGACC-3′	Abdullahi et al. (2023)
	S-CytbF	5′-GRCAAATGTCWTTATGATG-3′	
	R-S-CytbR	5′-AYTCAACKATATCTTGTCC-3′	
	RF-CYT2A	5′-AYAGAGCTCCWAGAACWTTAG-3′	Isa and Kim (2022)
	RF-CYT2B	5′-GAAACACCTAAWGGGTTACTTGA-3′	

ACT, actin; *GAPDH*, glyceraldehydes-3-phosphate dehydrogenase; *ITS*, internal transcribed spacer; *CYT b*, cytochrome b.

reads were paired, and consensus sequences calculated in MEGA11 (Tamura et al., 2021). Sequence alignments of the individual loci were prepared using MAFFT v. 7 and manually edited in MEGA11. Maximum-likelihood (ML) was used for phylogenetic inferences of the concatenated alignments. Bootstrap analysis was performed with 1,000 replications for branch stability.

***In vitro* fungicide sensitivity test.** First, the response of the 72 *Colletotrichum* species isolates to the pyraclostrobin (a.i. 25%, WG) was evaluated using *in vitro* sensitivity assay. The fungicide was serially diluted in SDW to a concentration of 2,000, 400, 80, 16, 3.2 μg/ml and used to amend the PDA in a 60 cm Petri plate at 1%, resulting in final concentrations 20, 4, 0.8, 0.16, and 0.032 μg/ml. A 3 mm mycelial disc taken from the edge of a 7-day old culture of the isolates was transferred on the amended PDA plates. Untreated PDA plates served as control and three replications were maintained for each isolate including the control. Petri plates were sealed with parafilm and incubated at 25°C in the dark. Five days after incubation, radial mycelium growth was measured and the diameter of each colony was evaluated by subtracting the 3-mm plug from two perpendicular diameters and then averaging them. The mycelial growth inhibition ratio (MGIR%) for each isolate at each test concentration was calculated as the difference between the radial growth of non-amended control (C) and the radial growth of each test concentration (T) divided by C expressed as a percentage as follows: MGIR (%) = [(C - T) / C] × 100.

The half maximal effective concentration (EC_{50}) values were calculated by linear regression of the probit-transformed relative inhibition on $\log_{10}$-transformed fungicide concentration using Excel. Later, cross resistance assays with mandestrobin (a.i. 40%, SC), trifloxystrobin (a.i. 22%, SC), and kresoxim-methyl (a.i. 44.2%, SC) were conducted with 10 selected isolates. The *in vitro* sensitivity assay was carried out as described above for pyraclostrobin. The Spearman's correlations coefficients were calculated, and linear regressions were computed on log-transformed EC_{50} values.

Allele-specific detection of the G143A mutation and sequencing of the partial *cyt b* gene. Resistant and sensitive allele-specific primer pairs R-CytbF/R-S-CytbR and S-CytbF/R-S-CytbR were used for the detection of the G143A point mutation on the cyt b gene from the gDNA of all the 72 isolates (Table 2). PCR amplification was performed with an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C

for 5 min. To confirm the amplification of the specific fragments by the allele-specific primer pairs, the PCR products were separated by electrophoresis in 1% agarose gel in TBE buffer for 40 min at 100 volt and viewed using transilluminator. Thereafter, 10 isolates were selected for the cytochrom b (*cyt b*) gene analysis. The partial *cyt b* gene flanking the 129, 137, and 143 amino acid positions was amplified using the primer pair RF-CYT1A/RF-CYT2B from cDNA. Total RNA was extracted using Hybrid-R™ RNA extraction kit (GeneAll Biotechnology), following manufacturer's recommendation. cDNA was synthesized from a 20 µl reaction mixture containing 11 µl SDW, 4 µl Reverse transcription 5X master mix (Elpis Biotech.) and 5 µl RNA, incubated at 37°C for 1 hr, followed by further incubation at 94°C for 5 min. and the PCR amplification from the cDNA was performed with the same conditions described above, and agarose gel electrophoresis also as described above. The PCR products were purified using the Expin™ SV PCR mini (GeneAll Biotechnology) purification kit and sent for sequencing at Macrogen for the presence or absence of the target site mutations.

Results

***Colletotrichum* species isolated from strawberry crown rot.** Phylogenetic analysis was conducted using ML method to compare the *ACT*, *GAPDH*, *TUB2*, *ITS* nucleotide sequences of the 72 isolates (Fig. 1). Based on the concatenated gene sequences, 64 isolates clustered with *C. fructicola* reference strains FLJ6 and CHQ-2 with with a bootstrap value of 97%, while eight isolates clustered with *C. siamense* reference strains TQ01 and PCGXNN4, with a boostrap value of 97%. The distribution of these species showed that *C. fructicola* was isolated from all the six regions, while *C. siamense* was isolated from Chungnam and Chungbuk only (Table 3).

Sensitivity of *Colletotrichum* spp. isolates to pyraclostrobin. The sensitivity assays with pyraclostrobin (a.i. 20%, WG) was assessed on PDA using a mycelial growth inhibition assay at 20, 4, 0.8, 0.16, and 0.032 µg/ml. All 72 isolates including 64 isolates identified as *C. fructicola* and eight isolates identified as *C. siamense* could grow on all the tested fungicide concentrations, with mycelial growth inhibition ratio of less than 50% at 20 µg/ml (Fig. 2A, Fig. 3A). The average and maximum EC_{50} values of the 2018, 2020, and

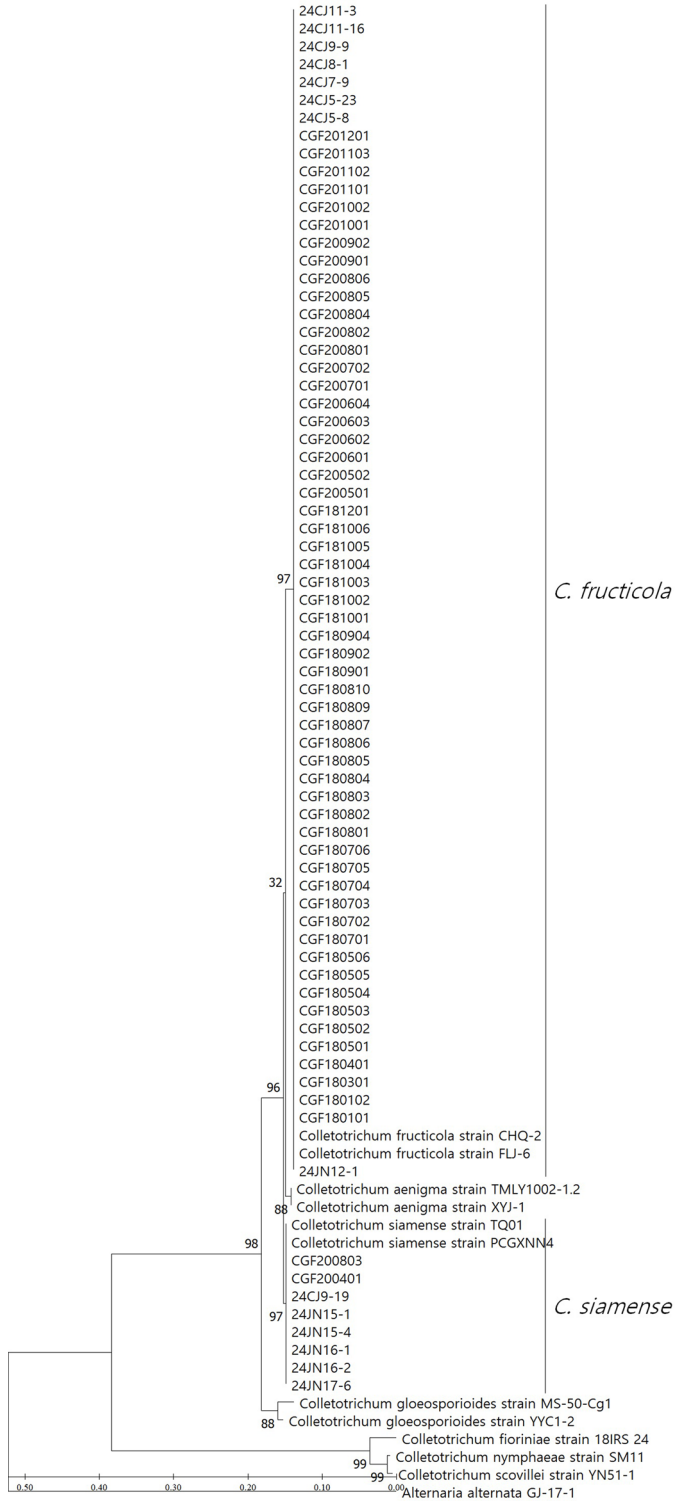

Fig. 1. Maximum likelihood phylogenetic tree based on the concatenated dataset (*GAPDH*, *ACT*, *TUB2*, and *ITS*) sequences showing phylogenetic relationship among *Colletotrichum* spp. isolated from strawberry crown rot in Korea. Bootstrap scores greater than 70 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site. Species are indicated with a black vertical line, with their names listed at the right. *Alternaria alternata* was used as an outgroup.

Table 3. Number of *Colletotrichum* spp. isolates collected, year, region and average pyraclostrobin EC_{50} values of the isolates

Year	Region	*Colletotrichum* spp.	Number of isolates	EC_{50} (μg/ml)		
				Min	Mean	Max
2018	Chungnam	*C. fructicola*	27	13	>100	>100
	Gyeongnam	*C. fructicola*	4			
	Gyeongbuk	*C. fructicola*	3			
	Jeonbuk	*C. fructicola*	1			
	Jeollanam	*C. fructicola*	1			
2020	Chungnam	*C. fructicola*	17	20	>100	>100
		C. siamense	2			
	Gyeongnam	*C. fructicola*	1			
	Jeonbuk	*C. fructicola*	1			
	Jeollanam	*C. fructicola*	1			
2024	Chungbuk	*C. fructicola*	8	58	>100	>100
		C. siamense	6			

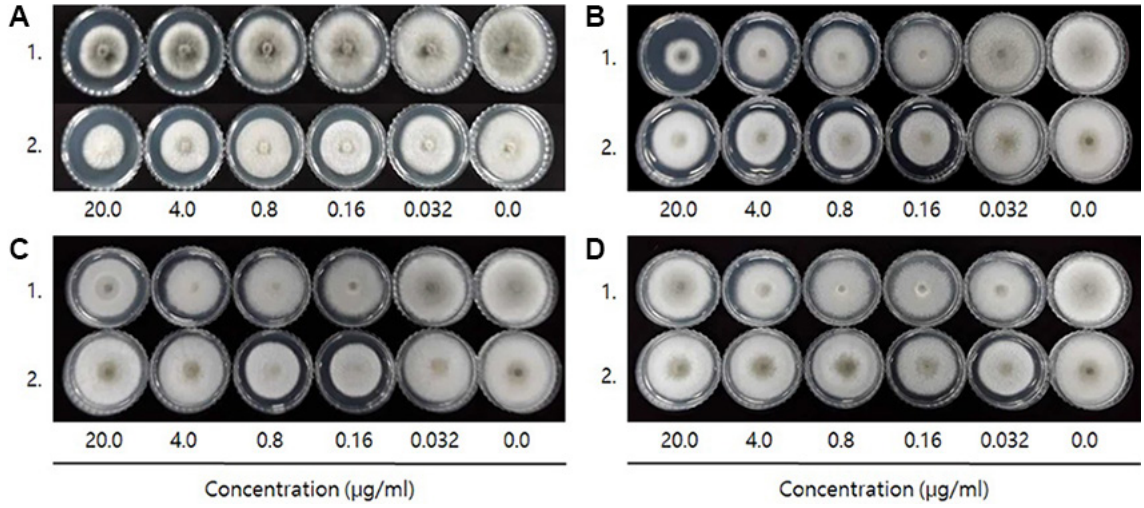

Fig. 2. Mycelial growth of *Colletotrichum* species on potato dextrose agar medium amended with pyraclostrobin (A), mandestrobin (B), trifloxystrobin (C), and kresoxim-methyl (D). The images (A-D) represent the one isolate (CGF181006) of *C. fructicola* (1) and the other isolate (CGF200401) of *C. siamense* (2).

2024 isolates were greater than 100 μg/ml, but the minimum EC_{50} values were 13, 20, and 58 μg/ml, respectively, indicating resistance of all the isolates to pyraclostrobin (Table 3).

Presence of the G143A target site modification on the *cyt b* gene. The resistant allele-specific primer pair R-CytbF/R-S-CytbR amplified the specific fragment (81 bp) from the *cyt b* gene of all the 72 isolates. There was no amplification with the sensitive allele-specific primerS-CytbF/R-S-CytbR. The amplification of the fragment by R-CytbF/R-S-CytbR is shown in Fig. 4 (bands of only 21 isolates shown here). The RF-CYT1A/RF-CYT2B primer pair amplified the partial fragments (325 bp) flanking the 129, 137, and 143 codons of *cyt b* of 10 selected isolates (Fig. 5). Analysis of the sequences revealed the presence of the G143A target site modifications from glycine to alanine, which was detected with the resistant allele-specific primers. The F129L and G137R mutations were not detected in the *cyt b* gene (Fig. 6).

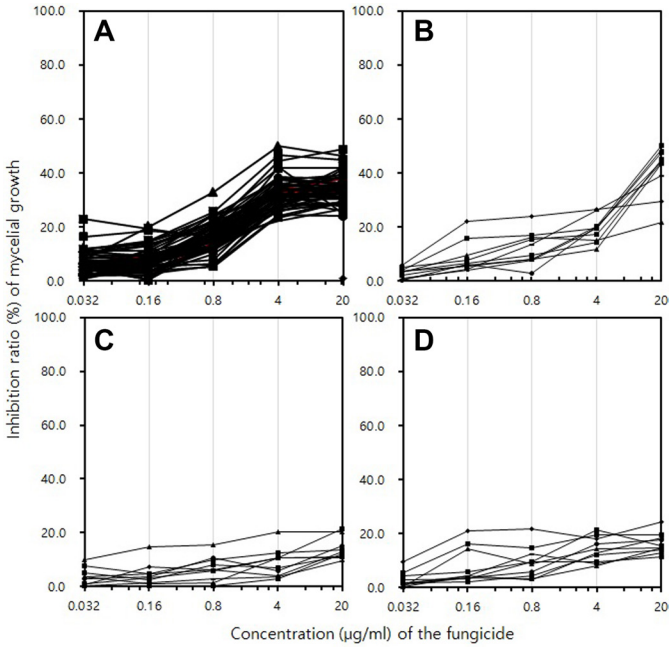

Fig. 3. Effect of the fungicides on the mycelial growth of isolates of *Colletotrichum* species on potato dextrose agar (PDA) medium. The effect of the fungicide was compared by calculating the effect of inhibiting the mycelial growth of isolates of *Colletotrichum* spp. using the following formula. Inhibition ratio (%) of mycelial growth = (1 - diameter of colony on the PDA medium amended with the fungicide / diameter of colony on the PDA medium amended without the fungicide) × 100. Among the quinone outside inhibiting fungicide, pyraclostrobin (A), mandestrobin (B), trifloxystrobin (C), and kresoxim-methyl (D) were used in this experiment.

Cross resistance of *Colletotrichum* spp. to QoI fungicides. *In vitro* sensitivity assay was conducted with three other QoI fungicides (mandestrobin, trifloxystrobin, and kresoxim-methyl) to test cross resistance of 10 selected *Colletotrichum* spp. isolates. Similar to the pyraclostrobin result, the mycelial growths of the 10 isolates on PDA were not inhibited by the three fungicides at all the tested concentrations (Fig. 2B-D). Mycelial growth inhibition ratio of all the isolates was less than 50% for mandestrobin (Fig. 3B) and less than 50% for trifloxystrobin and kresoxim-methyl at 20 μg/ml (Fig. 3C, D). The Spearman's correlations of the EC_{50} values of the isolates showed positive correlations with high coefficient of determination between pyraclostrobin and mandestrobin (R^2=0.790), pyraclostrobin and trifloxystrobin (R^2=0.665), pyraclostrobin and kresoxim-methyl (R^2=0.768), mandestrobin and trifloxystrobin (R^2=0.511), mandestrobin and kresoxim-methyl (R^2=0.934), and between trifloxystrobin and kresoxim-methyl (R^2=0.676) (Fig. 7).

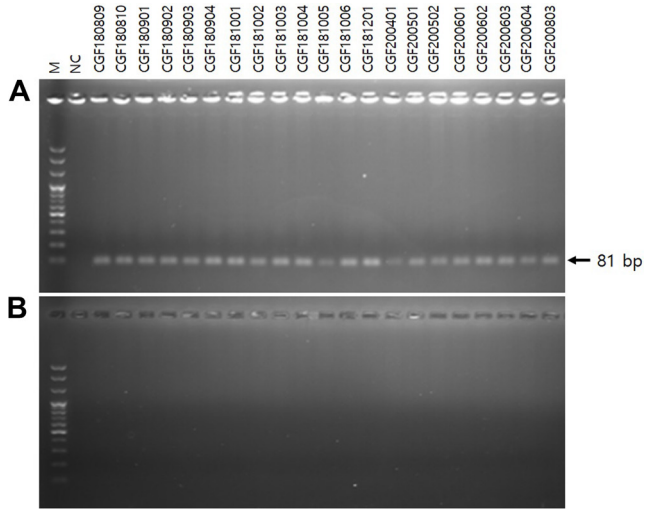

Fig. 4. Allele-specific PCR for isolates of *Colletotrichum* spp. causing strawberry anthracnose sensitive and resistant to pyraclostrobin. Primer pairs were used in this study, which were S-cytbF/R-S-CytbR and R-cytbF/R-S-CytbR for surceptible and resistant isolates to pyraclostrobin, respectively. (A) Gel electrophoresis using PCR primer as R-cytbF/R-S-CytbR for detecting isolates resistant to pyraclostrobin, (B) gel electrophoresis using PCR primer as S-cytbF/R-S-CytbR for detecting isolates sensitive to the fungicide. When using each primer pair for allele-specific PCR, a PCR product of 81 bp should be detected for both pyraclostrobin susceptible and resistant isolates. In this experiment, 21 isolates of *Colletotrichum* spp. were used. M, DNA ladder; NC, negative control.

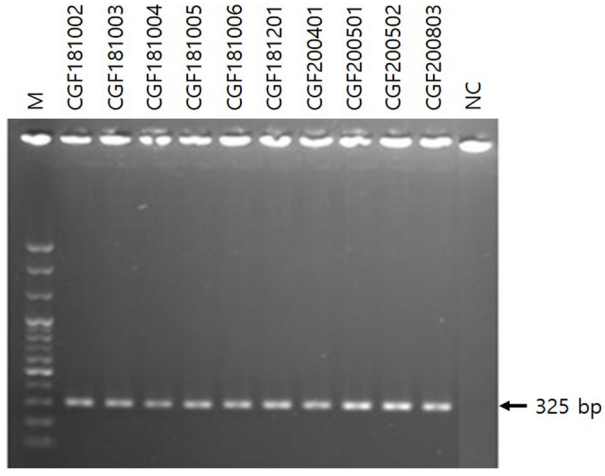

Fig. 5. Amplication of cytochrome b gene of *Colletotrichum* spp. causing strawberry anthracnose by using primer pair RF-CYT1A/RFCYT2B. As a result, a PCR product of 325 bp was amplified. Ten isolates of *Colletotrichum* spp. were used in this experiment. M, DNA ladder; NC, negative control.

Discussion

Anthracnose crown rot caused by *Colletotrichum* spp. is a

major production constraint in strawberry production in Korea. Molecular characteriation of 72 isolates collected in 2018, 2020, and 2024 revealed that two species of the *C. gloeosporioides* species complex, *C. fructicola* and *C. siamense* were associated with the strawberry crown rot. *C. fructicola* has been associated with anthracnose crown rot of strawberry in Korea for almost two decades but was initially reported as *C. gloeosporioides* epitype until 2013 after taxonomic re-evaluation by Nam et al. (2013). The first report of *C. siamense* from strawberry anthracnose crown rot was in 2022 (Nam et al., 2022), but the result of the present study showed that the pathogen was present among the 2020 isolates. This result is consistent with previous reports that *Colletotrichum* species of the *C. gloeosporioides* species complex are the predominant pathogens of anthracnose crown rot of strawberry in Korea (Kim et al., 1992; Nam et al., 2013, 2022). Similar reports of pathogen profile of strawberry crown rot was obtained in China, with the predominant species belonging to the *C. gloeosporioides* species complex (Han et al., 2016; Jayawardena et al., 2016; Zhang et al., 2020). There are indications that the predominance of the *Colletotrichum* spp. is region/

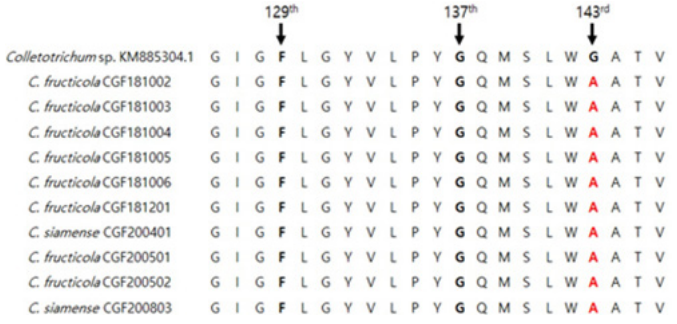

Fig. 6. Sequences of deduced amino acid of cytochrome b gene of *Colletotrichum* spp. The 143rd amino acid of *Coletotrichum* sp. KM885304.1, used as a reference isolate, was glycine (G), but in all other isolates used, glycine was substituted with alanine (A).

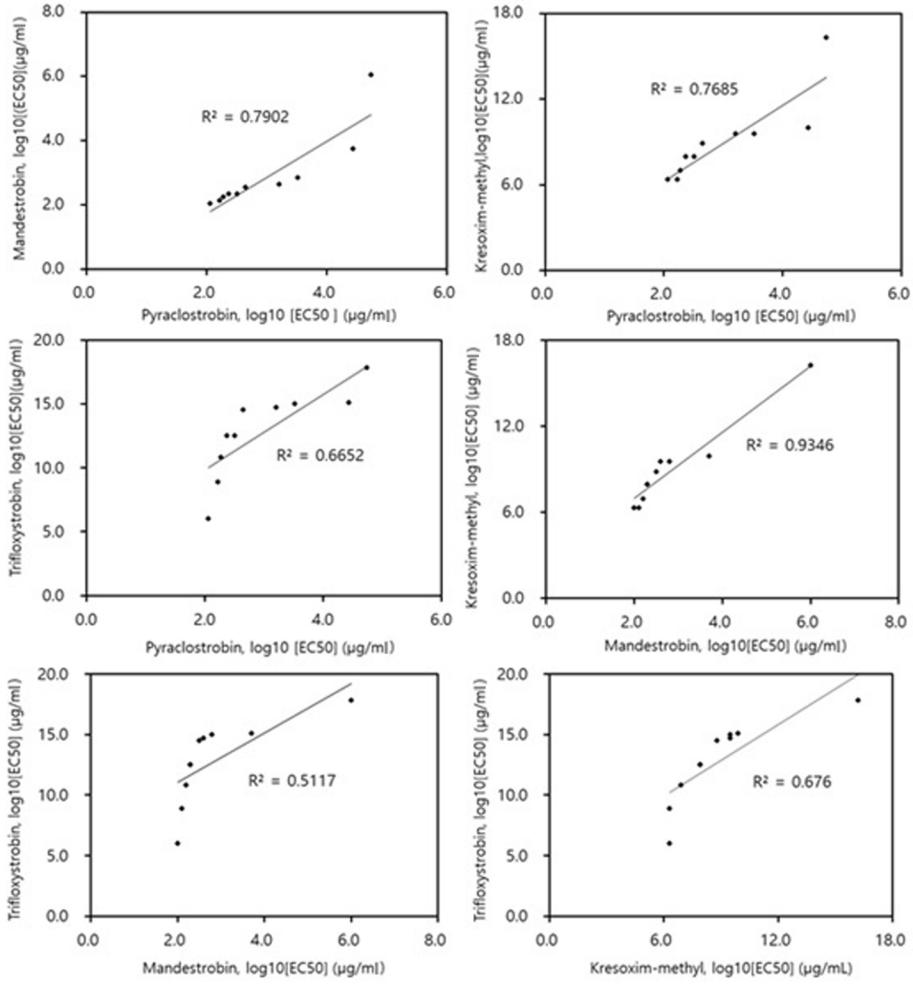

Fig. 7. Cross-resistance of *Colletotrichum* spp. isolates to quinone outside inhibitor fungicides. Spearman's correlations coefficients were calculated and linear regressions (black line) were computed on log-transformed EC_{50} values. R^2 is a coefficient of determination.

country specific. In parts most of USA, *Colletotrichum* species of the *C. acutatum* species complex are more prevalent in strawberry anthracnose crown rot (Daugovish et al., 2009; Haack et al., 2018).

Fungicide application remains an important component in the integrated disease management of strawberry crown rot in Korea. The efficacy of the QoI fungicides in the control of anthracnose crown rot has been demonstrated in many studies, both *in vitro* and *in vivo* (Nam et al., 2011, 2014). Although QoI fungicides have been among the most effective against anthracnose in strawerry, they are also highly vulnerable to fungal populations developing resistance to them. This resistance problem is due to the site-specific mode of action of these fungicides compounded by their continuous use. In this study, the *C. fructicola C. siamense* isolates were all resistant to the four QoI fungicides, indicating that these fungicides may not be suitable for the control of strawberry anthracnose crown rot, particularly in Korea. Resistance of *Colletotrichum* species to QoI fungicides has also been reported in various crops in Korea including *C. acutatum* and *C. gloeosporioides* from pepper (Isa and Kim, 2022; Kim et al., 2019; Park and Kim, 2022), *C. fructicola* and *C. fioriniae* from peach (An, 2023), *C. aenigma* and *C. fructicola* from apple (Abdullahi et al., 2023; Kim et al., 2023), *C. fructicola* and *C. gloeosporioides* from jujube (Cho, 2023), among others. However, in the aforementioned reports, sensitive isolates to QoI fungicides were also reported. This is in clear contrast to the findings of this study. In particular, all the *C. siamense* isolates collected from bitter rot infected apples from some orchards across Korea were sensitive to QoI fungicides (Abdullahi et al., 2023), whereas the *C. siamense* isolates from strawberry crown rot in this study were resistant. This contrasting responses of *C. siamense* to QoI fungicides suggest the role of host/cultivar in the sensitivity of *Colletotrichum* spp. QoI resistance problem among *Colletotrichum* spp. from strawberry anthracnose has been widely reported in some countries, such as *C. gloeosporioides* in Japan (Inada et al., 2008), *C. acutatum* in the USA (Forcelini et al., 2016; Haack et al., 2018), and *C. siamense* and *C. fructicola* in China (Zhang et al., 2020). Some of these reports have suggested alternative fungicides, such as those with broad-spectrum and multi-site activity, as well as protective fungicide and biofungicides in the management of strawberry anthracnose.

In conclusion, this study identified two *Colletotrichum* spp., *C. fructicola* and *C. siamense* from the *C. gloeosporioides* species complex causing strawberry crown rot in six regions in Korea. The fungicide sensitivity assay and *cyt b* gene sequence analysis showed that all the isolates were resistant to the fungicides, thus suggesting a high QoI fungicide resistance risk associated with anthracnose crown rot management in strawberry.

Conflicts of Interest

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

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