J. Pestic. Sci. **47**(4), 172–183 (2022)
DOI: 10.1584/jpestics.D22-030

JOURNAL OF PESTICIDE SCIENCE

Regular Article

Sensitivity of *Colletotrichum gloeosporioides* species complex (CGSC) isolated from strawberry in Taiwan to benzimidazole and strobilurin

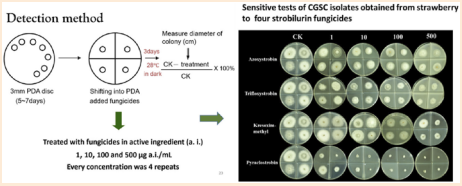
Sheng-Chi Chu,[1,5] Kuo-Hsi Lin,[2] Tsung-Chun Lin,[3] Chinnapan Thanarut[4] and Wen-Hsin Chung[5,6,*]

[1] *Miaoli District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Miaoli County 363, Taiwan, ROC*
[2] *Tungs' Taichung MetroHarbor Hospital, No.699, Section 8, Taiwan Boulevard, Wuqi District, Taichung City 435 Taiwan, ROC*
[3] *Plant Pathology Division, Taiwan Agricultural Research Institute, Council of Agriculture, Executive Yuan, Taichung City 413, Taiwan, ROC*
[4] *Faculty of Agricultural Production, Division of Pomology Maejo University, Chiangmai, Thailand*
[5] *Department of Plant Pathology, National Chung Hsing University, 145 Xingda Road, Taichung City 402, Taiwan, ROC*
[6] *Innovation and Development center of sustainable Agriculture (IDCSA), National Chung Hsing University, 145 Xingda Road, Taichung City 402, Taichung, Taiwan, ROC*

(Received May 25, 2022; Accepted October 4, 2022)

Supplementary material

Colletotrichum gloeosporioides species complex (CGSC) is the major pathogen causing strawberry anthracnose in Taiwan. Benzimidazoles and strobilurins are common fungicides used to control strawberry anthracnose. A total of 108 CGSC isolates were collected from five major strawberry-producing areas in Taiwan. The half-maximal effective concentration (EC_{50}) values of most CGSC isolates for benomyl (59 isolates), carbendazim (70 isolates), and thiabendazole (63 isolates) were higher than 500 μg a.i./mL. Strobilurin tests showed that the EC_{50} values of most CGSC isolates for azoxystrobin (66 isolates), kresoxim-methyl (42 isolates), and trifloxystrobin (56 isolates) were higher than 500 μg a.i./mL. However, most CGSC isolates were sensitive to pyraclostrobin at 100 μg a.i./mL. Fungicide tests indicated that CGSC isolates show multi-resistance to benzimidazoles and strobilurins. Benzimidazole-resistant isolates were associated with a point mutation in codon 198 of the β-tubulin gene, and strobilurin-resistant isolates did not correspond with mutation in the *cyt b* gene or alternative oxidase activity.



Keywords: anthracnose, fungicide-resistance, mechanisms, multi-resistance.

Introduction

Colletotrichum spp., the causal agents of strawberry anthracnose, are aggressive pathogens that can damage all parts of strawberry plants, including leaves, stems, petioles, vines, roots, and crowns, especially in hot and humid climates.[1)] Moreover, within seven days, crown infection increases the frequency of seedling/plant wilt and death from lack of water and causes severe losses.[2)] Anthracnose is an important limiting factor in strawberry cultivation worldwide.[3)] In Florida, USA, strawberry anthracnose caused up to 80% of seedling deaths in nurseries and field yield losses of more than 50%.[4)] Similarly, anthracnose was shown to cause 30–40% and 20% losses in seedlings and plants after transplanting, respectively, in Taiwan.[5)]

Several species of *Colletotrichum* that cause strawberry anthracnose have been reported worldwide, including *C. acutatum* (Ca), *C. boninense* (Cb), *C. denatium* (Cd), *C. fragariae* (Cf), and *C. gloeosporioides* (Cg).[6,7)] Among these *Colletotrichum* species, Cg and Ca are the major pathogens causing anthracnose worldwide.[8)] Currently, Cg,[9)] Ca,[10)] and Cb[11)] are considered species complexes. Species diversity depends on geographical or cultural conditions.[12)] In Taiwan, *C. gloeosporioides* species complex (CGSC) is a major pathogen that causes strawberry anthracnose.[13)] The current study indicated that CGSC in strawberries in Taiwan are composed of *C. fructicola* and *C. siamense*, with *C. siamense* being dominant.[6)]

The main strategy for controlling strawberry anthracnose is to use agro-chemicals.[14)] Several fungicides, such as benzimidazoles, strobilurins, and imazalil, are often recommended for

* To whom correspondence should be addressed.
E-mail: wenchung@nchu.edu.tw
Published online November 10, 2022

controlling strawberry anthracnose.[14–17] However, fungicide resistance has been reported in strawberry production areas.[18–20] Strobilurin-resistant Cg and Ca isolates from strawberry have been detected frequently in the field, leading to disease control failures in China,[21] the USA,[22] and Japan.[23] In Taiwan, several kinds of fungicides are recommended for controlling strawberry anthracnose, including benzimidazoles (thiabendazole), demethylation inhibitors (propiconazole, tebuconazole, and difenoconazole), strobilurins (trifloxystrobin and pyraclostrobin), and chlorothalonil (otserv2.tactri.gov.tw). Among these fungicides, strobilurins (quinone outside inhibitors QoIs) and benzimidazoles are at high risk of developing resistant isolates in the field.[24] Peng[25] reported that CGSC isolated from strawberries showed lower sensitivity to benzimidazoles and strobilurins. However, the number of CGSC isolates that Peng[25] used in the study was small and did not represent the actual situation in Taiwan's strawberry fields.

Presently, the CGSC includes 23 phylogenic species.[9] Several reports have indicated that CGSC shows different reactions to strobilurins and benzimidazoles. For example, CGSC members *C. siamense* and *C. aenigma* from apples in Japan remain sensitive to benomyl and strobilurin fungicides; meanwhile, *C. fructicola* often develops resistance to benomyl and strobilurin fungicides.[26] *C. perseae* and *C. fructicola*, which cause grape ripe rot in Nagano Prefecture, Japan, were often found to be resistant to strobilurin fungicides.[27] Azoxystrobin and pyraclostrobin sprayed preventively on strawberry fruit inoculated with Ca from Florida failed to control resistant isolates.[22] *C. siamense* and *C. fructicola* isolates from strawberries in eastern China are insensitive to azoxystrobin.[21] The mycelial growth inhibition of Ca isolates from post-bloom fruit drop of citrus in Brazil at a 1,000 μg/mL carbendazim concentration in sensitivity assays was only 59.7 to 67.6%.[28] Strawberry isolates of *C. siamense*, *C. fructicola*, and *C. nymphaeae* from China were insensitive to carbendazim, and their mycelial growth was not completely inhibited on potato dextrose agar supplemented with 500 μg/mL of carbendazim.[29] In Taiwan, Peng[25] did not provide detailed information on the efficacy of benzimidazoles and strobilurins against strawberry CGSC between species. Thus, sensitive information on CGSC members from strawberry to benzimidazole and strobilurin fungicides is lacking. The purposes of this study were to 1) examine the response of CGSC isolates from strawberry to benzimidazoles and strobilurins, including species responses to two fungicides, cross-resistance, and geographical differences; and 2) analyze the possible mechanisms of benzimidazole- and strobilurin-resistant CGSC isolates.

Materials and methods

1. Collection of Colletotrichum *spp. isolates*

Taoyuan No. 1 (Feng-Xiang) was the most popular cultivar, especially from 2014 to 2018, and more than 90% of this cultivar was infected by *Colletotrichum* spp. and showed necrosis or crown rot. Therefore, Taoyuan No. 1 showing necrosis or crown rot were collected from the main cultivation areas in Taiwan, including Taipei City (Neihu District), Hsinchu County (Kansai Township), Miaoli County (Dahu, Shitan, Taian, and Gongguan Townships), Taichung City (Houli District), and Nantou County (Guoshing Township). To isolate *Colletotrichum* spp., the surfaces of the diseased part, including healthy and necrotic tissues, were sterilized with 70% ethanol, rinsed three times with sterilized distilled water (SDW), and dried with sterilized filter paper. The sterilized tissues were loaded onto sterilized triangle glass rods in a petri dish or sealed box, and the appropriate humidity was maintained and cultured at 28°C with a 12 hr light/dark cycle for acervulus induction. After three to four days, the acervulus formed in the diseased tissues, and single spores were picked up for purification and cultured on potato dextrose agar (PDA, Difco™; Becton Dickinson and Co., Franklin Lakes, NJ, USA) for further study. On the other hand, the mycelial disc (5 mm) of single spore isolates was cut and put into a 2 mL cryogenic vial (cryogenic vial; Nalge Co., Rochester, NY, USA) containing 1 mL of sterile water (ddH_2O) or 10% glycerol and stored at 4°C or −80°C, respectively. A total of 108 isolates of *Colletotrichum* spp. were collected from five main cultivation areas in Taiwan, including eight isolates from Taipei City (Neihu District), 16 isolates from Hsinchu County (Kansai Township), 54 isolates from Miaoli County (Dahu, Shitan, Taian, and Gongguan Township), eight isolates from Taichung City (Houli District), and 22 isolates from Nantou County (Guoshing Township) (Fig. 1).

2. Identification of Colletotrichum *spp.*

For species confirmation, the single spore isolates of *Colletotrichum* spp. were primarily identified using internal transcribed spacer ribosomal DNA (ITS rDNA). *Colletotrichum* spp. isolates

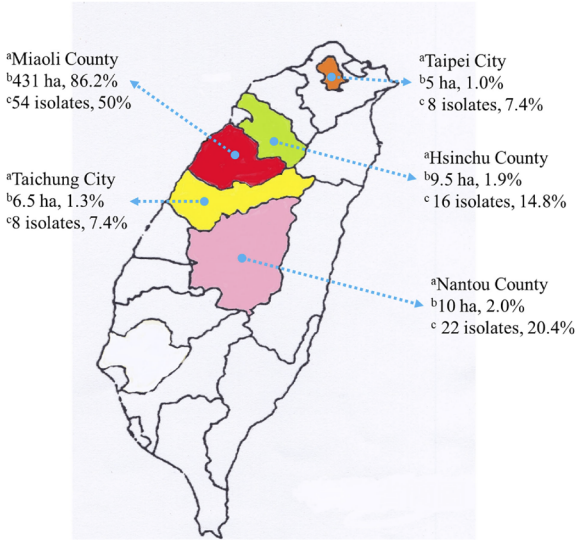


Fig. 1. The five main strawberry cultivation areas and CGSC isolates number in Taiwan. [a] Including Taipei City, Hsinchu County, Miaoli County, Taichung City, and Nantou County. [b] Cultivation area (ha) and proportion (%). [c] Isolates' numbers of *Colletotrichum gloeosporioides* species complexes (CGSC) and proportions (%).

cultured on PDA at 28°C with a 12 hr light/dark cycle after five days of incubation were used to extract total DNA. DNA was extracted as described by Chung *et al.*[30] Primers ITS1/ITS4[31] were used to amplify the ITS region. The PCR conditions were as described by Chung *et al.*[32] After the PCR products were recovered and purified using a gel elution kit, they were sent to Tri-I Biotech Inc. (Taichung City, Taiwan) for sequencing. Sequence results were compared with the gene database registered on the National Center for Biotechnology Information (NCBI) website. The CGSC isolates of *Colletotrichum* spp. were further confirmed using ITS sequences. Based on a blast of the NCBI website, the identities of all isolates were more than 99.5% compatible with the CGSC.

3. *Pathogenicity of* Colletotrichum gloeosporioides *species complex* (*CGSC*)

Isolates identified as CGSC were used for a pathogenicity test. CGSC isolates were transferred onto PDA and incubated at 28°C with a 12 hr light/dark cycle. The spores were suspended in SDW after five days of incubation and adjusted to 10^6 spores/mL as inoculum. In this study, 2.5-inch pots of the susceptible strawberry cultivar Taoyuan No. 1 seedlings were used, and wounds were created by removing one or two old leaves from the crown. Then, 1 mL of 10^6 spores/mL spore suspension was dropped on the wound site, and the inoculated strawberry seedlings were incubated in plastic bags with high humidity (>90%) at 28°C with a 12 hr light/dark cycle for two days. Then, the seedlings were transferred to a growth chamber at 28°C with over 70% relative humidity and with a 12 hr light/dark cycle. The symptoms of the seedlings were observed after 10 days of incubation, and seedlings were re-isolated to confirm and complete Koch's postulate.

4. *Phylogenetic analysis of CGSC isolates*

To understand the CGSC isolates in this study, those showing pathogenicity were analyzed. Several DNA regions were analyzed, including the intergenic regions of *Apn2* and *MAT1-2-1* (ApMAT), *actin* (ACT), *chitin synthase* (CHS-1), *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) genes, and internal transcribed spacer (ITS) region sequences. Total DNA was extracted in accordance with a previously described method. The primers used for amplification were reported by Weir *et al.*[9] (Table 1). Each 25 μL PCR mixture contained 20 ng DNA template, 1× PCR Dye Master Mix II (GMbiolab Co, Ltd., Taichung, Taiwan), and 0.25 μM primers.

The PCR parameters were as follows: denaturation at 95°C for three min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 53–59°C for 30 sec, and polymerization at 72°C for 60 sec, followed by a final extension at 72°C for five min. PCR was performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR products were subjected to electrophoresis in 1.5 to 2.0% agarose gels and visualized using a Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After confirming the sequence length, the amplicons were gel-purified, cloned into the pGEM®-T Easy vector (Promega Co., Madison, WI, USA), and sequenced using dideoxy terminator sequencing (Genomics Ltd., Taipei, Taiwan). Sequencing results were compared with gene databases registered with the National Center for Biotechnology Information on the NCBI website. A phylogenetic tree was generated using Bayesian inference (BI) and bootstrap support from the maximum likelihood method (ML) and maximum parsimony (MP) and based on the five sequences of *ApMAT*, *ACT*, *CHS-1*, *GAPDH*, and ITS. BI analysis with four Markov Chain Monte Carlo (MCMC) chains was run from a random tree for 106 generations or until the average standard deviation of split frequencies was below 0.01 using MrBayes v.3.2.7.[33] ML and MP were performed with 1,000 bootstrap replicates using MEGA X.[34] The support values of BI (posterior probability ≥0.95), ML (bootstrap ≥50%), and MP (bootstrap ≥50%) were provided at the node.

5. *Mycelia-based test of the CGSC isolates to benzimidazoles and strobilurins*

Three benzimidazoles and four strobilurins were used in this study. Benzimidazoles, including benomyl (50% WP; Sinon Corporation, Taichung, Taiwan), thiabendazole (41.8% SC; Sinon Corporation), and carbendazim (50% WP; Sinon Corporation); and strobilurins, including kresoxim-methyl (44.2% SC; BASF, Taipei, Taiwan), azoxystrobin (23% SC; Syngenta, Taipei, Taiwan), trifloxystrobin (43.7% SC; Bayer, Taipei, Taiwan), and pyraclostrobin (23.6% EC; BASF, Taipei, Taiwan), were used to examine the reaction of CGSC isolates from strawberry. In this study, serial concentrations of 1, 10, 100, and 500 μg a.i./mL were used to determine the effective concentration for 50% inhibition (EC_{50}). First, a 5,000 μg a.i./mL stock solution of four strobilurin fungicides was prepared. The fungicide stock solution was mixed with PDA, and the concentration was adjusted to 1, 10, 100, and 500 μg a.i./mL. The colony margin site of the CGSC isolates cultured in PDA at 28°C with a 12 hr light/dark cycle for five days was cut (3 mm) and transferred into a new PDA containing strobilurin fungicides. Each isolate had four replicates at different concentrations, and the control group used a blank PDA. The Petri dish was placed at 28°C in the dark. The diameters of the colonies were measured after five days, and the mycelial growth inhibition rates were calculated using the following formula:

$$\text{Inhibition rate (\%): (CK-Treatment)/CK} \times 100.$$

The EC_{50} value was determined using the Probit-MSChart program, supported by Dr. Hsin-Chi (Professor of the Department of Entomology of National Chung Hsing University).

6. *Germination-based test of CGSC isolates treated with strobilurins*

To compare the difference between the responses of spores and mycelia to strobilurins, a spore germination test was conducted and compared with a mycelial growth test. Fifteen isolates showing different responses to strobilurins were selected for use in

this study (Table 6). In the spore germination test, the PDA was replaced with 2% water agar (WA; Difco™, Becton Dickinson and Co., Franklin Lakes, NJ, USA), and WA containing 1, 10, 100, and 500 μg a.i./mL strobilurins was prepared following the PDA method.

To prepare the spore suspension, the selected CGSC isolates were cultured on PDA at 28°C without light for 10 days, followed by illumination for one day with short-wavelength blue light 400–450 nm, and then the spores were washed with SDW. The 300 μL spore suspension containing 1,000 spores was loaded in WA mixed with strobilurins and plated uniformly. The inoculated WA was then incubated at 28°C in the dark for 20 hr and stained with cotton blue. The stained WA was observed using a light microscope (Leica, Wetzlar, Germany). One hundred spores of each isolate in different concentrations of strobilurins were observed under 200× magnification in the field of view. Three replicates were performed for each isolate at different concentrations. A WA culture without pesticides was added as a control.

7. Cyt b gene analysis of CGSC isolates with different reactions to strobilurins

A point mutation in codon 143 of *cyt b* gene has been reported as a major mechanism for resistance to strobilurin fungicides.[35] CGSC isolates showing low/intermediate/high sensitivity to strobilurins were selected to amplify the *cyt b* gene, including codon 143. The primers used were GCCBF1 (5′-TTCTTGGGTTATGTTTTACCTTA-3′) and RSCBR2 (5′-AACAATATCTTGTCCAATTCATGG-3′).[23] Initial DNA denaturation was performed at 94°C for two min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for one min, and extension at 72°C for 1.5 min; and final extension at 72°C for 8.5 min. Amplification products were assessed by electrophoresis in a 1% agarose gel with 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA buffer) plus 2.5 μL of ethidium bromide. After the PCR products were recovered using a gel elution kit, they were sent to Tri-I Biotech Inc. for sequencing. Sequence results were compared with the gene database registered with the National Center for Biotechnology Information on the NCBI website.

8. Reaction of alternative oxidase (AOX) in CGSC isolates with different sensitivities to strobilurins

AOX is considered an alternative pathway to respiration for maintaining the life of cells when adversity hinders the respiratory electron transport chain.[36] This pathway can be inhibited by salicylhydroxamic acid (SHAM) and propyl gallate.[37] To determine the role of AOX in strobilurin-resistant CGSC isolates, SHAM was mixed with azoxystrobin or trifloxystrobin. The concentration of SHAM (Merck KGaA Co., Darmstadt, Germany) was 10 mg/L, and that of strobilurin fungicides was 100 μg a.i./mL.[25] A mycelial growth inhibition test was performed as described above. CGSC isolates showing low, intermediate, and high sensitivity to strobilurins were selected to examine AOX activities.

9. β-tubulin gene analysis of CGSC isolates with different reactions to benzimidazoles

Previous studies have indicated that point mutations at codons 198 and 200 in the β-tubulin gene are associated with resistance to benzimidazoles.[32] For β-tubulin gene amplification, the primer pair C′ (5′-GAGGAATTCGACCGNATGATG-3′)/F(5′-GACGTTGTTGGGNATCCA-3′)[38] was used to amplify β-tubulin gene fragments of CGSC isolates showing sensitivity or resistance. The following was used for PCR reaction: dH_2O 15 μL, 10X buffer (Invitrogen, Brazil) 2.5 μL, Mg^{2+} (Invitrogen) 2.5 μL, dNTP (GeneMark, Taiwan) 2 μL, primer 0.5 μL, 500 U Taq DNA polymerase (Invitrogen) 1 μL per 8 tubes, and template DNA 2 μL. The PCR conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, 54°C for 30 sec, 72°C for 2 min, and a final extension at 72°C for 5 min.[38] After the PCR products were recovered using a gel elution kit, they were sent to Tri-I Biotech Inc. for sequencing. Sequence results were compared with the gene database registered with the National Center for Biotechnology Information on the NCBI website.

Results

1. Pathogenicity and phylogenic analysis of CGSC

Based on the morphology and ITS identification, all 108 isolates identified as CGSC were used for the pathogenicity test. The results showed that all isolates caused necrosis or crown rot symptoms in Taoyuan No. 1. The tissues of seedlings showing symptoms were re-isolated to confirm and complete Koch's postulate. To understand the species of CGSC isolates, the 91 CGSC isolates showing pathogenicity were analyzed using multiple sequences. As shown in Fig. 2, 88 CGSC taxa formed a large polytomy and were close to *C. siamense*. The other three CGSC isolates (MLDH92, HCKS12, and MLST125) were closely related to *C. fructicola*. The multigene phylogenetic analysis concluded that the strains isolated in this study were closest to *C. siamense* and *C. fructicola*.

2. Mycelia-based tests of the CGSC isolates treated with benzimidazoles and strobilurins

According to the fungicide test, most CGSC isolates had low sensitivity to benzimidazoles (Table 1). The EC_{50} values of 38 and 59 CGSC isolates for benomyl were 100–500 μg a.i./mL and>500 μg a.i./mL, respectively. The EC_{50} values of 21 and 70 CGSC isolates for carbendazim were 100–500 μg a.i./mL and>500 μg a.i./mL, respectively. The EC_{50} values of 32 and 63 CGSC isolates for thiabendazole were 100–500 μg a.i./mL and>500 μg a.i./mL, respectively. In the strobilurin test, most CGSC isolates had low sensitivities to azoxystrobin, kresoxim-methyl, and trifloxystrobin; meanwhile, most CGSC isolates were sensitive to pyraclostrobin (Table 2). The numbers of CGSC isolates with EC_{50} values greater than 500 μg a.i./mL for azoxystrobin, kresoxim-methyl, and trifloxystrobin were 66, 42, and 56, respectively. In contrast, the numbers of CGSC isolates with EC_{50} values ranging from 100–500 μg a.i./mL and more than 500 μg a.i./mL for pyraclostrobin were four

and zero, respectively. In this study, we also analyzed the relationship between the isolation area and the fungicide reaction (Table 3). The CGSC isolates obtained from Taipei and Taichung were more sensitive to benzimidazoles and strobilurins, except pyraclostrobin, than those from the other three areas.

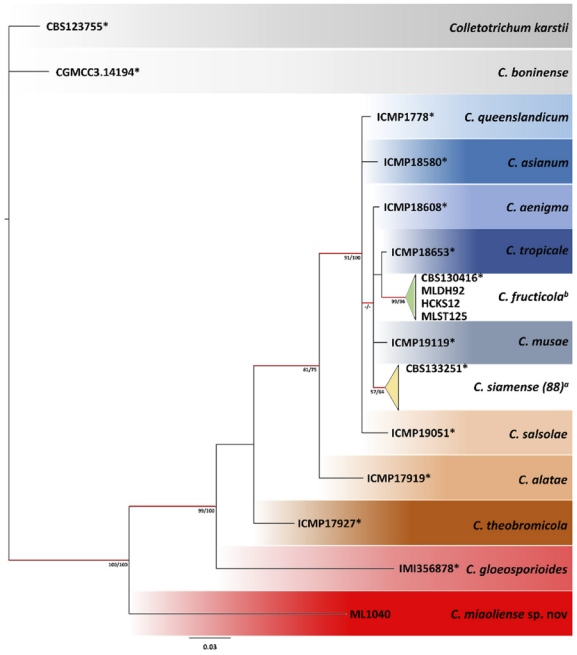


Fig. 2. Genetic tree analysis of 91 isolates of CGSC, using *ApMAT*, *ACT*, *ITS*, *CHS-1*, and *GAPDH* gene sequence for analysis using Bayesian inference (BI) *C. boninense* CBS123755 as an outgroup. Thick red lines indicate Bayesian posterior probabilities (PP) above 95%. Maximum likelihood bootstrap support values (MLB ≥50%) and maximum parsimony bootstrap supports (MPB ≥50%) are given at the nodes (MLB/MPB). *=ex-holotype or ex-epitype culture. ([a] 88 CGSC isolates were close to *C. siamense*; [b] three CGSC isolates (MLDH92, HCKS12 and MLST125) were close to *C. fructicola*)

Here, we used sensitivity (%) to show the EC_{50} values of isolates lower than 100 μg a.i./mL. The sensitivity percentage of EC_{50} values<100 μg a.i./mL showed that the CGSC isolates obtained from Hsinchu, Miaoli, and Nantou were below 50%. To confirm the possible mechanism of resistance to benzimidazoles and strobilurins, CGSC isolates that showed sensitivity/resistance to the two fungicides were selected for further study.

3. *Germination-based tests of CGSC isolates treated with strobilurins*

We aimed to understand whether strobilurin fungicides had different effects on mycelia and spores. According to the test results, mycelia were more sensitive than spores to strobilurins for certain CGSC isolates (Table 4). In particular, MLDH67, MLGG168, and TPNH20 isolates showed low EC_{50} values (<1 μg a.i./mL) for azoxystrobin in the mycelial test and higher EC_{50} values of 299.8, 355.3, and 32.2 μg a.i./mL, respectively, in the spore germination test. Similarly, the three isolates showed low EC_{50} values for trifloxystrobin and pyraclostrobin in the mycelial test and high EC_{50} values in the spore germination test.

4. *Cyt b gene analysis of CGSC isolates to strobilurins*

Fifteen CGSC isolates that showed low/intermediate/high sensitivity to the three strobilurin fungicides were selected for *cyt b* gene analysis. A DNA fragment of approximately 75 bp that included codon 143 of the *cyt b* gene was amplified. NCBI gene bank analysis showed that the similarity of the *cyt b* gene to *C. cereale* TCGC-5.34 (EF636930) was 98%. The analyzed CGSC isolates, including those of *C. siamense* and *C. fructicola*, aligned, and the mutation in codon 143 was not found in these CGSC isolates that showed low or high sensitivities to strobilurins.

Table 1. Distribution of CGSC isolates at different benzimidazole EC_{50} values

Fungicide	Number of CGSC isolates at different EC_{50} (μg a.i./mL)[a)]					Total
	<1	1–10	10–100	100–500	>500	
Benomyl	0	4	7	38	59	108
Carbendazim	0	7	10	21	70	108
Thiabendazole	3	3	7	32	63	108

[a)] EC_{50}: Concentration at which the inhibitive response is present in 50 percent of the population.

Table 2. Distribution of CGSC isolates at different strobilurin EC_{50} values

Fungicide	Number of CGSC isolates at different EC_{50} (μg a.i./mL)[a)]					Total
	<1	1–10	10–100	100–500	>500	
Azoxystrobin	0	3	11	28	66	108
Kresoxim- methyl	4	10	17	35	42	108
Pyraclostrobin	24	31	49	4	0	108
Trifloxystrobin	7	10	10	25	56	108

[a)] EC_{50}: Concentration at which the inhibitive response is present in 50 percent of the population.

Table 3. Sensitivity of the *Colletotrichum gloeosporioides* species complex to different fungicides from different areas

Fungicide	Place collected (number)	Number of isolates in each EC_{50} (μg a.i./mL) range					Sensitive (%)[a]
		<1	1–10	10–100	100–500	>500	
Kresoxim-methyl	Taipei (8)	2	3	0	2	1	62.5
	Hsinchu (16)	1	2	4	4	5	43.8
	Miaoli (54)	0	1	7	20	26	14.8
	Taichung (8)	1	2	3	1	1	75.0
	Nantou (22)	0	2	3	8	9	22.7
Azoxystrobin	Taipei (8)	0	1	3	4	0	50.0
	Hsinchu (16)	0	3	3	2	8	37.5
	Miaoli (54)	0	0	1	11	42	3.7
	Taichung (8)	0	0	2	5	1	25.0
	Nantou (22)	0	1	2	6	13	13.6
Trifloxystrobin	Taipei (8)	2	2	2	2	0	75.0
	Hsinchu (16)	1	3	4	3	5	50.0
	Miaoli (54)	1	0	0	13	40	5.6
	Taichung (8)	2	3	1	2	0	75.0
	Nantou (22)	1	2	3	5	11	27.3
Pyraclostrobin	Taipei (8)	2	3	2	1	0	87.5
	Hsinchu (16)	4	5	7	0	0	100
	Miaoli (54)	10	12	31	1	0	98.1
	Taichung (8)	3	4	0	1	0	87.5
	Nantou (22)	5	7	9	1	0	95.5
Benomyl	Taipei (8)	0	1	2	3	2	37.5
	Hsinchu (16)	0	1	1	5	9	12.5
	Miaoli (54)	0	0	1	20	33	1.9
	Taichung (8)	0	1	2	2	3	37.5
	Nantou (22)	0	1	1	8	12	9.1
Thiabendazole	Taipei (8)	1	1	2	1	3	50.0
	Hsinchu (16)	0	0	1	5	10	6.3
	Miaoli (54)	0	0	1	18	35	3.7
	Taichung (8)	1	1	2	2	2	50.0
	Nantou (22)	1	1	1	6	13	13.6
Carbendazim	Taipei (8)	0	3	2	2	1	62.5
	Hsinchu (16)	0	1	2	3	10	18.8
	Miaoli (54)	0	0	1	10	43	1.9
	Taichung (8)	0	1	3	2	2	50.0
	Nantou (22)	0	2	2	4	14	18.2

[a] Sensitive (%): The percentage of EC_{50}<100 μg a.i./mL.

5. Reaction of AOX in CGSC isolates with different sensitivities to strobilurins

According to the results, the rates of inhibiting mycelial growth were higher in PDA with azoxystrobin/trifloxystrobin and SHAM than in PDA with only azoxystrobin/trifloxystrobin (Table 5). The MLDH63 (2.1±0.2%), MLDH68 (1.8±0.1%), and MLTA183 (2.3±0.3%) isolates with lower inhibition rates of mycelial growth in azoxystrobin showed low inhibition rates after SHAM was added. Similarly, the three isolates with low rates of inhibiting mycelial growth by trifloxystrobin also showed low inhibition rates after the addition of SHAM. The results demonstrated that AOX activity could not support isolates with low sensitivity to azoxystrobin or trifloxystrobin to overcome strobilurin fungicides.

6. β-tubulin gene analysis of CGSC isolates with different reactions to benzimidazoles

Fifteen CGSC isolates with low, intermediate, and high sensitivities to benzimidazoles were selected for β-tubulin gene analysis (Table 6). A DNA fragment of approximately 570 bp that included codons 198 and 200 of the β-tubulin gene was amplified. The results showed that the β-tubulin gene similarity with *C. gloeosporioides* f. sp. *aeschynomene* (U14138) was 98%. In this study, isolates with higher EC_{50} values showed a point mutation in

Table 4. Susceptibility of 15 strawberry anthracnose isolates to the strobilurin fungicides azoxystrobin, trifloxystrobin, and pyraclostrobin in terms of mycelial growth and spore germination

Isolate	EC_{50} (μg a.i./mL)[a]					
	Azoxystrobin		Trifloxystrobin		Pyraclostrobin	
	Mycelium	Spore	Mycelium	Spore	Mycelium	Spore
MLDH63	>500	>500	>500	>500	5.5±0.3	49.1±0.8
MLDH68	>500	>500	>500	>500	8.9±0.1	39.8±0.3
MLTA183	>500	>500	>500	>500	8.3±0.2	33.6±0.8
HCKS11	391.9±8.7[b]	>500	188.7±3.3	>500	2.5±0.2	54.4±1.9
MLDH62	422.3±10.3	447.4±8.8	290.8±4.7	>500	3.3±0.8	<1
HCKS15	449.5±11.2	>500	378.9±4.2	>500	4.2±0.2	55.8±2.5
NTGS35	55.3±2.8	>500	190.8±5.3	>500	<1	37.7±2.9
MLST115	43.1±1.6	347.3±3.8	219.2±4.3	>500	<1	43.5±1.6
MLST70	16.4±0.3	>500	210.3±3.3	356.4±9.7	<1	33.9±1.7
TCHU02	2.9±0.2	392.8±9.4	55.9±2.9	377.6±4.1	6.9±0.4	2.8±0.4
NTGS20	8.9±0.7	285.3±5.5	33.5±1.7	488.5±9.8	8.7±0.2	6.7±0.2
NTGS123	5.8±0.3	368.9±3.8	20.2±0.6	467.6±3.9	7.7±0.3	2.2±0.2
MLDH67	<1	299.8±2.7	22.9±0.8	225.8±4.1	<1	24.1±1.3
MLGG168	<1	355.3±3.9	31.9±0.7	>500	2.1±0.1	35.2±1.2
TPNH20	<1	32.2±0.8	19.6±0.2	>500	1.5±0.3	55.7±2.2

[a] EC_{50}: concentration at which the inhibitive response is present in 50 percent of the population. [b] Mean±standard error (n=4).

Table 5. Sensitivity of 15 CGSC isolates to commercial azoxystrobin (100 μg a.i./mL) or trifloxystrobin (100 μg a.i./mL) mixed with 10 μg a.i./mL SHAM at 28°C after three days of culture

CGSC isolate	Inhibition rate of mycelial growth (%)[a]			
	Azoxystrobin	Azoxystrobin+SHAM[b]	Trifloxystrobin	Trifloxystrobin+SHAM
MLDH63	2.1±0.2[c]	2.5±0.1	2.1±0.2	2.3±0.2
MLDH68	1.8±0.1	2.0±0.2	2.3±0.2	2.3±0.1
MLTA183	2.3±0.3	2.4±0.3	2.5±0.1	2.6±0.1
HCKS11	32.3±0.8	33.5±0.5	36.1±0.9	37.2±0.5
MLDH62	35.8±0.9	37.1±1.7	32.7±1.1	33.8±1.9
HCKS15	32.1±1.3	33.3±0.3	33.7±1.2	33.7±1.6
NTGS35	56.1±0.8	57.2±0.5	57.5±0.6	58.2±0.9
MLST115	62.7±1.0	62.9±0.6	52.7±0.3	53.3±0.5
MLST70	53.7±1.5	55.0±1.1	53.9±1.7	54.1±1.3
TCHU02	76.2±1.9	78.1±1.7	79.2±1.9	81.3±1.4
NTGS20	82.2±1.2	83.7±1.5	84.1±1.7	85.2±1.5
NTGS123	81.5±1.1	83.2±1.4	82.3±1.5	84.0±1.8
MLDH67	97.1±2.2	98.2±2.1	98.2±2.7	97.9±2.9
MLGG168	98.0±1.9	99.7±1.9	96.0±1.7	95.0±1.3
TPNH20	99.3±1.5	100±0	93.1±1.8	94.9±1.6

[a] Inhibition rate (%): (CK Treatment)÷CK×100%. [b] SHAM: Salicylhydroxamic acid (an inhibitor of alternative oxidase). [c] Mean±standard error (n=4).

codon 198 from CGA to GCG (A198G). Here, three *C. fructicola* isolates with 229.7±4.3 μg a.i./mL to 392.3±1.5 μg a.i./mL EC_{50} values for benzimidazoles also had point mutations in codon 198.

Discussion

A total of 108 isolates of *Colletotrichum* spp. were collected from the five main areas of strawberry cultivation in Taiwan. Based on multiple sequence analyses, the CGSC isolates from strawberries were identified as *C. siamense* and *C. fructicola*, where *C. siamense* was the dominant species (more than 96.7%). In Taiwan, CGSC infects more than 80 crop species.[39] Currently, the species composition of CGSC has been identified from only a few crops, including strawberries, mangoes, grapes, avocadoes, lychee *etc.*[5,40,41] In our study, *C. siamense* was the dominant species of CGSC from strawberries, which is consistent with the re-

Table 6. Mutation and deduced amino acid substitution in the partial sequence of the β-tubulin gene at codons 198 and 200 in isolates of CGSC with different responses to benzimidazole fungicides benomyl, carbendazim, and thiabendazole

CGSC isolate	EC_{50} (μg a.i./mL) value of benzimidazole			Sequence in codon (amino acid)	
	Benomyl	Carbendazim	Thiabendazole	198	200
MLDH63	>500	>500	>500	GCG (Ala)	TTC (Phe)
MLDH68	>500	>500	>500	GCG (Ala)	TTC (Phe)
MLTA183	>500	>500	>500	GCG (Ala)	TTC (Phe)
HCKS11	>500	>500	354.8±8.7	GCG (Ala)	TTC (Phe)
MLDH62	183.5±7.9[b)]	>500	410.6±11.6	GCG (Ala)	TTC (Phe)
HCKS15	397.2±5.4	459.5±11.9	395.5±9.9	GCG (Ala)	TTC (Phe)
NTGS35	492.3±1.1	472.1±6.1	246.1±13.3	GCG (Ala)	TTC (Phe)
MLDH92[a)]	379.4±1.2	332.9±2.5	186.9±5.9	GCG (Ala)	TTC (Phe)
MLST125[a)]	219.1±5.3	286.2±2.5	257.7±3.4	GCG (Ala)	TTC (Phe)
HCKS12[a)]	253.4±1.9	228.3±2.9	225.7±5.7	GCG (Ala)	TTC (Phe)
NTGS123	43.5±1.1	61.4±1.6	46.1±0.8	GAG (Glu)	TTC (Phe)
MLDH67	7.9±0.2	36.8±0.5	61.4±1.9	GAG (Glu)	TTC (Phe)
MLGG169	5.4±0.3	9.2±0.7	6.2±0.2	GAG (Glu)	TTC (Phe)
TPNH11	62.5±0.8	63.2±1.4	8.5±0.5	GAG (Glu)	TTC (Phe)
TCHU03	2.2±0.3	1.5±0.2	4.2±0.2	GAG (Glu)	TTC (Phe)

[a)] *C. fructicola*. [b)] Mean±standard error (n=4).

sults of Chung *et al.*[5)] Apart from strawberries, *C. siamense* has also been reported to cause anthracnose in different crops, such as mango and lychee.[41,42)] Only a few *C. fructicola* isolates have been obtained from strawberries. *C. fructicola* is a major species in other crops, including apples, pears, and cassava.[43–45)] According to the composition of *Colletotrichum* spp. from strawberries in Taiwan as reported by Chung *et al.*,[5)] in addition to CGSC, *C. miaoliense*, *C. karstii*, and *C. boninense* also cause anthracnose. However, these three *Colletotrichum* species were not detected at this time. In this study, CGSC isolates were obtained from the crown rot of strawberries. Thus, *C. siamense* may be the major pathogen that infects the crown and causes crown rot in strawberries.

The strobilurin test showed that the EC_{50} values of most CGSC isolates for azoxystrobin, kresoxim-methyl, and trifloxystrobin were higher than 500 μg a.i./mL. In contrast, most of the CGSC isolates were sensitive to pyraclostrobin and showed EC_{50} values under 100 μg a.i./mL. Certain reports also indicated that *Colletotrichum* spp. had higher sensitivity to pyraclostrobin than other strobilurin fungicides.[46,47)] Several reasons are suspected to be associated with the higher ability of pyraclostrobin to inhibit the mycelial growth of CGSC. First, pyraclostrobin, recommended in 2002 for controlling strawberry anthracnose, was released later than azoxystrobin, kresoxim-methyl, and trifloxystrobin. Second, the structures of the toxic groups of the four agents (toxophores) are different. For example, the toxic group of kresoxim-methyl and trifloxystrobin is (E)-methyl-methoxyimino acetate, and that of pyraclostrobin is methyl *N*-methoxycarbamate.[48)] Thus, the difference in chemical structure might affect the ability of the drug to act and result in different susceptibilities to pathogens. It is necessary to continually monitor CGSC sensitivity to pyraclostrobin in strawberry fields. In this study, three *C. fructicola* isolates showed a higher sensitivity to strobilurins than did *C. siamense*. Previous reports have indicated that *C. fructicola* from strawberries was resistant to azoxystrobin.[21)] The sensitivity of *C. fructicola* to strobilurins revealed that the Taiwanese isolates did not develop resistance to strobilurins. This result might also be associated with *C. fructicola*, which is not a dominant species because of its high sensitivity to strobilurins in strawberry fields. The dynamic variation between *C. siamense* and *C. fructicola* will be continually monitored.

Furthermore, the results revealed that mycelia were more sensitive to strobilurins than were spores of certain CGSC isolates. According to Bartlett *et al.*,[48)] the mechanism of action of strobilurin fungicides is to inhibit the progress of electron transfer in the granulosa gland and reduce the synthesis of ATP; therefore, it should have an inhibitory effect on spore germination and mycelial growth. Previous studies have also pointed out the sensitivity of strobilurin fungicides to other phytopathogenic fungi—such as *Calonectria pseudonaviculata*, *Alternaria alternate*, and *A. solani*[49,50)]—and the higher sensitivity of spore germination compared to mycelial growth. In this study, differences in the susceptibility of mycelia and spores to strobilurins were compared, and it was found that the susceptibility of the 15 isolates to strobilurins was greater than that of spore germination. Since the number of CGSC isolates tested for spore germination in this study was small, more isolates need to be tested to determine whether strobilurins have a greater effect on mycelial growth than do spores. In our study, to understand the susceptibility of strains to drugs in a quick and convenient way, analyzing a large number of isolates using mycelial growth tests is

more suitable.

In the benzimidazole test, most of the CGSC isolates from strawberry showed resistance to benzimidazoles. Moreover, isolates with low sensitivity to benomyl also showed low sensitivity to carbendazim and thiabendazole. Thus, cross-resistance exists between benzimidazoles. Benzimidazoles are high-risk fungicides that induce resistance in fungal pathogens, and CGSC is a group that is easily resistant to benzimidazoles.[24] In Taiwan, CGSC isolates have been reported to develop benzimidazole-resistant isolates in the field, such as in avocado, fig, jujube, and mango cultivation.[41] Our results also showed that CGSC isolates from strawberry developed benzimidazole resistance in strawberry cultivation. Although benzimidazoles are not the major fungicides used to control strawberry anthracnose in Taiwan, they are still used to control anthracnose in other crops. Moreover, *C. siamense* and *C. fructicola* also cause anthracnose in other crops.[19,42,45,47] Benzimidazole-resistant isolates might be transmitted to different areas or crops based on wind velocity and rain.

Comparing the relationship between geography and fungicide sensitivity, the CGSC isolates from Taipei and Taichung areas were more sensitive to strobilurin fungicides than were those from Hsinchu, Miaoli, and Nantou. This result demonstrates that sensitivity to strobilurins might be associated with different areas in Taiwan. Due to the areas of strawberry cultivation in Hsinchu, Miaoli, and Nantou being larger than those in Taipei and Taichung (Fig. 1), the frequency of fungicide application might be higher and induce a more drug-resistant population. Previous study has also revealed that the reactions of *Colletotrichum* spp. to fungicides varied depending on the collection area.[21] This phenomenon might correspond to farmers' management in different areas. Thus, it is necessary to understand each farmer's habit of fungicide application.

Sequence analyses showed that the CGSC isolates with different sensitivities to strobilurins did not have a mutation at codon 143 (GGT→GCT) in the *cyt b* gene; meanwhile, neither did AOX play a role in enhancing resistance to strobilurins. The mutation at codon 143 in the *cyt b* gene has been confirmed to be associated with strobilurin resistance in many fungal pathogens, including certain *Colletotrichum* spp.,[23,51] such as *A. alternata*[52] and *Leveillula taurica*.[53] In our study, CGSC isolates showed low sensitivity to strobilurins and were not associated with *cyt b* gene point mutations. Thus, the mutation at codon 143 in the *cyt b* gene might not be the main factor inducing low sensitivity of CGSC isolates to strobilurins in Taiwan. Certain reports have also revealed that some *Colletotrichum* spp. isolates did not have a mutation at codon 143 in the *cyt b* gene.[54,55] Avila-Adame *et al.*[54] indicated that mutations in codons 95, 130, and 141 might be associated with strobilurin resistance. Here, we did not find mutations in codon 95, 130, or 141. For AOX analysis, isolates slightly or highly sensitive to strobilurins only showed a few changes after culture on PDA medium. Previous studies have indicated that AOX activity can induce the growth of fungal isolates against strobilurins.[56,57] Two inhibitors—propyl gallate and SHAM—are commonly used to study the role of AOX in strobilurin resistance.[37] However, AOX activity did not assist CGSC isolates, which showed low sensitivity to the three strobilurin fungicides. Except for the *cyt b* gene mutation and AOX, the ATP binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters are considered other possible mechanisms associated with strobilurin resistance.[58] ABC transporters are located in the plasma and organelle membranes of eukaryotes; they act as drug efflux pumps to reduce the effects of chemicals.[59] Resistance mechanisms may include point mutation in the transcriptional factor, promoter rearrangement (insertion or deletion), and the constitutive overexpression of fungicide efflux transporters.[58] The *C. acutatum* ABC transporter (*CaABC1*) gene had up-regulated expression in kresoxim-methyl, thiophanate-methyl, iprobenfos, and hygromycin B.[60] It is better to understand the role of ABC and MFS transporters in CGSC isolates showing low sensitivities in Taiwan.

β-tubulin gene analysis demonstrated that CGSC isolates with low sensitivity to benzimidazoles have a point mutation in codon 198 (GAG→GCG), and the amino acid glycine (Gly) was substituted for alanine (Ala). Several point mutations have been confirmed to be related to the benzimidazole resistance of fungal isolates, including codons 167, 198, and 200.[61] In this study, the CGSC isolates showed low sensitivity to benzimidazoles with only a point mutation in codon 198 and were not found in codon 167 or 200. Chung *et al.*[32] reported that moderately resistant isolates of CGSC ($100<EC_{50}<500\,\mu$g a.i./mL) had mutations in codon 200, and resistant isolates of CGSC ($EC_{50}>500\,\mu$g a.i./mL) had a mutation in codon 198. Our results indicate that the EC_{50} values of CGSC isolates between 100 and 500 μg a.i./mL did not show a mutation in codon 200. Peng[25] also reported that there was no intermediate mutation in the expression of drug resistance genes in Taiwan mango and strawberry anthracnose fungi. Thus, the phenotype of moderately resistant isolates did not completely correspond with the genotype. Interestingly, the moderately resistant phenotype might show differences between CGSC isolates from different crops. In the future, the information we gleaned could contribute to farmers' developing a strategy to manage strawberry anthracnose by fungicides, thereby reducing the pressure of selection and eliminating fungicide-resistant groups.

Acknowledgements

We acknowledge Dr. Dai-Rong Wu for sharing plant materials. We thank Ms. Wen-Ching Huang and Mr. Ji-Feng Li (Miaoli District Agricultural Research and Extension Station, Taiwan) for their help with the inoculation trials. This work was financially supported by the Innovation and Development Center of Sustainable Agriculture of The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) and Council of Agriculture (COA), Taiwan (grant nos.108AS-8.4.1-MS-M1 and 109AS-8.4.2-MS-M1).

Funding

This work was financially supported by the Innovation and Development Center of Sustainable Agriculture of The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) and the Council of Agriculture (COA) (108AS-8.4.1-MS-M1 and 109AS-8.4.2-MS-M1).

Conflicts of interest/Competing interests

Not applicable

Ethics approval

Not applicable

Author contributions

Sheng-Chi Chu planned and performed the experiments, analyzed the data, created the figures, and drafted and edited the manuscript. Tsung-Chun Lin provided the samples and isolates. Kuo-Hsi Lin and Chinnapan Thanarut revised the manuscript. Wen-Hsin Chung obtained funding, planned experiments, analyzed the data, and revised the manuscript. All authors contributed to the manuscript and approved the submitted version.

Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Tables S1–S3 and Supplemental Figs. S1–S6), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Consent to participate

Not applicable

Consent for publication

Not applicable

Code availability

Not applicable

References

1) A. G. Buddie, P. Martínez-Culebras, P. D. Bridge, M. D. García, A. Querol, P. F. Cannon and E. Monte: Molecular characterization of *Colletotrichum* isolates derived from strawberry. *Mycol. Res.* **103**, 385–394 (1999).
2) L. Cai, T. Giraud, N. Zhang, D. Begerow, G. Cai and R. G. Shivas: The evolution of species concepts and species recognition criteria in plant pathogenic fungi. *Fungal Divers.* **50**, 121–133 (2011).
3) S. F. Hou, J. J. Liu, T. F. Xu, X. F. Li, X. F. Li, S. Li and H. Q. Wang: Simultaneous detection of three crown rot pathogens in field-grown strawberry plants using a multiplex PCR assay. *Crop Prot.* **156**, 105957 (2022).
4) C. M. Howard, J. L. Maas, C. K. Chandler and E. E. Albregts: Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. *Plant Dis.* **76**, 976–981 (1992).
5) P. C. Chung, H. Y. Wu, H. A. Ariyawansa, S. S. Tzean and C. L. Chung: First report of anthracnose crown rot of strawberry caused by *Colletotrichum siamense* in Taiwan. *Plant Dis.* **103**, 1775–1776 (2019).
6) P. C. Chung, H. Y. Wu, Y. W. Wang, H. A. Ariyawansa, H. P. Hu, T. H. Hung, S. S. Tzean and C. L. Chung: Diversity and pathogenicity of *Colletotrichum* species causing strawberry anthracnose in Taiwan and description of a new species, *Colletotrichum miaoliense* sp. nov. *Sci. Rep.* **10**, 14664 (2020).
7) K. J. Curry, M. Abril, J. B. Avant and B. J. Smith: Strawberry anthracnose: Histopathology of *Colletotrichum acutatum* and *C. fragariae*. *Phytopathology* **92**, 1055–1063 (2002).
8) S. Freeman, D. Minz, E. Jurkevitch, M. Maymon and E. Shabi: Molecular analysis of *Colletotrichum* species from almond and other fruits. *Phytopathology* **90**, 608–614 (2000).
9) B. S. Weir, P. R. Johnston and U. Damm: The *Colletotrichum gloeosporioides* species complex. *Stud. Mycol.* **73**, 115–180 (2012).
10) U. Damm, P. F. Cannon, J. H. C. Woudenberg and P. W. Crous: The *Colletotrichum acutatum* species complex. *Stud. Mycol.* **73**, 37–113 (2012b).
11) U. Damm, P. F. Cannon, J. H. C. Woudenberg, P. R. Johnston, B. S. Weir, Y. P. Tan, R. G. Shivas and P. W. Crous: The *Colletotrichum boninense* species complex. *Stud. Mycol.* **73**, 1–36 (2012a).
12) D. De Silva, P. W. Crous, P. K. Ades, K. D. Hyde and P. W. Taylor: Life styles of *Colletotrichum* species and implications for plant biosecurity. *Fungal Biol. Rev.* **31**, 155–168 (2017).
13) T. C. Lin, C. S. You, I. S. Wu, C. H. Tsai, J. N. Tsai, J. Z. Yu and R. J. Chang: Application of a bio-control agent for controlling strawberry anthracnose in Taiwan, In "International Symposium on Proactive Technologies for Enhancement of Integrated Pest Management of Key Crops," Taiwan Agricultural Research Institute, Taichung, Taiwan, pp 180–194, 2018.
14) X. Li, T. Jing, D. Zhou, M. Zhang, D. Qi, X. Zang, Y. Zhao, K. Li, W. Tang, Y. Chen, C. Qi, W. Wang and J. Xie: Biocontrol efficacy and possible mechanism of *Streptomyces* sp. H4 against postharvest anthracnose caused by *Colletotrichum fragariae* on strawberry fruit. *Postharvest Biol. Technol.* **175**, 111401 (2021).
15) B. G. De los Santos and F. R. Muñoz: Effect of different fungicides in the control of *Colletotrichum acutatum*, causal agent of anthracnose crown rot in strawberry plants. *Crop Prot.* **21**, 11–15 (2002).
16) G. Han, Q. Li, F. Sun and H. Li: Identification of pathogen caused strawberry anthracnose and their resistance to carbendazim and diethofencarb. *Zhejiang Agri. Sci.* **6**, 1169–1172 (2009).
17) X. F. Xu, T. Lin, S. K. Yuan, D. J. Dai, H. J. Shi, C. Q. Zhang and H. D. Wang: Characterization of baseline sensitivity and resistance risk of *Colletotrichum gloeosporioides* complex isolates from strawberry and grape to two demethylation-inhibitor fungicides, prochloraz and tebuconazole. *Australas. Plant Pathol.* **43**, 605–613 (2014).
18) L. F. Arauz: Mango anthracnose: Economic impact and current options for integrated management. *Plant Dis.* **84**, 600–611 (2000).
19) M. J. Hu, A. Grabke, M. E. Dowling, H. J. Holstein and G. Schnabel: Resistance in *Colletotrichum siamense* from peach and blueberry to thiophanate-methyl and azoxystrobin. *Plant Dis.* **99**, 806–814 (2015).
20) F. P. Wong and S. L. Midland: Sensitivity distributions of California populations of *Colletotrichum cereale* to the DMI fungicides propiconazole, myclobutanil, tebuconazole, and triadimefon. *Plant Dis.* **91**, 1547–1555 (2007).
21) L. Zhang, L. Song, X. Xu, X. Zou, K. Duan and Q. Gao: Characterization and fungicide sensitivity of *Colletotrichum* species causing strawberry anthracnose in eastern China. *Plant Dis.* **104**, 1960–1968 (2020).
22) B. B. Forcelini, T. E. Seijo, A. Amiri and N. A. Peres: Resistance in

strawberry isolates of *Colletotrichum acutatum* from Florida to Quinone-Outside Inhibitor fungicides. *Plant Dis.* **100**, 2050–2056 (2016).
23) M. Inada, H. Ishii, W. H. Chung, T. Yamada, J. Yamaguchi and A. Furuta: Occurrence of strobilurin-resistant strains of *Colletotrichum gloeosporioides* (*Glomerella cingulata*), the causal fungus of strawberry anthracnose. *J. Jap. Phytopathol.* **74**, 114–117 (2008).
24) FRAC: "FRAC Code List 2022: Fungal control agents sorted by cross resistance pattern and mode of action," Fungicide Resistance Action Committee, https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2022--final.pdf?sfvrsn=b6024e9a_2 (Accessed Mar. 2022)
25) M. Z. Peng: Master Thesis, Sensitivity of mango and strawberry anthracnose fungi to strobilurins (QoIs) and benzimidazoles in Taiwan. National Chung Hsing University, Taichung, Taiwan, 2008 (in Chinese with English abstract).
26) S. Yokosawa, N. Eguchi, K. Kondo and T. Sato: Phylogenetic relationship and fungicide sensitivity of members of the *Colletotrichum gloeosporioides* species complex from apple. *J. Gen. Plant Pathol.* **83**, 291–298 (2017).
27) S. Yokosawa, N. Eguchi and T. Sato: N. Eguchi, and T. Sato: Characterization of the *Colletotrichum gloeosporioides* species complex causing grape ripe rot in Nagano Prefecture, Japan. *J. Gen. Plant Pathol.* **86**, 163–172 (2020).
28) A. B. Gama, J. S. Baggio, C. S. Rebello, S. A. Lourenço, M. C. Gâsparoto, G. J. da Silva Junior., N. A. Peres and L. Amorim: Sensitivity of *Colletotrichum acutatum* isolates from citrus to carbendazim, difenoconazole, tebuconazole, and trifloxystrobin. *Plant Dis.* **104**, 1621–1628 (2020).
29) S. Zhong, J. Miao, X. Liu and G. Zhang: Characterization of *Colletotrichum* spp. sensitivity to carbendazim for isolates causing strawberry anthracnose in China. *Plant Dis.* **105**, 87–95 (2021).
30) W. H. Chung, W. C. Chung, M. T. Peng, H. R. Yang and J. W. Huang: Specific detection of benzimidazole resistance in *Colletotrichum gloeosporioides* from fruit crops by PCR-RFLP. *N. Biotechnol.* **27**, 17–24 (2010).
31) T. J. White, T. Truns, S. Lee and J. W. Taylor: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In "PCR Protocols: A Guide to Methods and Applications," ed. by M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, Academic Press, New York, pp. 315–322, 1990.
32) W. H. Chung, H. Ishii, K. Nishimura, M. Fukaya, K. Yano and Y. Kajitani: Fungicide sensitivity and phylogenetic relationship of anthracnose fungi isolated from various fruit crops in Japan. *Plant Dis.* **90**, 506–512 (2006).
33) F. Ronquist and J. P. Huelsenbeck: MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574 (2003).
34) S. Kumar, G. Stecher, M. Li, C. Knyaz and K. Tamura: MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).
35) D. Fernández-Ortuño, J. A. Torés, A. de Vicente and A. Pérez-García: Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *Int. Microbiol.* **11**, 1–9 (2008).
36) Z. Ma and T. J. Michailides: Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Prot.* **24**, 853–863 (2005).
37) A. D. Murphy and N. Lang-Unnasch: Alternative oxidase inhibitors potentiate the activity of atovaquone against *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **43**, 651–654 (1999).
38) C. Albertini, M. Gredt and P. Leroux: Mutations of the β-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. *Pestic. Biochem. Physiol.* **64**, 17–31 (1999).
39) S. S. Tzean, K. C. Tzeng, C. A. Chang, T. T. Tsay and S. F. Yen: "List of Plant Diseases in Taiwan, 5th Ed," Taiwan Phytopathological Society, Taipei, Taiwan, 2019.
40) H. Duan, H. R. Pang and C. C. Wang: Identification, pathogenicity and fungicide sensitivity of Colletotrichum isolates from five fruit crops in Taiwan. *Taiwan Pestic. Sci.* **5**, 91–111 (2018).
41) C. H. Duan, H. R. Pan and C. C. Wang: Molecular identification and fungicides sensitivity of *Colletotrichum* isolates from various fruit crops in Taiwan. *Taiwan Pestic. Sci* **6**, 71–104 (2019).
42) H. F. Ni, C. W. Huang, C. J. Wu, H. R. Yang, C. Y. Lin, J. Y. Chang and J. W. Chang: First report of pepper spot disease of lychee caused by *Colletotrichum siamense* in Taiwan. *J. Plant Pathol.* **99**, 808 (2017).
43) L. Casanova, L. Hernández, E. Martínez, A. C. Velho, M. F. Rockenbach, M. J. Stadnik, S. Alaniz and P. Mondino: First report of *Glomerella* leaf spot of apple caused by *Colletotrichum fructicola* in Uruguay. *Plant Dis.* **101**, 834 (2017).
44) H. N. Li, J. J. Jiang, N. Hong, G. P. Wang and W. X. Xu: First report of *Colletotrichum fructicola* causing bitter rot of pear (*Pyrus bretschneideri*) in China. *Plant Dis.* **97**, 1000 (2013).
45) C. A. D. Bragança, L. L. Silva, F. Haddad and S. A. S. Oliveira: First report of *Colletotrichum fructicola* causing anthracnose in cassava (*Manihot esculenta*) in Brazil. *Plant Dis.* **100**, 857–858 (2016).
46) Y. Y. Gao, L. F. He, B. X. Li, W. Mu, J. Lin and F. Liu: Sensitivity of *Colletotrichum acutatum* to six fungicides and reduction in incidence and severity of chili anthracnose using pyraclostrobin. *Australas. Plant Pathol.* **46**, 521–528 (2017).
47) H. M. Usman, Q. Tan, M. M. Karim, M. Adnan, W. X. Yin, F. X. Zhu and C. X. Luo: Sensitivity of *Colletotrichum fructicola* and *Colletotrichum siamense* of peach in China to multiple classes of fungicides and characterization of pyraclostrobin-resistant isolates. *Plant Dis.* **105**, 3459–3465 (2021).
48) D. W. Bartlett, J. M. Clough, J. R. Godwin, A. A. Hall, M. Hamer and B. Parr-Dobrzanski: Review: The strobilurin fungicides. *Pest Manag. Sci.* **58**, 649–662 (2002).
49) K. Maurer, R. S. Cowles and J. A. LaMondia: Sensitivity of *Calonectria pseudonaviculata*, the pathogen of boxwood blight, to strobilurin and demethylation inhibitor fungicides in connecticut. *J. Environ. Hortic.* **35**, 138–145 (2017).
50) L. Tymon and D. A. Johnson: Fungicide resistance of two species of *Alternaria* from potato in the Columbia basin of Washington. *Plant Dis.* **98**, 1648–1653 (2014).
51) L. Ren, S. F. Wang, X. J. Shi, J. Y. Cao, J. B. Zhou and X. J. Zhao: Characterisation of sensitivity of *Colletotrichum gloeosporioides* and *Colletotrichum capsici* causing pepper anthracnose, to picoxystrobin. *J. Plant Dis. Prot.* **127**, 657–666 (2020).
52) A. Duba, K. Goriewa, U. Wachowska and M. Wiwart: *Alternaria alternata* (Fr.) Keissl with mutation G143A in the *Cyt b* gene is the source of a difficult-to-control allergen. *Environ. Sci. Pollut. Res. Int.* **25**, 469–478 (2018).
53) S. Mosquera, L. H. Chen, B. Aegerter, E. Miyao, A. Salvucci, T. C. Chang, L. Epstein and I. Stergiopoulos: Cloning of the cytochrome *b* gene from the tomato powdery mildew fungus *Leveillula taurica* reveals high levels of allelic variation and heteroplasmy for the G143A mutation. *Front. Microbiol.* **10**, 1–13 (2019).
54) C. Avila-Adame, G. Olaya and W. Köller: Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin-related QoI fungicides. *Plant Dis.* **87**, 1426–1432 (2003).

55) A. Chechi, J. Stahlecker, M. E. Dowling and G. Schnabel: Diversity in species composition and fungicide resistance profiles in *Colletotrichum* isolates from apples. *Pestic. Biochem. Physiol.* **158**, 18–24 (2019).
56) P. Kongtragoul, K. Imamoto and H. Ishii: Resistance to Quinone-outside inhibitor (QoI) fungicides in *Colletotrichum* species isolated from anthracnose disease occurring in Thailand. *Curr. J. Appl. Sci. Technol.* **20**, 79–89 (2020).
57) Y. Matsuzaki, S. Kiguchi, H. Suemoto and F. Iwahashi: Antifungal activity of metyltetraprole against the existing QoI-resistant isolates of various plant pathogenic fungi. *Pest Manag. Sci.* **76**, 1743–1750 (2020).
58) M. Hu and S. Chen: Non-target site mechanisms of fungicide resistance in crop pathogens: A Review. *Microorganisms* **9**, 502 (2021).
59) J. Sharom: ABC multidrug transporters: Structure, function and role in chemoresistance. *Pharmacogenomics* **9**, 105–127 (2008).
60) S. Kim, S. Y. Park, H. Kim, D. Kim, S. W. Lee, H. T. Kim, J. H. Lee and W. Choi: Isolation and Characterization of the *Colletotrichum acutatum* ABC Transporter *CaABC1*. *Plant Pathol. J.* **30**, 375–383 (2014).
61) L. F. V. Furtado, C. D. S. Medeiros, L. W. Zuccherato, W. P. Alves, V. N. G. M. de Oliveira, V. J. da Silva, G. S. Miranda, R. T. Fujiwara and É. M. L. Rabelo: First identification of the benzimidazole resistance-associated F200Y SNP in the betatubulin gene in Ascaris lumbricoides. *PLoS One* **17**, 1–11 (2019).