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

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**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. Moreover, within seven days, crown infection increases the frequency of seedling/plant wilt and death from lack of water and causes severe losses. Anthracnose is an important limiting factor in strawberry cultivation worldwide. In Florida, USA, strawberry anthracnose caused up to 80% of seedling deaths in nurseries and field yield losses of more than 50%. Similarly, anthracnose was shown to cause 30–40% and 20% losses in seedlings and plants after transplanting, respectively, in Taiwan.

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). Among these *Colletotrichum* species, Cg and Ca are the major pathogens causing anthracnose worldwide. Currently, Cg, Ca, and Cf are considered species complexes. Species diversity depends on geographical or cultural conditions. In Taiwan, *C. gloeosporioides* species complex (CGSC) is a major pathogen that causes strawberry anthracnose. The current study indicated that CGSC in strawberries in Taiwan are composed of *C. fructicola* and *C. siamense*, with *C. siamense* being dominant.

The main strategy for controlling strawberry anthracnose is to use agro-chemicals. Several fungicides, such as benzimidazoles, strobilurins, and imazalil, are often recommended for...
controlling strawberry anthracnose. However, fungicide resistance has been reported in strawberry production areas. In Taiwan, several kinds of fungicides are recommended for controlling strawberry anthracnose, including benzimidazoles (thiabendazole), demethyllation inhibitors (propiconazole, tebuconazole, and di-fenoconazole), strobilurins (trifloxystrobin and pyraclostrobin), and chlorothalonil (observ2.tactri.gov.tw). Among these fungicides, strobilurins (quinone outside inhibitors QoIs) and benzimidazoles are at high risk of developing resistant isolates in the field. Peng reported that CGSC isolated from strawberries showed lower sensitivity to benzimidazoles and strobilurins. However, the number of CGSC isolates that Peng 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. Several reports have indicated that the 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. C. perseae and C. fructicola, which cause grape ripe rot in Nagano Prefecture, Japan, were often found to be resistant to strobilurin fungicides. Azoxystrobin and pyraclostrobin sprayed preventively on strawberry fruit inoculated with Ca from Florida failed to control resistant isolates. The mycelial growth inhibition of Ca isolates from post-bloom fruit inoculated with Ca from Florida failed to control resistant isolates. The mycelial growth inhibition of Ca isolates from post-bloom fruit inoculated with Ca from Florida failed to control resistant isolates. The mycelial growth inhibition of Ca isolates from post-bloom fruit inoculated with Ca from Florida failed to control resistant isolates.

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, 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 (ddH₂O) 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
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/ITS431 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.30 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 (EC50). 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:

\[
\text{Inhibition rate} \% = \frac{(\text{CK-Treatment})}{\text{CK}} \times 100.
\]

The EC50 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. 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′-TTCTTGGGTATGTTTACCTTA-3′) and RSCBR2 (5′-AACAAATATCTTGCTCAATTCAATGG-3′). 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-aceate, 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. This pathway can be inhibited by salicylhydroxamic acid (SHAM) and propyl gallate. 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. 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. For β-tubulin gene amplification, the primer pair C′ (5′-GAGGAATTCAAGGAGATTATGATG-3′)/F(5′-GACGTTTGAGGATCGCA-3′) was used to amplify β-tubulin gene fragments of CGSC isolates showing sensitivity or resistance. The following was used for PCR reaction: dH2O 15 µL, 10X buffer (Invitrogen, Brazil) 2.5 µL, MgCl2 (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 of94°C for 1 min, 54°C for 30 sec, 72°C for 2 min, and a final extension at 72°C for 5 min. 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 phylogenetic 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 EC50 values of 38 and 59 CGSC isolates for benomyl were 100–500 µg a.i./mL and >500 µg a.i./mL, respectively. The EC50 values of 21 and 70 CGSC isolates for carbendazim were 100–500 µg a.i./mL and >500 µg a.i./mL, respectively. The EC50 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 EC50 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 EC50 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.

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 TPNH120 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 TCG-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.
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. aescynomone (U14138) was 98%. In this study, isolates with higher EC<sub>50</sub> values showed a point mutation in

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

<sup>a</sup>Sensitive (%): The percentage of EC<sub>50</sub>&lt;100µg a.i./mL.
 Codon 198 from CGA to CGG (A198G). Here, three *C. fructicola* isolates with 229.7 ± 4.3 µg a.i./mL to 392.3 ± 1.5 µg a.i./mL *EC₅₀* 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. Currently, the species composition of CGSC has been identified from only a few crops, including strawberries, mangoes, grapes, avocadoes, lychee, etc. In our study, *C. siamense* was the dominant species of CGSC from strawberries, which is consistent with the re-

**Table 4.** Sensitivity of 15 strawberry anthracnose isolates to commercial azoxystrobin, trifloxystrobin, and pyraclostrobin in terms of mycelial growth and spore germination

| Isolate  | Azoxystrobin | Trifloxystrobin | Pyraclostrobin |
|----------|--------------|-----------------|----------------|
|          | *EC₅₀* (µg a.i./mL) | *EC₅₀* (µg a.i./mL) | *EC₅₀* (µg a.i./mL) |
| MLDH63   | >500         | >500            | 5.5±0.3        |
| MLDH68   | >500         | >500            | 8.9±0.1        |
| MLTA183  | >500         | >500            | 8.3±0.2        |
| HCKS11   | 391.9±8.7(ª) | >500            | 2.5±0.2        |
| MLDH62   | 422.3±10.3   | 447.4±8.8       | 3.3±0.8        |
| HCKS15   | 449.5±11.2   | >500            | 4.2±0.2        |
| NTGS35   | 55.3±2.8     | >500            | <1             |
| MLST70   | 43.1±1.6     | 347.3±3.8       | <1             |
| TCHU02   | 2.9±0.2      | 392.8±9.4       | 6.9±0.4        |
| NTGS20   | 8.9±0.7      | 285.3±5.5       | 8.7±0.2        |
| NTGS123  | 5.8±0.3      | 368.9±3.8       | 7.7±0.3        |
| MLDH67   | <1           | 299.8±2.7       | <1             |
| MLGG168  | <1           | 355.3±3.9       | 2.1±0.1        |
| TPNH20   | <1           | 32.2±0.8        | 1.5±0.3        |

ª *EC₅₀* 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 (100µg a.i./mL) or trifloxystrobin (100µg a.i./mL) mixed with 10µg a.i./mL SHAM at 28°C after three days of culture

| CGSC isolate | Inhibition rate of mycelial growth (%)ª(ª) | A198G | A198G+SHAM | Trifloxystrobin | Trifloxystrobin+SHAM |
|--------------|------------------------------------------|-------|------------|-----------------|---------------------|
| MLDH63       | 2.1±0.2                                  | 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       | 97.2±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).
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. 41–43) 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. 44–46) According to the composition of Colletotrichum spp. from strawberries in Taiwan as reported by Chung et al., 5 C. miaoilense, 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 EC₉₀ 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 EC₉₀ values under 100 μg a.i./mL. Certain reports also indicated that Colletotrichum spp. had higher sensitivity to pyraclostrobin than other strobilurin fungicides. 47–49) 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-methoxy carbamate. 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., 45) 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 alternata, and A. solani— 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 on 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 important.

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 | Benomyl | Carbendazim | Thiabendazole | Sequence in codon (amino acid) |
|--------------|---------|-------------|--------------|-------------------------------|
| 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+1 | > 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+1     | 379.4±1.2 | 332.9±2.5   | 186.9±5.9    | GCG (Ala) TTC (Phe)          |
| MLST125+1    | 219.1±5.3 | 286.2±2.5   | 257.7±3.4    | GCG (Ala) TTC (Phe)          |
| HCKS12+1     | 253.4±1.9 | 228.3±2.9   | 225.7±5.7    | GCG (Ala) TTC (Phe)          |
| NTGS12       | 43.5±1.1  | 61.4±1.6    | 461.0±8.0    | 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)          |

Note: ± indicates standard error (n = 4).
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. In Taiwan, CGSC isolates have been reported to develop benzimidazole-resistant isolates in the field, such as in avocado, fig, jujube, and mango cultivation. 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. 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 studies have also revealed that the reactions of Colletotrichum spp. to fungicides varied depending on the collection area. 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.; such as A. alternata and Leveillula taurica. 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. Avila-Adame et al. 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. Two inhibitors—propyl gal-

late and SHAM—are commonly used to study the role of AOX in strobilurin resistance. 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. ABC transporters are located in the plasma and organelle membranes of eukaryotes; they act as drug efflux pumps to reduce the effects of chemicals. Resistance mechanisms may include point mutation in the transcriptional factor, promoter rearrangement (insertion or deletion), and the constitutive overexpression of fungicide efflux transporters. The C. acutatum ABC transporter (CaABC1) gene had up-regulated expression in kresoxim-methyl, thiophanate-methyl, iprobenfos, and hygromycin B. 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. 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. reported that moderately resistant isolates of CGSC (100<EC50<500 µg a.i./mL) had mutations in codon 200, and resistant isolates of CGSC (EC50>500 µg a.i./mL) had a mutation in codon 198. Our results indicate that the EC50 values of CGSC isolates between 100 and 500 µg a.i./mL did not show a mutation in codon 200. Peng 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.

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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 Tharanat 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

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Not applicable

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