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Diversity and Resistance to Thiophanate-Methyl of *Colletotrichum* spp. in Strawberry Nursery and the Development of Rapid Detection Using LAMP Method

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Abstract: Anthracnose is a devastating fungal disease in strawberry nurseries. Multiple *Colletotrichum* species are responsible for strawberry anthracnose. In this study, 105 *Colletotrichum* isolates were obtained from strawberry seedlings with anthracnose symptoms in fifteen nurseries located in Zhejiang province, China, and were classified based on multilocus sequence and morphological characteristic analyses. Analysis of ITS, *ACT*, *CAL*, *CHS-1*, and *GAPDH* revealed that four species within *C. gloeosporioides* species complex, including *C. siamense* (56 isolates, 53.3%), *C. fructicola* (37 isolates, 35.2%), *C. gloeosporioides* (7 isolates, 6.7%), and *C. aenigma* (5 isolates, 4.8%), were detected in diseased seedlings. Thiophanate-methyl is one of the benzimidazole fungicides, and has long been used to control strawberry anthracnose in China. Here, thiophanate-methyl resistance of *Colletotrichum* isolates was determined by the minimum inhibitory concentration (MIC) method. Our results indicated that the resistance frequency was up to 96.2%, containing 94.3% of highly resistant isolates. Only four sensitive isolates (two *C. fructicola*, one *C. gloeosporioides*, and one *C. siamense* isolates) and two moderately resistant isolates (one *C. aenigma* isolate and one *C. siamense* isolate) were detected. Our data indicated that the high resistance was mainly caused by the E198A mutation in the *β-tubulin* protein. In addition, F200Y (TTC→TAC) in the *β-tubulin* protein were detected in two moderately resistant isolates. Based on the point mutation at codon 198 (GAG→GCG) in the *β-tubulin* gene of *Colletotrichum* isolates, we developed a loop-mediated isothermal amplification (LAMP) assay to rapidly detect the E198A mutants. Collectively, our study indicated that four species within the *C. gloeosporioides* species complex were associated with anthracnose symptoms in strawberry nurseries in Zhejiang province, and serious resistance was widespread in each *Colletotrichum* species.

Keywords: anthracnose; strawberry nursery; *Colletotrichum gloeosporioides* species complex; multilocus sequence analysis; E198A mutation

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1. Introduction

Strawberry is one of the most consumed berries and contains various nutritive compounds, such as minerals, vitamins, fatty acids, and dietary fiber. It has been reported that phenolic acids and organic acids from strawberries are associated with protective effects against chronic diseases [1,2]. Unfortunately, fungal diseases, such as anthracnose, powdery mildew, and gray mold commonly occur in strawberry fields, and significantly decrease strawberry production [3]. Anthracnose is a destructive disease in the strawberry field that can affect the health of the plant at any stage, particularly during the seedling and transplanting stage [4]. For example, the incidence of anthracnose at the seedling stage reaches 50 to 80% under disease-favorable conditions of wetness and temperature, resulting in severe seedling death and yield loss [5,6].

Strawberry anthracnose is caused by *Colletotrichum* spp., including the *C. gloeosporioides* species complex and *C. acutatum* species complex [7–10]. *Colletotrichum* isolates can infect all parts of the strawberry plant, including the leaves, petioles, stolons, crown, and fruits [4]. To distinguish between different characterizations of infection, strawberry anthracnose was classified as anthracnose fruit rot (AFR), anthracnose root and crown necrosis (ARN), anthracnose crown rot (ACR), and leaf anthracnose (LA) [11–13]. The etiology of anthracnose is complex; multilocus phylogenetic assays coupled with morphological data have confirmed that multiple *Colletotrichum* species are responsible for AFR, ARN, ACR, and LA [5,7,11,14,15]. *Colletotrichum acutatum*, *C. fructicola*, *C. gloeosporioides*, *C. siamense*, *C. fragariae*, and *C. nymphaeae* cause AFR and ACR [7,11,13,16]. The prevalence and species of *Colletotrichum* causing anthracnose vary in different countries. *C. nymphaeae*, *C. fioriniae*, *C. siamense*, and *C. lineola* are associated with anthracnose in mid-Atlantic strawberry fields [14], whereas the *C. gloeosporioides* species complex (*C. fructicola*, *C. siamense*, *C. gloeosporioides*, and *C. aenigma*) are exclusively found in strawberry fields located in the Zhejiang province of China [9].

Usually, strawberry seedlings are vegetatively propagated from the runners of mother plants, and planting disease-free seedlings is the most important control method to prevent strawberry anthracnose outbreaks in production fields [17]. Due to a lack of anthracnose-resistant strawberry cultivars, growers always treat transplants by dipping them in suspensions of fungicide to prevent pathogens from infecting root and crown tissue [12]. Fungicides with a single-site mode of action, such as azoxystrobin and pyraclostrobin (quinine outside inhibitors [QoIs]), difenoconazole and tebuconazole (demethylation inhibitors [DMIs]), and carbendazim and thiophanate-methyl (benzimidazole fungicides [MBCs]), are used for controlling strawberry anthracnose in the field [12,14,18]. However, with the continuous use of fungicides, field *Colletotrichum* isolates have built up resistance, causing the fungicides to be ineffective [8,19].

MBC fungicides, which inhibit microtubule assembly in pathogens by binding to the *β-tubulin* protein, are used to control a broad range of fungal pathogens in many host plants, including gray mold, powdery mildew, and anthracnose [20–22]. MBC fungicides were first introduced in the 1960s for controlling plant diseases, and now their effectiveness has become remarkably reduced in field practice because of MBC fungicide-resistant population isolates. *β-tubulin* amino acid substitutions, including H6Y (histidine→tyrosine), F167Y (phenylalanin→tyrosine), E198A/K/V/G/L (glutamate→alanine/lysine/valine/glycine/leucine), F200Y (phenylalanine→tyrosine), and L240Y (leucine→tyrosine), have been reported to confer MBC fungicide resistance to many fungal species [20,21,23–25]. In previous research, point mutations at codon 198 or 200 of the *β-tubulin* gene were commonly found in MBC fungicide-resistant *Colletotrichum* isolates of strawberry anthracnose [8,14,26]. Generally, traditional methods are adopted to detect MBC fungicide resistance, such as determination of the 50% effective concentration value (EC_{50}) and minimum inhibition concentration (MIC), [8,26]. Meanwhile, several molecular methods have been developed to detect MBC fungicide resistance, such as PCR-RFLP (PCR restriction fragment length polymorphism), real-time PCR, and LAMP (loop-mediated isothermal amplification) [22,27,28]. The molecular methods can detect MBC fungicide resistance much more rapidly than traditional methods, especially the LAMP assay [28].

Zhejiang province is an important region for producing strawberry seedlings and strawberries in southern China, and the strawberry industry brings economic benefits to the local growers. However, seedling death caused by *Colletotrichum* spp. is a common and severe problem in strawberry nurseries [29]. Considering the vegetative propagation in strawberries, the health of transplants is important for the entire strawberry life cycle, and determination of the *Colletotrichum* species associated with anthracnose at the strawberry seedling stage is useful for managing the disease. The current study was conducted to (i) determine the *Colletotrichum* species in transplants sampled from strawberry nurseries and (ii) assess the resistance to thiophanate-methyl of *Colletotrichum* isolates obtained from strawberry seedlings and investigate the molecular mechanisms of thiophanate-methyl resistance.

2. Materials and Methods

2.1. Isolation of Colletotrichum Isolates

Strawberry seedlings with black spindle lesions on the petiole, with irregular cankers on the stem, crown rot, and wilting (Figure 1), were collected from fifteen commercial nurseries located in Zhejiang province, from July to September in 2021. Ten diseased seedlings were sampled from each nursery. Tissue isolation and monoconidial culture were conducted based on a previous study [30]. Diseased tissues from diseased plants were surface disinfected in 70% ethanol for 30 s and then in 3% NaOCl for 60 s, followed by three rinses in sterile water. After air drying, sterile tissues were cut into small pieces, and then placed on potato dextrose agar (PDA) supplemented with 50 mg/L of streptomycin sulfate. Fungus colonies with typical characteristics of *Colletotrichum* were transferred to new PDA plates for sporulation. Spores were collected and transferred onto 2% water agar plates, supplemented with 50 mg/L streptomycin sulfate, and then a single colony of a spore was transferred to PDA. In total, 105 single-conidial *Colletotrichum* isolates were obtained, and then maintained on PDA slants at 4 °C.

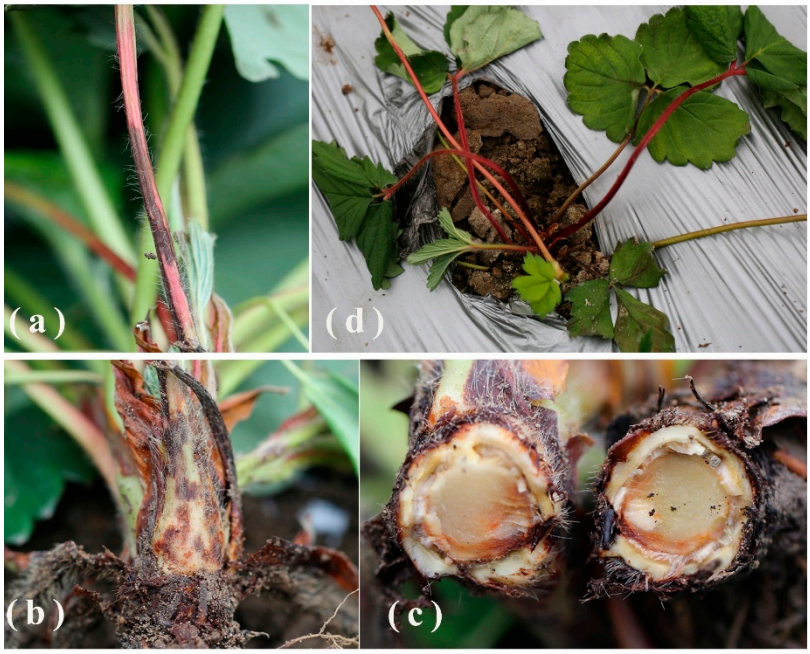

Figure 1. Anthracnose symptoms caused by *Colletotrichum* spp. in strawberry seedlings: (**a**) black spindle-shaped lesion on petiole; (**b**) irregular canker on stem; (**c**) crown rot; and (**d**) wilting.

2.2. Genomic DNA Extraction, PCR Amplification and Sequencing

Colletotrichum isolates were cultivated on solid PDA plates for 6 days at 25 °C. The mycelia of each isolate were harvested for DNA extraction, according to the procedure of the fungal genomic DNA kit (Sangon Biotech, Shanghai, China). Five loci, including internal transcribed spacer (ITS), partial acting (*ACT*), calmodulin (*CAL*), chitin synthase (*CHS-1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were amplified using the primer pairs ITS-1F/ITS4 [31], ACT-512F/ACT-783R [32], CL1C/CL2C [10], CHS-79F/CHS-354R [32], and GDF/GDR [33], respectively. PCR reaction reagents and PCR amplification protocol were the same as those described by Chen et al. [9].

PCR products were separated on 1% agarose gel and the target bands were cut. After purification, the products were sequenced at the Sangon (Sangon Biotech, Shanghai, China). The resulting DNA sequences were assembled by primer pair of each gene using DNAStar v. 5.0 software. Sequences generated in this study were shared in the GenBank database of the National Center for Biotechnology Information (Table S1).

2.3. Multilocus Phylogenetic Analysis

For initial determination of the closest matching species and species complex, ITS, *ACT*, *CHS-1*, *CAL*, and *GAPDH* gene sequences from each isolate were blasted against the GenBank database. Then, the *Colletotrichum* isolates obtained in this study were identified by a phylogenetic analysis method, as previously described [9]. The referenced standard isolates are listed in Table S1. *Colletotrichum boninense* (CBS: 123755) was used as the outgroup [10]. All sequences were aligned using MAFFT v.7 with default parameter settings [34]. If necessary, sequences were manually adjusted in MEGA v. 6.06 [35]. The modified sequences were concatenated in Sequence Matrix 1.8. Modeltest3.7.win, Winpaup4b10-console, and Mrmodeltest2, as implemented in MrMTgui, and were used to estimate the best model of nucleotide substitution [36]. Bayesian inference (BI) was used to construct phylogenies using MrBayes v. 3.1.2 [37]. Six simultaneous Markov chains were run for 1,000,000 generations each, and trees were sampled every 100th generation. The first 2000 trees, representing the burn-in phase of the analyses, were discarded, and the remaining 8000 trees were used to calculate the posterior probability (PP) in the majority rule consensus tree. Phylogenetic trees were drawn using TreeView [38]. The alignments and trees were deposited in TreeBase.

2.4. Morphological Characterization

Morphological features of *Colletotrichum* isolates, including colony characterization and growth rate, were studied on the PDA medium, according to Fu [39]. One or two isolates of each *Colletotrichum* species were randomly selected for morphological observation. Fresh mycelial discs (5 mm diameter) cut from the growing edge of 4-day-old cultures were transferred onto a PDA medium plate for culturing in the dark at 25 °C. Colony diameters of 5-day-old cultures were measured to calculate growth rate. Shape, color, and density of colonies were recorded after 7 d. Moreover, the shape, color, and size of conidiomata, conidia, and appressoria were observed using a light microscope (Nikon Eclipse 80i or Nikon SMZ25, Tokyo, Japan), and 50 conidia were randomly selected and measured to determine their size. Ten microliters of conidial suspensions (1×10^6 conidia) were dropped onto an unbreakable plastic cover slip (22 × 22 mm) (Thermos Fisher Scientific, Waltham, MA, USA), and then incubated in the dark at 25 °C. After incubating for 12–16 h, conidial appressoria were induced, and the size of 30 appressoria were measured.

2.5. Characterization of Resistance of Colletotrichum spp. to Benzimidazole Fungicides

Thiophanate-methyl (97.0% active ingredient) was kindly provided by Zhejiang Welldone Chemical Co., Ltd., Hangzhou, China, dissolved in acetone to 50 mg/mL. Assessment of benzimidazole-resistance was based on the inhibition of mycelial growth, measured by the minimum inhibitory concentration (MIC) method. The *Colletotrichum* isolates were cultivated on solid PDA plates, amended with 0, 5, 20, and 100 mg/L thiophanate-methyl, for 3 days in the dark at 25 °C. After 3 days, the diameter of the isolates grown on the PDA plate without thiophanate-methyl reached 30–40 mm. The evaluation grouped resistance levels into four representative phenotypes by reaction, as described in a previous study [8]: sensitive (S) strains grew on PDA amended without thiophanate-methyl and showed MIC values < 5 mg/L; low-resistance (LR) strains grew on PDA amended with 5 mg/L of thiophanate-methyl and showed MIC values = 5–20 mg/L; moderate-resistance (MR) strains grew on PDA amended with 20 mg/L of thiophanate-methyl and showed MIC values = 20–100 mg/L; and high-resistance (HR) strains grew on PDA amended with 100 mg/L of thiophanate-methyl and showed MIC values > 100 mg/L. Each isolate had three replications, and the resistance assessment was performed three times.

2.6. LAMP Primer Design and Optimization for Detection of the E198A Mutants

According to previous studies, the point mutation E198A in the *β-tubulin* protein is responsible for the high resistance of *Colletotrichum* isolates to MBC fungicides [5,14]. In this study, we developed a LAMP assay to detect the E198A mutation (E198A-LAMP).

In the LAMP assay system, a set of four primers, including two outer primers (F3/B3) and two inner primers (FIP/BIP), were necessary [28]. To screen a set of optimal primes for detecting the E198A mutation, four sets of LAMP primers (SET1, SET2, SET3, SET4) were designed using Primer explorerV5 software (http://primerexplorer.jp/e/ (accessed on 23 May 2020), and then FIPs were mismatched on a point mutation at codon 198 (GAG→GCG) in the *β-tubulin* gene. The primers were synthesized by Sangon Biotech (Shanghai, China); primer sequences are shown in Table 1 and Figure 2.

Table 1. Primers used in this study.

Primer Name	Primer Set	Type	Sequence (5′→3′)
F3	-	Forward	CCTACAACGCCACTCTCTCC
B3	-	Backward	CGCAGGTCAGAGTTCAGC
FIP1	SET1	Forward outer	ACAGCTTGAGGGTACGCATGCA-*TGGTCGAGAACTCCGACGCG*
FIP2	SET2	Backward outer	ACAGCTTGAGGGTACGCATGCA-*TGGTCGAGAACTCCGACCCC*
FIP3	SET3	Forward inner	ACAGCTTGAGGGTACGCATGCA-*TGGTCGAGAACTCCGACTCG*
FIP4	SET4	Backward inner	ACAGCTTGAGGGTACGCATGCA-*TGGTCGAGAACTCCGACACG*
BIP	-	Forward outer	CAACCCCTCTTACGGCGACCT-*TGACCCGGGAAACGCAG*
BETU-F1		Forward	CAGGTTCTCGATGTTGTCCG
BETU-R1		Backward	CCATCATGTTCTTGGGGTCG

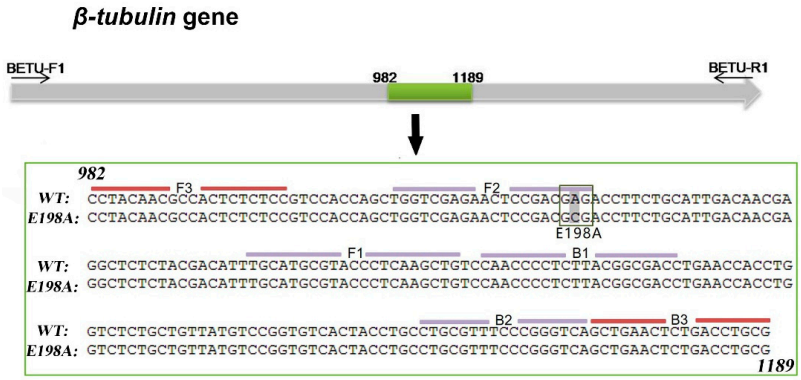

Figure 2. Schematic representation of the site of the *β-tubulin* gene fragment containing codon 198 and the E198A-LAMP assay.

The LAMP assay was performed in a dry thermostat with a total of 25 µL. The components of the LAMP reaction mixture contained 10× Thermopol (Vazyme, Nanjing, China), 4 mM Mg^{2+} (Sangon), 1 mM dNTP (Vazyme), 1.6 µM FIP/BIP, 0.3 µM F3/B3, 0.6 M betine (Sigma, Shanghai, China), 150 µM Hydroxynaphthol blue (HNB, Sigma, China), 0.16 U/µL Bst DNA Polymerase Large Fragment (Vazyme), and 100 ng/µL sample DNA template, which were adjusted as previously described [28]. In the E198A-LAMP assay system, sensitive isolate LA18-3 and E198A mutant LA18-2 were chosen to test as the negative control template and positive template, respectively. Isolates LA18-3 and LA18-2 were collected in 2018. According to the results of MIC testing and sequencing, LA18-2 was highly resistant to thiophanate-methyl and had a point mutation E198A in the *β-tubulin* protein; LA18-3 was sensitive to thiophanate-methyl and did not have a mutation.

The LAMP reactions were conducted at 65 °C for 60 min and then placed at 80 °C for 10 min. After the reaction was completed, if the color of HNB remained violet, it was considered a negative reaction; if the color of HNB turned sky blue, it was considered a

positive reaction. Each treatment was set up in triplicate and the experiment was performed three times.

2.7. LAMP Assay Used for Detection of E198A-Resistance Genotype

A total of 105 *Colletotrichum* isolates were detected by E198A-LAMP assay. The DNA of *Colletotrichum* isolates and optimal LAMP primers were added into a LAMP reaction mixture, then the LAMP assay was performed and assessed as described in the previous section. Each treatment was set up three times and the experiment was repeated three times.

2.8. Analysis of β-Tubulin Gene Sequences of Colletotrichum Isolates

The partial *β-tubulin* gene containing codon 198 and codon 200 from the genomes of 105 isolates was amplified by primer pair BETU-F1 (CAGGTTCTCGATGTTGTCCG) and BETU-R1 (CCATCATGTTCTTGGGGTCG), designed according to the *β-tubulin* gene sequences of *C. gloeosporioides*, downloaded from the GenBank database (GenBank no. U14138.1). The PCR reaction and amplification protocol are described in Liu et al. [40]. All PCR products were purified, and then sequenced at the Sangon Biotech. The sequences were aligned using DNAstar v.5.0 software.

3. Results

3.1. Isolation and Identification of Colletotrichum Species

A total of 150 strawberry seedlings, suspected to be infected by *Colletotrichum* spp., were collected for isolation, and 105 fungal isolates with a colony morphology resembling that of *Colletotrichum* were obtained.

The 105 representative isolates (Table S1), together with 11 reference isolates from previously described species (Table S1), were subjected to multilocus phylogenetic analyses with concatenated *ACT*, *CAL*, *CHS-1*, *GAPDH*, and ITS sequences. The concatenated alignment of *ACT*, *CAL*, *CHS-I*, *GAPDH*, and ITS contained 1239 characteristics (gene boundaries, *ACT*: 1-152, CAL: 153-434, *CHS-I*: 435-646, *GAPDH*: 647-849, and ITS: 850-1239). The results showed that isolates clustered together with four species in *C. gloeosporioides* complexes, including 5 isolates of *C. aenigma*, 7 of *C. gloeosporioides*, 37 isolates of *C. fructicola*, and 56 isolates of *C. siamense* (Figure 3).

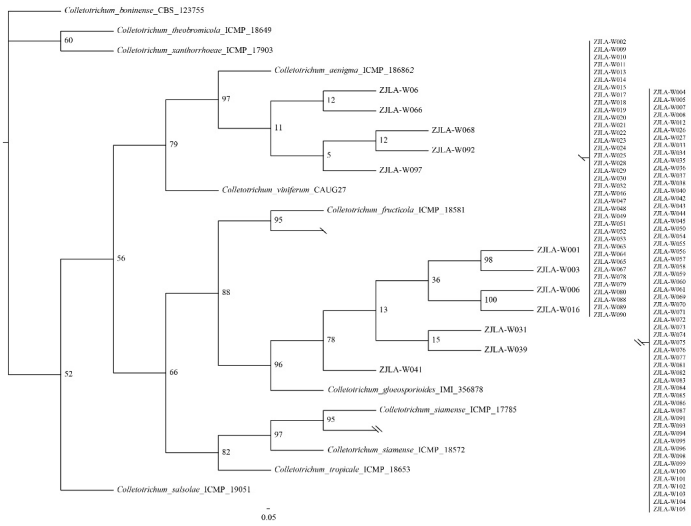

Figure 3. Bayesian inference phylogenetic tree of the *Colletotrichum gloeosporioides* species complex of isolates obtained from strawberry nurseries located in Zhejiang province. The tree was constructed with a Bayesian analysis of concatenated *actin* (*ACT*), *calmodulin* (*CAL*), *chitin synthase* (*CHS-1*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and internal transcribed spacer (ITS). The Markov chain was run for 1,000,000 generations, each locus having a separate model of DNA evaluation. *C. boninense* (CBS: 123755) was used as an outgroup. The scale bar shows 0.05 expected changes per site.

3.2. Morphology Description

Four *Colletotrichum* species were morphologically similar to one another (Figure 4). *C. aenigma* (representative isolate ZJLA-W062) on PDA was flat with the entire margin, with a gray-green surface, reverse gray in the center, light gray margin, and produced orange pigment. The isolates of *C. fructicola* (representative isolate ZJLA-W009) colonies on PDA was flat with the entire margin, with an olivaceous surface, reverse black in the center, and light gray margin. *C. gloeosporioides* were divided into two groups in this study according to morphology. Group I colonies (2 isolates, representative isolate ZJLA-W016) on PDA was flat with the entire margin and white, with conidia in mass orange formed beneath the mycelium. Group II colonies (5 isolates, representative isolate ZJLA-W039) on PDA was flat with the entire margin, with a gray-green surface, cottony, dense, and reverse black to gray. *C. siamense* (representative isolate ZJLA-W005) on PDA was flat with the entire margin, with an atrovirens surface and reverse black.

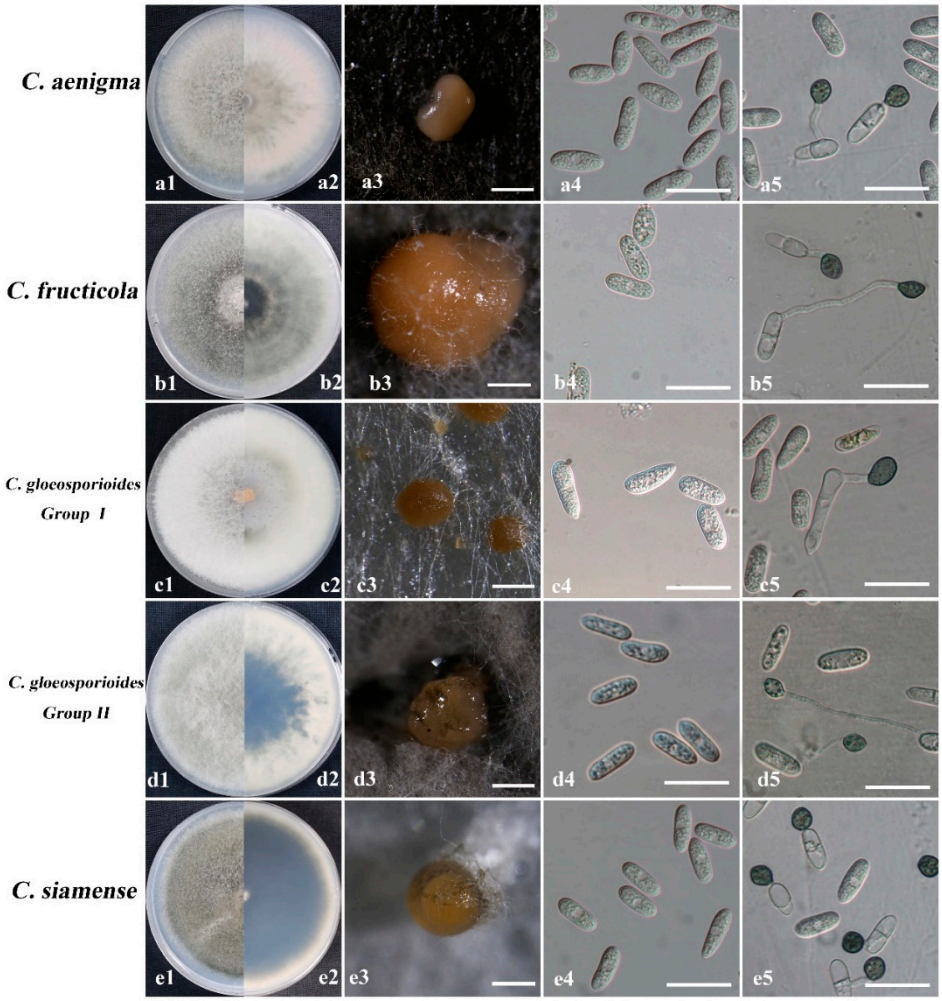

Figure 4. Culture characteristics and microscopic features of the four species within *C. gloeosporioides* complex: (**a1**–**e1**) upper side of 7-day-old colonies on PDA medium; (**a2**–**e2**) reverse side of colonies on PDA medium; (**a3**–**e3**) conidiomata; (**a4**–**e4**) conidia; and (**a5**–**e5**) appressoria. Scale bars: **a3**–**e3** = 500; **a4**–**e4** = 20; **a5**–**e5** = 20 μm.

All four *Colletotrichum* species had similar conidia and conidiomata morphology. All isolates of each *Colletotrichum* species had conidia in mass orange formed on the surface or beneath the mycelium. Their conidia were hyaline, aseptate, smooth-walled, cylindrical, with both ends round or one end slightly acute, and usually broader toward one side. Conidiomata morphology, conidia sizes, and appressoria sizes are described in detail in Table 2.

Table 2. Morphological data of *Colletotrichum* isolates on PDA medium.

Species [1] /Isolate No.	Colony Morphology [2]	Growth Rate (mm/d) [3]	Conidiomata [4]	Conidia [5]		Appressoria [5]	
				Length (μm)	Width (μm)	Length (μm)	Width (μm)
C. aenigma /ZJLA-W062	R, C, GG(c) to LG(m)	10.7	O, S	14.77 ± 0.35 (11.24–21.29)	5.65 ± 0.13 (3.28–7.26)	6.97 ± 0.51 (6.29–8.15)	4.90 ± 0.16 (4.60–5.22)
C. fructicola /ZJLA-W009	R, C, O	10.8	O, S	13.65 ± 0.20 (10.63–17.27)	5.61 ± 0.10 (3.54–7.34)	7.13 ± 0.37 (4.96–8.50)	5.28 ± 0.13 (4.78–5.67)
C. gloeosporioides /ZJLA-W016	R, C, W	13.8	O, U	13.91 ± 0.25 (10.38–18.24)	4.84 ± 0.09 (3.54–6.64)	6.61 ± 0.72 (5.40–7.79)	5.67 ± 0.19 (4.51–7.08)
C. gloeosporioides /ZJLA-W039	R, C, GG	13.2	O, S	13.63 ± 0.32 (9.71–16.29)	4.82 ± 0.12 (3.63–6.55)	6.57 ± 0.37 (5.60–8.23)	5.25 ± 0.17 (4.31–6.19)
C. siamense /ZJLA-W005	R, C, A	10.6	O, S	12.68 ± 0.21 (9.83–15.02)	4.89 ± 0.09 (3.81–6.22)	6.65 ± 0.12 (5.49–8.32)	5.62 ± 0.11 (4.51–6.96)

[1] The species was identified via multilocus phylogenetic analysis in this study. [2] Surface of colony: R, regular; C, circular; GG, gray-green; LG, light gray; O, olivaceous; A, atrovirens; W: white; c, c: center; m: margin. [3] Values are the mean of triplicate from three independent experiments. [4] O, conidia in mass orange; S, conidiomata formed surface of mycelium; U, conidiomata formed under mycelium. [5] Data are mean ± standard error.

3.3. Thiophanate-Methyl Resistance of Colletotrichum spp. Isolates Are a Severe Problem in Strawberry Nurseries

Thiophanate-methyl resistance of 105 single-conidial isolates was determined by MIC method. The results showed that four isolates could not normally grow on PDA amended with thiophanate-methyl; 99 isolates grew on PDA amended with 100 mg/L thiophanate-methyl, and one *C. aenigma* isolate and one *C. siamense* isolate grew on PDA amended with 20 mg/L thiophanate-methyl (Table 3). These data indicated that the frequency of highly resistant isolates and moderately resistant isolates were 94.3 and 1.9%, respectively.

Table 3. Thiophanate-methyl resistance phenotypes and genotypes of *Colletotrichum* spp. isolates.

Species [1]	N [2]	Resistance Level by MIC [3]				Positive in LAMP [4]	Amino Acid Substitution [5]	
		S	LR	MR	HR		E198A	F200Y
C. aenigma	5	0	0	1	4	4	4	1
C. *fructicola*	37	2	0	0	35	33	33	2
C. gloeosporioides	7	1	0	0	6	5	5	1
C. siamense	56	1	0	1	54	53	53	2
Total	105	4	0	2	99	95	95	6

[1] The species was identified via multilocus phylogenetic analysis in this study. [2] Number of samples. [3] Resistance of *Colletotrichum* spp. isolates to thiophanate-methyl, detected by MIC method, S, sensitive isolates, MIC values < 5 mg/L; LR, low-resistance isolates, MIC values = 5–20 mg/L; MR, moderate-resistance isolates, MIC values = 20–100 mg/L; high-resistance isolates, MIC values > 100 mg/L. [4] Isolates carrying the E198A mutation, detected by the E198A-LAMP assay. [5] Amino acid substitution in *β-tubulin* protein. E198A represents a mutation in codon 198 where glutamic acid (E) is replaced by alanine (A); F200Y represents a mutation in codon 200 where tyrosine (Y) is replaced by alanine (A).

3.4. Point Mutations in Codon 198 and 200 of the β-Tubulin Gene Are Responsible for High Resistance to MBC Fungicides in Strawberry Anthracnose at the Seedling Stage

Template DNA from isolate LA18-2, a Ben-resistant isolate carrying the E198A mutation, and a Ben-sensitive isolate LA18-3 were used to visually assess the four sets of mismatched LAMP primers, based on color changes of the HNB (Figure 5a). The reaction mixture containing template DNA from the isolate LA18-2 and primer set SET2 (F3/ FIP2/ BIP/ B3) changed from violet to blue, which distinguished the E198A genotype of the *Colletotrichum* species from sensitive isolates. Then, the primer set SET2 was

used to detect the E198A genotype in a E198A-LAMP assay. The results showed that 95 isolates were positive in the LAMP assay, and sequence analysis confirmed that the 95 positive isolates carried *β-tubulin* E198A (Table 3). Meanwhile, four highly resistant and two moderately resistant isolates contained a point mutation at codon 200 (TTC→TAC), resulting in a phenylalanine(F) to tyrosine(Y) substitution that was detected by sequencing (Table 3, Figure 5b).

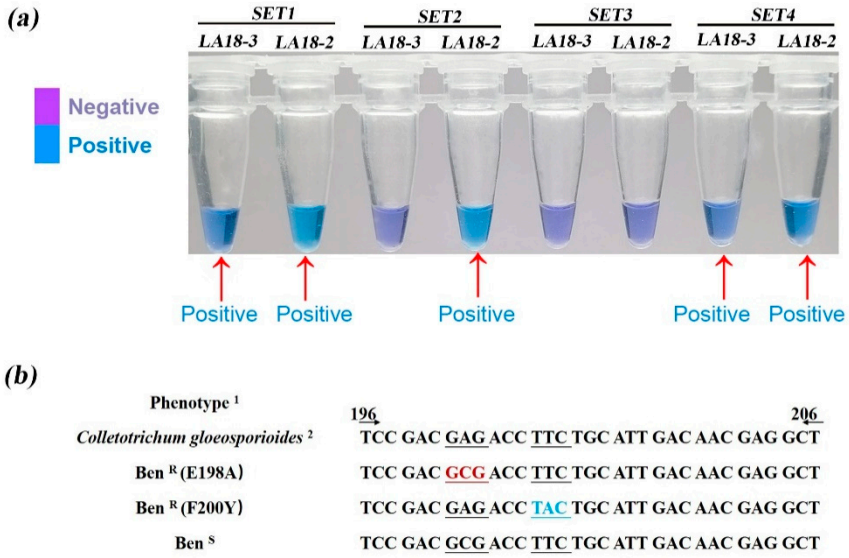

Figure 5. Genotypes of thiophanate-methyl-resistant isolates of *Colletotrichum* obtained from strawberry nurseries: (**a**) LAMP primer set screening and assessment were based on HNB-visualized changes in colors. Positive samples are indicated by red arrows: LA18-3, a sensitive isolate without the E198A mutation; LA18-2, a highly resistant isolate carrying the E198A mutation. (**b**) Three genotypes of *β-tubulin* gene and alignment of partial gene sequences of the *β-tubulin* gene: [1], thiophanate-methyl phenotype of *Colletotrichum* species; [2], partial *β-tubulin* gene sequences of *C. gloeosporioides*, downloaded from the GenBank database (GenBank no. U14138.1); 196 and 206 indicated that codon 196 and codon 206 at *β-tubulin* gene, respectively; the base in red or in blue indicated that a point mutation at codon 198 and codon 200, respectively; E198A, codon at 198 change from GAG to GCG, resulting in a glutamic acid(E) to alanine(A) substitution; F200Y, codon at 200 change from TTC to TAC, resulting a phenylalanine(F) to tyrosine(Y) substitution; Ben R, the isolates carrying point mutations E198A or F200Y were resistant to thiophanate-methyl.

4. Discussion

Anthracnose caused by the *Colletotrichum* species is a devastating disease in strawberry nurseries, resulting in seedling death. In 2021, we collected 105 isolates from 150 diseased strawberry seedlings, and then *Colletotrichum* species were identified by using multilocus phylogenetic and morphological characteristic analyses. A subset of the 105 isolates represented four species within the *C. gloeosporioides* complex: *C. siamense* (56 isolates, 53.3%), *C. fructicola* (37 isolates, 35.2%), *C. gloeosporioides* (7 isolates, 6.7%), and *C. aenigma* (5 isolates, 4.8%). In this study, we detected a high resistance level and high resistance frequency of *Colletotrichum* isolates to thiophanate-methyl, reinforcing the idea that strawberry growers should avoid using MBC fungicides to protect seedlings in nurseries, and new fungicides with different modes of action should be introduced for field use.

Colletotrichum species infect a wide variety of crops and fruits, and the genus is denoted as the world's eighth most important group of plant-pathogenic fungi [41]. The identification of *Colletotrichum* species that cause anthracnose is a major research focus [9,26,39,42]. According to previous reports, *Colletotrichum* isolates infect strawberry plant and induce brown or black sunken lesions on leaves, petioles, and stolons. Meanwhile, the pathogens also cause crown rot and wilting of plants [12,17,26]. Following the classic symptoms of anthracnose,

we sampled 150 diseased seedlings from 15 strawberry nurseries and obtained 105 isolates with features that were characteristic of *Colletotrichum* isolates.

The morphological characteristics of *Colletotrichum* species are associated with factors of the cultivation environment, such as wetness, temperature, light, and growth media. In addition, species within the *Colletotrichum* complex have not been distinguished from one another because of their similar morphological characteristics [6,9]. Therefore, we jointly used multilocus phylogenetic and morphological characteristic analyses to identify the pathogen of anthracnose [10,39,42]. In this study, ITS, *ACT*, *CAL*, *CHS-I*, and *GAPDH* gene sequences were concatenated and used to construct phylogenies using MrBayes. Consistent with previous studies [9], *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, and *C. siamense* were associated with anthracnose symptoms in strawberry seedlings in our study. Although it has been confirmed that *C. murrayae*, *C. nymphaeae*, *C. fioriniae*, *C. acutatum*, and *C. fragariae* can cause strawberry anthracnose [7,12,14,43,44], we did not detect any of these species in the diseased strawberry seedlings. Sampling time, strawberry cultivars, production practices, and geographic location may have affected the pathogen diversity of anthracnose [9,13,14].

Lacking anthracnose-resistant strawberry varieties, fungicides are commonly applied to control anthracnose during all stages of strawberry production [12,45]. Since the 1990s, MBC fungicides, including thiophanate-methyl and carbendazim, have been the main fungicide group used for controlling strawberry anthracnose in the field. However, the rapid development of MBC fungicide resistance affected the control efficacy of these treatments [5,14]. The resistance frequency of *Colletotrichum* spp. isolated from strawberry nurseries to thiophanate-methyl was as high as 96.2% in our study. A previous research reported that *Colletotrichum* species may exhibit significant differences in their response to fungicides; *C. gloeosporioides* species complex were sensitive to MBC fungicides, whereas *C. acutatum* species complex were resistant [26]. However, MBC fungicide resistance has been detected in *C. fructicola* and *C. siamense* (within the *C. gloeosporioides* species complex) [14,22]. Our study also detected that the thiophanate-methyl resistance frequency of *C. aenigma* and *C. gloeosporioides* (within the *C. gloeosporioides* species complex) was 100 and 85.7%, respectively.

In this study, most *Colletotrichum* isolates were highly resistant to thiophanate-methyl. Several studies demonstrated that point mutations at codon 198 or 200 of *β-tubulin* gene were the main cause of MBC fungicide resistance in various pathogens, and that the E198A mutation was responsible for HR level [5,14,20,22]. Generally, the LAMP assay was used to detect the pathogen of disease or the fungicide resistance frequency of the pathogen [28,46–48]. In this study, we developed a LAMP assay for detecting the E198A mutation (E198A-LAMP). Out of the 99 HR isolates that were determined by the MIC method, 95 isolates were positive in the E198A-LAMP assay. Sequence analysis showed that the 95 positive isolates carried the point mutation E198A in the *β-tubulin* protein, and the four HR isolates negative in LAMP assay carried a F200Y mutation. These results indicated that the E198A-LAMP assay could accurately detect HR resistant isolates with an E198A mutation. The MIC method is a cornerstone for monitoring the thiophanate-methyl resistance of *Colletotrichum* spp. However, it is not suitable for field applications because of its long detection cycles. LAMP assays can be completed within a shorter time frame and the results can be visually assessed. As previously reported, a LAMP assay for detecting fungicide resistance could be finished on field within 1.5 h, without thermal cycling equipment [40]. Therefore, our E198A-LAMP assay could be used to detect resistance of *Colletotrichum* spp. to fungicides in strawberry greenhouses to guide the application of benzimidazole fungicides.

According to our study, four species within the *C. gloeosporioides* species complex were associated with anthracnose symptoms in strawberry seedlings. Monitoring of resistance to thiophanate-methyl showed that most isolates were resistant and mainly caused by a point mutation E198A in the *β-tubulin* protein. A severe resistance indicates that this fungicide group is no longer suitable for control anthracnose in strawberry nurseries. Fortunately,

Colletotrichum isolates are sensitive to DMIs [18], which indicate that DMIs may be used as an alternatives to prevent the anthracnose in strawberry nurseries. For further research, the E198A-LAMP assay should be used to detect MBC fungicide resistance of *Colletotrichum* isolates obtained from strawberry plants at other stages and avoid fungicide inefficiency caused by resistance. The evolution pattern of genotypes in resistant isolates should also be investigated, to develop new control strategies using combinations of different fungicides in the control of thiophanate-methyl resistant *Colletotrichum* isolates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112815/s1, Table S1: *Colletotrichum* spp. used in multi-gene analysis in this study.

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References

1. Miller, K.; Feucht, W.; Schmid, M. Bioactive Compounds of Strawberry and Blueberry and Their Potential Health Effects Based on Human Intervention Studies: A Brief Overview. *Nutrients* **2019**, *11*, 1510. [CrossRef]
2. Giampieri, F.; Forbes-Hernandez, T.Y.; Gasparrini, M.; Alvarez-Suarez, J.M.; Afrin, S.; Bompadre, S.; Quiles, J.; Mezzeti, B.; Battino, M. Strawberry as a Health Promoter: An Evidence Based Review. *Food Funct.* **2015**, *6*, 1386–1398. [CrossRef]
3. Lu, D.; Cao, T.; Huang, Y.; Gao, J.; Hong, W. Investigation On the Occurrence Rules of Strawberry Anthracnose, Grey Mould and Powdery Mildew. *Zhejiang Agric. Sci.* **2018**, *59*, 2177–2178.
4. Howard, C.M.; Maas, J.L.; Chandler, C.L.; Albregts, E.A. Anthracnose of Strawberry Caused by the *Colletotrichum* complex in Florida. *Plant Dis.* **1992**, *76*, 976–981. [CrossRef]
5. Zhong, S.; Miao, J.; Liu, X.; Zhang, G. Characterization of *Colletotrichum* spp. Sensitivity to Carbendazim for Isolates Causing Strawberry Anthracnose in China. *Plant Dis.* **2021**, *105*, 87–95. [CrossRef]
6. Jian, Y.; Li, Y.; Tang, G.; Zheng, X.; Khaskheli, M.I.; Gong, G. Identification of *Colletotrichum* Species Associated with Anthracnose Disease of Strawberry in Sichuan Province, China. *Plant Dis.* **2021**, *105*, 3025–3036. [CrossRef]
7. Han, Y.C.; Zeng, X.G.; Xiang, F.Y.; Ren, L.; Chen, F.Y.; Gu, Y.C. Distribution and Characteristics of *Colletotrichum* spp. Associated with Anthracnose of Strawberry in Hubei, China. *Plant Dis.* **2016**, *100*, 996–1006. [CrossRef]
8. Lin, T.; Xu, X.F.; Dai, D.J.; Shi, H.J.; Wang, H.D.; Zhang, C.Q. Differentiation in Development of Benzimidazole Resistance In *Colletotrichum gloeosporioides* Complex Populations From Strawberry and Grape Hosts. *Australas. Plant Pathol.* **2016**, *45*, 241–249. [CrossRef]
9. Chen, X.Y.; Dai, D.J.; Zhao, S.F.; Shen, Y.; Wang, H.D.; Zhang, C.Q. Genetic Diversity of *Colletotrichum* spp. Causing Strawberry Anthracnose in Zhejiang, China. *Plant Dis.* **2020**, *104*, 1351–1357. [CrossRef]
10. Weir, B.S.; Johnston, P.R.; Damm, U. The *Colletotrichum gloeosporioides* species complex. *Stud. Mycol.* **2012**, *73*, 115–180. [CrossRef]
11. Rahman, M.; Ojiambo, P.; Louws, F. Initial Inoculum and Spatial Dispersal of *Colletotrichum gloeosporioides*, the Causal Agent of Strawberry Anthracnose Crown Rot. *Plant Dis.* **2015**, *99*, 80–86. [CrossRef]
12. Forcelini, B.B.; Seijo, T.E.; Amiri, A.; Peres, N.A. Resistance in Strawberry Isolates of *Colletotrichum acutatum* from Florida to Quinone-Outside Inhibitor Fungicides. *Plant Dis.* **2016**, *100*, 2050–2056. [CrossRef]
13. Hu, S.; Zhang, Y.; Yu, H.; Zhou, J.; Hu, M.; Liu, A.; Wu, J.; Wang, H.; Zhang, C. *Colletotrichum* Spp. Diversity Between Leaf Anthracnose and Crown Rot From the Same Strawberry Plant. *Front. Microbiol.* **2022**, *13*, 860694. [CrossRef]
14. Luo, Q.; Schoeneberg, A.; Hu, M. Resistance to Azoxystrobin and Thiophanate-Methyl Is Widespread in *Colletotrichum* spp. Isolates From the Mid-Atlantic Strawberry Fields. *Plant Dis.* **2021**, *105*, 2202–2208. [CrossRef] [PubMed]
15. Smith, B.J.; Black, L.L. Morphological, Cultural, and Pathogenic Variation among *Colletotrichum* species Isolated From strawberry. *Plant Dis.* **1990**, *74*, 69–76. [CrossRef]

16. Jayawardena, R.S.; Huang, J.K.; Jin, B.C.; Yan, J.Y.; Li, X.H.; Hyde, K.D.; Bahkali, A.H.; Yin, S.L.; Zhang, G.Z. An Account of *Colletotrichum* species Associated with Strawberry Anthracnose in China Based on Morphology and Molecular data. *Mycosphere* **2016**, *7*, 1147–1163. [CrossRef]
17. Furuta, K.; Nagashima, S.; Inukai, T.; Masuta, C. Construction of a System for the Strawberry Nursery Production towards Elimination of Latent Infection of Anthracnose Fungi by a Combination of PCR and Microtube Hybridization. *Plant Pathol. J.* **2017**, *33*, 80–86. [CrossRef]
18. Zhang, L.; Song, L.; Xu, X.; Zou, X.; Duan, K.; Gao, Q. Characterization and Fungicide Sensitivity of *Colletotrichum* Species Causing Strawberry Anthracnose in Eastern China. *Plant Dis.* **2020**, *104*, 1960–1968. [CrossRef]
19. Torres-Calzada, C.; Tapia-Tussell, R.; Higuera-Ciapara, I.; Martin-Mex, R.; Nexticapan-Garcez, A.; Perez-Brito, D. Sensitivity of *Colletotrichum truncatum* to Four Fungicides and Characterization of Thiabendazole-Resistant Isolates. *Plant Dis.* **2015**, *99*, 1590–1595. [CrossRef]
20. Vielba-Fernandez, A.; Vicente, A.; Perez-Garcia, A.; Fernandez-Ortuno, D. Monitoring Methyl Benzimidazole Carbamate-Resistant Isolates of the Cucurbit Powdery Mildew Pathogen, *Podosphaera xanthii*, Using Loop-Mediated Isothermal Amplification. *Plant Dis.* **2019**, *103*, 1515–1524. [CrossRef]
21. Duan, Y.B.; Yang, Y.; Wang, J.X.; Chen, C.J.; Steinberg, G.; Fraaije, B.A.; Zhou, M.G. Simultaneous Detection of Multiple Benzimidazole-Resistant *beta*-Tubulin Variants of *Botrytis cinerea* using Loop-Mediated Isothermal Amplification. *Plant Dis.* **2018**, *102*, 2016–2024. [CrossRef] [PubMed]
22. Chung, W.H.; Chung, W.C.; Peng, M.T.; Yang, H.R.; Huang, J.W. Specific Detection of Benzimidazole Resistance in *Colletotrichum* gloeosporioides From Fruit Crops by PCR-RFLP. *New Biotechnol.* **2010**, *27*, 17–24. [CrossRef] [PubMed]
23. Ma, Z.; Yoshimura, M.A.; Michailides, T.J. Identification and Characterization of Benzimidazole Resistance in *Monilinia fructicola* From Stone Fruit Orchards in California. *Appl. Environ. Microbiol.* **2003**, *69*, 7145–7152. [CrossRef]
24. Duan, Y.B.; Yang, Y.; Wang, J.X.; Liu, C.C.; He, L.L.; Zhou, M.G. Development and Application of Loop-mediated isothermal amplification for Detecting theHighly Benzimidazole-resistant Isolates in *Sclerotinia sclerotiorum*. *Sci. Rep.* **2015**, *5*, 17278. [CrossRef] [PubMed]
25. Carter, H.E.; Cools, H.J.; West, J.S.; Shaw, M.W.; Fraaije, B.A. Detection and Molecular Characterisation of *Pyrenopeziza brassicae* Isolates Resistant to Methyl Benzimidazole Carbamates. *Pest Manag. Sci.* **2013**, *69*, 1040–1048. [CrossRef]
26. Han, Y.C.; Zeng, X.G.; Xiang, F.Y.; Zhang, Q.H.; Guo, C.; Chen, F.Y.; Chen, G.Y. Carbendazim Sensitivity in Populations of *Colletotrichum gloeosporioides* complex Infecting Strawberry and Yams in Hubei Province of China. *J. Integr. Agric.* **2018**, *17*, 1391–1400. [CrossRef]
27. Duan, Y.B.; Yang, Y.; Li, T.; Zhao, D.; Cao, J.; Shi, Y.Y.; Wang, J.X.; Zhou, M.G. Development of A Rapid and High-throughput Molecular Method for Detecting the F200Y Mutant Genotype in Benzimidazole-resistant Isolates of *Fusarium asiaticum*. *Pest Manag. Sci.* **2016**, *72*, 2128–2135. [CrossRef]
28. Wu, J.Y.; Hu, X.R.; Zhang, C.Q. Molecular Detection of QoI Resistance in *Colletotrichum gloeosporioides* Causing Strawberry Anthracnose Based on Loop-Mediated Isothermal Amplification Assay. *Plant Dis.* **2019**, *103*, 1319–1325. [CrossRef]
29. Xie, L.; Zhang, J.Z.; Wan, Y.; Hu, D.W. Identification of *Colletotrichum* spp. Isolated From Strawberry in Zhejiang Province and Shanghai City, China. *J. Zhejiang Univ. Sci. B* **2010**, *11*, 61–70. [CrossRef]
30. Gunnell, P.S.; Gubler, W.D. Taxonomy and Morphology of *Colletotrichum* Species Pathogenic to Strawberry. *Mycologia* **1992**, *84*, 157–165. [CrossRef]
31. White, T.J.; Bruns, T.; Lee, S.; Taylor, J.W. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
32. Carbone, I.; Kohn, L.M. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]
33. Templeton, M.D.; Rikkerink, E.H.; Solon, S.L.; Crowhurst, R.N. Cloning and Molecular Characterization of the *Glyceraldehyde-3-phosphate dehydrogenase-encoding* gene and cDNA From the Plant Pathogenic Fungus *Glomerella cingulata*. *Gene* **1992**, *122*, 225–230. [CrossRef]
34. Katoh, K.; Standley, D.M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef]
35. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
36. Vaidya, G.; Lohman, D.J.; Meier, R. SequenceMatrix: Concatenation Software for the Fast Assembly of Multi-Gene Datasets with Character Set and Codon Information. *Cladistics* **2011**, *27*, 171–180. [CrossRef]
37. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian Phylogenetic Inference under Mixed Models. *Bioinformatics* **2003**, *19*, 1572–1574. [CrossRef]
38. Page, R.D. TreeView: An Application to Display Phylogenetic Trees on Personal Computers. *Comput. Appl. Biosci.* **1996**, *12*, 357–358.
39. Fu, M.; Crous, P.W.; Bai, Q.; Zhang, P.F.; Xiang, J.; Guo, Y.S.; Zhao, F.F.; Yang, M.M.; Hong, N.; Xu, W.X.; et al. *Colletotrichum* species Associated with Anthracnose of *Pyrus* spp. in China. *Persoonia* **2019**, *42*, 1–35. [CrossRef]

40. Liu, Y.H.; Yuan, S.K.; Hu, X.R.; Zhang, C.Q. Shift of Sensitivity in *Botrytis cinerea* to Benzimidazole Fungicides in Strawberry Greenhouse Ascribing to the Rising-lowering of E198A Subpopulation and its Visual, On-site Monitoring by Loop-mediated Isothermal Amplification. *Sci. Rep.* **2019**, *9*, 11644. [CrossRef]
41. Dean, R.; Van-Kan, J.A.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A.D.; Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, J.E.; Foster, G.D. The Top 10 Fungal Pathogens in Molecular Plant Pathology. *Mol. Plant Pathol.* **2012**, *13*, 414–430. [CrossRef]
42. Yan, J.; Jayawardena, R.; Goonasekara, I.; Wang, Y.; Zhang, W.; Liu, M.; Huang, J.B.; Wang, Z.Y.; Shang, J.J.; Peng, Y.L.; et al. Diverse Species of *Colletotrichum* associated with Grapevine Anthracnose in China. *Fungal Divers.* **2014**, *71*, 233–246. [CrossRef]
43. He, L.; Li, X.; Gao, Y.; Li, B.; Mu, W.; Liu, F. Characterization and Fungicide Sensitivity of *Colletotrichum* spp. from Different Hosts in Shandong, China. *Plant Dis.* **2019**, *103*, 34–43. [CrossRef] [PubMed]
44. Curry, K.J.; Abril, M.; Avant, J.B.; Smith, B.J. Strawberry Anthracnose: Histopathology of *Colletotrichum acutatum* and *C. fragariae*. *Phytopathology* **2002**, *92*, 1055–1063. [CrossRef] [PubMed]
45. Miller-Butler, M.A.; Smith, B.J.; Babiker, E.M.; Kreiser, B.R.; Blythe, E.K. Comparison of Whole Plant and Detached Leaf Screening Techniques for Identifying Anthracnose Resistance in Strawberry Plants. *Plant Dis.* **2018**, *102*, 2112–2119. [CrossRef] [PubMed]
46. Zhang, X.; Harrington, T.C.; Batzer, J.C.; Kubota, R.; Peres, N.A.; Gleason, M.L. Detection of *Colletotrichum acutatum* Sensu Lato on Strawberry by Loop-Mediated Isothermal Amplification. *Plant Dis.* **2016**, *100*, 1804–1812. [CrossRef]
47. Martinez-Valladares, M.; Valderas-Garcia, E.; Gandasegui, J.; Skuce, P.; Morrison, A.; Aguero, V.C.G.; Cambra-Pelleja, M.; Balana-Fouce, R.; Roko-Vazquez, F.A. *Teladorsagia circumcincta* Beta Tubulin: The Presence of the E198L Polymorphism on Its Own is Associated with Benzimidazole Resistance. *Parasites Vectors* **2020**, *13*, 453–455. [CrossRef]
48. Qin, Y.; Duan, X.; Peng, Y.; Rui, Y. Rapid Detection of a Novel B1-beta-lactamase Gene, *blaAFM-1* Using a Loop-mediated Isothermal Amplification (LAMP) assay. *Ann. Clin. Microbiol. Antimicrob.* **2021**, *20*, 80–87. [CrossRef]