Characterization and Fungicide Sensitivity of *Colletotrichum* spp. from Different Hosts in Shandong, China

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Abstract

Anthrachnose, caused by *Colletotrichum* species, can severely infect the fruits and leaves of more than 30 plants and thus results in great yield and quality losses. To identify the major *Colletotrichum* species infecting walnut fruits, strawberry leaves, grape fruits, and tea leaves in Shandong Province, China, 101 strains were collected and isolated. The morphological characteristics of all isolates were observed, and multilocus phylogenetic analyses (ITS, *GAPDH*, *ACT*, *TUB2*, *CAL*, *CHS-1*, and *HIS3*) were conducted on the representative isolates. The strains were identified as five *Colletotrichum* species, namely, *C. gloeosporioides* sensu stricto, *C. fructicola*, *C. camelliae*, *C. acutatum* sensu stricto, and *C. viniferum*. Among them, *C. viniferum* was reported for the first time from walnut fruits and strawberry leaves in Shandong Province, China. Corresponding

leaves or fruits were used as a model to clarify the pathogenicity of these isolates. The results showed that *C. fructicola* obtained from strawberry leaves was more aggressive than *C. viniferum*. All of the isolates obtained from various hosts were highly sensitive to pyraclostrobin, difenoconazole, fludioxonil, tebuconazole, pyrisoxazole, and tetramycin in terms of mycelial growth inhibition (EC₅₀ values of 0.07 to 1.63 mg/liter). The fastest mycelial growth was observed in the temperature range of 25–28°C for all isolates. In addition, anthracnose symptoms occur frequently under these conditions. Overall, this study can improve the understanding of *Colletotrichum* species causing anthracnose in walnut fruits, strawberry leaves, grape fruits, and tea leaves and can provide a solid foundation for the effective control of this disease in different hosts.

Colletotrichum is one of the most disastrous plant pathogens worldwide (Cannon et al. 2012; Hyde et al. 2014; Nilsson et al. 2014; Yan et al. 2015), occurring mainly in a wide range of crops in tropical and subtropical areas (Hyde et al. 2009a, b, 2014). Anthracnose has also become increasingly prevalent in Shandong Province. It has lately become the most economically subversive disease, especially causing rot of walnut fruits and grape fruits, and leaf scorch disease of strawberries and tea plants.

Walnut is an important nut and woody oil tree species worldwide. It has important economic value and medicinal value (Mcgranahan et al. 1988). The U.S. Food and Drug Administration (FDA) made a milestone decision to give walnut a claim as a health product (Zhu et al. 2014). A previous study showed that the incidence of anthracnose was 90% and that the diseased fruit rate was above 30% in walnut production areas in Shandong Province (Liu 2013). Strawberries are cultivated in temperate zones and certain subtropical areas (Lin et al. 2016). Currently, strawberries are a major crop cultivated in China, and China has become the largest producer and exporter of strawberries in the world (Xie et al. 2010). Anthracnose can occur throughout the production cycle, especially in the seedling and transplanting periods of the strawberry plants (Smith 2008). Grapes are one of the most diffusely cultivated fruit worldwide, whether for wine production or fruit consumption (Hong et al. 2008). During the ripening process of grape fruits, heavy rainfall and warm summer conditions in most of the grape growing areas in China have resulted in the loss of production due to anthracnose (Lu 2005). The ripe rot of grapes caused by *Colletotrichum* spp. has become a serious disease in most grape growing areas, particularly in Shandong, China. The tea plant, a popular nonalcoholic beverage crop, is cultivated in tropical and subtropical areas. China, which was one of the first countries to begin cultivating tea trees, plants the largest variety of tea plants over the widest area in the world (Chen and Yang 2011). In recent years,

plantation acreage and total output have experienced rapid growth in China. Because of the high planting density, anthracnose frequently occurs, causing severe damage such as fruit necrosis, stem rot, leaf scorch, and defoliation (Hyde et al. 2009a; Qu et al. 2011), ultimately leading to serious losses in yield (Guo et al. 2014).

Due to overlapping characteristics, morphological classification of species is arduous in this genus. Combining morphological and molecular tools can provide a robust and precise identification of fungi at the species level. At present, the majority of studies conducted regarding this disease principally focus on morphology combined with multilocus phylogenetic analysis (Damm et al. 2012a; Diao et al. 2017; Silva et al. 2016; Weir et al. 2012), which provides a more reliable resolution (Cai et al. 2009; Hyde et al. 2014). The sensitivities to fungicides of the species *C. siamense*, *C. fructicola*, *C. fioriniae*, *C. theobromicola*, *C. acutatum* sensu stricto, and *C. gloeosporioides* s.s. are well known to differ (Freeman et al. 1998; Munir et al. 2016; Peres et al. 2004; Valero et al. 2010). Recently, Hu et al. (2015) reported a substantial difference in the fungicide sensitivity of *C. siamense* and *C. fructicola*, two species belonging to the *C. gloeosporioides* species complex that cause anthracnose on blueberry and peach plants. No complete research is found in the current reference literature regarding anthracnose on walnuts, grapes, strawberries, and tea plants. Therefore, it is imperative to identify phylogenetic species within *Colletotrichum* complexes and to clarify their pathogenicity and fungicide sensitivity to enhance the management of anthracnose.

The objectives of this research are as follows: (1) to identify the species of *Colletotrichum* causing anthracnose on different hosts by morphological characteristics and multilocus phylogenetic analyses, (2) to determine the pathogenicity and growth characteristics of different *Colletotrichum* species, and (3) to determine the inhibitory effect of fungicides against mycelial growth of different *Colletotrichum* species.

Materials and Methods

Collection and isolates. In 2016, a total of 101 *Colletotrichum* spp. isolates were collected from different plants in Jinan, Tai'an, and Qindao of Shandong Province, China. Isolates were collected from commercial orchards where the anthracnose was serious and yield losses were heavy. Among them, the numbers of strains collected from walnut fruits (Liuzhong village, Jinan, China, 117.07 E, 36.69 N), strawberry leaves (High-tech district, Qindao, China,

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120.33 E, 36.07 N), grape fruits (Daiyue district, Tai'an, China, 117.13 E, 36.18 N), and tea leaves (Feicheng, Tai'an, China, 116.76 E, 36.24 N) were 17, 21, 38, and 25, respectively. Small pieces of disease lesion were disinfested with 70% ethanol for 30 s, washed three times with distilled sterile water, and dried with sterilized paper. Tissues were plated on potato dextrose agar (PDA) medium and incubated at 25°C for 24 h in the dark for 2 days. Fungal colonies with the typical characteristics of *Colletotrichum* spp. were transferred to new plates containing PDA medium. Isolates were preserved on PDA slants at 4°C in the dark.

Morphological characterization. Mycelial plugs (8 mm) were transferred from the edge of actively growing cultures to fresh PDA plates. The plates were kept at 25°C in the dark. The morphological characteristics of each isolate, including colony, conidial, and appressorial characteristics, were evaluated. Colony characteristics were determined after incubation for 8 days. Conidia morphology and production of appressoria were observed on water agar plates (WA; 20 g dextrose, 10 g agar, 1 liter distilled water) after 3 days of incubation by slide culture technology (Johnston and Jones 1997). The lengths and widths of 50 randomly chosen conidia and appressoria were then measured (Du et al. 2005). Isolates obtained from each host were divided into one to two morphological groups based on colony color and spore and appressoria shape. In total, 24 model isolates that contained all morphotypes and were collected from different host plants were selected for further study. Five to seven randomly selected isolates from each host were selected.

Isolation of fungal DNA. Mycelia were collected from cultures grown on PDA and ground after lyophilization. They were then macerated with Buffer FG1 (Omega Biotech) in 1.5-ml centrifuge tubes. Fungal genomic DNA was extracted with an E.Z.N.A. Fungal DNA Mini Kit (Omega Biotech) according to the manufacturers' instructions.

Molecular characterization: Multigene amplification and DNA sequencing. PCR amplification was performed for the following loci: the internal transcribed spacer regions (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), β -tubulin 2 (*TUB2*), calmodulin (*CAL*), chitin synthase 1 (*CHS-1*), and histone 3 (*HIS3*). The primers used in this study are shown in Table 1. PCR was performed in an EasyCycler Gradient 96-well Thermal cycler (Biometra, Goettingen, Germany) in a total volume of 25 μ l. The 25- μ l reaction mixture comprised 12.5 μ l 2 $\times$ Taq Master Mix (Novoprotein Scientific Inc.), 0.5 μ l of each primer (20 μ M), 1 μ l of template DNA, and 10.5 μ l of ddH₂O. PCR amplification protocols were performed as described by Damm et al. (2012a, b) and Silva et al. (2012), but the annealing temperatures were 54.9°C for ITS and *ACT*; 56°C for *CAL*, *CHS-1*, and *HIS3*; and 61.4°C for *GAPDH* and *TUB2*. The PCR products were electrophoresed on 1% agarose (HyAgarose LE Agarose, HydraGene Co., Ltd.) gel in 1.0 $\times$ TAE (Tris-acetic acid-EDTA) buffer stained with GelRed (Biotium, USA) and visualized in a UV-transilluminator. A DNA gel Recovery Kit (BioTeke) was used for rubber recovery. The recovered products were sequenced by Biosune Biotechnology (Shanghai) Co., Ltd.

Sequence alignment and phylogenetic analysis. The DNA sequences generated with forward and reverse primers were used to acquire consistent sequences using the BioNumerics version 5.1 software (Damm et al. 2012a), and their accession numbers are listed in Table 2. Sequences with high similarities were chosen and used in the analyses. A BLAST sequence identity search (Weir et al. 2012) was carried out to compare data from our isolate with those of other fungi deposited in the GenBank database and subsequently aligned using MUSCLE version 6 software (Edgar 2004). The alignments were edited manually using MEGA version 6 software (Tamura et al. 2013). Maximum likelihood (ML) and neighbor-joining (NJ) analyses combined with the combined datasets were analyzed using MEGA version 6 software. Phylogenetic trees were generated using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees were unlimited, with branches of zero length collapsed, and all multiple parsimonious trees were saved. Clade stability was assessed using a bootstrap analysis with 1,000 replicates, each with 10 replicates of random stepwise addition of taxa.

Pathogenicity assay. Representative materials (walnut fruits, strawberry leaves, grape fruits, and tea leaves) were used as the test material in the pathogenicity tests. Pathogenicity tests were conducted using the representative strains of each species. Isolates of each species were inoculated on detached leaves or fruits. The tested leaves or fruits were disinfected with 70% ethanol for 60 s, rinsed three times with sterile distilled water, and then air-dried on disinfectant paper towels at room temperature. Conidial suspensions were obtained from 7-day-old colonies by adding 5–10 ml of sterile distilled water into each culture, scraping the mycelia from plates, and filtering with two layers of gauze. The concentration of the conidia suspension was determined using a hemocytometer and adjusted to a final concentration of 1×10^5 ml⁻¹. Leaves or fruits were wounded with a sterile dissecting needle that had been modified to produce a puncture of 0.5 mm in depth (Biggs and Miller 2001; Velho et al. 2015). The wounded leaves or fruits were placed in sterilized plastic containers and then were inoculated by pipetting 10 μ l of conidial suspension onto each wound. After inoculation, the leaves or fruits were incubated in a growth chamber with 85% relative humidity (photoperiod of 12–12 h light/dark) at 25°C for 12 days, and then the lesion diameters were measured. Eight uninoculated leaves or fruits were used as the control. Eight leaves or fruits were tested for each isolate, and the experiment was repeated three times. To compare the differences in pathogenicity of each isolate, variance analyses were performed using the least significant difference (LSD) test in SPSS software (Version 13.0 for Windows) ($P = 0.05$).

Fungicide sensitivity assays on mycelial growth. Technical grade pyraclostrobin (95% active ingredient [a.i.]; Hubei Kang Bao Tai Fine-Chemical Co., Ltd., Hubei, China), difenoconazole (95% a.i.; Rainbow Chemical Co., Ltd., Shandong, China), fludioxonil (95% a.i.; Rainbow Chemical Co., Ltd., Shandong, China), tebuconazole (97% a.i.; Rainbow Chemical Co., Ltd., Shandong, China),

Table 1. Primers used for PCR and sequencing in this study

Gene	Product name	Primer	Direction	Sequence (5'-3')	Reference
ITS	Internal transcribed spacer	ITS1	Forward	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns 1993
		ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al. 1990
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GDF1	Forward	GCCGTCAACGACCCCTTCATTGA	Guerber et al. 2003
		GDR1	Reverse	GGGTGGAGTCGTACTTGAGCATGT	Guerber et al. 2003
ACT	Actin	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn 1999
		ACT-783R	Reverse	TACGAGTCCTTCTGGCCCAT	Carbone and Kohn 1999
TUB2	β -tubulin 2	T1	Forward	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik 1997
		Bt-2b	Reverse	ACCCTCAGTGATGACCCCTTGGC	Glass and Donaldson 1995
CAL	Calmodulin	CL1A	Forward	GATCAAGGAGGCCTTCTC	O'Donnell et al. 2000
		CL2A	Reverse	TTTTTGATCATGAGTTGGAC	O'Donnell et al. 2000
CHS-1	Chitin synthase 1	CHS-79F	Forward	TGGGGCAAGGATGCTTGAAGAAG	Carbone and Kohn 1999
		CHS-354R	Reverse	TGGAAGAACCATCTGTGAGAGTTG	Carbone and Kohn 1999
HIS3	Histone 3	CYLH3F	Forward	AGGTCCACTGGTGGCAAG	Crous et al. 2004
		CYLH3R	Reverse	AGCTGGATGTCCTTGACTG	Crous et al. 2004

pyrisoxazole (25% EC; Shenyang Sciencreat Chemical Co., Ltd., Liaoning, China), and tetracycline (15% a.i.; Liaoning Wkioc Bioengineering Co., Ltd., Liaoning, China) were used in the in vitro sensitivity assay. Stock solutions of pyraclostrobin, difenoconazole, and tetracycline were used in the tests by dissolving them in acetone to yield the concentration of 10,000 mg/liter, while fludioxonil and tebuconazole were dissolved in methanol to obtain a concentration of 10,000 mg/liter. Stock solutions were stored at 4°C in the dark until use. The stock solutions were diluted to a series of concentrations and were added to cooled molten potato dextrose agar (PDA) (approximately 50°C; approximately 15 ml per plate) after autoclaving. Salicylhydroxamic acid (SHAM) was added to the PDA amended with pyraclostrobin to yield a final concentration of 100 mg/liter. SHAM was used to inhibit the alternative oxidase respiration pathway. PDA plates with 0.2% organic solvent or SHAM were used as the control. Mycelial plugs (8 mm) cut from the active colony margins were placed upside down on PDA plates. After incubation for

5 days at 25°C in the dark, the diameter of each colony was measured in two perpendicular directions, and the mean diameter was obtained by subtracting the original diameter of the inoculation plugs. The experiment was repeated twice with three replicates. The percent inhibition was calculated as [(growth of the control – growth of the experimental)/growth of the control] × 100. The EC₅₀ values were calculated by performing a regression of the probit-transformed percent inhibition against the log₁₀ fungicide concentrations, and the correlation coefficients (*r*) were determined using the statistical algorithms of SPSS software (Version 13.0 for Windows).

Temperature effects on growth rate. To evaluate the effect of temperature on the growth of representative *Colletotrichum* spp. collected from different hosts, mycelial plugs (8 mm) were cut from the active colony margins on PDA and transferred onto the center of 9-cm plates containing approximately 15 ml of PDA. The cultures were separately incubated at five different temperatures (21, 23, 25, 28, and 30°C) in the dark for 8 days. The mean colony diameter (minus

Table 2. Strains of *Colletotrichum* spp. isolated from four hosts studied. Details are provided about host and locality, and GenBank accession numbers of the sequences generated.

Species	Culture	Host	Locality	GenBank accession number						
				ITS	GAPDH	ACT	TUB2	CAL	CHS	HIS3
<i>C. acutatum</i> sensu stricto	CBS 112996 ^a	<i>Carica papaya</i>	Australia	JQ005776	JQ948677	JQ005839	JQ005860	-	JQ005797	JQ005818
	CBS 144.29	<i>Capsicum annuum</i>	Sri Lanka	JQ948401	JQ948732	JQ949722	JQ950052	-	JQ949062	JQ949392
	CBS 110735	<i>Pinus radiata</i>	South Africa	JQ948354	JQ948685	JQ949675	JQ950005	-	JQ949015	JQ949345
	CBS 127539	<i>Aspalathus linearis</i>	South Africa	JQ948377	JQ948708	JQ949698	JQ950028	-	JQ949038	JQ949368
	HTTJ1	<i>Juglans regia</i> L.	China	MG561705	MG561689	MG561655	MG561717	MG561665	MG561677	-
	HTTJ3	<i>Juglans regia</i> L.	China	MH092012	MH113902	MH113867	MH113914	MH113878	MH113890	-
	JS1A7	<i>Camellia sinensis</i>	China	KU251586	KU252040	KU251667	KU252192	KU251828	KU251934	-
	JS1A46	<i>Camellia sinensis</i>	China	KU251585	KU252039	KU251666	KU252191	KU251827	KU251933	-
	LC1363	<i>Camellia sinensis</i>	China	KJ955080	KJ954781	KJ954362	KJ955229	KJ954633	-	-
	CSTJ2	<i>Camellia sinensis</i>	China	MG561702	MG561686	MG561652	MG561714	MG561662	MG561674	-
<i>C. camelliae</i>	CSTJ8	<i>Camellia sinensis</i>	China	MG561703	MG561687	MG561653	MG561715	MG561663	MG561675	MG561697
	CSTJ13	<i>Camellia sinensis</i>	China	MG561704	MG561688	MG561654	MG561716	MG561664	MG561676	-
	CSTJ20	<i>Camellia sinensis</i>	China	MH092010	MH113900	MH113865	MH113912	MH113876	MH113888	-
	CSTJ23	<i>Camellia sinensis</i>	China	MH092011	MH113901	MH113866	MH113913	MH113877	MH113889	-
	C1316.21	<i>Tetragastris panamensis</i>	Panama	JX010173	JX010032	JX009581	JX010409	JX009674	JX009874	-
	CAUG21	<i>Capsicum</i> sp.	China	KP145434	KP145406	KP145322	KP145462	KP145350	KP145378	-
	CCON5	<i>Gossypium hirsutum</i> L.	China	KJ781342	KJ781334	KJ781309	KJ781350	KJ781317	KJ781326	-
	LF647	<i>Camellia sinensis</i>	China	KJ955190	KJ954891	KJ954463	KJ955337	KJ954741	-	-
	CMTJ1	<i>Fragaria ananassa</i>	China	MG561700	MG561684	MG561650	MG561712	-	MG561672	MG561696
	CMTJ7	<i>Fragaria ananassa</i>	China	MH092005	MH113895	MH113860	MH113907	-	MH113883	MH113872
<i>C. fructicola</i>	CMTJ9	<i>Fragaria ananassa</i>	China	MH092006	MH113896	MH113861	MH113908	-	MH113884	MH113873
	CMTJ18	<i>Fragaria ananassa</i>	China	MH092008	MH113898	MH113863	MH113910	-	MH113886	MH113874
	CCOW3	<i>Gossypium hirsutum</i> L.	China	KJ781337	KJ781329	KJ781304	KJ781345	KJ781312	KJ781321	-
	CDLG4	<i>Hemerocallis fulva</i>	China	JQ400009	JQ400016	JQ399995	JQ400023	-	JQ400002	-
	DNCL022	<i>Syzygium samarangense</i>	Thailand	KF242097	KF242159	KF157804	KF254853	KF254849	-	-
	GZAAS5.09516	<i>Citrus reticulata</i>	China	JQ247627	JQ247604	JQ247652	JQ247639	JQ247593	-	-
	IMI 356878	<i>Citrus sinensis</i>	Italy	JX010152	JX010056	JX009531	JX010445	JX009731	JX009818	-
	LJTJ13	<i>Capsicum</i> sp.	China	KP748204	KP823783	KP823751	KP823863	KP823821	-	-
	LJTJ87	<i>Capsicum</i> sp.	China	KT936448	KP943521	KT936437	-	KT936431	-	-
	LN-48	<i>Juglans regia</i>	China	KY302640	KY302636	KY302628	-	-	KY302632	-
<i>C. gloeosporioides</i> sensu stricto	sxwzg14	<i>Proteaceae</i>	China	KC293560	KC293720	KC293600	KC293640	KC293680	-	-
	PTTJ1	<i>Vitis</i> spp.	China	MG561707	MG561691	MG561657	MG561719	MG561667	MG561679	-
	PTTJ7	<i>Vitis</i> spp.	China	MG561708	MG561692	MG561658	MG561720	MG561668	MG561680	-
	PTTJ11	<i>Vitis</i> spp.	China	MG561709	MG561693	MG561659	MG561721	MG561669	MG561681	-
	PTTJ14	<i>Vitis</i> spp.	China	MG561710	MG561694	MG561660	MG561722	MG561670	MG561682	MG561699
	PTTJ22	<i>Vitis</i> spp.	China	MG561711	MG561695	MG561661	MG561723	MG561671	MG561683	-
	PTTJ24	<i>Vitis</i> spp.	China	MH092015	MH113905	MH113870	MH113917	MH113881	MH113893	-
	PTTJ38	<i>Vitis</i> spp.	China	MH092016	MH113906	MH113871	MH113918	MH113882	MH113894	-
	IMI 350839	<i>Psidium guajava</i>	India	JQ948270	JQ948600	JQ949591	JQ949921	-	JQ948931	JQ949261
	B27	<i>Vitis vinifera</i>	China	KF156852	KF377483	KF377520	KF288971	-	KF288996	-
<i>C. guajavae</i>	C1-3	<i>Vitis vinifera</i>	China	KF156840	KF377469	KF377506	KF288965	-	KF288982	-
	D1-1	<i>Vitis vinifera</i>	China	KF156842	KF377471	KF377508	KF288966	-	KF288984	-
	CMTJ4	<i>Fragaria ananassa</i>	China	MG561701	MG561685	MG561651	MG561713	-	MG561673	-
	CMTJ13	<i>Fragaria ananassa</i>	China	MH092007	MH113897	MH113862	MH113909	-	MH113885	-
	CMTJ21	<i>Fragaria ananassa</i>	China	MH092009	MH113899	MH113864	MH113911	-	MH113887	-
	HTTJ6	<i>Juglans regia</i> L.	China	MH092013	MH113903	MH113868	MH113915	MH113879	MH113891	MH113875
	HTTJ12	<i>Juglans regia</i> L.	China	MH092014	MH113904	MH113869	MH113916	MH113880	MH113892	-
	HTTJ15	<i>Juglans regia</i> L.	China	MG561706	MG561690	MG561656	MG561718	MG561666	MG561678	MG561698

^a Ex-type culture. Strains/sequences studied in this paper are in bold font.

the original diameter of the inoculation plug) was measured. The experiment was repeated twice with three replicates. The growth rate was computed as the mean daily growth rate.

Results

Morphological characterization. The 101 isolates were divided into six morphological types based on colony morphology and color, spore and appressoria shape, and mycelial morphology. Two groups

came from walnut fruits, two groups from strawberry leaves, one group from grape fruits, and the other group from tea leaves.

Isolates collected from walnut fruits had light gray aerial mycelium and belonged to group 1 of *C. viniferum* (Fig. 1A1). The reverse side of the colony was pale olivaceous gray with a whitish margin (Fig. 1B1). The vegetative hyphae were hyaline, smooth-walled, and septate (Fig. 1C1). Conidia were hyaline, smooth-walled or minutely verruculose, aseptate, guttulate, cylindrical to clavate, with both

Fig. 1. Cultural characteristics and morphology of *Colletotrichum* spp. isolated from different hosts in China. **A1-E1**, group 1; **A2-E2**, group 2; **A3-E3**, group 3; **A4-E4**, group 4; **A5-E5**, group 5; **A6-E6**, group 6. **A**, upper of colony on PDA; **B**, reverse of colony on PDA; **C**, micrographs of mycelium; **D**, micrographs of conidia; **E**, micrographs of appressoria. Scale bars = 20 μ m.

ends rounded (Fig. 1D1). Appressoria were single, medium to dark brown, aseptate, and ovoid (Fig. 1E1).

Colonies of isolates collected from walnut fruits belonging to group 2 of *C. acutatum* s.s. were charcoal gray and had a flat center with white aerial hyphae at the margin (Fig. 1A2). The reverse side of the colony was densely arranged with olivaceous gray in the middle area (Fig. 1B2). Mycelia were smooth-walled, septate, and branched, and the partitive membranes of the hyphae were shorter than in group 1 (Fig. 1C2). Conidia were principally straight, with a rounded and a pointed end (Fig. 1D2). Appressoria were medium brown, single or in small dense clusters, roughly round to irregular in outline, and crenate at the margins (Fig. 1E2).

Colonies of the group 3 isolates which corresponded to *C. fructicola* from strawberry leaves produced an abundance of light gray aerial mycelium with white margins (Fig. 1A3), and the reverse side was pale olivaceous gray with concentric rings (Fig. 1B3). Mycelia were medium to dark brown, septate, and smooth-walled (Fig. 1C3). Conidia were cylindrical or clavate, with both ends rounded or one end acute (Fig. 1D3). The appressoria were medium to dark brown and formed singly, with ellipsoidal to irregular outlines (Fig. 1E3).

The isolates from strawberry leaves of group 4 corresponded to *C. viniferum* and had gray, dense, cottony mycelia on the upper surface (Fig. 1A4), while the color of the mycelia on the reverse side were white (Fig. 1B4). In addition, mycelia were connected with tiny mycelia that were dark brown and septate (Fig. 1C4). Conidia were cylindrical to clavate, straight, with each apex rounded, containing one oil globule (Fig. 1D4). Appressoria were single, subglobose to cylindrical, or clavate with a medium to dark brown color (Fig. 1E4).

The group 5 isolates obtained from grape fruits of *C. gloeosporioides* s.s. exhibited dense light gray aerial mycelium (Fig. 1A5), and the reverse side seemed olivaceous to black, radial, with white margins (Fig. 1B5). Mycelia were medium to dark brown, smooth-walled, and septate (Fig. 1C5). Conidia were cylindrical to clavate, with one to two oil globules (Fig. 1D5). Appressoria were single, ovoid or cylindrical, smooth-walled, and seemed medium to dark brown (Fig. 1E5).

The mycelia of the isolates from tea leaves in group 6 corresponded to *C. camelliae* and were white, fluffy, and cottony, with

abundant aerial mycelia (Fig. 1A6). The reverse side was white to gray, with melanin in the center of the bottom (Fig. 1B6). Mycelia were light brown, smooth-walled, septate, and mostly branched (Fig. 1C6). Conidia were hyaline, ovoid to cylindrical, and guttulate (Fig. 1D6). Appressoria were multicell, puce, and mostly irregular in outline. They were always lobed, crenate, and catenate (Fig. 1E6).

The sizes of conidia and appressoria of the 24 representative isolates were determined and compared (Table 3). Isolates collected from tea leaves had the longest conidia, while isolates obtained from strawberry leaves had the shortest conidia. Meanwhile, conidia of isolates obtained from tea leaves were the widest, and those from walnut fruits were the narrowest. The length/width ratios of conidia in isolates from walnut fruits were higher than that of other hosts. In terms of appressoria, isolates from tea leaves had the longest appressoria, but those from walnut fruits of group 1 were the shortest. Similarly, appressoria of isolates from tea leaves were the widest, and appressoria of isolates from walnut fruits of group 1 were the narrowest. However, the length/width ratios of appressoria of isolates collected from walnut fruits were lower than those of other hosts, with a numerical value close to one.

Multilocus phylogenetic analyses. Twenty-four representative isolates collected from four different host plants and twenty-four previously characterized reference strains were analyzed by multilocus phylogenetic analyses (Table 2). Multilocus phylogenetic analysis was carried out by combining ITS, *GADPH*, *ACT*, *TUB2*, *CAL*, *CHS-1*, and *HIS3* sequences with *Colletotrichum guajavae* (IMI 350839) as the outgroup taxa (Fig. 2). The dataset was composed of 3,285 characters including the alignment gaps. The topologies of phylogenetic trees were structured with the ML and NJ methods and were basically concordant with each other, manifesting that the evolutionary relationship of the test strains was statistically supported. The phylogenetic tree showed that the 24 representative isolates belonged to five distinct clades. The isolates obtained from grape fruits clustered with *C. gloeosporioides* s.s., while those from tea leaves clustered with *C. camelliae*. Four strains from strawberry leaves corresponded to *C. fructicola*. The strains HTTJ1 and HTTJ3 created a separate branch with a 99% bootstrap value (NJ) and was a sister taxon to *C. acutatum* s.s. Furthermore, three strains from

Table 3. Mean conidium and appressoria of length, width, and length/width ratio among isolates from different hosts^a

Isolates ^b	Host	Morphological group	Conidial			Appressoria		
			Length (μm)	Width (μm)	Length/width ratio	Length (μm)	Width (μm)	Length/width ratio
HTTJ1	Walnut	Group 2	16.63 (0.59)	5.45 (0.23)	3.07 (0.11)	15.65 (0.68)	13.98 (0.95)	1.14 (0.06)
HTTJ3	Walnut	Group 2	16.37 (0.31)	5.63 (0.64)	2.91 (0.25)	15.63 (0.63)	14.01 (0.32)	1.13 (0.08)
HTTJ6	Walnut	Group 1	16.72 (0.38)	5.39 (0.25)	3.11 (0.17)	10.47 (0.54)	9.73 (0.47)	1.09 (0.03)
HTTJ12	Walnut	Group 1	16.29 (0.46)	5.51 (0.12)	2.97 (0.08)	10.65 (0.48)	9.88 (0.39)	1.08 (0.10)
HTTJ15	Walnut	Group 1	16.99 (0.93)	5.70 (0.23)	3.00 (0.19)	10.32 (1.48)	9.05 (1.65)	1.14 (0.03)
CMTJ1	Strawberry	Group 3	14.30 (0.58)	5.86 (0.26)	2.44 (0.11)	24.15 (0.75)	17.63 (0.34)	1.37 (0.02)
CMTJ4	Strawberry	Group 4	15.22 (0.56)	6.21 (0.31)	2.50 (0.16)	14.68 (0.96)	12.55 (0.25)	1.17 (0.04)
CMTJ7	Strawberry	Group 3	14.56 (0.39)	5.88 (0.21)	2.48 (0.26)	23.48 (0.36)	18.03 (0.37)	1.30 (0.32)
CMTJ9	Strawberry	Group 3	14.38 (0.34)	5.67 (0.32)	2.53 (0.12)	23.89 (0.54)	17.92 (0.52)	1.33 (0.08)
CMTJ13	Strawberry	Group 4	15.45 (0.36)	6.56 (0.52)	2.36 (0.23)	15.38 (0.34)	12.56 (0.53)	1.23 (0.17)
CMTJ18	Strawberry	Group 3	14.67 (0.43)	5.87 (0.58)	2.50 (0.27)	24.01 (0.48)	17.95 (0.36)	1.34 (0.18)
CMTJ21	Strawberry	Group 4	15.31 (0.73)	6.12 (0.39)	2.50 (0.37)	15.81 (0.37)	13.28 (0.42)	1.20 (0.09)
PTTJ1	Grape	Group 5	16.93 (1.61)	6.39 (0.83)	2.65 (0.25)	19.74 (0.72)	13.90 (1.34)	1.42 (0.74)
PTTJ7	Grape	Group 5	17.12 (1.33)	5.78 (0.31)	2.83 (0.18)	22.34 (1.37)	17.54 (1.15)	1.32 (0.17)
PTTJ11	Grape	Group 5	17.07 (1.73)	6.33 (0.21)	2.72 (0.22)	18.57 (0.46)	14.97 (0.63)	1.26 (0.08)
PTTJ14	Grape	Group 5	16.83 (0.96)	6.04 (0.38)	2.79 (0.36)	19.73 (0.25)	13.89 (1.42)	1.42 (0.93)
PTTJ22	Grape	Group 5	17.20 (1.76)	6.72 (0.73)	2.56 (0.37)	19.35 (0.36)	13.92 (0.36)	1.39 (0.56)
PTTJ24	Grape	Group 5	16.84 (0.83)	6.48 (0.62)	2.60 (0.31)	19.42 (0.38)	14.27 (0.53)	1.36 (0.11)
PTTJ38	Grape	Group 5	16.86 (0.72)	6.59 (0.54)	2.56 (0.16)	18.83 (0.51)	13.92 (0.62)	1.36 (0.32)
CSTJ2	Tea plant	Group 6	18.22 (0.49)	7.89 (0.35)	2.33 (0.11)	27.81 (0.89)	18.60 (1.35)	1.52 (0.08)
CSTJ8	Tea plant	Group 6	18.33 (0.58)	7.38 (0.74)	2.48 (0.16)	29.72 (0.48)	18.46 (1.04)	1.61 (0.82)
CSTJ13	Tea plant	Group 6	17.67 (0.39)	7.50 (0.52)	2.40 (0.18)	36.63 (0.36)	23.74 (0.52)	1.55 (0.05)
CSTJ20	Tea plant	Group 6	18.47 (0.56)	7.63 (0.37)	2.42 (0.35)	28.67 (0.83)	18.36 (0.72)	1.56 (0.34)
CSTJ23	Tea plant	Group 6	17.93 (0.49)	7.73 (0.53)	2.32 (0.23)	29.47 (0.52)	18.53 (0.63)	1.59 (0.16)

^a Numbers in parentheses represent standard error of the mean based on least square means ($P = 0.05$).

^b Isolates were selected as representative of each group and host plant.

strawberry leaves and three strains from walnut fruits formed a clade with *C. viniferum*.

Pathogenicity tests. In addition to the isolates from walnut fruits, other representative isolates of *Colletotrichum* produced typical symptoms of anthracnose on detached leaves or fruits (Fig. 3). These tested *Colletotrichum* species collected from four hosts showed different infection incidences. *C. fruticola* isolates obtained from strawberry leaves were more virulent than *C. viniferum* isolates from strawberry leaves. Only *C. fruticola* isolates collected from strawberry leaves had sporulation abilities (data not shown). However, seven isolates collected from grape fruits showed no significant differences in lesion length and width ($P > 0.05$). There was no

significant difference in toxicity among all *C. camelliae* strains to tea leaves. Two species from walnuts did not infect the walnut fruits; we considered that this was caused by the thicker epidermis of walnut fruits.

Sensitivity of *Colletotrichum* spp. to fungicides. The sensitivities of isolates from four different host plants to the fungicides were determined using the mycelial growth method (Table 4). No significant differences in sensitivities were observed among all tested isolates for the same fungicide ($P > 0.05$). The independent variables fungicide, species, and plant all generally had effects on the EC₅₀ of six fungicides against mycelial growth of *Colletotrichum* spp. (Table 4). All of the six fungicides had high activity against

Fig. 2. Maximum likelihood (ML) consensus tree based on a 7-gene combined dataset (ITS, GAPDH, ACT, TUB2, CAL, CHS-1, HIS3) showing phylogenetic affinities of *Colletotrichum* isolates from different hosts in this study. Bootstrap support values (ML $\geq 50\%$) and NJ bootstrap support values (NJ $\geq 50\%$) are displayed at the nodes (ML/NJ). *Colletotrichum guajavae* IMI 350839 was used as the out-group. Different background colors represent different species (*C. gloeosporioides* sensu stricto is covered with green, *C. fruticola* is covered with blue, *C. camelliae* is covered with yellow, *C. acutatum* sensu stricto is covered with purple). The species of this study are marked with small round dots (Strains from grape fruits are marked with purple, strains from strawberry leaves are marked with green, strains from walnut fruits are marked with blue, strains from tea leaves are marked with red.).

the mycelial growth of all isolates, with the highest EC₅₀ values at 1.63 mg/liter.

Based on all representative strains from the same plant, fludioxonil was the most effective against isolates from walnut fruits, difenoconazole was the most effective against isolates from strawberry leaves, and pyraclostrobin was the most effective against isolates from tea leaves (Table 4). The sensitivities of isolates from grape fruits to fungicides was inconsistent. The sensitivities of five isolates (*C. camelliae*) of tea leaves to six fungicides were similar, whereas the sensitivities of seven isolates (*C. gloeosporioides* s.s.) of grape fruits to fungicides were different (Table 4).

Based on isolates, the EC₅₀ values of pyraclostrobin and tebuconazole against the mycelial growth of PTTJ7 were lower than those of any other fungicides in this study (mean EC₅₀ = 0.08, 0.07 mg/liter)

Fig. 3. Average length and width represent disease severity of anthracnose caused by *Colletotrichum* spp. from different hosts. Values are mean and standard error (SE), with columns and bars indicating mean and SE.

Table 4. Mean half maximal effective concentration (EC₅₀) values of mycelial growth of *Colletotrichum* spp. from four hosts to six fungicides^a

Isolate	EC ₅₀ (mg/liter)					
	Pyrac	Difen	Fludi	Tebuc	Pyris	Tetra
HTTJ1	0.43 (0.11)	0.45 (0.18)	0.21 (0.10)	0.58 (0.13)	0.41 (0.19)	0.30 (0.12)
HTTJ3	0.78 (0.03)	0.32 (0.17)	0.28 (0.06)	0.31 (0.13)	0.89 (0.12)	0.38 (0.11)
HTTJ6	0.54 (0.10)	0.44 (0.12)	0.25 (0.09)	0.56 (0.06)	0.45 (0.07)	0.33 (0.07)
HTTJ12	0.48 (0.02)	0.51 (0.09)	0.26 (0.01)	0.49 (0.11)	0.61 (0.22)	0.40 (0.08)
HTTJ15	0.89 (0.33)	0.25 (0.10)	0.22 (0.02)	0.24 (0.09)	0.97 (0.21)	0.23 (0.03)
CMTJ1	0.26 (0.09)	0.16 (0.08)	0.30 (0.12)	0.39 (0.17)	0.39 (0.13)	0.17 (0.07)
CMTJ4	0.67 (0.17)	0.08 (0.01)	0.40 (0.13)	0.36 (0.12)	0.53 (0.17)	0.41 (0.13)
CMTJ7	0.32 (0.03)	0.19 (0.08)	0.44 (0.11)	0.38 (0.04)	0.58 (0.10)	0.29 (0.01)
CMTJ9	0.42 (0.07)	0.11 (0.02)	0.38 (0.06)	0.41 (0.02)	0.55 (0.08)	0.30 (0.09)
CMTJ13	0.59 (0.12)	0.15 (0.01)	0.37 (0.07)	0.45 (0.02)	0.47 (0.01)	0.42 (0.11)
CMTJ18	0.37 (0.03)	0.20 (0.04)	0.47 (0.10)	0.48 (0.11)	0.38 (0.02)	0.33 (0.05)
CMTJ21	0.38 (0.07)	0.23 (0.03)	0.52 (0.16)	0.39 (0.11)	0.48 (0.05)	0.26 (0.07)
PTTJ1	0.24 (0.03)	0.18 (0.03)	0.26 (0.06)	0.86 (0.33)	1.48 (0.45)	0.08 (0.03)
PTTJ7	0.08 (0.01)	0.35 (0.21)	0.15 (0.04)	0.07 (0.02)	0.86 (0.32)	0.26 (0.11)
PTTJ11	0.21 (0.06)	0.48 (0.16)	0.14 (0.08)	0.38 (0.17)	1.63 (0.43)	1.32 (0.55)
PTTJ14	0.52 (0.18)	0.20 (0.09)	0.86 (0.34)	0.33 (0.18)	0.33 (0.18)	0.12 (0.03)
PTTJ22	0.57 (0.15)	0.41 (0.18)	0.25 (0.13)	0.10 (0.07)	1.06 (0.34)	0.33 (0.12)
PTTJ24	0.25 (0.08)	0.38 (0.09)	0.19 (0.03)	0.38 (0.08)	1.00 (0.23)	0.83 (0.22)
PTTJ38	0.52 (0.11)	0.48 (0.08)	0.22 (0.10)	0.43 (0.12)	0.92 (0.16)	0.52 (0.13)
CSTJ2	0.28 (0.09)	0.35 (0.14)	1.21 (0.32)	0.51 (0.13)	0.73 (0.24)	0.29 (0.09)
CSTJ8	0.26 (0.06)	0.33 (0.16)	1.48 (0.54)	0.53 (0.18)	0.65 (0.26)	0.30 (0.11)
CSTJ13	0.29 (0.08)	0.31 (0.03)	1.50 (0.44)	0.55 (0.24)	0.69 (0.28)	0.31 (0.15)
CSTJ20	0.26 (0.05)	0.36 (0.17)	1.38 (0.54)	0.58 (0.33)	0.74 (0.23)	0.31 (0.09)
CSTJ23	0.27 (0.03)	0.38 (0.09)	1.51 (0.37)	0.52 (0.34)	0.66 (0.38)	0.35 (0.21)

^aFungicides pyraclostrobin (Pyrac), difenoconazole (Difen), fludioxonil (Fludi), tebuconazole (Tebuc), pyrisoxazole (Pyris), tetramycin (Tetra). Numbers in parentheses represent standard error of the mean based on least square means ($P = 0.05$).

(Table 4). Moreover, the EC₅₀ values of pyrisoxazole and tetramycin against the mycelial growth of PTTJ11 were higher than those of any other isolates in the study (mean EC₅₀ = 1.63, 1.32 mg/liter) (Table 4).

Based on the mean EC₅₀ value of the same host, the mean EC₅₀ of the mycelial growth of *Colletotrichum* spp. from tea leaves against fludioxonil was higher than that of any other fungicides, and the mean EC₅₀ of *Colletotrichum* spp. from strawberry leaves against difenoconazole was lower than that of other fungicides in this study (Fig. 4). High error lines indicated that the sensitivities of different strains from the same crop to fungicides are greatly different (Fig. 4). The fungicide (difenoconazole, tetramycin) sensitivity of mycelial growth among different hosts was, on average, significantly higher than the sensitivity to pyrisoxazole (Fig. 4).

Effect of temperature on mycelial growth. In this test, the mycelial growth of the representative isolates was significantly influenced by temperature alteration ($P < 0.05$) (Fig. 5). The optimal temperature for mycelial growth was found to be 25°C for the isolates collected from walnut fruits and tea leaves, but it was 28°C for the

Fig. 4. Mean EC₅₀ values of *Colletotrichum* spp. from four hosts to pyraclostrobin (Pyrac), difenoconazole (Difen), fludioxonil (Fludi), tebuconazole (Tebuc), pyrisoxazole (Pyris), tetramycin (Tetra). Comparison of sensitivity among different hosts. Values are mean and standard error (SE), with columns and bars indicating mean and SE.

Fig. 5. Average growth rate of *Colletotrichum* species among different hosts at five selected temperatures. Values are mean and standard error (SE), with columns and bars indicating mean and SE.

isolates collected from strawberry leaves and grape fruits (Fig. 5). The growth rate of strains from strawberry leaves and grape fruits at 25°C was not significantly different from that at 28°C ($P > 0.05$) (Fig. 5). These results indicated that the optimal temperature for mycelial growth of isolates was 25–28°C regardless of the hosts that they were collected from (Fig. 5).

Discussion

Recent reports suggested that anthracnose affected a wide range of plants, and the species were complex (Damm et al. 2012a, b; Weir et al. 2012). This study aimed to compare and identify the *Colletotrichum* species isolated from different hosts in Shandong Province, China. According to the morphological characteristics and multilocus phylogenetic analyses, the 101 isolates were categorized into six groups, namely, group 1 (14 strains from walnut fruits, 13.9%), group 2 (3 strains from walnut fruits, 2.9%), group 3 (13 strains from strawberry leaves, 12.9%), group 4 (8 strains from strawberry leaves, 8.0%), group 5 (38 strains from grape fruits, 37.6%), group 6 (25 strains from tea leaves, 24.7%).

Regarding *Colletotrichum* complexes, many morphologically similar species were reported in economic crops and ornamental plants (Hyde et al. 2009a, b). However, when morphological detection was conducted to classify the experimental isolates into six groups, seven *Colletotrichum* species were actually identified by multilocus phylogenetic analysis (Liu et al. 2016). Therefore, the identification of *Colletotrichum* species by morphological characteristics alone cannot be considered reliable. At present, the most powerful identification of *Colletotrichum* complexes was achieved by the incorporation of the morphological analysis and multilocus phylogeny (Crouch et al. 2006, 2009; Damm et al. 2012a, b; Guerber et al. 2003; Weir et al. 2012). In this study, the morphological groups based on colony characteristics, conidial morphology, and appressorium morphology were entirely in line with the results of the multilocus phylogenetic analysis deduced from the molecular data. Furthermore, either the maximum likelihood (ML) or neighbor-joining (NJ) analysis was sufficiently high to classify them into separate species.

The name *Colletotrichum gloeosporioides* s.s. was the first to be applied to *Colletotrichum* isolates connected with diseases of *Citrus* in Italy (Penzig 1882). *C. gloeosporioides* s.s., a biologically and genetically diverse species, threatened at least 470 different host genera (Sutton 1980). *C. gloeosporioides* s.s. was previously reported to infect many mature fruits, particularly in the tropical zone (Freeman et al. 1998; Lin et al. 2016; Sangeetha and Rawal 2008). In recent years, *Colletotrichum* species were increasingly prevalent in the temperate monsoon climate zone, e.g., the Shandong, Hebei, and Shanxi provinces in China (Jayawardena et al. 2016; Zhu et al. 2014). Our research showed that all of the strains collected from grapes formed the same clade of *C. gloeosporioides* s.s. *C. fructicola*, a non-host-specific pathogen, threatens a wide range of hosts (Liu et al. 2016; Weir et al. 2012) and belongs to *C. gloeosporioides* sensu lato. It has been reported to cause strawberry anthracnose in China, Canada, and the United States (Jayawardena et al. 2016; Weir et al. 2012). In the current study, 13 of 101 isolates obtained from strawberry leaves were described as *C. fructicola* based on morphological characteristics and multigene phylogenetic analysis.

C. acutatum s.s. is widely reported to infect walnuts (Liu et al. 1995), strawberries (Than et al. 2008), apples (Víchová et al. 2012), peppers (Harp et al. 2014), and grapes (Samuelian et al. 2014). In our study, the three strains isolated from Jinan, Shandong Province, China, were identified as *C. acutatum* s.s., whose colony, conidia, and appressoria morphology were obviously different from those of the other strains. Their genetic relationship was more distant than those of other strains. As far as we know, the earliest record of anthracnose was described from living leaves of tea (Willis et al. 1899). *C. camelliae* is still widely listed as the causal agent of the anthracnose of tea plants. (Liu et al. 2015) and (Weir et al. 2012) reported that *C. camelliae* was found in samples of tea anthracnose in different provinces in China. In our research, five representative strains obtained from tea leaves formed a separate clade with *C.*

camelliae in the phylogenetic tree. Morphological characteristics of the isolates collected from tea leaves were consistent with those reported by Liu et al. (2015). Pathogenic species associated with strawberry anthracnose are very extensive, including *C. coccodes* (Cannon et al. 2012), *C. cuscuteae* (Damm et al. 2012a), *C. fioriniae* (Damm et al. 2012a), *C. fructicola* (Weir et al. 2012), *C. godetiae* (Damm et al. 2012a), *C. nymphaeae* (Damm et al. 2012a), *C. salicis* (Damm et al. 2012a), and *C. simmondsii* (Damm et al. 2012a). This article was the first report of *C. viniferum* causing strawberry anthracnose in Shandong Province, China. *C. viniferum* had formerly been isolated from pathogenic sites of grapes in China (Peng et al. 2013; Yan et al. 2015). Likewise, our research was the first to identify the anthracnose of walnuts caused by *C. viniferum* in Shandong Province, China, which indicates that *C. viniferum* has an extensive host range.

In this study, isolates obtained from different hosts were inoculated on detached leaves or fruits. Different *Colletotrichum* species revealed various degrees of pathogenicity by artificial inoculation on leaves or fruits. According to current data, the virulence of *C. fructicola* isolates collected from strawberry leaves toward strawberry leaves was significantly different from that of *C. viniferum* isolates. More importantly, isolates of *C. fructicola* from strawberry leaves were more aggressive than those of *C. viniferum*. Nevertheless, isolates of *C. fructicola* produced a large number of spores on strawberry leaves. Interestingly, *C. viniferum* isolates had no sign of spore production.

For growers, an effective and durable management strategy for controlling fungal diseases relies on timed applications of fungicides that have different modes of action. This research determined the sensitivity of *Colletotrichum* complex species to fungicides with different modes of action, such as pyraclostrobin, difenoconazole, fludioxonil, tebuconazole, pyrisoxazole, and tetramycin. Isolates from different hosts of the five *Colletotrichum* spp. revealed EC₅₀ values of 0.07 to 1.63 mg/liter for all the fungicides. Although the EC₅₀ values were relatively low, there was a slight difference between species. In previous studies, the *C. acutatum* species were more tolerant to fungicides compared with the *C. gloeosporioides* species (Greer et al. 2011; Jayasinghe and Fernando 1998; Munir et al. 2016). This phenomenon was not observed in this study. To better understand the species, further experiments need to be done in the future. Additionally, the EC₅₀ values measured in this study are low, and fungicides may produce better control efficacy against *Colletotrichum* spp. in the field. Nevertheless, more field studies are needed in the future.

The effect of temperature on the growth rate of *Colletotrichum* spp. in vitro was also evaluated. The results showed that colony growth is the fastest in the temperature range of 25–28°C. Prior to this study, the maximum growth rate occurred in the range of 25–30°C for *Colletotrichum* spp. isolates (Munir et al. 2016; Xu et al. 2017). Temperature plays an indispensable role in the infection process of anthracnose. Chongo and Bernier (2000) confirmed that the number of lesions and disease severity of anthracnose on lentils increased with temperature, reaching the highest damage at a temperature of 28°C. Once the optimum growth temperature for the pathogen is reached, anthracnose may frequently occur, and thus this is the time to apply fungicides.

In the present study, we investigated the dissimilarity of *Colletotrichum* species obtained from four hosts in Shandong Province, China. Isolates of each host had only one or two species to be identified. The limited sample numbers and the limited host scope of the species investigated may be the main factors. Future work will be required to probe species collected from different geographical regions and the pathogenicity of *Colletotrichum* species reported on multiple hosts. This research is the first to report molecular and phenotypic characterization and fungicide sensitivity differences among species of different hosts from representative orchards in Shandong Province, China. According to the current data, we found that *C. viniferum* was a new species on walnut fruits and strawberry leaves, and its sensitivity to fungicides was not significantly different from that of other species. Therefore, we deduce that the fungicides

used in this paper can be extensively used to prevent anthracnose on various hosts.

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