Identification, Virulence and Fungicide Sensitivity of *Colletotrichum gloeosporioides* s.s. Responsible for Walnut Anthracnose Disease in China

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Abstract

Walnut (*Juglans regia* L.) is an economically important woody nut and edible oil tree all over the world. However, walnut production is limited by walnut anthracnose, which is a disastrous disease that causes significant yield losses. Studying the etiology of anthracnose on walnut and the pathogens' virulence and sensitivities to fungicides would be beneficial for effective control. This study was conducted to identify the pathogen of walnut anthracnose and reveal the population diversity of pathogens through virulence, sensitivities to fungicides, and genetic variation. A total of 13 single-spore *Colletotrichum* isolates were collected from walnut anthracnose-diseased fruits and leaves from 13 walnut commercial orchards in Henan, Hubei, Shandong, and Shaanxi provinces in China. The isolates were identified as *Colletotrichum gloeosporioides* sensu stricto (s.s.) according to multilocus phylogenetic analyses (internal transcribed spacer, actin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase), morphological as well as cultural characters, and pathogenicity. When the same walnut tissue was inoculated with different isolates, the disease lesion size was different. The results showed that the virulence of all isolates was considerably different, and the differences were not correlated with geographic origins. The virulence to walnut leaves and fruits inoculated with the same isolate was significantly different. Based on the virulence to walnut leaves and fruits, the 13 isolates were divided into three groups. Virulence of 69.2% of the isolates to walnut fruits was higher than that to leaves; 15.4% of isolates had no

difference in pathogenicity, and the virulence to walnut leaves was higher for 15.4% of isolates. Tebuconazole, difenoconazole, flusilazole, and carbendazim inhibited the growth of fungal mycelia, and the concentration for 50% of maximal effect (EC₅₀) values were 0.4 to 20.5, 0.6 to 2.6, 0.2 to 1.6, and 0.002 to 0.2 µg/ml, respectively, with average values of 6.5 ± 6.9, 1.5 ± 0.6, 0.9 ± 0.4, and 0.1 ± 0.05 µg/ml, respectively. All isolates were more sensitive to difenoconazole, flusilazole, and carbendazim than tebuconazole ($P < 0.01$). Isolate sensitivities to the same fungicide were different. Isolates SL-31 and TS-09 were the least sensitive to carbendazim and tebuconazole, respectively, and the resistance ratios were 87.3 and 51.6, respectively. Sensitivities to difenoconazole and flusilazole were largely consistent among all isolates, and the resistance ratios were from 1 to 4.6 and from 1 to 7, respectively. Therefore, difenoconazole and flusilazole could be chosen for disease control. The differences of pathogenicity and fungicide sensitivity were not correlated with geographic regions. These results indicated that there was high intraspecific diversity of populations in *C. gloeosporioides* s.s. that caused walnut anthracnose. For effective management, the targeted control strategy should be implemented based on the different geographic regions.

Keywords: coelomycete, diversity, juglans, multilocus phylogeny, pathogenicity, resistance ratio

Walnut (*Juglans regia* L.) is an important woody nut and edible oil tree throughout the world. Walnut trees are planted largely for their nuts, which are mainly used for food and oil expression (Blomhoff et al. 2006; Cortés et al. 2006). Walnut production is threatened by several insects and diseases (Khan and Kundoo 2018; Tian et al. 1991; Wang and Tang 2018). Among these diseases, anthracnose disease caused by *Colletotrichum* spp. is a crucial constraint to walnut production in many walnut-planting areas in the world, and it often results in huge yield losses and even total losses in some walnut orchards (Da Lio et al. 2018; Wang et al. 2016). In Shandong province, China, walnut anthracnose occurred from late June to harvest.

The spots were about 5 to 10 mm in diameter in mid- to late July. Some spots were merged into large necrotic areas in the middle of August, and the fruits and leaves failed to develop normally or dropped prematurely. The fruits and leaves infected in August would shed in a large area before harvest (late August to early September) (Zhu et al. 2014).

The genus *Colletotrichum* Corda is one of the most important and destructive plant pathogens worldwide. From the perspective of scientific and economic significance, the genus *Colletotrichum* was considered as the eighth most important group of phytopathogenic fungi worldwide (Dean et al. 2012) and could attack >3,200 species of monocot and dicot plants (Manire et al. 2002; O'Connell et al. 2012), including many crops of cereals, fruits, vegetables, and ornamentals (Cannon et al. 2012; Damm et al. 2010; Honger et al. 2016; Huang et al. 2016). Several species of the genus *Colletotrichum* have been isolated from *J. regia*, such as *Colletotrichum gloeosporioides* sensu lato in Korea, Japan, New Zealand, and South Africa (Chen 2003; Cho and Shin 2004; Crous et al. 2000; Gadgil et al. 2005; Gorter 1977; Kobayashi 2007; Pennycook 1989); *Colletotrichum* sp. in Mexico (Alvarez 1976); *C. gloeosporioides* from walnut fruits in Slovenia, which caused spots on green walnut fruits (Juhászová et al. 2005); *Colletotrichum godetiae* in Australia (Damm et al. 2012); *Colletotrichum acutatum* in Australia (Simmonds 1966); and *C. acutatum* sensu lato (s.l.; geographic origin and pathogenicity were unknown) (Sreenivasaprasad and Talhinhas 2005). In France, *Colletotrichum fioriniae*, *Colletotrichum nymphaeae*, *C. godetiae*, and *C. gloeosporioides* s.l. were isolated from walnut leaves, nuts, and buds, and they were confirmed to be the pathogens of walnut

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Funding: This work was supported by National Key Research and Development Program of China grant 2017YFD0600104, Priority Academic Program Development of Jiangsu Higher Education Institutions grant PAPD, and Science and Technology Development Project in Shandong Province grant 2012GGB22004.

*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures and one supplementary table are published online.

The author(s) declare no conflict of interest.

Accepted for publication 20 December 2019.

anthracnose, which were identified by multigene phylogenetic analysis (Da Lio et al. 2018). In Brazil, *C. nymphaeae* caused walnut anthracnose (Savian et al. 2019). In China, *C. gloeosporioides* sensu lato (Chen 2003; Qu et al. 2011), *C. acutatum* (Liu et al. 1995), *C. fiorinae* (Zhu et al. 2015), *Colletotrichum siamense* (Wang et al. 2017), *Colletotrichum fructicola* (Wang et al. 2018), and *Colletotrichum viniferum* (He et al. 2019) were isolated from walnut. The pathogen of walnut anthracnose was not only *C. gloeosporioides* but also, *C. fructicola* and *C. siamense* (Wang et al. 2017, 2018) in China. *C. fiorinae* could infect walnut leaves, and the symptoms were similar to anthracnose (Zhu et al. 2015). *C. viniferum* only infected walnut leaves, and the symptoms were not typical (He et al. 2019).

There was a difference in pathogenicity of *Colletotrichum* isolates. Zhang et al. (2014a) considered that the pathogenicity of the genus *Colletotrichum* from different sources to the same litchi cultivar was different. The virulence of *C. siamense* to walnut fruits was not consistent with that to leaves (Wang et al. 2017). Chemical control methods are the most commonly used strategies against anthracnose. These reports showed that the *C. gloeosporioides* causing grape ripe rot was resistant to thiophanate-methyl and diethofencarb (Chen et al. 2013). *C. gloeosporioides* isolates from mango, litchi, longan, etc. in Hainan province showed high resistance to carbendazim (Zhang et al. 2014b). However, any differences in fungicide sensitivities of isolates causing walnut anthracnose remain unknown. There were abundant interspecies and intraspecific diversities of populations. The genetic diversity of grape anthracnose pathogens in Yunnan province was complex and inconsistent with geographic origins, host specificities, colony or spore morphologies, and pathogenicity to red globe grape (Deng et al. 2015). The diversity of populations of the isolates, which caused anthracnose in tea and other woody plants in Fujian province, was consistent with host specificities, not with geographic origins (Liu et al. 2017). The genetic differences of walnut anthracnose pathogens are largely unknown.

The objectives of this study were (i) to identify the species of the genus *Colletotrichum* causing walnut anthracnose using multilocus phylogenetic analyses and morphological characteristics, (ii) to determine the pathogenicity of the isolates obtained from walnut fruits

and leaves in China, and (iii) to examine the sensitivity of isolates to four fungicides (tebuconazole, difenoconazole, flusilazole, and carbendazim) using in vitro methods.

Materials and Methods

Plant materials, pathogen isolation, and purification. From 2013 to 2014, walnut fruits and leaves with anthracnose symptoms were collected from 13 walnut orchards in Henan, Hubei, Shaanxi, and Shandong provinces in China (Table 1).

Pure cultures were obtained from diseased walnut fruits and leaves as well as from single-spore isolation. The walnut fruits and leaves were surface sterilized with 70% ethanol, and margins of the healthy and diseased tissues were cut into 3- to 4-mm² segments. The segments were sterilized with 1% NaOCl for 60 s and 70% ethanol for 60 s, and they were washed in sterile distilled water three times. Five segments were placed on potato dextrose agar (PDA; 1.5%, Difco-BD Diagnostics) and incubated at 28°C. Seven days later, hyphae were transplanted to PDA plates for sporulation. Using serial dilution, monospore isolates were obtained (Choi et al. 1999; Than et al. 2008) and stored at 4°C on PDA slants. Most of the isolates were stored and preserved in the China Forestry Culture Collection Center (Table 2).

Morphological analysis. The isolates were cultured on PDA at 28°C for 5 days. The colony color, conidial masses, and zonation were observed and documented. A drop of sterile water was placed on a glass slide, and then, three acervuli were placed on the slide and gently crushed under a cover glass; a few drops of cedar oil were added under the cover glass to decrease water evaporation. Appressoria developed on the surface of the glass slide. Acervuli, conidia, appressoria, conidiophores, and conidiogenous cells were observed under a compound microscope (Nikon Eclipse 50i), and photomicrographs were taken with a high-resolution QImaging camera system (QImaging); the sizes of conidia and appressoria were measured by cellSens standard software (Olympus).

DNA extraction, amplification, sequencing, and phylogenetic analyses. Mycelia (growing for 5 days on PDA) were dried with sterile filter paper and collected in a sterile centrifuge tube. Mycelial powder was obtained by the trituration method in liquid nitrogen.

Table 1. The walnut anthracnose sample geographic distribution, walnut varieties, and damage degree

No.	Isolate	Orchard location	Longitude and latitude coordinate	Varieties	Damage degree ^z	Disease sample
1	SMX-42H	Nanlupo village, Cizhong town, Sanmenxia city, Henan province	N:34°44'07.7" E:111°18'07.4"	Cultivar Liaohe	++	Leaf
2	TS-09	Haojiazhuang village, Qiujiadian town, Taian city, Shandong province	N:36°10'01.1" E:117°14'53.3"	Cultivar Xiangling	++++	Fruit
3	SQ-141	Daoshanyu village, Shuangquan town, Jinan city, Shandong province	N:36°22'42.4" E:116°40'58.4"	Cultivar Xiangling	++++	Fruit
4	SL-31	Yanping village, Sanchahe town, Shangluo city, Shaanxi province	N:33°58'05.7" E:109°42'11.1"	Cultivar Liaohe	++	Fruit
5	ZZ-27	Shuitun village, Yicheng district, Zaozhuang city, Shandong province	N:34°46'25.8" E:117°39'19.9"	Cultivar Xiangling	+++	Fruit
6	SL-34	Kanghe village, Sanchahe town, Shangluo city, Shaanxi province	N:33°51'10.5" E:109°41'59.1"	Cultivar Liaohe	++	Fruit
7	YT-172	Zaoyuan village, Miaohou town, Qixia city, Shandong province	N:37°20'24.8" E:121°03'57.4"	Unknown	+++	Fruit
8	SL-30	Shanghe village, Chenyuan town, Shangluo city, Shaanxi province	N:33°25'32.5" E:109°08'47.3"	Cultivar Liaohe	+++	Fruit
9	SL-37	Chuanxing village, Yangxie town, Shangluo city, Shaanxi province	N:33°51'32" E:109°41'10.6"	Cultivar Liaohe	++	Fruit
10	LZ-02	Lizhuang village, Shuangquan town, Jinan city, Shandong province	N:36°24'56.8" E:116°40'58.4"	Cultivar Xiangling	+++	Fruit
11	ZQ-29	Duozhuang village, Zaoyuan town, Jinan city, Shandong province	N:36°45'14.0" E:117°26'36.4"	Cultivar Xiangling	+++	Fruit
12	ZZ-01	Xiaofeng village, Xincun town, Zhengzhou city, Henan province	N:34°22'43.6" E:113°43'10.9"	Cultivar Zhonglin	++	Fruit
13	HB-49	Sanxigou village, Chengguan town, Xiangyang city, Hubei province	N:31°52'02.1" E:111°15'04.0"	Cultivar Xiangling	+++	Fruit

^z ++, Incidence 5 to 10%; +++, incidence 10 to 30%; +++++, severe with >30% incidence (even total losses).

The mycelial powder was dissolved in cetyltrimethylammonium bromide (CTAB) buffer (2% [wt/vol] CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris HCl [pH 8.0], and 0.2% [wt/vol] β -mercaptoethanol) and then, incubated at 65°C for 30 min. Genomic DNAs of all isolates were extracted in conformity to the method described by Freeman et al. (1996). The PCR amplifications were conducted with the ribosomal internal transcribed spacer (ITS), actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and chitin synthase (CHS-1). Four loci were amplified: ribosomal DNA region using universal primers ITS1 and ITS4 for ITS (Gardes and Bruns 1993; White et al. 1990), GAPDH gene using primers GDF-1 and GDR1 (Templeton et al. 1992), CHS-1 gene using primers CHS-79F and CHS1-354R (Carbone and Kohn 1999), and ACT gene using primers ACT-512F and ACT-783R (Carbone and Kohn 1999). Information about primer sequences, amplicon size, and PCR conditions is listed in Table 3.

The PCR reaction system was (with the total reaction volume of 25 μ l) mixed with Taq DNA polymerase mix (12.5 μ l), primer-F (1.0 μ l), primer-R (1.0 μ l), genomic DNA (2.0 μ l), and ddH₂O (8.5 μ l). The PCR amplification procedure consisted of 36 cycles, and information about initial denaturation, denaturation, annealing, extension, and final extension are listed in Table 3. After PCR, all of the PCR amplification products were sequenced by Shanghai Personal Biotechnology Co., Ltd. All sequences have been deposited in GenBank (Table 2).

All ITS, GAPDH, ACT, and CHS-1 sequences were compared with the sequences of the already described species in the Q-Bank (www.q-bank.eu) by blasting, and the sequences of 46 *Colletotrichum* isolates were downloaded from GenBank (Table 2). *Colletotrichum boninense* MAFF 305972 was used as an outgroup, which was an ex-holotype culture that was isolated from *Crinum asiaticum* var. *sinicum*. The sequences of each locus were aligned

Table 2. Strains and sequences of the genus *Colletotrichum* used in this study

Species	Culture accession no.	Host	Country	GenBank accession numbers ^x			
				ITS	GAPDH	ACT	CHS-1
<i>Colletotrichum aenigma</i> ^y	ICMP 18608	<i>Persea americana</i>	Israel	JX010244	JX010044	JX009443	JX009774
<i>Colletotrichum alatae</i>	CBS 304.67	<i>Dioscorea alata</i>	India	JX010190	JX009990	JX009471	JX009837
<i>Colletotrichum aeshynomenes</i>	ICMP 17673	<i>Aeshynomene virginica</i>	U.S.A.	JX010176	JX009930	JX009483	JX009799
<i>Colletotrichum alienum</i>	ICMP 12071	<i>Malus domestica</i>	New Zealand	JX010251	JX010028	JX009572	JX009882
<i>Colletotrichum aotearoa</i>	ICMP 18537	<i>Coprosma</i> sp.	New Zealand	JX010205	JX010005	JX009564	JX009853
<i>Colletotrichum asianum</i>	ICMP 18580	<i>Coffea arabica</i>	Thailand	FJ972612	JX010053	JX009584	JX009867
<i>Colletotrichum boninense</i>	MAFF 305972	<i>Crinum asiaticum</i> var. <i>sinicum</i>	Japan	JX010292	JX009905	JX009583	JX009827
<i>Colletotrichum camelliae</i>	CGMCC 3.14925	<i>Camellia sinensis</i>	China	KJ955081	KJ954782	KJ954363	
<i>Colletotrichum chengpingense</i>	MFLUCC 15-0022	<i>Fragaria vesca</i>	China	KP683152	KP852469	KP683093	KP852449
<i>Colletotrichum clidemiae</i>	ICMP 18658	<i>Clidemia hirta</i>	U.S.A., Hawaii	JX010265	JX009989	JX009537	JX009877
<i>Colletotrichum conoides</i>	CAUG17	<i>Capsicum</i> spp.	China	KP890168	KP890162	KP890144	KP890156
<i>Colletotrichum cordylinicola</i>	ICMP 18579	<i>Cordyline fruticosa</i>	Thailand	JX010226	JX009975	HM470235	JX009864
<i>Colletotrichum endophytica</i>	MFLUCC 130418	<i>Pennisetum purpureum</i>	Thailand	KC633854	KC832854	KF306258	
<i>Colletotrichum fructicola</i>	CFCC 51553	<i>Juglans regia</i>	China	KX913950	KX913956	KX913947	KX913953
<i>Colletotrichum gloeosporioides</i>	ICMP 17821	<i>Citrus sinensis</i>	Italy	JX010152	JX010056	JX009531	JX009818
<i>Colletotrichum gloeosporioides</i>	ICMP 18738	<i>Carya illinoensis</i>	Australia	JX010151	JX009976	JX009542	JX009797
<i>Colletotrichum gloeosporioides</i>	ICMP 12939	<i>Citrus</i> sp.	New Zealand	JX010149	JX009931	JX009462	JX009747
<i>Colletotrichum gloeosporioides</i>	ICMP 12066	<i>Ficus</i> sp.	New Zealand	JX010158	JX009955	JX009550	JX009888
<i>Colletotrichum gloeosporioides</i>	ICMP 18730	<i>Citrus</i> sp.	New Zealand	JX010157	JX009981	JX009548	JX009861
<i>Colletotrichum gloeosporioides</i>	ICMP 12938	<i>Citrus sinensis</i>	New Zealand	JX010147	JX009935	JX009560	JX009746
<i>Colletotrichum gloeosporioides</i>	ICMP 18694	<i>Mangifera indica</i>	South Africa	JX010155	JX009980	JX009481	JX009796
<i>Colletotrichum gloeosporioides</i>	ICMP 18678	<i>Pueraria lobata</i>	U.S.A.	JX010150	JX010013	JX009502	JX009790
<i>Colletotrichum gloeosporioides</i>	ICMP 18695	<i>Citrus</i> sp.	U.S.A.	JX010153	JX009979	JX009494	JX009779
<i>Colletotrichum gloeosporioides</i>	ICMP 18697	<i>Vitis vinifera</i>	U.S.A.	JX010154	JX009987	JX009557	JX009780
<i>Colletotrichum gloeosporioides</i>	ICMP 19121	<i>Citrus limon</i>	Italy	JX010148	JX010054	JX009558	JX009903
<i>Colletotrichum gloeosporioides</i>	SMX-42H=CFCC 52827 ^z	<i>Juglans regia</i>	China	MF554884	MF554870	MF554844	MF554853
<i>Colletotrichum gloeosporioides</i>	TS-09 = CFCC 52838 ^z	<i>Juglans regia</i>	China	MF554878	MF554872	MF554846	MF554855
<i>Colletotrichum gloeosporioides</i>	SQ-141 = CFCC 52833 ^z	<i>Juglans regia</i>	China	MF554883	MF554871	MF554845	MF554854

(Continued on next page)

^x ACT, actin; CHS-1, chitin synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITS, internal transcribed spacer.

^y Ex-type strains or authentic cultures are in bold.

^z Strains collected in this study.

using MEGA 7.0, the gaps were considered as missing data, and the alignments were adjusted manually for maximum alignment and maximum sequence similarity. Four genes per region were concatenated in an order of “ITS-GAPDH-ACT-CHS-1” using Fasta alignment joiner software (http://users-birc.au.dk/palle/php/fabox/alignment_joiner.php). Phylogenetic analyses were performed with the concatenated sequence dataset using MEGA 7.0 software (Kumar et al. 2016), and two multilocus phylogenetic trees were generated with neighbor-joining method or maximum parsimony method. The phylogenetic trees were drawn to scale, and the optimal tree with the sum of branch length was measured according to the number of substitutions per site to evaluate the relative stability of the branches.

Pathogenicity and virulence in leaves and fruits. Pathogenicity experiments were conducted at the experimental station of the Research Center for the Prevention and Control of Invasive Forest Pests of Shandong Province. Healthy fruits (cultivar Xiangling, 1 month

old) and leaves (fully expanded) were inoculated in vitro with plugs ($\phi = 5$ mm) of isolates cultured for 5 days. Thirteen isolates were used (Table 2). In total, 280 fruits and 168 leaves were rinsed with running tap water for 60 s, surface sterilized with 70% ethanol, and rinsed with sterile distilled water three times. All of the fruits and leaves were punctured with a red-hot needle ($\phi = 0.5$ mm) on the surface. The leaf was inoculated with two hyphal plugs, and the fruit was inoculated with one hyphal plug; the needle-wounded leaves and fruits were inoculated with blank agar plug as a control. Each isolate was inoculated to 20 fruits and 12 leaves; an additional 20 fruits and 12 leaves were treated with blank agar plugs as controls. All treatments and controls were placed into sterile tissue culture bottles ($\phi = 92$ mm, $h = 274$ mm, $v = 690$ ml) containing two layers of wet paper towels to keep them moist at 28°C under a 12-h light/dark cycle. Anthracnose symptoms were evaluated at the seventh day after inoculation. The fungus was reisolated from symptomatic lesions using the aforementioned method. The morphological characters

Table 2. (Continued from previous page)

Species	Culture accession no.	Host	Country	GenBank accession numbers ^x			
				ITS	GAPDH	ACT	CHS-1
<i>Colletotrichum gloeosporioides</i>	SL-31 = CFCC 52830 ^z	<i>Juglans regia</i>	China	MF554879	MF554867	MF554840	MF554857
<i>Colletotrichum gloeosporioides</i>	ZZ-27 = CFCC 52837 ^z	<i>Juglans regia</i>	China	MF554887	MF554876	MF554850	MF554863
<i>Colletotrichum gloeosporioides</i>	SL-34 = CFCC 52831 ^z	<i>Juglans regia</i>	China	MF554890	MF554868	MF554842	MF554858
<i>Colletotrichum gloeosporioides</i>	YT-172 = CFCC 52836 ^z	<i>Juglans regia</i>	China	MF554889	MF554874	MF554848	MF554861
<i>Colletotrichum gloeosporioides</i>	SL-30 = CFCC 52829 ^c	<i>Juglans regia</i>	China	MF554880	MF554866	MF554841	MF554856
<i>Colletotrichum gloeosporioides</i>	SL-37 = CFCC 52832 ^z	<i>Juglans regia</i>	China	KY302642	KY302639	KY302631	KY302635
<i>Colletotrichum gloeosporioides</i>	LZ-02 = CFCC 52834 ^z	<i>Juglans regia</i>	China	MF554881	MF554865	MF554839	MF554852
<i>Colletotrichum gloeosporioides</i>	ZQ-29 = CFCC 52824 ^z	<i>Juglans regia</i>	China	KY302643	KY302638	KY302630	KY302634
<i>Colletotrichum gloeosporioides</i>	ZZ-01 ^z	<i>Juglans regia</i>	China	MF554886	MF554875	MF554849	MF554862
<i>Colletotrichum gloeosporioides</i>	HB-49 = CFCC 52826 ^z	<i>Juglans regia</i>	China	MF554882	MF554864	MF554838	MF554851
<i>Colletotrichum grevilleae</i>	CBS 132879	<i>Grevillea</i> sp.	Italy	KC297078	KC297010	KC296941	KC296987
<i>Colletotrichum grossum</i>	CAUG7	<i>Capsicum</i> spp.	China	KP890165	KP890159	KP890141	KP890153
<i>Colletotrichum hebeiense</i>	MFLUCC 13-0726	<i>Vitis vinifera</i>	China	KF156863	KF377495	KF377532	KF289008
<i>Colletotrichum henanense</i>	CGMCC 3.17354	<i>Camellia sinensis</i>	China	KJ955109	KJ954810		KM023257
<i>Colletotrichum horii</i>	NBRC7478	<i>Diospyros kaki</i>	Japan	GQ329690	GQ329681	JX009438	JX009752
<i>Colletotrichum jiangxiense</i>	CGMCC 3.17363	<i>Camellia sinensis</i>	China	KJ955201	KJ954902	KJ954471	
<i>Colletotrichum kahawae</i>	ICMP17816	<i>Coffea arabica</i>	Kenya	JX010231	JX010012	JX009452	JX009813
<i>Colletotrichum musae</i>	ICMP 116870	<i>Musa</i> sp.	U.S.A.	JX010146	JX010050	JX009433	JX009896
<i>Colletotrichum nupharicola</i>	CBS470.96	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	U.S.A.	JX010187	JX009972	JX009437	JX009835
<i>Colletotrichum proteae</i>	CBS 132882	<i>Protea</i> sp.	South Africa	KC297079	KC297009	KC296940	KC296986
<i>Colletotrichum psidii</i>	CBS 145.29	<i>Psidium</i> sp.	Italy	JX010219	JX009967	JX009515	JX009901
<i>Colletotrichum queenslandicum</i>	ICMP 1778	<i>Carica papaya</i>	Australia	JX010276	JX009934	JX009447	JX009899
<i>Colletotrichum salsolae</i>	ICMP 19051	<i>Salsola tragus</i>	Hungary	JX010242	JX009916	JX009562	JX009863
<i>Colletotrichum siamense</i>	CFCC 51940	<i>Juglans regia</i>	China	KY242352	KY242347	KY242336	KY242342
<i>Colletotrichum syzygicola</i>	MFLUCC 10-0624	<i>Syzygium samarangense</i>	Thailand	NR152278	KF242156	KF157801	
<i>Colletotrichum theobromicola</i>	CBS 124945	<i>Theobroma cacao</i>	Panama	JX010294	JX010006	JX009444	JX009869
<i>Colletotrichum ti</i>	ICMP 4832	<i>Cordyline</i> sp.	New Zealand	JX010269	JX009952	JX009520	JX009898
<i>Colletotrichum tropicale</i>	ICMP 18653	<i>Theobroma cacao</i>	Panama	JX010264	JX010007	JX009489	JX009870
<i>Colletotrichum viniferum</i>	GZAAS 5.08601	<i>Vitis vinifera</i> ‘Shuijing’	China	JN412804	JN412798	JN412795	
<i>Colletotrichum wuxiense</i>	CGMCC 3.17894	<i>Camellia sinensis</i>	China	KU251591	KU252045	KU251672	KU251939
<i>Colletotrichum xanthorrhoeae</i>	BRIP 45094	<i>Xanthorrhoea preissii</i>	Australia	JX010261	JX009927	JX009478	JX009823

of the reisolates, such as colony, acervuli, conidia, appressoria, conidiophores, and conidiogenous cells, were compared with those of the inoculated isolates to complete Koch's postulates.

The lesion sizes and areas on fruits or leaves were measured at the 10th day after inoculation to evaluate virulence. The virulence of the isolates was compared using one-way analysis of variance, and means were compared by Tukey's honestly significant difference (HSD) test at the 1% significance level using IBM SPSS Statistics 20 software.

In vitro sensitivity of isolates to four fungicides. The inhibitive activities of tebuconazole, difenoconazole, flusilazole, and carbendazim to the isolates were estimated using a mycelium growth rate method in the laboratory.

Tebuconazole (97% a.i.; provided by Jiangsu Changlong Agrochemical Co., Ltd.) and difenoconazole (95.8% a.i.; provided by Qingdao Hansen Biologic Science Co., Ltd.) were dissolved in acetone; flusilazole (92% a.i.) and carbendazim (97.5% a.i.; provided by Qingdao Hansen Biologic Science Co., Ltd.) were dissolved in cyclohexanone and 1% (wt/wt) hydrochloric acid, respectively. The concentration of all solutions was 10,000 $\mu\text{g/ml}$, and they were stored at 4°C.

Four fungicides were added to autoclaved PDA cooled to ca. 50°C. PDA media were amended to result in 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu\text{g/ml}$ a.i. of tebuconazole, flusilazole, or difenoconazole and 0.125, 0.25, 0.5, 1.0, and 2.0 $\mu\text{g/ml}$ a.i. of carbendazim. PDA medium without fungicide was used as control. Approximately 15 ml of PDA medium was decanted to each plate ($\phi = 90$ mm). The isolates were cultured on PDA without fungicides at 28°C for 5 days, and the hyphae

were cut into plugs ($\phi = 5$ mm) with a cork borer. The plugs were placed at the center of PDA plates.

Additionally, one plug per plate was cultured at 28°C for 3 days. Each treatment and control had four replicates, and the experiments were performed twice. The colony diameters were measured in a cross pattern. The original plug diameter was subtracted and mean of the colony diameters was calculated. All data were analyzed using IBM SPSS Statistics 20 software, and concentration for 50% of maximal effect (EC_{50}) values were estimated by the probit analysis. The frequency distribution of EC_{50} of the 13 isolates was plotted using PROC FREQ and tested for normality (Fan et al. 2016).

Results

Description of symptoms in the field. In major walnut production areas, walnut anthracnose disease mainly damaged leaves and fruits. When the leaves were infected, the spots were nearly round or irregular, water soaked, and brown to dark brown. The spots expanded along the veins and formed long strip lesions. In a later stage, the older lesions became necrotic and perforated, and this resulted in early defoliation (Fig. 1A). When the fruits were infected, some sub-circular or irregular spots appeared in the initial stage. The spots were water soaked, sunken, and brown to black (Fig. 1B). Some spots enlarged and merged into large necrotic areas. In the necrotic areas, some acervuli were in concentric rings and oozed gelatinous pink conidial masses (Fig. 1C). When the disease was serious, multiple spots merged and expanded to the whole fruit (Fig. 1D), and the pathogen reached the immature endocarp (nut shell). The fruits failed to

Fig. 1. Disease symptoms of walnut anthracnose on fruits and leaves of walnuts in the field. **a**, Disease symptoms on walnut leaves. **b to d**, Disease symptoms on walnut fruits. **e**, Disease symptoms on walnut kernels.

develop normally; most fruits dropped prematurely, and the kernels (nutmeat) were inedible (Fig. 1E). The degree of the severity of walnut pericarp was in substantial agreement with walnut kernels. With the walnut pericarp spot expansion, the risk of damage to walnut kernels increased. At the initial infection period, the spots were only in walnut pericarp, not in kernels, at this time, and the walnut kernels quality was not influenced by walnut anthracnose. When the walnut pericarp spot area was up to one-fourth of the whole fruit, the kernels were infected, and some spots were in kernels. At this point, the quality and quantity of the kernels were reduced (Fig. 2).

Isolate collection. The incidence was different in the 13 orchards, the highest incidence was in orchards 2 and 3, and the walnut variety was Xiangling (Table 1). A total of 59 isolates were obtained from diseased fruits and leaves. In orchard 7, the genera *Colletotrichum* and *Fusarium* were isolated. The genera *Colletotrichum* and *Alternaria*

were isolated in orchards 4, 6, and 8, and the genera *Colletotrichum* and *Xanthomonas* were in orchards 10 and 11. One *Colletotrichum* isolate was selected to represent an orchard (Table 1), and 13 isolates were preserved.

Morphological characteristics. On PDA, aerial mycelium was dense and cottony; the colonies were milky, pale gray to medium gray toward the center, in reverse, gray, and olivaceous gray to blue gray at the center, with sporadic dark gray flecks on the edge (Fig. 3A to C). Colonies were with the entire margin, and orange conidial masses were visible in acervuli. Sexual morph was absent. Asexual morph was present on PDA. Hyphae were hyaline to medium brown, smooth, septate, and branched. Acervuli were observed (Fig. 3D). Setae were not observed. Conidiophores were hyaline, smooth walled, and sometimes branched. Conidiogenous cells were hyaline, smooth, and cylindrical, often with a septum (Fig. 3G and H).

Fig. 2. The various degrees of the severity of inner and outer symptoms of walnut anthracnose on fruit. **A,** The various severity degrees of walnut anthracnose on pericarp. **B,** The corresponding various severity degrees on walnut kernels.

Fig. 3. The morphological characteristics of *Colletotrichum gloeosporioides* sensu stricto isolated from walnut anthracnose fruits on potato dextrose agar. **a to c,** Cultural characters. **a,** Isolate SQ-141. **b,** Isolate SL-31. **c,** Isolate LZ-02. **d,** Acervulus. Scale bar = 20 μ m. **e,** Conidia. Scale bar = 10 μ m. **f,** Appressoria. Scale bar = 10 μ m. **g,** Conidiophores and conidiogenous cells. Scale bar = 10 μ m. **h,** Acervulus, conidiophores, and conidiogenous cells. Scale bar = 10 μ m.

Conidia were straight and mostly cylindrical with broadly rounded ends that were sometimes slightly and gradually acute to the end (Fig. 3E). The size was $(14.8 - 16.7 - 17.3 - 19.5) \times (4.3 - 5.0 - 5.3 - 6.1) \mu\text{m}$ ($\bar{x} = 16.9 \pm 1.0 \times 5.2 \pm 0.4$, $n = 51$), and $L/W = 3.3$. Appressoria were simple or in a small group, subglobose or ellipsoid, and rarely irregular (Fig. 3F). The size was $(5.2 - 6.7 - 7.6 - 8.9) \times (2.7 - 5.0 - 5.9 - 7.1) \mu\text{m}$ ($\bar{x} = 7.2 \pm 1.1 \times 5.4 \pm 1.0$, $n = 26$), and

$L/W = 1.3$. Consequently, the isolates were identified as *C. gloeosporioides*.

Phylogenetic analyses. Using the Blast option in the Q-Bank, all ITS, GAPDH, ACT, and CHS-1 sequences were compared with the sequences of the known species.

Compared with the sequences of *C. gloeosporioides* sensu stricto (s.s.) isolates ICMP 17821 and ICMP 12939, the similarity of ITS

Fig. 4. Phylogenetic tree of isolates of walnut anthracnose, *Colletotrichum gloeosporioides* sensu stricto, with allied taxa calculated with concatenated sequence data of internal transcribed spacer, glyceraldehyde-3-phosphate dehydrogenase, actin, and chitin synthase using maximum parsimony method (1,000 bootstrap replicates; bootstrap values indicated at nodes). *Colletotrichum boninense* MAFF 305972 is the outgroup. The scale bar indicates the number of expected changes per site. The isolates in bold are ex-types.

was from 99.3 to 100%; the CHS-1 sequences of all isolates showed 99.2 to 100% similarity. The similarity of GAPDH was from 89.9 to 98.6%, and the ACT sequences of isolates SL-30, LZ-02, SQ-141, ZZ-27, YT-172, SL-31, HB-49, SL-37, SL-34, SMX-42H, and ZQ-29 showed 100 and 99.6% similarity with *C. gloeosporioides* s.s. isolates ICMP 17821 and ICMP 12939, respectively (Supplementary Table S1).

To compare with the sequences of 46 isolates downloaded from GenBank, ITS, GAPDH, ACT, and CHS-1 of all isolates were concatenated with the Fasta alignment joiner software. The aligned sequence was from 98.0 to 99.5% homologous to ex-type *C. gloeosporioides* s.s. ICMP 17821. The multilocus phylogenetic trees were generated using neighbor-joining and maximum parsimony methods. There were a total of 1,061 sites in the final dataset, and conserved sites, variable sites, parsimony informative sites, and singleton sites were 715, 312, 163, and 140, respectively. In the maximum parsimony phylogenetic tree, the tree length was 465, the consistency index was 0.56, and the retention index was 0.84. The composite indices were 0.57 for all sites and 0.47 for parsimony-informative sites. The isolates tested were in the same cluster with *C. gloeosporioides* s.s. with 98% bootstrap support (Fig. 4). In the neighbor-joining phylogenetic tree, the sum of the branch length was 0.57. All 13 isolates studied were clustered with *C. gloeosporioides* s.s., including its ex-type with 100% bootstrap support (Supplementary Fig. S1).

The multilocus phylogenetic analyses of four genes as well as morphological characters, including colony, conidia, appressoria, conidiophores, and conidiogenous cells, demonstrated that all of the isolates were *C. gloeosporioides* s.s.

Pathogenicity and virulence to leaves and fruits. Walnut fruits and leaves were inoculated with 13 isolates. The infection occurred at the inoculation sites on leaves or fruits (Supplementary Fig. S2A

to M), and symptoms resembled the anthracnose disease symptoms in the field (Fig. 1). The fruits and leaves in the controls had no symptoms 7 days after inoculation with blank agar plugs (Supplementary Fig. S2N). The typical symptom appeared as a roughly circular or irregular spot, and the infections were water soaked, sunken, and brown to black. The black lesions expanded and turned into necrotic areas. The fungus was reisolated from lesions using the aforementioned methods. Koch's postulates were completed, and the results indicated that all 13 isolates obtained were the walnut anthracnose pathogen.

There were significant differences ($P < 0.01$) among the 13 isolates that caused anthracnose disease on walnut leaves or fruits. The lesion areas on leaves were from 0.44 ± 0.03 to 1.42 ± 0.21 cm². Isolate ZQ-29 showed the highest virulence, whereas isolate YT-172 was the weakest in pathogenicity to walnut leaves. At the $P < 0.01$ level, all 13 isolates were divided into seven groups (Fig. 5). Based on the fruit lesion size, the isolate SQ-141 was the most virulent, and the lesion size was $2.53 \pm 0.25 \times 1.97 \pm 0.25$ cm. Isolates LZ-02, SL-34, and HB-49 showed the weakest virulence, and the lesion sizes were $0.73 \pm 0.05 \times 0.63 \pm 0.05$, $0.77 \pm 0.11 \times 0.67 \pm 0.05$, and $0.77 \pm 0.05 \times 0.67 \pm 0.05$ cm, respectively. There were no significant differences ($P < 0.01$). The 13 isolates were distributed into five groups (Fig. 5).

The disease lesion on leaves and fruits inoculated with the same isolate showed considerable differences. The 13 isolates were divided into three groups by comparing the virulence to walnut leaves and fruits. In group 1, the virulence to walnut fruits was higher than that to leaves, and nine isolates (69.2%) were in group 1. In group 2, there was no difference in pathogenicity, including isolates LZ-02 and ZQ-29. In group 3, the virulence of isolates to walnut leaves was higher, and isolates ZZ-01 and HB-49 were clustered in group 3.

Fig. 5. Results of inoculations with *Colletotrichum gloeosporioides* sensu stricto (s.s.): mean fruit lesion size (centimeters) and leaves lesion area (centimeters²) caused by *C. gloeosporioides* s.s. isolates associated with walnut anthracnose. Bars above columns are the standard errors. Bars with the same pattern and letter (symbols) do not show significant differences according to Tukey's honestly significant difference (HSD) test ($P < 0.01$).

In vitro sensitivity of *C. gloeosporioides* s.s. isolates to four fungicides. Four fungicides inhibited the growth of fungal mycelium. Tebuconazole, difenoconazole, flusilazole, and carbendazim EC₅₀ values were 6.5 ± 6.9, 1.5 ± 0.6, 0.9 ± 0.4, and 0.1 ± 0.05 µg/ml, respectively (Table 4). *C. gloeosporioides* s.s. was more sensitive to difenoconazole, flusilazole, and carbendazim than to tebuconazole ($P < 0.01$). The toxicities of carbendazim, flusilazole, and difenoconazole to *C. gloeosporioides* s.s. were not different ($P < 0.01$).

The result confirmed that the resistance ratios of all of the isolates to four fungicides were different (Fig. 6). The sensitivities to carbendazim and tebuconazole were significantly different among all isolates. The resistance ratios to carbendazim were from 1 to 87.33; isolate HB-49 was the most sensitive one, whereas isolate SL-31 was the least sensitive to carbendazim. The resistance ratios to tebuconazole were from 1 to 51.59. Isolate SL-31 was the most sensitive one, and isolate TS-09 was the least sensitive one of all isolates. The sensitivities to difenoconazole and flusilazole were largely consistent among all isolates, and the resistance ratios were from 1 to 4.55 and 7, respectively.

There was a significant difference in resistance ratio of carbendazim among the isolates. Therefore, difenoconazole and flusilazole could be used for disease control.

Discussion

Tode was the first one to observe the genus *Colletotrichum* in 1790, and Corda (1831) legitimately described it in 1831. Since 2009, Cai et al. (2009) have proposed a *Colletotrichum* systematics approach based on a multigene phylogeny, comparing the isolates under study with type specimens. A clearly defined phylogenetic lineage should be combined with decipherable polyphasic characters, including morphology, physiology, pathogenicity, cultural characteristics, and secondary metabolites. The taxonomy of the genus *Colletotrichum* has been significantly changed, and it has gone through numerous revisions, with the addition, typification, and synonymy of many species. Some new species were introduced; some species were segregated, and some species were abolished. As of 2019, there are 213 accepted species in the genus *Colletotrichum* (Dar et al. 2018; de Silva et al. 2019; Douanla-Meli and Unger 2017; Fu et al. 2019; Gan et al. 2019; Jayawardena et al. 2016, 2017; Lee and Jung 2018; Marin-Felix et al. 2017; Sharma et al. 2017; Sun et al. 2019; Xu et al. 2018). Most members of the genus *Colletotrichum* are important plant pathogens. *Colletotrichum* spp. may cause walnut anthracnose. Before 2015, the pathogen was identified as *C. gloeosporioides* based on morphologic characters or ITS data analysis in China (Qu et al. 2011; Xiao et al. 2010; Zhu et al. 2014). *C. gloeosporioides* is a species complex and includes 38 closely related species (Jayawardena et al. 2016; Marin-Felix et al. 2017; Weir et al. 2012). These reports failed to determine the species to which the pathogens belonged in *C. gloeosporioides* s.l. Subtle differences in colony morphology exist among the sibling species, whereas the colonies of different isolates of the same species are morphologically different. Different *Colletotrichum* species could infect the same host, and the same species could infect different hosts. Several mycologists who worked on *Colletotrichum* taxonomy opined that the taxonomy system of the genus *Colletotrichum* based on morphological characteristics, host range, and ITS data was confusing and that

identification results were problematic (Than et al. 2008; Weir et al. 2012; Yang et al. 2009). Proper identification of the pathogen is the basis for developing effective management strategies. Therefore, it is very important to accurately distinguish the species from each other. In this paper, by using multigene phylogeny, morphologic characters, and cultural properties, we confirmed that *C. gloeosporioides* s.s. was one of the species of the genus *Colletotrichum* responsible for walnut anthracnose in China.

Several *Colletotrichum* species have been associated with walnut anthracnose, such as *C. siamense*, *C. fruticola*, *C. fioriniae*, *C. gloeosporioides* s.s., *C. nymphaeae*, *C. godetiae*, and *C. gloeosporioides* s.l. (Da Lio et al. 2018; Savian et al. 2019; Wang et al. 2017, 2018; Zhu et al. 2015). It is safe to state that walnut anthracnose is a complex disease. With more and more walnut anthracnose samples collected from more walnut orchards in wider geographic areas in China, it is possible that more *Colletotrichum* species or new species responsible for walnut anthracnose will be determined or described. Da Lio et al. (2018) obtained *C. fioriniae* and *C. godetiae* from nuts and buds in the same orchard, and they all caused walnut anthracnose. Multiple *Colletotrichum* species can occur in the same orchard and from different walnut isolation sites. Whether multiple species may occur in the same walnut tissues remains

Table 4. Toxicity of four fungicides to *Colletotrichum gloeosporioides* sensu stricto

Fungicides	EC ₅₀ ^a (µg/ml)	95% CI ^b (µg/ml)
Tebuconazole	6.5 ± 6.9 A ^c	2.3–10.6
Difenoconazole	1.5 ± 0.6 B	1.1–1.8
Flusilazole	0.9 ± 0.4 B	0.6–1.1
Carbendazim	0.1 ± 0.05 B	0.05–0.11

^a EC₅₀: concentration for 50% of maximal effect.

^b 95% CI.

^c Means having a common letter in the same column are not significantly different according to Tukey's honestly significant difference (HSD) test ($P < 0.01$).

Fig. 6. The resistance ratio of *Colletotrichum gloeosporioides* sensu stricto isolates to four fungicides: carbendazim, flusilazole, difenoconazole, and tebuconazole.

Table 3. Primers used in this study with sequence, amplicon size, sources, and PCR conditions

Gene	Primer	Sequence	Size (bp)	Initial denaturation		Denaturation		Annealing		Extension		Final extension		Reference
				°C	min	°C	min	°C	min	°C	min	°C	min	
ITS ²	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	500	95	5	94	0.5	58	0.5	72	1.5	72	7	Gardes and Bruns 1993; White et al. 1990
ITS	ITS4	5'-TCCTCCGCTTATTGATATGC-3'												
ACT	ACT-512F	5'-ATGTGCAAGGCCGGTTTCGC-3'	300	95	5	94	0.5	59	0.5	72	1.5	72	7	Carbone and Kohn 1999
ACT	ACT-783R	5'-TACGAGTCCTTCTGGCCCAT-3'												
GAPDH	GDF-1	5'-GCCGTCACGACCCCTTCATTGA-3'	300	95	5	94	0.5	56	0.5	72	1.5	72	7	Templeton et al. 1992
GAPDH	GDR1	5'-GGGTGGAGTCGTACTTGAGCATGT-3'												
CHS-1	CHS1-79F	5'-TGGGGCAAGGATGCTTGGGAAGAAG-3'	300	95	5	94	0.5	56	0.5	72	1.5	72	7	Carbone and Kohn 1999
CH-1	CHS1-354R	5'-TGGGAAGAACATCTGTGAGAGTTG-3'												

² ACT, actin; CHS-1, chitin synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITS, internal transcribed spacer.

unclear, and additional studies are necessary in the future. It is necessary to conduct further studies on infection time; infecting walnut independently or collaboratively; and disease occurrence patterns of *C. siamense*, *C. fruticola*, and *C. gloeosporioides* s.s.

There was a high variability of virulence among the isolates of the same species (*C. siamense*, *C. godetiae*, and *C. fioriniae*), which caused walnut anthracnose (Da Lio et al. 2018; Wang et al. 2017). In this paper, virulence varied among isolates of *C. gloeosporioides* s.s. based on inoculation on walnut leaves or fruits. However, the virulence of the isolates to walnut fruits was different between *C. gloeosporioides* s.s. and *C. siamense*. The virulence of *C. siamense* isolates to walnut fruits was similar, whereas the virulence of *C. gloeosporioides* s.s. isolates was significantly different. Even if the isolates were from the same geographic origin, the virulence of the isolates to walnut leaves or fruits showed a great discrepancy, and it appeared that the virulence differences were not correlated with geographic origins. Compared with the virulence to walnut leaves and fruits, *C. gloeosporioides* s.s. and *C. siamense* were more aggressive to walnut fruits than leaves. From the perspective of pathogen characters, the results probably explained why walnut anthracnose caused serious damage in some orchards, whereas much less damage occurred in other orchards. However, the results need be further confirmed by artificially inoculating living walnut trees in the fields. The virulence of isolates from different tissues in the same orchards remains unknown and needs further study.

For controlling walnut anthracnose, the main management strategy was to rely mainly on timely application of fungicides. We determined the sensitivities of *C. gloeosporioides* s.s. isolates to fungicides, including difenoconazole, tebuconazole, flusilazole, and carbendazim, in this paper. Although the number of isolates was relatively restricted, the results still showed that there were differences in resistance ratio to carbendazim and tebuconazole among all isolates. The resistance ratios to carbendazim and tebuconazole were from 1 to 87.3 and from 1 to 51.6, respectively. Isolates SL-31, SL-34, SL-30, and SL-37 were from Shangluo county, Shaanxi province, and the resistance ratios to tebuconazole were 1, 6, 16.2, and 38.9, respectively. Carbendazim and tebuconazole were extensively used to control walnut anthracnose disease, and the differences of sensitivities were perhaps consistent with frequency of the carbendazim or tebuconazole usage locally. The sensitivities of *C. gloeosporioides* and *C. acutatum* to carbendazim were different (Cao et al. 2017). Meanwhile, our study indicated that isolates of *C. gloeosporioides* s.s. displayed differential sensitivities to carbendazim. The results reflected the sensitivities of *C. gloeosporioides* s.s. causing walnut anthracnose to fungicides to some extent. To increase the control effect, it is important for practitioners to ascertain the isolate sensitivities to fungicides. Based on the EC₅₀ values and resistance ratio, difenoconazole and flusilazole would be expected to provide better control than carbendazim and tebuconazole in a given plantation. However, the efficacies need to be verified with more field trials in the future. For controlling walnut anthracnose effectively, it is imperative to identify the pathogen accurately. Meanwhile, it is also necessary to determine fungicide sensitivities of the different isolates of the same fungal taxon. Fungicides should be cautiously chosen for the disease management, and fungicides with different mechanisms should be used in rotation.

In conclusion, the 13 isolates from the orchards were *C. gloeosporioides* s.s. All of the isolates had been confirmed to be the pathogen of walnut anthracnose in China. The isolates from different geographic regions showed differences in virulence and fungicide sensitivities. The results preliminarily indicated that there was a high intraspecific diversity among the populations of *C. gloeosporioides* s.s. The study provided crucial information for managing this disease in China. For example, the targeted control strategy should be implemented in accordance with the different geographic regions to reduce the losses caused by *C. gloeosporioides* s.s.

Acknowledgments

We are grateful to Li-ying Chen, Hua-bing Ma, Xing-hong Liu, Chun-hua Duan, Yin Liu, Jing Wang, and Qun Zhu for their assistance in sample collections,

and we express our appreciation to Dr. James LaMondia (the Connecticut Agricultural Experiment Station, United States) for reviewing the revised manuscript.

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