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Research Article

Characterization of *Colletotrichum siamense* causing crown rot of strawberry in Jingzhou, Hubei Province

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Abstract

Crown rot (*Colletotrichum siamense*) of strawberry is a severe disease in Jingzhou, Hubei Province, China. Pathogen identification is the basis for disease prevention and resistance breeding. Nearly fifty isolates were achieved from 192 crown rot samples in this study. A number of 21 isolates were characterized as the pathogen of crown rot of strawberry by Koch's postulates, and they were identified as *Colletotrichum* spp. by conidia morphology observation. The 21 isolates were divided into three groups based on colony morphology, and SCR-7, SCR-11 and SCR-16 belonged to each group were clustered into one group with *C. siamense* isolates by phylogenetic analysis of actin, β-Tubulin 2 and calmodulin sequences. In addition, the isolates of *C. siamense* SCR-7 showed the strongest pathogenicity of the three which caused highest values of lesion length and leaf temperature, and lowest leaf water content, photosynthetic rate, stomatal conductivity and even transpiration rate values. This study contributes to updating the *Colletotrichum* species associated with strawberry of China.

Keywords: anthracnose; crown rot; *Colletotrichum siamense*; phylogenetic; pathogenicity

Introduction

Strawberry (*Fragaria × ananassa* Duch.) is an important perennial herbaceous berry in China, which achieved 120 thousand hectares cultivation area and more than 3.060 million tons (National Bureau of Statistics, 2019). Crown rot is a serious disease, which threaded strawberry cultivation worldwide (Noh *et al.*, 2018; Zhang *et al.*, 2020). The pathogen of crown rot is complex, which include *Colletotrichum*, *Phytophthora*, *Neopetalotiopsis* and many other fungi (Noh *et al.*, 2018; Park *et al.*, 2019; Zhang *et al.*, 2020).

The incidence of strawberry crown rot was as high as 45.4% in 2012 in Hubei Province (Han *et al.*, 2014), and the damage of this disease is becoming more serious in recent years. Crown rot leads to the collapse of the drainage tissue, and the aboveground part wilts, and then quickly withers, and finally the aboveground parts die in summer and autumn with hot and rainy (Xie *et al.*, 2010; Jayawardena *et al.*, 2016). Identifying the pathogen and taking pathogenicity assays are important for control of strawberry crown rot. *C. siamense* was identified as the pathogen of strawberry crown rot in Wuhan (Han *et al.*, 2014), but the pathogen had not been isolated and identified in Jingzhou City, which is located 200 kilometres away from Wuhan. Whether the pathogen was the same as Wuhan or mixed infection by other pathogens? Whether the pathogenicity of pathogen was different?

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In this study, a combination of morphological and phylogenetic analysis was used to identify the pathogen causing strawberry crown rot. Mycelial growth rate on PDA plate of each isolate, the lesion size on crown of each isolate treatment, the relative water content, photosynthetic rates, stomatal conductivity, transpiration rates, leaf temperature, and intercellular CO_2 concentration of leaf with isolate treatment were measured for pathogenicity assays. This study clarified the pathogen types of strawberry crown rot disease in Jingzhou, Hubei Province, China and compared isolate pathogenicity, which would provide a theoretical basis for the disease control and strawberry disease resistance breeding.

Materials and Methods

Pathogen isolation

One hundred and ninety-two crown samples were achieved from crown root rot with wilting 'Benihoppe' strawberry seedlings in Jingzhou, Hubei Province, China. The samples of adjacent tissues of rot and health were cut into 0.5 cm × 0.5 cm pieces and placed into 0.1% aqueous mercuric chloride for 1 min, and then washed 3 times with sterile water for 1 min each time. The diseased tissues were placed on potato dextrose agar (PDA) plate with 28 °C under continuous light for 3 days. After that, a small amount of hyphae was picked from the edge of the grown colony to inoculate a new PDA plate for 3 days (Han *et al.*, 2014). Nine healthy and consistent one-year old 'Benihoppe' strawberry seedlings were inoculated for each isolate. The seedlings were cultivated in three pots, and three seedlings in each pot. The seedlings of one pot were taken as one biological replicate, so three biological replicates were used for pathogen identification. The crowns were surface-sterilized with 70% alcohol for 10 s, and then rinsed three times with sterile water. The crowns were wounded using a sterilized toothpick, a single mycelial plug (4 mm in diameter) of isolates was placed onto the wound, and a plug of sterile PDA medium was placed onto the wound as the controls (Wang *et al.*, 2011). The symptoms of each isolate were observed after 1 week after inoculation, the seedlings which appeared wilting and crown root rot same as previous host were isolated again. At last, fifteen isolates were verified as the pathogen of strawberry in Jingzhou City.

Morphology identification of pathogens

Fifteen isolates were inoculated on PDA medium and incubated at 28 °C for 10 days, and then the colony morphology was observed. A small amount of conidia from the pile of conidia on the PDA plate was picked using a sterile toothpick, suspended in sterile water, and then the morphology of the pathogenic fungal conidia was observed via an optical microscope (Leica M125., Germany) and the length and width of 20 conidia were measured. Three types of isolates with different colony morphology were chose from fifteen isolates. Holes were punched from edge of the three isolates colony using a 0.5 cm hole puncher, and the hyphae blocks were inoculated in the center of the PDA plate. The PDA plate was placed in a constant temperature incubator at 18 °C for 4 days in the dark, and then colony diameter was measured. The pathogens were characterized as Colletotrichum based on the taxonomic criteria of morphological characteristics (colony characters, conidial and setal morphology and production of a teleomorph) according to that described by Gunnell and Gubler (1992).

Molecular identification of pathogens

The hyphae of three isolates were quick-frozen with liquid nitrogen, and ground into powder and taken as template for PCR amplification directly. The PCR amplification of actin (*ACT*), beta-tubulin 2 (*TUB2*) and calmodulin (*CAL*) of three isolates were carried out, respectively. The primers and PCR amplification program was following the method of Han *et al.* (2014). The annealing temperature of PCR amplification of three genes was different. The PCR annealing temperatures were 59 °C, 55 °C and 58 °C for *CAL*, *TUB2* and *ACT*, respectively (Han *et al.,* 2014). After detection for agarose gel electrophoresis, the PCR products were

sequenced by Qingke Biotechnology Co., Ltd. using PCR amplification primers corresponding to each gene, after that the sequence were uploaded to the GenBank database (Table 1).

Taking the corresponding gene sequences of *C. hippeastri* and *C. boninense* as the outgroup (Damm *et al.*, 2012), MEGA 4.0 software was used to perform phylogenetic evolution analysis based on the *CAL*, *TUB2*, and *ACT* gene sequences of each strain (Tamura *et al.*, 2007; Liu *et al.*, 2013). The neighbour-joining (NJ) method was used to construct phylogenetic tree, repeat 1,000 times. Bootstrap value of each branch in the NJ phylogenetic tree was calculated.

Table 1. Strains and sequence used in this study

Species	Strain	GenBank accession number		
		ACT	*TUB2*	*CAL*
Uncharacteristic	SCR-7	MZ436924	MZ436927	MZ436930
	SCR-11	MZ436925	MZ436928	MZ436931
	SCR-16	MZ436926	MZ436929	MZ436932
Colletotrichum aenigma	ICMP18608	JX009443	JX010389	JX009683
C. aeschynomenes	ATCC201874	JX009483	JX010392	JX009721
C. alatae	ICMP17919	JX009471	JX010383	JX009738
C. alienum	ICMP18691	JX009580	JX010385	JX009664
C. aotearoa	ICMP18532	JX009544	JX010421	JX009614
C. asianum	ICMP18696	JX009576	JX010384	JX009723
C. boninense	ICMP10338	JQ005507	JQ005593	JQ005680
C. clidemiae	ICMP18706	JX009476	JX010439	JX009639
C. cordylinicola	ICMP18579	HM470235	JX010440	HM470238
C. fructicola	ICMP18613	JX009491	JX010388	JX009675
C. gloeosporioides	ICMP17821	JX009531	JX010445	JX009731
C. hippeastri	CBS125377	JQ005753	JQ005580	JQ005666
C. horii	ICMP12942	JX009533	JX010375	JX009603
C. kahawae	ICMP18539	JX009523	JX010434	JX009635
C. musae	ICMP17817	JX009432	JX010395	JX009689
C. nupharicola	ICMP17938	JX009486	JX010397	JX009661
C. psidii	ICMP19120	JX009515	JX010443	JX009743
C. queenslandicum	ICMP1778	JX009447	JX010414	JX009691
C. salsolae	ICMP19051	JX009562	JX010403	JX009696
C. theobromicola	ICMP17958	JX009498	JX010381	JX009598
C. ti	ICMP5285	JX009553	JX010441	JX009650
C. tropicale	ICMP18672	JX009480	JX010396	JX009722
C. xanthorrhoeae	ICMP17903	JX009478	JX010448	JX009653
Glomerella cingulata	ICMP18542	JX009488	JX010429	JX009628
C. siamense	ICMP12567	JX009541	JX010387	JX009697
	ICMP17795	JX009506	JX010393	JX009703
	ICMP18121	JX009460	JX010402	JX009715
	CBS112983	KC296929	KC297100	KC296961
	CBS113199	KC296930	KC297090	KC296962
	Zhd-3	KF110981	KF055337	KF110984
	Zhd-4-1	KF110982	KF055338	KF110985
	Zhd-5	KF110983	KF055339	KF110986

Pathogenicity assays

The plant materials in a single factor experiment were used for pathogenicity assays. Seventy-two one-year old healthy and consistent 'Benihoppe' strawberry seedlings, grown in twenty-four pots with three in one

pot, were divided into four groups equally for inoculation with *C. siamense* SCR-7, SCR-11, SCR-16 and nontoxic medium (control) as previous. Three seedlings grown in each pot were achieved mixed and taken as one biological replicate, thus six biological replicates were used for pathogenicity assays. After 1 week seedlings inoculation of each isolate (inoculated on PDA medium and incubated at 28 °C for 1 week), the lesion size was measured. Fourth fully expanded top leaf was used for leaf gas exchange including photosynthetic rates (Pn), stomatal conductivity (gs), transpiration rates (E), leaf temperature, and intercellular CO_2 concentration (Ci) was determined using a Li-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, USA) from 9:30 am to 10:30 am following the method of Huang *et al.* (2020). Relative water content (RWC) of the fourth fully expanded top leaf was estimated with the following formula (Zou *et al.,* 2015) after photosynthetic parameters measurement: RWC (%) = (FW–DW)/(SW–DW) × 100, where FW stands for fresh weight, DW for dry weigth at 75 °C for 48 h, and SW for saturated weight after leaf rehydration for 24 h.

Statistical analysis

All the data were analysed with analysis of variation (SAS, v 8.1, Cary, USA), and significant differences between treatments were compared by Duncan's multiple range tests at P = 0.05 level.

Results

Isolation of fungi

Fifty isolates were achieved from one hundred and ninety-two crown samples which exhibited typical rots symptoms (Figure 1). Twenty-one isolates were confirmed as pathogen of strawberry crown rot by Koch's postulates. On PDA, the colony colour of twenty-one showed three different types, grey colonies on the front and red wheel in back; white colonies with red rings on the front and yellow wheel in back; grey colonies on the front and black speck in back (Figure 2a; 2b). The conidia morphology of three isolates with different colonial morphologies was nearly the same. Most of the conidia were narrowly obovate, straight or occasionally slightly curved and with obtuse ends (means ± SE, length 17.12±0.12μm; width 4.05±0.32μm) (Figure 2c). The pathogens were characteristic as *Colletotrichum* based on taxonomic criteria of morphological characteristics.

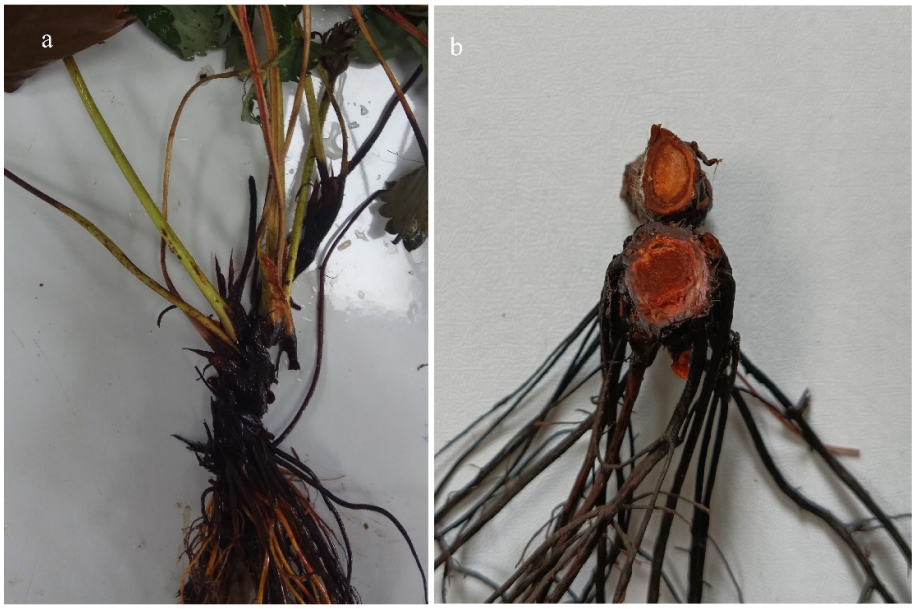

Figure 1. Symptoms of strawberry crown rot and root rot

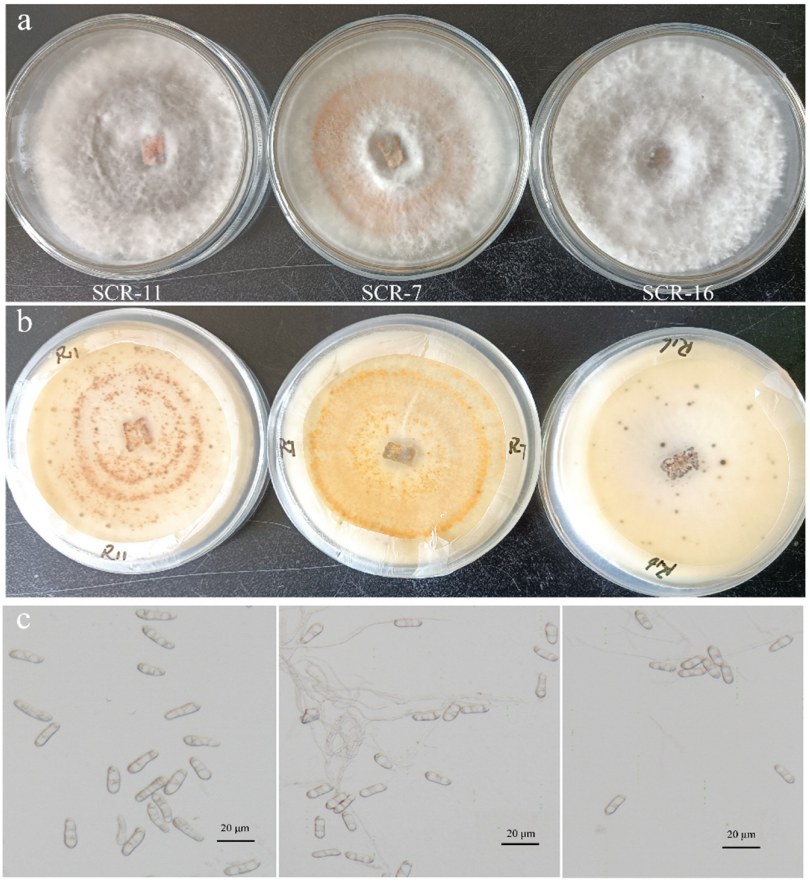

Figure 2. The characteristics of biological morphology
Figure 2a and 2b showed the colony morphology of SCR-7, SCR-11, and SCR-16 on potato dextrose agar after 10 days of incubation in 28 °C from above and below, respectively; Figure 2c showed conidia under optical microscope.

Phylogenetic analysis

CAL, *ACT* and *TUB2* sequence data were achieved from three isolates with different colonial morphology, respectively. The sequence data of our isolates of *Colletotrichum* and recent publications on *Colletotrichum* strains were used for phylogenetic tree construction for pathogen characteristic and phylogenetic analysis. The combined gene alignment of *CAL*, *ACT* and *TUB2* for three isolates supported those pathogens were *C. siamense* named *C. siamense* strain SCR-11, *C. siamense* strain SCR-7 and *C. siamense* strain SCR-16. Three groups of *C. siamense* were shown in phylogenetic tree. Our isolates were clustered together in group I, and three isolates were clustered with *C. siamense* strain ICMP:12567 and *C. siamense* strain Zhd-4-1 (Figure 3).

Pathogenicity studies

The inoculated strawberry seedlings began wilting after 10 d of inoculation and showed growth retardation. Necrosis was observed in crown of the wilted plants. The longitudinal section of the crown showed red brown. The lesion length ranged 0.6 to 1.2 cm, and the *C. siamense* SCR-7 grew faster significantly than other isolates on 2 weeks after inoculation. However, the lesion width caused by the three isolates showed no difference (Figure 4).

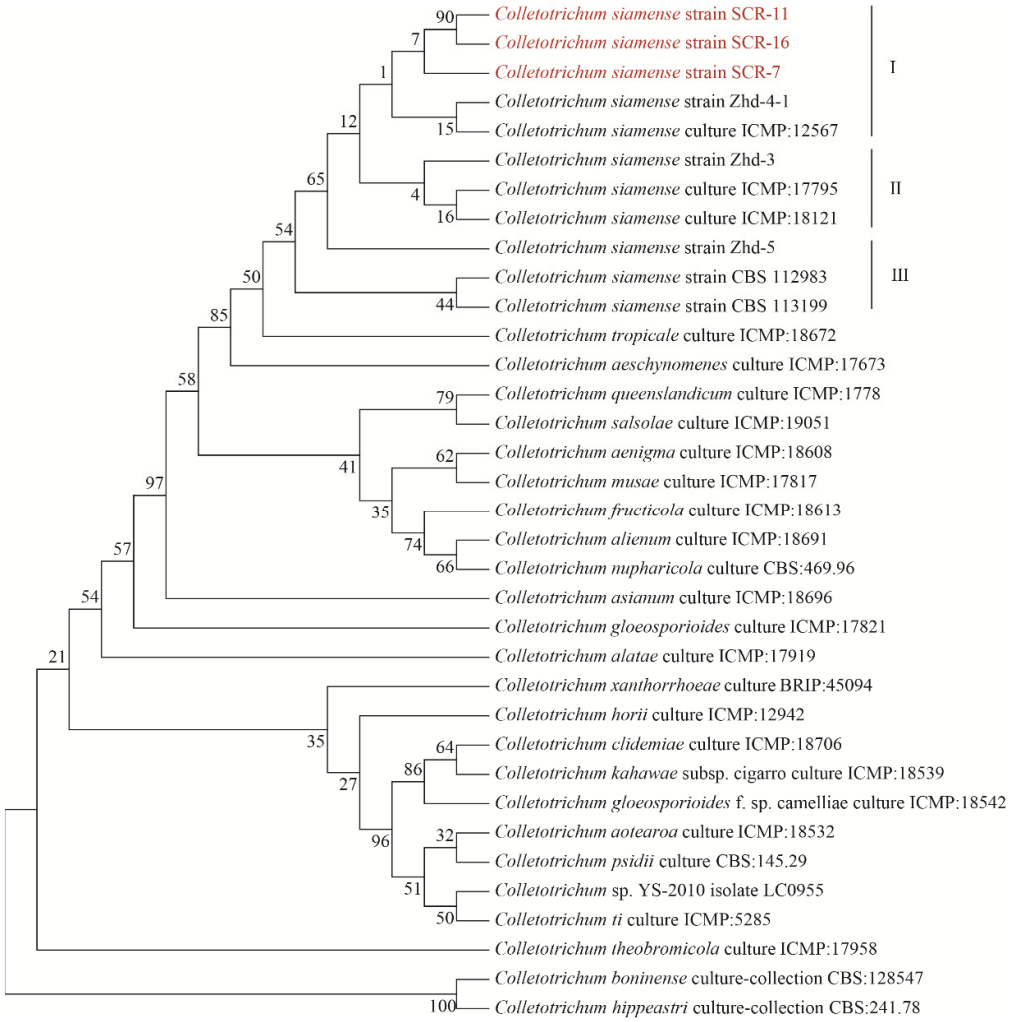

Figure 3. Phylogenetic tree of *Colletotrichum gloeosporioides* species complex based on the *ACT*, *CAL*, and *TUB2* sequences

As the crown rot caused wilting, leaf water content and temperature were measured for characterizing pathogenicity of the three isolates. The inoculation of three isolates exhibited lower leaf water content than that in control, and *C. siamense* SCR-7 remarkably reduced leaf water content than other isolates. Corresponding to leaf water content, *C. siamense* SCR-7 inoculation promoted leaf temperature to 35.3 °C which was significantly higher than other two isolates and control (Figure 5).

Photosynthetic parameters were also determined for analyzing pathogenicity of the three isolates because the crown rot caused wilting. Compared with control, the Pn, E, and gs were all decreased significantly in seedlings inoculated with *C. siamense*. In addition, the seedlings inoculated with *C. siamense* SCR-7 exhibited the lowest Pn (8.47 ± 0.24 μmol/m^2/s) and gs (53.47± 4.21μmol/m^2/s) value, and the lowest E value was possessed by the seedlings inoculated with *C. siamense* SCR-7 (1.41 ± 0.14 μmol/m^2/s) and *C. siamense* SCR-11(1.36 ± 0.11 μmol/m^2/s). Compared with control, inoculation with *C. siamense* remarkably increased the Ci values. And the seedlings inoculated with *C. siamense* SCR-7 and *C. siamense* SCR-11 showed the highest Ci value of the four treatments, which were 166.25 ± 5.71 μmol/m^2/s and 159.75 ± 4.58 μmol/m^2/s, respectively (Figure 6).

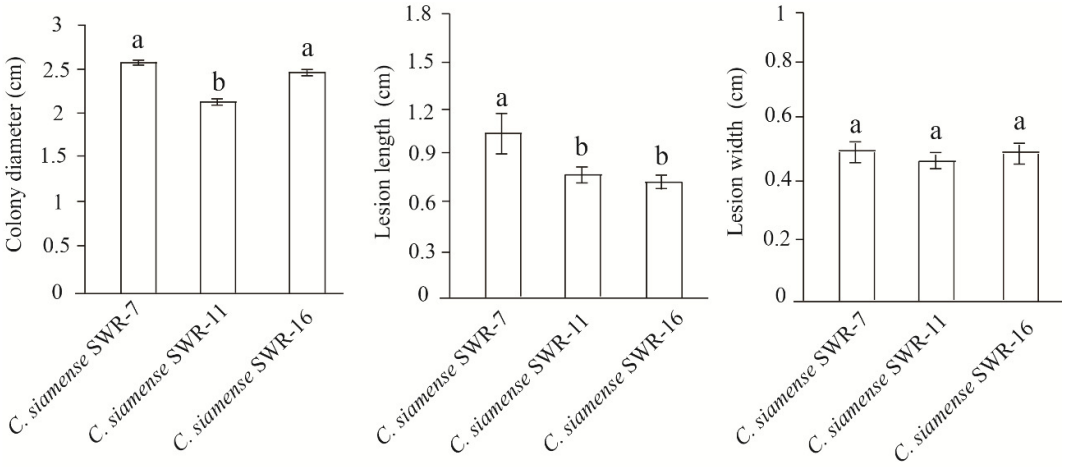

Figure 4. Mycelial growth indexes of different *Colletotrichum siamense* isolates
Colony diameter of different *C. siamense* isolates on PDA; Lesion length and lesion width of different *C. siamense* isolates on strawberry crown. Data (Means ± SE, n = 6) followed by different letters above the bars among treatments indicate significant differences at the 5% level.

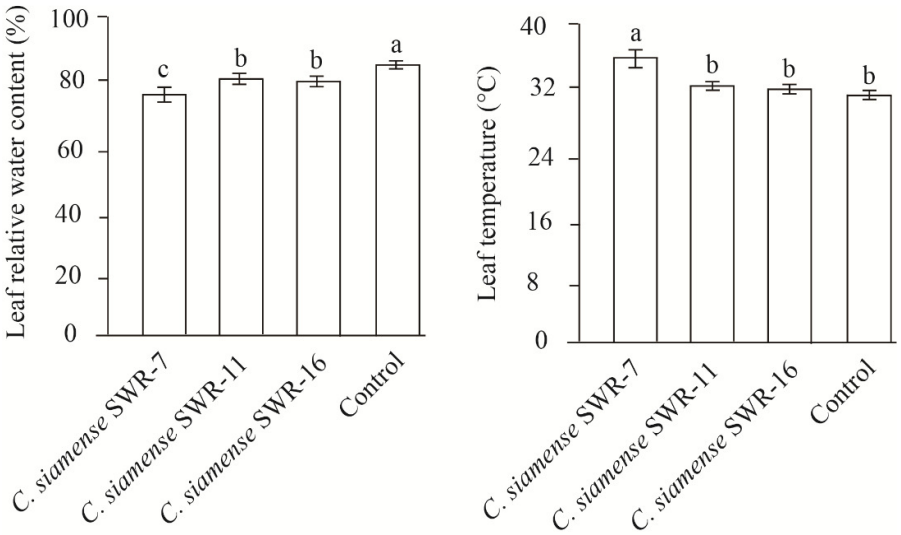

Figure 5. Effects of different *Colletotrichum siamense* isolates on relative water content and temperature of strawberry leaf
Data (Means ± SE, n = 6) followed by different letters above the bars among treatments indicate significant differences at the 5% level.

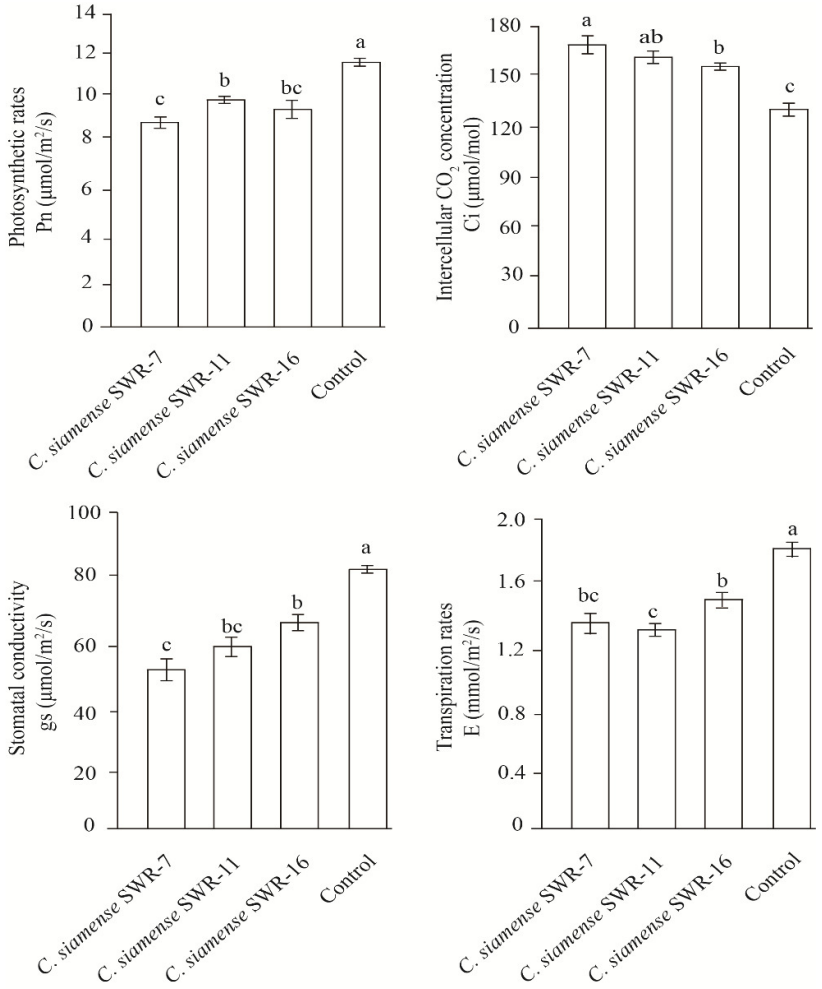

Figure 6. Effects of different *Colletotrichum siamense* isolates on strawberry leaf photosynthetic parameters including photosynthetic rates (Pn), stomatal conductivity (gs), transpiration rates (E), leaf temperature, and intercellular CO_2 concentration (Ci)

Data (Means ± SE, n = 6) followed by different letters above the bars among treatments indicate significant differences at the 5% level.

Discussion

Pathogens causing strawberry root and crown rot included *Colletotrichum*, *Phytophthora*, *Neopestalotiopsis* and *Macrophomina* (Noh *et al.*, 2018; Park *et al.*, 2019; Zhang *et al.*, 2020; Chung *et al.*, 2020). The pathogen of *Colletotrichum* sp. was worldwide and cause crown rot heavily (He *et al.,* 2019). Earlier studies have shown that there were three pathogens of strawberry anthracnose, namely, *C. gloeosporioides*, *C. acutatum* and *C. fragariae* (Peres *et al.*, 2005). Among them, *C. gloeosporioides* and *C. fragariae* mainly infected root crown, petiole and stolon of strawberry to cause root crown rot, which was collectively referred to as Anthracnose Crown Rot; *C. acutatum* mainly infected fruits to cause fruit anthracnose, and rarely infected root caps (Peres *et al.*, 2005). Recently it was reported that *C. gloeosporioides* was the main pathogen of strawberry anthracnose (Jacobs *et al.*, 2019), and *C. gloeosporioides* was a compound species including 22 species and 1 subspecies (Weir *et al.*, 2012). Among them, *C. gloeosporioides*, *C. fructicola*, *C. siamense*, *C.*

aenigma and *C. changpingense* were identified to causing crown rot (Han *et al.*, 2016; Jayawardena *et al.*, 2016; Adhikari *et al.*, 2019). Our study showed twenty-one isolates were ensured as pathogen of strawberry crown rot by Koch's postulates (Figure 1). On PDA, twenty-one isolates showed three different types of colonies colour, which were grey colonies on the front and red wheel in back, white colonies with red rings on the front and yellow wheel in back, grey colonies on the front and black speck in back (Figure 2; Figure 3). The conidia morphology of three types of fungi with different colonial morphology was nearly the same. Thus the *C. siamense* was the pathogen of strawberry crown rot in Jingzhou Hubei province, same as the crown rot pathogen in Wuhan (Han *et al.*, 2014). However, the three isolates of our study showed distant phylogenetic relationships with *C. siamense* strain Zhd-5, *C. siamense* strain Zhd-4-1 and *C. siamense* strain Zhd-3 identified in Wuhan. This suggested the *C. siamense* presented high diversity due to different region and environment.

C. siamense is capable of causing crown rot disease which is characterized by reddish brown rhizomes with less roots and wilted leaves. According to the comparation of lesion length, lesion width, indexes of leaf relative water content, and temperature and photosynthetic parameters, the pathogenicity of three type's *C. siamense* isolates was different. The isolates of *C. siamense* SCR-7 showed the strongest pathogenicity which caused the highest values of lesion length and leaf temperature (Figure 4), lowest leaf relative water content (Figure 5), and lowest Pn, gs even E value of the three isolates (Figure 6), which suggested not only lesion index but also relative water content and photosynthetic parameters could evaluate the pathogen pathogenicity (Tan *et al.*, 2005; Yang *et al.*, 2014; Calonnec *et al.*, 2018; Xue *et al.*, 2018). The isolates of *C. siamense* SCR-7 and SCR-16 exhibited the highest mycelial growth rate, but *C. siamense* SCR-16 showed lower pathogenicity than *C. siamense* SCR-7. This result suggested us there was an inevitable connection between mycelial growth rate on PDA flat and pathogenicity on plant tissue, which might contribute to the growth process of the isolates was different between PDA flat and strawberry crown as previous study stated (Irzykowska *et al.*, 2008; Irani *et al.*, 2011; Wang *et al.*, 2011; Lu *et al.*, 2018). In addition, the different pathogenicity of three isolates suggested us further clarifications of *C. siamense* and systemic pathogenicity analysis are needed.

Conclusions

The objective of this study was to identify the pathogen causing crown rot on strawberry in Jingzhou, China, and to provide a basis for disease prevention and resistance breeding. Twenty-one isolates were characteristic as pathogen of crown rot of strawberry by Koch's postulates, and all of them were identified as *Colletotrichum* spp. Based on conidia morphology observation. Twenty-one isolates were divided into three groups based on colony morphology, and isolates SCR-7, SCR-11 and SCR-16 were belonged to each group, respectively. Three isolates SCR-7, SCR-11 and SCR-16 were characterized as *C. siamense* by actin, β-Tubulin 2 and calmodulin gene sequences. In addition, the pathogenicity of three types of *C. siamense* isolates was different. The isolates of *C. siamense* SCR-7 showed the strongest pathogenicity which caused the highest values of lesion length and leaf temperature, and the lowest leaf water content, Pn, gs even E value of the three isolates. The results of biological characteristics and multigene loci analysis based on the sequences of *TUB2*, *ACT*, and *CAL* concluded that the crown rot disease of strawberry occurred in Jingzhou was caused by *C. siamense*.

Authors' Contributions

BS conceived and designed the experiments, supervised and revised the manuscript. CL conducted part of the experiments and wrote the original manuscript. YH conducted part of the experiments.

All authors read and approved the final manuscript

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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