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Molecular Characterization, Pathogenicity and Biological Characterization of *Colletotrichum* Species Associated with Anthracnose of *Camellia yuhsienensis* Hu in China

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Abstract: *Camellia yuhsienensis* Hu, a species of tea oil tree with resistance to anthracnose, is widely used to breed disease-resistant *Camellia* varieties. In 2019, anthracnose symptoms were observed on Ca. *yuhsienensis* for the first time. However, the species and biological characteristics of *Colletotrichum* spp. isolated from Ca. *yuhsienensis* (YX-*Colletotrichum* spp.) have not been elucidated. In this study, five isolates (YX2-5-2, 2YX-3-1, 2YX-5-1, 2YX-8-1-1 and 2YX-8-1-2), which were consistent with the morphological characteristics of *Colletotrichum* spp., were obtained from Ca. *yuhsienensis*. A phylogenetic analysis demonstrated that YX2-5-2, 2YX-3-1 and 2YX-8-1-2 belonged to first clade along with *Colletotrichum fructicola*. 2YX-8-1-1 belonged to the second clade along with *Colletotrichum siamense*. 2YX-5-1 belonged to the third clade with *Colletotrichum camelliae*. Pathogenicity tests revealed that the pathogenicity of YX-*Colletotrichum* spp. was stronger than that of *Colletotrichum* spp. isolated from *Camellia oleifera* (GD-*Colletotrichum* spp.). Biological characteristics illustrated that the mycelial growth of YX-*Co. camelliae* (2YX-5-1) was slower than that of GD-*Co. camelliae* when the temperature exceeded 20 °C. In addition, in the presence of ions, the mycelial growth of YX-*Co. fructicola* (YX2-5-2) and YX-*Co. siamense* (2YX-8-1-1) was also slower than that of GD-*Co. fructicola* and GD-*Co. siamense*. Furthermore, the ability of YX-*Colletotrichum* spp. to utilize lactose and mannitol was weaker than that of GD-*Colletotrichum* spp., while the ability to utilize NH_4^+ was generally stronger than that of GD-*Colletotrichum* spp. This is the first report of anthracnose of Ca. *yuhsienensis* induced by *Co. fructicola*, *Co. siamense* and *Co. camelliae* in China. These results will provide theoretical guidance for the study of the pathogenesis and control of anthracnose on Ca. *yuhsienensis*.

Keywords: tea oil tree; *Camellia yuhsienensis* Hu; *Colletotrichum*; anthracnose; morphology; pathogenicity

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1. Introduction

The tea oil tree generally refers to the *Camellia* genus, which has rich seed oil content that is produced and highly valuable [1]. The genus includes such species as Ca. *oleifera*, Ca. *yuhsienensis*, *Camellia vietnamensis* and Ca. *oleifera* var. *monosperma*, among others [2,3]. Tea oil extracted from the seed of tea oil tree is rich in unsaturated fatty acids and vitamin E and has unique nutritional value [4]. Thus, the tea oil tree is as famous as coconut, palm and olive, and is also known as one of the four major woody oil plants in the world [5,6]. Moreover, the United Nations Food and Agriculture Organization (FAO) recommended tea oil as a high-quality and healthy vegetable oil owing to its nutritional value and excellent storage quality [7]. In 2020, the area in China planted with tea oil trees reached 45,333.3 km^2;

the output of tea oil reached 627,000 tons, and the output value of tea oil industry reached 18 billion U.S. dollars, indicating that tea oil is highly valuable [8].

Anthracnose of the tea oil tree is an important factor that limits the yield of tea oil [9,10]. *Colletotrichum* spp. primarily infects the leaves and fruits of the tea oil tree, leading to a 20% to 40% fruit drop and up to 40% seed loss [11]. It can also lead to the death of branches and even entire plants, causing substantial economic losses and seriously damaging the safety of edible oil in China [12]. In addition, *Colletotrichum* spp. are also important pathogens of a variety of plants, such as tea plants (*Camellia sinensis*) and apple trees (*Malus domestica*), among others [13–15]. *Colletotrichum* spp. are also regarded as among the top 10 plant pathogenic fungi in the field of molecular plant pathology because of their strong pathogenicity and wide spread [16].

The species and biological characteristics of *Colletotrichum* spp. vary according to the host. The different species and biological characteristics of *Colletotrichum* spp. cause great obstacles to the targeted control of anthracnose. A previous study revealed that the destructive pathogen that causes anthracnose of the tea oil tree (*Ca. oleifera*) is in the *Colletotrichum gloeosporioides* species complex [17].Li (2016) [18] further isolated 406 strains of *Colletotrichum* spp. from Ca. *oleifera* in 10 provinces of China, including *Co. fructicola*, *Co. siamense*, *Co. gloeosporioides*, *Co. camelliae* and *C. horii*, with *Co. fructicola* the most widely distributed. Fu (2019) [19] isolated 488 strains of *Colletotrichum* spp. from pear in seven provinces of China. It was found that *Co. fructicola* was the most distributed in Asian pear (*Pyrus pyrifolia*), and *Co. siamense* was the most distributed in European pear (*P. communis*), indicating that the species and pathogenicity of *Colletotrichum* spp. vary on different species of pear. Lu (2018) [20] isolated *Co. camelliae* and *Co. fructicola* from Ca. *sinensis*, and their biological characteristics showed that they were substantially different. Consequently, the identification of the species and biological characteristics of *Colletotrichum* spp. on the host is the basis of targeted control of anthracnose.

Breeding and planting resistant plants is an important measure to control anthracnose [21,22].

Camellia yuhsienensis Hu, a species of tea oil tree, was once widely cultivated in central China because of its high quality, yield and strong resistance to anthracnose. [23–30]. Consequently, Ca. *yuhsienensis*, as a wild relative of Ca. *oleifera*, is widely used to breed varieties of tea oil tree [23].

Unfortunately, anthracnose symptoms have been observed on the leaves of Ca. *yuhsienensis* for the first time. Therefore, anthracnose of Ca. *yuhsienensis*, as a new disease, merits urgent study. The aim of the present study was to investigate the cause of anthracnose associated with Ca. *yuhsienensis*. Following surveys, morphological studies and DNA phylogenies were used to identify the disease causal agent. Moreover, pathogenicity and biological characterization studies were performed to determine the virulence of diverse fungal isolates in Ca. *yuhsienensis* and provide guidance for the targeted control of Ca. *yuhsienensis* anthracnose. In summary, this study provides a theoretical basis for further understanding the pathogenic mechanism of tea oil tree anthracnose and provides theoretical guidance for the prevention and control of tea oil tree anthracnose.

2. Materials and Methods

2.1. Fungal Isolates and Plant Material

Colletotrichum spp. isolated from Ca. *oleifera*, Guangdong Province, China (GD-*Colletotrichum* spp.) were all obtained from the Key Laboratory of National Forestry and Grassland Administration for the Control of Diseases and Pests of South Plantation, Changsha, China.

The infected leaves of Ca. *yuhsienensis* were collected from a Ca. *yuhsienensis* plantation in Youxian, Hunan Province, China (113.3°2′16″ E, 26.7°15′14″ N).

Three-year-old specimens of Ca. *yuhsienensis* were used as the experimental material. Trees were originally obtained from the Key Laboratory of Cultivation and Protection for

Non-Wood Forest Trees of Ministry of Education, Changsha, Hunan Province, China, and transplanted into a greenhouse (28 °C, 12 h light, 90% humidity).

2.2. Molecular Characterization

Colletotrichum spp. Were incubated on potato dextrose broth (PDB) at 28 °C for 5 days. The genomic DNA of *Colletotrichum* spp. Was extracted from the mycelia using a Plant Genomic DNA Extraction Kit DP305 (TIANGEN, Biotech, China). The DNA samples were used as the templates for PCR amplification. The partial actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase (CHSI) and manganese-superoxide dismutase (SOD2) genes were amplified by PCR [31,32]. The PCR products were sequenced by Tsingke Biotechnology Co., Ltd., Changsha, China. A Maximum Likelihood (ML) phylogenetic tree based on the combined ACT, CHSI, SOD2 and GAPDH sequences using MEGA 5.05 (https://www.megasoftware.net, accessed on 4 November 2021; AZ, USA) was established.

2.3. Morphological Characterization

Colletotrichum spp. Were cultured on potato dextrose agar (PDA) at 5-day post-inoculation (dpi). A 5 mm mycelial plug was transferred from the edge of actively growing cultures to fresh PDA plates. Morphological characters, including the shape and color of the colony and mycelia, were visually observed at 5 dpi [33]. Microscopic characters were examined by microscopy (Eclipse Ni-U; Nikon, Tokyo, Japan) at 10 dpi.

2.4. Koch's Postulates Verification

Attached Ca. *yuhsienensis* leaves were washed with deionized water and then sterilized with 1% sodium hypochlorite for 3 min. Nonwounded tests were conducted by inoculating leaves with a YX2-5-2 conidial suspension (1×10^6 conidia/mL) that were used as the treated samples, and leaves inoculated with sterile water were used as the controls. Wounding tests were conducted by scratching the leaves with sterilized needles and then inoculating them with a YX2-5-2 conidial suspension (1×10^6 conidia/mL). Leaves inoculated with sterile water were used as the controls. Finally, anthracnose symptoms were photographed after 5 dpi. Both tests were repeated three times.

2.5. Pathogenicity Tests

Nonwounded and unattached Ca. *yuhsienensis* or Ca. *oleifera* leaves were washed with deionized water and then sterilized with 1% sodium hypochlorite for 3 min. Pathogenicity tests were conducted by scratching the leaves with sterilized needles and then inoculating them with a conidial suspension (1×10^6 conidia/mL). Leaves inoculated with sterile water were used as the controls. The inoculated samples were placed in 12 cm plastic Petri dishes and cultured in an incubator for 2 (For Ca. *oleifera*) or 4 (For Ca. *yuhsienensis*) days at 28 °C. Finally, the diameter of the lesions was measured. Each isolate was measured in triplicate.

2.6. Effect of Temperature and pH on Mycelial Growth

Mycelial plugs (5 mm) from PDA were placed in the center of PDA plates and cultured in incubators set at different temperatures (10, 15, 20, 25, 28, 30, and 35 °C) for 5 days. Moreover, 5 mm mycelial plugs from PDA were placed in the center of PDA plates adjusted to a range of pH values from 3.0 to 10.0 for 5 days in an incubator at 28 °C. Na_2HPO_4–citric acid buffer was used to prepare PDA with pH values of 3.0–8.0, while Na_2CO_3-$NaHCO_3$ buffer was used to prepare PDA at pH values 9.0 and 10.0. Finally, the colony diameter was measured. Each isolate was measured in triplicate.

2.7. Effect of Carbon and Nitrogen Sources on Mycelial Growth

Czapek-Dox Agar (3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.01 g/L FeSO4, and 30 g/L sucrose) was used as the basic medium. To analyze the carbon sources, sucrose in the basic medium was replaced by the same quantity of

glucose, mannitol, lactose, or soluble starch. To analyze the nitrogen source, sodium nitrate ($NaNO_3$) in the basic medium was replaced by the same quantity of casein tryptone, urea, ammonium chloride (NH_4Cl), or ammonium nitrate (NH_4NO_3). Mycelial plugs (5 mm) from PDA were transferred onto the media containing different carbon or nitrogen sources. The cultures were incubated at 28 °C for 5 days. The colony diameter was measured, and each experiment was conducted in triplicate.

3. Results and Discussion

3.1. Symptom Characteristics

In April 2019, typical anthracnose symptoms were first observed on Ca. *yuhsienensis* in a plantation in Youxian, Hunan Province, China (113.3°2′16″ E, 26.7°15′14″ N). Most of the diseased leaves had wounds, such as those caused by insect bites [34–36]. Therefore, *Colletotrichum* spp. are more likely to infect leaves through these wounds. It is common for resistant plants to be infected through wounds. For instance, Silva (2021) [37] found that on the relatively resistant host *Capsicum chinense* PBC932, pathogenicity was dependent on both the inoculation method (with or without wounding) and the stage of maturity of the fruit. It was difficult to infect PBC932 with *Colletotrichum* spp. without injury but relatively easy to infect PBC932 with injury.

However, five unwounded Ca. *yuhsienensis* leaves also showed symptoms of anthracnose (Figure 1B). The isolate YX2-5-2 was reinoculated on Ca. *yuhsienensis*, and the same symptoms occurred, confirming Koch's postulates (Figure 1C,D). All of the diseased leaves had irregular grayish brown spots with dark brown edges and dark brown undersides, similar to previous reports [38,39]. Ca. *yuhsienensis* is famous for its resistance to anthracnose, and there have been no reports of anthracnose on Ca. *yuhsienensis* to date. Therefore, the phenomenon that the healthy leaves of Ca. *yuhsienensis* were infected by *Colletotrichum* spp. attracted our attention. Five leaves of Ca. *yuhsienensis* were collected to obtain the pathogens.

Figure 1. Typical symptoms of anthracnose on *Camellia yuhsienensis*. (**A**) No symptoms were observed on control leaves treated with sterilized water; (**B**) Anthracnose symptoms on Ca. *yuhsienensis* in a plantation in Youxian, Hunnan Province, China; (**C**) Anthracnose symptoms were seen on attached, unwounded leaves treated with a conidial suspension (1×10^6 conidial/mL) of isolate YX2-5-2; (**D**) Anthracnose symptoms were seen on attached wounded leaves treated with a conidial suspension (1×10^6 conidial/mL) of isolate YX2-5-2.

3.2. Cultural and Morphological Characteristics

Five isolates (YX2-5-2, 2YX-3-1, 2YX-5-1, 2YX-8-1-1 and 2YX-8-1-2) were obtained from Ca. *yuhsienensis* for the first time. Their morphological characteristics are shown in Figure 2. Few differences in colony morphology were clearly observed among the five isolates. Figure 2 shows that the upper side of these colonies on PDA was fluffy, cottony and white at first, then became light gray, whereas the reverse side slowly turned dark gray. Thus, the color of the upper side of the colony was generally lighter than that of the reverse side. There were significant differences in the rate of mycelial growth between 2YX-5-1 and the other isolates. The mycelial growth of 2YX-5-1 was generally slower than that of the other four isolates (Table 1). The conidia were all hyaline, guttulate, smooth, one-celled, and cylindrical (Figure 2). In addition, the conidial sizes of 2YX-8-1-1 and 2YX-5-1 were larger than those of other isolates (Table 1). An interesting phenomenon was observed that larger conidia can result in slower mycelial growth. A similar phenomenon has been described in a previous study; the conidia of isolate C07046 was larger than those of isolate C96002, and the mycelial growth was slower than that of C96002 even though both C07046 and C96002 are isolates of *Colletotrichum coccodes* [40]. In conclusion, the characteristics of the five isolates were consistent with the morphological characteristics of *Colletotrichum* spp. [31,41].

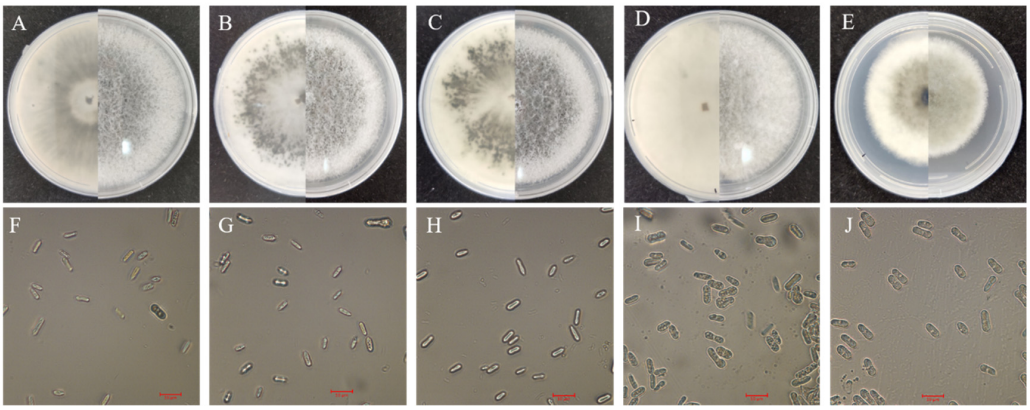

Figure 2. (**A**–**E**) Colony morphology of isolates YX2-5-2, 2YX-8-1-2, 2YX-3-1, 2YX-8-1-1 and 2YX-5-1 on PDA after 5 days at 28 °C, respectively; (**F**–**J**) Conidia of isolates YX2-5-2, 2YX-8-1-2, 2YX-3-1, 2YX-8-1-1 and 2YX-5-1, respectively; scale bar = 10 μm.

Table 1. Summary of the morphological data of the five isolates.

Isolates	Conidial Size (μm)	Mycelial Growth (mm/d)
YX2-5-2	7.27 ± 0.52 × 1.81 ± 0.31	13.9 ± 0.6
2YX-8-1-2	7.27 ± 0.48 × 2.42 ± 0.27	13.7 ± 0.3
2YX-3-1	7.27 ± 0.55 × 1.81 ± 0.21	13.1 ± 0.8
2YX-8-1-1	9.09 ± 0.45 × 3.64 ± 0.43	13.4 ± 0.3
2YX-5-1	9.09 ± 0.36× 3.64 ± 0.39	8.5 ± 0.9

3.3. Phylogenetic Analysis

For molecular identification, internal transcribed spacer (ITS), partial actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), manganese-superoxide dismutase (SOD2) and chitin synthase (CHS-1) genes/region of all the isolates were successfully amplified and sequenced (Table 2). The sequences at individual loci were insufficient to separate closely related species within the *Co. gloeosporioides* species complex. Thus, the five genes/region of each isolate were combined in the order of ITS-ACT-GAPDH-SOD2-CHS-1. A phylogenetic tree of these five isolates in this study indicated that there were three well separated clades (Figure 3). YX2-5-2, 2YX-3-1 and 2YX-8-1-2 belonged to first clade along with *Co. fructicola* ICMP 18581, ICMP 17,921 and ICMP 18646. 2YX-8-1-1 belonged to the second clade along with *Co. siamense* ICMP 18642. Lastly, 2YX-5-1 belonged to the third

clade with *Co. camelliae* ICMP 10646, ICMP 10,643 and ICMP 18542. These results suggest that *Co. fructicola* is probably the most distributed species on Ca. *yuhsienensis*. Li (2016) [18] also found that *Co. fructicola* was the dominant species on Ca. *oleifera*. Wang (2020) [38] further discovered that *Co. fructicola* was common and a widely distributed species on the leaves of Ca. *oleifera*, which indicated that the tea oil tree is probably the most susceptible to *Co. fructicola*. The anthracnose of Ca. *yuhsienensis* was first discovered with a small rate of incidence and a few samples, and the disease has not yet been found in other areas. Consequently, the deduction above merits further verification with more samples in the future. However, this result is still helpful for the study of anthracnose on Ca. *yuhsienensis*.

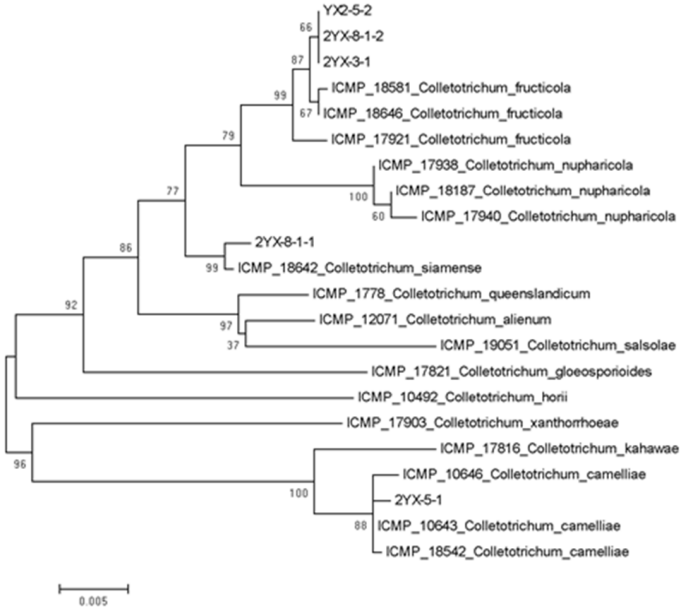

Figure 3. A Maximum Likelihood phylogenetic tree using isolates YX2-5-2, 2YX-8-1-2, 2YX-3-1, 2YX-8-1-1, 2YX-5-1 and type strains of the *Colletotrichum gloeosporioides* complex based on the combined CHS-1, ACT, ITS, SOD2 and GAPDH gene sequences.

Table 2. GenBank accession number of the five isolates.

Isolate	Gene Name	Genbank Accession Number
YX2-5-2	GAPDH	MW398864
YX2-5-2	ACT	MW398863
YX2-5-2	CHSI	MW886232
YX2-5-2	SOD2	MW398866
YX2-5-2	ITS	MW398865
2YX-8-1-2	GAPDH	MZ224482
2YX-8-1-2	ACT	MZ224483
2YX-8-1-2	CHSI	OL310498
2YX-8-1-2	SOD2	MZ224480
2YX-8-1-2	ITS	MZ224481
2YX-8-1-1	GAPDH	MW398861
2YX-8-1-1	ACT	MW398860
2YX-8-1-1	CHSI	OL310500
2YX-8-1-1	SOD2	MZ048745.1
2YX-8-1-1	ITS	MW398862
2YX-5-1	GAPDH	MZ048746
2YX-5-1	ACT	MW924872
2YX-5-1	CHSI	MW924874
2YX-5-1	SOD2	MW924873
2YX-5-1	ITS	MW911446
2YX-3-1	GAPDH	MW924878
2YX-3-1	ACT	MW924879
2YX-3-1	CHSI	OL310499
2YX-3-1	SOD2	MW924877
2YX-3-1	ITS	MW924880

3.4. Pathogenicity Tests

To explore the reason why the *Colletotrichum* spp. isolated from Ca. *yuhsienensis*, Youxian, China (YX-*Colletotrichum* spp.) could infect Ca. *yuhsienensis* without requiring an injury to the tree, the *Colletotrichum* spp. isolated from Ca. *oleifera*, Guangdong, China (GD-*Colletotrichum* spp.) and YX-*Colletotrichum* spp. were used for pathogenicity tests. First, GD-*Colletotrichum* spp. infected Ca. *yuhsienensis* without injury for 15 days. There were no typical symptoms of anthracnose, proving that Ca. *yuhsienensis* could not be infected by GD-*Colletotrichum* spp. Therefore, we suspected that some changes may have taken place in the biological characteristics, such as pathogenicity, optimal temperature, pH, carbon and nitrogen source, of the YX-*Colletotrichum* spp., so that they could infect Ca. *yuhsienensis* without injury, while GD-*Colletotrichum* spp. could not.

Secondly, wounded leaves of Ca. *yuhsienensis* were used for pathogenicity. The results are shown in Figure 4. Different species of *Colletotrichum* differ in their degree of pathogenicity to Ca. *yuhsienensis*. The pathogenicity of *Co. fructicola* was the weakest, in which the diameter of lesion formed by GD-*Co. fructicola* was 2.45 mm and those of YX-*Co. fructicola* (YX2-5-2, 2YX-3-1, 2YX-8-1-2) were 4.97 mm, 4.6 mm, and 4.95 mm, respectively. *Co. camelliae* was the most aggressive at causing infection, in which the diameter of the lesion formed by GD-*Co. camelliae* was 4.95 mm and that by YX- *Co. camelliae* (2YX-5-1) was 7.83 mm (Figure 4G). These results showed that the pathogenicity of *Co. fructicola* was weaker than that of the other species of *Colletotrichum* spp., which is consistent with previous studies [13,20,38,42]. Lu (2018) [20] concluded that the difference in appressorium development between *Co. camelliae* and *Co. fructicola* led to pathogenic variation between these two species. However, a phenomenon was observed that YX-*Co. camelliae* (2YX-5-1) with larger conidia and slower mycelial growth may be more pathogenic. Choi (2011) [40] also found that the pathogenicity of isolate C07046 with larger conidia and slower mycelial growth was stronger than that of isolate C96002, even though they are all members of *Co. coccodes*.

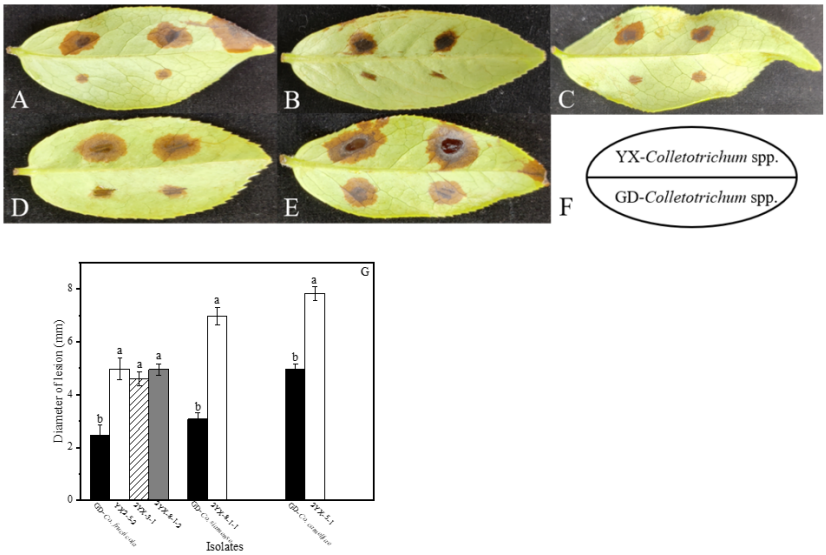

Figure 4. Pathogenicity of GD-*Colletotrichum* spp. (isolated from *Camellia oleifera*) and YX-*Colletotrichum* spp. (isolated from Ca. *yuhsienensis*) on Ca. *yuhsienensis*. (**A**–**C**) Lesion development by YX-Co. *fructicola* (YX2-5-2, 2YX-3-1, 2YX-8-1-2) and GD-*Co. fructicola* was photographed at 4 days post infiltration (dpi); (**D**) Lesion development by YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense* was photographed at 4 dpi; (**E**) Lesion development by YX-*Co. camelliae* (2YX-5-1) and GD-*Co. camelliae* was photographed at 4 dpi; (**F**) Schematic diagram of the pathogenicity test in which the conidia of GD-*Colletotrichum* spp. were infiltrated into the underside panel of the leaf, while those of YX-*Colletotrichum* spp. were infiltrated into the upper side panel of the same leaf; (**G**) Lesion diameters were measured at 4 dpi. The error bars represent standard deviations based on six biological replicates. Lesion diameters followed by the same lowercase letter are not significantly different at $p < 0.05$ using an ANOVA.

Significantly, in contrast to the previous experimental results, only one isolate of YX-*Co. camelliae* (2YX-5-1) and YX-*Co. siamense* (2YX-8-1-1) with stronger pathogenicity was obtained, while three isolates of YX-*Co. fructicola* with weaker pathogenicity were obtained. Li (2016) [18] also found that although *Co. camelliae* had strong pathogenicity, the prevalent *Colletotrichum* spp. on Ca. *oleifera* was *Co. fructicola*, which indicated that the strong pathogenicity may be owing to the loss of other abilities, such as transmission and mycelial growth. This phenomenon is also consistent with the law of infectious diseases in animals. For instance, influenza has strong transmissibility, but the mortality rate is poor. In contrast, Creutzfeldt -Jacob disease has high mortality but poor transmission. In addition, these results also indicated that during the same incubation time, the diameter of lesions caused by YX-*Colletotrichum* spp. were generally larger than those of GD-*Colletotrichum* spp. (Figure 4). Thus, the pathogenicity of YX-*Colletotrichum* spp. to Ca. *yuhsienensis* was stronger than that of *Colletotrichum* spp.

The pathogenicity of *Colletotrichum* spp. to different hosts varies. For example, Han (2016) [43] found that *Co. fructicola* was more pathogenic to strawberry than other *Colletotrichum* spp., which differs from the results of this study. Owing to the fact that Ca. *yuhsienensis* is more resistant to anthracnose than Ca. *oleifera*, we hypothesized that YX-*Colletotrichum* spp. was also more pathogenic on Ca. *oleifera* than GD-*Colletotrichum* spp. [44]. Thus, wounded leaves of Ca. *oleifera* were used for pathogenicity. Figure 5 shows similar results. *Co. fructicola* was the weakest pathogen. The diameter of the lesion formed by GD-*Co. fructicola* was 2.75 mm and by those of YX-*Co. fructicola* (YX2-5-2, 2YX-3-1, 2YX-8-1-2) were 5.67 mm, 4.33 mm, and 4.75 mm, respectively. *Co. camelliae* was the most pathogenic. The diameter of the lesion formed by GD-*Co. camelliae* was 3.83 mm and that of YX-*Co. camelliae* (2YX-5-1) was 8.0 mm (Figure 5G). The fact that the pathogenicity of *Co. fructicola* was weaker than that of the other *Colletotrichum* spp. is also consistent with the results. The diameter of lesions caused by YX-*Colletotrichum* spp. were generally larger than those of GD-*Colletotrichum* spp. during the same amount of incubation (Figure 5).

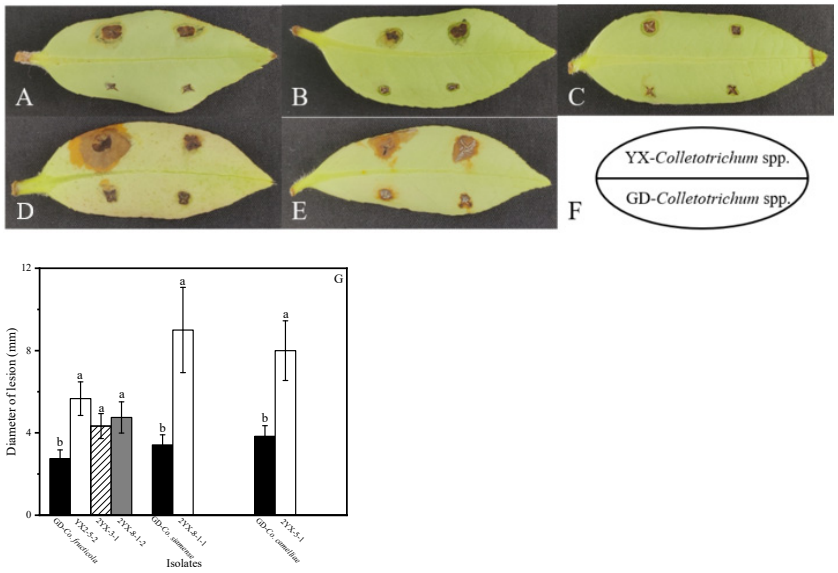

Figure 5. Pathogenicity of GD-*Colletotrichum* spp. and YX-*Colletotrichum* spp. on *Camellia. oleifera*. (**A**–**C**) Lesion development by YX-Co. *fructicola* (YX2-5-2, 2YX-3-1, 2YX-8-1-2) and GD-Co. *fructicola* was photographed at two days post inoculation (dpi); (**D**) Lesion development by YX-Co. *siamense* (2YX-8-1-1) and GD-Co. *siamense* were photographed at 2 dpi; (**E**) Lesion development by YX-Co. *camelliae* (2YX-5-1) and GD-*Co. camelliae* were photographed at 2 dpi; (**F**) Schematic diagram of pathogenicity. The conidia of GD-*Colletotrichum* spp. were infiltrated into the underside panel of the leaf, while the YX-*Colletotrichum* spp. was infiltrated into the upper side panel of the same leaf; (**G**) Lesion diameters were measured at 2 dpi. The error bars represent standard deviations based on six biological replicates. Lesion diameters followed by the same lowercase letter are not significantly different at $p < 0.05$ using an ANOVA.

These results prove that whether the host was Ca. *oleifera* or Ca. *yuhsienensis*, the pathogenicity of YX-*Colletotrichum* spp. was stronger than that of GD-*Colletotrichum* spp., which could be the reason why YX-*Colletotrichum* spp. could infect Ca. *yuhsienensis* without injury. Different plants respond differently to pathogens, which leads to different growth environments after the pathogen has colonized. For instance, Wang (2018) [45] found two varieties of Ca. *sinensis* Zhongcha 108 and Longjing 43 with different resistances to anthracnose. Among them, Zhongcha 108, having strong resistance could be due to the important role of H_2O_2. When the same isolate of *Colletotrichum* spp. infected Zhongcha 108, only the *Colletotrichum* spp. that were more resistant to H_2O_2 could colonize. The formation of resistance to H_2O_2 requires the cooperation of multiple metabolic pathways of *Colletotrichum* spp. During this process, the biological characteristics of *Colletotrichum* spp. may change. Similarly, increased pathogenicity of YX-*Colletotrichum* spp. may also cause changes in some biological characteristics. Consequently, the other biological characteristics of YX-*Colletotrichum* spp. were studied. Owing to the strong pathogenicity of YX2-5-2 in YX-*Co. fructicola*, YX2-5-2 was selected as the representative isolate of YX-*Co. fructicola* for the convenience of the follow-up experiments.

3.5. Effect of Temperature and pH on Mycelial Growth

Figure 6 indicates that the mycelia of isolates grew more quickly as the temperature increased. When the temperature reached 25 °C~30 °C, the diameter of colonies decreased with an increase in temperature. Lima (2015) [46] also found that both high and low temperatures significantly affected the development of the pathogen in vitro and in vivo, and that a high temperature (35 °C) can completely inactivate the virulence of *Colletotrichum acutatum*, while low temperatures (≤2 °C) can inactivate the virulence of *Co. gloeosporioides*. The results illustrate that the optimal temperature of different isolates differed, but the growth trend was similar. The optimal temperature of GD-*Co. fructicola* and YX-*Co. fructicola* (YX2-5-2) was 30 °C. The optimal temperature of GD-*Co. siamense* was 30 °C, while the optimal temperature of YX-*Co. siamense* (2YX-8-1-1) was 28 °C. The optimal temperature of YX-*Co. camelliae* (2YX-5-1) was 25 °C, while the optimal temperature of GD-*Co. camelliae* was 30 °C (Table 3). These results prove that the optimal growth temperature of GD-*Colletotrichum* spp. and YX-*Colletotrichum* spp. were slightly different. This could be because GD-*Colletotrichum* spp. and YX-*Colletotrichum* spp. originated from different latitudes. GD-*Colletotrichum* spp. was isolated from Guangdong Province in southern China, while YX-*Colletotrichum* spp. was isolated from Youxian, Hunan Province in central China. Therefore, the optimal growth temperature of GD-*Colletotrichum* spp. may be slightly higher than that of YX-*Colletotrichum* spp. A similar phenomenon was also found by Han, in that isolates of *Colletotrichum nymphaeae*, which are only distributed in areas of higher altitude (1,000 m), were highly sensitive to higher temperatures [43].

However, Figure 6C indicates an interesting phenomenon, in that the mycelial growth of YX-*Co. camelliae* (2YX-5-1) was slower than that of GD-*Co. camelliae* when the temperature exceeded 20 °C. The results of 3.1 and 3.2 show that YX-*Co. camelliae* with stronger pathogenicity has mycelia that grow more slowly than those of the other species of YX-*Colletotrichum* spp. A previous study also displayed the strong pathogenicity and slow mycelial growth of *Co. camelliae* [38]. Therefore, we hypothesized that the increased pathogenicity of YX-*Co. camelliae* (2YX-5-1) was at the expense of its mycelial growth rate. Xue (2019) [47] also discovered a similar phenomenon, that the mycelial growth of *Colletotrichum fioriniae* with stronger pathogenicity was slower than that of *Co. fructicola*. Li (2008) [48] also discovered that the mycelial growth of *Colletotrichum gloeosporioides* with stronger pathogenicity was slower than that of *Colletotrichum gloeosporioides* with weaker pathogenicity. Thus, these phenomena further proved the previous hypothesis that the improvement in pathogenicity may come at the expense of other adaptive abilities.

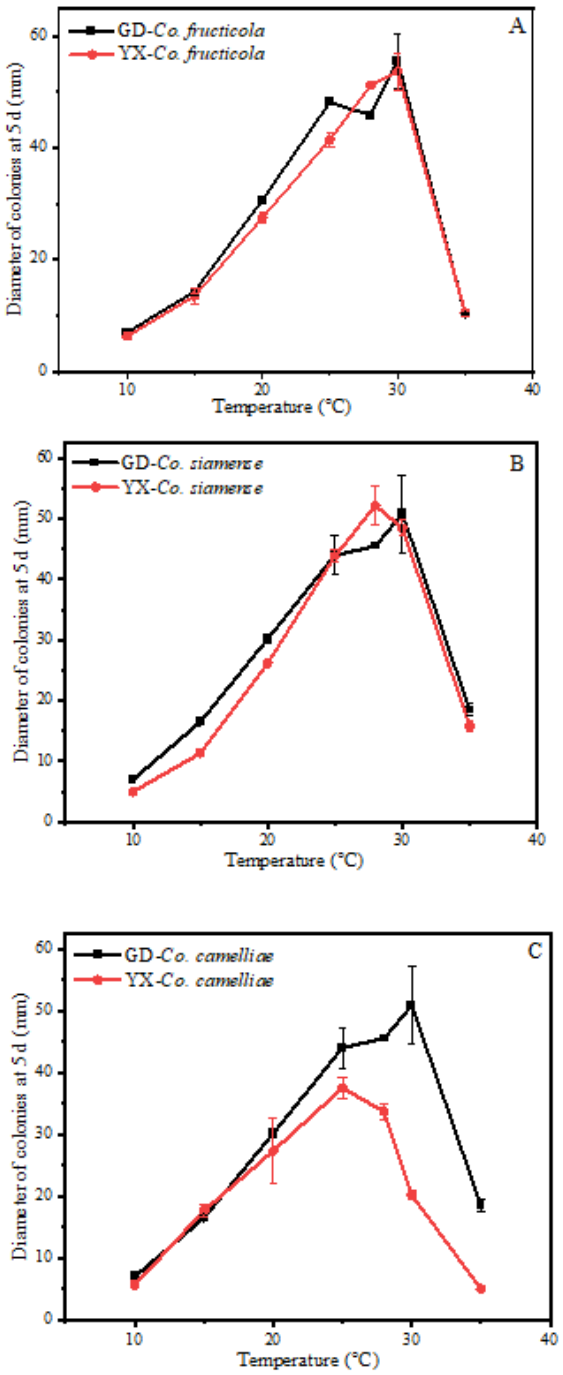

Figure 6. Effect of temperature on the growth of (**A**) YX-*Co. fructicola* (YX2-5-2) and GD-*Co. fructicola*; (**B**) YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense*; (**C**) YX-*Co. camelliae* (2YX-5-1) and GD-*Co. camelliae*. The error bars represent standard deviations based on three biological replicates.

Table 3. Optimum mycelial growth temperature of the six isolates.

Isolates		Optimal Temperatures (°C)
YX-*Colletotrichum* spp.	YX-*Co. fructicola*	30
	YX-*Co. siamense*	28
	YX-*Co. camelliae*	25
GD-*Colletotrichum* spp.	GD-*Co. fructicola*	30
	GD-*Co. siamense*	30
	GD-*Co. camelliae*	30

Figure 7 illustrates that all of *Colletotrichum* spp. could grow at pH 3~10, but the growth was greatly limited when the pH was 3 or 10. The optimal pH for the growth of the six isolates was between 6 and 8, which indicates that *Colletotrichum* spp. can grow in acidic, alkaline and neutral environments. He (2016) [49] also found that the optimal growth pH of *Colletotrichum truncatum* was 5~8.

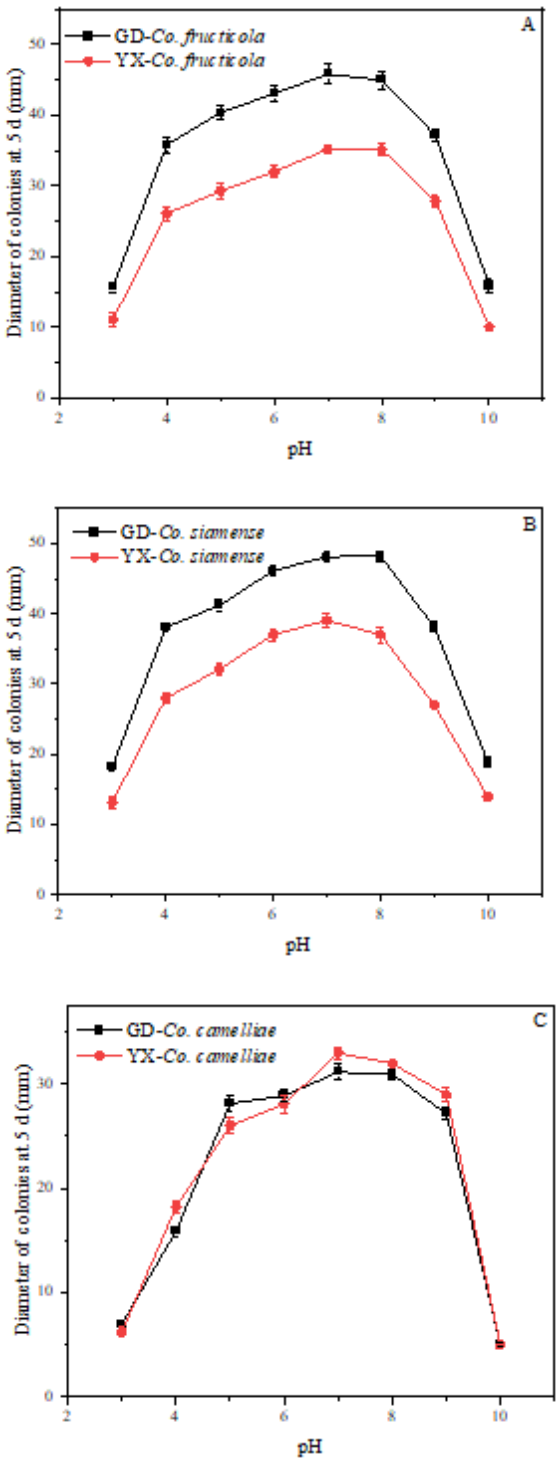

Figure 7. Effect of pH on the mycelial growth of (**A**) YX-*Co. fructicola* (YX2-5-2) and GD-*Co. fructicola*; (**B**) YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense*; (**C**) YX-*Co. camelliae* (2YX-5-1) and GD-*Co. camelliae*. The error bars represent standard deviations based on three biological replicates.

A previous study indicated that *Colletotrichum* spp. alkalinizes its surroundings during the colonization of host tissue [50,51]. Tardi-Ovadia (2017) [52] also found that the pH of the area infected by *Co. coccodes* and *Helminthosporium solani* increased from the native pH of approximately 6.0 for potatoes to 7.4 to 8.0, which proved that *Colletotrichum* spp. grow better in an alkaline environment. However, De Costa (2014) [53] found that the optimal pH for the mycelial growth of *Colletotrichum musae* was 4.5. These results prove that different species of *Colletotrichum* respond differently to environmental factors, such as pH. Consequently, it is necessary to explore the biological characteristics of YX-*Colletotrichum* spp.

An interesting phenomenon appeared in that the mycelial growth of YX-*Co. fructicola* (YX2-5-2) and YX-*Co. siamense* (2YX-8-1-1) was slower than that of GD-*Co. fructicola* and GD-*Co. siamense* at pH 3~10. We hypothesized that the existence of ions inhibits their growth. We further hypothesized that the enhancement of their pathogenicity may come at the cost of the reduction of ion resistance, just as YX-*Co. camelliae* (2YX-5-1) increased its pathogenicity at the cost of its ability to grow.

3.6. Effect of Carbon and Nitrogen Sources on Mycelial Growth

When glucose, sucrose and mannitol were used as carbon sources, there was no significant difference in the mycelial growth of GD-*Co. fructicola* and YX-*Co. fructicola* (YX2-5-2) (Figure 8A). When lactose was used as the carbon source, the mycelial growth of YX-*Co. fructicola* (YX2-5-2) decreased, and when soluble starch was used as the carbon source, the mycelial growth of YX-*Co. fructicola* (YX2-5-2) increased (Figure 8A). There was no significant difference between YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense* in the utilization of glucose, sucrose and soluble starch, but YX-*Co. siamense* (2YX-8-1-1) was less effective than GD-*Co. siamense* at utilizing lactose and mannitol (Figure 8B). Furthermore, compared with GD-*Co. camelliae*, YX-*Co. camelliae* (2YX-5-1) did not differ significantly in the utilization of glucose, sucrose and soluble starch, but YX-*Co. camelliae* (2YX-5-1) was significantly less effective than GD-*Co. camelliae* in the utilization of lactose and mannitol (Figure 8C). Therefore, there was no significant difference in the utilization of glucose, sucrose and soluble starch between YX-*Colletotrichum* spp. and GD-*Colletotrichum* spp. Nevertheless, the ability of YX-*Colletotrichum* spp. to utilize lactose and mannitol was generally less than that of GD-*Colletotrichum* spp. The reason for this phenomenon could be that Ca. *yuhsienensis* was difficult to infect and colonize. Thus, YX-*Colletotrichum* spp. may sacrifice some functions not normally used, such as the utilization of mannitol and lactose, to improve its pathogenicity.

When urea, NH_4Cl, casein tryptone and $NaNO_3$ were used as nitrogen sources, there was no significant difference in the mycelial growth of GD-*Co. fructicola* and YX-*Co. fructicola* (YX2-5-2). When NH_4NO_3 was used as the nitrogen source, the mycelial growth of YX-*Co. fructicola* (YX2-5-2) increased (Figure 9A). There was no significant difference between YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense* in the utilization of urea and $NaNO_3$, but YX-*Co. siamense* (2YX-8-1-1) was less effective than GD-*Co. siamense* at utilizing casein tryptone (Figure 9B). In addition, YX-*Co. siamense* (2YX-8-1-1) was significantly more effective than GD-*Co. siamense* at utilizing NH_4Cl and NH_4NO_3 (Figure 9B). YX-*Co. camelliae* (2YX-5-1) was less effective than GD-*Co. camelliae* at utilizing casein tryptone and $NaNO_3$ but slightly more effective at utilizing NH_4NO_3 (Figure 9C). These results illustrate that the utilization of NH_4^+ of YX-*Colletotrichum* spp. was generally stronger than that of GD-*Colletotrichum* spp., while the utilization of casein tryptone was generally less effective than that of *Colletotrichum* spp. We hypothesized that there are fewer proteins or amino acids that can be directly used in the leaves of Ca. *yuhsienensis*, but more NH_4^+, resulting in the stronger utilization of NH_4^+ by YX-*Colletotrichum* spp. Prusky (2001) [54] also believed that NH_4^+ was an important pathogenic factor of *Colletotrichum*. Therefore, the increased utilization of NH_4^+ may increase the pathogenicity of YX-*Colletotrichum* spp.

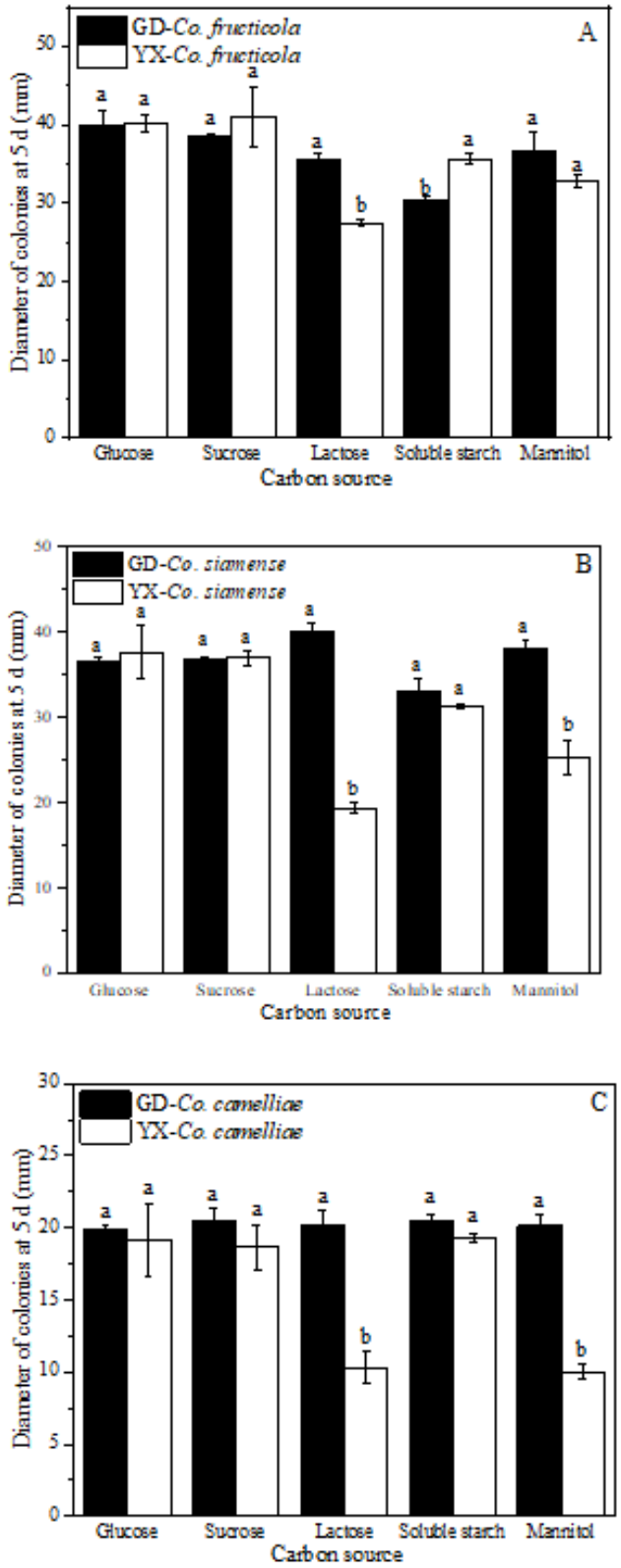

Figure 8. Effect of carbon sources on the growth of (**A**) YX-*Co. fructicola* (YX2-5-2) and GD-*Co. fructicola*; (**B**) YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense*; (**C**) YX-*Co. camelliae* (2YX-5-1) and GD-*Co. camelliae*. The error bars represent standard deviations based on three biological replicates. Diameters of colonies followed by the same lowercase letter are not significantly different at $p < 0.05$ using an ANOVA.

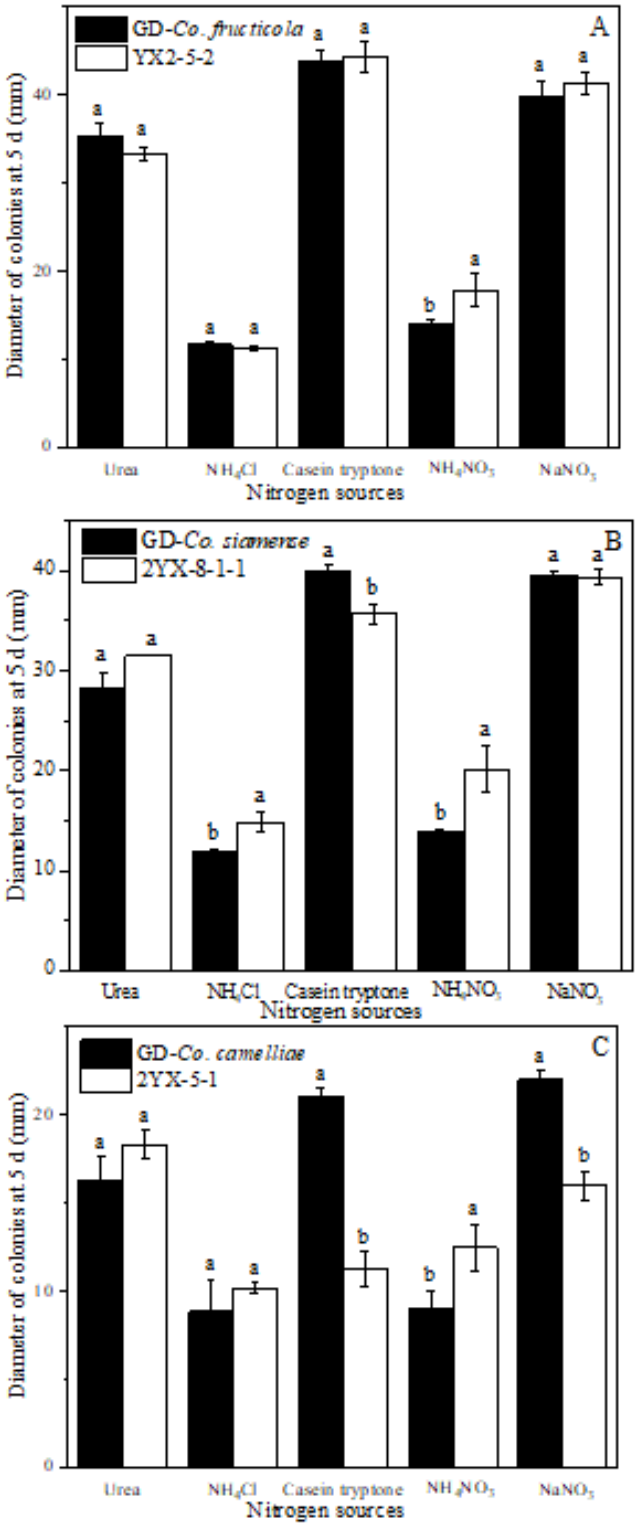

Figure 9. Effect of nitrogen sources on the growth of (**A**) YX-*Co. fructicola* (YX2-5-2) and GD-*Co. fructicola*; (**B**) YX-*Co. siamense* (2YX-8-1-1) and GD-*Co. siamense*; (**C**) YX-*Co. camelliae* (2YX-5-1) and GD-*Co. camelliae*. The error bars represent standard deviations based on three biological replicates. Diameters of colonies followed by the same lowercase letter are not significantly different at $p < 0.05$ using an ANOVA.

4. Conclusions

This study presents the first research on anthracnose in Ca. *yuhsienensis* leaves caused by *Colletotrichum* spp. in Hunan Province, China. Five isolates (YX2-5-2, 2YX-3-1, 2YX-5-1, 2YX-8-1-1 and 2YX-8-1-2), having the morphological characteristics of *Colletotrichum* spp., were obtained from Ca. *yuhsienensis*. A phylogenetic analysis demonstrated that YX2-5-2, 2YX-3-1 and 2YX-8-1-2 belonged to *Co. fructicola*, 2YX-8-1-1 belonged to *Co. siamense* and 2YX-5-1 belonged to *Co. camelliae*. A pathogenicity test indicated that the pathogenicity of YX-*Colletotrichum* spp. to Ca. *yuhsienensis* and Ca. *oleifera* was stronger than that of GD-*Colletotrichum* spp. The results of biological characteristics indicated that the mycelial growth and ionic resistance of YX-*Colletotrichum* spp. were generally less than that of *Colletotrichum* spp. Further research illustrated that the ability of YX-*Colletotrichum* spp. to utilize lactose and mannitol was less than that of *Colletotrichum* spp., while the ability to utilize NH_4^+ was generally greater than that in *Colletotrichum* spp. In summary, the results will provide theoretical guidance for the study of the pathogenesis and control schemes of anthracnose in Ca. *yuhsienensis*.

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