Quality Assurance and Safety of Crops & Foods, 2022; 14(1): 92–101

Isolation and identification of *Colletotrichum* as fungal pathogen from tea and preliminary fungicide screening

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Received: 4 November 2021; Accepted: 6 February 2022: Published: 24 March 2022
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RESEARCH ARTICLE

Abstract

The increasing incidence of anthracnose disease in tea plant caused by *Colletotrichum* has become an important global concern. It is the cause of withered leaves of the tea plant, leading to a considerable decrease in the economic yield of tea, and thereby threatening the sustainable development of tea industries. In this study, *Colletotrichum* infected tea leaves were collected from three separate regions: Zhouning, Longyan, and Ningde in Fujian province, China. The pathogen was isolated from the leaves and identified based on morphology following Koch's postulates and DNA sequencing of the nuclear rDNA internal transcribed spacer (ITS) region, beta-tubulin 2 (β-Tub2), the large sub-unit of the nuclear ribosomal RNA gene (LSU), Glutamine synthetase (GS), and an oligonucleotide primer (CgInt). A total of six strains were identified with different cultural, morphological, and molecular characteristics. Furthermore, multi-locus phylogenetic analysis showed the six strains that belong to *C.fructicola*. Finally, a preliminary screening of nine chemical fungicides to inhibit the strain N by toxicity test identified 40% prochloraz at 0.1 μg mL^{-1} as the most effective method. The phylogenetic tree analysis revealed a close relationship between the identified strains, and the strains were classified based on cultural, morphological, and molecular characteristics. The findings of this study add to our understanding of *C.fructicola*, which will aid in the development of preventive measures, the improvement of tea quality, and the assurance of safe production.

Keywords: anthracnose; *Colletotrichum*; fungicide screening; isolation and identification; tea

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the most economically important crops in the world. Tea enriched with various beneficial bioactive constituents have been proven to effectively reduce the risk of several diseases in human beings (Rahmani *et al.*, 2015). However, the biotic stress in tea that reduces its economic yield has become a major concern in tea-growing regions globally. According to the preliminary statistics, the occurrence and prevalence of diseases in tea were vary by region. It is more serious in the southern region than in the northern region of China. (Liu *et al.*, 2013).

Anthracnose disease caused by *Colletotrichum theaesinensis* is found widely in the tea regions globally, and is one of the main diseases of tea plants in China (Liu *et al.*, 2015; Lu *et al.*, 2018; Wang *et al.*, 2016; Weir *et al.*, 2012). During 2011–2012, anthracnose caused by *C. gloeosporioides* was observed in about half of the tea plant fields in the Yellow Mountain region in the Anhui Province of China. The symptoms of the disease were observed with

 ISSN 1757-837X online, DOI 10.15586/qas.v14i1.1000

small water-soaked lesions in young leaves at the initial stage. As the disease progresses, the lesions become larger and necrotic, finally leading to serious losses in yield (Guo *et al*., 2014). The disease is also found in the tea regions in Yabukita, Japan, and has been reported to harm tea seedlings and machine-picked or trimmed leaves (Yoshida *et al*., 2010). The trend of its increasing incidence in tea regions, and the harmful effects on the quality and quantity of economic yield has imposed a raised an alarm on the sustainable development of tea industries. *C.camelliae* and *C.fructicola* were the species most often isolated, and were proposed as the dominant pathogens of tea (Lu *et al*., 2018; Wang *et al*., 2016). Therefore, it is expected that the taxonomic study of *Colletotrichum* species in tea plants would be of great significance for the prevention and control of anthracnose disease in tea.

Morphological characteristics and understanding of its host range are important for identifying the strains and distinguishing the relationships in *Colletotrichum* species. It has been shown that the same anthracnose fungus has a wide host range. Therefore, the occurrence of homologous synonyms over the years has always led to controversy in the taxonomy of this genus (Noireung *et al*., 2012). With the rapid development of molecular biology techniques, phylogeny has become an important approach to decipher the exact fungal taxonomy. In particular, the application of polygenic locus phylogeny has played a significant role in identifying the different species of anthracnose fungus and deciphering their taxonomic relationships. At present, the anthracnose causing fungi in proteaceae (Liu *et al*., 2013), guava (Oliveira *et al*., 2018), chili (Diao *et al*., 2017), strawberry (Hirayama *et al*., 2018), mango, and papaya (Oliveira *et al*., 2018) have been systematically studied.

In this study, we aim to isolate and identify the main pathogenic isolate of *Colletotrichum* in Zhouning, Longyan, and Ningde (Fujian province, China), as well as evaluate the main pathogen's susceptibility to nine commercially available chemical fungicides. This results would aid in the development of preventive measures, the improvement of tea quality, and the assurance of safe production.

Materials and Methods

Tea leaf sample collection

Colletotrichum infected tea leaves were collected from the tea plantations of Fujian Zhouning Guikelai Organic Tea Co., Ltd. (North latitude 27°6′18″, east longitude 119°20′0″), Fujian Longyan Zhangping County Hung Ding Tea Co., Ltd. (North latitude 25°3′6″, east longitude 117°16′16″), and Fujian Ningde Jiulongfeng Agricultural Development Co., Ltd. (North latitude 26°43′5″, east longitude 119°28′11″) from July 2016 to December 2017. Fujian Zhouning Guikelai Organic Tea Co., Ltd., Fujian Longyan Zhangping County Hung Ding Tea Co., Ltd., and Fujian Ningde Jiulongfeng Agricultural Development Co., Ltd. approved the field site access to this work. The collection sites and times were marked and recorded.

Isolation, purification, and morphological properties of fungal tea pathogen strains

The collected tea leaves with the symptom of the disease were rinsed for 30 min, dried, disinfected with 75% alcohol and 1% mercuric chloride, and rinsed three times with sterile water. The leaves with the lesions were then cut in the dimension of 1 cm×1 cm and inoculated into potato dextrose agar (PDA) containing 1% ampicillin (antibiotic), and placed at 26°C for cultivation (Cai *et al*., 2009). The pathogen was identified following the guidelines described in the Fungus Identification Handbook (Wei, 1979) after isolation and purification. The purified *Colletotrichum* strains were separately inoculated into PDA, oat agar (OA), carrot glucose agar (CA), and Czapek-Dox media (Czapek), and cultivated at 26°C for 7 days. The colony diameter was measured using the cross intersect method, and their biological characteristics including growth pattern, change in color, and spore production were observed.

Tie-back measurement of pathogenicity

For the measurement of pathogenicity, the wound inoculation method following Koch's postulates was used. Several fresh and healthy tea leaves of the same size (7 cm × 4 cm) were selected and rinsed with sterile water, disinfected with 75% alcohol, and placed in a culture dish containing cotton balls or filter papers soaked in sterile water. After separation and purification, a puncher was used to obtain 5 mm fungal tablets from the purified pathogens, which were subsequently inoculated onto the wounds made on the leaves by puncturing it through a sterile inoculation needle. The punctured leaves without the pathogen inoculations were used as the control group. All samples were then kept in a moist environment at a stable 26°C.

After five days, the disease symptoms were recorded, and the pathogenic fungal strains were isolated on the emergence of new scabs (Fang, 2001).

PCR detection

The total genomic DNA of the six strains were extracted using Rapid Plant Genomic DNA Isolation

Kit (Sangon Biotech, Shanghai, China), and stored at −20°C. The ITS, LSU, β-tub, GS, and CgInt were amplified (Table 1). The protocols for amplification was carried out in 25 μL reaction volumes comprising 2.5 μL 10×Taq PCR buffer, 0.5 μL dNTP mix (10 mM each), 0.3 μL Taq DNA polymerase (5 U μL^{-1}), 1 μL genomic DNA, 1 μL of each primer (10 μM), and 18.7 μL ddH_2O. The sample was then amplified in a T100 PCR meter using the following reaction conditions. Predenaturation at 94°C for 10 min, denaturation at 94°C for 5 s, annealing for 45 s (the temperature for each primer are shown in Table 1), extension at 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 10 min. The PCR products resolved by 1% sepharose electrophoresis were sent to Fuzhou Boshang (Shangchen) Biotech Co., Ltd. for sequencing (Liu, 2013; Mills *et al*., 1992). The phylogeny tree for each gene of the fungal strains was constructed by using the neighbor-joining bootstrap method (1000 repetitions).

Preliminary of tested fungicides against the pathogenic strain N

The N strain was used to assess the efficiency of the chemical fungicides. The inhibitory activity of the strain was determined by using nine different fungicides (Table 2). A 5 mm tablet of the selected strain was prepared and inoculated into the center of each medium containing the fungicides at different concentrations. The control group was treated with sterile water, placed at 26°C, and cultured for five days. The cross intersect method was used to measure the growth diameter of the colony and determine the inhibition rate of the concentrations of each fungicide (Marais, 1990). The inhibition rate was calculated using the equation mentioned below:

$$\text{Inhibition rate} = (d_{Control} - d)/d_{Control} \times 100\%$$

where, $d_{Control}$ is the colony diameter of the control group, and d represents the colony diameter of the groups treated with different fungicides.

Using the logarithmic value of the processing concentration ($\mu g\ mL^{-1}$) as the horizontal axis and the corresponding inhibition rate as the ordinate, the toxicity equation was constructed as follows: y = ax + b. The correlation coefficient (r) and the effective concentration (EC_{50}) was calculated, and finally compared the effects of different fungicides on the growth of strain N based on their EC_{50} values.

Results

Identification and characteristics of the isolates

In total, six *Colletotrichum* isolates were obtained from the tea growing regions in China (Zhouning, Ningde and Longyan). These isolates were isolated from the diseased tissues. In the main tea regions of the East Fujian province, *Colletotrichum* was widely distributed.

The mycelia of the pathogenic strains were initially observed to be white on the PDA medium, and then became sparse to dense velvet over time. The center of the colony was gray, light-brown, or yellow, and the back was wine red (Figure 1). The conidiophores were either oval or long oval, and black particles were present on the surface. Some particles were binuclear. No gap was observed between the spores. According to Wei Jingchao's Fungus

Table 1. Specific primers and annealing temperatures.

Primer name	Primer sequences (5′–3′)	Annealing temperature (°C)
ITS1	TCCGTAGGTGAACCTGCGG	55
ITS4	TCCTCCGCTTATTGATATGC	
LSU-F	GCATATCAATAAGCGGAGGAAAAG	57
LSU-R	GGTCCGTGTTTCAAGACGG	
β-Tub2F	GGTAACCAAATCGGTCCTGCTTTC	57
β-Tub2R	ACCCTCAGTGTAGTGACCCTTGGC	
GS-F	ATGGCCGAGTACATCTGG	58
GS-R	GAACCGTCGAAGTTCCAC	
CgInt	GGCCTCCCGCCTCCGGGCGG	57
ITS4	TCCTCCGCTTATTGATATGC	

Table 2. List of chemical fungicides used in this study.

Fungicide	Production factory
75% Chlorothalonil wettable powders	Guangdong Zhongxun Agri-science Corporation
10% Difenoconazole water dispersible granule	Qingdao John Sheng Biological Technology Co., Ltd.
50% Cyprodinil water-dispersible granule	Jixi Nonghua Biological Technology Co., Ltd.
70% Cao Tuo thiophanate-methyl	Jiangxi Zhongxun Agrochemical Co., Ltd.
50% Chloroisobromine cyanuric acid	Hebei Shangrui Chemical Co., Ltd.
25% Triadimefon wettable powders	Chengdu Kelilong Biochemical Co., Ltd.
80% Carbendazol	Jiangsu Taicang agrochemical Co., Ltd.
40% Prochloraz water emulsion	Dongguan Ruidefeng Biological Technology Co., Ltd.
50% Azoxystrobin suspension	Shandong Haixun Biochemical Co., Ltd.

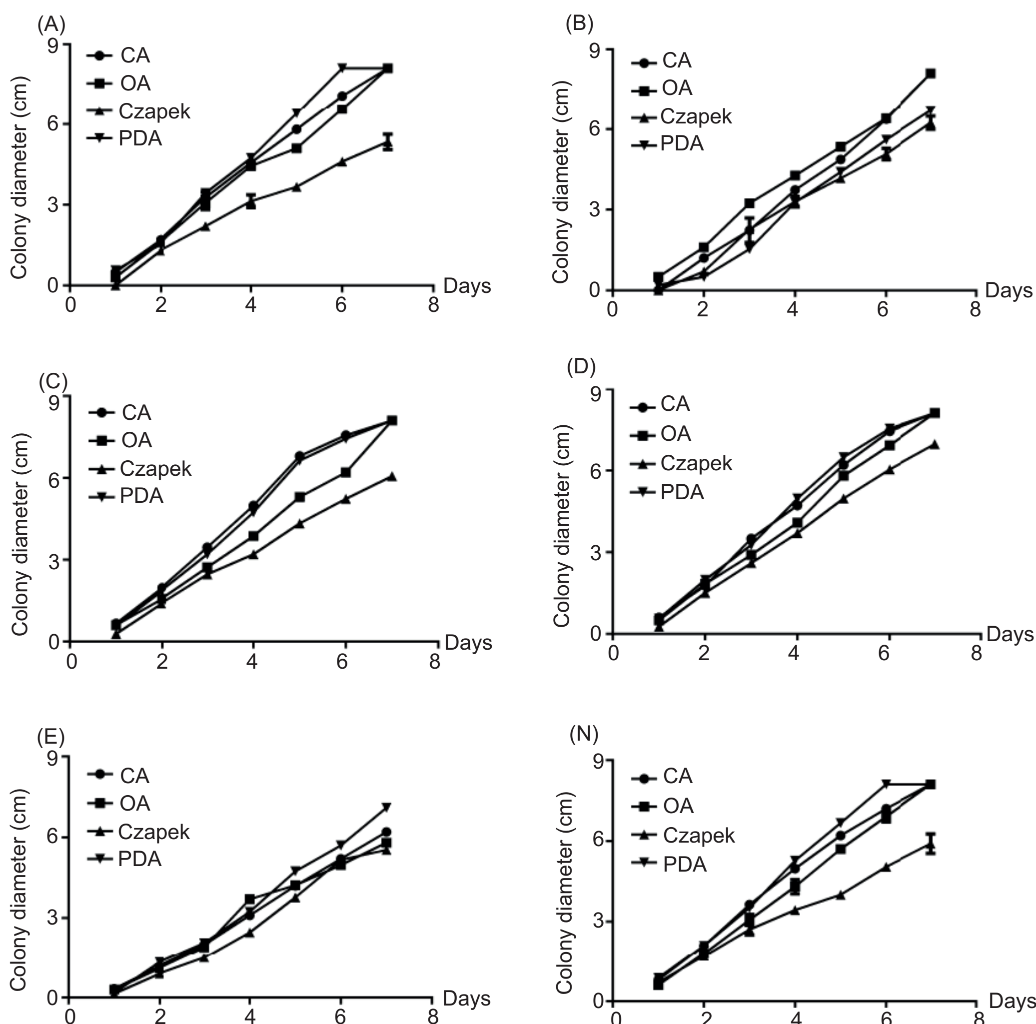

Figure 1. Effects of different media on anthrax mycelium (n = 3). carrot glucose agar (CA), oat agar (OA), Czapek-Dox media (Czapek) and potato dextrose agar (PDA).

Identification Handbook, the pathogen strains preliminarily belong to *Colletotrichum*.

As shown in Figure 1, strains A, D, E, and N exhibited rapid growth on the PDA, and slow growth on the Czapek-Dox medium, indicating that the PDA medium is the most suitable for the growth of these strains. On the contrary, B and C strains exhibited rapid growth on the OA and CA medium.

The purified pathogenic strains (A, B, C, D, E, and N) cultivated on the CA medium for seven days revealed round colonies with neat edges and blanket forms. The orangeexudates secreted in the medium were the spores with a flocculent surface. The mycelia of strains A, B, and D were dense, and the mid-front was grayish brown, and the back was rice white. In contrast, the mycelia of the strains C, E, and N were sparse, and the mid-front and back were grayish green, as shown in Figure 2. After cultivating in an OA medium for seven days, round colonies of the strains with neat edges were formed. The mycelia were sparse and transparent, and were attached to the medium. The exudations in the middle and back of the spores were colorless. After cultivating the strains on PDA medium for seven days, the colonies formed were round with neat edges, and were flocculent. The orange secretions in the middle were bulging spores. The strains A, B, and C exhibited melanin pigmentation on their back parts. The front parts of strains A, C, E, and N were taupe, whereas those of strains B and D were beige. The strains cultivated in the Czapek medium for seven days formed irregular colonies. The hyphae were dense, tapetum, and white. The mycelia with beige back grew slowly in the Czapek medium. The spores of the strain A, B, C, D, E, and N showed black particles on the surface.

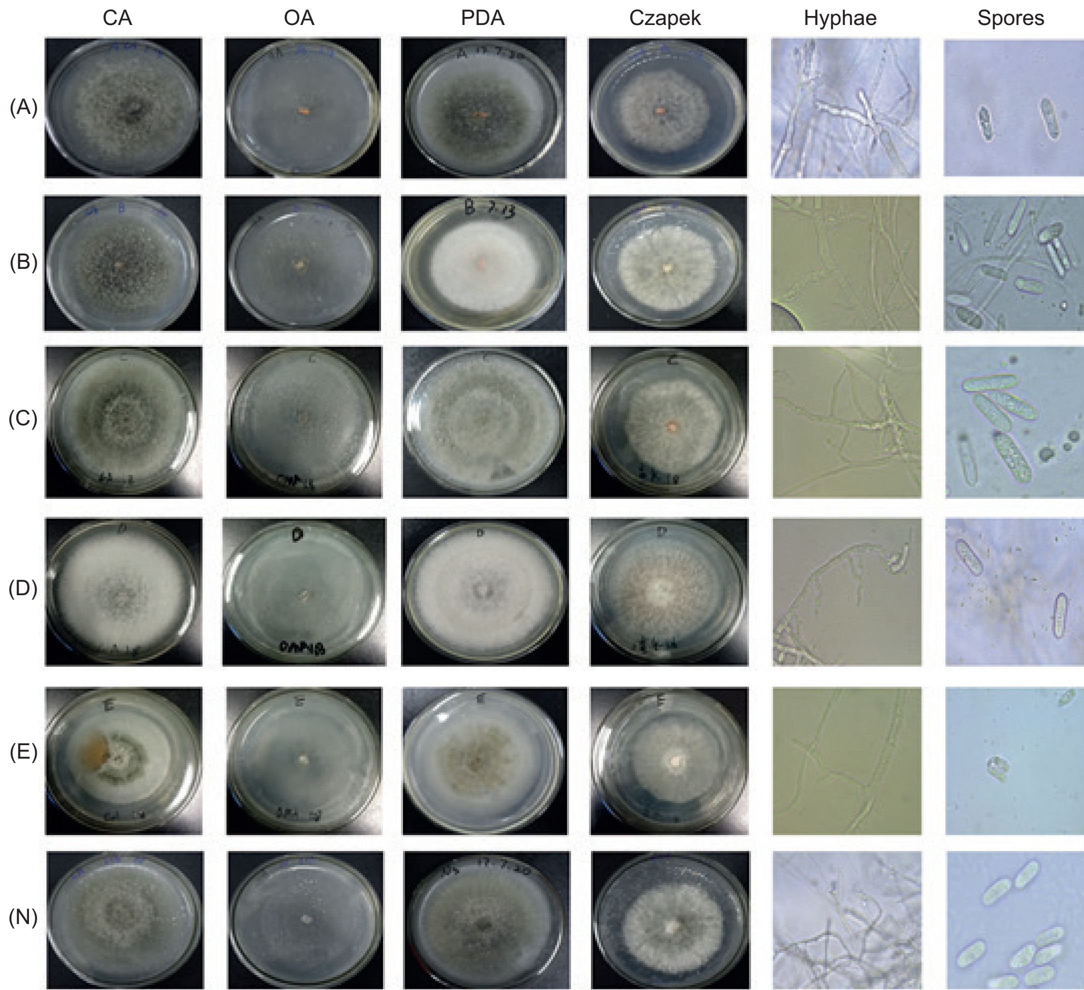

Figure 2. Morphological identification of anthrax (A, B, C, D, E, N).

Determination of pathogenicity of the isolated strains

The fungal pathogens were inoculated into the healthy tea leaves. It was inoculated continuously for five days, the disease symptoms were observed in a small point at the center of the puncture, which formed brown or black-brown disease spots with the increase in the number of days of cultivation. The symptoms were similar to those of the natural anthracnose disease in the field. Then, the samples were isolated by the conventional tissue separation method. The culture and morphological characteristics of the purified strains and the strains used for the inoculation were the same. The pathogenic strains were preliminarily determined by observing the spores under a microscope. Finally, the same pathogen was separated from the leaves with typical symptoms and it was observed that it fulfilled Koch's postulates. Based on the symptoms, the morphological characteristics, and pathogenicity, these six strains were identified as *Colletotrichum*. The N strain was capable of developing the most serious characteristic symptoms of anthracnose as compared to the other strains, whereas the control leaf remained symptomless, which revealed that the N strain was the major pathogen.

Specific sequence PCR amplification

The amplified products of the ITS, LSU, β-tub, GS, and CgInt domains from the six strains using the specific primers are shown in Figure 3. The obtained amplicon sizes of ITS *(*~500 bp), LSU (~500 bp), β-tub 2(500~750 bp), GS (500~750 bp), and CgInt (~1000 bp) matched the expectations. However, some strains failed to amplify the exact product sizes as shown in Figure 4.

Building an evolutionary tree

Bi-directional sequencing was used to determine the sequences, and the sequences ITS, LSU, β-tub, GS, and CgInt were compared using the Basic Local Alignment

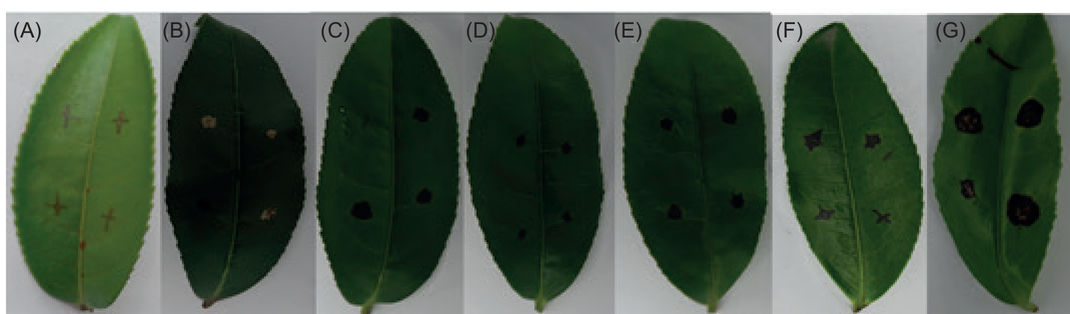

Figure 3. 6 Strains infect the tea leaves. (A) is CK; (B-G) were A, B, C, D, E, N strains that infect the tea leaves.

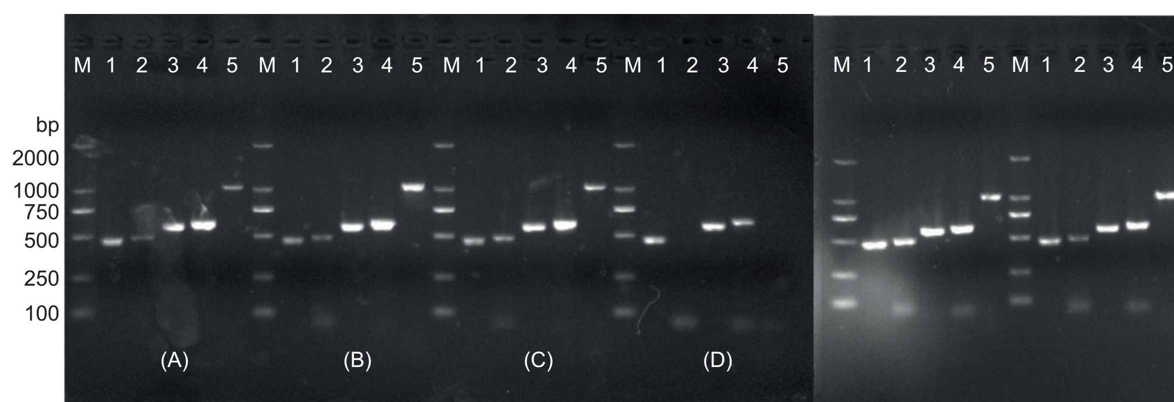

Figure 4. Primer amplification electrophoresis figure of the specificity of *Colletotrichum*. M is the marker; 1, 2, 3, 4, and 5 are the amplified fragments of ITS, LSU, β-tub 2, GS, and CgInt, respectively. A, B, C, D, E, and N are the six objective strains.

Search Tool (BLAST) program in the GenBank database. The accession numbers were as follows: 090620, 090621, 094064, 094065, 147730, 147731, 147732, 147733, 147734, 273211, 188770, 188771, 188772, 147824, 147825, 147826, and 149118. The sequences of the genus and species with high similarity with the sequences of the strains were downloaded from the database, and a phylogenetic tree was built using MEGA7.0. The neighbor-joining bootstrap method (1000 repetitions) was adopted for the analysis. As shown in Figure 5, the evolutionary tree belonged to *C.fructicola*, and different species were located on different branch ends. The strains with close relationships were gathered together on different levels. These results indicate the accuracy of the identification methods. From cultural, morphological, molecular, and pathogenicity, the characteristics of the isolated strains of fungal tea pathogen, the strains were classified under *Colletotrichum* as *C.fructicola*.

Preliminary screening of the chemical control of anthracnose pathogen

The efficacies of nine the fungicides on the inhibition of mycelial growth of the N strain was assessed by measuring the growth diameter of their colonies, which revealed different inhibitory effects of the fungicides on the isolated strains (Table 3). Specifically, the treatment with 40% prochloraz at 0.1 μg mL^{-1} (minimum concentration) did not form any colony on the plate, indicating that it was the most effective fungicide against N strains of anthracnose fungus. EC_{50} of 50% azoxystrobin suspension and 50% cyprodinil water dispersible granule were 1.63 μg mL^{-1}, 17.54 μg mL^{-1}, which exhibited a good inhibition effect, whereas 80% carbendazol exhibited the worst effect. However, the average value of the measurement was larger than that of the control group, and the inhibition effect was lacking, possibly because the configuration concentration was extremely low. Therefore, the minimum concentration will have to be re-optimized in the future.

Discussion

Colletotrichum is a commonly reported genus, and it causes anthracnose in numerous plants worldwide (Diao *et al*., 2017; Hirayama *et al*., 2018; Kumita *et al*., 2021; Oliveira *et al*., 2018). Morphologically-based identification of *Colletotrichum* species has always been problematic, because there are few reliable characters and many of these characters are plastic, and dependent upon

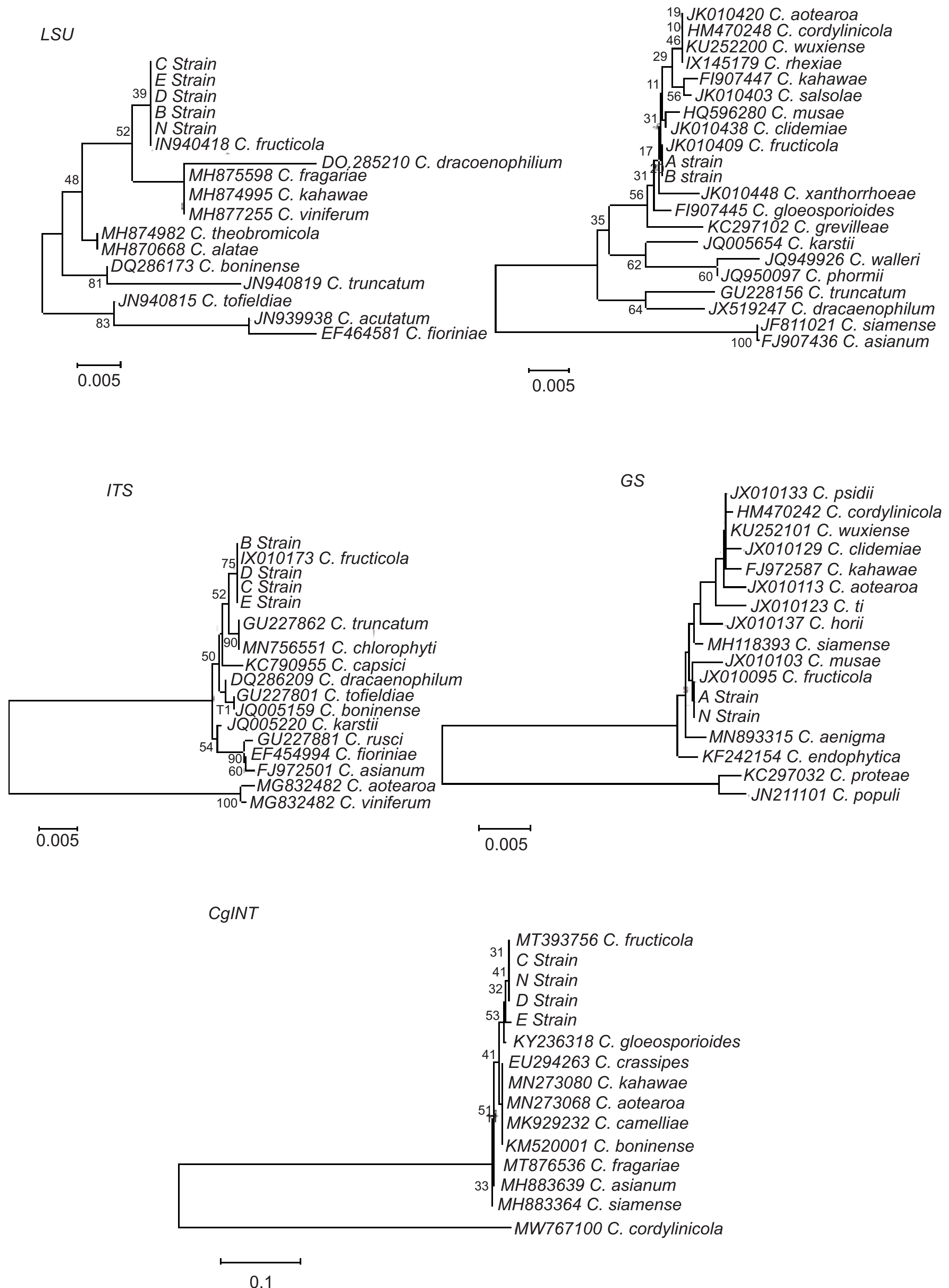

Figure 5. Phylogenetic tree on the basis of the gene sequence analysis

Table 3. Measurement of inhibitory effects of different fungicides against the growth of N strain in the laboratory conditions.

Sl no.	Fungicide	Concentration (μg/mL)	Inhibition rate (%)	Regression	EC_{50} (μg/mL)	Correlation coefficient
1	75% Chlorothalonil wettable powders	40.0	35.22 ± 0.36	y = 0.1306x + 0.1428	543.34	0.9929
		100.0	40.59 ± 0.69			
		130.0	41.30 ± 0.52			
		160.0	43.42 ± 0.48			
2	10% Difenoconazole water dispersible granule	66.7	20.51 ± 0.85	y = 0.3977x − 0.4771	286.34	0.9473
		100.0	33.52 ± 0.08			
		200.0	50.35 ± 0.13			
		500.0	55.73 ± 0.28			
3	50% Cyprodinil water dispersible granule	0.1	2.26 ± 0.17	y = 0.2143x + 0.2334	17.54	0.9831
		1.0	19.24 ± 0.82			
		10.0	51.91 ± 0.65			
		100.0	62.80 ± 0.35			
4	70% Cao Tuo Thiophanate-methyl	0.1	17.54 ± 0.19	y = 0.016x + 0.1762	1.7E	0.7044
		1.0	16.55 ± 0.27			
		10.0	16.83 ± 0.23			
		100.0	22.77 ± 0.5			
5	50% Chloroisobromine cyanuric acid water soluble powder	200.0	7.59 ± 0.95	y = 0.6326x − 1.4306	1126.68	0.8971
		400.0	13.96 ± 0.48			
		600.0	26.45 ± 0.51			
		800.0	49.32 ± 0.43			
6	25% Triadimefon wettable powders	0.1	16.55 ± 0.2	y = 0.0731x + 0.2057	10616.75	0.8555
		1.0	19.38 ± 0.25			
		10.0	20.37 ± 0.18			
		100.0	40.59 ± 0.33			
7	80% Carbendazol	533.0	0			
		800.0	0			
		1600.0	0			
		4000.0	0			
8	40% Prochloraz water emulsion	0.1	100%			
		1.0	100%			
		10.0	100%			
		100.0	100%			
9	50% Azoxystrobin suspension	0.1	37.06 ± 0.1	y = 0.1169x + 0.4751	1.63	0.9843
		1.0	47.52 ± 0.35			
		10.0	55.45 ± 0.48			
		100.0	73.40 ± 0.39			

methods and experimental conditions. Therefore, DNA sequence analysis has become an important auxiliary approach which has been widely used in the identification and diversity analysis of several fungal species. The six strains isolated in this study demonstrated distinguishing morphological and cultural characteristics. Furthermore, the amplification of the ITS, LSU, β-tub2, GS, and CgInt gene domains from these strains amplified the clear bands as expected. Subsequent sequencing and homologous alignment analysis of the PCR products showed that the degree of similarity between the sequencing results obtained in this study and sequence homology reported by other authors was >97% (Taylor *et al.*, 2000). The phylogenetic tree based on the sequences of these genes showed that the strains A, B, C, D, E, and N could be clustered to *C.fructicola*.

The study of fungal classification and identification by nucleic acid sequence analysis, especially in the fungal ribosome transcription spacer (rDNA-ITS), has been

widely used. ITS is undeniably the best gene used for the classification, and the preferred sequence for fungus bar code engineering (Nilsson *et al*., 2008). The hypervariable regions in ITS are extremely similar. Hence, for some complex species, ITS cannot provide enough differentiation and support the result. Moreover, the sequences of the different strains in the GenBank are uploaded by different researchers obtained from different studies worldwide, where there is a possibility of errors depending on the works. Therefore, a focus on the other gene sequences with sufficient interspecies resolution such as actin (ACT), calmodulin (CAL), 3-glyceraldehyde phosphate dehydrogenation (GAPDH), chitin synthase 1 (CHS-1), β-TUB2, LSU, and GS have gained increasing research interest. Liu *et al.* used the morphological identification and the sequence of seven genes to reveal that strains of the *C.gloeosporioides* complex associated with *Proteaceae* belong to four known species (*C.alienum*, *C.aotearoa*, *C.kahawae*, *C.siamense*) and two new taxa (*C. proteae* and *C. grevilleae*) (Liu *et al*., 2013). They then unraveled the phylogenetic diversity of 144 *Colletotrichum* isolates associated with symptomatic and asymptomatic tissues of *C. sinensis,* and other *Camellia* spp. from the seven provinces of China and seven isolates obtained from other countries. Based on the multi-locus phylogenetic analyses and phenotypic characters, 11 species were distinguished (Liu *et al*., 2015). Wang *et al*. (2016) collected 106 *Colletotrichum* isolates from 15 main tea production provinces in China. A multi-locus phylogenetic analysis coupled with morphological identification showed that the isolates belonged to 11 species, including six known species, three new record species, one novel species, and one indistinguishable strain. It is believed that the identification of strains by morphological characteristics, combined with the phylogenetic analysis based on multiple genes, unravels the evolution process of the species closely and determines their taxonomic status more accurately and scientifically. In this study, six isolated strains were accurately and rapidly identified as *C.fructicola* based on the traditional morphological and molecular methods. The data has been deposited in the National Center for Biotechnology Information (NCBI) database, which could facilitate the comparative analyses of the experimental results of different research teams in different countries.

The pathogenicity of the six strains were tested on the tea leaves. The N strain was capable of developing the most serious characteristic symptoms of anthracnose than the other strains, which revealed that the N strain was the major pathogen. Among the nine tested chemical fungicides, three fungicides (40% prochloraz water emulsion, 50% azoxystrobin suspension, and 50% cyprodinil water dispersible granule) were significant, while the remaining six revealed comparatively weak inhibitory effects. The 40% prochloraz showed the greatest activity in inhibiting the growth of the N strain. Prochloraz is an imidazole fungicide which is widely used around the world in gardening and agriculture to control the growth of the fungi (Vingaard *et al*., 2006). Gutiérrez-Alonso *et al*. (2003) showed that *C.gloeosporioides* is highly sensitive to this fungicide because it acts over the synthesis of ergosterol, the main component of the plasma membrane, so that its absence increases the permeability of the membrane, thereby interrupting the fungal growth (Walsh *et al*., 2004). Reyes-Estebanez *et al.* (2019) showed that only prochloraz inhibited up to 83% of *C.gloeosporioides* growth, which was the best result of inhibition among the treatments.

Conclusions

In this study, the six strains of fungal pathogen isolated from the tea leaves which were identified by cultural, morphological, molecular approaches, and pathogenicity as *C.fructicola*. Strain N was selected for fungicide susceptibility with the efficacy of 40% prochloraz at 0.1 μg mL^{-1} as the most effective method for the prevention and treatment of *C. fructicola*.

Competing Interests

The authors declare there are no competing interests.

Funding Statement

This study was supported by the Postdoctoral Science Foundation of Fujian Agricultural and Forestry University, and the project of Ningde Normal University (2020Z01, 2020Q101, and 2019T02).

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