

Characterisation of *Colletotrichum siamense* TD1 causing anthracnose leaf spots of *Camellia tamdaoensis* Ninh et Hakoda at the Tam Dao National Park

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Abstract:

Camellia L. is a precious medicinal plant with 16 different species and high economic value. In Tam Dao National Park, Vinh Phuc, *Camellia* L. is rich in ingredients. Anthracnose is a severe disease caused by fungi on the leaves of *Camellia* L., however, research of the fungus causing anthracnose on leaves of *Camellia* L. in Vietnam remains limited. In this study, anthracnose-infected leaves of *Camellia tamdaoensis* Ninh et Hakoda in Tam Dao National Park were collected, and the causative agent was isolated. Eleven fungal strains that cause anthracnose were obtained through gene sequence identification of the internal transcribed spacer (*ITS*) region. One fungal strain was selected to isolate DNA and determine the gene sequence of the *ITS* region. Based on this *ITS* sequence, the causative agent on leaves of *C. tamdaoensis* Ninh et Hakoda was *Colletotrichum siamense* TD1. The *ITS* sequence was determined to be 522 bp in length, 52.30% of guanine-cytosine (GC) content, and was registered on GenBank with accession number OK560718. Pathogenicity tests were conducted to fulfil Koch's postulates. This is the first report of anthracnose leaf spots of *C. tamdaoensis* Ninh et Hakoda caused by *C. siamense* TD1 in Vietnam. The results of anthracnose fungi provide helpful information for disease management and are necessary for future studies on selecting disease-resistant varieties of *Camellia* L.

Keywords: anthracnose, *Camellia tamdaoensis* Ninh et Hakoda, *Colletotrichum siamense*, golden camellia, Tam Dao National Park.

Classification numbers: 3.1, 3.4

1. Introduction

Golden camellia belongs to the *Camellia* L. genus, *Theaceae* family. They are shrubs and small-sized trees that are naturally distributed in Vietnam. Its benefits are well-known in traditional medicine for improving human health. In Tam Dao, a large number of *Camellia* species were discovered in the National Park and natural zone [1]. Especially, *C. tamdaoensis* Ninh et Hakoda is one of the many unique plants scattered in the forest of this mountain. In recent years, anthracnose has become one of the most severe fungal diseases globally and severe anthracnose symptoms can be seen on the leaves of the *Camellia* genus. The first observed symptom appears as random chlorotic spots on leaves after which they coalesce and form large spots. The visual spots become dark brown or black depending on damage level [2]. In the past, morphological characters and host ranges were mainly used to classify the *Colletotrichum* species [3]. They were conidial, conidiophore, and acervuli morphology or sensitive to benomyl [4, 5]. However, it is challenging to identify taxonomies based on the morphological differences of *Colletotrichum* species because their morphological characteristics were strongly affected by both laboratory and

natural conditions. Currently, molecular tools give biologists many advantages to rapidly recognise *Colletotrichum* species at the genus level [3]. The nuclear *ITS* regions have been used to separate *Colletotrichum* species [4]. In this study, we aimed to collect a leaf with a disease related to anthracnose of *C. tamdaoensis* Ninh et Hakoda and describe the symptoms of this disease. Then, the fungal pathogen was isolated on a specific medium and molecular identification was performed based on sequencing the *ITS* sequence. Consequently, *C. tamdaoensis* Ninh et Hakoda was reinfected with the fungus to test pathogenicity ability.

2. Materials and methods

2.1. Collection of the disease leaf and describe the symptoms

C. tamdaoensis Ninh et Hakoda plant with anthracnose leaf spots were obtained from the Tam Dao National Park, Tam Dao district, Vinh Phuc province in May 2021. The symptoms of anthracnose disease were observed with an increase from small brown spots to black necrotic spots appearing on the leaf and stem. Diseased leaves of *C. tamdaoensis* Ninh et Hakoda were taken from these infected plants, packed in a

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cooler box, and transported to the laboratory of The Institute for Science and Application, Hanoi Pedagogical University 2.

2.2. Fungal isolation

Disease spots on leaves of *C. tamdaoensis* Ninh et Hakoda with typical symptoms of anthracnose were cut off and washed with 75% (v/v) ethanol for 30 s, with 5% (v/v) sodium hypochlorite for 1 min, which was followed by rinsing with sterilised water three times, and finally laid to dry on sterile filter paper [6, 7]. The explants were placed onto Petri plates containing Potato dextrose agar (PDA) medium and incubated at 25°C. Isolates were subcultured aseptically to fresh PDA.

2.3. Extraction of fungal DNA and PCR amplification for ITS and sequencing

The total DNA from fungal isolates was extracted by cetyltrimethylammonium bromide (CTAB) method where mycelia served as explants [8]. Amplification of the *ITS* region was performed using the primers *ITS1* and *ITS4* [9]. Primers were synthesised by Phusa Co., Ltd. (Can Tho, Vietnam). Amplification was performed in a PCR Thermal Cycler (TP600, Takara). The PCR products were purified and sequenced at the National Key Laboratory of Gene Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

2.4. Molecular analysis

The sequence of *ITS Colletotrichum* isolate in this study was aligned using Bioedit software (version 7.2.5) and submitted to GenBank under accession number OK560718. To study it in more detail, nine *ITS* sequences of *Colletotrichum* species were retrieved from NCBI and used to compare the GC content and length, and to construct the phylogenetic tree using Molecular evolutionary genetics analysis (MEGA) (ver 7.0) with the maximum likelihood method with 1,000

bootstrap replicates. The species-level of the isolate was identified based on phylogenetic approaches [10].

2.5. Pathogenicity test

Colletotrichum sp. was tested on detached leaves of *C. tamdaoensis* Ninh et Hakoda under artificial conditions. *Colletotrichum* sp. was incubated on PDA plates for 7 days at 25°C. Sterile distilled water was adjusted to the conidial suspension to 2.0×10^6 conidia/ml. Fresh young leaves without disease were used for inoculation. The leaves were washed with running water, were sterilised with 75% (v/v) ethanol, and finally rinsed in sterile water. Afterward, detached healthy leaves were placed on a plastic box with moist tissue papers. A sterilised needle was used to make wounds on the surface of the fine leaf. Each wound was inoculated with a 100 μ l conidial suspension, and sterile distilled water served as the control.

The enamel basins were sealed by plastic film and incubated in a growth chamber at 25°C. Virulence was assessed by measuring lesion length at 8 days post inoculation (dpi) in two perpendicular directions on each leaf.

3. Results

3.1. Symptoms of leaf camellia species disease and isolation

Typical anthracnose symptoms in *C. tamdaoensis* Ninh et Hakoda were clearly expressed on leaves whose necrosis spots were dark brown or black. The results showed that the symptoms occurred on two sides of the diseased leaves (Figs. 1A, B). Firstly, the disease spots usually appear small and brown. Then, these brown spots spread and the color changes. These symptoms on the disease leaf of *C. tamdaoensis hakodae* in this study were the same as the previously described study on *Camellia chrysantha* (Hu) [2].

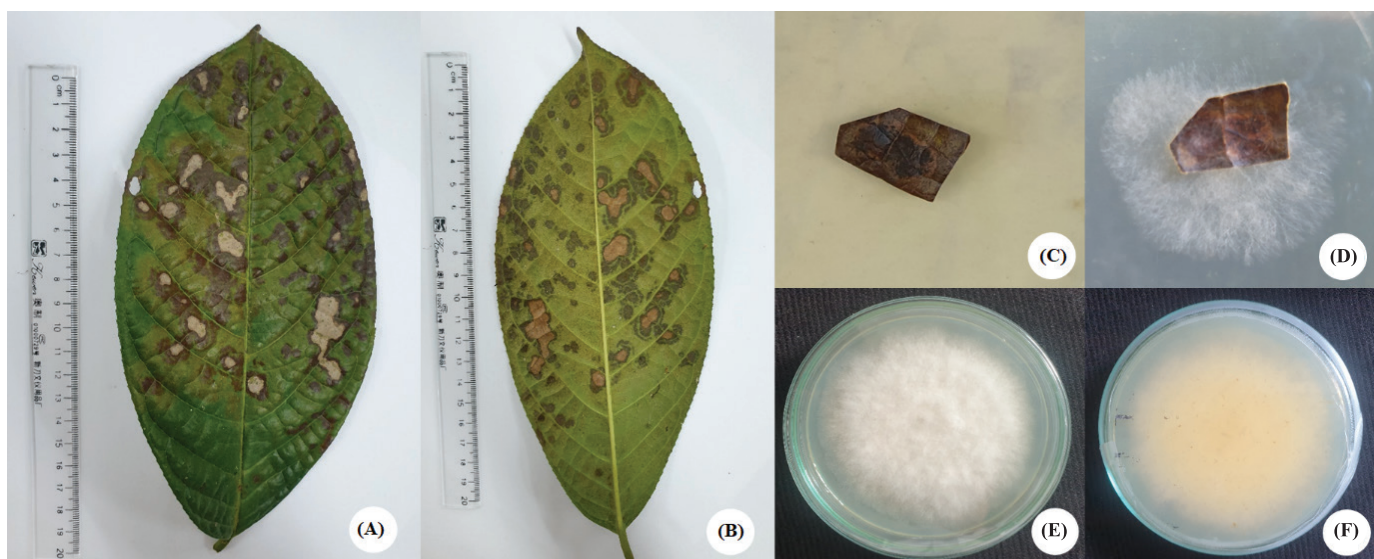


Fig. 1. Symptoms on diseased leaves and fungal isolation from *C. tamdaoensis* Ninh et Hakoda. (A, B) Upper and lower of disease leaf; (C) Leaf section put on PDA medium; (D) Mycelia growth; (E, F) Upper and lower of fungal isolate implied anthracnose after 7 days culture.

Eighteen leaf sections were surface-sterilised, put on PDA media, and inoculated at 25°C in the incubator. After 7 days of culture, we obtained 11 fungal-like isolates. They had the same morphology of mycelium with white color (Figs. 1C, D, E, F). One of them was selected to study further. According to previous publications, anthracnose is caused by many different fungal agents such as *C. siamense* causing disease on *C. sinensis* [11] and *C. fructicola* causing disease on *Citrus sinensis* [12]. However, in *C. chrysanth*, anthracnose was caused by *C. siamense* and *C. fructicola* [2]. The initial classification agent that caused anthracnose in this study belonged to *Collectotricum* sp.

3.2. Molecular characteristics of ITS from fungal agent caused to Anthracnose

The length and GC content of ITS: This study used ITS sequences of ten species/strains of *Collectotricum* from GenBank to analyse the length and GC content. The results, given in Table 1, showed that the lengths of ITS fragments range from 522 bp of *Collectotricum* sp. species to 603 bp of the *Collectotricum siamense* strain Cg131. The GC content also increased from 51.41 to 52.34%. In *Collectotricum* sp., the GC content was 52.30%, which was similar to the analysed ITS sequences. The high GC levels usually indicate gene stability.

Table 1. Molecular comparison of ITS sequences from the isolated fungal agent that caused anthracnose in *C. tamdaoensis* Ninh et Hakoda.

Order	Species/strains	GenBank accession	Length (bp)	GC content (%)
1	<i>Collectotricum siamense</i> TD1	OK560718	522	52.30
2	<i>Collectotricum siamense</i> strain rb12	MT434638.1	593	51.43
3	<i>Collectotricum siamense</i> strain rb10	MT434636.1	593	51.43
4	<i>Collectotricum siamense</i> strain Hainan21	MG830366.1	563	52.22
5	<i>Collectotricum siamense</i> strain Cg131	KU642474.1	603	51.41
6	<i>Collectotricum siamense</i> isolate GQHZJ20	MN296085.1	564	51.95
7	<i>Collectotricum siamense</i> isolate GQHZJ19	MN296084.1	556	52.34
8	<i>Collectotricum queenslandicum</i> isolate RHCOL1	KT372377.1	599	51.92
9	<i>Collectotricum eremochloae</i> strain CBS 129661	MH865527.1	541	53.97
10	<i>Collectotricum endophyticum</i> clone EIPP	MK330020.1	593	51.43
11	<i>Collectotricum boninense</i> isolate PL9	MK685133.1	586	51.88

This evidence demonstrated that the anthracnose causing-fungal agent may belong to the *Collectotricum* genus.

Genetical relationship of *Collectotricum* based on ITS RNA ribosomal: In *Camellia oleifera* in China, the most common cause of anthracnose disease in fruit consists of 5 strains including *C. fructicola*, *C. camelliae*, *C. aenigma*, *C. siamense*, and *C. gloeosporioides* while in leaves is *C. fructicola* [13].

In this study, no anthracnose symptoms were observed on *C. tamdaoensis* Ninh et Hakoda leaves while 11 strains were isolated from symptomatic leaves, which had similar mycelium morphology. The ITS sequence of the fungal agent in this study was amplified by PCR using ITS 1 and ITS 4 primers. This product was sequenced and used to build a phylogenetic tree using MEGA 7 software. The results demonstrated that the isolate belonged to *Collectotricum siamense* with 96-99% homology to other *Collectotricum siamense* species (Fig. 2). To our knowledge, there has been no publication describing *Collectotricum siamense* on golden tea *C. tamdaoensis* Ninh et Hakoda at the Tam Dao National Park.

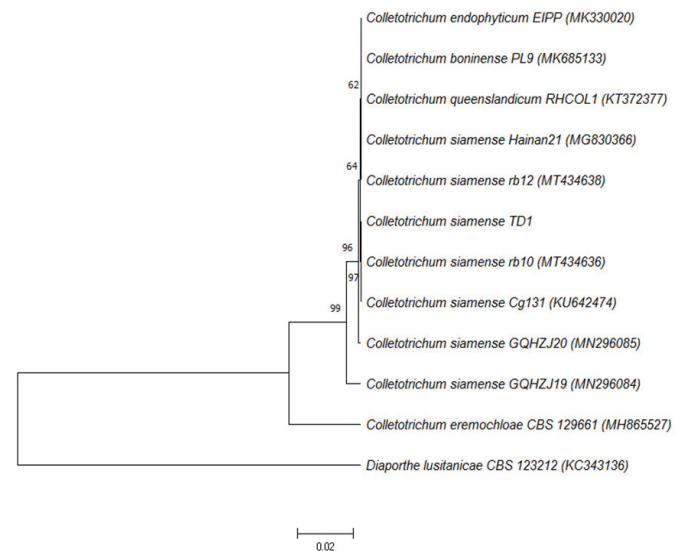


Fig. 2. Phylogenetic tree using the DNA ribosomal ITS of *Collectotricum* isolated from *Camellia hakodae* disease symptom leaf in Tam Dao, Vinh Phuc. *C. hakodae* isolate was labeled with the red rectangle. Expressed value at the phylogenetic tree was a bootstrap of 1000 replications. 0.02 bar was nucleotide substitution rate units. *Diaporthe lusitanicae* was used as the outgroup.

3.3. Virulence test

Collectotricum sp. was grown and infected back to the detached leaf of *C. tamdaoensis* Ninh et Hakoda. Similar symptoms to those observed in the field developed on all infected leaves (Fig. 3). The controls showed no symptoms.



Fig. 3. Pathogenicity test back on the healthy leaf of *C. tamdaoensis* Ninh et Hakoda. (A) Whole plant, (B, C) Lesions caused by *Colletotrichum siamense* TD1, on upper and lower sides, respectively.

This result demonstrated that the isolate from *C. tamdaoensis* Ninh et Hakoda disease caused anthracnose.

4. Conclusions

A fungal strain causing anthracnose disease has been isolated from *C. tamdaoensis* Ninh et Hakoda collected the Tam Dao National Park. Diseased leaves showed typical anthracnose symptoms such as necrotic spots, which were initially brown, then spreading and turning black. The *ITS* sequence fragment of the pathogen was determined to have a length of 522 bp and GC content of 52.30%. By genetic relationship analysis based on the *ITS* sequence, *Colletotrichum siamense* TD1 was identified as the causative agent. The pathogenicity of *Colletotrichum siamense* TD1 was tested on leaves by artificial inoculation. The necrotic spot symptoms appeared after 7 days, which was similar to those collected in the wild.

CRedit author statement

Hong Viet La: Conceptualisation, Methodology, Reviewing and Editing; Duc Hoang Le: Doing experiments; Data analysis; Thao Thanh Thi Duong: Doing experiments, Data curation; Bang Phi Cao: Writing - Original draft preparation; Ha Duc Chu: Data analysis, Investigation.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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