

Article

Identification and Characterization of *Nectria pseudotrachia* Associated with Camellia Canker Disease in China

Xiansheng Geng¹, Ying Liu¹, Jiyuan Li^{1,*}, Zhihong Li¹, Jinping Shu¹ and Guiyang Wu²

¹ Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Hangzhou 311400, China; gxsh2001@163.com (X.G.); liuying1996517928@163.com (Y.L.); lizhihong927@126.com (Z.L.); shu_jinping001@163.com (J.S.)

² Agricultural Technology Service Station of Fenghua City, Ningbo 315502, China; wuguiyang611013@sina.cn

* Correspondence: jiyuan_li@126.com

Abstract: *Camellia japonica* is a native tree species with high economic value that is widely cultivated in southern China. In recent years, canker disease has been observed in camellia plantations in Zhejiang Province, China, with the disease incidence rate in some plantations exceeding 20%. Canker disease severely affects the trunks and branches of *C. japonica* in China, but the causal agent has not yet been identified. In this study, the pathogen was isolated from infected *C. japonica* tissues through a conventional tissue isolation approach. Species identification was conducted using morphological methods combined with multilocus phylogenetic analysis. Pathogenicity was tested based on Koch's postulates. The results showed that the pathogen could be isolated from the diseased bark of *C. japonica* 'Hongluzhen'. The pathogen was identified as *Nectria pseudotrachia* based on morphological, cultural, and molecular traits. The inoculation of the pathogen into *C. japonica* 'Hongluzhen' caused necrotic lesions on healthy seedlings, and the fungus *N. pseudotrachia* could be re-isolated from such lesions. Therefore, *N. pseudotrachia* is the causal agent of canker disease affecting *C. japonica* in China.

Keywords: *Camellia japonica*; canker disease; pathogen; Koch's postulates



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1. Introduction

Camellia japonica L. is an evergreen shrub or tree in the family Theaceae that is native to China, South Korea, and Japan [1,2]. Over the last 200 years, *C. japonica* has been gradually introduced into Europe, the Americas, and Oceania, and has become a popular garden plant in most warm-temperate regions of the world [2,3]. *C. japonica* is a multipurpose tree species with high ornamental, economic, and medicinal value. *C. japonica* is popular due to its bright color and beautiful flowers, which are responsible for its high ornamental value. The content of unsaturated fatty acids, such as oleic acid, in the seeds of *C. japonica* accounts for more than 90% of the total fatty acid content, giving this species high economic value [4]. *C. japonica* plants have been found to contain triterpenes, saponins, flavonoids, tannins, fatty acids, and phenolic compounds, which exhibit various biological activities, including antioxidant, antiaging, antiviral, anti-inflammatory, antihyperuricemic, antiosteoporotic, and antiallergic activities [1,5,6].

Canker disease was first described in *C. japonica* in the United States in 1924 [7] and was subsequently observed in the United Kingdom [8], Spain [9] and China. The causal agent of this disease was reported to be *Glomerella cingulate* (Stonem) Spauld & Schrenk in the United States [10] and in the United Kingdom [8] and to be *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips in Spain [9]. However, the pathogen causing this disease in China has not yet been reported. In recent years, canker disease in *C. japonica* has been found in many areas of Zhejiang Province, China. Among these infections, *C. japonica* plantations in Ningbo and Jinhua are the most seriously impacted, with more than 20% of camellia plants being infected. In Zhejiang Province, canker disease has only been observed

on the trunk and branches of *C. japonica*. Mildly infected plants exhibit large necrotic lesions on the bark, while severe infection can cause the death of the entire branch or even the entire tree. Canker disease has become the most serious disease affecting *C. japonica*.

This study aims to estimate the incidence rate of canker disease in two camellia plantations in Zhejiang Province, to isolate the pathogen from infected *C. japonica* tissues through a conventional tissue isolation approach, to identify the fungal species that are associated with camellia canker disease in China based on morphology combined with multilocus phylogenetic analysis, and to determine the pathogenicity of *Nectria pseudotrachia* Berk. & M.A. Curtis on the stems of *C. japonica* using Koch's postulates.

2. Materials and Methods

2.1. Study Site and Field Surveys of Canker Disease in *C. japonica*

Two camellia plantations, located in Ningbo (29°39'19" N, 121°29'32" E) and Jinhua (29°7'36" N, 119°35'21" E) in Zhejiang Province, China, were selected as the study sites. There are 15 cultivars of camellia planted at the Ningbo camellia plantation, including 8 cultivars of *C. japonica*, 4 cultivars of *C. hiemalis* Nakai, 2 cultivars of *C. sasanqua* Thunb., and 1 cultivar of *C. uraku* (Mak.) Kitamura. There are eight cultivars of camellia planted at the Jinhua camellia plantation, including three cultivars of *C. japonica* and five cultivars of *C. hiemalis*. The area of each camellia plantation is about 2 hectares in size, and the area of *C. japonica* on each plantation is more than 0.2 hectares. The *C. japonica* plants range from 10 to 20 years of age, and the planting density is about 4000 plants per hectare. In June 2020, the study investigated the incidence and symptoms of canker disease in two camellia plantations. The preliminary situation of canker disease occurrence in all of the cultivars at the two plantations was obtained by consulting the local managers and through our own systematic observation, and then the incidence and symptoms of canker disease in *C. japonica* 'Hongluzhen' were investigated using a sampling method. Three sampling plots with an area of 20 m × 20 m were randomly selected from a *C. japonica* 'Hongluzhen' planting plot at each plantation, and 20 trees in each plot were investigated to calculate the rate of disease incidence. The disease incidence rate was calculated according to the method proposed by Linaldeddu et al. [11].

A scalpel was used to scrape the diseased *C. japonica* 'Hongluzhen' bark, which was transported to the laboratory so that the surface characteristics could be analyzed and the pathogenic fungi could be isolated.

2.2. Fungal Isolation

A total of 15 bark samples from diseased and healthy plants were collected from two camellia plantations. To isolate the causal agent, the samples were washed under running water for 2 h, and the margin of each infected sample was cut into small sections. The surfaces of these sections were sterilized through immersion in 70% ethanol for 30 s, followed by 10% sodium hypochlorite solution for 10 min, and the sections were then rinsed five times in sterile water and were blotted dry using sterile filter paper [12]. The dry sections were transferred to potato dextrose agar (PDA) medium supplemented with streptomycin (200 mg/L) and were incubated at 25 °C. Mycelial fragments were collected from the growing colony margin, sub-cultured onto synthetic nutrient-poor agar medium (SNA) and PDA, and incubated at 25 °C.

2.3. Morphological Characterization

The morphology of the colonies of the pathogenic fungi that formed on the PDA and SNA plates was observed, and colony sizes were measured with a millimeter ruler. The morphological structures of hyphae and conidia were observed with a microscope. Some samples were stained with Melzer's reagent [13] or lactophenol cotton blue staining solution (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) prior to observation. The size and morphology of the conidia and synnemata were determined using the Keyence VHX-5000 digital microscope (Keyence, Osaka, Japan).

2.4. DNA Extraction, Amplification, Sequencing, and Phylogenetic Analysis

For genomic DNA extraction, the mycelium was harvested from the PDA plates and was ground into a powder using a TissueLyser-48 grinder. Total genomic DNA was extracted from the powder with a universal genomic DNA Extraction Kit (Takara MiniBEST Universal Genomic DNA Extraction Kit ver. 5.0; Takara Biomedical Technology Co., Ltd., Beijing, China). Polymerase chain reaction (PCR) amplification of the large subunit ribosomal DNA (LSU rDNA), internal transcribed spacer (ITS) rDNA, and the *tef1- α* region were conducted with the primer pairs LR0R/LR5 [14], ITS5/ITS4 [15,16], and EF1-728F/EF1-986R [17], respectively. The PCR mixture for all regions contained 1 μ L genomic DNA, 10 μ L 2 \times PCR Mix (Tiangen Biotech Co., Ltd., Beijing, China), 0.5 μ L of each primer, and 8 μ L double-distilled sterilized water. The PCR conditions for all of the regions were 94 $^{\circ}$ C for 5 min followed by 35 cycles of 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s, followed by a final extension of 72 $^{\circ}$ C for 10 min. The PCR products were sent to Shangya Biotechnology Co., Ltd. (Hangzhou, China) for sequencing, and representative sequences were deposited in GenBank (Table 1).

Table 1. Specimens used in phylogenetic analyses and their GenBank accession numbers.

Species	Strain ¹	Geographic Origin	GenBank Accession No.		
			ITS	LSU	<i>tef1-α</i>
<i>Nectria antarctica</i>	A.R.2767	USA	HM484556	HM484560	HM484516
<i>N. aurantiaca</i>	CBS 308.34	UK	JF832628	JF832682	JF832519
<i>N. balansae</i>	A.R. 4446; CBS 123351	France	HM484552	GQ505996	HM484525
<i>N. balansae</i>	A.R. 4635	China	JF832653	JF832711	JF832522
<i>N. berberidicola</i>	A.R. 4662	France	JF832662	JF832712	JF832538
<i>N. cinnabarina</i>	A.R. 4477	France	HM484548	HM484562	HM484527
<i>N. dematiosa</i>	G.J.S. 94-37	USA	HM484557	HM484561	HM484534
<i>N. magnispora</i>	TPP-h122	Japan	JF832663	JF832683	JF832539
<i>N. mariae</i>	A.R. 4274	France	JF832629	JF832684	JF832542
<i>N. nigrescens</i>	A.R. 4211	USA	HM484707	HM484720	HM484672
<i>N. polythalamia</i>	A.R. 4586	New Zealand	JF832638	JF832695	JF832523
<i>N. pseudocinnabarina</i>	G.J.S. 09-1359	Venezuela	JF832642	JF832697	JF832533
<i>N. pseudotrichia</i>	LPNP	China	OK562743	OK562730	OL440127
<i>N. pseudotrichia</i>	CBS551.84; CBS 451.85	Japan; Honduras	HM484554	GQ506000	HM484532
<i>N. pseudotrichia</i>	MAFF 241394	Japan	JF832639	JF832705	JF832527
<i>N. pseudotrichia</i>	ICMP 2245	Japan	JF832640	JF832701	JF832532
<i>N. pseudotrichia</i>	MAFF 241452	Papua New Guinea	JF832649	JF832706	JF832531
<i>N. pseudotrichia</i>	CBS 652.83	Japan	JF832648	JF832703	JF832528
<i>N. pseudotrichia</i>	Y.H. 10-04	Venezuela	JF832650	JF832704	JF832529
<i>N. pseudotrichia</i>	G.J.S. 09-1329	USA	JF832647	JF832702	JF832530
<i>N. pseudotrichia</i>	CFCC 52122	Venezuela	MG231953	MG231974	MG232016
<i>N. pseudotrichia</i>	CFCC 52123	China	MG231954	MG231975	MG232017
<i>N. pseudotrichia</i>	CFCC 52124	China	MG231955	MG231976	MG232018
<i>N. novae-zealandiae</i>	HMAS 252485	China	KM026503	KM026504	KM026506
<i>N. ulmicola</i>	CFCC 52117	China	MG231959	MG231980	MG232022
<i>N. australiensis</i>	H13	China	GU075855	DQ119569	HM054076
<i>N. eustomatica</i>	CBS 121896	China	HM534896	HM534896	HM534875
<i>N. sordida</i>	G.J.S. 86-117	Croatia	HM484857	HM484868	HM484848
<i>N. balansae</i>	CFCC 52119	French Guiana	MG231956	MG231977	MG232019
<i>Cosmospora coccinea</i>	CBS 114050; AR2741	China	FJ474072	GQ505990	AY489629

¹ Strain sequenced in the study is shown in bold.

The sequences for the 3 loci (LSU rDNA, ITS rDNA, and *tef1- α*) that were obtained in this study and 84 sequences of the genus *Nectria* from GenBank (Table 1) were used for phylogenetic analysis. *Cosmospora coccinea* (CBS 114050, AR2741) sequences from GenBank were used as an outgroup. Multiple sequence alignment of each locus was performed using MAFFT v.7.313 [18] and were manually adjusted using PhyloSuite v.1.2.2 [19]. Alignments of the three loci were concatenated into a single dataset with PhyloSuite v.1.2.2. Nucleotide

substitution models for each locus, which were determined using PartitionFinder v.2 [20], were included for each gene partition. Phylogenetic analysis was performed using MrBayes v.3.2.6 [21]. Two Markov chain Monte Carlo (MCMC) analyses were run from random trees for 1,000,000 generations, with sampling every 100 generations. The initial 25% of the sampled trees were discarded as burn-in, and posterior probabilities were determined from the remaining trees. The resulting tree was visualized in FigTree v.1.4.3 and was edited with Adobe Illustrator CS6.

2.5. Pathogenicity Tests

In October 2020, stems and detached leaves from seedlings (15.7 ± 3.5 mm ground diameter) of *C. japonica* ‘Honggluzhen’ were inoculated with the representative isolate LPNP of *N. pseudotrachia*. Fungal agar plugs (5 mm in diameter) from the edge of actively growing cultures were placed into a wounded inoculation region, which were made using a sterile scalpel or dissecting needle, and the inoculation region was then covered with Parafilm to conserve moisture. Negative controls were inoculated with sterile PDA plugs, which were applied to wounds. The inoculated seedlings and detached leaves were maintained in a climate-controlled chamber at 28 °C with a 12 h photoperiod. At 14 and 28 days after inoculation, the necrotic lesions on the detached leaves and stems surrounding each inoculation region were individually observed with a VHX-5000 microscope.

At 28 days after inoculation, re-isolation was conducted from the margin of the necrotic tissue to recover the inoculated fungi and to meet Koch’s postulates. Additional isolation from the negative controls was conducted to verify that no endophytic *N. pseudotrachia* fungus was present. The re-isolation procedure is described in Section 2.2. The isolates were identified based on morphological characterization and ITS rDNA sequences.

3. Results

3.1. Field Surveys of Canker Disease in *C. japonica*

Field surveys indicated that canker disease occurred on both camellia plantations, and that the infections only occurred in *C. japonica* cultivar ‘Honggluzhen’. The disease incidence rates of *C. japonica* ‘Honggluzhen’ in Ningbo and Jinhua were $46.2\% \pm 9.4\%$ and $20.0\% \pm 0.0\%$, respectively.

The pathogenic fungus can infect the trunk and branches of *C. japonica* ‘Honggluzhen’. When the pathogen infects the trunk, it often causes the necrosis of inner bark and xylem surface tissues, revealing the xylem (Figure 1a). In natural conditions, it is difficult for a callus to form around the lesion, meaning that it is possible for the lesion to continuously expand, forming longer and larger lesions. When the branches are infected, the bark of the infected regions becomes dark and slightly sunken (Figure 1b), and light red synnematal structures are present on some of the portions of diseased bark. The synnema were erumpent through the epidermis, solitary, slightly spherical at the top, 284.0–414.0 µm in diameter, and had a long (1323.0–1584.0 µm) stipe at the base (Figure 1c).

3.2. Isolation, Morphological Structures and Cultural Characteristics of the Pathogenic Fungus of Camellia Canker

In this study, a total of 15 samples were collected, and Nectriaceae-like fungi were grown on PDA. The isolates grew quickly on PDA, with colony diameters of 8.1 ± 1.6 cm after 6 days of incubation at 25 °C. The upper side of the colonies on PDA was red at the center, and the color gradually lightened from the center to the margin of each colony (Figure 2a). The reverse side of each colony was red at the center and was creamy at the margin (Figure 2b). The isolates formed colorless colonies on SNA (Figure 2c). Vegetative hyphae were observed on SNA, but no aerial hyphae were present (Figure 2c). When the PDA plate was observed using the VHX-5000 microscope, the conidiophores, conidial mass, and conidia were observed in the center of each colony (Figure 2d). The conidia varied in size and morphology on PDA, with young conidia that were ellipsoidal to cylindrical in shape and that were generally $4.5\text{--}5.6 \mu\text{m} \times 2.6\text{--}3.3 \mu\text{m}$ in size, and mature conidia

that were ellipsoidal, cylindrical to allantoid in shape, and approximately $9.1\text{--}12.2\ \mu\text{m} \times 2.9\text{--}3.9\ \mu\text{m}$ in size. When the isolate was cultured on PDA for 20 days, a large number of synnemata were produced near the side wall of the Petri dish (Figure 2e). However, only a small number of synnemata were produced on SNA after 60 days (Figure 2f).



Figure 1. Disease symptoms and synnematal morphology of the pathogenic fungus. (a,b) Trunk canker and branch canker observed on *C. japonica* ‘Hongluzhen’; (c) synnemata on diseased bark.

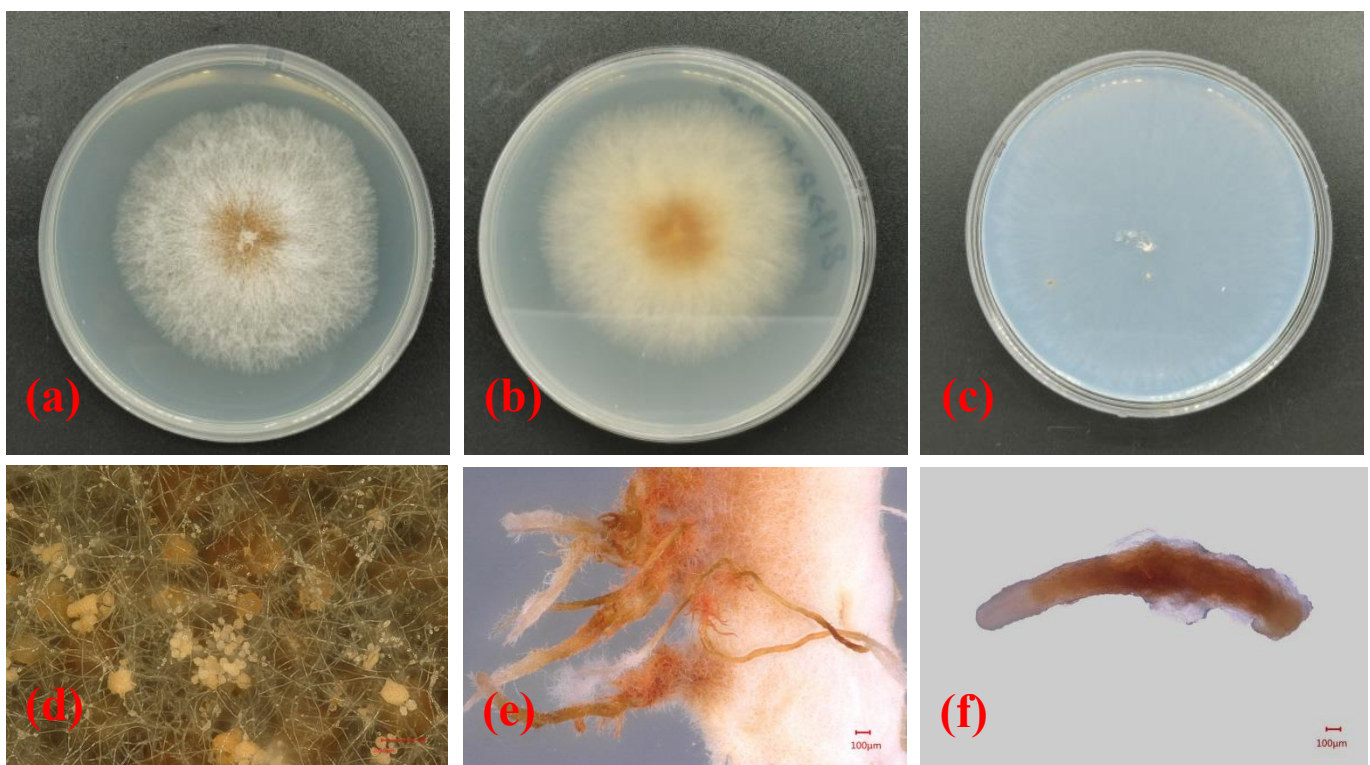


Figure 2. Morphological structures and cultural characteristics of *Nectria pseudotrichia*. (a,b) Upper and reverse sides of colonies on PDA, respectively; (c) upper side of colony on SNA; (d) conidial masses produced on PDA; (e,f) synnemata produced on PDA and SNA, respectively.

Based on the morphological characteristics of the mycelia, synnema, conidiophores, conidial mass, and conidia of the isolated pathogenic fungi as well as their cultural characteristics on PDA and SNA, the pathogen affecting camellia *C. japonica* in Zhejiang Province was preliminarily identified as *Nectria pseudotrachia*.

3.3. Phylogenetic Analysis

Phylogenetic analyses of 29 strains of *Nectria* were performed using the concatenated datasets for three loci. A total of 1561 characters (LSU: 1–799, ITS: 800–1308, *tef1- α* : 1309–1561) were included in the datasets. For Bayesian inference (BI) analysis, GTR + I + G, GTR + I + G, and SYM + G were selected as the best nucleotide substitution models for LSU rDNA, ITS rDNA, and *tef1- α* , respectively. The phylogenetic tree that was generated from BI analysis showed that the isolate LPNP clustered with 10 strains of *N. pseudotrachia* from Genbank and had a high posterior probability value (Figure 3). Multilocus phylogenetic analysis confirmed the assignment of the isolate LPNP as a strain of *N. pseudotrachia*. Multilocus phylogenetic analysis also revealed that within the *N. pseudotrachia* Clade, the LPNP strain and the two strains ICMP2245 and MAFF241394 from Japan clustered into a subclade, indicating that the genetic distance between these three strains is the closest (Figure 3).

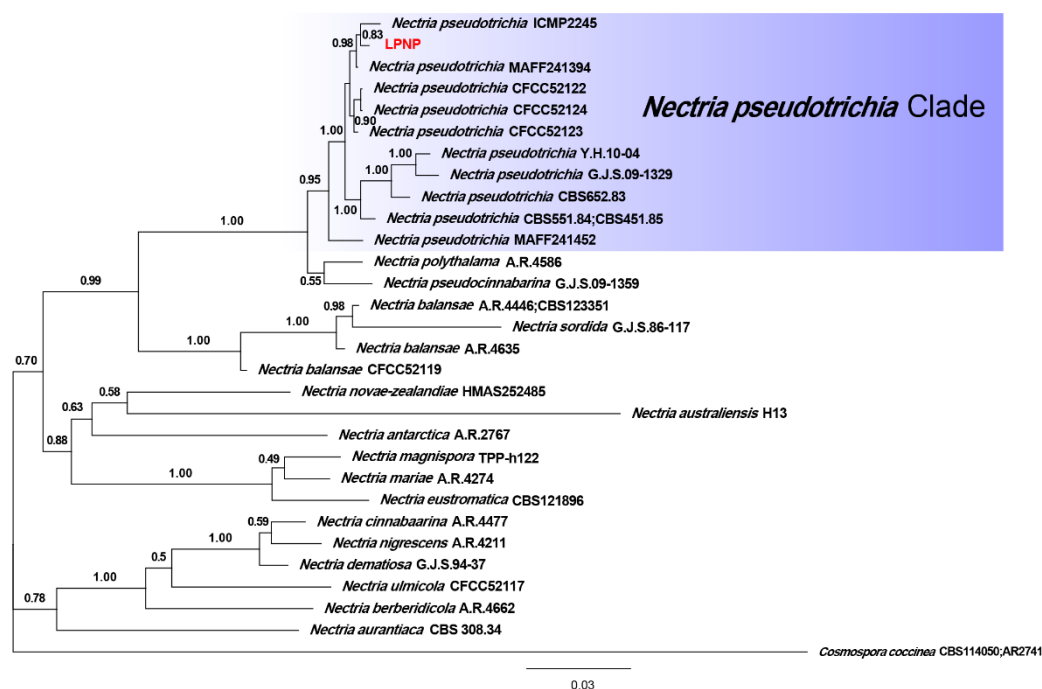


Figure 3. Phylogenetic tree inferred from BI analysis based on concatenated alignments of LSU rDNA, ITS rDNA, and *tef1- α* sequences. *Cosmospora coccinea* was used as an outgroup. Numbers on the nodes represent Bayesian posterior probabilities. The isolate obtained in the current study is highlighted in red.

3.4. Pathogenicity Tests

At 28 days after inoculation, symptoms were observed at the inoculation site on the stem of *C. japonica* ‘Hongluzhen’ (Figure 4a,b). The stems of the seedlings that had been inoculated with sterile PDA plugs produced a large amount of callus at the inoculated site, and the bark surface was flat with no necrotic lesions beneath the bark (Figure 4c,d). However, the stems of seedlings that had been inoculated with *N. pseudotrachia* produced a negligible callus at the inoculation site; the bark was slightly sunken, and necrotic lesions had formed beneath it (Figure 4a,b). In addition, at 14 days after inoculation, the detached leaves of the seedlings that had been inoculated with *N. pseudotrachia* showed no lesions

(Figure 4e), indicating that *N. pseudotrachia* had no pathogenicity to the leaves of *C. japonica* ‘Honggluzhen’. In the re-isolation experiment, a fungus could be isolated from the necrotic tissue of symptomatic seedlings, and the morphological characterization as well as the ITS rDNA sequence of the isolate were consistent with those of LPNP. This pathogen was not isolated from the bark around the inoculation sites of negative control seedlings.

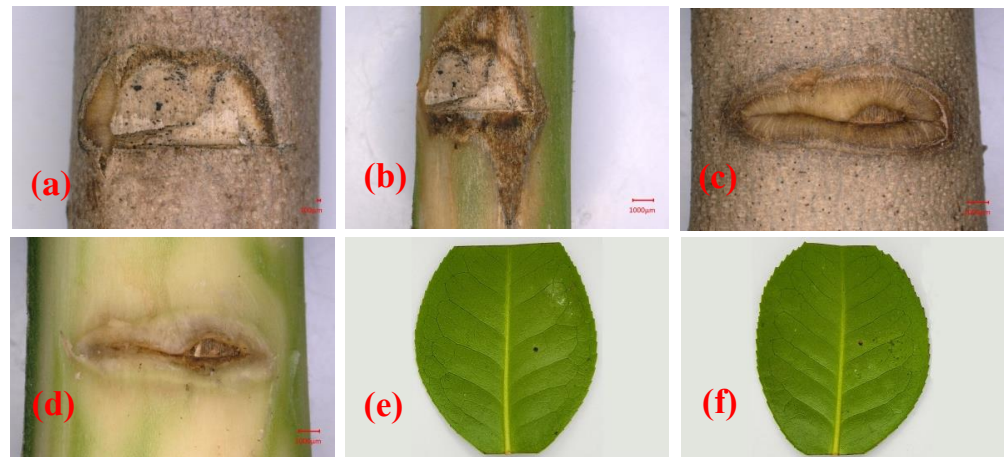


Figure 4. Pathogenicity analysis of *Nectria pseudotrachia* on the stems and detached leaves of *Camellia japonica* ‘Honggluzhen’. (a,b) Lesions on and beneath the bark of a camellia stem inoculated with *N. pseudotrachia*, respectively; (c,d) lesions on and beneath the bark of a camellia stem inoculated with a sterile PDA plug, respectively; (e,f) lesions on detached leaves inoculated with sterile PDA and *N. pseudotrachia*, respectively.

N. pseudotrachia can cause necrotic lesions on the stem of *C. japonica* and can be re-isolated from necrotic tissue, indicating that *N. pseudotrachia* is the causal agent of camellia canker disease in Zhejiang Province, China.

4. Discussion

Canker disease is characterized by a necrotic region, with swelling surrounding a sunken lesion or blister on the bark of trunks and branches that affect the underlying tissues [22]. The basic characteristic of canker disease is the generation of a visible necrotic area on the bark of infected trees [23]. Most of the pathogenic fungi that cause canker disease belong to a few genera of Ascomycetes, including *Nectria*, *Valsa*, *Cytospora*, *Dothiorella*, *Dothichiza*, *Botryodiplodia*, *Macrophoma*, *Diaporthe*, *Melanconium*, *Fusarium*, and *Trichoscyphella* [23–27].

Fungi in the genus *Nectria* are commonly found on broad-leaved trees and shrubs in the temperate regions of the northern hemisphere [28,29]. These fungi are characterized by the production of brightly colored fleshy perithecia on the bark [28,29]. Approximately 35 species have been described worldwide, with 11 species occurring in China [29]. *Nectria* causes canker disease on more than 60 species of trees and shrubs, including apple, pear, ash, holly, elm, aspen, birch, dogwood, maple, sassafras, sweet gum, and walnut. Infected trees often exhibit large areas of necrotic bark, which may lead to the death of infected branches or entire trees.

N. pseudotrachia belongs to the genus *Nectria*, and its anamorph is *Tubercularia lateritia* (Berk.) Seifert [30]. This fungus is distributed worldwide and has been isolated from more than 40 genera of trees in Asia, Africa, North America, South America, and Oceania [28,30–34]. Early researchers mostly found *N. pseudotrachia* on dead trees or branches, so it is considered to be a saprophytic tree fungus [30]. In 2003, Becker first reported that *N. pseudotrachia* can cause canker disease on *Pyrus pyrifolia* (Burm.) Nakai [31], and the pathogenicity of this fungus has gradually been recognized. This fungus has been reported to cause canker or dieback on *P. pyrifolia* [31], *Macadamia integrifolia* Maiden & Betche and *M. tetraphylla* L.A.S. Johnson [32], *Fragaria × ananassa* Duch. [33], and *Hevea*

brasiliensis (Willd. ex A. Juss.) Muell. Arg. [35]. In this study, synnemata of *N. pseudotrichia* were observed on the diseased bark of *C. japonica* in the field, and this fungus could be isolated from the diseased bark. To our knowledge, this is the first report of *N. pseudotrichia* infection in *Camellia*. When we inoculated the isolates onto seedlings of *C. japonica*, they caused disease in the camellia seedlings, and the fungus could be re-isolated from the diseased bark. Thus, *N. pseudotrichia* is the causal agent of camellia canker disease in Zhejiang Province, China. Further investigation is required for disease prevention and control measures according to the biological characteristics of *N. pseudotrichia* to reduce the losses caused by canker disease in *C. japonica*.

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Data Availability Statement: The gene sequences have been deposited in GenBank under the accession numbers OK562743, OK562730 and OL440127 for ITS rDNA, LSU rDNA and *tef1- α* , respectively.

Conflicts of Interest: The authors declare no conflict of interest.

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