Research

First Report of Dieback Caused by *Lasiodiplodia pseudotheobromae* on *Ormosia pinnata* in ChinaLuoye Li,¹ Mengying Lei,¹ Honghong Wang,¹ Xiaozhu Yang,¹ Mebeaselassie Andargie,^{2,†} and Shaobin Huang,^{1,†}¹ Collaborative Innovation Center of Plant Pest Management and Bioenvironmental Health Application Technology, Guangdong Eco-Engineering Polytechnic, Guangzhou 510520, China² Molecular Phytopathology and Mycotoxin Research, University of Göttingen, Göttingen, Germany

Abstract

Ormosia pinnata (Lour.) Merr. is an important tree used for landscape and plant recovery of barren slopes in China. During an investigation of plant disease on landscape trees in 2018, a dieback was observed on *O. pinnata* trees in Guangzhou, Guangdong Province, China. Symptoms were characterized by initial dryness of the twigs and eventual death of the whole branch of the tree. Isolations from symptomatic branches yielded 13 isolates including two main morphotypes. Pathogenicity tests showed that isolate *GDOPI* from Type I caused

dieback of *O. pinnata*. Based on morphological characteristics and molecular analysis of the internal transcribed spacer rDNA (ITS1-5.8S-ITS2) and partial sequence of the translation elongation factor 1 α (EF1- α), the fungus causing dieback on *O. pinnata* was identified as *Lasiodiplodia pseudotheobromae*. This is the first report of *L. pseudotheobromae* infecting *O. pinnata* in the world.

Keywords: dieback, *Lasiodiplodia pseudotheobromae*, ITS, EF1- α

The legume genus *Ormosia* Jacks. was established by G. Jackson (1811) and it comprises around 130 species, which are distributed in Southeast Asia, tropical America, and North Australia (Pennington et al. 2005). *Ormosia pinnata* (family Fabaceae) is an evergreen tree originating in Hainan, China. It is mainly distributed in Guangdong Province, Guangxi autonomous region, China, as well as Vietnam and Thailand. Because of its large crown and lush branches and leaves, it is often used as a landscape tree on streets and courtyards. Furthermore, it is often used as pioneer tree for vegetation restoration on the barren slopes of South China, since its root system can fix nitrogen, which improves soil fertility (Zhao et al. 2000).

During 2018, an investigation of plant disease on landscape trees was done in the four districts of Guangzhou (Tianhe, Baiyun, Huangpu, and Haizhu), Guangdong Province, China. A dieback on *O. pinnata* was observed for the first time in Tianhe district. Although few diseased trees were found, the symptoms were serious. Disease symptoms included black spots and necrosis on branches, branch withering, and eventual death of the diseased trees.

Lasiodiplodia pseudotheobromae (family Botryosphaeriaceae) is an important pathogen of a wide variety of plants covering a broad geographical distribution. It causes great loss to agriculture and forestry. For instance, it was reported to cause canker and shoot dieback on apple and resulted in the death of apple branches (Xue et al. 2019). It brought twig blight on blueberry and resulted

in the death of diseased branches within 7 to 10 days (Wang et al. 2016). It is also responsible for dieback on English walnut (Li et al. 2016), branch blight of *Bougainvillea spectabilis* (Li et al. 2015), and pedicel and peduncle discoloration of grapes (Dissanayake et al. 2015) in China. Furthermore, it caused cankers and dieback on Persian lime in Mexico (Bautista-Cruz et al. 2019), dieback of mango in Peru and Korea (Kwon et al. 2017; Rodriguez-Galvez et al. 2017), and trunk cankers in *Acacia mangium* in Venezuela (Castro-Medina 2014). In this paper, dieback on *O. pinnata* trees is reported for the first time. The objectives of the study were: (i) to isolate the causal pathogen and determine its virulence by pathogenicity tests on *O. pinnata* seedlings, and (ii) to identify the species of pathogens associated with dieback of *O. pinnata* using morphological and molecular approaches.

Materials and Methods

Plant sampling and fungal isolation. Ten symptomatic branches were collected from the diseased trees in Tianhe district of Guangzhou, Guangdong Province (113.33°E, 23.17°N). A piece of diseased tissue from each branch was used to isolate pathogen. Each infected tissue sample was surface-sterilized by soaking in 75% ethanol solution for 45 s, followed by three rinses in sterile distilled water, then dried on sterilized filter paper. The surface-sterilized diseased pieces were placed on potato dextrose agar (PDA) in Petri dishes and incubated at 25°C in the dark. To purify fungi, a hyphae tip was carefully picked from the edge of each colony using an elbow tweezer with a very small pointed tip under a dissecting microscope and transferred to new PDA for subculturing. Each isolate was purified at least twice by this method. The purified isolates were used for pathogenicity determination, morphological observation, and molecular identification.

Pathogenicity tests. Through artificial inoculation, pathogenicity tests were conducted on living seedlings of *O. pinnata*. From the two morphotypes, the representative isolates of each type (*GDOPI* and *GDOPI2*) were used to inoculate branches. Each fungal isolate was grown on PDA in the dark at 25°C for 5 to 7 days prior to inoculation. A mycelial plug inoculation method was used as reported previously by Kwon et al. (2017) and Bautista-Cruz et al. (2019). For wound inoculation, young healthy branches were surface

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disinfected by 75% alcohol and a small wound was made on each branch with a sterile knife. A piece of colonized PDA plug (8 mm in diameter) was placed into the wound and sealed with Parafilm (Bemis, U.S.A.) to keep moist until disease symptoms appeared. For inoculation without wounding, colonized PDA plugs were kept on unwounded branches and sealed with Parafilm. Branches of control seedlings were inoculated with PDA plugs without the pathogen. The plants were kept in a greenhouse for over 3 days until symptoms appeared and they were managed as normal. The experiments were conducted in 2018, 2019, and 2020. For each isolate or control, 30 seedlings of *O. pinnata* were used for pathogenicity tests in total.

Morphological analysis of the pathogen. Based on preliminary pathogenicity tests, the isolates of *GDOP1* and *GDOP2* were inoculated on PDA and incubated at 25°C in the dark for 7 days. Colony characteristics on PDA including mycelium growth, color, and sporulation were observed. In addition, water agar media and autoclaved *O. pinnata* twigs were used to induce sporulation following the method of Alves et al. (2008). Briefly, fungal isolates were grown on autoclaved *O. pinnata* twigs on 2% water agar to observe sporulation. Conidiomata and sporulation on the twig were observed 7 days later.

Fungal structures such as conidia obtained from pycnidia of the symptomatic branches were observed with a Leica DM4B microscope to elucidate the conidial morphology. Photomicrographs were recorded and 50 conidia measurements were made (Leica DM4B or Leica DM750). Conidiomata on dead or dying branches were also observed with a dissecting microscope (Leica SPAO).

DNA extraction, PCR amplification and sequencing. The pathogen's DNA was extracted from mycelium cultured on PDA plates using GV-filamentous fungi genomic DNA extraction kit (Beijing Dingguo Changsheng Biotechnology Co. Ltd, Beijing, China) according to the manual. Target regions of the internal transcribed spacer (*ITS*) of the nuclear rDNA (*ITS1-5.8S-ITS2*) using *ITS1* (5'-TCCGTAGGTGAACCTGCGG-3') and *ITS4* (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) primers were amplified using PCR. A 25 µl PCR mixture was prepared, which contained 2.5 µl of 10× PCR buffer, 0.5 µl of 10 µmol/liter of each primer, 0.2 µl of 5 U/µl ExTaq DNA Polymerase (Takara, China), 2 µl of dNTP mixture (2.5 mmol/liter of each), 1 µl DNA template, and ddH₂O to 25 µl. The PCR conditions for amplification of the *ITS*-rDNA region were initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s,

annealing at 58°C for 30 s, and extension at 72°C for 45 s; and final extension at 72°C for 7 min.

The *EF1-α* was amplified and sequenced with primers *EF1-688F* (5'-CGGTCACTTGATCTACAAGTGC-3') and *EF1-1251R* (5'-CCTCGAACTCACCAGTACCG-3') (Alves et al. 2008). The PCR mixture was the same as that of the *ITS*-rDNA, except that the primers were replaced by *EF1-α* primers. The PCR conditions for amplification of the *EF1-α* region were initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. PCR products were sequenced at IGE Biotechnology LTD in Guangzhou, China. The newly obtained sequences were deposited in the GenBank database with the accession numbers MN294610 and MN326014.

Sequence alignment and phylogenetic analysis. The resulting *ITS*-rDNA and *EF1-α* sequences were compared with the GenBank database using BLASTN at the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov>). Closely related and previously published sequences were selected for phylogenetic analyses. Analysis was done according to a previously published methodology by Wang et al. (2019). A phylogenetic tree was constructed using MrBayes 3.2. The MCMC (Markov Chain Monte Carlo) method was performed to estimate the posterior probabilities of the phylogenetic trees and generate a 50% majority rule consensus tree. TREEVIEW1.6 was used to display and edit the trees. Sequences of *D. seriata* and *D. mutila* were used as outgroup.

Results

Fungal isolation. A total of 13 isolates were obtained from diseased tissue samples. Morphologically, there were mainly two morphotypes (Type I and Type II). Six isolates of Type I and five isolates of Type II were identified, which accounted for 85% of the total isolates. After 4 days on PDA, the mycelium of Type I was dense and became grayish black, but that of Type II was rare and white. Under artificial culture condition, only Type II had sporulation. One isolate of each type (named *GDOP1* and *GDOP2*, respectively) was selected for the following study. The remaining two fungal isolates morphologically differed from each other, which might be from the air or other sources.

Pathogenicity tests. The isolates of *GDOP1* and *GDOP2* were selected to perform pathogenicity tests. Healthy branches were inoculated with both fungal isolates, *GDOP1* and *GDOP2*, respectively.

Fig. 1. Symptom of dieback disease on *O. pinnata* **A**, 3 days after inoculation with *GDOP1*; **B**, 8 days after inoculation with *GDOP1* isolate; **C**, 17 days after inoculation with *GDOP1* isolate; and **D**, 17 days after inoculation with *GDOP2* isolate.

At the third day after inoculation, the inoculation point of the wounded branch that was inoculated with *GDOP1* became black (Fig. 1A). Later, the black spots expanded rapidly, covering the branches; even the leaves on these branches eventually dried (Fig. 1B and C). Lastly, all the dried leaves fell and the branches themselves dried fully. The symptoms of these branches were consistent to those of diseased plants observed in the field. Even though the loci of the wounded branch inoculated with *GDOP2* became dark, the dark spots did not expand, and dryness was observed only on the area where inoculation was performed, and the branches grew normally (Fig. 1D). No symptoms were observed on unwounded or control branches. Similar results were obtained during the whole study period. From the branches inoculated with *GDOP1*, we successfully reisolated the same isolate as the original one. Thus, it was confirmed that *GDOP1* was the causal agent of dieback of *O. pinnata*.

Morphological characteristics. The mycelia of *GDOP1* grew very fast on PDA. Mycelia spread and covered the whole 9-cm Petri dish 3 days after inoculation. Aerial mycelia grew vigorously and occasionally grew up to the Petri dish cover. The mycelia were initially white. After 4 days of inoculation, the color deepened and eventually turned black (Fig. 2A). *GDOP1* did not produce spores on PDA, water agar media, or on autoclaved *O. pinnata* twigs on water agar.

Many conidiomata were observed on the diseased branches that were infected by *GDOP1*. The conidiomata were semi-immersed, near spherical, and black. The top of conidiomata protruded out of the plant epidermis (Fig. 2B). Two kinds of conidia were observed in these conidiomata. One was an immature type, hyaline and aseptate (Fig. 2D). The other was a mature type, dark brown, 1-median septate, and had a longitudinally striate appearance (Fig. 2E). Conidia of *GDOP1* were ellipsoid, 17.8 to 28.6×12 to 16.7 μm (avg. of 50 conidia = 23.5×13.8 μm , L/W ratio = 1.7, range from 1.32 to

2.28). These morphological characteristics of *GDOP1* corresponded to *L. pseudotheobromae* (Alves et al. 2008; Wang et al. 2016; Xue et al. 2019).

Molecular identification and phylogenetic analyses. The amplicon size of ITS and EF1- α sequence of *GDOP1* were 474 and 534 bp, respectively. These two sequences were deposited in GenBank (accession nos. MN294610 and MN326014). NCBI BLASTn search showed that *GDOP1* was most similar to *L. pseudotheobromae*. ITS sequence showed 99.79% similarity to that of published *L. pseudotheobromae* isolates CBS 116459 (type specimen of *L. pseudotheobromae*), CBS 116460, CBS 304.79, and CBS 374.54 while EF1- α sequence showed 99.67% similarity to that of CBS 116459, CBS 116460, and CBS 374.54 and 99.35% similarity to CBS 304.79. In the phylogenetic tree based on the ITS and EF1- α sequences, the isolate *GDOP1* clustered within a clade comprising reference isolates of *L. pseudotheobromae* (Fig. 3). Based on symptoms, pathogenicity, morphological characteristics, and molecular data, *GDOP1* (causing dieback of *O. pinnata*) was identified as *L. pseudotheobromae*.

Discussion

Dieback was newly found on *O. pinnata* in Guangzhou, China. Although few infected plants were observed in our survey, attention should be given to this disease since it has severe symptoms and may kill *O. pinnata* trees. In this study, the pathogen causing dieback of *O. pinnata* was identified as *L. pseudotheobromae* based on symptoms, pathogenicity, morphological characteristics, and molecular data. To our knowledge, this is the first report of *L. pseudotheobromae* causing dieback on *O. pinnata* in the world.

Dieback on *O. pinnata* seedlings was observed on almost all wounded branches (>90%) that were artificially inoculated by *GDOP1*. No symptoms were observed on unwounded branches in this study. These results showed that *GDOP1* invades *O. pinnata*

Fig. 2. Colony and conidial morphology of *GDOP1*. **A**, Colony morphology of a 15-day-old sample; **B**, conidiomata on diseased branches; **C**, uninfected healthy branch; **D**, immature conidia (whitish) with thin walls; **E**, mature conidia (dark brown) with septa and thick walls (scale bars: 20 μm); **F**, mycelia of *GDOP1* (scale bar: 200 μm).

trees mainly through wounds. This is consistent with previous studies (Xu et al. 2013).

L. pseudotheobromae was first described as a new species in 2008 (Alves et al. 2008). It is difficult to distinguish *L. pseudotheobromae* and *L. theobromae* just by their morphological characteristics. Therefore, DNA sequences of ITS and EF1- α genes were usually used to identify these species (Alves et al. 2008; Dou

et al. 2017; Xue et al. 2019; Zhang et al. 2019). In this study, not only did the morphology of *GDOI* correspond to *L. pseudotheobromae*, but also the sequences of ITS and EF1- α showed high homology to those of *L. pseudotheobromae*. Phylogenetic analyses also showed that *GDOI* was closest to *L. pseudotheobromae*. Therefore, the pathogen causing dieback on *O. pinnata* *GDOI* was determined as *L. pseudotheobromae*.

Fig. 3. Bayesian consensus tree generated from sequences of the combined ITS and TEF1- α dataset. Posterior probability values exceeding 50% are given on appropriate clades. Newly obtained sequences are indicated in bold.

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