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Pathogenicity of five Botryosphaeriaceae species isolated from *Tectona grandis* (teak): the pathogenic potential of *Lasiodiplodia* species

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Abstract

Botryosphaeriaceae species commonly cause cankers and diebacks on woody hosts widely threatening forests and plantations. In this study, pathogenicity tests on *Tectona grandis* were conducted for five Botryosphaeriaceae species, *viz*. *Barriopsis tectonae*, *Dothiorella tectonae*, *Lasiodiplodia brasiliense*, *L. pseudotheobromae* and *Sphaeropsis eucalypticola*. Detached and wounded *T. grandis* twigs were inoculated with 0.1 cm^3 agar plugs cut from the margins of actively growing colonies. Lesion development was recorded seven days after inoculation. Two isolates of *L. pseudotheobromae* (MFLUCC 12-0772 and MFLUCC 12-0796) associated with cankers and dieback were significantly pathogenic on *T. grandis*. However, each of these isolates was found only in one site amongst the 35 sites surveyed. Three species, *B. tectonae*, *D. tectonae* and *S. eucalypticola* were not pathogenic on *T. grandis*. It is supposed that these species might act as endophytes or saprobes on *T. grandis*. Two saprobic isolates *L. brasiliense* (MFLUCC 11-0414) and *L. pseudotheobromae* (MFLUCC 12-0053) were likely to be potential pathogens. This is the first report of the pathogenic potential of saprobic *L. brasiliense* and *L. pseudotheobromae* on teak

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in Thailand. The possible reasons for lesions caused by these two saprobes are discussed.

Key words – dieback – saprobes – Thailand – cankers

Introduction

Botryosphaeriaceae species are economically important forest, plantation and crop pathogens, which occur on a wide range of hosts (Pérez et al. 2009, Mehl et al. 2011, Sakalidis et al. 2011, Lynch et al. 2013, Dissanayake et al. 2016). They are commonly associated with dieback, canker, stem-end rot, shoot blight, wood necrosis, blue-stain of the sapwood and even cause plant death (Burgess et al. 2003, Phillips et al. 2005, Damm et al. 2007, Slippers & Wingfield 2007, Wunderlich et al. 2012, Dissanayake et al. 2016, Manawasinghe et al. 2016). For example, *Diplodia sapinea* is one of the most economically important pathogens of pine forests in South Africa, New Zealand and other parts of the Southern Hemisphere (de Wet et al. 2000, Smith et al. 2001, Reay et al. 2006).

Botryosphaeriaceae species have been reported to infect through wounds (Müller & von Arx 1954, Old & Davison 2000). They can also infect directly through lenticels, stomata or other natural openings on healthy plants (Brown & Hendrix 1981, Michailides 1991, Kim et al. 1999, Smith et al. 2001). It has been proposed that some Botryosphaeriaceae species have ability to live as endophytes and then shift into pathogens when the host is stressed. Therefore, these species are treated as opportunistic pathogens (Slippers & Wingfield 2007, Yan et al. 2018). Pathogenicity of this family has been studied on some hosts. Lynch et al. (2013) determined the pathogenicity of Botryosphaeriaceae species associated with coast live oak (*Quercus agrifolia*) decline in southern California. Sakalidis et al. (2011) examined the pathogenicity of eight Botryosphaeriaceae taxa associated with stem cankers and dieback of mango trees in the Kimberley region of Australia and concluded that *Lasiodiplodia* species were the most pathogenic fungus on that host. Mehl et al. (2014) studied Botryosphaeriaceae associated with dieback of *Schizolobium parahyba* in South Africa and Ecuador and tested their pathogenicity under greenhouse and field conditions. Yan et al. (2013) found that none of the 25 grape cultivars grown in China was resistant to *Botryosphaeria dothidea*, *Diplodia seriata*, *Lasiodiplodia theobromae* and *Neofusicoccum parvum* in Botryosphaeriaceae. Borges et al. (2015) reported that *Lasiodiplodia theobromae* caused canker of *Tectona grandis* in Brazil, which poses a serious threat to the commercial industry.

Tectona grandis is an important source for timber industry worldwide. However, little is known about their pathogens, specifically Botryosphaeriaceae species on *T. grandis* in Thailand. In this study, we tested the pathogenicity of *L. pseudotheobromae* associated with stem cankers and diebacks of *T. grandis* in Thailand. For this, five saprobic Botryosphaeriaceae species (Table 1) were tested to examine their pathogenic potential.

Materials & Methods

Symptoms and isolations

We selected seven Botryosphaeriaceae species isolated from trunk, branches, twigs and leaves of *Tectona grandis* in northern Thailand in our previous studies (Doilom et al. 2014, 2015, 2017). Those were *Barriopsis tectonae*, *Dothiorella tectonae*, *Lasiodiplodia brasiliense*, *L. pseudotheobromae*, *L. theobromae*, *Pseudofusicoccum adansoniae* and *Sphaeropsis eucalypticola*. Seven isolates (Table 1) are selected to conduct pathogenicity tests. The symptoms associated with *L. pseudotheobromae* on teak and the isolate MFLUCC 12-0796 grown on malt extract agar (MEA) are shown in Fig. 1.

Pathogenicity tests

Pathogenicity tests were carried out on detached twigs of *Tectona grandis* for seven Botryosphaeriaceae isolates (Table 1). Isolates were grown on 2% MEA at 25^{o}C for 7 days. We followed the modified inoculation techniques described in Damm et al. (2007) and Taylor et al.

(2009). Collected short twigs (2 cm diam.) were cut into pieces of 12 cm long. Surface disinfested in 70% ethanol for 30 s, 0.35% NaOCl for 2 min and 70% ethanol for 30 s. A small lateral incision was made through the bark and cambium layers with a sterile scalpel. Our tested species did not produce spores in culture. The agar plug inoculation method (Chen et al. 2014, Xu et al. 2015, Guan et al. 2016) is therefore used in this study. Agar plugs with 0.1 cm^3 diameter containing a thick layer of nutrient were cut from the edge of each actively growing colony and placed on each wound. Inoculated wounds were covered with a strip of Parafilm to prevent desiccation of the plug. Sterile MEA plugs were used as controls. Inoculated twigs were incubated in moist chambers at 27°C for seven days. There were five replicates for each isolate and the control. The lesion developments were measured with a ruler for statistical analysis and then photographed. Fungi were re-isolated from the advancing edge of twig lesions and sequenced.

Fig. 1 – Symptoms associated with *Lasiodiplodia pseudotheobromae*. a Cracking of bark and cankers spreading from base of tree. b, c Black discolouration of the inner bark. d, e Twig dieback. f Seven-day-old colony of *L. pseudotheobromae* (MFLUCC 12-0796) on MEA isolated from a trunk cankers symptom.

Re-isolation

Fungal isolates from inoculated twigs were re-isolated by cutting the interface between healthy and lesion parts into small pieces (1 cm^2). The plant tissues were surface disinfected in 70% ethanol for 1 min, sterile distilled water for 2 min, 3% NaOCl for 1 min and then sterile distilled water for 2 min. After dried on sterile filter paper, tissues were placed on WA containing 0.02 %

streptomycin and incubated at 25°C for 2–4 days. Hyphal tips with agar were transferred to new MEA until we got pure cultures.

Table 1 Isolates used in the pathogenicity test.

Taxa	Culture No.	Life mode	Substrate
Barriopsis tectonae	MFLUCC 12-0381	Saprobe	Dead branch
Dothiorella tectonae	MFLUCC 12-0382	Saprobe	Dead branch
Lasiodiplodia brasiliense	MFLUCC 11-0414	Saprobe	Dead branch
L. pseudotheobromae	MFLUCC 12-0053	Saprobe	Dead twig
L. pseudotheobromae	MFLUCC 12-0772	Associated with dieback	Twigs
L. pseudotheobromae	MFLUCC 12-0796	Associated with cankers	Trunk
Sphaeropsis eucalypticola	MFLUCC 13-0701	Saprobe	Dead branch

DNA extraction, PCR amplification, sequencing and nucleotide comparison

The genomic DNA was extracted using the Fungus DNA Extraction Kit (Bioer Technology Co., Hangzhou, China). Fresh mycelia collected from 5–7 days-old cultures were used to extract total genomic DNA according to the manufacturer's instructions. DNA amplification was performed by polymerase chain reaction (PCR). The internal transcribed spacer (ITS) operon of the ribosomal DNA, partial translation elongation factor 1-α (TEF) and partial β-tubulin (Tub2) genes were amplified and sequenced using the primer pairs ITS1/ITS4, EF1F/EF2R and Bt2a/Bt2b, respectively (White et al. 1990, Glass & Donaldson 1995, Jacobs et al. 2004). Amplifications were performed in a 25 μL reaction volume as described in Dong et al. (2020) and followed the PCR thermal cycles as specified in Doilom et al. (2015). PCR products were visualized on 1% agarose electrophoresis gels stained with Gel Red. The sequencing was carried out at Sangon Biological Engineering Technology and Services Co., Shanghai, China. Isolates were identified using morphological characters coupled with nucleotide comparisons performed in the BioEdit (Hall 1999).

Statistical analyses

Differences in lesion lengths were assessed with one-way analysis of variance (ANOVA). Given the *F*-test rejects the general hypothesis that the treatment means were the same, subsequent treatment means were compared using Fisher's Least Significant Difference (LSD). All the processes referred to the requirements of Kozak & Powers (2017). The corresponding statistical analyses were performed using the 'agricolae' package in R version 3.6 (de Mendiburu 2020).

Results

Pathogenicity tests

All isolates of *Lasiodiplodia brasiliense* and *L. pseudotheobromae* developed lesions on the detached twigs of *Tectona grandis*, with discolorations and necrosis on bark and cambium tissues (Figs 2a–d). *Lasiodiplodia brasiliense* (MFLUCC 11-0414) produced the longest lesion ($\bar{x}$ = 9.96±1.11 cm) (Fig. 3). The twigs inoculated with *Barriopsis tectonae* showed the smallest lesion (Fig. 2e). *Dothiorella tectonae* and *Sphaeropsis eucalypticola* did not produce lesions on the detached twigs (Figs 2f, g). No lesions were observed on the controls, apart from the initial wounding of the twigs. Furthermore, the statistical analysis showed that the lengths of lesions caused by four strains of two *Lasiodiplodia* species were significantly different from the other strains ($p<0.05$) (Fig. 3).

Fig. 2 – Lesion developments after 7 days of inoculation of each mycelium plug. a *Lasiodiplodia brasiliense* (MFLUCC 11-0414). b *Lasiodiplodia pseudotheobromae* (MFLUCC 12-0796). c *Lasiodiplodia pseudotheobromae* (MFLUCC 12-0772). d *Lasiodiplodia pseudotheobromae* (MFLUCC 12-0053). e *Barriopsis tectonae* (MFLUCC 12-0381). f *Dothiorella tectonae* (MFLUCC 12-0382). g *Sphaeropsis eucalypticola* (MFLUCC 13-0701). h Control.

Pathogen re-isolation

The re-isolated colonies of *Lasiodiplodia brasiliense* and *L. pseudotheobromae* were similar to the colonies used for inoculating, and thus those four isolates were sequenced for nucleotide comparison (Table 2). However, fungi re-isolated from *Barriopsis tectonae* inoculated shoots did not show any similarity. Those colonies were similar to *Diaporthe* species. Thus, the isolate was not selected for the statistical and molecular analyses.

Nucleotide comparison

Three re-isolations of *Lasiodiplodia pseudotheobromae* from inoculated twigs had identical ITS, TEF and Tub2 sequences with the isolations from symptomatic tissues. Similarly, the re-isolation of *L. brasiliense* had nearly identical sequence data with the isolations from symptomatic tissue (Table 2). The results confirmed that the lesions shown on the twigs were respectively caused by *L. brasiliense* and *L. pseudotheobromae*.

Table 2 Nucleotide comparison of isolations from symptomatic tissue and re-isolations from inoculated twigs.

	***Lasiodiplodia brasiliense* (MFLUCC 11-0414)**	***L. pseudotheobromae* (MFLUCC 12-0053)**	***L. pseudotheobromae* (MFLUCC 12-0772)**	***L. pseudotheobromae* (MFLUCC 12-0796)**
ITS	496/496 (100%)	454/454 (100%)	495/495 (100%)	499/499 (100%)
TEF	338/345 (98%)	385/385 (100%)	301/302 (100%)	333/333 (100%)
Tub2	404/404 (100%)	418/418 (100%)	409/409 (100%)	440/440 (100%)

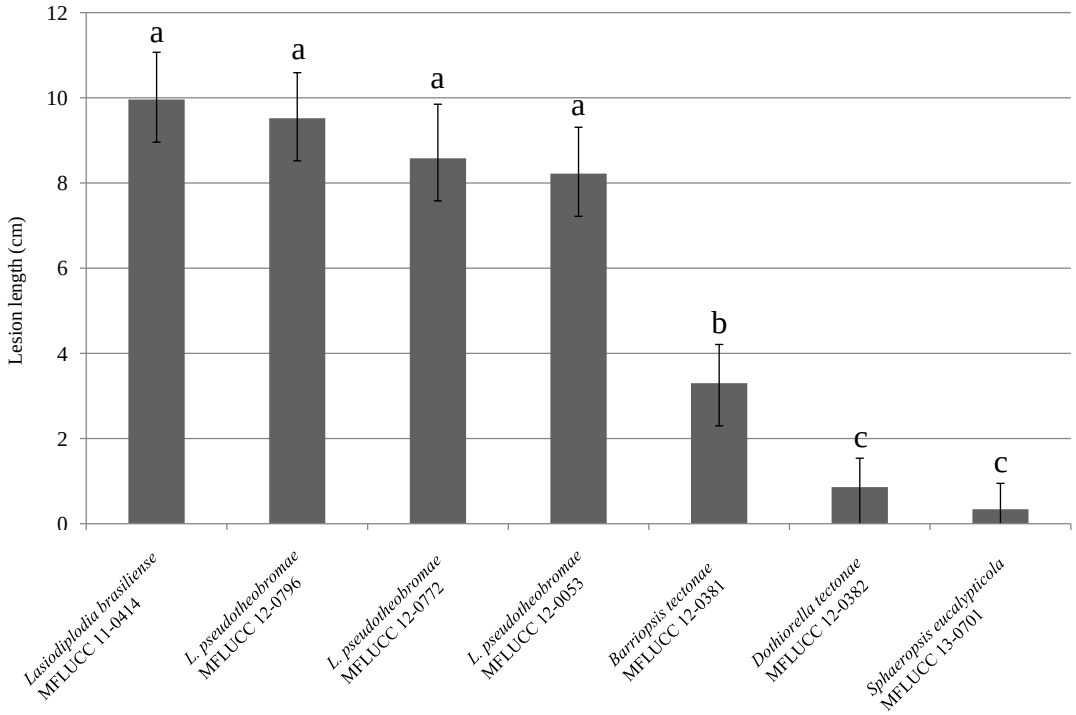

Fig. 3 – Mean lesion length values (cm) of different Botryosphaeriaceae species after 7 days of inoculation on detached twigs of *Tectona grandis*. Lesion length is based on average of five replicates. Different letters indicate significant differences ($p < 0.05$).

Discussion

We tested the pathogenicity of Botryosphaeriaceae species, which were isolated from branches, twigs and trunk of *Tectona grandis* (Table 1). Pathogenicity tests indicated that one isolate of *Lasiodiplodia brasiliense* and three isolates of *L. pseudotheobromae* were able to infect *T. grandis* by developing lesions.

Two isolates of *Lasiodiplodia pseudotheobromae* (MFLUCC 12-0772 and MFLUCC 12-0796) associated with cankers and dieback were confirmed as pathogenic on *Tectona grandis*. However, the disease severity of *L. pseudotheobromae* is low on *T. grandis*. Out of 35 surveyed sites, symptoms were encountered only in one natural forest and one plantation. The lesion lengths developed by two isolates provide the pathogenic potential of these taxa on *T. grandis*. *Barriopsis tectonae*, *Dothiorella tectonae* and *Sphaeropsis eucalypticola* did not develop any symptom on *T. grandis* twigs (Fig. 2).

Several Botryosphaeriaceae species survive as saprobes on dying barks (Tisserat 2004) or as endophytes in healthy plant tissues (Dissanayake et al. 2018, Jayawardena et al. 2018). These fungi are well known opportunistic pathogens, and the opportunistic nature of Botryosphaeriaceae is also shown in several studies (Slippers & Wingfield 2007, Manawasinghe et al. 2016, Osorio et al. 2017). Depending on these facts, we suppose that saprobes *Lasiodiplodia brasiliense* (MFLUCC 11-0414) and *L. pseudotheobromae* (MFLUCC 12-0053) isolated from *T. grandis* (Doilom et al. 2015) might have potential pathogenesis. This was confirmed by our preliminary pathogenicity tests. Yan et al. (2018) demonstrated the importance of high temperatures for the opportunistic infections of *Lasiodiplodia* species and other members of Botryosphaeriaceae. Therefore, we suppose that two saprobes are probably latent or facultative pathogenic fungi and they might be able to infect *T. grandis* under stressed environments, such as drought, late frosts, cold winds, hot winds, insect damage or pruning.

There are few studies conducted on pathogenic Botryosphaeriaceae species associated with *T. grandis* (Borges et al. 2015). Therefore, it is important to conduct proper studies to identify potential pathogens on teak as saprobes and endophytes. In addition, it is necessary to understand

the alternative life modes of these isolated taxa and establish and understand the relationships regarding the infection pathways. Such data can suggest appropriate management strategies.

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