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ORIGINAL ARTICLE

Pathogenicity of six *Calonectria* species isolated from five soil layers in a *Eucalyptus* plantation

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Abstract

The genus *Calonectria* includes species that are pathogens of many plants. Calonectria leaf blight is a serious threat to *Eucalyptus* plantations in China and elsewhere in the world. Some *Calonectria* recovered from the surface layer (<20 cm) of soils can cause typical leaf blight symptoms on *Eucalyptus* trees. However, pathogenicity tests of *Calonectria* species from deeper soil layers have not been conducted. We tested the pathogenicity of 42 isolates of *Calonectria aconidialis*, *C. chinensis*, *C. hongkongensis*, *C. ilicicola*, *C. kyotensis* and *C. orientalis* obtained from 0–20, 20–40, 40–60, 60–80 and 80–100 cm soil layers in one *Eucalyptus* plantation in southern China. All the *Calonectria* isolates were pathogenic to two *Eucalyptus* genotypes. For isolates of the same *Calonectria* species from different soil layers, there was no evidence of correlation between their pathogenicity and soil depth. The pathogenicity of some isolates found in deeper soil layers was stronger than isolates in shallower soil layers. This may increase the challenges of controlling the diseases caused by these fungi. This study performed the first pathogenicity test for *C. chinensis*, *C. kyotensis* and *C. orientalis* on plants, and the first pathogenicity test for *C. ilicicola* on *Eucalyptus*. Research results in this study helped to clarify the potential threats of *Calonectria* species in deeper soil layers posed to *Eucalyptus* plantations.

KEYWORDS
inoculation, leaf blight, plant pathogen, soil fungi, tree disease

1 | INTRODUCTION

The *Calonectria* genus includes destructive plant pathogens which have a wide host plant range from agriculture, horticulture and forestry distributed in tropical and subtropical regions (Crous, 2002; Daughtrey, 2019; Li et al., 2021; Liu et al., 2020; Lombard et al., 2010a; Vitale et al., 2013). Diseases caused by *Calonectria* include seedling damping-off, seedling rot, cutting rot, leaf spots, leaf blight, stem cankers, root and tuber rot (Chen et al., 2011; Crous, 2002; Freitas et al., 2019; Gai et al., 2017; Gehesquière et al., 2016; Lechat et al., 2010; Lombard et al., 2010b, 2011; Lopes et al., 2017; Old et al., 2003; Rodas et al., 2005; Wang & Chen, 2020; Wu & Chen, 2021).

Some *Calonectria* species are pathogens of *Eucalyptus* trees. *Calonectria* species primarily infect and cause leaf blight on the leaves of *Eucalyptus* tree plantation (Alfenas et al., 2015; Chen et al., 2011; Crous, 2002; Lombard et al., 2015; Rodas et al., 2005; Wang & Chen, 2020). This disease can reach outbreak proportions under conditions of high humidity and frequent rainfall (Chen et al., 2011; Crous, 2002; Old et al., 2003; Rodas et al., 2005). Calonectria leaf blight (CLB) is one of the most damaging diseases of *Eucalyptus* worldwide and it causes significant economic losses (Chen et al., 2011; Crous, 2002; Crous et al., 2004; Freitas et al., 2019; Li et al., 2017; Old et al., 2003; Rodas et al., 2005; Wang & Chen, 2020; Zhou & Wingfield, 2011).

Several *Calonectria* species have been isolated from diseased *Eucalyptus* trees and soils in *Eucalyptus* plantations in China (Chen et al., 2011; Li et al., 2017; Liu et al., 2020, 2021; Lombard et al., 2015; Wang & Chen, 2020; Wu & Chen, 2021). *Calonectria pseudoreteaudii*

is the most important causal agent of CLB in *Eucalyptus* plantations in China (Li et al., 2017; Liu et al., 2020; Lombard et al., 2015; Wang & Chen, 2020; Wu & Chen, 2021). This fungus has been isolated from diseased tissues of several *Eucalyptus* genotypes, which are planted in different geographic regions of southern China (Li et al., 2017; Liu et al., 2020; Lombard et al., 2010b, 2015; Wang & Chen, 2020; Wu & Chen, 2021). Inoculation studies indicated that *C. pseudoreteaudii* caused leaf blight and killed the *Eucalyptus* seedlings in a few days (Wang & Chen, 2020; Wu & Chen, 2021). Several *Calonectria* species have also been isolated from soils in the *Eucalyptus* plantations (Li et al., 2017; Liu et al., 2020, 2021; Lombard et al., 2015; Wang & Chen, 2020; Wu & Chen, 2021). Recent inoculations showed that *C. aconidialis*, *C. auriculiformis*, *C. hongkongensis*, *C. pseudoreteaudii* and *C. reteaudii* isolated from *Eucalyptus* plantation soils were all pathogenic to tested *Eucalyptus* genotypes causing leaf lesions and leaf blight on *Eucalyptus* seedlings within 3 days (Wu & Chen, 2021).

Some *Calonectria* species are soil-borne fungal pathogens, and they can survive in the soil for long periods by producing microsclerotia (Crous, 2002; Phipps et al., 1976; Sobers & Littrell, 1974; Thies & Patton, 1970). Microsclerotia of some *Calonectria* can exist in the soil without hosts for 15 years or longer (Sobers & Littrell, 1974; Thies & Patton, 1970). Anderson (1918) found *Calonectria* microsclerotia at 66 cm below the topsoil layer. Long-term survival and the deep soil presence of microsclerotia may increase the difficulties of prevention and management of diseases caused by *Calonectria* species.

Few studies have evaluated the distribution characteristics and pathogenicity of *Calonectria* in the soils of *Eucalyptus* plantations, and the research has typically focused on the surface soil (<20 cm) (Jessadarom et al., 2018; Wu & Chen, 2021). The pathogenicity of *Calonectria* isolates from different soil layers is unknown. Understanding the pathogenicity characteristics of *Calonectria* species in different soil layers will help clarify their potential threats to *Eucalyptus* plantations. A systematic research was recently conducted where six *Calonectria* species were isolated and identified from five different soil layers from one *Eucalyptus* plantation in China (Liu et al., 2021). The aims of the current study were to test the pathogenicity of these six *Calonectria* species on *Eucalyptus* seedlings, and to understand the pathogenicity variances among isolates of the same *Calonectria* species isolated from different soil layers.

2 | MATERIALS AND METHODS

2.1 | *Calonectria* isolates and *Eucalyptus* seedlings for inoculations

Based on the research results in Liu et al. (2021), forty-two previously studied *Calonectria* isolates representing six *Calonectria* species, which include *C. hongkongensis* (10 isolates), *C. aconidialis* (10 isolates), *C. kyotensis* (8 isolates), *C. ilicicola* (8 isolates), *C. chinensis* (2 isolates) and *C. orientalis* (4 isolates), were used in the present study (Table 1). These 42 isolates were identified based on phylogenetic analysis using DNA sequences for the translation elongation factor1-alpha (*tef1*), β-tubulin (*tub2*), calmodulin (*cmdA*) and histone H3 (*his3*) gene regions as well as morphological characteristics (Liu et al., 2021). Two isolates were selected for each *Calonectria* species in each soil layer (Table 1). In addition, *C. pseudoreteaudii* CSF13317, which was studied by Wang and Chen (2020) and identified as *C. pentaseptata* prior to the taxonomic revision of Liu et al. (2020), was selected as the positive control (Table 1). *Calonectria pseudoreteaudii* CSF13317 was originally isolated from leaf blighted *Eucalyptus* trees in southern China and was pathogenic to *Eucalyptus* genotypes (Wang & Chen, 2020). The 43 isolates are preserved in the culture collection of the Research Institute of Fast-growing Trees (RIFT)/China Eucalypt Research Centre (CERC) of the Chinese Academy of Forestry (CAF) in Zhanjiang, Guangdong Province, China. These 43 *Calonectria* isolates were subsequently transferred onto 2% malt extract agar (MEA) (20 g malt extract powder and 20 g agar powder per litre of water: malt extract powder was obtained from Beijing Shuangxuan microbial culture medium products factory, Beijing, China; the agar powder was obtained from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and maintained at room temperature for 7 days prior to inoculations.

Two *Eucalyptus* hybrid genotypes, the *E. urophylla* × *E. grandis* genotype CEPT1878 and the *E. urophylla* × *E. tereticornis* genotype CEPT1879, which are widely planted in southern China, were used for inoculations. Young plants were obtained in September 2021 from tissue culture and cultivated under greenhouse conditions (20–25 °C and a natural photoperiod). The *Eucalyptus* plants were 3 months old and approximately 30–40 cm high at the time of the inoculation trials. All the inoculated seedlings were of similar sizes.

2.2 | Pathogenicity tests

To examine the pathogenicity differences between the 42 isolates of six *Calonectria* species from different soil layers, mycelial plugs (5 mm in diameter) cut from the actively growing margins of 7-day-old MEA cultures were used to inoculate the leaves of *Eucalyptus* seedlings. For each *Eucalyptus* hybrid genotype, 10 mycelial plugs of each isolate were inoculated on the abaxial surface of 10 leaves of three *Eucalyptus* seedlings. No wounds were made on the inoculated leaves. Ten mycelial plugs of *C. pseudoreteaudii* isolate CSF13317 were inoculated on ten leaves of another three *Eucalyptus* seedlings as positive controls, and 10 leaves inoculated with sterile MEA plugs were treated in the same manner as negative controls. All the inoculated and control seedlings were kept in the moist plastic chambers and maintained in stable climatic conditions (humidity 65–75%; temperature 23–26°C) for 3 days. The entire experiment was repeated a second time using the same approach. Inoculations were performed during October 2021 at the South China Experimental Nursery (SCEN), located in Zhanjiang, Guangdong, China. Three days after inoculation, the most pathogenic isolate rotted the whole inoculated leaves. The plastic chambers were removed and all inoculated leaves were collected. To measure the lesion lengths of each

TABLE 1 Details of the tested *Calonectria* isolates used in this study

Calonectria species	Soil layer	Isolate No[a]	Genotype[b]	References
C. hongkongensis	0–20 cm	CSF20271	AAAA	Liu et al. (2021)
		CSF20272	AA--	Liu et al. (2021)
	20–40 cm	CSF20263	AA--	Liu et al. (2021)
		CSF20264	AA--	Liu et al. (2021)
	40–60 cm	CSF20268	AA--	Liu et al. (2021)
		CSF20269	AA--	Liu et al. (2021)
	60–80 cm	CSF20342	AA--	Liu et al. (2021)
		CSF20343	AA--	Liu et al. (2021)
	80–100 cm	CSF20293	AA--	Liu et al. (2021)
		CSF20294	AA--	Liu et al. (2021)
C. aconidialis	0–20 cm	CSF20492	AA--	Liu et al. (2021)
		CSF20496	AA--	Liu et al. (2021)
	20–40 cm	CSF20717	AA--	Liu et al. (2021)
		CSF20718	AA--	Liu et al. (2021)
	40–60 cm	CSF20583	AA--	Liu et al. (2021)
		CSF20584	AA--	Liu et al. (2021)
	60–80 cm	CSF20824	AA--	Liu et al. (2021)
		CSF20825	AA--	Liu et al. (2021)
	80–100 cm	CSF21224	AA--	Liu et al. (2021)
		CSF21225	AA--	Liu et al. (2021)
C. kyotensis	0–20 cm	CSF20261	AB--	Liu et al. (2021)
		CSF20262	AB--	Liu et al. (2021)
	20–40 cm	CSF20338	ABBA	Liu et al. (2021)
		CSF20341	AB--	Liu et al. (2021)
	40–60 cm	CSF21191	ABAA	Liu et al. (2021)
		CSF21192	AB--	Liu et al. (2021)
	60–80 cm	CSF21068	AB--	Liu et al. (2021)
		CSF21070	AB--	Liu et al. (2021)
C. ilicicola	0–20 cm	CSF20594	AAAB	Liu et al. (2021)
		CSF20595	AA--	Liu et al. (2021)
	20–40 cm	CSF21310	AAAB	Liu et al. (2021)
		CSF21312	AA--	Liu et al. (2021)
	40–60 cm	CSF21326	AA--	Liu et al. (2021)
		CSF21327	AA--	Liu et al. (2021)
	60–80 cm	CSF21215	BB--	Liu et al. (2021)
		CSF21216	BB--	Liu et al. (2021)
C. chinensis	0–20 cm	CSF20756	AAAA	Liu et al. (2021)
		CSF20759	AAAA	Liu et al. (2021)
C. orientalis	40–60 cm	CSF20605	AA--	Liu et al. (2021)
		CSF20607	AAAA	Liu et al. (2021)
	60–80 cm	CSF20614	AAAA	Liu et al. (2021)
		CSF20615	AAAA	Liu et al. (2021)
C. pseudoreteaudii	–[c]	CSF13317	AAAA	Wang and Chen, (2020)

[a]CSF, Culture Collection located at Research Institute of Fast-growing Trees (RIFT)/China Eucalypt Research Centre (CERC), Chinese Academy of Forestry, Zhanjiang, Guangdong Province, China.

[b]Genotype within each *Calonectria* species, determined by sequences of the *tef1*, *tub2*, *cmdA* and *his3* regions; '-' means not available.

[c]'–' represents *C. pseudoreteaudii* CSF13317 not isolated from any soil layer; this isolate was selected as the positive control.

leaf, two diameter measurements of each lesion, perpendicular to each other, were taken for each leaf, and the averages of diameter measurements (lengths) for each leaf were computed.

To confirm Koch's postulates, re-isolations were conducted, for which small pieces of the discoloured leaf (approximately 2 mm × 2 mm) were cut from the edges of the lesions and placed on 2% MEA at room temperature. Re-isolations were performed for four randomly selected leaves of each *Eucalyptus* genotype inoculated by each *Calonectria* isolate, and for all 10 leaves of each *Eucalyptus* genotype inoculated by the negative control. The identities of re-isolated fungi were verified by culture morphological characteristics, the fruiting structures and disease symptoms produced on inoculated leaves compared to the original fungi used for the inoculations. Results were analysed using SPSS Statistics 26 software (IBM Corp., Armonk, NY, USA) by an analysis of variance (ANOVA).

3 | RESULTS

Three days after inoculations, all treated seedlings (Figure 1a–d, i–t) and positive control seedlings (Figure 1e, f) displayed similar symptoms including leaf lesions and leaf blight. No disease symptoms were observed on the leaves of the negative control seedlings (Figure 1g, h). The fungi shared the same morphological characteristics with the originally inoculated *Calonectria* that were successfully re-isolated from the leaf lesions, but no *Calonectria* were re-isolated from the negative control leaves. Consequently, Koch's postulates were fulfilled. The leaves were assessed 3 days after inoculation. The ANOVA results showed that the results of the replicated pathogenicity tests were significantly different ($p < .05$). Thus, the data of each experiment were analysed separately.

The inoculation results showed that the pathogenicity of some isolates from the same *Calonectria* species between the two experiments was similar (Figures 2 and 3, Table S1). For example, *C. aconidialis* CSF20825 produced the longest lesions on *Eucalyptus* genotype CEPT1878 in both experiments, and isolate *C. hongkongensis* CSF20272 produced the shortest lesions on *Eucalyptus* genotype CEPT1879 in both experiments (Figures 2 and 3, Table S1).

The two experiments consistently showed the presence of isolate(s) of each *Calonectria* species produced significantly longer lesions ($p < .05$) than those on the positive control leaves, except for *C. orientalis* (Figures 2 and 3, Table S1). For example, lesions produced by some isolates of *C. hongkongensis* (CSF20264, CSF20293), *C. aconidialis* (CSF20496, CSF20717, CSF20824, CSF20825, CSF21224), *C. kyotensis* (CSF21068), *C. ilicicola* (CSF20595, CSF21215) and *C. chinensis* (CSF20756, CSF20759) were significantly longer ($p < .05$) than *C. pseudoreteaudii* CSF13317 on the two *Eucalyptus* hybrid genotypes in two experiments (Figures 1a–f, 2 and 3, Table S1).

The two experiments showed that the pathogenicities of some isolates within the same *Calonectria* species were significantly different ($p < .05$) (Figures 2 and 3, Table S1). For example, in both experiments, *C. aconidialis* CSF20825 produced significantly longer lesions ($p < .05$) than CSF20584, *C. hongkongensis* CSF20263 produced significantly longer lesions ($p < .05$) than CSF20272, and *C. kyotensis* CSF21068 produced significantly longer lesions ($p < .05$) than CSF21070 on the two tested *Eucalyptus* hybrid genotypes (Figures 1i–l, 2 and 3, Table S1).

For isolates of the same *Calonectria* species from different soil layers, both experiments showed that there was no evidence of correlation between pathogenicity and soil layer depth (Figures 2 and 3). Take *C. aconidialis* as an example, some isolates from shallower soil layers (isolate CSF20496 from the 0–20 cm soil layer, CSF20717 from the 20–40 cm soil layer, CSF20824 and CSF20825 from the 60–80 cm soil layer) produced significantly longer ($p < .05$) lesions than isolate from deeper soil layer (CSF21225 from the 80–100 cm soil layer) (Figures 2 and 3, Table S1). However, in some cases, isolate from the deeper soil layer (isolate CSF20825 from the 60–80 cm soil layer) produced significantly longer ($p < .05$) lesions than isolates from shallower soil layers (CSF20492 and CSF20496 from the 0–20 cm soil layer, CSF20718 from the 20–40 cm soil layer as well as CSF20583 and CSF20584 from the 40–60 cm soil layer) on two tested *Eucalyptus* genotypes in both experiments (Figures 1m–p, 2 and 3, Table S1).

Regarding two tested *Eucalyptus* genotypes, most of the tested *Calonectria* isolates produced significant longer lesions on genotype CEPT1878 than CEPT1879 in both experiments (Figures 1q–t, 2 and 3, Table S1). Except for isolates *C. aconidialis* CSF20584 and *C. kyotensis* CSF20262 in experiment one (Figure 2, Table S1), and *C. aconidialis* CSF20496 and CSF20583, *C. ilicicola* CSF20594, CSF21310 and CSF21327, and *C. orientalis* CSF20605 in experiment two (Figure 3, Table S1).

4 | DISCUSSION

The pathogenicities of 42 isolates belonging to six *Calonectria* species were tested through pathogenicity tests. The results showed that isolates of each *Calonectria* species from five soil layers could cause typical leaf lesion symptoms on the seedlings of two *Eucalyptus* hybrid genotypes. The inoculation results also showed that some isolates of *C. hongkongensis*, *C. aconidialis*, *C. kyotensis*, *C. ilicicola* and *C. chinensis* are more pathogenic than one tested isolate of *C. pseudoreteaudii*, which is highly pathogenic to *Eucalyptus* genotypes (Wang & Chen, 2020). These results indicated that the isolates are potential threats to *Eucalyptus* in southern China.

Pathogenicity assays, using different concentrations of conidia, have been used to study *Calonectria* that produce large numbers of conidia (Alfenas et al., 2016; Graça et al., 2009). However, for many *Calonectria* species, few or no conidia are produced in culture. Except for conidia suspension inoculation, other methods used for testing the pathogenicity of *Calonectria* include inoculation of mycelia suspension and mycelial plugs (Guo et al., 2016; Wu & Chen, 2021). Detached leaves were inserted into the margins of a colony by Guo et al. (2016). In this study, most of the isolates used for inoculations failed to produce conidia on the culture media. Thus, mycelial plugs were used to inoculate attached leaves of *Eucalyptus* seedlings. This

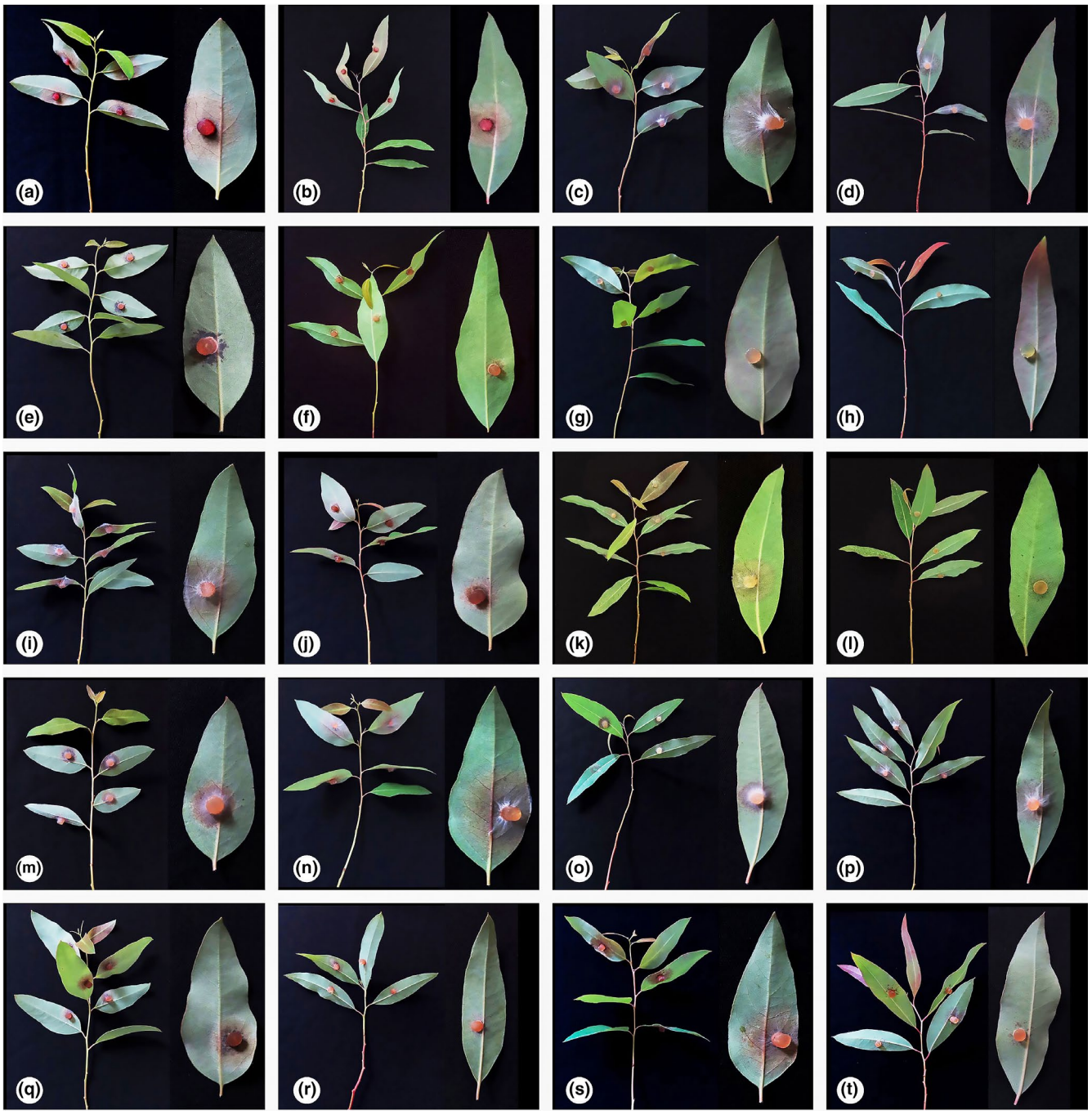

FIGURE 1 Symptoms on seedlings of *E. urophylla* × *E. grandis* hybrid genotype CEPT1878 and *E. urophylla* ×E. *tereticornis* hybrid genotype CEPT1879 inoculated by species of *Calonectria* mycelial plugs and MEA plugs. (a, b): CEPT1878 (a) and CEPT1879 (b) inoculated by *C. hongkongensis* CSF20264. (c, d): CEPT1878 (c) and CEPT1879 (d) inoculated by *C. aconidialis* CSF20824. (e, f): Disease symptoms were observed on leaves of CEPT1878 (e) and CEPT1879 (f) inoculated by *C. pseudoreteaudii* CSF13317 mycelial plugs (positive controls). (g, h): No disease symptoms were observed on leaves of CEPT1878 (g) and CEPT1879 (h) inoculated by sterile MEA plugs (negative controls). (i, j): CEPT1878 inoculated by *C. kyotensis* CSF21068 (i) and CSF21070 (j). (k, l): CEPT1879 inoculated by *C. kyotensis* CSF21068 (k) and CSF21070 (l). (m, n): CEPT1878 inoculated by *C. aconidialis* CSF20492 (m) and CSF20825 (n). (o, p): CEPT1879 inoculated by *C. aconidialis* CSF20492 (o) and CSF20825 (p). (q, r): CEPT1878 (q) and CEPT1879 (r) inoculated by *C. hongkongensis* CSF20294. (s, t): CEPT1878 (s) and CEPT1879 (t) inoculated by *C. aconidialis* CSF20718. a, b, k, l, s and t are from experiment one; c–j and m–r are from experiment two

method clearly revealed the pathogenicity of the *Calonectria* spp. on the tested *Eucalyptus* genotypes.

For the pathogenicities of six *Calonectria* species isolated from soils, except for *C. aconidialis* and *C. hongkongensis* tested to be pathogenic to *Eucalyptus* seedlings in the previous study (Wu & Chen, 2021), no pathogenicity tests on *Eucalyptus* have been conducted for the other four species. To our knowledge, this study conducted the first pathogenicity test for *C. chinensis*, *C. kyotensis*

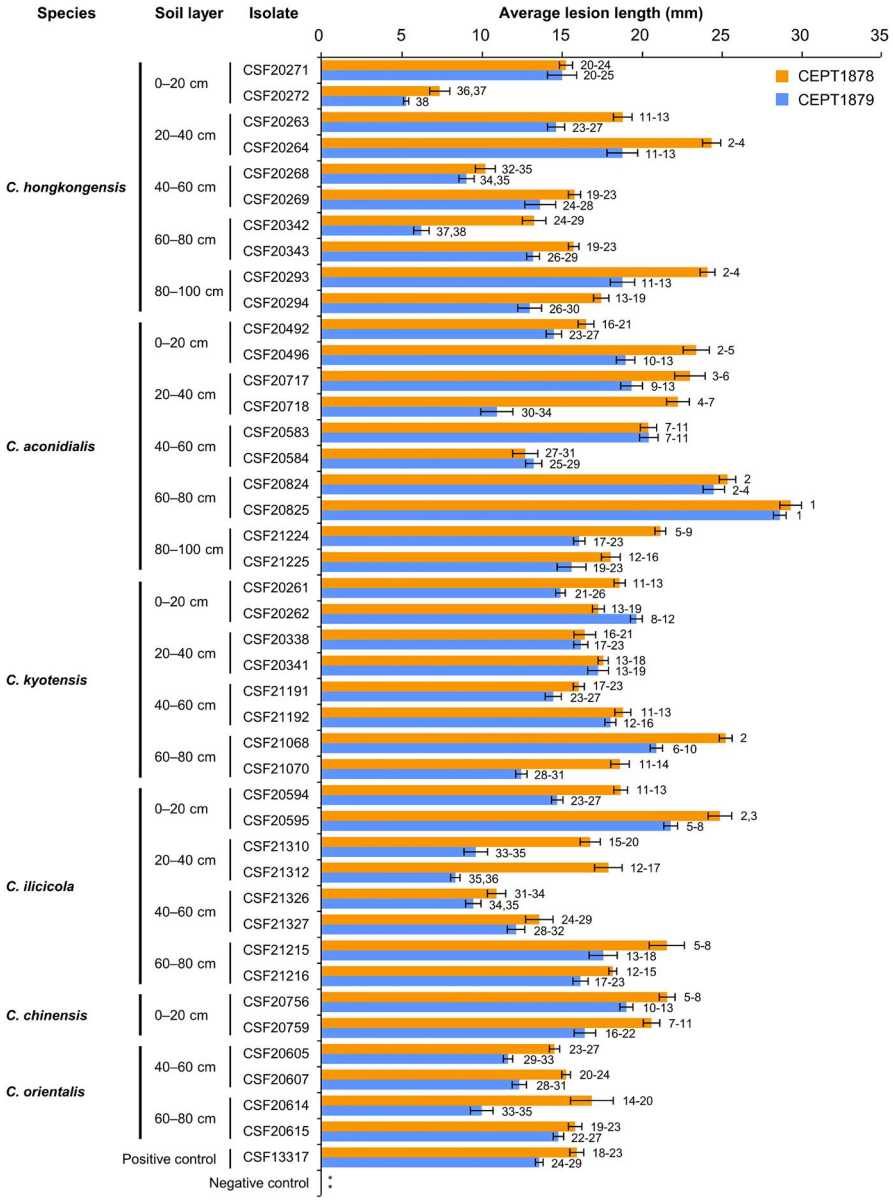

FIGURE 2 Pathogenicity results of experiment one. Column chart indicating the average lesion length (mm) on leaves resulting from inoculation trials of two *Eucalyptus* hybrid genotypes inoculated with 42 isolates of six *Calonectria* species, positive and negative controls. Horizontal bars represent the standard error of the means. Different numbers on the right of bars indicate treatment means that are significantly different ($p = .05$). '*' represents no lesions produced by the negative controls

and *C. orientalis* on plants. This was also the first pathogenicity test for *C. ilicicola* on *Eucalyptus* genotypes, and *C. ilicicola* was proved as an aggressive pathogen of peanut and soybean in many regions (Bell & Sobers, 1966; Crous, 2002; Gai et al., 2017; Ochi et al., 2011). Recent studies indicated that a large number of *Calonectria* species occur in the soils of *Eucalyptus* plantations in China (Li et al., 2017; Liu et al., 2020, 2021; Lombard et al., 2015; Wang & Chen, 2020; Wu & Chen, 2021). However, it is unclear if these species also occur on *Eucalyptus* tissues. It is necessary to determine if these species have already caused damage to *Eucalyptus* and to understand the dispersal and infection cycle of these species in further studies.

This study expands our understanding of the pathogenicity of *Calonectria* species isolated from soils and highlights the distribution of these potential pathogens in *Eucalyptus* plantations in China. To our knowledge, this study presented the first pathogenicity test for *Calonectria* species isolated from different soil layers. For isolates within the same *Calonectria* species, their pathogenicities seemed not to be influenced by the depths of soil layer. Statistical analysis results showed that some isolates distributed in deeper soil layers produced significantly longer lesions than isolates of the same *Calonectria* species from shallower soil layers. This may increase the challenges of controlling the diseases caused by these fungi. We also found that the resistances of the two *Eucalyptus* hybrid genotypes were significantly different for most of the tested isolates. Research in previous studies in China and Brazil also showed that the resistances of *Eucalyptus* genotypes to *Calonectria* isolates are significant different (Alfenas et al., 2016; Wang & Chen, 2020; Wu & Chen, 2021). These findings suggest that it may be possible to screen for disease-tolerant *Eucalyptus* genotypes to reduce the adverse effects of the diseases caused by *Calonectria*.

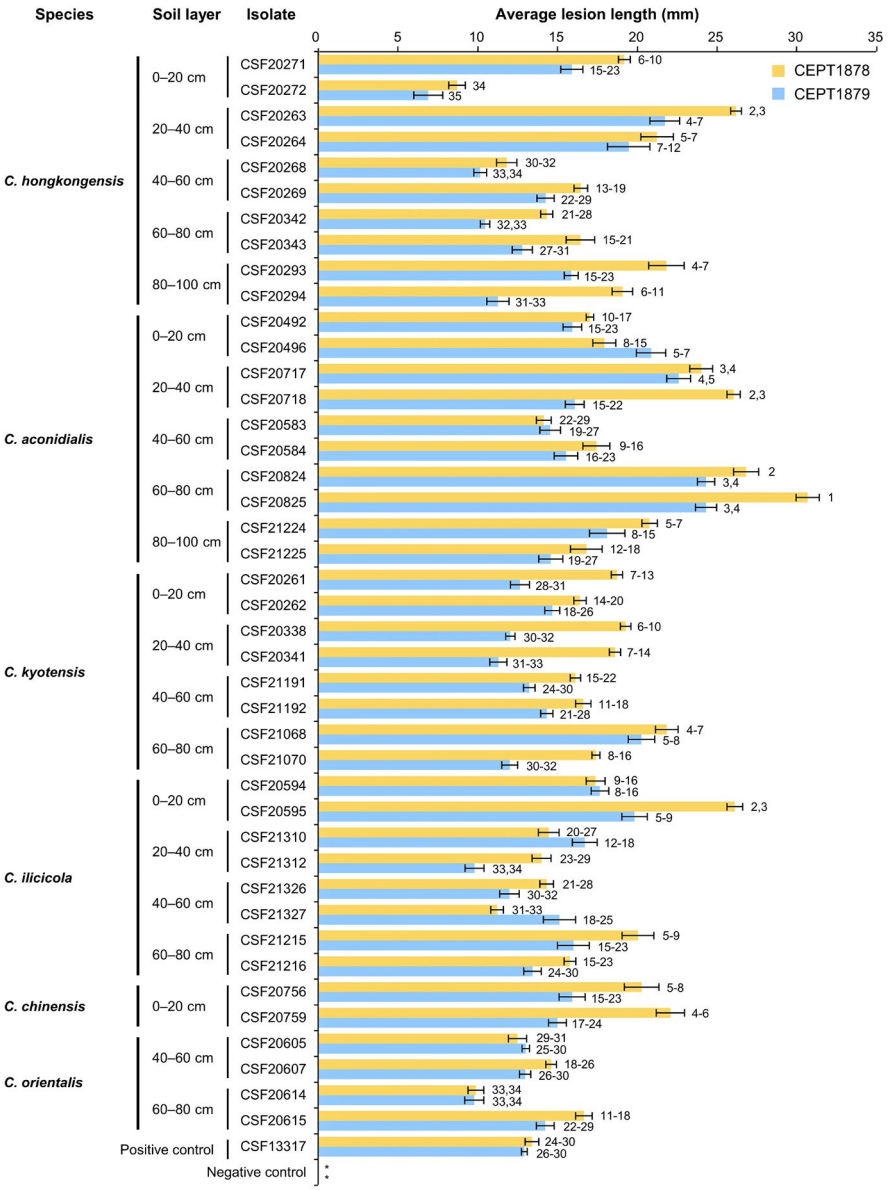

FIGURE 3 Pathogenicity results of experiment two. Column chart indicating the average lesion length (mm) on leaves resulting from inoculation trials of two *Eucalyptus* hybrid genotypes inoculated with 42 isolates of six *Calonectria* species, positive and negative controls. Horizontal bars represent the standard error of the means. Different numbers on the right of bars indicate treatment means that are significantly different ($p = .05$). '*' represents no lesions produced by the negative controls

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

ShuaiFei Chen planned and designed the research; LingLing Liu performed laboratory work; LingLing Liu and ShuaiFei Chen analysed data; and LingLing Liu and ShuaiFei Chen wrote the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jph.13096.

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