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ORIGINAL RESEARCH ARTICLE

Chrysoporthe cubensis emerges causing wilt on *Eucalyptus* mini-stumps in Brazil

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Abstract
A fungus that resembles *Chrysoporthe* sp. was found associated with *Eucalyptus* mini-stumps in clonal mini-gardens in Brazil causing severe losses. The symptoms observed were wilt that evolves into partial or complete drying and death of the canopy, and lesion in the xylem of the mini-stumps. The primary objectives of this research were (i) to determine the causal agent of the wilt disease using morphological and molecular analyses; (ii) to assess the pathogenicity of the isolates on various commercial clones of *Eucalyptus*; and (iii) to evaluate the impact of temperature on the pathogen's development. Through phylogenetic analyses of sequences from the Internal Transcribed Spacer, actin, and β-tubulin genes, *Chrysoporthe cubensis* was identified as the causal agent responsible for the wilt disease. All six tested *Eucalyptus* clones exhibited susceptibility to the pathogen, with clone CNB 007 demonstrating higher susceptibility and clones CNB 005 and CNB 030 displaying comparatively lower susceptibility. Furthermore, the development of the isolates varied depending on the *Eucalyptus* clone, with higher temperatures favouring pathogen growth. Notably, the less susceptible clones exhibited greater sensitivity to elevated temperatures compared to the more susceptible ones. This study represents the first report of *C. cubensis* causing wilt disease on *Eucalyptus* mini-stumps worldwide.

Keywords Clonal mini-garden disease. Cryphonectriaceae. Etiology. *Eucalyptus* canker

Introduction

Brazil has 9.55 million hectares of planted forests for industrial purposes, and approximately 78% of this area is occupied by *Eucalyptus* plantations (Ibá 2021). The country leads the global ranking of forest productivity with an average production of 35.7 m³/ha/year, a value equivalent to twice that of countries in the northern hemisphere. The main destinations for Brazilian planted forest woods are the pulp and paper industry, steel and charcoal, panels, and laminate flooring (Ibá 2021).

To optimize the production of clonal seedlings, new methodologies of vegetative propagation were developed and popularized in *Eucalyptus*, such as mini-cutting and micro-cutting techniques (Xavier and Silva 2009). These techniques improved the commercialization of clonal genotypes that were difficult to root (Ferreira et al. 2004), allied to other advantages of vegetative propagation in clonal mini-gardens such as lower costs of implantation, transportation, and maintenance, as well as greater ease of harvest, irrigation control, and rooting speed (Mafia et al. 2005).

Notwithstanding the recent advances in forest biotechnology, *Eucalyptus* cloning carried out in mini-gardens still faces some challenges, such as the losses caused by diseases favoured by the high temperature and humidity of the forest nurseries. Recently, fungal structures resembling those in the Cryphonectriaceae were found associated with several *Eucalyptus* mini-stumps in clonal mini-gardens in Brazil, for the first time. The symptoms observed were wilt that evolved into partial or complete drying of the canopy and subsequent death of the mini-stumps. The internal symptom was characterized by an upward lesion along the stem, and no fungal structures were observed in fresh samples.

Chrysoporthe Gryzenh. & M.J. Wingf. is a genus of fungi belonging to the family Cryphonectriaceae, order Diaporthales, phylum Ascomycota. The genus encompasses

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various and important pathogens known for causing typical symptoms of canker in species of the Myrtaceae and Melastomataceae families (Barreto et al. 2006; Gryzenhout et al. 2006; Myburg et al. 2003). Initially, the causal agent of *Eucalyptus* canker disease was described as *Diaporthe cubensis* (Bruner 1917), which was later transferred to the genus *Cryphonectria*, as *Cryphonectria cubensis* (Bruner) Hodges, due to similar cultural and morphological characteristics with *Cryphonectria* species (Hodges et al. 1980). Subsequently, based on morphological characters and phylogenetic analyses, isolates of *Cr. cubensis* were shown to be distinct from *Cryphonectria* (Gryzenhout et al. 2004; Myburg et al. 2003). As a result, the genus *Chrysoporthe* was created to accommodate the single species *Ch. cubensis* (Bruner) Gryzenhout & M.J. Wingf., (Gryzenhout et al. 2004).

In additional to *Ch. cubensis*, nine other *Chrysoporthe* species are currently known: *Ch. austroafricana*, *Ch. colombiana*, *Ch. deuterocubensis*, *Ch. doradensis*, *Ch. hodgesiana*, *Ch. inopina*, *Ch. puriensis*, *Ch. syzygiicola*, and *Ch. zambiensis* (Gryzenhout et al. 2004, 2005, 2006; Chungu et al. 2010; van der Merwe et al. 2010; Oliveira et al. 2021; Suzuki et al. 2023). All these species were recorded in association with *Eucalyptus*, except *Ch. colombiana*, *Ch. hodgesiana*, *Ch. inopina*, *Ch. puriensis*, and *Ch. syzygiicola* that were found in the Melastomataceae (Gryzenhout et al. 2004, 2006; Oliveira et al. 2021; Suzuki et al. 2023). It was, however, demonstrated by inoculation that *Ch. colombiana*, *Ch. puriensis*, and *Ch. syzygiicola* are able to cause symptoms on *Eucalyptus* (Chungu et al. 2010; Oliveira et al. 2021; Suzuki et al. 2023). On the other hand, only *Ch. cubensis*, *Ch. puriensis*, and *Ch. doradensis* have been reported in Brazil (Hodges et al. 1976; Soares et al. 2018; Oliveira et al. 2021). Furthermore, there is strong evidence that *Ch. cubensis* and *Ch. puriensis* are native to Brazil, which is supported by the fact that these species occur commonly on native *Tibouchina* spp. and have a high level of genetic diversity (Oliveira et al. 2022).

Chrysoporthe cubensis is able to infect *Eucalyptus* at different ages. In young plants, the fungus may cause occasional death due to girdling of stem at the base of the trunk. In older plants, the symptoms vary from lesions with diverse degrees of bark cracking to typical cankers at different heights of the trunk (Ferreira 1989). A typical canker is a deep lesion bordered by calluses, as a consequence of cambium death in an attempt to prevent girdling of the stem (Alfenas et al. 2009). Canker caused by *Ch. cubensis* significantly impacted the development of *Eucalyptus* plantations in the tropics and southern hemisphere in the 1970s (Ferreira 1989), which motivated the first studies on the disease and boosted genetic breeding of *Eucalyptus* to obtain resistant species to the pathogen. Despite the extensive research on breeding, *Eucalyptus* canker caused by *Ch. cubensis* is still one of the most harmful diseases in commercial plantations in Brazil (Ferreira and Milani 2004; Gryzenhout et al. 2009).

To date, there are no studies that demonstrate the occurrence of *Chrysoporth*e spp. associated with *Eucalyptus* mini-stumps worldwide. Based on this, efforts to disclose the identity of the wilt disease and to evaluate how different clones react to the causal agent are crucial to the definition of control strategies in clonal mini-gardens. Therefore, the aims of the present study were (i) to elucidate the causal agent of the wilt disease on *Eucalyptus* mini-stumps through morphological/molecular analyses and pathogenicity tests on healthy eucalypt seedlings; (ii) to evaluate the susceptibility of seedlings of different commercial clones of *Eucalyptus* to the pathogen; and (iii) to evaluate the influence of temperature on the development of the disease.

Materials and methods

Fungal isolates and preservation of the cultures

Symptomatic mini-stumps of five hybrid clones of *E. urophylla* × *E. grandis* (CNB 005, CNB 007, CNB 010, CNB 029, and CNB 030) were collected from the clonal mini-garden at a forest company in the state of Minas Gerais, located in the Southeast region of Brazil. Symptoms included wilt, canopy drying, xylem lesion, and death (Fig. 1.A-H). Fungal structures were rarely found on mini-stumps surfaces, only after 72 h of incubation of segments from the bark (Fig. 1.I). All samples were first examined at 20× magnification under a stereomicroscope Motic® SMZ-140 for observation of fungal structures and longitudinal cuts were made with a sterilized scalpel, from the root system to the tip of the stem, in order to find internal symptoms.

For fungal isolation, surface disinfection of symptomatic stem fragments was done by stepwise washing in 70% ethanol for 30 s and 2% sodium hypochlorite solution for two minutes. Fragments were transferred to Potato Dextrose Agar (PDA) and incubated at 28 °C with a 12-hour photoperiod until mycelial growth. Additional isolates obtained out of symptomatic mini-stumps from clonal mini-gardens in the states of São Paulo and Mato Grosso do Sul were provided from the Collection of Laboratório de Patologia Florestal. All pure cultures obtained from hyphal tips on PDA were preserved and deposited in the Octavio de Almeida Drumond Collection (COAD) at Universidade Federal de Viçosa (UFV), Brazil under collection numbers COAD 3400–3401, COAD 3421–3423 and COAD 3478–3486 (Table 1).

Fig. 1 *Chrysoporthe cubensis* on *Eucalyptus* mini stumps. (**A**-**B**) Losses due to wilt caused by *Ch. cubensis* in a clonal-mini garden; (**C**) asymptomatic mini-stump; (**D**) early stage of drying; (**E**) late stage of drying; (**F**-**G**) lesion in the xylem; (**H**) mini-canker in inoculated plant; (**I**) Conidiomata exuding conidia on the surface of mini-stump bark

DNA extraction and amplification

The fungal isolates were grown on PDA at 28 °C under a 12-hour photoperiod for 7–10 days to obtain sufficient fungal biomass for DNA extraction. Mycelia was scraped off with a sterilized wooden toothpick and transferred to a 2 mL microcentrifuge tube. The extraction was performed by mechanical disruption using stainless steel beads in an L-Beader-3 (Loccus Biotecnologia, SP, Brazil). Total DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA) according to the manufacturer's instructions.

The primer pairs ITS1 and ITS4 (White et al. 1990), Bt1a and Bt1b (Glass and Donaldson 1995), and ACT-512 and ACT-783R (Carbone and Kohn 1999) were used to amplify the nuclear rDNA internal transcribed spacers (ITS1-5.8 S-ITS2 = ITS), β-tubulin (TUB), and actin (ACT) genes, respectively. All amplification reactions were prepared in a final volume of 12.5 µl. PCR was performed with 6 µL of Dream Taq TM PCR Master Mix 2× (MBI Fermentas, Vilnius, Lithuania);5 µmol/l of each forward and reverse primer; 0.5 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO); 1 mg/µL Bovine Serum Albumin (BSA, Sigma–Aldrich,);30 ng of genomic DNA and 2.5 µL

Table 1 GenBank accession numbers of DNA sequences of *Chrysoporthe* and outgroup species used in the phylogenetic analyses

Species	Isolate/Strain[1]	Country	Host	Genbank acession number[2]		
				ACT	*TUB*	ITS
Chrysoporthe cubensis	**COAD 3400**	Brazil: Minas Gerais, Belo Oriente	*Eucalyptus urophylla* × *Eucalyptus grandis*	OP524136	OP524159	–
	COAD 3401	Brazil: Minas Gerais, Belo Oriente	*Eucalyptus urophylla* × *Eucalyptus grandis*	OP524137	OP524149	OP846021
	COAD 3421	Brazil: Minas Gerais, Belo Oriente	*Eucalyptus urophylla* × *Eucalyptus grandis*	OP524138	OP524150	OP846023
	COAD 3422	Brazil: Minas Gerais, Belo Oriente	*Eucalyptus urophylla* × *Eucalyptus grandis*	OP524139	OP524148	OP846022
	COAD 3423	Brazil: Minas Gerais, Belo Oriente	*Eucalyptus urophylla* × *Eucalyptus grandis*	OP524140	OP524151	–
	COAD 3478 / LPF 2121	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	OP524141	OP524152	–
	COAD 3479 / LPF 2305	Brazil: Minas Gerais, Araxá	*Eucalyptus* sp.	OP524143	OP524153	–
	COAD 3480 / LPF 2347	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	OP524144	OP524154	–
	COAD 3481 / LPF 2374	Brazil: São Paulo, Agudos	*Eucalyptus* sp.	OP524142	OP524155	–
	COAD 3482 / LPF 2375	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	OP524147	–	–
	COAD 3483 / LPF 2378	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	OP524146	–	–
	COAD 3484 / LPF 2379	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	OP524145	OP524156	–
	COAD 3485 / LPF 2381	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	–	OP524157	–
	COAD 3486 / LPF 2382	Brazil: Mato Grosso do Sul, Três Lagoas	*Eucalyptus* sp.	–	OP524158	
	CMW 10,028	Colombia	*Miconia rubiginosa*	QG290161	GQ290175	–
	CMW 10,669	Republic of Congo	*Eucalyptus* sp.	GQ290171	GQ290177	–
	CMW 12,734	Mexico	*Rhyncanthera mexicana*	GQ290159	AH015646	–
	CMW 14,394 / CBS 118,654 (ET)	Brazil	*Eucalyptus* sp.	GQ0165	GQ290178	DQ368773
C. puriensis	CT 10 / LPFCT	Brazil	*Tibouchina granulosa*	–	MN590040	MN590028
	CT 13 / CML3738 (T)	Brazil	*Tibouchina granulosa*	–	MN590041	MN590029
C. deuterocubensis	CMW 12,745	Singapore	*Tibouchina urvilleana*	GQ290160	GQ290183	JN942340
	CMW 17,178	Thailand	*Tibouchina urvilleana*	GQ290164	DQ368785	JN942339
	CMW 2631	Australia	*Eucalyptus marginata*	GQ290174	GQ290184	GQ290157
	CMW 8650 (T)	Indonesia	*Syzygium aromaticum*	GQ290172	AY084024	–
C. hodgesiana	CMW 10,641 (T) / CBS 115,854	Colombia	*Tibouchina semidecandra*	–	AY 692,326	JN942329
	CMW 9995	Colombia	*Tibouchina semidecandra*	GQ290162	AY956978	AY956969
	CMW 10,625	Colombia	*Miconia theaezans*	GQ290170	AY262391	JN942328
C. syzygiicola	CMW 29,940 (T)	Zambia	*Syzygium guineense*	–	FJ805230	JN942335
	CMW 29,942	Zambia	*Syzygium guineense*	–	FJ805232	FJ655007
C. zambiensis	CMW 29,928 (T) / CBS 124,503	Zambia	*Eucalyptus grandis*	–	FJ858709	JN942333
	CMW29930	Zambia	*Eucalyptus grandis*	–	FJ858711	–
C. austroafricana	CMW 10,192	South Africa	*Syzygium cordatum*	GQ290163	GQ290176	–
	CMW 2113 (T)	South Africa	*Eucalyptus grandiss*	–	AF 273,067	JN942338
	CMW 9327	South Africa	*Tibouchina granulosa*	GQ290173	GQ290185	–
C. inopina	CMW 12,727 (T)	Colombia	*Trichilia lepidota*	GQ290169	GQ290180	DQ368777
	CMW 12,729	Colombia	*Trichilia lepidota*	GQ290166	DQ368808	DQ368778
	CMW 12,731	Colombia	*Trichilia lepidota*	GQ290168	GQ290182	DQ368779
C. doradensis	CE 37 (T) / CBS 115,735	Brazil	*Eucalyptus* sp.	KX603776	KX639099	NR_165948

Table 1 (continued)

Species	Isolate/Strain[1]	Country	Host	Genbank acession number[2]		
				ACT	*TUB*	ITS
	CE 38	Brazil	*Eucalyptus* sp.	KX603777	KX639100	–
	CE 45	Brazil	*Eucalyptus* sp.	KX603778	KX639101	–
	CMW 11,286 (T)	Ecuador	*Eucalyptus grandis*	–	AY 214,217	–
Amphilogia gyrosa	CMW 10,469 (T)	New Zealand	*Elaeocarpus dentatus*	–	AF525707	AF452116
	YMJ 91,123,101	Taiwan	*Elaeocarpus japonicus*	EF025600	EF025615	EF026147

[1]Culture collections: CBS-KNAW Culture Collection, Westerdijk Fungal Biodiversity Institute (CBS); Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria (CMW); Coleção Octávio de Almeida Drumond (COAD); Laboratório de Patologia Florestal collection (LPF); Coleção Micológica de Lavras (CML); culture collection of the Forest Pathology Laboratory (LPF) of Universidade Federal de Lavras. Isolates obtained in the present study are in boldface. [2] Accession numbers for the sequences of the genomic loci Actin (*ACT*), ß-tubulin (*TUB*) and Internal Transcribed Spacer (ITS) deposited in the GenBank. Type or ex-type specimens are indicated by a (T) after the scientific name

of nuclease-free water. Amplification was performed with an initial denaturing step at 94 °C for 5 min, followed by 38 cycles of denaturation at 94 °C for 30 s, primer annealing at 54 °C for 30 s (for both *TUB* and ITS), or 60 °C for 30 s (*ACT*), and extension at 72 °C for 45 s, with an additional final extension at 72 °C for 7 min. The presence of PCR products was confirmed by 0.8% agarose gel electrophoresis. The amplified fragments were purified and sequenced by Macrogen Inc., South Korea.

Data editing and phylogenetic analyses

The sequences were edited and manually checked with the BioEdit software program version 7.2 (Hall 2014). Nucleotide arrangements with ambiguous positions were clarified by analyzing the sequences obtained with forward and reverse primers. Based on a BLAST search to check for similarity, 29 additional reference sequences were obtained from the NCBI GenBank database (Table 1). One dataset was built for each locus using the sequences obtained in this study, as well as the representative for the other known species of *Chrysoporthe* and *Amphilogia gyrosa* (outgroup), and then aligned individually using the MAFFT algorithm (Katoh and Standley 2013) implemented in Aliview software version 1.28 (Larsson 2014). The alignment concatenation (ITS + *TUB* + *ACT*) was performed in Mesquite version 3.61 (Maddison and Maddison 2018). Substitution models were determined separately for each gene partition using jModelTest 2.1.10 (Darriba et al. 2012), in which they were selected according to the Akaike Information Criterion (AIC).

Bayesian Inference (BI) trees were generated employing the Markov Chain Monte Carlo (MCMC) algorithm. The phylogenetic analyses were performed through CIPRES Science Gateway (Miller et al. 2010) using MrBayes 3.2.6 on the XSEDE tool (Ronquist and Huelsenbeck 2003). Four MCMC chains were run simultaneously from random trees for ten million generations and sampled every 1,000 generations. The first 2,500 trees were discarded as the burn-in phase of each analysis. The posterior probabilities were determined from the remaining trees. Additionally, Maximum Likelihood (ML) analyses were generated for each separate gene and multi-locus alignment using RAxML-HPC ver. 8.2.12 (Stamatakis 2014). The chain robustness was assessed through the bootstrap re-sampling strategy with 1,000 bootstrap test replicates. The trees obtained from single genes and multi-locus alignments were compared along with their performance in species recognition. The resulting trees were visualized in FigTree (Rambaut 2009) and exported to Inkscape v. 0.91 (www.inkscape.org) for editing of the layout.

Pathogenicity test

The pathogenicity of the five isolates from the state of Minas Gerais (COAD 3400–3423) was assessed on 6-month-old seedlings of six commercial hybrid clones of *E. urophylla* × *E. grandis* (CNB 005, CNB 007, CNB 010, CNB 029, CNB 030, and CNB 032). At the base of the seedling stem, a 1 cm wound was made with a scalpel to remove the bark and expose the xylem. A mycelium plug of 5 mm diameter from a 7-day-old culture was placed facing the wound to allow the contact between mycelium and the xylem. To avoid desiccation, the wound was covered with plastic film for 30 days, according to the method described by Ferreira and Milani (2004). Control treatment consisted of a sterilized PDA plug.

For each combination of *Eucalyptus* clone and isolate (including control), 10 plants were inoculated, resulting in a total of 360 plants divided into 36 treatments of clone × isolate combinations. Inoculated plants were kept under greenhouse conditions for 70 days. All seedlings were evaluated by measuring the lesion length in the xylem. Koch's postulates were performed by re-isolating the fungi from

the inoculated areas, and the cultures were compared to the original ones. The experiment was repeated once.

Effect of temperature on pathogenicity of Ch. cubensis on Eucalyptus clones

The effect of temperature was tested on 6-month-old seedlings of two commercial hybrid clones of *E. urophylla* × *E. grandis* (CNB 010 and CNB 032), which were inoculated with five isolates: COAD 3400–3401 and COAD 3421–3423. Inoculations were performed similar to those of the pathogenicity tests. For each combination of *Eucalyptus* clone and isolate (including control), 10 plants were inoculated, resulting in a total of 120 plants divided into 12 treatments of clone × isolate. Subsequent to the inoculation, half of the plants of each treatment were transferred to a growth chamber at 19 °C and the other half to 28 °C to compare the effect of temperature on disease development. At the end of 70 days, all seedlings were evaluated by measuring the lesion length in the xylem and the treatments were compared to each other. Koch's postulates were fulfilled by re-isolating the fungi from the inoculated areas, and the cultures were compared to the original ones. The experiment was not repeated.

Experimental design and data analysis

Tests for pathogenicity and the influence of temperature on disease development were carried out in a completely randomized design, with 10 biological replicates. The effects of the main factors for the pathogenicity test data (clones) and temperature tests (clones and temperature, and the interaction between them) were evaluated in a linear mixed modeling framework. Isolates and replicates were treated as random effects in our model. The model was fitted with the lmer function of the package lmer4 (Bates et al. 2015) of the software R version 4.2.2 (R Core Team 2022). Data were subjected to a type III analysis of variance with the Anova function on the car package (Fox and Weisberg 2019). The emmeans package was used to obtain the estimated marginal means and respective 95% confidence intervals (Lenth 2021). The function cld from multcomp package was used for multiple comparisons of treatment means via the Tukey test at 5% significance (Hothorn et al. 2008).

Results

Fungal isolates and preservation of the cultures

Fourteen isolates resembling species of *Chrysoporthe* were obtained from symptomatic mini-stumps of *Eucalyptus* in three states of Brazil, of which five were obtained in this study, one from each *Eucalyptus* clone, and nine from the Collection of Laboratório de Patologia Florestal (Table 1). Symptoms included wilt, canopy drying, xylem lesion, and death (Fig. 1.A-H). Fungal structures were rarely found on mini-stumps surfaces, only after 72 h of incubation of segments from the bark (Fig. 1.I).

Phylogenetic analyses

Phylogenetic analyses were performed on single-locus and multi-loci datasets of *ACT, TUB* and ITS. Single-locus trees (Suppl. material 1) showed low topological divergence, congruence for species delimitation, and high support value for the *Ch. cubensis* clade. The single-locus tree based on *TUB* sequences was the best in delimitating all the currently accepted species of *Chrysoporthe*. The ITS region alone did not accurately delineate *Ch. cubensis*, *Ch. austroafrican*a, *Ch. syzygiicola*, *Ch. deuterocubensis*, *Ch. doradensis*, *Ch. zambiensis*. ACT failed only in separating *Ch. deuterocubensis* from *Ch. inopina*. The concatenated dataset consisted of 27 sequences of known species of *Chrysoporthe*, 14 isolates included in this study and two sequences of *Amphilogia gyrosa* as the outgroup. The final alignment was 1370 in length (457, 273, 640 for *TUB*, *ACT*, and ITS, respectively) of which 1210 sites were conserved, 126 variable, 63 parsimony informative, and 62 singletons. The best nucleotide substitution models for the Bayesian Inference were HKY for *ACT* and HKY + I for *TUB* and ITS, according to the Akaike Information Criterion (AIC). According to the multi-locus trees obtained through BI and ML methods, all the isolates collected in this study grouped with the ex-type strain of *Ch. cubensis* (Fig. 2).

Pathogenicity test

All isolates of *Ch. cubensis* were pathogenic to *Eucalyptus* regardless of the clone, although there was a significant variation in lesion length for each isolate ($P < 0.05$) (Fig. 3A). There was also a significant difference in lesion length among the *Eucalyptus* clones, regardless of the isolate ($P < 0.05$) (Fig. 3B). Comparing all *Eucalyptus* clones, CNB 007 was the most susceptible, whereas CNB 005 and CNB 030 were the least susceptible. CNB 010, CNB 029, and CNB 032 were moderately susceptible (Fig. 3B).

In all plants, a lesion developed in the xylem and the fungus was recovered from the inoculated tissue (Fig. 1.F-G). Most inoculated plants did not present any external symptoms or fungal structures, however, the formation of mini-cankers was observed in few plants. The fungal colonies recovered from the lesions exhibited white and fluffy growth when younger and orange when older, which is the

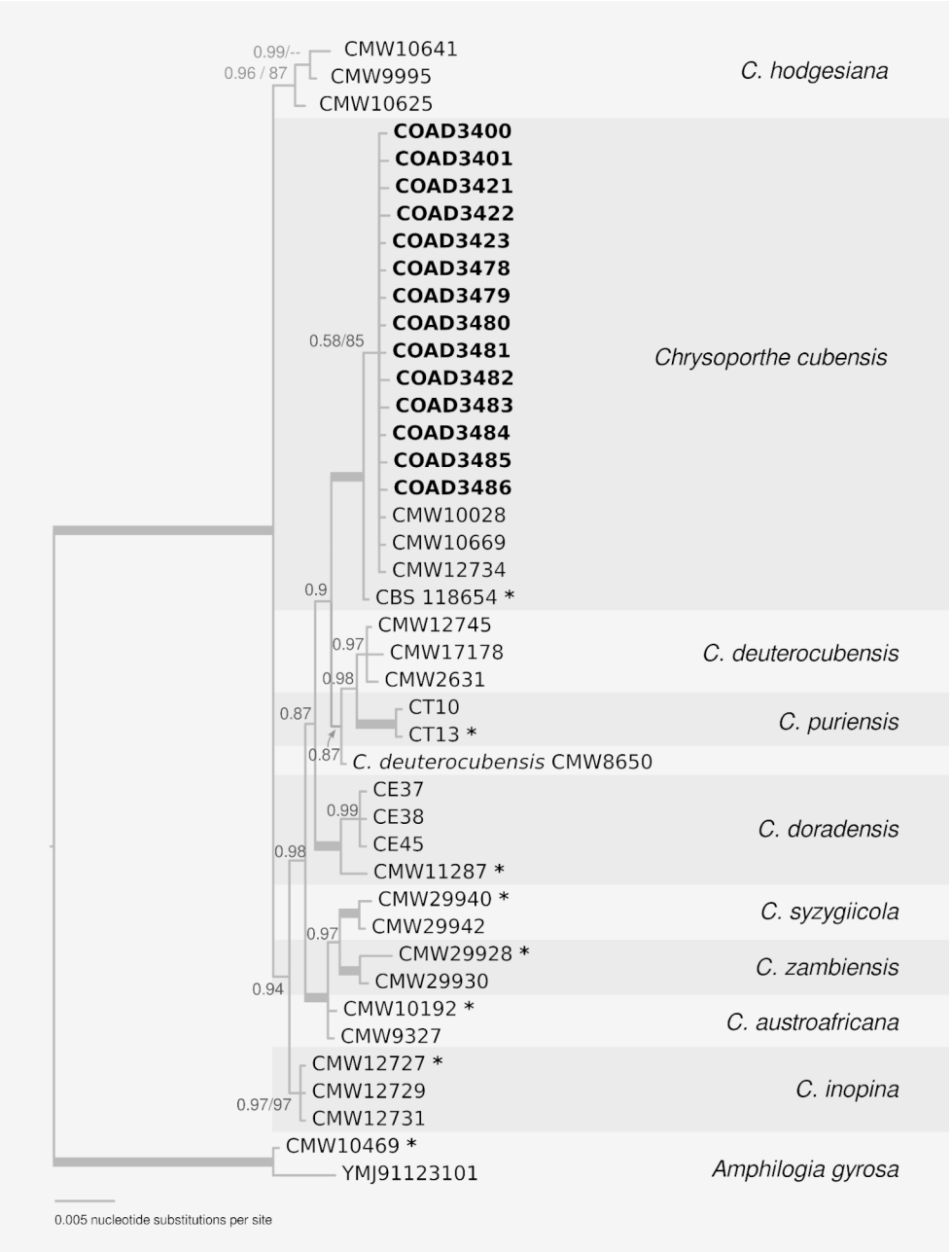

Fig. 2 Multilocus phylogenetic tree based on Bayesian Inference (BI) using the alignment of combined sequences (*TUB* + *ACT* + ITS) of *Chrysoporthe* species. Bayesian posterior probabilities and bootstrap values for BI and Maximum Likelihood higher than 0.5/80 are shown respectively at the nodes (BI/ML). Thickened branches denote Bayesian posterior probabilities higher than 0.99 and Maximum Likelihood bootstrap support higher than 90%. The tree was rooted with *Amphilogia gyrosa* CMW 10,469 and YMJ91123101. Ex-type strains are emphasized with an asterisk (*). The isolates obtained in this study are in bold

same pattern of the colonies used for inoculation, fulfilling Koch's postulates. No symptoms were observed in the control plants.

Effect of temperature on pathogenicity of ch. Cubensis on Eucalyptus clones

The development of the disease occurred on both clones regardless of the temperature and isolates. There was no significant interaction between *Eucalyptus* clones and temperature (P = 0.831). However, significant differences were observed for the effects of the factors clone (P = 0.02) and temperature (P = 0.005) individually. Regardless of the temperature, the clone CNB 010 was more susceptible to the pathogen (Fig. 4). Overall, a temperature of 28 °C favored the progress of the disease more so than did 19 °C (Fig. 4).

Discussion

This study reports *Ch. cubensis* as the causal agent of wilt on *Eucalyptus* mini-stumps. It is the first record of *Chrysoporth*e species causing wilt disease on *Eucalyptus* mini-stump worldwide.

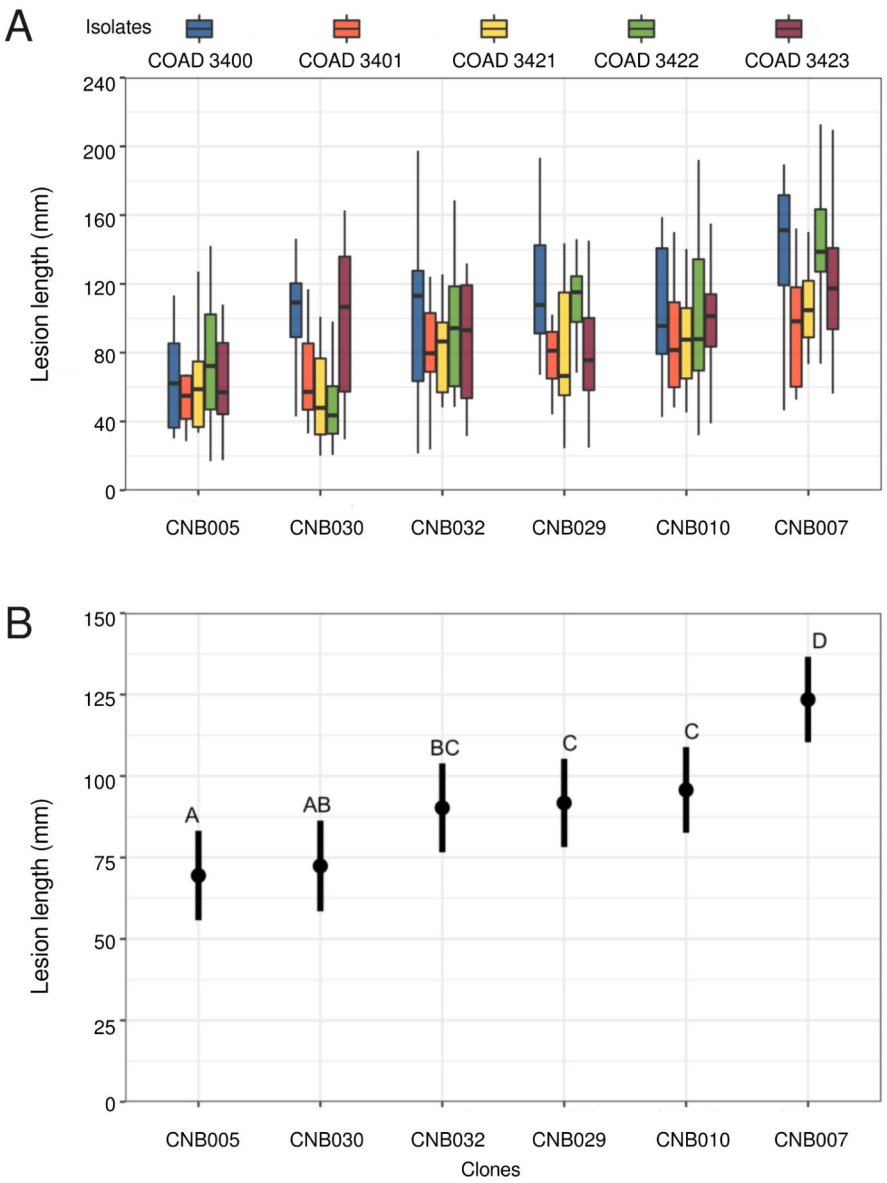

Fig. 3 **A**, Distribution of the length of lesion caused by *Chrysoporthe cubensis* isolates on seedlings of *Eucalyptus urophylla* × *Eucalyptus grandis* clones under greenhouse conditions. **B**, Estimates of the marginal means and respective 95% confidence intervals by a multilevel model fitted to lesion length data

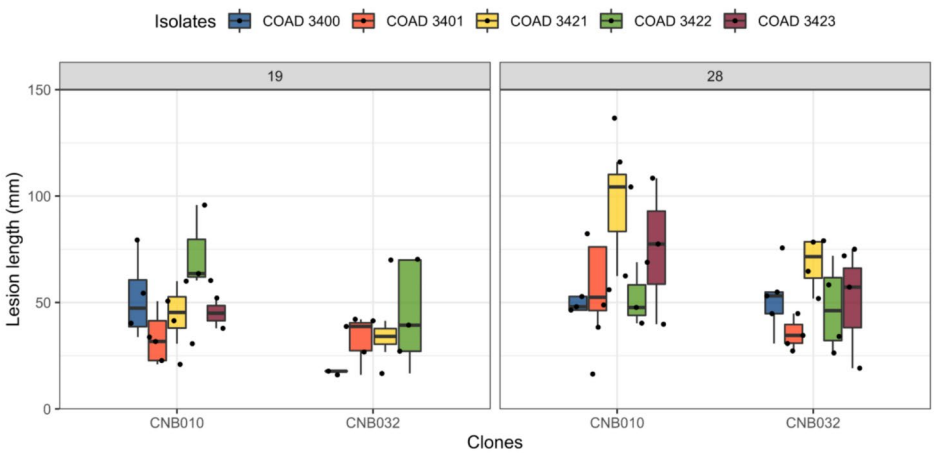

Fig. 4 Distribution of the length of lesions caused by *Chrysoporthe cubensis* isolates on seedlings of two clones (CNB 010 and CNB 032) of *Eucalyptus urophylla* × *Eucalyptus grandis* incubated under 19 and 28°C

A previous report of *Ch. cubensis* on *Eucalyptus* mini-stumps exists, but is associated with death by stem girdling. It was also based exclusively on morphological identification and Koch's postulates were not fulfilled (Alfenas et al. 2009).

The most common symptomatology presented by trees and shrubs infected by *Chrysoporthe* spp. are stem canker, girdling of the stem, cracking of the bark, dying branches and presence of fruiting bodies typical (Chungu et al. 2010; Ferreira et al. 1989; Oliveira et al. 2021). Therefore, *Ch. cubensis* related to wilt on *Eucalyptus* mini-stumps is a new symptom amid others considered a hallmark for that pathogen. Pathogen structures (conidiomata) have rarely been found associated with the bark.

Phylogenetic analyses of the *ACT* gene pointed to the presence of two distinct, but closely related, groups of *Ch. cubensis* isolates. The first one, characterized by COAD 3400–3401 and COAD 3421–3423, represented the isolates obtained from samples collected at the clonal mini-garden in the state of Minas Gerais. The second group, within which the first group is nested, contains the isolates COAD3478-3486, representing the isolates collected in two different locations in the states of São Paulo and Mato Grosso do Sul. *TUB* and ITS could, however, not differentiate these two clades of *Ch. cubensis*. Although it may seem interesting, a recent study on the structure and genetic variability of *Ch. cubensis* concluded that it was not strongly influenced by geographical origin of the isolates in Brazil (Oliveira et al. 2022). Individual and multi-locus trees showed a typical topology delineating known *Chrysoporthe* species.

With regards to the pathogenicity test, the greater susceptibility of clones CNB 007 to the wilt followed by CNB 010 was also observed in a survey carried out by the Company where these *Eucalyptus* clones have been propagated (C. S. Abreu, personal communication). This is despite the fact that susceptibility at the clonal mini-garden it was evaluated using the incidence of wilting plants, whereas our findings were based on the length of the xylem lesion. It is noteworthy that the wilt on *Eucalyptus* mini-stumps arises just after the outset of collections of minicuts, which sheds light on the role played by this mechanical damage in the development of the disease.

The fact that the aggressiveness of the *Ch. cubensis* isolate differs depending on the *Eucalyptus* clone demonstrates that the clones present different levels of susceptibility. Similar results were obtained when isolates of *Ch. cubensis* were inoculated on *Eucalyptus* trees belonging to different genotypes since there is a high inter and intra-specific genetic variability among *Eucalyptus* genotypes (Guimarães et al. 2010; Van Heerden et al. 2005).

The aggressiveness of *Ch. cubensis* on *Eucalyptus* plants was directly related to temperature: the higher the temperature, the greater the susceptibility to the pathogen. However, the higher temperature had a greater influence on lesion development in CNB 032 than in CNB 010, which indicates that less susceptible clones may be more influenced by higher temperatures. The positive effect of temperature on disease development is supported by the geographic distribution of the pathogen in tropical and subtropical areas between latitudes 30° North and South, and its predominance in areas with high temperatures and rainfall (Hodges et al. 1979).

This work revealed the causal agent of a new disease in mini-stumps of *Eucalyptus* that has negatively impacted their longevity and caused significant losses, leading to implications such as the need to replace the dead mini-stumps with new healthy ones, subsequently downgrading productivity and increasing costs. However, a question that has not yet been answered is what the possible inoculum source of *Ch. cubensis* would be considering that the *Eucalyptus* canker disease does not occur on the extensive *Eucalyptus* plantations that surround the clonal mini-garden where this study was performed. Thus, a hypothesis that needs to be investigated is whether *Ch. cubensis* is present as an endophytic fungus on *Eucalyptus* mini-stumps. *Chrysoporthe cubensis* and other Cryphonectriaceae have been found as asymptomatic fungal endophytes in Melastomataceae trees and shrubs that grow alongside commercial *Eucalyptus* plantations in Colombia (Granados et al. 2020). If this is also the case on *Eucalyptus* mini-stumps, the stress that results from often collection of minicuts may lead to the development of the wilt disease.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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