UNIVERSIDADE FEDERAL DE VIÇOSA

Chrysoporthe wilt in eucalyptus mini-stumps: detection, identification of the pathogen, and efficacy of triazole fungicides for disease management

Priscila Raiane Assunção de Andrade
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2025

PRISCILA RAIANE ASSUNÇÃO DE ANDRADE

Chrysoporthe wilt in eucalyptus mini-stumps: detection, identification of the pathogen, and efficacy of triazole fungicides for disease management

Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Gleiber Quintao Furtado

VIÇOSA - MINAS GERAIS
2025

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
A553c
2025

Andrade, Priscila Raiane Assunção de, 1994-
Chrysoporthe wilt in eucalyptus mini-stumps: detection, identification of the pathogen, and efficacy of triazole fungicides for disease management / Priscila Raiane Assunção de Andrade. – Viçosa, MG, 2025.
1 tese eletrônica (102 f.): il. (algumas color.).

Texto em inglês.
Orientador: Gleiber Quintão Furtado.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Fitopatologia, 2025.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2025.770
Modo de acesso: World Wide Web.

1. Fungos fitopatogênicos. 2. *Chrysoporthe sp*. 3. Eucalipto - Doenças e pragas - Controle. 4. Fungicidas. 5. Triazóis. I. Furtado, Gleiber Quintão, 1974-. II. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-Graduação em Fitopatologia. III. Título.

CDD 22. ed. 632.467

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

PRISCILA RAIANE ASSUNÇÃO DE ANDRADE

Chrysoporthe wilt in eucalyptus mini-stumps: detection, identification of the pathogen, and efficacy of triazole fungicides for disease management

Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: February 13, 2025.

Assent:

Priscila Raiane Assunção de Andrade
Author

Gleiber Quintao Furtado
Adviser

To my grandfather Antônio

To my parents Lázara and Ivan

ACKNOWLEDGMENTS

I am grateful, first and foremost, to God. I am immensely grateful for life and for all the experiences and experiences that have given me, unique opportunities for growth and improvement as a human being.

To my parents Ivan and Lázara, I express my sincere gratitude for all the effort they have dedicated to building who I am and for supporting my choices.

I would like to thank my grandfather Antônio for everything he represented and still represents, and for being a great friend and an inspiration to me. Life was undoubtedly better simply because I was with you.

To my siblings Arlei and Gabriela, I am grateful for their care and constant support and, above all, for always believing that I could achieve anything.

My sincere thanks to Thalyta, my love, best friend and great motivator, for her patience, dedication, companionship, unconditional love and care.

I thank my family and friends for every conversation, every exchange, every experience.

I would like to express my gratitude to Professor Gleiber Quintão Furtado for the opportunity, guidance, and all his teachings and contributions over the past few years.

I am grateful to all the professors in the Department of Plant for each lesson they have taught me.

I would like to sincerely thank the team at the Plant Disease Clinic and the Forest Pathology Laboratory. Each of them has certainly helped to improve my career path. I am very grateful to Delfin, Célio, and Marcela. Thank you for your countless contributions throughout this process.

I would like to thank the Graduate Program in Plant Pathology and the Department of Plant Pathology of the Universidade Federal de Viçosa and all the employees and outsourced workers who contribute so much to this institution.

I would like to thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for fostering science, technology, and innovation in the state of Minas Gerais and Brazil.

I would also like to thank Celulose Nipo-Brasileira S/A for establishing

partnerships that contribute to the development of research in Forest Pathology.
This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).

Where there is a will,

there is a way.

George Herbert

ABSTRACT

ANDRADE, Priscila Raiane Assunção de, D.Sc., Universidade Federal de Viçosa, February, 2025. **Chrysoporthe wilt in eucalyptus mini-stumps: detection, identification of the pathogen, and efficacy of triazole fungicides for disease management**. Adviser: Gleiber Quintao Furtado.

The fungus *Chrysoporthe cubensis*, in addition to causing canker in trees, has also been reported in eucalyptus mini-stumps to cause wilting that can progress to partial or complete canopy drying of the plants. Considering the limited knowledge about Chrysoporthe wilt in mini-stumps, the objectives of this study were: (1) to confirm the identity of the pathogen through phylogenetic analyses using a larger number of isolates; (2) to determine whether *Chrysoporthe* sp. occupies an endophytic niche in both asymptomatic rooted cuttings and mini-stumps of eucalyptus; (3) to assess the spread of disease from symptomatic mini-stumps to mini-cuttings and rooted cuttings; (4) to evaluate the sensitivity of the pathogen to triazole fungicides and the efficacy of these molecules in controlling the disease in eucalyptus both preventively and curatively; and (5) to develop a LAMP assay for detecting *Chrysoporthe* spp. Of the 42 selected isolates obtained from asymptomatic and symptomatic mini-stumps, 41 were identified as *C. cubensis*, while one isolate, recovered from a symptomatic mini-stump, was identified as *Chrysoporthe doradensis*. This is the first report of *C. doradensis* in eucalyptus mini-stump in the world. Disease spread through vegetative propagation in clonal mini-gardens, from mini-stumps to mini-cuttings and to rooted cuttings, was not confirmed. *Chrysoporthe* sp. could not be recovered from eucalyptus rooted cuttings derived from rooted mini-cuttings collected from symptomatic mini-stumps. The EC50 test showed that *C. cubensis* isolates are highly sensitive to triazole fungicides at low doses: cyproconazole (0.0770 µg/mL), difenoconazole (0.0065 µg/mL), ipconazole (0.0868 µg/mL), and tebuconazole (0.0376 µg/mL). The fungicides application towards the mini-stumps stem did not result in macroscopic symptoms of phytotoxicity. Curative application of fungicides (72 h after pathogen inoculation) resulted in a 26.94% smaller necrotic lesion in the stem compared to the preventive application (72 h before pathogen inoculation) for all fungicides. The application of the molecules 15 days after pathogen inoculation was ineffective in controlling the disease. However, the fungicides ipconazole and tebuconazole were able to reduce the average number of fruiting bodies on the stem surface by 53.7% and 56.9%, respectively. For the LAMP assay, a primer set (FIP, BIP, B3, and F3) was developed based on the TEF 1a gene. The optimal conditions for

amplification were a 2:1 ratio of FIP/BIP to F3/B3 primers, temperatures of 65 and 68°C, and incubation times of 75 and 90 minutes. For positive reaction, the color of the reaction changed from pink to yellow. The primer set was able of detecting *C. cubensis* and *C. doradensis*. No amplification of non- target fungi, such as *Botrytis* sp., *Fusarium* sp., and *Trichoderma* sp., was observed, providing evidence of specificity for *Chrysoporthe* species. This study provides important updates into the detection of *Chrysoporthe* spp., as well as the etiology and control of Chrysoporthe wilt in eucalyptus mini-stumps, aimed at mitigating the impact of the disease in clonal mini-gardens.

Keywords: *Chrysoporthe cubensis*; *Chrysoporthe doradensis*; Chemical control; Endophytic niche; Eucalyptus; LAMP

RESUMO

ANDRADE, Priscila Raiane Assunção de, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Murcha de Chrysoporthe em miniestacas de eucalyptus: detecção, identificação do patógeno e eficácia de fungicidas triazóis no manejo na doença**. Orientador: Gleiber Quintao Furtado.

O fungo *Chrysoporthe cubensis*, além de causar cancro em árvores, também foi relatado em minicepas de eucalipto causando murcha, a qual progride para a seca parcial ou total da copa das plantas. Considerando o limitado conhecimento sobre a murcha de Chrysoporthe em minicepas, os objetivos deste estudo foram: (1) confirmar a identidade do patógeno por meio de análises filogenéticas usando um número maior de isolados; (2) determinar se *Chrysoporthe* sp. ocupa um nicho endofítico em mudas assintomáticas e minicepas de eucalipto; (3) avaliar a disseminação da doença de minicepas sintomáticas para miniestacas e mudas; (4) avaliar a sensibilidade do patógeno a fungicidas triazóis e a eficácia dessas moléculas no controle da doença em eucalipto, tanto preventiva quanto curativamente; e (5) desenvolver a técnica de LAMP para detectar *Chrysoporthe* spp. A partir dos 42 isolados selecionados obtidos de minicepas assintomáticas e sintomáticas, 41 foram identificados como *C. cubensis*, enquanto um isolado, obtido de minicepa sintomática, foi identificado como *Chrysoporthe doradensis*, o que consistiu no primeiro relato de desta espécie em minicepa de eucalipto no mundo. A disseminação da doença por meio da propagação vegetativa, a partir de minicepas para miniestacas e para mudas, não foi confirmada. *Chrysoporthe* sp. não pôde ser recuperado de mudas de eucalipto oriundas de miniestacas enraizadas coletadas de minicepas sintomáticas. O teste EC50 mostrou que os isolados de *C. cubensis* são altamente sensíveis aos fungicidas triazóis em baixas doses: ciproconazol (0,0770 µg/mL), difenoconazol (0,0065 µg/mL), ipconazol (0,0868 µg/mL) e tebuconazol (0,0376 µg/mL). A aplicação de fungicidas no caule das minicepas não resultou em sintomas macroscópicos de fitotoxicidade. A aplicação curativa de fungicidas (72 h após a inoculação do patógeno) resultou em uma lesão necrótica no caule 26,94% menor em comparação com a aplicação preventiva (72 h antes da inoculação do patógeno) para todos os fungicidas testados. A aplicação das moléculas 15 dias após a inoculação do patógeno foi ineficaz no controle da doença. No entanto, os fungicidas ipconazol e tebuconazol foram capazes de reduzir o número médio de corpos de frutificação na superfície do caule em 53,7% e 56,9%, respectivamente. Para o ensaio de LAMP, um conjunto de primers (FIP, BIP, B3 e F3) foi desenvolvido com base no gene TEF 1a. As condições ótimas

para amplificação foram uma proporção de 2:1 de primers FIP/BIP para F3/B3, temperaturas de 65 e 68 °C e tempos de incubação de 75 e 90 minutos. Para a reação positiva, a cor da reação mudou de rosa para amarelo. O conjunto de primers foi capaz de detectar *C. cubensis* e *C. doradensis*. Nenhuma amplificação de fungos não-alvo, como *Botrytis* sp., *Fusarium* sp. e *Trichoderma* sp., foi observada, fornecendo evidências de especificidade para espécies de *Chrysoporthe*. Este estudo traz importantes atualizações sobre a detecção de *Chrysoporthe* spp., etiologia e o controle da murcha de Chrysoporthe em minicepas de eucalipto que contribuem para mitigar o impacto da doença em minijardins clonais.

Palavras-chave: *Chrysoporthe cubensis*; *Chrysoporthe doradensis*; Controle químico; Eucalyptus; LAMP; Nicho endofítico

SUMMARY

GENERAL INTRODUCTION

The forestry sector, which encompasses silviculture, forest-based industries, and forest ecosystem services, holds socio economic significance in Brazil by generating foreign exchange, direct and indirect jobs, providing essential bioproducts for human activities, and contributing to the Brazilian Gross Domestic Product (GDP), among others. From the year 2000 onwards, Brazil established itself internationally in the planted forest sector as a result of the expansion of plantation areas and the continuous investment in technological development within the forestry production chain (Gonçalves et al. 2013; Hurmekoski et al. 2019; Ibá 2023). In 2022, the planted tree sector in Brazil covered 9.94 million hectares and generated approximately 260 billion reais (about 49.7 billion US dollars), which accounted for 1.3% of this production chain's contribution to the GDP. Among the planted trees, eucalyptus occupies 76% of the forest cultivation area in the country (Ibá 2023). A large number of *Eucalyptus* species are native to Australia, but due to their economic importance, they have been extensively planted in other geographic regions to provide raw materials for the production of timber, paper, pulp, and alternative energy as an alternative to the exploitation of tropical forests (Barradas et al. 2016; Grattapaglia & Kirts 2008).

Although silviculture and the use of improved genotypes have contributed to the increase in eucalyptus productivity over the past decades (Gonçalves et al. 2013; Grattapaglia & Kirst 2008), the occurrence of phytosanitary problems related to the incidence of phytopathogens represents a challenge for the forestry sector (Graziosi et al. 2019; Wingfield et al. 2013).

Recently, a high incidence of wilt was observed in mini-stumps of hybrid eucalyptus (*Eucalyptus grandis* × *Eucalyptus urophylla*) in a clonal mini-garden. This wilt progressed to dieback of the aerial parts and ultimately led to the death of the plants. Internally, ascending lesions originating from the collar were noted. Symptoms appeared after the initiation of mini- cutting collection from the forming mini-stumps, and phylogenetic analyses have attributed the causal agent of this disease to the fungal species *Chrysoporthe cubensis* (Martins et al. 2023). *Chrysoporthe cubensis* is recognized as an important pathogen in the forestry sector, being the etiological agent of eucalyptus canker and affecting other species within the Myrtaceae and Melastomataceae families (Ferreira & Milani 2002; Hodges 1980; Rodas et al. 2005; Wingfield 1989). This pathogen was first described as *Diaporthe cubensis* (Bruner 1917) and later transferred to *Cryphonectria* due to the similarity of the fungal cultures and the presence of orange stroma tissue (Hodges 1980). However, Hodges' (1980) proposal was not

sufficient to resolve the questions regarding the taxonomic positioning of this fungus within the scientific community. A significant milestone in taxonomy occurred with the proposal of a new genus within the Diaporthales, named *Chrysoporthe*, based on morphological and phylogenetic studies conducted by Gryzenhout et al. (2004). Currently, the genus *Chrysoporthe* includes, in addition to *C. cubensis*, the following species: *C. austroafricana*, *C. doradensis*, *C. inopina*, *C. deuterocubensis*, *C. syzygiicola*, *C. hodgesiana*, *C. zambiensis*, *C. puriensis*, *C. colombiana*, and the most recently described species, *C. brasiliensis* (Chungu et al. 2010; Gryzenhout et al. 2004; Gryzenhout et al. 2005; Gryzenhout et al. 2006; Oliveira et al. 2021; Van der Merwe et al. 2010; Silva et al. 2024; Suzuki et al. 2023).

The most commonly observed symptoms in trees and shrubs infected by *Chrysoporthe* spp. include stem canker, ring barking, bark cracking, branch dieback, and frequent presence of typical fruiting bodies (Chungu et al. 2010; Ferreira 1989; Oliveira et al. 2021). However, in the clonal mini-garden, wilt in mini-stumps constitutes a new symptom, and typical structures of the pathogen (ascomata and conidiomata) have not been found associated with the bark of diseased mini-stumps (Martins et al. 2023). Additionally, the disease has also not been observed in the nearby forest stands, raising questions about the source of the primary inoculum. This has led to inquiries regarding the process of propagule dispersal and the subsequent spread of the disease. The discovery of endophytic niches occupied by Chryphonectriaceae in Mozambique and Colombia in the last decade (Granados et al. 2020; Mausse-Sitoe et al. 2016) may be crucial for understanding the biology of the pathogen and the infection process of *Chrysoporthe* spp. in the clonal mini-garden.

Regarding the control of diseases caused by species of *Chrysoporthe*, the primary measure employed following the emergence of eucalyptus canker in Brazilian commercial plantations was the selection and subsequent vegetative propagation of hybrid materials that exhibited some level of resistance (Wingfield 2003). Since then, management has largely depended on the planting of resistant clonal materials; however, there are commercially available genetic materials that show high susceptibility to the disease. In the case of mini-stump wilt, the adopted approach involves the removal of diseased plants, which affects the longevity of the clonal mini-garden, reduces productivity, and increases the cost of the rooted cuttings production process (Martins et al. 2023). This situation highlights the need for new strategies for the control of this harmful disease on eucalyptus mini-garden.

In plant disease management, it is recommended to integrate cultural practices, the use of tolerant or resistant genotypes, the employment of biocontrol agents, and the application of fungicides (Beckerman et al. 2023; Jørgensen et al. 2017). Conceptually, fungicides comprise

a class of pesticides able of inhibiting the growth or killing fungi due to interference at the cellular level in critical processes of fungal metabolism (McGrath 2004). Fungicides can protect crops by blocking multiple biochemical pathways or by selectively interfering with a specific enzyme associated with fungal biosynthetic pathways (Lamberth 2022; Neupane et al. 2022; Zubrod et al. 2019).

Although some fungicides are registered in Brazil for the management of fungal diseases in eucalyptus cultivation, there are no products registered targeting *Chrysoporthe* spp. (Brazilian Ministry of Agriculture, Livestock and Food Supply 2024), and scientific studies on this approach are scarce. Ambrose et al. (2023) demonstrated that *C. deuterocubensis* shows *in vitro* sensitivity to prochloraz manganese chloride and dimethomorph, and that the preventive and curative application of fungicides can reduce the average length of lesions. However, this study focused on isolates of *C. deuterocubensis* obtained from *E. grandis* × *E. urophylla* plantations in Malaysia. Therefore, there is a gap regarding of the sensitivity of *C. cubensis* isolates to fungicides and the efficacy of fungicidal molecules in controlling wilt and eucalyptus canker under Brazilian conditions.

The accurate and early diagnosis of Chrysoporthe wilt in Eucalyptus mini-stumps, as with other plant diseases, is crucial for effective management (Mahlein 2016). In this genus, accurate pathogen identification based solely on morphological characteristics has become increasingly challenging. Due to the limitations of traditional fungal taxonomy methods, more reliable and rapid detection approaches have been developed (Baldi & La Porta 2020; Luchi et al. 2020). Consequently, molecular methods have become standard in pathogen detection and quantification. Currently, the classification of *Chrysoporthe* spp. relies on the amplification of pathogen DNA regions via the Polymerase Chain Reaction (PCR) technique, followed by sequencing of the amplified region and subsequent phylogenetic reconstruction based on DNA sequence data, particularly the internal transcribed spacer (ITS) and β-tubulin regions (Hariharan & Prasannath 2021; Suzuki et al. 2023;). While this approach enables precise pathogen identification, limitations associated with equipment requirements and procedural complexities hinder the rapid detection of the pathogen in field samples (Gomez-Gutierrez & Goodwin 2022).

In this context, an alternative technique with significant potential for detecting *C. cubensis* is Loop-Mediated Isothermal Amplification (LAMP). First introduced by Notomi et al. (2000), LAMP is highly efficient, specific, and significantly faster than conventional PCR and has been applied across various biological disciplines. Furthermore, LAMP operates under isothermal conditions, eliminating the need for thermal cycling (Feng et al. 2019; Dai et al.

2016). This technique is particularly advantageous due to its simplicity, cost-effectiveness, and applicability in field diagnostics without requiring advanced laboratory equipment (Yang et al. 2025).

Thus, the objectives of this study were (1) to confirm the identity of the pathogen through phylogenetic analyses using a larger number of isolates; (2) to determine whether *Chrysoporthe* sp. occupies an endophytic niche in both asymptomatic rooted cuttings and mini-stumps of eucalyptus; (3) to assess the spread of disease from symptomatic mini-stumps to mini-cuttings and rooted cuttings; (4) to evaluate the sensitivity of the pathogen to triazole fungicides and the effectiveness of these molecules in controlling the disease in eucalyptus both preventively and curatively; and (5) to develop a LAMP assay for detecting *Chrysoporthe* spp.

References

Ambrose, A., Peter Remun, A. B., Shamsul Kamar, N. A., Mohamad Pazi, A. M., Wan Azhar, W. M. A., Awing, N. H., Liam, J., & Terhem, R. (2023). Evaluation of fungicides as protective and curative treatments against canker disease of *Eucalyptus urograndis* caused by *Chrysoporthe deuterocubensis* in Malaysia. Forests, 14, 2337. https://doi.org/10.3390/f14122337

Barradas, C., Phillips, A. J., Correia, A., Diogo, E., Bragança, H., & Alves, A. (2016). Diversity and potential impact of Botryosphaeriaceae species associated with *Eucalyptus globulus* plantations in Portugal. European Journal of Plant Pathology, 146, 245-257. https://doi.org/10.1007/s10658-016-0910-1

Beckerman, J., Palmer, C., Tedford, E., & Ypema, H. (2023). Fifty years of fungicide development, deployment, and future use. Phytopathology®, 113, 694-706. https://doi.org/10.1094/PHYTO-10-22-0399-IA

Brazilian Ministry of Agriculture, Livestock and Food Supply. (2024). Phytosanitary agrochemicals system – Ministry of Agriculture, Livestock and Food Supply, Brazil, 2024. https://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons

Bruner, S. C. (1917) Una enfermedad gangrenosa de lós eucaliptos. https://www.biodiversitylibrary.org/page/51662817

Chungu, D., Gryzenhout, M., Muimba-Kankolongo, A., Wingfield, M. J., & Roux, J. (2010). Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia. Mycological Progress, 9, 379-393. https://doi.org/10.1007/s11557-009-0646-9

Ferreira, F. A., & Milani, D. (2002). Diagnose visual e controle das doenças abióticas e bióticas do eucalipto no Brasil. Mogi Guaçu: International Paper.

Ferreira, F. A. (1989) Patologia florestal: principais doenças florestais no Brasil. Viçosa: UFV.

Gomez-Gutierrez, S. V., & Goodwin, S. B. (2022). Loop-mediated isothermal amplification

for detection of plant pathogens in wheat (*Triticum aestivum*). Frontiers in Plant Science, 13, 857673. https://doi.org/10.3389/fpls.2022.857673

Gonçalves, J. L. M., Alvares, C. A., Higa, A. R., Silva, L. D., Alfenas, A. C., Stahl, J., Ferraz, S. F. B., Lima, W. P., Brancalion, P. H. S., Hubner, A., Bouillet, J. P. D., Laclau, J. P., Nouvellon Y., & Epron, D. (2013). Integrating genetic and silvicultural strategies to minimize abiotic and biotic constraints in Brazilian eucalypt plantations. Forest Ecology and Management, 301, 6-27. https://doi.org/10.1016/j.foreco.2012.12.030

Granados, G. M., McTaggart, A. R., Rodas, C. A., Roux, J., & Wingfield, M. J. (2020). Species of Cryphonectriaceae occupy an endophytic niche in the Melastomataceae and are putative latent pathogens of *Eucalyptus*. European Journal of Plant Pathology, 156, 273-283. https://doi.org/10.1007/s10658-019-01887-9

Grattapaglia, D., & Kirst, M. (2008). Eucalyptus applied genomics: from gene sequences to breeding tools. New Phytologist, 179, 911-929. https://doi.org/10.1111/j.1469-8137.2008.02503.x

Graziosi, I., Tembo, M., Kuate, J., & Muchugi, A. (2019). Pests and diseases of trees in Africa: A growing continental emergency. Plants, People, Planet, 2, 14-28. https://doi.org/10.1002/ppp3.31

Gryzenhout, M., Myburg, H., Van der Merwe, N. A., Wingfield, B. D., & Wingfield, M. J. (2004). *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Studies in Mycology, 50, 119-142.

Gryzenhout, M., Myburg, H., Wingfield, B. D., Montenegro, F., & Wingfield, M. J. (2005). *Chrysoporthe doradensis* sp. nov. pathogenic to *Eucalyptus* in Ecuador. Fungal Diversity, 20, 39-57.

Gryzenhout, M., Rodas, C. A., Portales, J. M., Clegg, P., Wingfield, B. D., & Wingfield, M. J. (2006). Novel hosts of the Eucalyptus canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. Mycological Research, 110, 833-845. https://doi.org/10.1016/j.mycres.2006.02.010

Hodges, C. S. (1980). The taxonomy of *Diaporthe cubensis*. Mycologia, 72, 542-548. https://doi.org/10.1080/00275514.1980.12021216

Hurmekoski, E., Lovrić, M., Lovrić, N., Hetemäki, L., & Winkel, G. (2019). Frontiers of the forest-based bioeconomy – A European Delphi study. Forest Policy and Economics, 102, 86-99. https://doi.org/10.1016/j.forpol.2019.03.008

Ibá - Indústria Brasileira de Árvores (2023). Relatório Anual 2023.

Jørgensen, L.N., Van den Bosch, F., Oliver, R. P., Heick, T. M., & Paveley, N. D. (2017). Targeting fungicide inputs according to need. Annual Review of Phytopathology, 55, 181-203. https://doi.org/10.1146/annurev-phyto-080516-035357

Lamberth, C. (2022). Latest research trends in agrochemical fungicides: any learnings for pharmaceutical antifungals? ACS Medicinal Chemistry Letters, 13, 895-903. https://doi.org/10.1021%2Facsmedchemlett.2c00113

Mahlein, A. K. (2016). Plant disease detection by imaging sensors–parallels and specific demands for precision agriculture and plant phenotyping. Plant Disease, 100, 241-251. https://doi.org/10.1094/PDIS-03-15-0340-FE

Martins, M. D., Guterres, D. C., Andrade, P. R. A., Machado, F. J., & Furtado, G. Q. (2023). *Chrysoporthe cubensis* emerges causing wilt on *Eucalyptus* mini-stumps in Brazil. Australasian Plant Pathology, 52, 517-527. https://doi.org/10.1007/s13313-023-00940-w

Mausse-Sitoe, S. N., Rodas, C. A., Wingfield, M. J., Chen, S., & Roux, J. (2016). Endophytic Cryphonectriaceae on native Myrtales: Possible origin of Chrysoporthe canker on plantation-grown *Eucalyptus*. Fungal Biology, 120, 827-835. https://doi.org/10.1016/j.funbio.2016.03.005

McGrath, M. T. (2004). What are Fungicides? The Plant Health Instructor, 10, 109-115. 10.1094/PHI-I-2004-0825-01

Neupane, K., Alexander, L., & Baysal-Gurel, F. (2022). Management of *Phytophthora*

cinnamomi using fungicides and host plant defense inducers under drought conditions: A case study of flowering dogwood. Plant Disease, 106, 475-485. https://doi.org/10.1094/PDIS-04-21-0789-RE

Oliveira, M. E. S., Van der Merwe, N. A., Wingfield, M. J., Wingfield, B. D., Soares, T. P. F., Kanzi, A. M., & Ferreira, M. A. (2021). *Chrysoporthe puriensis* sp. nov. from *Tibouchina* spp. in Brazil: an emerging threat to Eucalyptus. Australasian Plant Pathology, 50, 29-40. https://doi.org/10.1007/s13313-020-00745-1

Rodas, C. A., Gryzenhout, M., Myburg, H., Wingfield, B. D., & Wingfield, M. J. (2005). Discovery of the Eucalyptus canker pathogen *Chrysoporthe cubensis* on native *Miconia* (Melastomataceae) in Colombia. Plant Pathology, 54, 460-470. https://doi.org/10.1111/j.1365-3059.2005.01223.x

Silva, G. A., Oliveira, M. E. S., Rêgo, G. M. S., Wingfield, B. D., Wingfield, M. J., & Ferreira, M. A. (2024). *Chrysoporthe brasiliensis* sp. nov. pathogenic to Melastomataceae in southeast Brazil. Fungal Biology, 128, 1790-1799. https://doi.org/10.1016/j.funbio.2024.04.001

Suzuki, H., Marincowitz, S., Rodas, C. A., Wingfield, B. D., & Wingfield, M. J. (2023). First report of two *Chrysoporthe* species, *Chrysoporthe doradensis* and *Chrysoporthe colombiana* sp. nov. from *Henriettea seemannii* pathogenic to Eucalyptus in Colombia. Mycological Progress, 22, 44. https://doi.org/10.1007/s11557-023-01891-8

Van der Merwe, N. A., Gryzenhout, M., Steenkamp, E. T., Wingfield, B. D., & Wingfield, M. J. (2010). Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. Fungal Biology, 114, 966-979. https://doi.org/10.1016/j.funbio.2010.09.007

Wingfield, M. J. (1989). First record of Cryphonectria canker of Eucalyptus in South Africa. Phytophylactica, 21, 311-313.

Wingfield, M. J. (2003). 2003 Daniel McAlpine Memorial Lecture Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from Cryphonectria

canker. Australasian Plant Pathology 32, 133–139. https://doi.org/10.1071/AP03024

Wingfield, M. J., Roux, J., Slippers, B., Hurley, B. P., Garnas, J., Myburg, A. A., & Wingfield, B. D. (2013). Established and new technologies reduce increasing pest and pathogen threats to Eucalypt plantations. Forest Ecology and Management, 301, 35-42. https://doi.org/10.1016/j.foreco.2012.09.002

Yang, L., Sun, Y., Sun, L., Wang, Z., Feng, J., & Liang, Y. (2025). Application of Loop-Mediated Isothermal Amplification in Plant Pathogen Detection. Phytopathology®, 115,6-13. https://doi.org/10.1094/PHYTO-10-23-0391-KC

Zubrod, J. P., Bundschuh, M., Arts, G., Brühl, C. A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J. J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., & Schäfer, R. B. (2019). Fungicides: An overlooked pesticide class? Environmental Science & Technology, 53, 3347-3365. https://doi.org/10.1021/acs.est.8b04392

CHAPTER 1

Chrysoporthe wilt in eucalyptus mini-stumps: Pathogen detection and identification in symptomatic and asymptomatic hosts, and disease spread from mini-stems to mini-cuttings and rooted cuttings

P. R. A. Andrade[1], H. W. Abreu[1], M. G. V. Kiti[1], G. Q. Furtado[1]*

[1]Department of Plant Pathology, Universidade Federal de Viçosa, 36570-900, Viçosa, Minas Gerais, Brazil.

*Corresponding author: gfurtado@ufv.br

Abstract

The fungus *Chrysoporhe cubensis*, known as the causal agent of canker in eucalyptus plantation, was recently uncovered causing severe losses in eucalyptus mini-stump in a clonal mini-garden in Brazil. The symptomatic mini-stumps exhibited wilting, which gradually progressed to canopy drying and eventually resulted in plant death. This study aimed to investigate the occupation of endophytic niches by *Chrysoporthe* spp. in eucalyptus mini-stumps and rooted cuttings, confirm the pathogen identity through phylogenetic analyses using a larger number of isolates, and assess the potential spread of disease through the mini-cutting technique. We demonstrated that *Chrysoporthe* sp. occupies an endophytic niche on eucalyptus mini-stumps in the hybrid clones (*Eucalyptus grandis* × *Eucalyptus urophylla*) CNB007 and CNB010. Disease spread through vegetative propagation in clonal minigardens, from mini-stumps to mini-cuttings and rooted cuttings, was not confirmed. Furthermore, the pathogen could not be recovered from eucalyptus rooted cuttings derived from mini-cuttings collected from symptomatic mini- stumps. A pathogenicity test to confirm the ability of *Chrysoporthe* sp. isolates from asymptomatic mini-stumps showed that all of them induced necrotic lesions in the stems of eucalyptus rooted cuttings. Additionally, 42 selected isolates obtained from asymptomatic and symptomatic mini-stumps were identified through phylogenetic analyses, with 41 belonging to *C. cubensis* and one belonging to *Chrysoporthe doradensis*, which was recovered from a symptomatic mini-stump. This is the first report of *C. doradensis* in eucalyptus mini-stumps in the world; however, further studies are needed to determine the ecological and epidemiological importance of this species as a causal agent of wilt in eucalyptus mini-stumps.

1 Introduction

Chrysoporthe Gryzenhout & M.J. Wingf, a genus of fungi in the Cryphonectriaceae family, accommodates important pathogenic species of trees from two families within the order Myrtales: Melastomataceae and Myrtaceae (Gryzenhout et al. 2004; Rodas et al. 2005). Some of these species, such as *Chrysoporthe cubensis*, *Chrysoporthe doradensis*, and *Chrysoporthe austroafricana*, are well distributed in tropical and subtropical regions and cause severe damage to eucalyptus (Heath et al. 2006; Nakabonge et al. 2006; Pegg et al. 2010). In Brazil, significant economic damage to eucalyptus plantation was observed in the 1970s due to the high incidence of eucalyptus canker, a disease caused by *C. cubensis* (Ferreira 1989).

The symptoms of canker in eucalyptus include stem girdling and cambium death in plants less than one year old, which cause these plants to die. Trees that are two years old show sunken areas in the stem and cracking of the bark. Another common symptom is the typical canker: a well-defined, deep lesion surrounded by calluses, which is associated with the death of a larger section of the cambium (Hodges et al. 1976). Additionally, the symptomatology of eucalyptus canker includes formation of pycnidia and perithecia scattered on the damaged bark (Chungu et al. 2019; Ferreira & Milani 2002). To tackle eucalyptus canker, efforts focused on selecting hybrid progenies (*Eucalyptus grandis* × *Eucalyptus urophylla*) resistant to the disease, followed by vegetative propagation of these materials through cloning. This approach, used during the 1980s, was crucial to the success of eucalyptus vegetative propagation, which continues to this day (Alfenas et al. 2009; Ferreira 1989; Wingfield 2003).

Recently, *C. cubensis* was reported for the first time causing severe losses in eucalyptus mini- stumps in clonal mini-garden in Brazil (Martins et al. 2023). Interestingly, the symptomatic mini-stumps presented wilting, which progressed to crown dieback and subsequent death, which differs from the known symptoms of eucalyptus canker in eucalyptus plantation. Fruiting bodies were not observed on the examined mini-stumps. Additionally, canker was not considered a prevalent disease in the surrounding eucalyptus plantations near the clonal mini-garden, which could pose a threat to the mini-stumps and serve as a potential inoculum source of *C. cubensis* (Martins et al. 2023). Martins et al. 2023 suggested that *C. cubensis* likely occupies an endophytic niche in eucalyptus mini-stumps. This hypothesis is supported by Mausse-Sitoe et al. (2016), who mention that the occupation of endophytic niches by Cryphonectriaceae may contribute to disease spread, with cuttings, whether rooted or not, being a potential source of propagation. The species *C. austroafricana* and *Celoporthe woodiana* were associated with asymptomatic infections of native Myrtales from

Mozambique. Additionally, Granados et al. (2020) confirmed the occupation of endophytic niches by *Aurapex penicillata*, *C. cubensis*, and *Chrysoporthe inopina* in Melastomataceae in Colombia.

Although the contributions of both studies to the understanding of the biology of *Chrysoporthe* species, the occurrence of asymptomatic infections in eucalyptus mini-stumps remains a poorly understood topic. Another aspect that is still little explored for members of Cryphonectriaceae is the potential fungal spread from plants or plant parts that are infected but visually asymptomatic, with particular attention to rooted cuttings (Mausse-Sitoe et al. 2016; Roberts et al. 2020). Therefore, the present study aimed to investigate the occupation of endophytic niches by *Chrysoporthe* spp. in eucalyptus mini-stumps and rooted cuttings, the putative spread of disease through the mini-cutting technique, and to confirm the identity of *Chrysoporthe* species associated with symptomatic and asymptomatic eucalyptus mini-stumps using phylogenetic analyses.

2 Materials and Methods

2.1 Recovery of *Chrysoporthe* spp. in mini-stumps, mini-cuttings, and eucalyptus rooted cuttings

2.1.1 Symptomatic and asymptomatic mini-stumps

Eucalyptus mini-stumps, approximately two and three years old and representing two clones (CNB007 and CNB010), were collected from a clonal mini-garden located in the municipality of Belo Oriente, Minas Gerais, Brazil. For each clone and age group, ten mini-stumps showing wilt symptoms and ten asymptomatic mini-stumps were selected (Figure 1). The samples were packed in plastic bags and sent to the Forest Pathology Laboratory at the Universidade Federal de Viçosa (UFV). Initially, the samples were analyzed for the presence of *Chrysoporthe* spp. fruiting bodies on the bark using a dissecting microscope. The samples were then washed with running water and soap to remove substrate and dirt adhered to the roots and other plant organs, and subsequently dried at room temperature. The indirect isolation of fungi present in the internal tissues of the plant stems was carried out according to the fungal isolation protocol for tissues of woody plants described by Alfenas & Mafia (2016).

At this stage, the outer bark tissues were carefully removed using sterilized tweezers and a scalpel, and fragments approximately 5 mm in size from the newly exposed inner tissue

were obtained with a scalpel. For each sample, fragments were collected from three stem sections: the basal portion (region above the root), the median portion (mid-stem region), and the upper portion (canopy formation region). The collected fragments were incubated on three culture media: Potato Dextrose Agar (PDA), PDA supplemented with rose bengal (50 mg L^{-1}), and Water Agar (WA). All culture media were supplemented with the antibiotic chloramphenicol (100 mg L^{-1}). Three fragments were evenly distributed on each Petri dish and incubated at 25°C in the dark and monitored daily for mycelial growth and the development of cultures with morphological characteristics similar to *Chrysoporthe* spp. as well as the presence of fungal structures typical of this genus.

Once detected, the mycelium and/or spores were transferred to Potato Carrot Agar (PCA) culture medium. Monosporic or single-hypha cultures of *Chrysoporthe* spp. representatives were obtained and preserved through periodic subculturing on PCA medium, Castellani's method (water storage), silica gel, and freezing using an aqueous glycerol solution (15%) at – 80°C (Alfenas & Mafia 2016). The isolates were deposited in the Octávio Almeida Drummond Collection (COAD) at UFV.

2.1.2 Detection in asymptomatic mini-cuttings

Twenty-five eucalyptus mini-cuttings from both clones were obtained from a clonal mini- garden and transported to the laboratory under refrigerated conditions in a cooler box. The sample processing involved removing of leaf blades and petioles, leaving only the main stem, and obtaining 5 mm-long fragments using a sterilized scalpel. Fragments were superficially disinfected by washing them with 70% ethanol and 2.5% sodium hypochlorite solutions for one and two minutes, respectively. Any excess sodium hypochlorite solution was removed by washing the fragments with autoclaved distilled water for one minute, followed by removal of excess water using sterile filter paper (Alfenas & Mafia 2016).

The fragments were plated on PDA, PDA with rose bengal (50 mg L^{-1}), and WA media (2%), all supplemented with chloramphenicol (100 mg L^{-1}). The effectiveness of surface disinfestation was confirmed by plating the rinse water from the surface disinfestation process on PDA, with adjustments to the methodology adopted by Saunders and Kohn (2009). The absence of microorganism growth from planting five aliquots of 25 µL of water, evenly distributed on each Petri dish containing the mentioned culture media, indicated satisfactory disinfestation. For each mini-cutting, two fragments were placed on each Petri dish (90 x 15 mm) containing one of the culture media. The Petri dishes were incubated in the dark at 25°C

and monitored daily for the growth of fungal structures with morphology similar to *Chrysoporthe* spp.

2.1.3 Detection in asymptomatic eucalyptus rooted cuttings

Eucalyptus rooted cuttings approximately 3 months old, showing no apparent symptoms of infection by *Chrysoporthe* spp., with twenty rooted cuttings from each clone (CNB010 and CNB007), were analyzed in the laboratory. The analysis involved inspecting, under a dissecting microscope, the external surface (bark) and internal tissues (inside the stem) of the rooted cuttings for the presence of symptoms and fungal structure. Subsequently, the same indirect isolation procedure described for the mini-stumps was adopted in order to detect the pathogen within the internal plant tissues.

2.2 Disease spread by mini-cuttings

Eucalyptus mini-cuttings (clone CNB007) ranging from 4 to 8 centimeters in length and with one to two pairs of leaves were collected from mini-stumps with or without wilt symptoms caused by *Chrysoporthe* spp. in a clonal mini-garden, totaling 130 mini-cuttings for each condition. Within the first thirty minutes after collection, the mini-cuttings were subjected to the rooting process. This step was carried out in containers of the tube-type (50 cm^3) filled with a substrate composed of rice husk, vermiculite, and coconut fiber, under mist irrigation. The mini-cuttings undergoing the rooting process were placed in rooting chambers and monitored from the 15th to the 20th day after collection for root system development. The number of rooted mini-cuttings was determined, and the result was expressed as the total rooting percentage.

The rooted cuttings were subjected to acclimatization in the shade for approximately 5 to 10 days. After this period, they were acclimatized to open air for growth and hardening, which lasted for 20 to 30 days and 15 to 20 days, respectively. Once the rooted cuttings were ready for shipment, they were placed in trays and sent to the laboratory for processing. The processing was carried out within the first 48 hours after the rooted cuttings were received and involved a visual inspection (both with the naked eye and under a dissecting microscope) for the presence of wilt symptoms and mini-cankers.

The plants were dissected vertically to analyze the presence of necrosis in the internal tissues of the stem. The number of samples showing both external and internal symptoms was

recorded. Additionally, indirect isolation of fungi present inside the stems of the rooted cuttings was carried out. In this step, 15% of the rooted cuttings obtained from both asymptomatic and symptomatic mini-stumps were randomly sampled. Fragments (approximately 5 mm) were removed from the stem interior using a flame-sterilized scalpel and superficially sterilized as described in section 2.1.2.

Three tissue fragments from each sample were plated on a PDA medium. The incubation was conducted at 25°C in the dark, and the Petri dishes were analyzed daily for the presence of mycelial growth of *Chrysoporthe* spp. over a period of twenty days.

Additionally, 25% of the rooted cuttings were placed in a humid chamber, as per Alfenas and Mafia (2016). Dissecting microscope observations were made every five days for a month to detect the presence of fruiting bodies of the pathogen.

2.3 Pathogenicity test of isolates recovered from asymptomatic mini-stumps

Chrysoporthe sp. isolates recovered from asymptomatic mini-stumps were evaluated for their pathogenicity on eucalyptus rooted cuttings (clone CNB007). Three-month-old rooted cuttings were transplanted into polyethylene pots containing 2 kg of a commercial substrate mixture with 300 grams of osmocote + 300 grams of single superphosphate per 50 kg of substrate. The rooted cuttings were kept in a greenhouse at ambient temperature and irrigated daily for three months.

After this period, a 5-mm diameter bark disc was removed approximately five centimeters above the plant collar region using a sterilized cork borer. Then, a mycelial disc with the same diameter as the wound was removed from the edge of the active mycelial growth of the pathogen isolates grown on PDA in the dark for seven days. The mycelial disc was placed on the wound caused by the bark removal. Control treatments included inoculation with only PDA, following the same procedure described earlier, and inoculation with isolate COAD 3401, whose pathogenicity was preliminarily confirmed by Martins et al. (2023). The inoculation site was covered with plastic film to prevent contamination and desiccation, and it was left for 30 days (Oliveira et al. 2021; Rêgo et al. 2023).

The disease incidence was evaluated at 15 and 45 days after inoculation, determined by the presence of internal necrosis starting from the inoculation point. Disease quantification was performed by determining the lesion length on the plant stem using a digital caliper (Rauf et al. 2020). Koch's postulate was fulfilled by removing fragments from the transition area between healthy and diseased tissue and subsequently plating them on PDA. The fungal cultures

grown from the plated fragment were compared to the original cultures of the tested isolates for pathogenicity.

The experimental trial was conducted in a Completely Randomized Design, with three replications for each evaluated isolate. Each replication consisted of one eucalyptus rooted cutting. The experiment was independently repeated once to confirm reproducibility. The data obtained were subjected to Bartlett's test to analyze the homogeneity of variances and the Shapiro-Wilk test to analyze the normality of residuals. The data were subjected to Analysis of Variance (ANOVA), and the means were compared using Tukey's test at a 5% significance level. All statistical analyses, as well as the graphical presentation of the results, were performed using the R statistical software (R Core Team 2023).

2.4 Molecular identification of the isolates

In order to determine the identity of the *Chrysoporthe* spp. isolates recovered, 42 isolates were randomly selected from asymptomatic (n=5) and symptomatic (n=37) mini-stumps and identified through phylogenetic analyses.

2.4.1 DNA extraction

The isolates were grown on PDA at 25°C for ten days. The mycelium from the cultures was scraped using a sterilized wooden stick and transferred to 1.5 mL Eppendorf tubes, following an adaptation of the methodology used by Wang and Chen (2023). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, WI, USA), according to the manufacturer's protocol. The yield and quality of the extracted DNA were estimated using the DS-11 spectrophotometer (DeNovix, Wilmington, Delaware, USA). The DNA concentration was adjusted to 30 ng/µL.

2.4.2 PCR amplification and sequencing of gene regions

The polymerase chain reaction (PCR) technique was used to amplify two partial regions (BT1 and BT2) of the gene encoding the β-tubulin protein, using two sets of primers developed by Glass and Donaldson (1995): Bt1a/Bt1b and Bt2a/Bt2b. Each PCR reaction consisted of 19 µL, including 9.5 µL of Dream Taq polymerase (Thermo Fisher, USA), 2 µL of Bovine Serum Albumin (BSA, Sigma–Aldrich), 0.5 µL of dimethyl sulfoxide (DMSO, Sigma–

Aldrich, St. Louis, MO), 0.75 µL of each primer, 3.5 µL of nuclease-free water, and 2.0 µL of genomic DNA adjusted to 30 ng/µL.

The amplification conditions for the BT1 region were as follows: 95°C for 4 minutes; 95°C for 40 seconds; 38 cycles at 55°C for 45 seconds; 72°C for 1 minute; and 72°C for 7 minutes. For the BT2 region, the conditions were: 94°C for 5 minutes; 94°C for 30 seconds; 38 cycles at 60°C for 30 seconds; 72°C for 1 minute; and 72°C for 10 minutes. The PCR products were visualized by agarose gel electrophoresis (1%) and purified using 3.6 µL of ExoSAP-IT PCR Product Cleanup (Affymetrix, Cleveland, Ohio, USA). The amplified fragments were sequenced using the Sanger method by Macrogen Inc. (Seoul, South Korea).

2.4.3 Phylogenetic analyses

DNA sequences were assembled using the DNA Dragon software (Sequentix, Germany), with ambiguous positions manually resolved. Consensus sequences were compared to the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLASTn, National Center for Biotechnology Information - NCBI) for preliminary identification of the isolates. Subsequently, a sequence matrix of *Chrysoporthe* spp. representative isolates was compiled for each gene region (Table 2). *Amphilogia gyrosa* sequences were used as the outgroup. The sequence matrix alignment was performed using the Muscle program (Edgar 2004) implemented in the Aliview software (Larsson 2014).

Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted using RAxML 8.2.9 (Stamatakis 2014) and MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003), respectively. For ML analysis, 1000 bootstrap replicates were used to estimate branch support values in the phylogenetic tree. For BI analysis, evolutionary models were estimated for each partition using jModelTest2 (Darriba et al. 2012) and chosen according to corrected Akaike information criteria (AIC). A burn-in of 25% of the sampled trees was discarded, with the remaining trees used to estimate Bayesian posterior probabilities.

Both phylogenetic analyses were conducted on the CIPRES Science Gateway 3.1 (Miller et al. 2010), and the resulting phylogenetic trees were visualized using the FigTree 1.4.2 software. Tree editing was performed in Inkscape (Free Software Foundation, USA). Additionally, the alignments were concatenated using the SequenceMatrix (Vaidya, Lohman & Meier 2011). The PartitionFinder2 tool, implemented in the CIPRES Science Gateway, was used to analyze congruence between the loci (Lanfear et al. 2016).

3 Results

3.1 Recovery of *Chrysoporthe* sp. from eucalyptus mini-stumps, mini-cuttings, and rooted cuttings

Seventy and 109 isolates of *Chrysoporthe* sp. were recovered, respectively, from two-year-old and three-year-old symptomatic mini-stumps (Figure 2A). Processing of asymptomatic mini-stumps resulted in no recovery of the pathogen from two- year-old mini-stumps. From three-year-old ones, eight and two isolates were obtained from clones CNB007 and CNB010, respectively (Figure 2B). From two-year-old mini-stumps, the pathogen was recovered in 90% of symptomatic samples from both clones, but it was not recovered from any asymptomatic samples. From three-year-old mini-stumps, the pathogen was recovered in 90% of symptomatic samples from both clones. The recovery of the pathogen was lower in asymptomatic samples, with 20% and 30% from mini-stumps belonging to clones CNB010 and CNB007, respectively (Figure 3).

With respect to the distribution of the pathogen within the stems of mini-stumps, in symptomatic ones, regardless of age, the pathogen was predominantly detected in the basal, median, and upper portions. The occurrence of the fungus in the basal and median portions, as well as in the median and upper portions, was also notable. However, in only 10% of the samples, *Chrysoporthe* sp. was exclusively recovered from the upper portion of the stem of both ages for clone CNB007 (Table 1). In three-year-old asymptomatic mini-stumps, the pathogen was recovered under the following conditions: upper, basal and median, and basal and upper portions (CNB010); as well as upper and middle portions (CNB007). The pathogen was not recovered from all three stem portions analyzed in any of the samples from two-year-old mini- stumps (Table 1). Similarly, no pathogen was recovered from asymptomatic eucalyptus mini-cuttings and rooted cuttings, regardless of the eucalyptus clone.

3.2 Disease spread by mini-cuttings

In the first rooted cuttings selection, the rooting rates of mini-cuttings obtained from healthy and wilt- symptomatic mini-stumps were 77.84% and 73.86%, respectively (Figure 4). Necrotic lesions inside the stems of eucalyptus rooted cuttings were not observed in any of the 90 rooted cuttings derived from healthy mini-stumps or the 150 rooted cuttings derived from wilt-symptomatic mini-stumps. No fungal mycelium similar to *Chrysoporthe* spp. was

observed in fragments plated on PDA medium. No fruiting bodies of the pathogen were observed from samples maintained in a humid chamber.

3.3 Pathogenicity test of isolates recovered from asymptomatic mini-stumps

All five isolates of *C. cubensis* recovered from the internal tissues of asymptomatic mini-stumps induced necrotic lesions in inoculated eucalyptus plants. At 15 dai, all isolates recovered from asymptomatic mini-stumps, except for isolate COAD 3920, induced lesions larger than those of the negative control and statistically similar to the positive control (Figure 5A). All isolates exhibited mean lesion lengths statistically similar to the positive control at 45 dai (Figure 5B). The isolates were recovered from inoculated plants, confirming their pathogenicity.

3.4 Molecular identification of the isolates

The sequence database contained 80 taxa, including 36 sequences representing 11 species of the genus *Chrysoporthe*, 42 sequences generated in the present study, and 2 sequences of *Amphilogia gyrosa* used to root the phylogenetic tree. The final alignment, generated by concatenating the two regions of the gene encoding for β-tubulin, consisted of 745 nucleotides, with 359 for BT1 and 386 for BT2. Of the 745 nucleotides, 576 were conserved sites, and 90 were variable. The informative sites for parsimony included 78 nucleotides, with 12 nucleotides corresponding to singletons. The best nucleotide substitution model for Bayesian Inference analysis, selected by the Akaike Information Criterion, was GTR+I for BT1 and GTR+G for BT2.

Based on the concatenated phylogenetic tree generated by BI and ML methods, 41 isolates obtained in this study clustered with isolates of *C. cubensis*, including the ex-type strain of this species. Additionally, one isolate formed a monophyletic group with isolates of *C. doradensis*, including the ex-type strain (Figure 6).

4 Discussion

For the first time, the detection and identification of *C. cubensis* in asymptomatic eucalyptus mini-stumps, as well as the spread of disease from symptomatic mini-stems to mini-cuttings and rooted cuttings, were investigated, revealing new insights into the etiology of

Chrysoporthe wilt.

The colonization of endophytic niches by fungi from the Cryphonectriaceae family in plants of the order Myrtales has been confirmed. For instance, *Aurapex penicillata* and two species of *Chrysoporthe* (*C. cubensis* and *C. inopina*) were recovered from healthy trees of the Melastomataceae family naturally growing in Colombian forests (Granados et al. 2020). *Chrysoporthe austroafricana* and *Celoporthe woodiana* were also recovered from asymptomatic Myrtales hosts (Mausse-Sitoe et al. 2016). Generally, an aspect frequently raised in these studies is the role of asymptomatic infections in native trees in the dissemination of diseases to eucalyptus trees planted in shared landscapes.

The *C. cubensis* isolates recovered from asymptomatic mini-stumps were analyzed for their ability to induce diseases in eucalyptus plants. The results showed that all isolates were pathogenic. Thus, this study provides the first evidence that *C. cubensis* has the ability to colonize the tissues of eucalyptus mini-stumps without inducing symptoms of wilt and necrosis, as previously mentioned by Martins et al. 2023. Therefore, this finding suggests that *Chrysoporthe* spp. may be occupying an endophytic niche in eucalyptus mini-stumps in Brazil. This is particularly intriguing, given that *C. cubensis* is recognized as aggressive necrotrophic pathogens in eucalyptus plants (Ferreira & Milani 2002).

Fungi occupying endophytic niches can be pathogens with the ability to adapt to their hosts to colonize plant tissues without being detected early (Carroll 1988; Photita et al. 2004). One question raised was whether the *C. cubensis* isolates occupying endophytic niche required more time to induce symptoms in the plants, which could explain their recovery from asymptomatic tissues. To address this, we analyzed the pathogenicity of the isolates recovered from asymptomatic tissues and found that they were able of inducing typical necrotic lesions on plant stems. However, our results did not support this possibility, as necrotic lesions were observed within the first 15 days after inoculation, consistent with the positive control from a proven pathogenic isolate. Moreover, our results support the suggestion that *Chrysoporthe* species may exhibit biological aspects similar to other canker-inducing pathogens, such as species of the genus *Botryosphaeria*. For instance, *Botryosphaeria dothidea* survives latently in tree trunks for extended periods, making detection challenging. Changes in environmental conditions and host niche, commonly associated with periods of plant stress, contribute to infections becoming detectable, marked by the appearance of symptoms in infected plants (Dai et al. 2017; Marsberg et al. 2017).

Additionally, it is well known that the transition from endophytism to necrotrophy appears to occur relatively easily in nature, particularly for pathogens classified as necrotrophic,

such as some members of the Sclerotiniaceae family. The simplistic perception that necrotrophic fungi rapidly kill host cells has given way to an understanding of a multifaceted and sophisticated infection process. Evidence shows that necrotrophic fungi colonize plant tissues without inducing any symptoms during the initial phases of infection (Alkan & Fortes 2015; Delaye, García-Guzmán & Heil 2013; Van Kan et al. 2014).

In light of the above, it has been observed that the incidence of wilt in eucalyptus mini-stump increases with rising temperatures, coinciding with the summer season in Brazil (Martins et al. 2023; Caroline Abreu, personal communication). Furthermore, eucalyptus mini-stumps are continuously subjected to mini-cutting collection for vegetative propagation (cloning) which might cause physiological changes that disrupt the balance between the endophytic colonization and an apparently healthy host. Confirming this possibility requires more detailed studies. Another hypothesis that remains unexplored is whether plants that host the pathogen during endophytic niche exploration contribute to inoculum maintenance and disease dissemination in clonal mini-gardens, especially during maintenance pruning and mini-cutting collection.

This study also revealed that a higher number of *Chrysoporthe* isolates were obtained from three-year-old eucalyptus mini-stumps. Additionally, pathogen detection in asymptomatic samples occurred only in mini-stumps of this age, with no detection in two-year-old samples. These observations might be related to the longer exposure time of the host to inoculum in the mini-garden and to constant pruning that contribute to physiological stress and/or disease spread.

When analyzing the pathogen's distribution in plant stems, we observed that the pathogen was relatively well-distributed along the stem, especially in symptomatic mini-stumps. However, in some symptomatic and asymptomatic mini-stump samples, the pathogen was recovered only from the upper stem portion. For eucalyptus canker, it is known that infection can occur at different heights of the tree trunk and that cankers are more commonly found at the tree base (Ferreira & Milani 2002; Ferreira 1989). The recovery of the fungus solely from the upper stem and not from the base raised questions about the pathways of entry into host tissue in mini- gardens, highlighting the potential role of disease spread through pruning shear during the collection of mini-cuttings for vegetative propagation. Based on these findings, two questions can be raised: Does mini-cutting collection create wounds that could facilitate the fungal infection in mini-stumps, or is the pruning shear in charge of disease spread from one mini-stump to another?

It is scientifically proven that the vegetative propagation of apple trees plays a critical

role in the dissemination of *Neonectria ditissima* inoculum (Børve, Dalen & Stensvand 2019). Therefore, it would not be surprising if the vegetative propagation of eucalyptus through mini- cuttings also represented a critical moment for the dissemination of Chrysoporthe wilt. Interestingly, however, efforts to reveal spread of disease from symptomatic mini-stumps to mini-cuttings and rooted cuttings in the present study failed. Nonetheless, the absence of detection does not necessarily imply the absence of the pathogen, as its distribution within host tissues may not be uniform.

Moreover, conventional techniques, such as isolation and identification based on morphological and molecular characterization, have limitations. In addition to being labor-intensive and time-consuming, these techniques have low fungal detection limits, potentially underestimating the fungal community present in plant tissues (Momtaz, Hardy & Bayliss 2024). Thus, fungal detection through culture-dependent methods may result in plating fragments that do not include the target organism, even if it is present in the host plant (Mausse-Sitoe et al. 2016). In this context, it is suggested that molecular techniques based on nucleic acid detection be employed in complement to conventional methods in order to achieve higher resolution in the detection of fungi directly within host tissues, particularly in the context of endophytic niche occupation. Among the potential approaches that may be useful for detecting *Chrysoporthe* species are the Polymerase Chain Reaction (PCR), both conventional and real-time (qPCR), Next Generation Sequencing (NGS), and Loop Mediated Isothermal Amplification (LAMP). These techniques enable qualitative or quantitative detection of fungal nucleic acids, even when the target organism exhibits low biomass within plant tissues (Bullington & Larkin 2015; Malapi-Wight et al. 2016; Naqvi et al. 2025; Sun & Guo 2012).

Regarding the identification of isolates based on the phylogenetic species criterion, all isolates were identified as *C. cubensis*, except COAD 3945. This result was expected, as this species had previously been associated with mini-stump wilt in eucalyptus within the same clonal mini- garden (Martins et al. 2023). However, among the 42 fungi identified, the isolate COAD 3945 was identified as belonging to the species *Chrysoporthe doradensis*, which is a novel finding. To date, this species has been reported causing canker in *Tibouchina* ssp., *Henriettea seemannii*, and others, in Ecuador, Brazil, and Colombia (Rodas et al. 2005; Soares et al. 2018; Suzuki et al. 2023).

Since the isolate identification process included sampling one isolate per mini-stump, it is impossible to infer co-infections by *C. cubensis* and *C. doradensis* within the same plant, although this hypothesis cannot be ruled out. On the other hand, our results confirm that both species coexist within a relatively small area. Therefore, the epidemiological implications of

this pathosystem, as well as potential impacts on mini-stump wilt management, require further study. Species within the same genus may differ in pathogenicity and aggressiveness and exhibit varied responses to preventive and curative measures (Ramos et al. 2024).

Therefore, it is concluded that *C. cubensis* occupies an endophytic niche in eucalyptus mini-stumps of clones CNB007 and CNB010, whereas *C. doradensis* was not recovered occupying this niche in asymptomatic mini-stumps. For eucalyptus rooted cuttings of both clones, as well as for rooted cuttings obtained from the rooting of mini-cuttings derived from mini-stumps exhibiting wilt symptoms, the occupation of the endophytic niche by *Chrysoporthe* species was not confirmed. Regarding the identity of the isolates, *C. cubensis* was the predominantly recovered species from both asymptomatic and symptomatic tissues of eucalyptus mini-stumps, with 41 out of 42 isolates characterized through phylogenetic analyses belonging to this species, and one isolate identified as *C. doradensis*. It is noteworthy that this is the first study reporting *C. doradensis* as the etiological agent of wilt in eucalyptus mini-stumps worldwide.

References

Alkan, N., & Fortes, A. M. (2015). Insights into molecular and metabolic events associated with fruit response to post-harvest fungal pathogens. Frontiers in Plant Science, 6, 889. https://doi.org/10.3389/fpls.2015.00889

Alfenas, A. C., & Mafia, R. G. (2016). Métodos em Fitopatologia (2nd ed.). Viçosa: Editora UFV.

Alfenas, A. C., Zauza, E. A. V., Mafia, R. G., & Assis, T. F. (2009). Clonagem e doenças do eucalipto. Viçosa: Editora UFV.

Børve, J., Dalen, M., & Stensvand, A. (2019). Development of *Neonectria ditissima* infections initiated at grafting of apple trees. European Journal of Plant Pathology, 155, 1225-1239. https://doi.org/10.1007/s10658-019-01851-7

Bullington, L. S., & Larkin, B. G. (2015). Using direct amplification and next-generation sequencing technology to explore foliar endophyte communities in experimentally inoculated western white pines. Fungal Ecology, 17, 170-178.

https://doi.org/10.1016/j.funeco.2015.07.005

Carroll, G. (1988). Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology, 69, 2-9. https://doi.org/10.2307/1943154

Chungu, D., Siyingwa, J., Ng'andwe, P., & Chitala Chungu, B. (2019). Lesion size induced by *Chrysoporthe* fungal pathogens varies between *Eucalyptus* species and geographic locations in Zambia. Southern Forests: a Journal of Forest Science, 81, 39-44. https://doi.org/10.2989/20702620.2018.1488207

Dai, D. J., Wang, H. D., Wang, Y. P., & Zhang, C. Q. (2017). Management of Chinese hickory (*Carya cathayensis*) trunk canker through effective fungicide application programs and baseline sensitivity of *Botryosphaeria dothidea* to trifloxystrobin. Australasian Plant Pathology, 46, 75-82. https://doi.org/10.1007/s13313-017-0465-4

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and high-performance computing. Nature Methods, 9, 772. https://doi.org/10.1038/nmeth.2109

Delaye, L., García-Guzmán, G. & Heil, M. (2013). Endophytes versus biotrophic and necrotrophic pathogens—are fungal lifestyles evolutionarily stable traits?. Fungal Diversity 60, 125–135. https://doi.org/10.1007/s13225-013-0240-y

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32, 1792-1797. https://doi.org/10.1093/nar/gkh340

Ferreira, F. A., & Milani, D. (2002). Diagnose visual e controle das doenças abióticas e bióticas do eucalipto no Brasil. Mogi Guaçu: International Paper.

Ferreira, F. A. (1989). Patologia florestal-principais doenças florestais no Brasil. Viçosa: SIF.

Glass, N. L., & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from\ filamentous ascomycetes. Applied and Environmental Microbiology, 6, 1323-1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995

Granados, G. M., McTaggart, A. R., Rodas, C. A., Roux, J., & Wingfield, M. J. (2020). Species of Cryphonectriaceae occupy an endophytic niche in the Melastomataceae and are putative latent pathogens of *Eucalyptus*. European Journal of Plant Pathology, 156, 273-283. https://doi.org/10.1007/s10658-019-01887-9

Gryzenhout, M., Myburg, H., Van der Merwe, N. A., Wingfield, B. D., & Wingfield, M. J. (2004). *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Studies in Mycology, 50, 119-142.

Heath, R. N., Gryzenhout, M., Roux, J., & Wingfield, M. J. (2006). Discovery of the canker pathogen *Chrysoporthe austroafricana* on native *Syzygium* spp. in South Africa. Plant Disease, 90, 433-438. https://doi.org/10.1094/PD-90-0433

Hodges, C. S., Reis, M. S., Ferreira, F. A., & Henfling, J. D. M. (1976). O cancro do eucalipto causado por *Diaporthe cubensis*. Fitopatalogia Brasileira, 1, 129–170.

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Molecular Biology and Evolution, 34, 772–773. https://doi.org/10.1093/molbev/msw260

Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics, 30, 3276-3278. https://doi.org/10.1093/bioinformatics/btu531

Malapi-Wight, M., Demers, J. E., Veltri, D., Marra, R. E., & Crouch, J. A. (2016). LAMP detection assays for boxwood blight pathogens: a comparative genomics approach. Scientific Reports, 6, 26140. https://doi.org/10.1038/srep26140

Martins, M. D., Guterres, D. C., Andrade, P. R. A., Machado, F. J., & Furtado, G. Q. (2023). *Chrysoporthe cubensis* emerges causing wilt on *Eucalyptus* mini-stumps in Brazil. Australasian Plant Pathology, 52, 517-527. https://doi.org/10.1007/s13313-023-00940-w

Mausse-Sitoe, S. N., Rodas, C. A., Wingfield, M. J., Chen, S., & Roux, J. (2016). Endophytic

Cryphonectriaceae on native Myrtales: Possible origin of Chrysoporthe canker on plantation-grown *Eucalyptus*. Fungal Biology, 120, 827-835. https://doi.org/10.1016/j.funbio.2016.03.005

Marsberg, A., Kemler, M., Jami, F., Nagel, J. H., Postma-Smidt, A., Naidoo, S., Wingfield M. J., Crous, P. W., Spatafora, J. W., Hesse, C. N., Robbertse, B., & Slippers, B. (2017). *Botryosphaeria dothidea*: a latent pathogen of global importance to woody plant health. Molecular Plant Pathology, 18, 477-488. https://doi.org/10.1111/mpp.12495

Momtaz, F., Hardy, G., & Bayliss, K. L. (2024). Fungal communities associated with postharvest strawberries in Western Australia. Plant Pathology, 74, 210-219. https://doi.org/10.1111/ppa.14010

Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. New Orleans: IEEE. https://doi.org/10.1109/GCE.2010.5676129

Nakabonge, G., Roux, J., Gryzenhout, M., & Wingfield, M. J. (2006). Distribution of Chrysoporthe canker pathogens on *Eucalyptus* and *Syzygium* spp. in Eastern and Southern Africa. Plant Disease, 90, 734-740. https://doi.org/10.1094/PD-90-0734

Naqvi, S. A. H., Abbas, A., Hasnain, A., Bilal, Z., Hakim, F., Shabbir, M., Amin, A., & Iqbal, M. U. (2025). Advancing fungal phylogenetics: integrating modern sequencing, dark taxa discovery, and machine learning. Archives of Microbiology, 207, 1-27. https://doi.org/10.1007/s00203-025-04392-2

Oliveira, M. E. S., Van der Merwe, N. A., Wingfield, M. J., Wingfield, B. D., Soares, T. P. F., Kanzi, A. M., & Ferreira, M. A. (2021). *Chrysoporthe puriensis* sp. nov. from *Tibouchina* spp. in Brazil: an emerging threat to *Eucalyptus*. Australasian Plant Pathology, 50, 29-40. https://doi.org/10.1007/s13313-020-00745-1

Pegg, G. S., Gryzenhout, M., O'Dwyer, C., Drenth, A., & Wingfield, M. J. (2010). The Eucalyptus canker pathogen *Chrysoporthe cubensis* discovered in eastern Australia. Australasian Plant Pathology, 39, 343-349. https://doi.org/10.1071/AP10004

Photita, W., Lumyong, S., Lumyong, P., McKenzie, E. H. C., & Hyde, K. D. (2004). Are some endophytes of *Musa acuminata* latent pathogens?. Fungal Diversity, 16, 131-140.

Ramos, D. G. B., Amaral, A. G. G., Duarte, I. G., da Silva, A. C., dos Santos Vieira, W. A., Castlebury, L. A., & Câmara, M. P. S. (2024). Endophytic species of *Colletotrichum* associated with cashew tree in northeastern Brazil. Fungal Biology, 128, 1780-1789. https://doi.org/10.1016/j.funbio.2024.04.002

Rauf, M. R. B. A., McTaggart, A. R., Marincowitz, S., Barnes, I., Japarudin, Y., & Wingfield, M. J. (2020). Pathogenicity of *Chrysoporthe deuterocubensis* and *Myrtoporthe bo*denii gen. et sp. nov. on *Eucalyptus* in Sabah, Malaysia. Australasian Plant Pathology, 49, 53-64. https://doi.org/10.1007/s13313-019-00676-6

Rêgo, G. M. S., de Souza, I. A. L., Silva, G. A., de Oliveira, M. E. S., & Ferreira, M. A. (2023). *Chrysoporthe puriensis* causing canker and mortality in *Pleroma mutabile* in the Atlantic Forest, Brazil. Forest Pathology, 53, e12803. https://doi.org/10.1111/efp.12803

Roberts, M., Gilligan, C. A., Kleczkowski, A., Hanley, N., Whalley, A. E., & Healey, J. R. (2020). The effect of forest management options on forest resilience to pathogens. Frontiers in Forests and Global Change, 3, 7. https://doi.org/10.3389/ffgc.2020.00007

Rodas, C. A., Gryzenhout, M., Myburg, H., Wingfield, B. D., & Wingfield, M. J. (2005). Discovery of the Eucalyptus canker pathogen *Chrysoporthe cubensis* on native *Miconia* (Melastomataceae) in Colombia. Plant Pathology, 54, 460-470. https://doi.org/10.1111/j.1365-3059.2005.01223.x

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19, 1572-1574. https://doi.org/10.1093/bioinformatics/btg180

Saunders, M., & Kohn, L. M. (2009). Evidence for alteration of fungal endophyte community assembly by host defense compounds. New Phytologist, 182, 229-238. https://doi.org/10.1111/j.1469-8137.2008.02746.x

Soares, T. P., Ferreira, M. A., Mafia, R. G., Oliveira, L. S., Hodges, C. S., & Alfenas, A. C.

(2018). Canker disease caused by *Chrysoporthe doradensis* and *C. cubensis* on *Eucalyptus* sp. and *Tibouchina* spp. in Brazil. Tropical Plant Pathology, 43, 314-322. https://doi.org/10.1007/s40858-018-0238-9

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30, 1312-1313. https://doi.org/10.1093/bioinformatics/btu033

Sun, X., & Guo, L. D. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. Mycology, 3, 65-76. https://doi.org/10.1080/21501203.2012.656724

Suzuki, H., Marincowitz, S., Rodas, C. A., Wingfield, B. D., & Wingfield, M. J. (2023). First report of two *Chrysoporthe* species, *Chrysoporthe doradensis* and *Chrysoporthe colombiana* sp. nov. from *Henriettea seemannii* pathogenic to Eucalyptus in Colombia. Mycological Progress, 22, 44. https://doi.org/10.1007/s11557-023-01891-8

Vaidya, G., Lohman, D. J., & Meier, R. (2011). SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics, 27, 171-180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Van Kan, J. A., Shaw, M. W., & Grant-Downton, R. T. (2014). *Botrytis* species: relentless necrotrophic thugs or endophytes gone rogue?. Molecular Plant Pathology, 15, 957-961. https://doi.org/10.1111/mpp.12148

Wang, W., & Chen, S. (2023). Identification and pathogenicity of *Aurifilum* species (Cryphonectriaceae, Diaporthales) on *Terminalia* species in Southern China. MycoKeys, 98, 37-58. https://doi.org/10.3897/mycokeys.98.104719

Wingfield, M. J. (2003). 2003 Daniel McAlpine Memorial Lecture Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from Cryphonectria canker. Australasian Plant Pathology, 32, 133–139. https://doi.org/10.1071/AP03024

Figure 1. Eucalyptus mini-stumps showing wilt symptoms (A), dieback (B), and necrotic lesion in the upper portion of the stem in symptomatic mini-stumps (C).

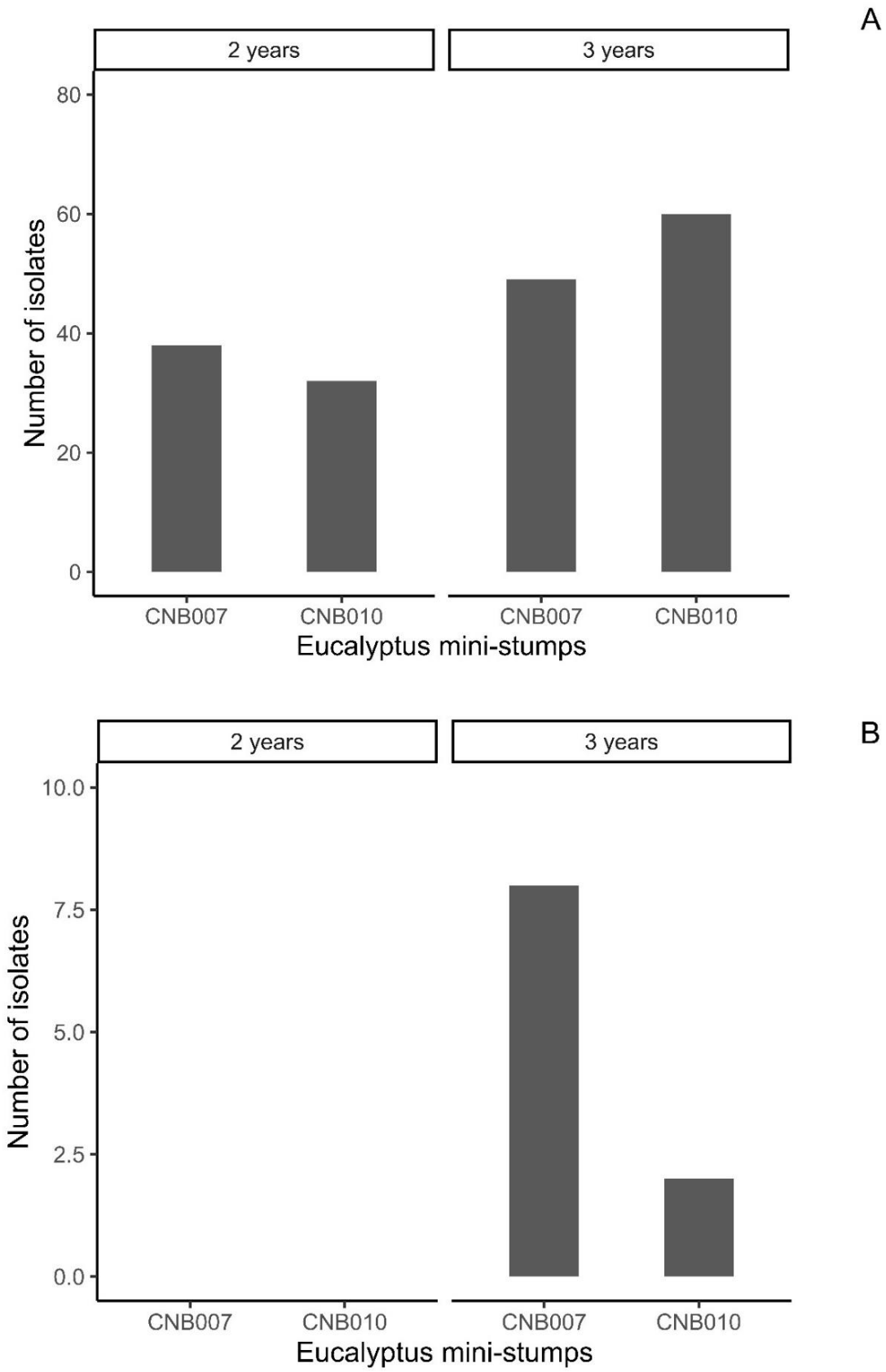


Figure 2. Number of *Chrysoporthe* sp. isolates recovered from two-year-old and three-year-old symptomatic (A) and asymptomatic (B) mini-stumps belonging to CNB007 and CNB010 eucalyptus clone.

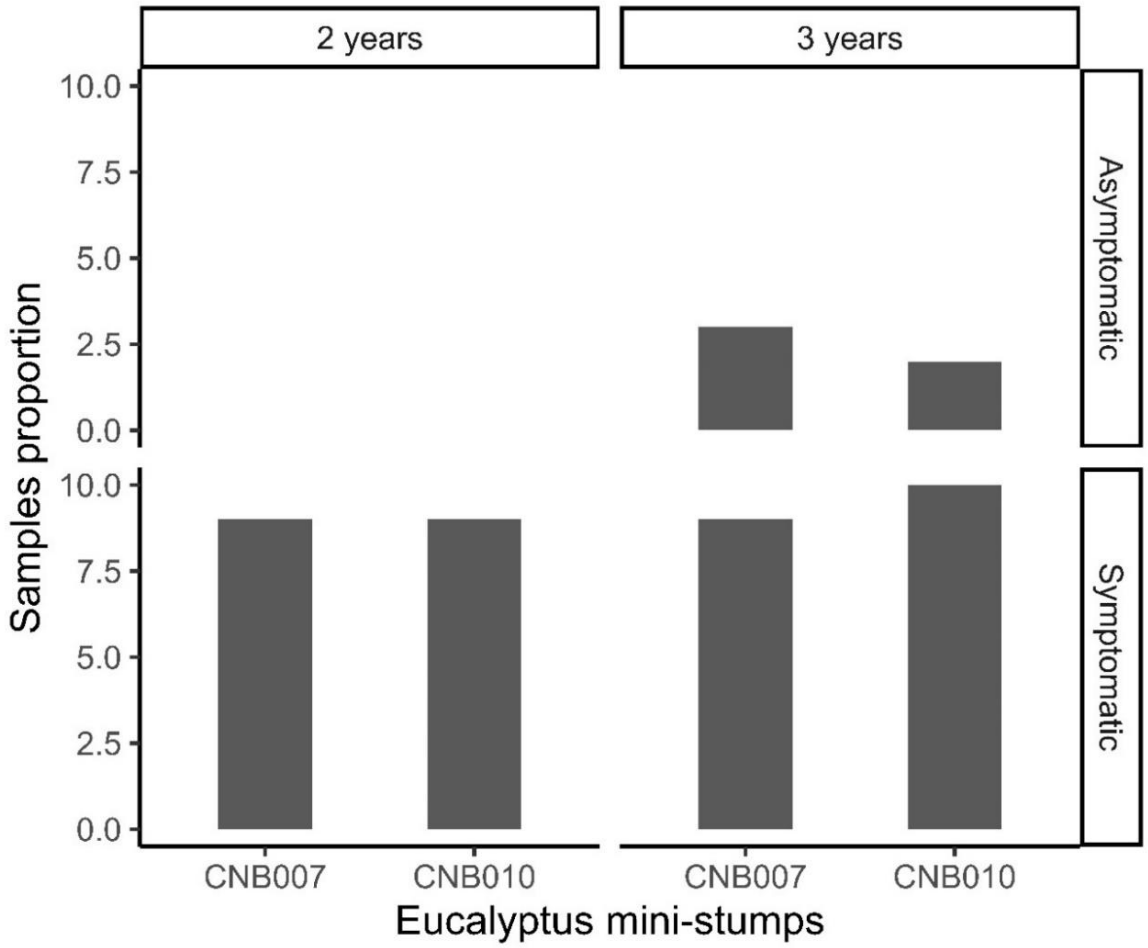


Figure 3. Number of eucalyptus mini-stump samples (clones CNB007 and CNB010), asymptomatic and symptomatic, from which *Chrysoporthe* sp. was recovered.

Table 1. Detection of *Chrysoporthe* sp. from basal, median and upper stem portion of asymptomatic and symptomatic eucalyptus mini-stumps of clones CNB007 and CNB010.

Mini-stumps	**Age (years)**	**Total number of mini-stumps sampled**	**Number of mini-stumps/stem portions in which *Chrysoporthe* sp. was recovered**						
			Basal, Median and Upper	Basal and Median	Median and Upper	Basal and Upper	Basal	Median	Upper
CNB007 asymptomatic	2	10	0	0	0	0	0	0	0
	3	10	0	1	0	1	0	0	1
CNB010 asymptomatic	2	10	0	0	0	0	0	0	0
	3	10	0	0	0	0	0	1	1
CNB007 symptomatic	2	10	3	2	3	0	0	0	1
	3	10	4	2	1	0	0	1	1
CNB010 symptomatic	2	10	4	0	3	0	0	2	0
	3	10	4	6	0	0	0	0	0

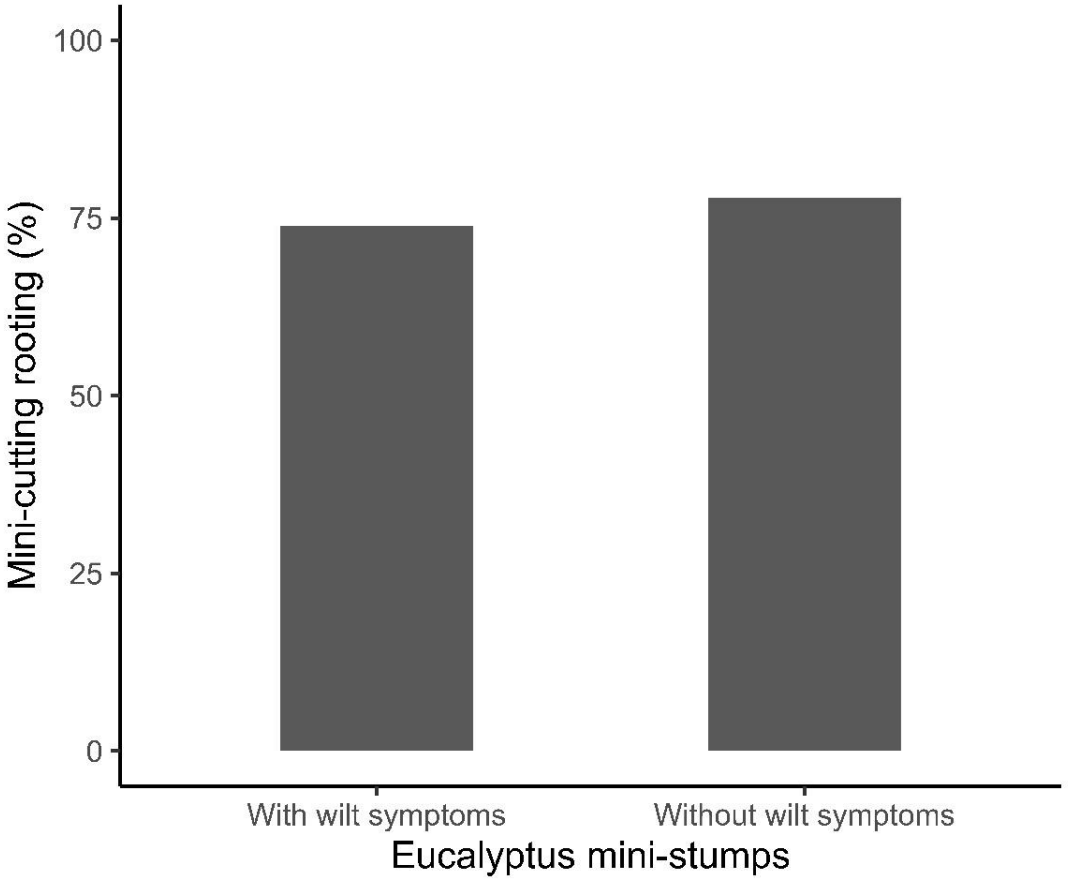


Figure 4. Percentage of mini-cuttings rooting sourced from symptomatic and asymptomatic eucalyptus mini-stumps.

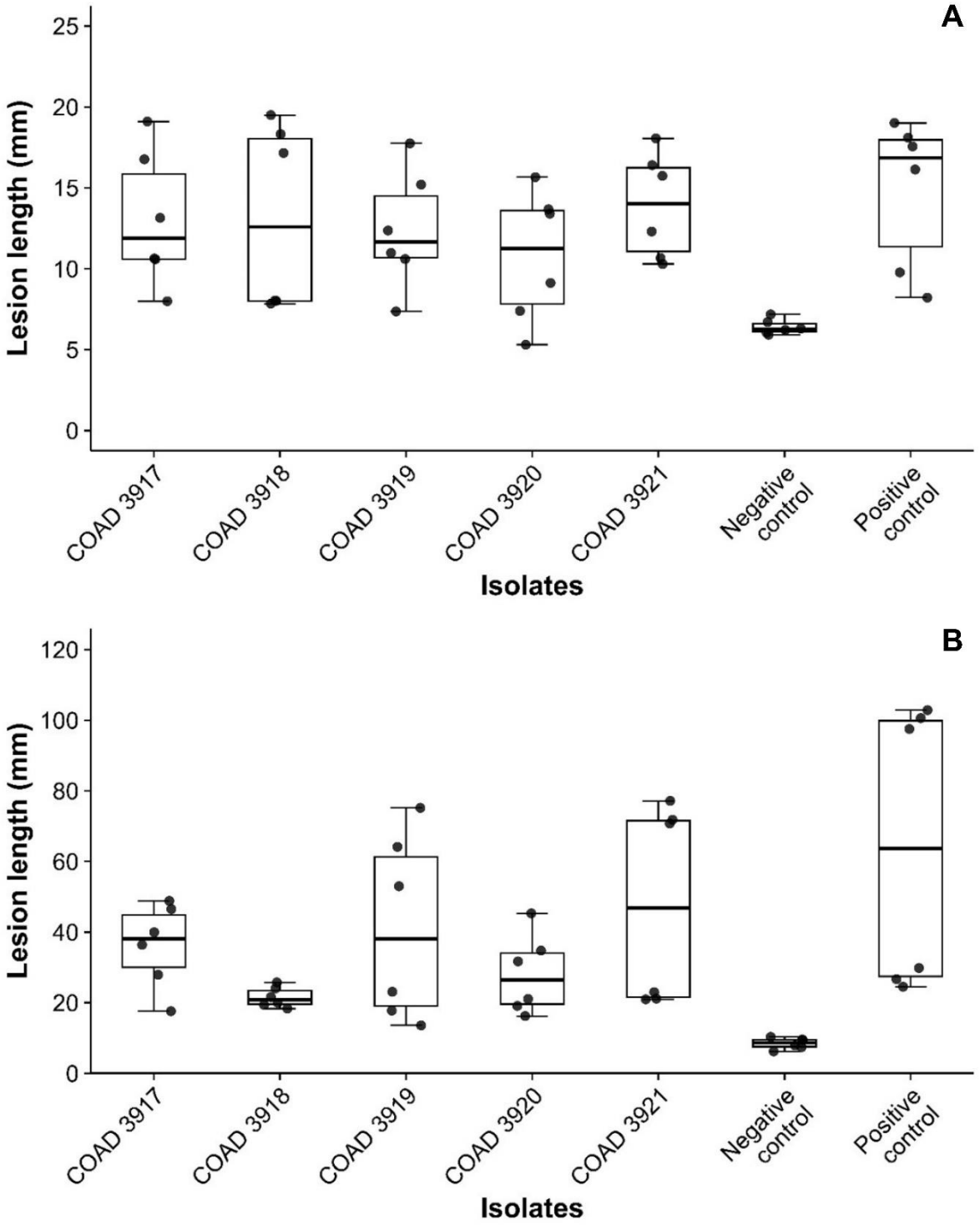


Figure 5. Pathogenicity test of *Chrysoporthe cubensis* isolates recovered from asymptomatic eucalyptus mini-stumps at 15 (A) and 45 (B) days after inoculation. The negative control consists of rooted cuttings inoculate with PDA. The positive control consists of rooted cuttings inoculated with an isolate (COAD 3401) whose pathogenicity has been previously.

Table 2. GenBank accession number of partial gene sequences for the *Chrysoporthe* species used in the phylogenetic analysis and the outgroup *Amphilogia gyrosa*. Information generated in this study is in bold. Type (T) or ex-type (ET) specimens are indicated in superscript after isolate identification.

Species name	**Isolate identification[1]**	**Host/Substrate**	**Geographic origin**	**GenBank acession numbers[2]**	
				TUB1	***TUB2***
Chrysoporthe austroafricana	CMW 2113^{T} = CBS 112916	*Eucalyptus grandis*	South Africa	AF273067	AF273462
C. austroafricana	CMW 9328	*Tibouchina granulosa*	South Africa	AF273061	AF273456
C. austroafricana	CMW 9327	*Tibouchina granulosa*	South Africa	AF273060	AF273455
C. brasiliensis	LPFMIL06	*Miconia theaezans*	Brazil	OP714218	-
C. brasiliensis	LPFMIL104B	*Rhynchanthera grandiflora*	Brazil	OP714219	-
C. brasiliensis	LPFTILU104D	*Rhynchanthera grandiflora*	Brazil	OP714224	-
C. colombiana	CMW 55973^{T}	*Henriettea seemannii*	Colombia	OQ336269	OQ336269
C. colombiana	CMW 56173	*Henriettea seemannii*	Colombia	OQ336270	OQ336270
C. colombiana	CMW 56176	*Henriettea seemannii*	Colombia	OQ336271	OQ336271
C. cubensis	CMW 9996 = CBS 115731	*Miconia rubiginosa*	Colombia	AY214220	AY214256
C. cubensis	CMW 10638 = CBS 115746	*Eucalyptus grandis*	Colombia	AY 956971	AY 956972
C. cubensis	CMW10778^{ET} = CBS 118654	*Eucalyptus grandis*	Cuba	GQ290178	

C. cubensis	CMW 10028	*Miconia rubiginosa*	Colombia	GQ290175	GQ290186
C. cubensis	CE12	*Eucalyptus* sp.	Brazil	KX639088	KX639105
C. cubensis	CMW 12734	*Rhynchanthera mexicana*	Mexico	AH015646	GQ290191
C. cubensis	**COAD 3917**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3918**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3919**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3920**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3921**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3922**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3938**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-

C. cubensis	**COAD 3939**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3940**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3941**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3942**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3943**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3946**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3947**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3948**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-

C. cubensis	**COAD 3949**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3950**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3951**	***Eucalyptus urophylla* ×**	**Brazil**	-	-
		Eucalyptus grandis			
C. cubensis	**COAD 3952**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3953**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3954**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3956**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3957**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-

C. cubensis	**COAD 3958**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3959**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3960**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3923**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3936**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3937**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3924**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3925**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3926**	***Eucalyptus urophylla* × *Eucalyptus grandi***	**Brazil**	-	-

C. cubensis	**COAD 3927**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3928**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3929**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3930**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3931**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3932**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3933**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. cubensis	**COAD 3934**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-

C. cubensis	**COAD 3935**	***Eucalyptus urophylla* × *Eucalyptus grandis***	**Brazil**	-	-
C. deuterocubensis	CMW 12745	-	Singapore	GQ290183	AH015651
C. deuterocubensis	CMW 2631	*Eucalyptus marginata*	Australia	GQ290184	AH012043
C. deuterocubensis	CMW 8650	*Syzygium aromaticum*	Indonesia	AY084024	GQ290193
C. doradensis	CMW 9124	*Eucalyptus deglupta*	Ecuador	DQ224040	DQ224041
C. doradensis	CMW 11286^{T}	*Eucalyptus grandis*	Ecuador	AY214217	AY214253
C. doradensis	CMW 55974	*H. seemannii*	Colombia	OQ336267	OQ336267
C. doradensis	**COAD 3945**	***Eucalyptus urophylla* × *Eucalyptus grandi***	**Brazil**		
C. hodgesiana	CMW 9994 = CBS 115729	*T. semidecandra*	Colombia	AH014903	AH014903
C. hodgesiana	CMW 10625 = CBS 115744	*M. theaezans*	Colombia	AH014900	AH014900
C. inopina	CMW 12727^{T}	*T. lepidota*	Colombia	AH015657	AH015657
C. inopina	CMW 12729	*T. lepidota*	Colombia	AH015656	AH015656
C. inopina	CMW 12731	*T. lepidota*	Colombia	AH015655	AH015655
C. puriensis	LPFCT13 ^{T}	*Pleroma granulosum*	Brazil	MN590041	-
C. puriensis	LPFTCL01	*P. candolleanum*	Brazil	MN590042	-
C. puriensis	LPFTGCD01	*P. granulosum*	Brazil	MN590049	-
C. syzygiicola	CMW 29940^{T}	*Syzygium guineense*	Zambia	FJ805230	FJ805236

C. syzygiicola	CMW 29941	*S. guineense*	Zambia	FJ805231	FJ805237
C. syzygiicola	CMW 29942	*S. guineense*	Zambia	FJ805232	FJ805238
C. zambiensis	CMW 29928^{T}	*E. grandis*	Zambia	FJ858709	FJ805233
C. zambiensis	CMW 29929	*E. grandis*	Zambia	FJ858710	FJ805234
C. zambiensis	CMW 29930	*E. grandis*	Zambia	FJ858711	FJ805235
Amphilogia gyrosa	CMW 10469^{T} = CBS 112922	*Elaeocarpus dentatus*, *El. hookerianus* and *El. glandulifer*	New Zealand	AF525707	AF525714
A. gyrosa	CMW 10470	*El. dentatus*	New Zealand	AF525708	AF525715

[1]Collection of cultures - CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa. CBS: CBS-KNAW Culture Collection at Westerdijk Fungal Biodiversity Institute (CBS), The Netherlands. COAD: Coleção Octávio de Almeida Drumond at Universidade Federal de Viçosa, Brazil.

[2]Accession number of nucleotide sequences for the TUB1 and TUB2 gene regions deposited in GenBank.

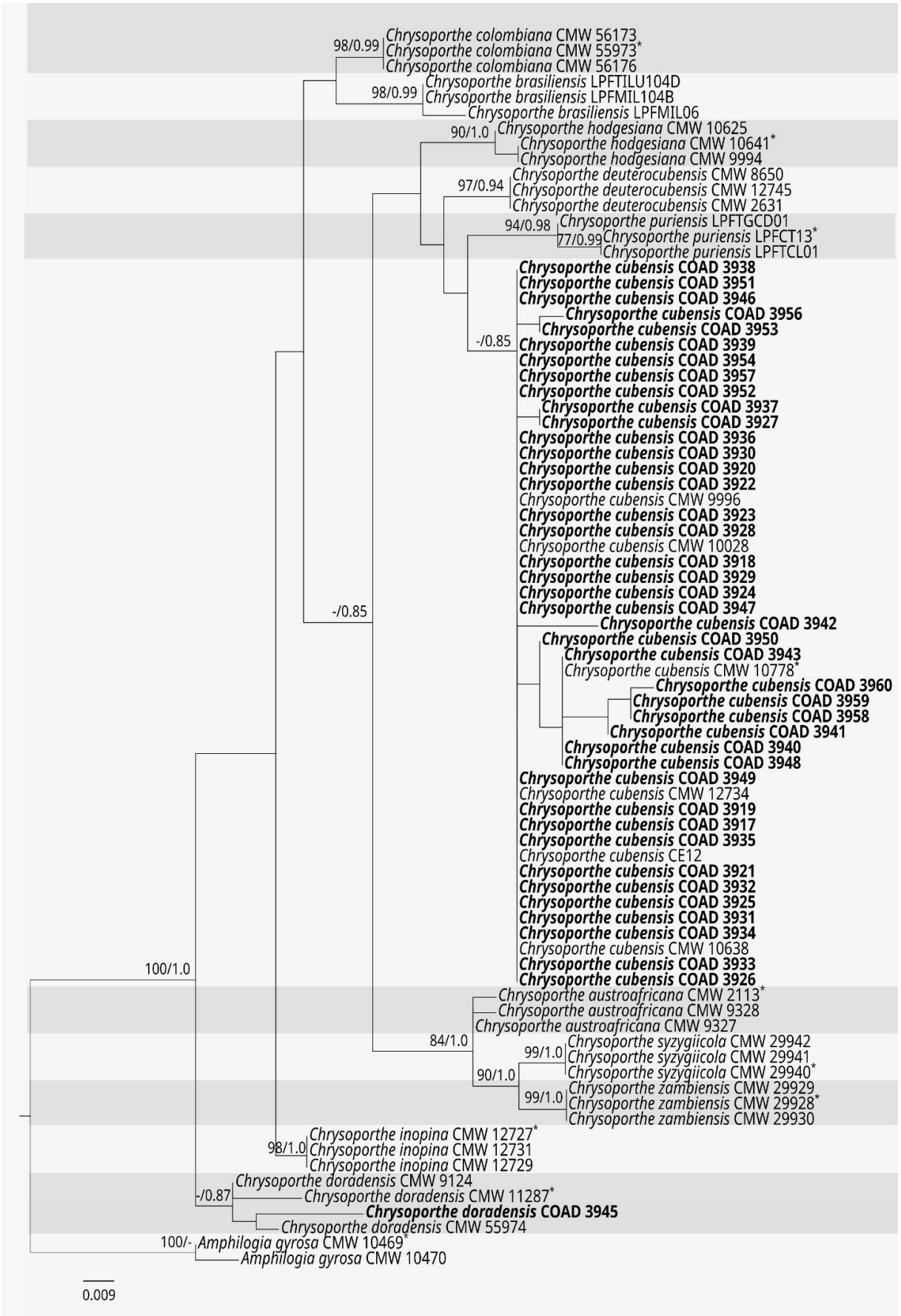


Figure 6. Multilocus phylogenetic tree based on Bayesian Inference (BI) and Maximum

Likelihood (ML) using the alignment of combined sequences (*Bt1* and *Bt2*) of *Chrysoporthe* spp. Bayesian posterior probabilities ≥ 0.85 and bootstrap values ≥ 80 are shown at the nodes (ML/BI), respectively. Asterisk indicate sequence from type and ex-type specimens. The tree was rooted with *Amphilogia gyrosa* (CMW 10469 and CMW 10470). The isolates obtained in this study are emphasized in bold.

CHAPTER 2

Potential use of DMI fungicides against *Chrysoporthe cubensis*, the causal agent of wilting in eucalyptus mini-stumps

P. R. A. Andrade[1], M. G. V. Kiti[1], H. W. Abreu[1], F. J. Machado[1], G. Q. Furtado[1]*

[1]Department of Plant Pathology, Universidade Federal de Viçosa, 36570-900, Viçosa, Minas Gerais, Brazil.

*Corresponding author: gfurtado@ufv.br

Abstract

Chrysoporthe spp. are known as important etiological agents of canker in eucalyptus plantations. Recently, *Chrysoporthe cubensis* was recorded causing wilt and posing a threat to the productivity of eucalyptus mini-stumps in Brazil. Considering that there is no information on the use of fungicides to control Chrysoporthe wilt in clonal mini-gardens, the objective of this study was to investigate the sensitivity of *C. cubensis* to four triazole fungicides and their efficacy in controlling the wilt in eucalyptus rooted cuttings, both preventively and curatively. It was found that the isolates are highly sensitive to the triazole fungicides cyproconazole (0.0770 µg/mL), difenoconazole (0.0065 µg/mL), ipconazole (0.0868 µg/mL), and tebuconazole (0.0376 µg/mL). The drench application of the fungicides, directed at the stem and root of eucalyptus rooted cuttings, did not result in macroscopic phytotoxicity symptoms at doses of 5, 10, 15, and 20 g of active ingredient per 100 liters of water. Based on *in vivo* experiments, the curative application of fungicides (72 hours after pathogen inoculation) resulted in a 26.94% smaller necrotic lesion within the stem compared to the preventive application (72 hours before pathogen inoculation) for all tested triazole molecules. In another *in vivo* assay, the application of the molecules 15 days after pathogen inoculation was ineffective in controlling the disease. However, the fungicides ipconazole and tebuconazole were able to reduce the average number of fruiting bodies on the outer stem surface, emerging from diseased tissue, by 53.7% and 56.9%, respectively. The findings of this study suggest that triazole fungicides, when applied curatively, could be a useful strategy for managing Chrysoporthe wilt in eucalyptus clonal mini-gardens in order to reduce the development and reproduction of the pathogen.

1 Introduction

Species of *Chrysoporthe* are well known pathogens of trees belonging to the families Myrtaceae and Melastomataceae (Gryzenhout et al. 2004; Roux et al. 2003; Silva et al. 2023; Suzuki et al. 2023). Among these, *Chrysoporthe cubensis*, the causal agent of eucalyptus canker, posed a significant threat to forest plantations in Brazil during the 1970s (Alfenas et al. 2009; Ferreira 1989). Eucalyptus canker is a field disease that affects trees of various ages. Historically, economic damage has been observed in geographic regions characterized by high humidity and elevated temperatures, climatic conditions typical of tropical and subtropical areas (Hodges et al. 1976; Rodas et al. 2005; Trivi et al. 2013).

The symptomatology of canker disease includes stem girdling, vascular cambium death, and plant mortality in eucalyptus trees less than one year old. In trees older than two years, the main symptoms are bark cracking and the development of either superficial or sunken lesions at various heights along the trunk. The occurrence of typical cankers—characterized by deep lesions bordered by callus tissue, resulting from the death of larger portions of the cambium—is also a key symptom (Alfenas et al. 2009; Hodges et al. 1976). The selection of resistant progenies and advances in the cloning of resistant genotypes have contributed to the success of eucalyptus cultivation in Brazil in recent decades (Ferreira 1989; Guimarães et al. 2010).

Later, *C. cubensis* was reported causing wilt in eucalyptus mini-stumps in Brazil, which progressed to partially or completely desiccation, and eventually leading mini-stumps to death. Internally, necrotic lesions can be observed within the stem of the host plants (Martins et al. 2023). To deal with Chrysoporthe wilt in eucalyptus clonal mini-garden, the main management approach involves the elimination of symptomatic mini-stumps, which results in frequent replacement of mini-stumps, a reduced operational lifespan for the mini-garden, and higher labor demand (C. S. Abreu, personal communication).

Although there is a well-established understanding of the importance of integrated plant disease management (Mukhtar, Vagelas, & Javaid 2023), the Brazilian eucalyptus industry remains heavily reliant on genetic control to address infections caused by *C. cubensis*. This highlights the need for new management strategies, such as the adoption of chemical control methods. Chemical fungicides are effective tools for managing fungal diseases in many hosts (Brent & Hollomon 2007). However, there are currently no fungicide molecules registered to target *C. cubensis* in Brazil (Brazilian Ministry of Agriculture, Livestock and Food Supply 2024). There is only one study on the use of fungicides in managing eucalyptus canker.

However, it focused on the potential of chemical control for the causal agent *Chrysoporthe deuterocubensis* and was conducted in Malaysia (Ambrose et al. 2023). To date, no studies on the sensitivity of *Chrysoporthe* spp. to fungicides have been conducted in Brazil. Moreover, the lack of information on the efficacy of this approach makes it difficult to integrate chemical control into integrated management programs for eucalyptus canker and mini-stump wilt.

Among the fungicides available on the market are demethylation inhibitors (DMI, FRAC Code 3), which are used globally to manage diseases targeting various biological agents in a wide range of hosts. The molecules in this group act by inhibiting the biosynthesis of membrane sterols, with the primary target being the fungal 14-α-demethylase (FRAC 2024; Jefcoate 1978; Mehl et al. 2019). These fungicides inhibit fungal growth by affecting membrane integrity and interfering with the function of membrane-bound proteins. This inhibition results primarily from the accumulation of methylated sterol precursors and the depletion of membrane sterols (Ruge et al. 2005; Yan et al. 2011; Yang et al. 2009).

Thus, this study aimed to investigate the sensitivity of *C. cubensis* isolates to four triazole fungicides and their efficacy in controlling Chrysoporthe wilt applied both preventively and curatively.

2 Material and method

2.1 Pathogen isolates and fungicides

In order to assess the sensitivity of *C. cubensis* to fungicides, seven isolates (COAD 3400, COAD 3401, COAD 3423, COAD 3478, COAD 3480, COAD 3481 and COAD 3483) were used. The isolates were obtained from mini-stumps collected in the municipality of Belo Oriente, Três Lagoas and Agudos in the Brazilian States of Minas Gerais, Mato Grosso do Sul and São Paulo (Supplementary Figure 1). The isolates were identified through morphological and molecular approaches by Martins et al. (2023) and deposited in the Octávio de Almeida Drummond Collection (COAD) at the Universidade Federal de Viçosa, Brazil. The isolates were reactivated on PDA (Potato Dextrose Agar) and incubated at 25°C for five days.

The fungicides tested in this study were: ipconazole, difenoconazole, cyproconazole and tebuconazole. Information on trade names, manufacturers, solvents used for fungicide dilution, and the concentrations analyzed can be found in Table 1. The fungicides were diluted in autoclaved distilled water, except for tebuconazole, which was diluted in acetone to obtain a stock solution of 2,000 μg mL^{-1}. The stock solutions were stored at 4 °C in the dark until use.

2.2 *In vitro* sensitivity of active ingredients

The *in vitro* fungitoxicity assay was carried out based on the mycelial growth rate of the pathogen in culture medium amended with increasing concentrations of fungicide (Figure 1). Each fungicide molecule was diluted from the stock solution to obtain the following concentrations: 0.001, 0.010, 0.100, 1.00, and 10.00 µg/mL, which were into cooled (45 to 50 °C) autoclaved PDA, before solidification. As a control, distilled water or acetone (0.6%) were added to the PDA, depending on how the stock solution was prepared.

Mycelial plugs (5.5 mm) of *C. cubensis*, removed from the edge of 3-day-old colony, were transferred to Petri dishes (90 x 15 mm) containing 15 mL of PDA amended with fungicides. The plates were incubated at 25°C in the dark for 72 hours. According to the method described by Anderson et al. (2020), the fungal colony diameter was measured in two perpendicular directions using a digital caliper. The initial mycelial plug diameter (5.5 mm) was subtracted from each measurement, and both values were used to calculate the average colony diameter.

The experiment was conducted in a completely randomized design, with five replicates per treatment. Each experimental unit was represented by a Petri dish. The experiment was conducted twice, independently.

2.3 Phytotoxicity evaluation of the molecules in eucalyptus

Eucalyptus rooted cuttings of the clone CNB010 were transplanted into polyethylene pots containing 2 kg of a commercial substrate mixture with 300 g of Osmocote and 300 g of simple superphosphate for every 50 kg of substrate. The rooted cuttings were kept at room temperature in a greenhouse and irrigated daily.

Twenty days after transplantation, the active ingredients cyproconazole, ipconazole, difenoconazole, and tebuconazole were applied. For each molecule, four doses of active ingredient were tested: 5, 10, 15, and 20 g of active ingredient per 100 L of water. In preparing the spray solution, the commercial formulations described in item 2.1 were used, with the exception of the fungicide tebuconazole. For this fungicide, the commercial product Tebufort® (UPL OpenAg™) was used. The fungicides were applied by using 50 mL of a fungicide solution per plant. The solution was distributed around the plant collar, at a height of 4 centimeters above the plant collar. For each combination of active ingredients and dose, three replicates were used,

with each experimental unit being a single eucalyptus plant. The control treatment consisted of applying only distilled autoclaved water under the same conditions as the fungicide solutions. After application, the pots were randomly placed in the greenhouse.

The plants were visually assessed daily for phytotoxicity symptoms caused by systemic pesticides, described by Alfenas et al. (2009). Symptoms such as chlorosis, leaf deformation, excessive sprouting, necrosis, leaf curling, and reduced plant growth were monitored over a period of 14 days after inoculation.

2.4 Efficacy of preventive and curative applications

Eucalyptus rooted cuttings of the clone CNB010 were cultivated as described in section 2.3. Approximately two months after transplanting, the fungicide ipconazole, difenoconazole, cyproconazole, and tebuconazole were applied to the plants. The fungicides were applied at two intervals: 72 hours before inoculation or 72 hours after inoculation with *C. cubensis*.

The spray solution was prepared using the same commercial formulations as those used in the in vitro sensitivity assay, except for tebuconazole, which was tested using Tebufort® (UPL OpenAg™). The solution was prepared by diluting the products in autoclaved distilled water, standardizing the dose to 20 g of active ingredient per 100 L of water for each fungicide. A volume of 50 mL of the solution was applied to each plant via drench. The solution was distributed at the plant's basal position, approximately four centimeters above the collar, to ensure the solution reached the cultivation substrate. In the control treatment, only autoclaved distilled water was applied. The experiment was conducted in a randomized block design with six replicates per treatment and each replicate corresponded to a plant. The assay was independently repeated once.

For inoculation purposes, *C. cubensis* isolate (COAD 3423) was cultivated on PDA at 25°C in the dark; and 5.5-mm mycelial plugs were taken from the edge of 7-day-old colony e and transferred to the freshly exposed wounds in the stems. Wounds were created using a 5.5-mm- diameter sterilized cork borer in the basal portion of the stem, approximately 10 centimeters above the collar region. The inoculation site in the stem was covered with plastic film to prevent desiccation and contamination. Thirty days after inoculation, the stems of the plants were opened with the aid of a scalpel, and the length of the necrotic lesion was measured with a digital caliper.

2.5 Curative action against ongoing colonization of *C. cubensis*

To test the curative fungicide effect against Chysoporthe wilt, three-month-old eucalyptus rooted cuttings of clone CNB010 were grown in greenhouse (as described in item 2.3) for two months after transplanting and then challenged with the COAD 3423 isolate. The inoculation of pathogen was carried out in section 2.4.

Fifteen days after pathogen inoculation, the cyproconazole, difenoconazole, ipconazole, and tebuconazole were applied to the plants. The methodology for fungicide application was the same as described in section 2.4. The evaluation was performed 30 days after pathogen inoculation. The number of fruiting bodies per stem was determined by counting them under a dissecting microscope (Olympus SZX7). The total length of the necrotic lesion inside the stem was determined using a digital caliper. The lesion length was assessed as mentioned previously.

The experiment was conducted in a randomized block design with six replicates per treatment. Each replicate consisted of one pot containing a single plant. Two independent trials were conducted.

2.6 Statistical analysis

The EC_{50} calculation was based on dose-response curve fitting from the linear regression adjustment between the percentage of inhibition of mycelial growth in relation to the control (not treated with fungicide) and the fungicide concentrations transformed to a logarithmic scale ($\log 10$) (Machado et al. 2024).

The lesion length data obtained in the efficacy trial of preventive and curative applications were log-transformed to meet the assumptions of a normal distribution. Analysis of variance (ANOVA) ($p = 0.05$) was performed to evaluate the factorial interaction (2×5) between the factor's application timing and fungicides, based on the transformed data. Tukey's post-hoc test ($p = 0.05$) was used to compare the means among the levels of the fungicide factor. Data from both independent trials were combined due to the absence of significant differences between them, as determined by the analysis of variance ($p = 0.387$).

The lesion length and the number of fruiting bodies per stem data obtained from the curative action trial against Chrysoporthe wilt in ongoing colonization were log-transformed to meet the assumptions of normal distribution. ANOVA was conducted, and Tukey's post-hoc test was applied to compare the treatment means (fungicides and control) for both response

variables (p = 0.05). ANOVA indicated no significant differences between the two independent trials (p = 0.1370), which justified the combination of data from both trials. All statistical analyses and graphical representations were performed using RStudio software, version 4.3.1 (R Core Team 2023).

3 Results

3.1 *In vitro* sensibility

The estimation of EC_{50} values for the triazole fungicides, estimated for the seven *C. cubensis* isolates, resulted in the ranges of 0.0028 - 0.0625, 0.0155 - 0.3371, 0.0028 - 0.0150, 0.0440 - 0.1806 µg/mL for tebuconazole, ipconazole, difenoconazole, cyproconazole, respectively. The EC_{50} values for difenoconazole showed a narrower range compared to those obtained for the other active ingredients (Figure 2). The average EC_{50} values per fungicide were 0.0065, 0.0770, 0.0376 and 0.0868 µg/mL for difenoconazole, cyproconazole, tebuconazole, and ipconazole, respectively. The average EC50 values were not significantly different among the four fungicides (data not shown).

3.2 Phytotoxicity evaluation of the molecules in eucalyptus

The application of fungicides to eucalyptus rooted cuttings did not result in visible symptoms of phytotoxicity in the aerial parts at 4 and 12 days after application, regardless of the dose applied (Supplementary Figure 2).

3.3 Efficacy of preventive and curative applications

The interaction among the fungicides and the timing of application was not significant (p-value = 0.474). On the other hand, analysis of variance revealed a significant difference between the factor levels for both application timing (p = 0.00003) and fungicides (p = 0.00278).

The analysis of the application timing factor revealed that applying the molecules 72 hours after inoculation resulted in smaller lesions compared to preventive application (Figure 3A; Figure 5). On average, lesions were 26.94% smaller when fungicides were applied after

pathogen inoculation (Supplementary Figure 3A). All fungicides, except for difenoconazole—which showed intermediate values—differed significantly from the control and reduced the average lesion length. For treatments with cyproconazole and ipconazole, lesion length was reduced by 28.33% and 29.04%, respectively, while tebuconazole resulted in a 29.33% reduction (Figure 3B, Supplementary Figure 3B).

3.4 Curative action against ongoing colonization of *C. cubensis*

The analysis of variance among treatments showed that the application of triazole fungicides to ongoing infections did not significantly affect the lesion length in the stem ($p = 0.848$) (Figure 4A, Supplementary Figure 3C). However, plants treated with the fungicides tebuconazole and ipconazole displayed a lower number of fruiting bodies per stem compared to the control. The reduction in the number of fruiting bodies for tebuconazole and ipconazole was 56.9% and 53.7%, respectively. The treatments corresponding to the curative application of cyproconazole and difenoconazole exhibited an intermediate response, not differing from the control treatment or from the treatments that resulted in a lower number of fruiting bodies per stem (Figure 4B, Supplementary Figure 3D).

4 Discussion

Although sterol biosynthesis inhibitors represent the main group of commercial fungicides and have been used to control fungal diseases since the 1970s (Price et al. 2015; Yin et al. 2023), there is a gap in knowledge regarding the sensitivity of *Chrysoporthe* spp. to these fungicides worldwide. A prior study investigated the sensitivity of *Chrysoporthe* spp. to fungicides using isolates of *C. deuterocubensis* from Malaysia. In that study, the molecules belonged to the chemical groups dithiocarbamate (thiram and mancozeb) and imidazoles (manganese chloride prochloraz) were tested, as well as the multisite copper hydroxide and the oomycide morpholine (dimethomorph). *In vitro*, *C. deuterocubensis* was sensitive to manganese chloride prochloraz, dimethomorph, and thiram, and in field conditions, all fungicides reduced lesion length when applied both preventively and curatively (Ambrose et al. 2023). Therefore, according to the literature, the present study appears to be the first to investigate the sensitivity of *C. cubensis* to fungicides.

This study showed that 50% of the mycelial growth of *C. cubensis* was inhibited at active ingredient concentrations below 0.4 µg/mL, regardless of the fungicide-isolate

combination. While the isolates COAD 3478, COAD 3401, and COAD 3480 exhibited differential EC_{50} responses to certain molecules, no significant variation in their overall sensitivity was observed. The high fungitoxicity of sterol biosynthesis inhibitor fungicides against *C. cubensis* isolates may be due to the limited exposure of the pathogen to these active ingredients, given the success of genetic resistance and the lack of registered fungicides to control eucalyptus canker in Brazil. Since epidemics of eucalyptus canker posed a threat to the health of commercial eucalyptus plantations, genetic resistance to the disease has been adopted as the main strategy. Furthermore, the application of fungicides in the forestry sector is more the exception than the rule, as these compounds are typically applied only in special circumstances, such as outbreaks of rust (*Austropuccinia psidii*) in commercial plantations with highly susceptible genotypes (Alfenas et al. 2009; Ferreira 1989).

Three of the four molecules tested (difenoconazole, tebuconazole and cyproconazole) are registered for eucalyptus diseases, providing evidence that these products are safe for these plants. However, the positioning of fungicides in eucalyptus intended to target fungi of the genera *Austropuccinia*, *Botrytis*, *Botryosphaeria*, *Calonectria* and *Rhizoctonia*, with recommendations for aerial applications (Ministry of Agriculture, Livestock and Supply of Brazil 2024). Conversely, in this study, fungicides were applied to the plant collar and substrate in order to promote the acropetal movement of the molecules, in an attempt to reduce pathogen colonization within plant tissues. For this reason, before evaluating the *in vivo* efficacy of these fungicides, we assessed their phytotoxic potential in eucalyptus plants. The absence of visible phytotoxicity symptoms in leaves, branches and stems, even at the highest tested dose (20 g a.i./100 L of water), suggests that the application of this dose would not cause significant damage to the plants.

The need to manage stem diseases, such as canker and wilt, drives the search for molecules able of controlling these diseases. However, the management of such diseases has shown low efficacy (Mondello et al. 2018; Twizeyimana et al. 2013). One example is *Botryosphaeria dothidea*, the causal agent of pecan canker in Chinese hickory (*Carya cathayensis*), for which cultural and chemical control strategies have demonstrated limited efficacy (Dai et al. 2017). However, the present study demonstrated promising effect of triazole fungicides in controlling Chrysoporthe wilt, as the *in vivo* and *in vitro* results were convergent regarding the fungitoxicity of the analyzed molecules. Although further studies are necessary to better elucidate the factors contributing to the reduction in necrotic lesion length induced by

C. cubensis, the application of fungicides toward the plant collar and soil may have played a decisive role in this response. This proposition aligns with recommendations for

delivering fungicides to woody plants via trunk and/or soil, including trunk injections, for managing diseases caused by lignicolous fungi (Bertsch et al. 2013). Another important aspect is the likely successful redistribution of fungicide molecules from the roots and collar to the upper portions of the plant through xylem-mediated movement. Their resulting systemic activity may have partially inhibited the progression of *C. cubensis* infection in host tissues (Shahinasi et al. 2017).

The fact that difenoconazole was less effective compared to the other fungicides is in line with the variation in performance of molecules within the same chemical group, depending on their physicochemical properties, formulation characteristics, application timing, and location (Kleczewski, Butts-Willmsmeyer & Scanlan 2020; Klittich 2014). In a study conducted by Liu et al. (2023) limited translocation of difenoconazole was observed in rice plants compared to other triazoles. Although rice is taxonomically distant from eucalyptus, the molecule's behavior – remaining concentrated in the roots – appears to be similar in this study. However, more in- depth studies are needed to precisely determine the redistribution of this fungicide in eucalyptus plants.

Regarding the timing of fungicide application, our findings align with the understanding that control efficacy is closely related to the application window of the molecules. Although most triazole fungicides are positioned preventively for greater efficacy, our results demonstrate that curative application was more effective in reducing lesion length in the stem (Kleczewski, Butts-Willmsmeyer & Scanlan 2020; Pathan et al. 2020). This outcome is supported by the well-documented curative effects of triazole fungicides (Corkley, Fraaije & Hawkins 2022). Moreover, a similar response was observed for *Cryphonectria parasitica*, a fungus closely related to *Chrysoporthe*, with higher efficacy of the curative application of epoxiconazole when applied 21 days after inoculation (Trapiello, González-Varela & González, 2015).

A likely explanation for a better efficacy of curative application of triazole is the rapid redistribution along the transpiration following application, which may have slowed lesion progression when the infection process was still confined to small areas of host tissue. In contrast, during preventive application, maximum redistribution may have occurred before pathogen arrival, followed by gradual degradation of the molecules over time (Liu et al. 2023; Trapiello, González-Varela & González 2015). Therefore, the concept of fungicide application window is also useful to explain the lack of efficacy observed when fungicides were applied to ongoing infections in the present study.

We also observed that the late application of the fungicides ipconazole and

tebuconazole in ongoing infections resulted in a reduction in the number of fruiting bodies produced during fungal sporulation. While these findings suggest that the application of these compounds could potentially reduce inoculum, the practical implications of this observation for the management of Chrysoporthe wilt and eucalyptus canker require further investigation. Although our results show a reduction in the number of fruiting bodies, it remains unclear whether fungicide treatment can interfere with the germination and infectivity of fungal propagules, which can be abundantly produced in lesioned tissues and contribute to the dissemination of diseases under field conditions (Ferreira & Milani 2002).

It is important to note that in certain situations, curative applications have been discouraged, as they are often associated with the use of higher doses to contain infection, and higher doses may promote the selection and increase the frequency of resistant isolates within the pathogen population. Therefore, the potential curative application of triazole fungicides in the management of diseases caused by *C. cubensis* should not overlook these aspects. Beyond efficacy in disease control, other factors must be considered when employing fungicides in the management of diseases caused by *Chrysoporthe* in eucalyptus cultivation. These include cost-benefit analysis, the most effective application rate, applicability to different stages of rooted cuttings production and forest plantation, the risk posed by these compounds to non- target organisms and the environment, among others. Additionally, the compatibility of these molecules with industry certification requirements cannot be ignored. Based on the four molecules analyzed in this study and the *List of Highly Hazardous Pesticides* approved in 2024, cyproconazole is classified as highly restricted for FSC certification. No restrictions are conferred on the other molecules in this document (Forest Stewardship Council 2024; Mikaberidze et al. 2017).

In summary, our findings provide the most up-to-date status on the sensitivity of *C. cubensis* to triazole fungicides in Brazil and contribute to the knowledge of the potential of chemical control as a strategy for managing diseases caused by this species in eucalyptus. Furthermore, the results suggest that triazole fungicides, which are not registered for this biological target in Brazil, represent a chemical group with potentially effective active ingredients.

However, considering that the results of this study on the efficacy of DMI fungicides are limited to greenhouse conditions, future research is necessary to validate the efficacy of triazole fungicides in mini-garden. Therefore, this is only the first step toward integrating chemical control-based strategies into an integrated management program for eucalyptus diseases caused by *C. cubensis*.

References

Alfenas, A. C., Zauza, E. A. V., Mafia, R. G., & Assis, T. F. (2009). Clonagem e doenças do eucalipto. Viçosa: Editora UFV.

Ambrose, A., Peter Remun, A. B., Shamsul Kamar, N. A., Mohamad Pazi, A. M., Wan Azhar, W. M. A., Awing, N. H., Liam, J., & Terhem, R. (2023). Evaluation of Fungicides as Protective and Curative Treatments against Canker Disease of *Eucalyptus urograndis* Caused by *Chrysoporthe deuterocubensis* in Malaysia. Forests, 14, 2337. https://doi.org/10.3390/f14122337

Anderson, N. R., Freije, A. N., Bergstrom, G. C., Bradley, C. A., Cowger, C., Faske, T., Hollier, C., Kleczewski, N., Padgett, G. B., Paul, P., Price, T., & Wise, K. A. (2020). Sensitivity of *Fusarium graminearum* to metconazole and tebuconazole fungicides before and after widespread use in wheat in the United States. Plant Health Progress, 21, 85-90. https://doi.org/10.1094/PHP-11-19-0083-RS

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., & Fontaine, F. (2013). Grapevine trunk diseases: complex and still poorly understood. Plant Pathology, 62, 243-265. https://doi.org/10.1111/j.1365-3059.2012.02674.x

Brent, K. J., & Hollomon, D. W. (1995). Fungicide Resistance in Crop Pathogens: How Can It Be Managed?. FRAC Monigr. No. 1, 2nd ed. Brussels, Belgium: Fungicide Resistance Action Committee, Fungicide Resistance Action Committee.

Brazilian Ministry of Agriculture, Livestock and Food Supply. (2024). Phytosanitary agrochemicals system – Ministry of Agriculture, Livestock and Food Supply, Brazil, 2024. https://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons

Corkley, I., Fraaije, B., & Hawkins, N. (2022). Fungicide resistance management: Maximizing the effective life of plant protection products. Plant Pathology, 71, 150-169. https://doi.org/10.1111/ppa.13467

Dai, D. J., Wang, H. D., Wang, Y. P., & Zhang, C. Q. (2017). Management of Chinese

hickory (*Carya cathayensis*) trunk canker through effective fungicide application programs and baseline sensitivity of *Botryosphaeria dothidea* to trifloxystrobin. Australasian Plant Pathology, 46, 75-82. https://doi.org/10.1007/s13313-017-0465-4

Forest Stewardship Council - FSC publishes the updated Lists of Highly Hazardous Pesticides. (2024). Available at: [https://fsc.org/en/newscentre/standards/fsc-publishes-the-updated-lists-of-highly-hazardous-pesticides]. Accessed on January 10, 2025.

FRAC – Brazil. Comitê de Ação a Resistência a Fungicidas. Modo de Ação de Fungicidas. 2024. Available at: [https://www.frac-br.org/modo-de-acao]. Accessed on December 5, 2024.

Ferreira, F. A. (1989). Patologia florestal-principais doenças florestais no Brasil. Viçosa: SIF.
Ferreira, F. A., & Milani, D. (2002). Diagnose visual e controle das doenças abióticas e bióticas do eucalipto no Brasil. Mogi Guaçu: International Paper.

Gryzenhout, M., Myburg, H., Van der Merwe, N. A., Wingfield, B. D., & Wingfield, M. J. (2004). *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Studies in Mycology, 50, 119-142.

Guimarães, L. M. D. S., Resende, M. D. V. D., Lau, D., Rosse, L. N., Alves, A. A., & Alfenas, A. C. (2010). Genetic control of *Eucalyptus urophylla* and *E. grandis* resistance to canker caused by *Chrysoporthe cubensis*. Genetics and Molecular Biology, 33, 525-531. https://doi.org/10.1590/S1415-47572010005000069

Hodges, C. S., Reis, M. S., Ferreira, F. A., & Heffling, J. D. M. (1976). O cancro do eucalipto causado por *Diaporthe cubensis* Bruner. Fitopatologia Brasileira, 1,129-169.

Jefcoate, C. R. (1978). Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. Methods In enzymology, 52, 258-279. https://doi.org/10.1016/S0076-6879(78)52029-6

Kleczewski, N. M., Butts-Willmsmeyer, C., & Scanlan, C. (2020). Assessing the curative and protective impacts of select fungicides for control of powdery mildew of wheat. Plant Disease, 104, 1195-1200. https://doi.org/10.1094/PDIS-08-19-1754-RE

Klittich, C. J. (2014). Fungicide mobility and the influence of physical properties. Retention, uptake, and translocation of agrochemicals in plants. American Chemical Society.

Liu, J., Cheng, J., Zhou, C., Ma, L., Chen, X., Li, Y., Sun, X., Yan, X., Geng, R., Wan, Q., & Yu, X. (2023). Uptake kinetics and subcellular distribution of three classes of typical pesticides in rice plants. Science of The Total Environment, 858, 159826. https://doi.org/10.1016/j.scitotenv.2022.159826

Machado, F. J., Silva, C. N., Paiva, G. F., Feksa, H. R., Tessmann, D. J., Mizubuti, E. S., & Del Ponte, E. M. (2024). Sensitivity to tebuconazole and carbendazim in *Fusarium graminearum* species complex populations causing wheat head blight in southern Brazil. Tropical Plant Pathology, 49, 157-167. https://doi.org/10.1007/s40858-023-00616-2

Martins, M. D., Guterres, D. C., Andrade, P. R. A., Machado, F. J., & Furtado, G. Q. (2023). *Chrysoporthe cubensis* emerges causing wilt on Eucalyptus mini-stumps in Brazil. Australasian Plant Pathology, 52, 517-527. https://doi.org/10.1007/s13313-023-00940-w

Mehl, A., Schmitz, H., Stenzel, K., & Bloomberg, J. (Eds.). (2019). CHAPTER 5: DMI Fungicides (FRAC Code 3): Sensitivity Status of Key Target Pathogens, Field Versus Laboratory Resistance, and Resistance Mechanisms. In Fungicide Resistance in North America, Second Edition (pp. 51-68). The American Phytopathological Society.

Mikaberidze, A., Paveley, N., Bonhoeffer, S., & van den Bosch, F. (2017). Emergence of resistance to fungicides: the role of fungicide dose. Phytopathology, 107, 545-560. https://doi.org/10.1094/PHYTO-08-16-0297-R

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., Clément, C., Mugnai, L., & Fontaine, F. (2018). Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. Plant Disease, 102, 1189-1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Mukhtar, T., Vagelas, I., & Javaid, A. (2023). New trends in integrated plant disease management. Frontiers in Agronomy, 4, 1104122. https://doi.org/10.3389/fagro.2022.1104122

Pathan, A. K., Cuddy, W., Kimberly, M. O., Adusei-Fosu, K., Rolando, C. A., & Park, R. F. (2020). Efficacy of fungicides applied for protectant and curative activity against myrtle rust. Plant Disease, 104, 2123-2129. https://doi.org/10.1094/PDIS-10-19-2106-RE

Price, C. L., Parker, J. E., Warrilow, A. G., Kelly, D. E., & Kelly, S. L. (2015). Azole fungicides–understanding resistance mechanisms in agricultural fungal pathogens. Pest Management Science, 71, 1054-1058. https://doi.org/10.1002/ps.4029

R Core Team. (2023). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rodas, C. A., Gryzenhout, M., Myburg, H., Wingfield, B. D., & Wingfield, M. J. (2005). Discovery of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* on native *Miconia* (Melastomataceae) in Colombia. Plant Pathology, 54, 460-470. https://doi.org/10.1111/j.1365-3059.2005.01223.x

Roux, J., Myburg, H., Wingfield, B. D., & Wingfield, M. J. (2003). Biological and Phylogenetic Analyses Suggest that Two *Cryphonectria* spp. Cause Cankers of Eucalyptus in Africa. Plant Disease, 87, 1329-1332. https://doi.org/10.1094/PDIS.2003.87.11.1329

Ruge, E., Korting, H. C., & Borelli, C. (2005). Current state of three-dimensional characterisation of antifungal targets and its use for molecular modelling in drug design. International Journal of Antimicrobial Agents, 26, 427-441. https://doi.org/10.1016/j.ijantimicag.2005.09.006

Shahinasi, E., Brahushi, F., Devolli, A., & Kodra, M. (2017). The ecotoxicology of pesticides group of triazole and their use to control apple scab (*Venturia inaequalis*). Journal of Hygienic Engineering and Design, 18, 36-42.

Silva, G. A., Moreira, S. I., & Ferreira, M. A. (2023). New Melastomataceae hosts of *Chrysoporthe* species in Brazil. Journal of Phytopathology, 171, 716-722. https://doi.org/10.1111/jph.13232

Suzuki, H., Marincowitz, S., Rodas, C. A., Wingfield, B. D., & Wingfield, M. J. (2023). First report of two *Chrysoporthe* species, *Chrysoporthe doradensis* and *Chrysoporthe colombiana* sp. nov. from *Henriettea seemannii* pathogenic to Eucalyptus in Colombia. Mycological Progress, 22, 44. https://doi.org/10.1007/s11557-023-01891-8

Trapiello, E., González-Varela, G., & González, A. J. (2015). Chestnut blight control by agrochemicals in *Castanea sativa* under managed conditions. Journal of Plant Diseases and Protection, 122, 120-124. https://doi.org/10.1007/BF03356540

Trivi, L. H. M., Jasso Mata, J., López Upton, J., Alvarado Rosales, D., & Fierros González, A. M. (2013). Impacto de *Chrysoporthe cubensis* (Bruner) Gryzenhout & M J. Wingfield en dos áreas semilleras de *Eucalyptus urophylla* ST Blake en Santiago Yaveo, Oaxaca. Revista Mexicana de Ciencias Forestales, 4, 64-75.

Twizeyimana, M., McDonald, V., Mayorquin, J. S., Wang, D. H., Na, F., Akgül, D. S., & Eskalen, A. (2013). Effect of fungicide application on the management of avocado branch canker (formerly Dothiorella canker) in California. Plant Disease, 97, 897-902. https://doi.org/10.1094/PDIS-06-12-0518-RE

Yan, X., Ma, W. B., Li, Y., Wang, H., Que, Y. W., Ma, Z. H., Talbot, N. J., & Wang, Z. Y. (2011). A sterol 14α-demethylase is required for conidiation, virulence and for mediating sensitivity to sterol demethylation inhibitors by the rice blast fungus *Magnaporthe oryzae*. Fungal Genetics and Biology, 48, 144-153. https://doi.org/10.1016/j.fgb.2010.09.005

Yang, J., Zhang, Q., Liao, M., Xiao, M., Xiao, W., Yang, S., & Wan, J. (2009). Expression and homology modelling of sterol 14α-demethylase of *Magnaporthe grisea* and its interaction with azoles. Pest Management Science, 65, 260-265. https://doi.org/10.1002/ps.1680

Yin, Y., Miao, J., Shao, W., Liu, X., Zhao, Y., & Ma, Z. (2023). Fungicide resistance: Progress in understanding mechanism, monitoring, and management. Phytopathology®, 113, 707-718. https://doi.org/10.1094/PHYTO-10-22-0370-KD

Active ingredients	Trade names	Manufactures	Type of formulation	Solvents	Fungicides concentrations (µg/mL)
Ipconazole	Rancona® 450 FS	UPL OpenAg TM	Concentrated Suspension	Distilled water	0.001, 0.010, 0.100, 1.00, and 10.00
Difenoconazole	Score®	Syngenta Crop Protection	Emulsifiable concentrate	Distilled water	0.001, 0.010, 0.100, 1.00, and 10.00
Cyproconazole	ALTO®100	Syngenta Crop Protection	Soluble concentrate	Distilled water	0.001, 0.010, 0.100, 1.00, and 10.00
Tebuconazole	technical, 94%	-	-	Acetone	0.001, 0.010, 0.100, 1.00, and 10.00

Table 1. Information on the active ingredients, trade names, manufactures, type of formulation, solvents used and concentrations of the fungicides.

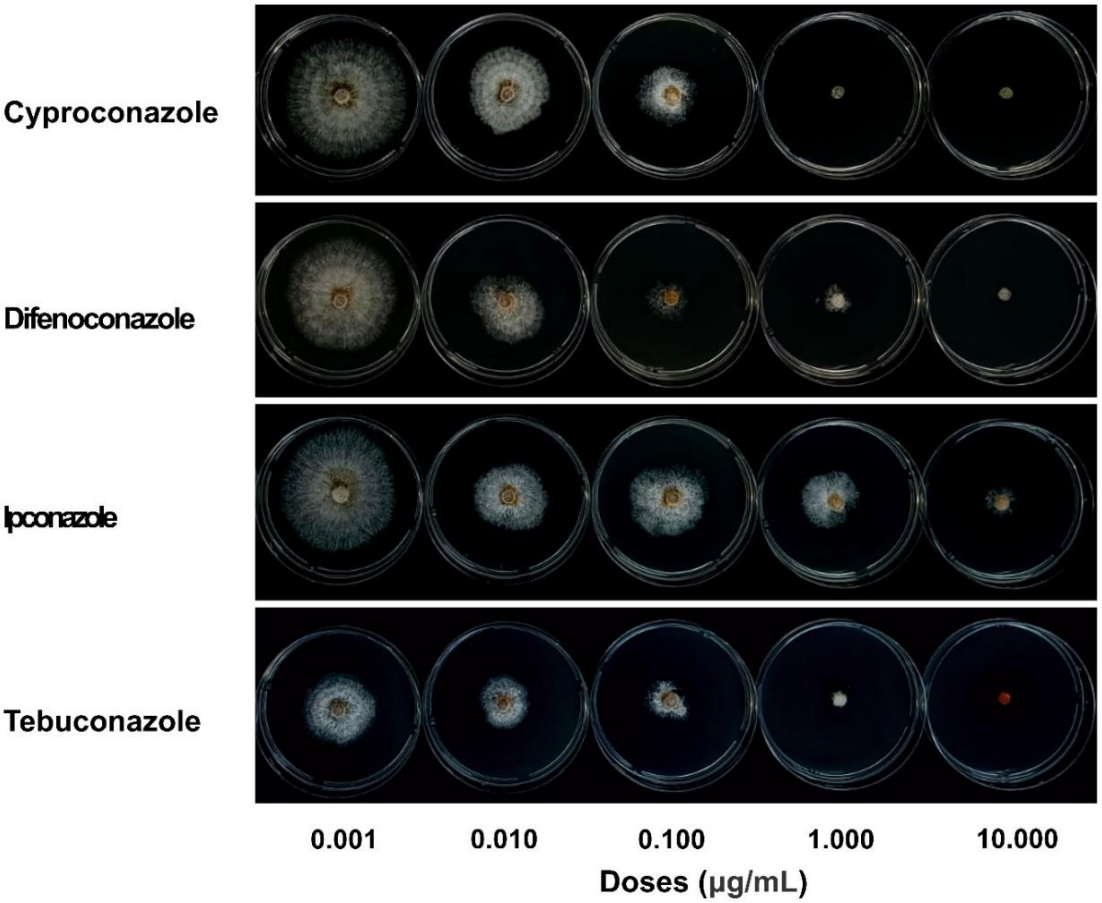


Figure 1. Mycelial growth inhibition of *Chrysoporthe cubensis* in PDA amended with the fungicides cyproconazole, difenoconazole, ipconazole, and tebuconazole at concentrations of 0.001, 0.010, 0.100, 1.000, and 10.000 µg/mL.

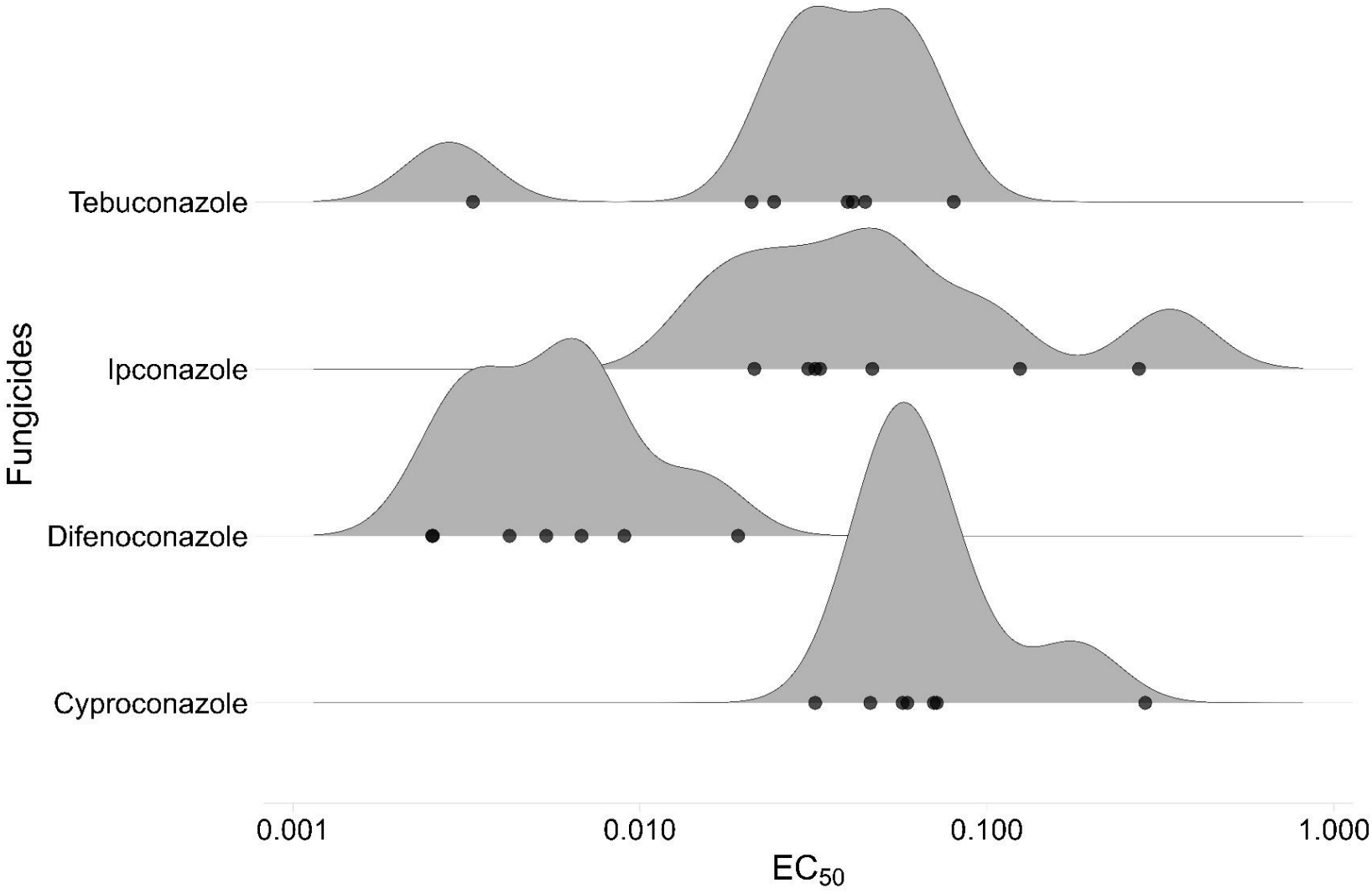


Figure 2. Density plots of the distribution of the effective concentration (µg/mL) of triazole fungicides (tebuconazole, ipconazole, difenoconazole and cyproconazole) that inhibit mycelial growth by 50% (EC50) for seven isolates of *Chrysoporthe cubensis*.

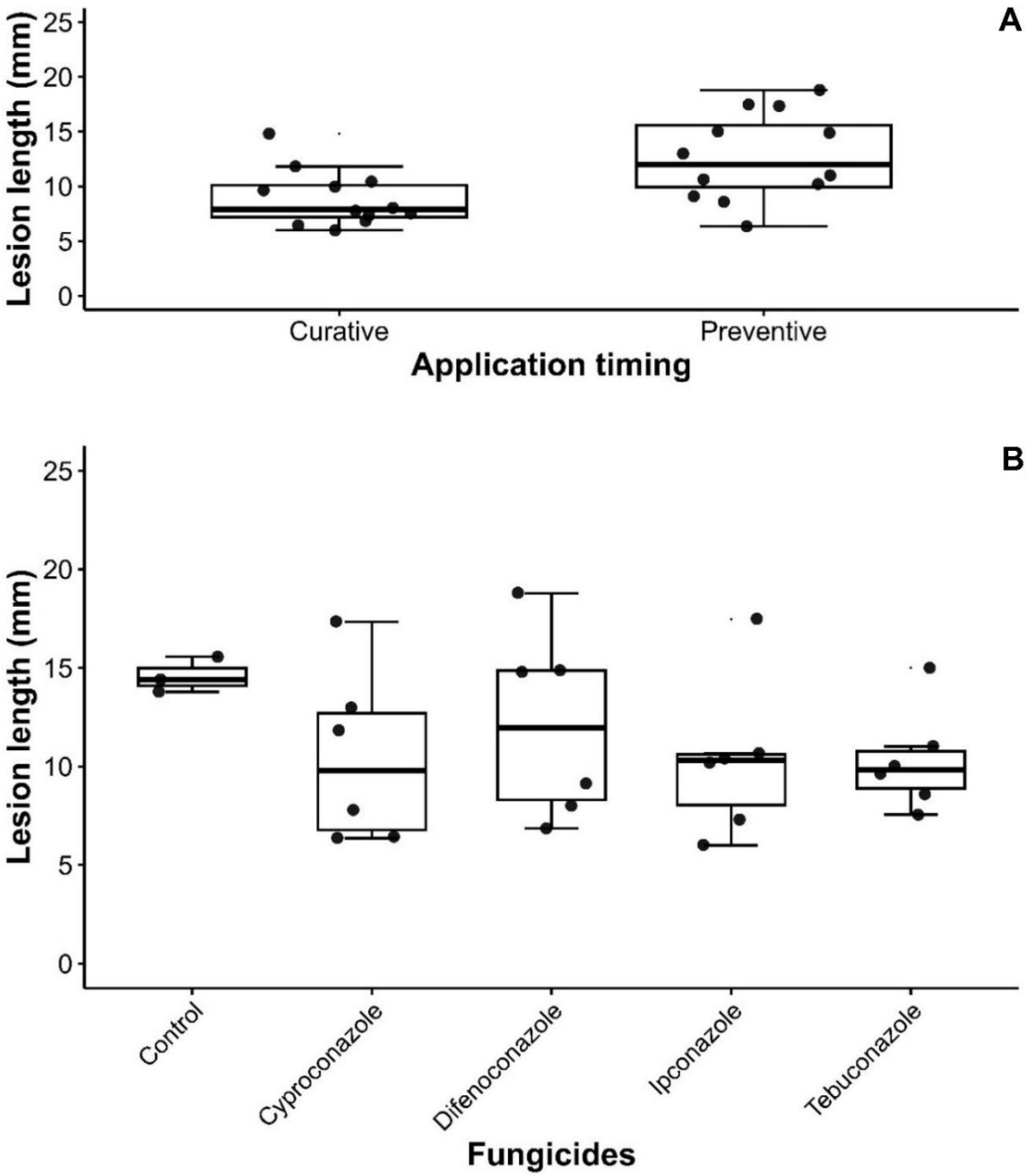


Figure 3. Boxplots representing the variation in lesion length induced by *Chrysoporthe cubensis*, thirty days after inoculation, in eucalyptus plants treated curatively and preventively (A) with triazole fungicides (B). The horizontal line represents the median, and the lower and upper boxes surrounding the median correspond to the first and third quartiles. The dots represent the values obtained in each replicate. Dots that exceed the lower and upper whiskers are outliers.

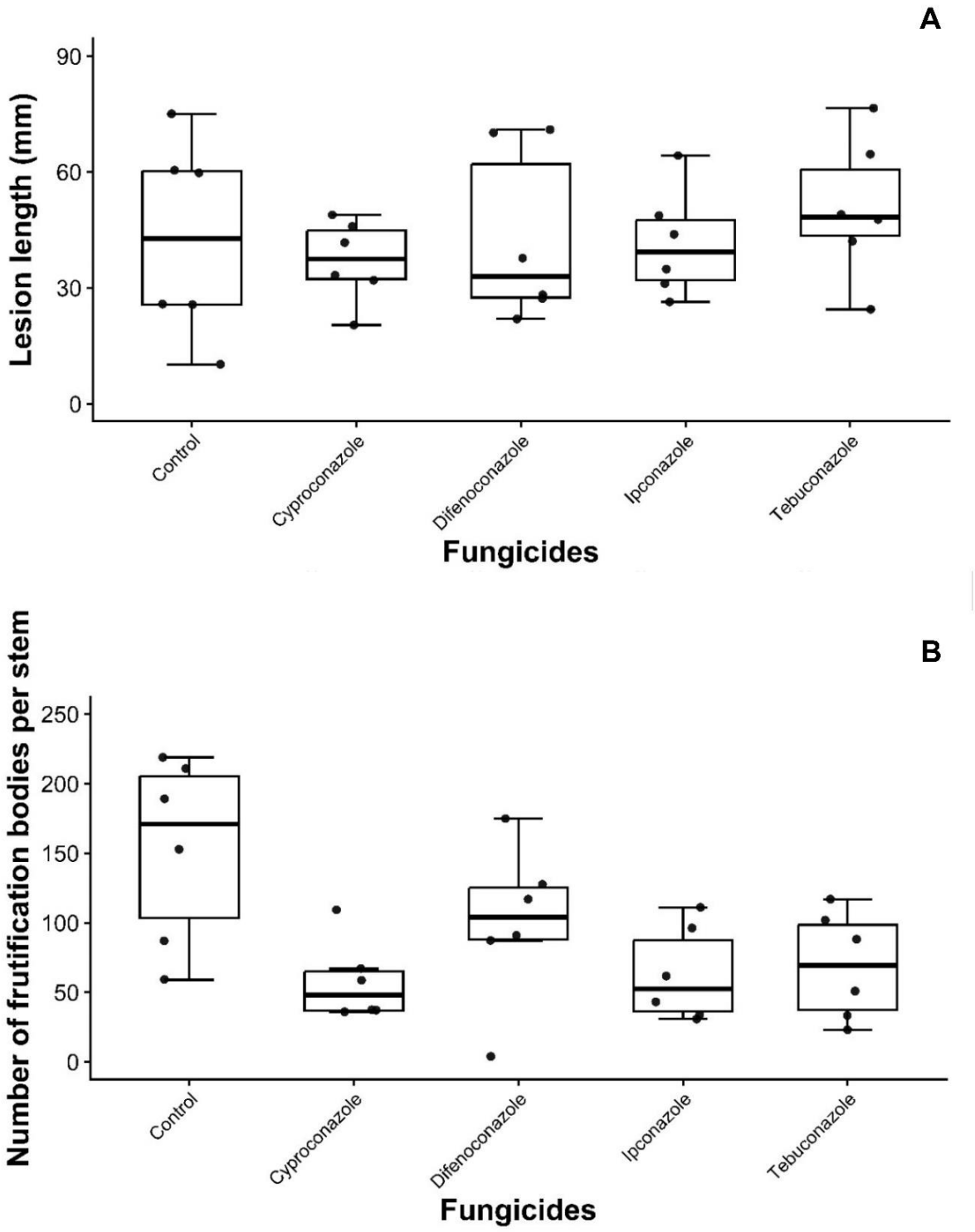


Figure 4. Boxplots representing the variation in lesion length and number of fruiting bodies per stem in eucalyptus plants inoculated with *Chrysoporthe cubensis* and treated with triazole fungicides 15 days after inoculation. The horizontal line represents the median, and the lower and upper boxes surrounding the median correspond to the first and third quartiles. The dots represent the values obtained in each replicate. Dots that exceed the lower and upper whiskers are outliers.

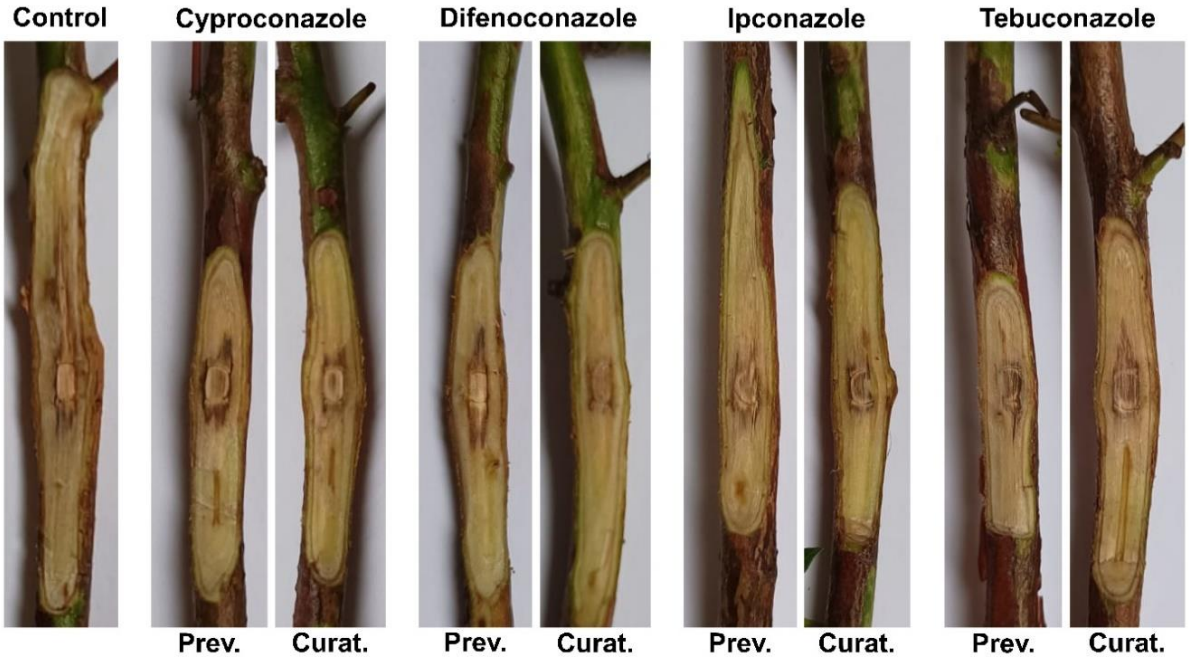
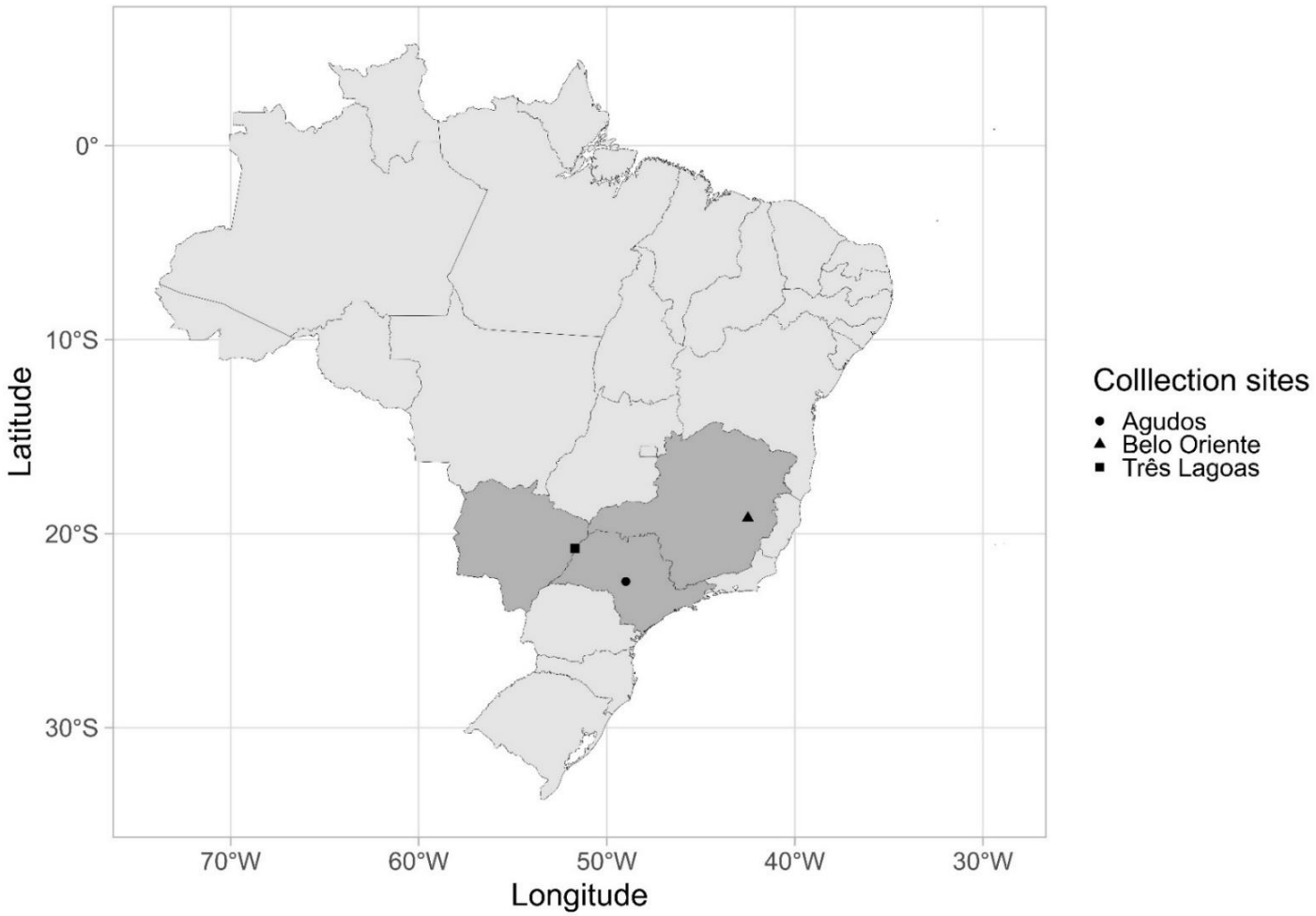
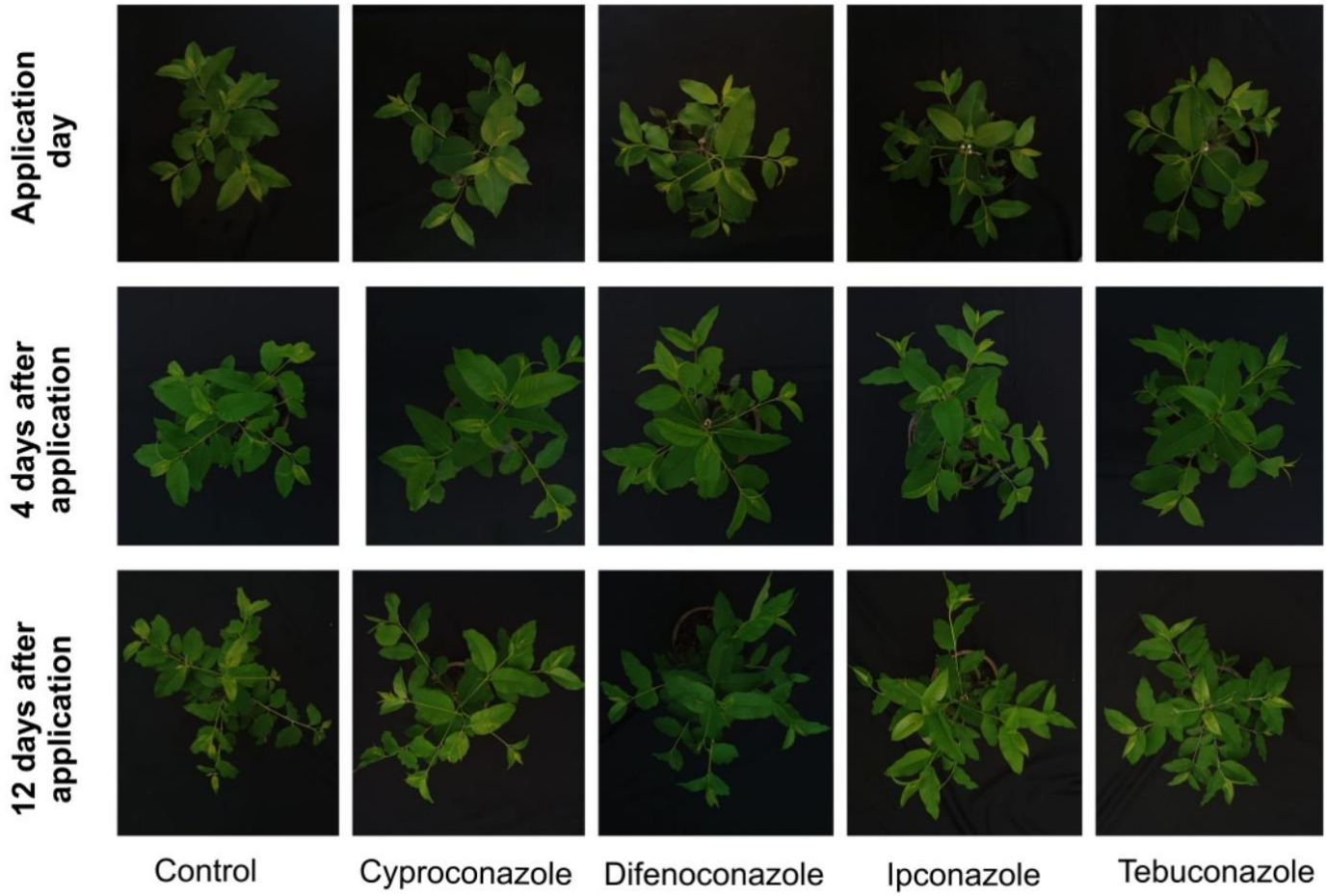


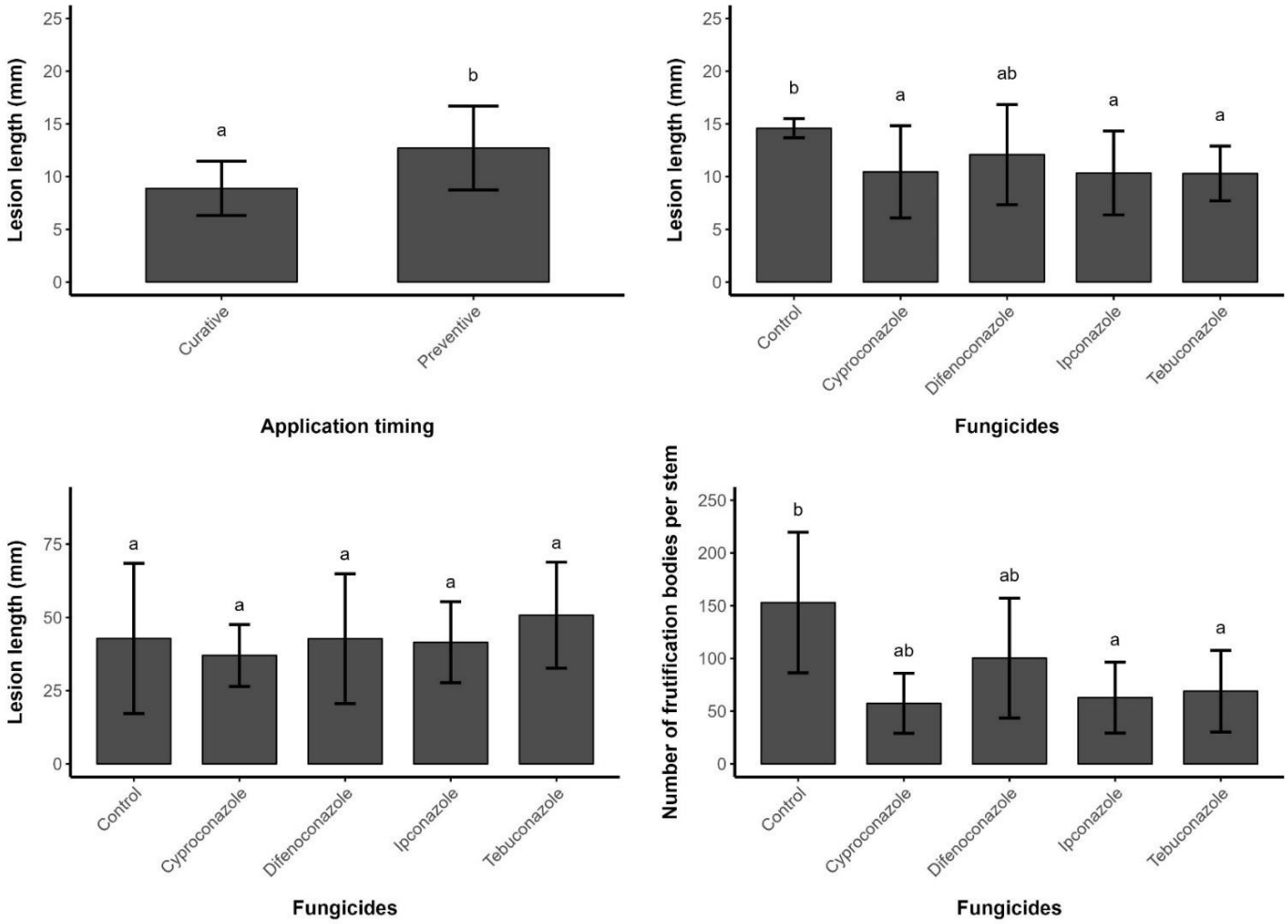
Figure 5. Necrotic lesions caused by *Chrysoporthe cubensis* inside the stem of eucalyptus plants treated preventively (Prev.) and curatively (Curat.) with triazole fungicides.



Supplementary Figure 1. Map of Brazil showing collection sites of *Chrysoporthe cubensis* isolates in the states of Minas Gerais (Belo Oriente), Mato Grosso do Sul (Três Lagoas) and São Paulo (Agudos). The isolates COAD 3400, COAD 3401 and COAD 3423 were obtained from mini-stumps from Belo Oriente; the isolates COAD 3478, COAD 3480 and COAD 3483 are from Três Lagoas and COAD 3481 is from Agudos. Municipality are indicated by different formats.



Supplementary Figure 2. Absence of visible symptoms of phytotoxicity in the aerial parts of eucalyptus plants at four and twelve days after fungicide application.



Supplementary Figure 3. Average lesion length in eucalyptus plants treated with triazole fungicides: effect of application timing (A) and triazole fungicide type (B). Average lesion length (C) and average number of fruiting bodies per stem (D) in eucalyptus plants treated with fungicides 15 days after pathogen inoculation.

CHAPTER 3

Colorimetric LAMP assay for detection of *Chrysoporthe* spp.

P. R. A. Andrade[1], E. A. Lopes[2], G. Q. Furtado[1]*

[1]Department of Plant Pathology, Universidade Federal de Viçosa, 36570-900, Viçosa, Minas Gerais, Brazil.

[2]Institute of Agrarian Sciences, Universidade Federal de Viçosa, 38810-000, Rio Paranaíba, Minas Gerais, Brazil.

*Corresponding author: gfurtado@ufv.br

Abstract

Loop-Mediated Isothermal Amplification (LAMP) is a rapid, simple, highly sensitive, and specific technique with great potential for field diagnostics. No LAMP assay has been previously described for the detection of *Chrysoporthe* species. Consequently, this study aimed to design primers and develop a colorimetric LAMP assay for detecting *Chrysoporthe* spp. Four LAMP primers (FIP, BIP, F3, and B3) were designed based on a partial region of the translation elongation factor 1-α (TEF1α) gene, and the technique was combined with color indicators for result interpretation. The optimal conditions for colorimetric LAMP assays were a 2:1 ratio of FIP/BIP to F3/B3 primers, a temperature of 65/68°C, and an amplification time of 75 to 90 minutes. Colorimetric LAMP assays using the developed primers specifically detected nucleic acid (DNA) samples from *Chrysoporthe cubensis* and *Chrysoporthe doradensis*.

To date, eleven species within this genus *Chrysoporthe* (Cryphonectriaceae) have been described infecting trees and mini-stumps (eucalyptus) of the order Myrtales in tropical and subtropical regions, namely: *C. austroafricana*, *C. brasiliensis*, *C. colombiana*, *C. cubensis*, *C. deuterocubensis*, *C. doradensis*, *C. hodgesiana*, *C. inopina*, *C. puriensis*, *C. syzygiicola*, and *C. zambiensis* (Chungu et al. 2010; Gryzenhout et al. 2004; Gryzenhout et al. 2005; Gryzenhout et al. 2006; Martins et al. 2023; Oliveira et al. 2021; Silva et al. 2024; Suzuki et al. 2023; Van der Merwe et al. 2010).

Following a prevailing trend in fungal taxonomy, nucleic acid sequence-based methods have

become essential for the rapid and reliable delimitation of *Chrysoporthe* species. Currently, in addition to morphological characteristics, the description of *Chrysoporthe* spp. is primarily based on phylogenetic reconstruction using DNA sequence data, particularly from the internal transcribed spacer (ITS) and the gene encoding β-tubulin (Baldi & La Porta 2020; Hariharan & Prasannath 2021; Luchi, Ioos & Santini 2020; Suzuki et al. 2023;).

One of the most widely used methods for diagnosing plant pathogens is based on Polymerase Chain Reaction (PCR), a highly reliable and versatile technique that can be adapted to various needs. However, despite its advantages, this technique has certain limitations, such as the requirement for specialized, high-cost equipment and highly skilled personnel (Ferrara et al. 2020; Gomez- Gutierrez & Goodwin 2022).

A diagnostic technique that has been applied across various fields of biology and shows potential for detecting *C. cubensis* is Loop-Mediated Isothermal Amplification (LAMP). The simplicity of the technique, its minimal resource requirements, and its potential for field application using a water bath or a heat block under isothermal conditions have made it an increasingly studied method (Feng et al. 2018; Ristaino et al. 2020).

Initially developed by Notomi et al. (2000), this technique is based on PCR but employs a *Bacillus stearothermophilus* (*Bst*) polymerase enzyme. This enzyme, which possesses strand displacement activity, can amplify the template nucleic acid without requiring the denaturation step commonly needed for polymerases. The practical implication of this characteristic is that *Bst* enables amplification under isothermal conditions, typically at an optimal temperature between 60 and 65°C. Additionally, the technique requires a set of four to six primers, resulting in high specificity for the target DNA/RNA. The required primers include two external primers (F3 – forward external primer, and B3 – reverse external primer), two internal primers (FIP – forward internal primer, and BIP – reverse internal primer), and two loop primers (Loop forward and Loop backward) (Donoso & Valenzuela 2018; Wong et al. 2018; Becherer et al. 2020).

The LAMP technique offers several advantages, including high efficiency and specificity, as well as ease and speed of execution compared to conventional PCR. Additionally, LAMP reactions can be performed under isothermal conditions (Dai et al., 2016; Feng et al. 2019), providing a reliable and rapid opportunity for field monitoring and timely decision-making regarding disease management (King et al. 2018; Kogovšek et al. 2015; Thiessen et al. 2016). Another positive aspect of this technique is the use of colorimetric methods that simplify result interpretation (Okiro et al. 2019).

The LAMP technique appears to be a promising approach for detecting *Chrysoporthe*

species for reasons described by Xu et al. (2021) in their study on the potential application of this method for detecting *Valsa mali*. First, the presence of these species in endophytic niches underscores the relevance of simple, sensitive, and reliable diagnostic techniques able of detecting the pathogen before infection symptoms become visible. Another important factor concerns the characteristics of the host plants, as DNA extracted from woody plant tissues may contain PCR inhibitors, making field detection using conventional PCR more challenging.

However, despite the potential of the LAMP technique, no efforts had been made to develop LAMP primers for the detection of *Chrysoporthe* species. Therefore, this study aimed to design LAMP primers and develop a colorimetric LAMP assay for detecting *Chrysoporthe* spp.

Seventy-nine DNA sequences from the partial coding region of translation elongation factor 1- α (TEF1α), representing species within the family Cryphonectriaceae, were obtained from the National Center for Biotechnology Information (NCBI) and aligned using the Muscle algorithm implemented in MEGA software (version 12.0.7). The sequence matrix included eight sequences of *Chrysoporthe* spp., representing six species: *C. austroafricana*, *C. cubensis*, *C. inopina*, *C. hodgesiana*, *C. zambiensis*, and *C. syzygiicola*. The alignment comprised 1,180 nucleotides, of which 162 were conserved, 664 were variable, 437 were parsimony-informative, and 187 were singletons. Between positions 151 and 801, polymorphisms shared among *Chrysoporthe* spp. were identified. This region was selected for primer design.

LAMP primers were designed using PrimerExplorerV5 (http://primerexplorer.jp/e/index.html), with default settings. Primer specificity was confirmed by comparing primer sequences against the NCBI database using the BLAST tool. The designed LAMP primers were synthesized by Invitrogen. Primers were resuspended in Tris-EDTA buffer (10 nM Tris-HCl, pH 8.0, 1 mM EDTA) as per the manufacturer's instructions after synthesis.

LAMP reactions were performed using the WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs®). Each 12.5 µl reaction contained 6.25 µl of Master Mix, 1.25 µl of the 10× concentrated primer mix, 1 µl of target DNA (10 ng/µl), and 4.0 µl of DNase/RNase-free water. For negative controls, DNase/RNase-free water was used in place of the target DNA in the same volume.

The optimal conditions for primer concentration, temperature, and reaction time were adjusted. First, the optimal primer ratio and temperature conditions were determined. In this stage, LAMP reactions were set up as previously described, evaluating three primer mixtures: 2:1, 4:1, and 8:1 ratios of FIP/BIP to F3/B3. To prepare the 2:1 mixture, 2 µM of FIP and 2 µM of BIP were used for every 1 µM of F3 and 1 µM of B3. Similarly, the 4:1 mixture consisted of

4 μM of FIP and 4 μM of BIP for every 1 μM of F3 and 1 μM of B3, while the 8:1 mixture consisted of 8 μM of FIP and 8 μM of BIP for every 1 μM of F3 and 1 μM of B3.

The reaction temperatures tested were 62°C, 65°C, and 68°C. In this step, the reaction time was standardized at 75 minutes, followed by a 5-minute increase to 85°C to inactivate Bst DNA polymerase and terminate the reaction. To optimize reaction time, LAMP reactions were conducted for 30, 45, 60, 75, and 90 minutes, while primer ratio and temperature were fixed at the optimal conditions determined in the previous step. Moreover, LAMP reactions were performed using *C. doradensis* template DNA, with 1 μl of DNA diluted to a concentration of 10 ng/μl.

The specificity of the designed primers was assessed by conducting LAMP reactions using DNA from *C. cubensis* and non-target fungi (*Botrytis* sp., *Fusarium* sp. and *Trichoderma* sp.). DNA from an isolate was used to represent each non-target fungal genus. LAMP reactions using nuclease-free water replacing fungal DNA was used as a negative control. Amplification reactions were performed under the previously optimized conditions, with modifications. The 12.5 μl reactions contained 0.75 μl of dimethyl sulfoxide and 3.25 μl of DNase/RNase-free water and the volume of the other reagents was maintained.

The LAMP primers set designed to amplify a specific region of the Tef-1α gene consisted of primers F3, B3, FIP, BIP, F2, F1c, B2, and B1c (Table 1). The binding sites of primers F3, B3, FIP and BIP, as well as the orientation of DNA synthesis, are shown in Figure 1.

The results indicated that the optimal conditions for LAMP reactions, determined using purified *C. cubensis* DNA, were a 2:1 ratio of FIP/BIP to F3/B3 primers and reaction temperatures of 65°C and 68°C, based on the observed color change from pink to yellow (Figure 2A). Regarding the optimal reaction time for isothermal amplification, a slight color change was observed after 45 minutes, while an intense yellow color was observed at 75 and 90 minutes (Figure 2B). Under the reaction condition for analyzing the amplification of Chrysoporthe species (primer ratio of 2:1, 65 °C for 75 minutes), chosen from the results of the condition optimization test, the LAMP assay successfully amplified the DNA of both *C. cubensis* and *C. doradensis* (Figure 2C).

For samples referring to control and DNA from non-target fungi (*Botrytis* sp., *Fusarium* sp. and *Trichoderma* sp.) negative reactions were observed (indicated by pink color). Only the sample referring to DNA from *C. cubensis* showed positive reactions (indicated by the yellow color) (Figure 2D).

The use of the LAMP technique for molecular diagnosis of plant pathogens has been

increasing (Sagcan & Kara 2019). LAMP-based assays have been developed for the detection of phytopathogens from various groups, including fungi (Liu et al. 2025; Megariti et al. 2024), bacteria (DeLude et al. 2022), phytoplasmas (Egito et al. 2024), viruses (Rafiq et al. 2021; Yilmaz, Adkins & Batuman 2023), nematodes (Babilônia et al. 2024; Suwanngam et al. 2025), and oomycetes (Ristaino et al. 2020). Efforts have led to the development of LAMP assays for the detection of important forest pathogens such as *Cronartium ribicola*, *Bretziella fagacearum*, *Fusarium circinatum*, *Heterobasidion irregulare*, *Elsinoë necatrix* (Kozhar et al. 2023; Novi et al. 2024; Meinecke et al. 2023; Sillo et al. 2018), among others. To the best of our knowledge, this is the first study focused on the development and validation of LAMP-based assays for the detection of *Chrysoporthe* spp., thus providing the first evidence of the potential application of this technique for the molecular diagnosis of species within this genus.

The successful application of LAMP for pathogen detection is closely related to the proper selection of the target gene region, where primer annealing occurs (Wang et al. 2021). In the present study, in addition to the Tef-1α gene, three other genes encoding β-tubulin I, the Second Largest Subunit of RNA polymerase II (RPB2), and the Nuclear Large Subunit Ribosomal RNA (LSU) were analyzed for LAMP primer design. A total of 11 primer sets were generated (data not shown), and the validated set presented the highest specificity for detecting *Chrysoporthe*, as determined by preliminary BLAST analysis. Regarding specificity, BLAST results demonstrated that the selected LAMP primer set specifically recognizes *Chrysoporthe* spp. (data not shown). Consequently, the detection of multiple species was expected and was confirmed by the positive LAMP reactions using DNA from *C. cubensis* and *C. doradensis*.

Furthermore, conventional LAMP requires four primers (F3, B3, FIP, and BIP), although two additional Loop primers can be designed to enhance the reaction (Nagamine, Hase & Notomi 2002).

In this study, four primers were designed for the amplification of *Chrysoporthe* spp. DNA via LAMP, which were sufficient to amplify the target DNA. However, the optimal amplification time observed exceeded 60 minutes, which is considered adequate in studies by Okiro et al. (2019) and Xu et al. (2021). Here, the complete color change from pink to yellow occurred between 75 and 90 minutes, which may be related to the absence of Loop primers, known to increase efficiency and accelerate the reaction (Duan et al. 2014; Gomez-Gutierrez & Goodwin 2022). The relationship between the presence of Loop primers and a reduced detection time in LAMP assays has been demonstrated by Alhamid, Tombuloglu, and Al-Suhaimi (2023).

The optimization of reaction time can influence the applicability of LAMP assays, as some of the main advantages of colorimetric LAMP are its ability to diagnose the target

pathogen in a single step and the visual interpretation of amplification results through color changes in the amplicons. These features contribute to the application of LAMP in field diagnostics (Buddhachat et al. 2021; Kozhar et al. 2023; Suwanngam et al. 2025). However, conducting LAMP assays under optimal conditions is crucial, as we observed that reactions incubated for 45 and 60 minutes exhibited slight color transitions. Similar observations were reported by Carvalho et al. (2024) when performing LAMP for the amplification of *Xanthomonas phaseoli* pv. *manihotis* with a reaction time of 45 minutes. From a practical perspective, such conditions may lead to uncertainty in visual interpretation during inspection, potentially requiring additional techniques such as electrophoresis for greater accuracy in result interpretation (Duan et al. 2014; Sridapan et al. 2024), which could compromise the portability of the technique.

In addition to reaction time, other adjustments to optimal amplification conditions are necessary. In this study, the optimal temperature observed was 65/68°C, which falls within the range considered efficient for LAMP (60°C–70°C) (Moehling et al. 2020). The optimal ratio of inner to outer primers was determined to be 2:1, a result similar to that observed by Zhang et al. (2018) when applying this technique for the detection of Milk Vetch Dwarf Virus (MDV). LAMP is commonly associated with the specific, rapid, and sensitive detection of target pathogens (Choudhary et al. 2022; Ocenar et al. 2019). However, a potential limitation of LAMP-designed primers is nonspecific amplification, which can lead to false-positive and/or false-negative results, thereby reducing the reliability of the technique (Grabicoski et al. 2020; Ristaino et al. 2020).

In this study, false-positive results were observed in negative control reactions conducted without pathogen template DNA (data not shown). To minimize false positives, we added dimethyl sulfoxide (6% of the total reaction volume) and reduced the amplification time from 90 to 75 minutes. Consequently, no amplification was observed in the negative control or in non-target fungi (*Botrytis* sp., *Fusarium* sp., and *Trichoderma* sp.), providing evidence of the need for further optimizations to enhance specificity in *Chrysoporthe* detection. These observations align with the understanding that prolonged incubation times increase false-positive detection, and that dimethyl sulfoxide is an organic additive able of reducing false-positive results (Kim et al. 2023; Liu et al. 2025).

In this study, the specificity of the designed LAMP primers was confirmed using nucleic acids from isolates belonging to the genera *Botrytis* sp., *Fusarium* sp., and *Trichoderma* sp. The successful application of these LAMP primers, however, requires an additional validation step: confirming the absence of nonspecific amplification of nucleic acids from fungi

that share common ancestors with *Chrysoporthe* spp., as well as from pathogenic or beneficial fungi and bacteria commonly present in eucalyptus plant tissues.

New tests are being conducted to evaluate the sensitivity of the developed primers. An important step is validating the applicability of the technique for field detection of *Chrysoporthe* spp., ensuring its portability. Detection assays for phytopathogens using this technique have proven effective for application in orchards, nurseries, and field conditions, given their ability to detect the target pathogen from biological samples such as soil and symptomatic and asymptomatic plant tissues (Egito et al. 2024; Grabicoski et al. 2020; Megariti et al. 2024).

One possible application of the LAMP technique for the detection of *Chrysoporthe* spp. in clonal mini-gardens is the amplification directly from macerated tissues of cuttings and seedlings, whether or not they exhibit symptoms of Chrysoporthe wilt. However, since diagnostic analyses based on tissue macerates may involve destructive sampling, an alternative approach would be to develop a representative sampling scheme for individuals within the plant population.

Therefore, studies validating the use of colorimetric LAMP for *Chrysoporthe* detection in symptomatic and asymptomatic mini-cuttings are highly relevant, as early detection—before symptom manifestation—can support decision-making and contribute to timely interventions for the management of Chrysoporthe wilt.

References

Alhamid, G., Tombuloglu, H., & Al-Suhaimi, E. (2023). Development of loop-mediated isothermal amplification (LAMP) assays using five primers reduces the false-positive rate in COVID-19 diagnosis. Scientific Reports, 13, 5066. https://doi.org/10.1038/s41598-023-31760-z

Babilônia, G. B., Lopes, E. A., Londe, M. L. A., Ribeiro, L. B., Oliveira, C. M. G., & Buonicontro, D. S. (2024). Unveiling the potential of loop-mediated isothermal amplification (LAMP) for plant-parasitic nematode identification: a review. Journal of Plant Diseases and Protection, 131, 665-679. https://doi.org/10.1007/s41348-024-00909-x

Baldi, P., & La Porta, N. (2020). Moleculares approaches for low-cost point-of-care pathogen detection in agriculture and forestry. Frontiers in Plant Science, 11, 570862. https://doi.org/10.3389/fpls.2020.570862

Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., & von Stetten, F. (2020). Loop-mediated isothermal amplification (LAMP)–review and classification of methods for sequence-specific detection. Analytical Methods, 12, 717-746. https://doi.org/10.1039/C9AY02246E

Buddhachat, K., Ritbamrung, O., Sripairoj, N., Inthima, P., Ratanasut, K., Boonsrangsom, T., & Sujipuli, K. (2021). One-step colorimetric LAMP (cLAMP) assay for visual detection of *Xanthomonas oryzae* pv. *oryzae* in rice. Crop Protection, 150, 105809. https://doi.org/10.1016/j.cropro.2021.105809

Carvalho, I. C. B., Silva Carvalho, A. M., Wendland, A., & Rossato, M. (2024). Colorimetric LAMP assay for detection of *Xanthomonas phaseoli* pv. *manihotis* in cassava through genomics: A new approach to an old problem. Plant Disease, 108, 2993-3000. https://doi.org/10.1094/PDIS-08-23-1507-RE

Choudhary, P., Goswami, S. K., Chakdar, H., Verma, S., Thapa, S., Srivastava, A. K., & Saxena, A. K. (2022). Colorimetric loop-mediated isothermal amplification assay for detection and ecological monitoring of *Sarocladium oryzae*, an important seed-borne pathogen of rice. Frontiers in Plant Science, 13, 936766. https://doi.org/10.3389/fpls.2022.936766

Chungu, D., Gryzenhout, M., Muimba-Kankolongo, A., Wingfield, M. J., & Roux, J. (2010). Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia. Mycological Progress, 9, 379-393. https://doi.org/10.1007/s11557-009-0646-9

Dai, T. T., Shen, H., & Zheng, X. B. (2016). Establishment and evaluation of a TEF1-α based loop-mediated isothermal amplification assay for detection of *Phomopsis longicolla*. Australasian Plant Pathology, 45, 335-337. https://doi.org/10.1007/s13313-016-0415-6

DeLude, A., Wells, R., Boomla, S., Chuang, S. C., Urena, F., Shipman, A., Rubas, N., Kuehu, D. L., Bickerton, B., Peterson, T., Dobhal, S., Arizala, D., Klair, D., Ochoa-Corona, F., Ali, M. E., Odani, J., Bingham, J. P., Jenkins, D. M., Fletcher, J., Stack, J. P., Alvarez, A. M., & Arif, M. (2022). Loop-mediated isothermal amplification (LAMP) assay for specific and rapid

detection of *Dickeya fangzhongdai* targeting a unique genomic region. Scientific Reports, 12, 19193. https://doi.org/10.1038/s41598-022-22023-4

Donoso, A., & Valenzuela, S. (2018). In-field molecular diagnosis of plant pathogens: recent trends and future perspectives. Plant Pathology, 67, 1451-1461. https://doi.org/10.1111/ppa.12859

Duan, Y., Ge, C., Zhang, X., Wang, J., & Zhou, M. (2014). A rapid detection method for the plant pathogen *Sclerotinia sclerotiorum* based on loop-mediated isothermal amplification (LAMP). Australasian Plant Pathology, 43, 61-66. https://doi.org/10.1007/s13313-013-0239-6

Egito, I. C. S., Rodrigues Alves, A., Carvalho, I. C. B., Costa Ferreira, L., & Rossato, M. (2024). Specific colorimetric LAMP assay for the detection of maize bushy stunt phytoplasma in corn through comparative genomics. Tropical Plant Pathology, 1-13. https://doi.org/10.1007/s40858-024-00638-4

Feng, W., Hieno, A., Kusunoki, M., Suga, H., & Kageyama, K. (2019). LAMP detection of four plant-pathogenic oomycetes and its application in lettuce fields. Plant Disease, 103, 298-307. https://doi.org/10.1094/PDIS-05-18-0858-RE

Feng, W., Nukaya, A., Satou, M., Fukuta, N., Ishiguro, Y., Suga, H., & Kageyama, K. (2018). Use of LAMP detection to identify potential contamination sources of plant-pathogenic *Pythium* species in hydroponic culture systems of tomato and eustoma. Plant Disease, 102, 1357-1364. https://doi.org/10.1094/PDIS-10-17-1679-RE

Ferrara, M., Logrieco, A. F., Moretti, A., & Susca, A. (2020). A loop-mediated isothermal amplification (LAMP) assay for rapid detection of fumonisin producing *Aspergillus* species. Food microbiology, 90, 103469. https://doi.org/10.1016/j.fm.2020.103469

Grabicoski, E. M. G., Jaccoud-Filho, D. D. S., Lee, D., Henneberg, L., & Pileggi, M. (2020). Real-time quantitative and ion-metal indicator LAMP-based assays for rapid detection of *Sclerotinia sclerotiorum*. Plant Disease, 104, 1514-1526. https://doi.org/10.1094/PDIS-07-19-1455-RE

Gryzenhout, M., Myburg, H., Van der Merwe, N. A., Wingfield, B. D., & Wingfield, M. J. (2004). *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Studies in Mycology, 50, 119-142.

Gryzenhout, M., Myburg, H., Wingfield, B. D., Montenegro, F., & Wingfield, M. J. (2005). *Chrysoporthe doradensis* sp. nov. pathogenic to *Eucalyptus* in Ecuador. Fungal Diversity, 20, 39-57.

Gryzenhout, M., Rodas, C. A., Portales, J. M., Clegg, P., Wingfield, B. D., & Wingfield, M. J. (2006). Novel hosts of the Eucalyptus canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. Mycological Research, 110, 833-845. https://doi.org/10.1016/j.mycres.2006.02.010

Gomez-Gutierrez, S. V., & Goodwin, S. B. (2022). Loop-mediated isothermal amplification for detection of plant pathogens in wheat (*Triticum aestivum*). Frontiers in Plant Science, 13, 857673. https://doi.org/10.3389/fpls.2022.857673

Hariharan, G., & Prasannath, K. (2021). Recent advances in molecular diagnostics of fungal plant pathogens: a mini review. Frontiers in Cellular and Infection Microbiology, 10, 600234. https://doi.org/10.3389/fcimb.2020.600234

Kim, S. H., Lee, S. Y., Kim, U., & Oh, S. W. (2023). Diverse methods of reducing and confirming false-positive results of loop-mediated isothermal amplification assays: A review. Analytica Chimica Acta, 341693. https://doi.org/10.1016/j.aca.2023.341693

King, K. M., Krivova, V., Canning, G. G. M., Hawkins, N. J., Kaczmarek, A. M., Perryman, S. A. M., Dyer, P. S., Fraaije, J. S., & West, J. S. (2018). Loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Pyrenopeziza brassicae* (light leaf spot of brassicas). Plant Pathology, 67, 167-174. https://doi.org/10.1111/ppa.12717

Kogovšek, P., Hodgetts, J., Hall, J., Prezelj, N., Nikolić, P., Mehle, N., Lenarčič, R., Rotter, A., Dickinson, M., Boonham, N., Dermastia, M., & Ravnikar, M. (2015). LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine. Plant Pathology, 64, 286-296. https://doi.org/10.1111/ppa.12266

Kozhar, O., Ibarra Caballero, J. R., Burns, K. S., & Stewart, J. E. (2023). Field ready: Development of a rapid LAMP-based colorimetric assay for the causal agent of white pine blister rust, *Cronartium ribicola*. Forest Pathology, 53, e12814. https://doi.org/10.1111/efp.12814

Liu, Y., Rasheed, U., Shan, B., Lu, Q., Chen, S., Meng, K., Qin, A., & Mo, G. (2025). A Rapid PCR-LAMP Assay for the Early Detection of *Lasiodiplodia theobromae* from Basal Stem Rot-Infected Passion Fruit Plants. Molecular Biotechnology, 1-13. https://doi.org/10.1007/s12033-024-01363-8

Luchi, N., Ioos, R., & Santini, A. (2020). Fast and reliable molecular methods to detect fungal pathogens in woody plants. Applied Microbiology and Biotechnology, 104, 2453-2468. https://doi.org/10.1007/s00253-020-10395-4

Martins, M. D., Guterres, D. C., Andrade, P. R. A., Machado, F. J., & Furtado, G. Q. (2023). *Chrysoporthe cubensis* emerges causing wilt on Eucalyptus mini-stumps in Brazil. Australasian Plant Pathology, 52, 517-527. https://doi.org/10.1007/s13313-023-00940-w

Megariti, M., Panagou, A., Patsis, G., Papadakis, G., Pantazis, A. K., Paplomatas, E. J., Tzima, A. K., Markakis, E. A., & Gizeli, E. (2024). Rapid real-time quantitative colorimetric LAMP methodology for field detection of *Verticillium dahliae* in crude olive-plant samples. Plant Methods, 20, 139. https://doi.org/10.1186/s13007-024-01251-x

Meinecke, C. D., Vos, L. D., Yilmaz, N., Steenkamp, E. T., Wingfield, M. J., Wingfield, B. D., & Villari, C. (2023). A LAMP assay for rapid detection of the pitch canker pathogen *Fusarium circinatum*. Plant Disease, 107, 2916-2923. https://doi.org/10.1094/PDIS-04-22-0972-SR

Moehling, T. J., Choi, G., Dugan, L. C., Salit, M., & Meagher, R. J. (2021). LAMP diagnostics at the point-of-care: Emerging trends and perspectives for the developer community. Expert Review of Molecular Diagnostics, 21, 43-61. https://doi.org/10.1080/14737159.2021.1873769

Nagamine, K., Hase, T., & Notomi, T. J. M. C. P. (2002). Accelerated reaction by loop-

mediated isothermal amplification using loop primers. Molecular and cellular probes, 16, 223-229. https://doi.org/10.1006/mcpr.2002.0415

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Research, 28, e63-e63. https://doi.org/10.1093/nar/28.12.e63

Novi, V. T., Aboubakr, H. A., Moore, M. J., Zarouri, A., Juzwik, J., & Abbas, A. (2024). A rapid LAMP assay for the diagnosis of oak wilt with the naked eye. Plant Methods, 20, 119. https://doi.org/10.1186/s13007-024-01254-8

Ocenar, J., Arizala, D., Boluk, G., Dhakal, U., Gunarathne, S., Paudel, S., Dobhal, S., & Arif, M. (2019). Development of a robust, field-deployable loop-mediated isothermal amplification (LAMP) assay for specific detection of potato pathogen *Dickeya dianthicola* targeting a unique genomic region. PLoS One, 14, e0218868. https://doi.org/10.1371/journal.pone.0218868

Okiro, L. A., Tancos, M. A., Nyanjom, S. G., Smart, C. D., & Parker, M. L. (2019). Comparative evaluation of LAMP, qPCR, conventional PCR, and ELISA to detect *Ralstonia solanacearum* in Kenyan potato fields. Plant Disease, 103, 959-965. https://doi.org/10.1094/PDIS-03-18-0489-RE

Oliveira, M. E. S., Van der Merwe, N. A., Wingfield, M. J., Wingfield, B. D., Soares, T. P. F., Kanzi, A. M., & Ferreira, M. A. (2021). *Chrysoporthe puriensis* sp. nov. from *Tibouchina* spp. in Brazil: an emerging threat to Eucalyptus. Australasian Plant Pathology, 50, 29-40. https://doi.org/10.1007/s13313-020-00745-1

Rafiq, A., Ali, W. R., Asif, M., Ahmed, N., Khan, W. S., Mansoor, S., Bajwa, S. Z., & Amin, I. (2021). Development of a LAMP assay using a portable device for the real-time detection of cotton leaf curl disease in field conditions. Biology Methods and Protocols, 6, bpab010. https://doi.org/10.1093/biomethods/bpab010

Ristaino, J. B., Saville, A. C., Paul, R., Cooper, D. C., & Wei, Q. (2020). Detection of *Phytophthora infestans* by loop-mediated isothermal amplification, real-time LAMP, and droplet digital PCR. Plant Disease, 104, 708-716. https://doi.org/10.1094/PDIS-06-19-1186-

RE

Sagcan, H., & Kara, N. T. (2019). Detection of Potato ring rot Pathogen *Clavibacter michiganensis* subsp. *sepedonicus* by Loop-mediated isothermal amplification (LAMP) assay. Scientific reports, 9, 20393. https://doi.org/10.1038/s41598-019-56680-9

Sillo, F., Giordano, L., & Gonthier, P. (2018). Fast and specific detection of the invasive forest pathogen *Heterobasidion irregulare* through a Loop-mediated isothermal AMPlification (LAMP) assay. Forest Pathology, 48, e12396. https://doi.org/10.1111/efp.12396

Silva, G. A., Oliveira, M. E. S., Rêgo, G. M. S., Wingfield, B. D., Wingfield, M. J., & Ferreira, M. A. (2024). *Chrysoporthe brasiliensis* sp. nov. pathogenic to Melastomataceae in southeast Brazil. Fungal Biology, 128, 1790-1799. https://doi.org/10.1016/j.funbio.2024.04.001

Sridapan, T., Jaturapaktrarak, C., Rujirawat, T., Jiaranaikulwanich, A., Yurayart, C., & Krajaejun, T. (2024). A colorimetric loop-mediated isothermal amplification assay (c-LAMP) for rapid detection of *Pythium insidiosum*. Heliyon, 10, e40478. https://doi.org/10.1016/j.heliyon.2024.e40478

Suwanngam, A., Schiffer, P. H., Sasnarukkit, A., Siripattanapipong, S., Jindapunnapat, K., Chinnasri, B., & Ruang-Areerate, T. (2025). Development of colorimetric and fluorescent closed tube LAMP assay using simplified extraction for diagnosis of *Meloidogyne enterolobii* in root tissues. Scientific Reports, 15, 160. https://doi.org/10.1038/s41598-024-83214-9

Suzuki, H., Marincowitz, S., Rodas, C. A., Wingfield, B. D., & Wingfield, M. J. (2023). First report of two *Chrysoporthe* species, *Chrysoporthe doradensis* and *Chrysoporthe colombiana* sp. nov. from *Henriettea seemannii* pathogenic to *Eucalyptus* in Colombia. Mycological Progress, 22, 44. https://doi.org/10.1007/s11557-023-01891-8

Thiessen, L. D., Keune, J. A., Neill, T. M., Turechek, W. W., Grove, G. G., & Mahaffee, W. F. (2016). Development of a grower-conducted inoculum detection assay for management of grape powdery mildew. Plant Pathology, 65, 238-249. https://doi.org/10.1111/ppa.12421

Van der Merwe, N. A., Gryzenhout, M., Steenkamp, E. T., Wingfield, B. D., & Wingfield, M.

J. (2010). Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. Fungal Biology, 114, 966-979. https://doi.org/10.1016/j.funbio.2010.09.007

Wang, T., Ji, H., Yu, Y., Wang, X., Cheng, Y., Li, Z., Chen, J., Guo, L., Xu, J., & Gao, C. (2021). Development of a Loop-Mediated Isothermal Amplification Method for the Rapid Detection of *Phytopythium vexans*. Frontiers in Microbiology, 12, 720485. https://doi.org/10.3389/fmicb.2021.720485

Wong, Y. P., Othman, S., Lau, Y. L., Radu, S., & Chee, H. Y. (2018). Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. Journal of Applied Microbiology, 124, 626-643. https://doi.org/10.1111/jam.13647

Xu, L., Wang, Y., Zhu, S., Li, J., Chang, Y., & Huang, L. (2021). Development and application of a LAMP assay for the detection of the latent apple tree pathogen *Valsa mali*. Plant Disease, 105, 1065-1071. https://doi.org/10.1094/PDIS-07-20-1449-RE

Yilmaz, S., Adkins, S., & Batuman, O. (2023). Field-portable, rapid, and low-cost RT-LAMP assay for the detection of tomato chlorotic spot virus. Phytopathology®, 113, 567-576. https://doi.org/10.1094/PHYTO-08-22-0319-R

Zhang, J., Liu, X., Li, W., Zhang, J., Xiao, Z., Zhou, Z., Liu, T., Li, Y., Wang, F., Zhang, S., & Yang, J. (2018). Rapid detection of milk vetch dwarf virus by loop-mediated isothermal amplification. Journal of Virological Methods, 261, 147-152. https://doi.org/10.1016/j.jviromet.2018.08.012

Primers names	Length (base pairs)	Tm (°C)	Sequences (5' - 3')
F3	18	60.79	TCCATCGTGGCCGATCTG
B3	20	60.07	AGCTGTGCAACAAGGAATGT
FIP[a]	38	-	GAGCGAAACGGCTGAACCCC ATCTGCCCTCTCACCCAA
BIP[b]	41	-	CGTGATCCACCTGATACACCGC TGATTGAGGTGGTGCATGG
F1c	20	65.54	GAGCGAAACGGCTGAACCCC
F2	18	59.47	ATCTGCCCTCTCACCCAA
B1c	22	64.82	CGTGATCCACCTGATACACCGC
B2	19	59.47	TGATTGAGGTGGTGCATGG

Table 1. Set of primers designed for the detection of *Chrysoporthe* spp. using the Loop-Mediated Isothermal Amplification (LAMP) technique. Tm indicates the melting temperature.

[a] FIP primer consists of the F1 complementary sequence and F2 sequence.

[b] BIP primer consists of the B1 complementary sequence and B2 sequence.

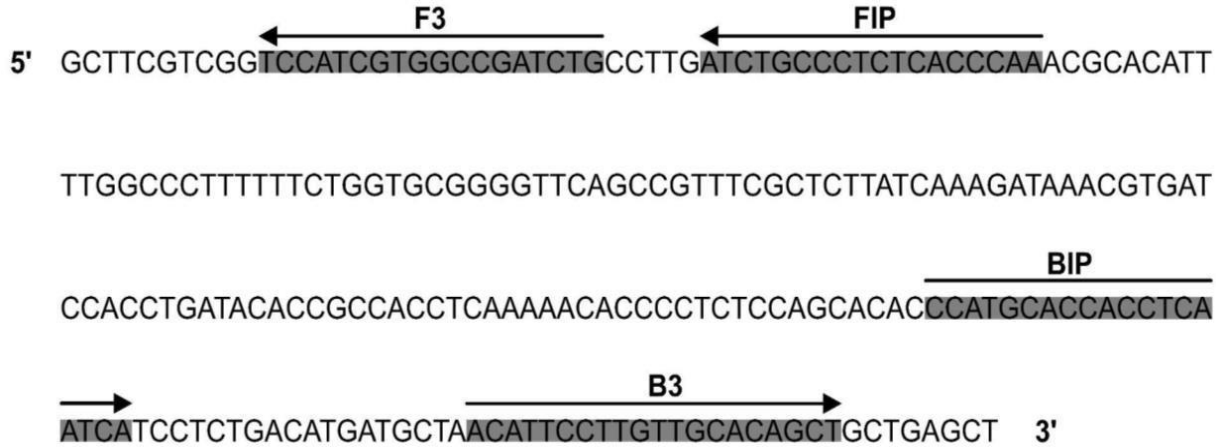


Figure 1. Sequence of the region of translation elongation factor 1-α (TEF1α) used to design the LAMP primer set. The primer annealing site is highlighted in gray and the arrows indicate the direction of extension.

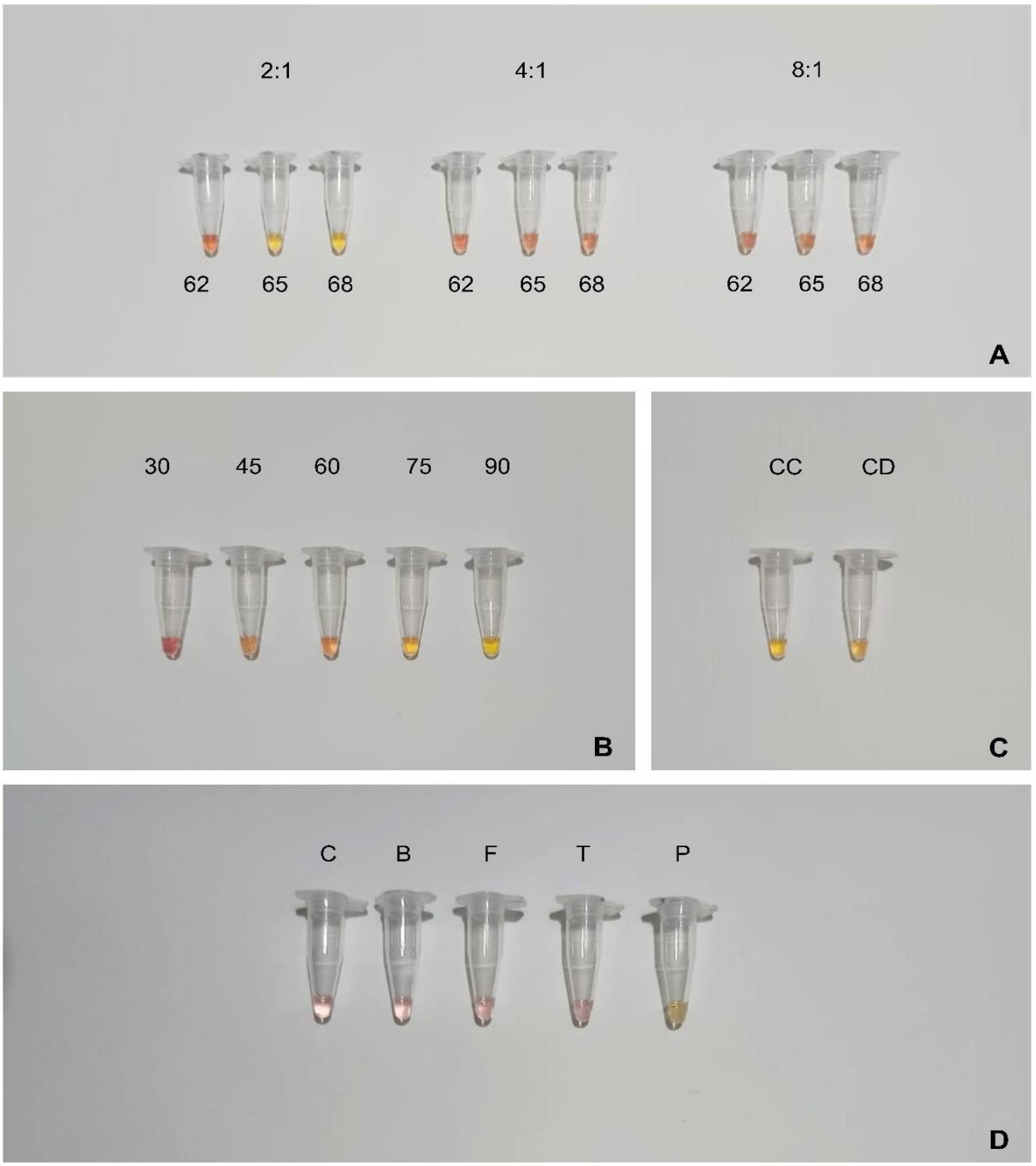


Figure 2. LAMP assays based on colorimetric assay for detection of *Chrysoporthe* spp. A – Optimization of the optimal temperature (62, 65 and 68 °C) and the ratio of BIP/FIP and F3/B3 primers (2:1, 4:1, 8:1). B – Optimization of time (30, 45, 60, 75 and 90 minutes) for pathogen detection. C – Validation of the detection of *Chrysoporthe cubensis* (CC) and *Chrysoporthe doradensis* (CD). D – Specificity of LAMP primers for detection of target DNA. Letters indicate the samples analyzed, as follows: C – control (reaction with DNase/RNase-free water), B – reaction with *Botrytis* sp. DNA, F - reaction with *Fusarium* sp. DNA, T - reaction with *Trichoderma* sp. DNA and P - reaction with *Chrysoporthe cubensis* DNA.

GENERAL CONCLUSIONS

Chrysoporthe cubensis was found occupying an endophytic niche in asymptomatic eucalyptus mini-stumps.

Chrysoporthe cubensis is the main fungal species causing Chrysoporthe wilt, while *Chrysoporthe doradensis* was found for the first time in association with the etiology of the disease in eucalyptus mini-stumps.

The spread of disease from mini-stumps, through vegetative propagation via the mini-cutting technique, to mini-cuttings and rooted cuttings has not been confirmed.

Chrysoporthe cubensis isolates are highly sensitive to the triazole fungicides cyproconazole, difenoconazole, ipconazole, and tebuconazole under in vitro conditions. The application of cyproconazole, ipconazole, and tebuconazole 72 hours after pathogen inoculation in eucalyptus plants reduces lesion length caused by *C. cubensis*. Additionally, tebuconazole and ipconazole effectively reduce the number of fruiting bodies per stem.

C. cubensis and *C. doradensis* can be detected using a colorimetric LAMP assay with a set of four primers (FIP, BIP, F3, and B3) at a concentration ratio of 2:1 (FIP/BIP: F3/B3) at 65°C for 75 to 90 minutes.