UNIVERSIDADE ESTADUAL DO CENTRO-OESTE, UNICENTRO-PR

OCCURRENCE OF *Apoharknessia eucalyptorum* IN BRAZIL: *In vitro* CULTIVATION, PATHOGENICITY AND SYMPTOMATOLOGY

Doctor Scientiae

ALEXANDRE TECHY DE ALMEIDA GARRETT

IRATI, PARANÁ

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Thesis presented to the Universidade Estadual do Centro-Oeste, as part of the requirements of the Programa de Pós-Graduação em Ciências Florestais, concentration area Sustainable Management of Forestry Resources, to obtain the title of Doctor.

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GENERAL ABSTRACT

Alexandre Techy de Almeida Garrett. Occurrence of *Apoharknessia eucalyptorum* in Brazil: *in vitro* cultivation, pathogenicity and symptomatology.

The number of diseases associated with *Eucalyptus* spp. in South America is increasing. In this context, the fungus *Apoharknessia eucalyptorum* was identified recently infecting leaves of *Eucalyptus dunnii* in Southern Brazil. Since 2004 the genus *Apoharknessia* was composed only by *A. insueta*. *A. eucalyptorum* was described only in 2017 and *A. eucalypti* in 2018. This genus is poorly understood, lacking information about dispersion, *in vitro* cultivation, pathogenicity, symptomatology, severity, and impacts. Thus, this study aimed to report the occurrence of *A. eucalyptorum* in Brazil, the fungus characteristics, the cultivation and sporulation *in vitro*, the inoculation, pathogenicity, symptomatology and the severity on leaves and trunks. The first chapter addresses the occurrence, and the identification in Brazil of *A. eucalyptorum* associated with leaf spots on *E. dunnii*, as well as the first description of pathogenicity. In the second chapter, colony characteristics, growth, and sporulation of *A. eucalyptorum* were evaluated at temperatures of 15, 20, and 25 °C on four culture media: malt extract agar (MEA); potato dextrose agar (PDA); V8 juice agar (V8); and bean dextrose agar (BEAN). The third chapter addresses the inoculation, symptomatology, pathogenicity, and the severity of *A. eucalyptorum* on leaves of *E. dunnii* and *Corymbia citriodora*, and trunks of *E. dunnii*. *A. eucalyptorum* was identified by ITS, ß-Tubulin and Calmodulin sequences, the morphological analysis confirmed the size of the conidia, and the presence of basal and apical appendages on the brown conidia; and the inoculations confirmed the pathogenicity of *A. eucalyptorum*. The better conditions for growth and sporulation were at 25 °C on PDA, BEAN, and MEA, with 24 hours light regime. The colonies characteristics changed with temperature and media tested. After inoculations, brown, circular or irregular lesions were observed occurring along the leaf margins, sparsely distributed, or coalescing throughout the leaf blade, on detached leaves with and without wounds. Severity ranged from 13 to 63% on *E. dunnii* and from 30 to 38% on *C. citriodora*. In this study, the pathogenicity of *A. eucalyptorum*, the symptomatology of the disease, and that the fungus can infect non-wounded leaf tissues is demonstrated. Moreover, this is the first report of the fungus on *C. citriodora*. Thus, the dispersion of *A. eucalyptorum* and the possible impacts of the pathogen on *Eucalyptus* spp. plantations should be monitored in Brazil.

Keywords: pathogenicity; severity; symptoms; incubation; leaf disease; canker; *Apoharknessia*.

RESUMO GERAL

Alexandre Techy de Almeida Garrett. Ocorrência de *Apoharknessia eucalyptorum* no Brasil: cultivo *in vitro*, patogenicidade e sintomatologia.

O número de doenças associadas com *Eucalyptus* spp. na América do Sul está aumentando. Neste contexto, o fungo *Apoharknessia eucalyptorum* foi identificado recentemente infectando folhas de *Eucalyptus dunnii* no Sul do Brasil. Desde 2004 o gênero *Apoharknessia* era composto apenas por *A. insueta*. *A. eucalyptorum* foi descrita somente em 2017 e *A. eucalypti* em 2018. Este gênero é pouco entendido, faltando informações sobre dispersão, cultivo *in vitro*, patogenicidade, sintomatologia, severidade e impactos. Deste modo, este estudo teve o objetivo de relatar a ocorrência de *A. eucalyptorum* no Brasil, suas características, o cultivo e a esporulação *in vitro*, a inoculação, patogenicidade, sintomatologia, e a severidade em folhas e caule de mudas. O primeiro capítulo trata da ocorrência e da identificação do fungo no Brasil associado com manchas foliares em *E. dunnii*, bem como a primeira descrição de patogenicidade da espécie. No segundo capítulo, características de colônia, crescimento e esporulação de *A. eucalyptorum* foram avaliadas em temperaturas de 15, 20, and 25 °C em quatro meios de cultura: extrato de malte ágar (MEA); batata dextrose ágar (PDA); Suco V8 ágar (V8); e feijão dextrose ágar (BEAN). O terceiro capítulo aborda a inoculação, sintomatologia, e a severidade de *A. eucalyptorum* em folhas de *E. dunnii* e *Corymbia citriodora*, e em caules de mudas de *E. dunnii*. O fungo *A. eucalyptorum* foi identificado por sequencias ITS, ß-Tubulina e Calmodulina, e análises morfológicas que confirmaram o tamanho e a presença de apêndices basais e apicais nos conídios de cor marrom, similares ao fungo estudado. As inoculações confirmaram a patogenicidade de *A. eucalyptorum*. As características das colônias variaram com as temperaturas e meios testados. As melhores condições para o crescimento e esporulação foram a 25 °C em PDA, BEAN, e MEA, com fotoperíodo de 24 horas. Após as inoculações, lesões de cor marrom, circulares ou irregulares foram observadas ocorrendo ao longo da margem das folhas, distribuídas esparsamente ou coalescendo pelo limbo foliar, em folhas destacadas com e sem ferimentos. A severidade variou de 13 a 63% em *E. dunnii* e de 30 a 38% em *C. citriodora*. Neste estudo, a patogenicidade de *A. eucalyptorum* foi comprovada, demonstrando a sintomatologia da doença, e que o fungo pode infectar tecidos foliares sem ferimentos. Além disso, este é o primeiro relato do fungo em *C. citriodora*. Portanto, a dispersão de *A. eucalyptorum* e os possíveis impactos do patógeno em plantios de *Eucalyptus* spp. devem ser monitorados no Brasil.

Palavras-chave: patogenicidade; severidade; sintomas; incubação; doença foliar; cancro; *Apoharknessia*.

GENERAL INTRODUCTION

Recognized as one of the biggest pulp and paper producers in the world, Brazil's wood production chain is based on vast areas of *Eucalyptus* spp. and *Pinus* spp. plantations (IBÁ, 2017). A variety of species have been adopted in different regions of the country based on wood characteristics and adaptation to climatic conditions. In Southern Brazil, forest stands were predominantly composed of *Pinus* spp., but recent expansion of the *Eucalyptus* spp. plantation area has been considerable. In the last five years, the eucalypt plantation areas increased 56% in Paraná State, 11% in Santa Catarina State, and 10% in Rio Grande do Sul State (IBÁ, 2017).

The increase of *Eucalyptus* spp. plantation area in Southern Brazil is related to demand for short-fiber in pulp and paper production, aiming at higher yield and shorter rotation of the eucalypt stands (DOBNER Jr. et al., 2017). Originating in Australia, the genus *Eucalyptus* is composed of hundreds of species (BROOKER, 2000) that are adapted to a wide range of climatic conditions. According to RIRDC (2009), among other species, those indicated for temperate and subtropical regions are: *Corymbia citriodora*, *Eucalyptus benthamii*, *Eucalyptus camaldulensis, Eucalyptus cladocalyx, Eucalyptus cloeziana, Eucalyptus dunnii, Eucalyptus fraxinoides, Eucalyptus globulus, Eucalyptus nitens, Eucalyptus pilularis, Eucalyptus saligna,* and *Eucalyptus viminalis*. In regions with subtropical climate in Australia, the most frequently planted species are *C. citriodora*, *E. dunnii*, *E. pilularis*, *E. cloeziana*, *E. grandis*, and the hybrid *E. grandis* x *E. camaldulensis*, whereas in temperate regions *E. globulus* is the most commonly planted species (CARNEGIE et al., 2005). As a consequence of different species adapted to subtropical climate, research and evaluation of adapted species have been conducted in Southern Brazil, which is characterized by a subtropical climate, with mild temperatures and occurrence of frosts in the winter.

In subtropical regions of Southern Brazil, the most frequently adopted species include *E. benthamii*, *E. dunnii*, *E. saligna*, *E. grandis*, and *E. viminalis* (AUER; SANTOS, 2011; PALUDZYSZYN FILHO; SANTOS; FERREIRA., 2006; SANTOS et al., 2001). To date, *Eucalyptus dunnii* and *Eucalyptus benthamii* are the principal species in Southern Brazil, as they are adapted to below freezing temperatures and frosts that occur in the region (HIGA; PEREIRA, 2003; PALUDZYSZYN FILHO; SANTOS, 2005), and are suitable for pulp and paper production (RIRDC, 2009; THOMAS et al., 2009; HIGA; PEREIRA, 2003).

Eucalyptus dunnii has its origin in warm and humid climates, where the number of frosts range from 20 to 60 per year. The species can adapt to different site, rainfall, and frost conditions, growing in areas with temperatures of 2 to 5 °C in the coldest month, and of 24 to

19 °C in the hottest month, and rainfall ranging from 1000 to 1600 mm per year. Commercial plantations of the species are common in Australia, China, South Africa, and mainly in South America, where the species is not susceptible to many pathogens, but silvicultural practices are necessary to maintain tree vigor (BOLAND et al., 2006; RIRDC, 2009; RAYMOND; THOMAS; HENSON, 2010). *Eucalyptus benthamii* has its origin in Western Australia along the basin of the Nepean River. The mean temperature in the winter is about 4 °C and 26 °C in summer, with occurrence of mild frosts and a mean rainfall of 1100 mm. Plantations of the species are found in China, South Africa, and South America (HIGA; PEREIRA, 2003). *Corymbia citriodora* occurs in the east Australia, in regions from 30 to 1100 m of altitude where light frosts are observed. The mean temperature in the coldest month is of 8 to 9 °C, and in the hottest month of 29 to 30 °C. The mean rainfall in the regions of origin range from 600 to 2000 mm per year, and plantations are mainly present in Australia, South Africa and South America (BOLAND et al., 2006; RIRDC, 2009; REIS et al., 2013).

In subtropical regions of China, the plantation areas of *E. dunnii* and *E. benthamii* have increased due to improved plant survival and volume at six years of age in comparison to other species (ARNOLD et al., 2015). In Brazil, *E. dunnii* can reach 1.2 m³ per tree in optimal conditions at 10 years, with a mean annual increment of 50 $m^3.ha^{-1}.year^{-1}$ (DOBNER Jr. et al., 2017). *Corymbia citriodora* lacks information about growth in Australia, but an increment of 20 $m^3.ha^{-1}.year^{-1}$ is reported (RIRDC, 2009). In Brazil, the species is not a primary species adopted in large plantation areas, lacking yet silvicultural information (REIS et al., 2013).

As monocultures comprising large plantation areas, the eucalypti stands are attacked by diseases that can limit the increment and quality of the trees. Several diseases have been reported as occurring on *Eucalyptus* spp. (MAÚSSE-SITOE et al., 2016; JIMU et al., 2015; CROUS et al., 2004; OLD; WINGFIELD; YUAN, 2003; KEANE et al., 2000; CROUS; KNOX-DAVIES; WINGFIELD, 1989a). Trees of *E. dunnii* and *E. benthamii* are hosts for several diseases, including *Botrytis cinerea*, *Calonectria candelabrum*, *Corynespora cassiicola*, *Crysoporthe cubensis*, *Oidium eucalypti*, *Pestalotiopsis* sp., *Austropuccinia psidii*, *Rhizoctonia* sp. (SCHULTZ et al., 2015; PÉREZ et al., 2014; REIS et al., 2014; SCHULTZ et al., 2012; AUER; SANTOS, 2011), and also leaf diseases caused by *Mycosphaerella* spp. and *Teratosphaeria* spp. (AUER; SANTOS 2011; HUNTER et al., 2009). The latter genera are considered among the most severe pathogens for eucalypts (BALMELLI et al., 2014; HUNTER et al., 2009) that can affect yield and tree survival (BARBER, 2004). The diseases caused by *Mycosphaerella* spp. and *Teratosphaeria* spp. were identified about ten years ago in Brazil (TEODORO et al., 2012; PASSADOR et al., 2013; GARRETT; CAMARGO; GARCIA, 2018a), demonstrating an increase in newly reported diseases in Southern Brazil. *Corymbia*

citriodora is host for diseases such *Calonectria* spp., *Rhizoctonia* spp., *Botrytis cinerea*, *Pestalotiopsis* spp., *Erythricium salmonicolor*, *Chrysoporthe cubensis*, *Valsa* sp. The species show resistance to the rust caused by *Austropuccinia psidii* and canker (SANTOS; AUER; GRIGOLETTI JUNIOR, 2001; ALFENAS et al., 2009; REIS et al., 2013).

It is important to highlight that the climate of subtropical regions favor the occurrence and impacts of pathogens, mainly due to humidity and frosts that benefit pathogen outbreaks (BERGAMIN FILHO; AMORIN, 2001). Associated with these conditions is an increase in the occurrence and severity of diseases linked to climate change (EVANS et al., 2008) or changes in ecological and genetic factors in the interactions between pathogens and plants (BURDON; THRALL; ERICSON, 2009). Furthermore, a disease can remain confined to a region, initially without causing negative impacts, until it emerges due to increased incidence, geographical dispersion, and severity (ANDERSON et al., 2004).

A new fungus that emerged as a disease is the fungus *Apoharknessia eucalyptorum*, that was isolated from leaves of *Eucalyptus dunnii* Maiden with leaf spots and leaf blight in Santa Catarina State, in Southern Brazil (GARRETT et al., 2018b). The genus *Apoharknessia* was first described by Lee, Groenewald, and Crous (2004), when the former coelomycetes species, *Harknessia insueta*, was analyzed using morphology, *in vitro* cultivation, and DNA. The *Apoharknessia* etymology was proposed due to the hyaline basal and apical appendages on brown conidia and its similarity with Harknessia.

Distinguishing between different Coelomycete species based only on morphological characteristics is difficult (CROUS et al., 2012b), but some features are helpful to distinguish Apoharknessia from Harknessia, as observed by Lee, Groenewald, and Crous (2004). Apoharknessia does not form fluffy mycelia but grows within the culture medium and sporulates within one week, sporulating on naked hyphae *in vitro* (LEE; GROENEWALD; CROUS, 2004). On the other hand, the related genus Harknessia, that belongs to the order Diaporthales that includes several eucalypt pathogens (MAHARACHCHIKUMBURA et al., 2015), has an aerial fluffy mycelium, sporulating in a longer period of time, and forming conidioma from the onset of cultivation. Although there are more than 40 Harknessia species with distinct morphology and colony characteristics, all have brown conidia with basal tubular appendages and striations (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2012a).

As morphological characteristics are helpful to distinguish Apoharknessia from Harknessia, studies on the *in vitro* cultivation of these species is the first step in their characterization. Cultivation provides information about size, color, and shape of the colonies and reproductive structures. This information is necessary for comparative morphology as the size of the conidia is important in species identification (CROUS; WINGFIELD; NAG RAJ,

1993; FURLANETTO; DIANESE, 1998; FARR et al., 2003; CROUS et al., 2017), for molecular studies as cultures for many fungi associated with eucalypts are still unavailable (SUMMERELL et al., 2006), and also to understand host-pathogen interactions (KOLEY; MAHAPATRA, 2015).

Harknessia has more information in the literature, and is represented by anamorphs associated with leaf, stem, and twig lesions on *Eucalyptus* spp. (YUAN; MOHAMMED, 1997; YUAN; WARDLAW; MOHAMMED, 2000; PARK et al., 2000) and leaf litter (LEE; GROENEWALD; CROUS, 2004). Some species are endophytic (CROUS et al., 2012a) and others are saprophytic or soil colonizers (ROSSMAN et al. 2007). Harknessia species occur associated with each other, with other pathogens, or on tissues damaged by insects (YUAN; WARDLAW; MOHAMMED, 2000; CROUS; ROGERS, 2001; CHEEWANGKOON et al., 2008; ALFENAS et al., 2009; CROUS et al., 2012a). Besides *Eucalyptus*, species of *Acacia*, *Araucaria*, *Cupressus*, *Liquidambar*, *Melaleuca*, *Podocarpus*, and *Quercus* are also hosts of Harknessia (SWART, 1972; SUTTON, 1980; SUTTON; PASCOE, 1989). Although Harknessia and Apoharknessia species are parasites of *Eucalyptus* spp., their pathogenicity, symptomatology, potential damage, and impacts have not been studied in depth (BROWN, 2000).

It is expected that DNA analyses will reduce problems related to the classification of Harknessia species and its hosts (CROUS et al., 2012a). This is valid for species of both Harknessia and Apoharknessia, as they are morphologically similar (SUMMERELL et al., 2006). Despite the fact that they are relatively well understood, the number of described Harknessia species continues to increase (CROUS et al., 2007). For example, in the study by Crous et al. (2017), the authors described *A. eucalyptorum* along with five new species of Harknessia, one occurring on *Eucalyptus* spp. Meanwhile, Marin-Felix et al. (2018) described another four new species, two of which occur on *Eucalyptus* spp., and *Harknessia corymbiae* which occurs on *Corymbia maculata*.

Harknessia species have been identified occurring on *E. dunnii* and on the genus *Corymbia*; however, to date, there has been no report of Apoharknessia on these species. On *E. dunnii* the following species have been observed: *H. hawaiiensis* (MASCHIO; AUER; GRIGOLETTI Jr., 1996); *H. pseudohawaiiensis* (CROUS et al., 2012a); and *H. fumaginea* (CARNEGIE, 2007). Whereas *H. rhabdosphaera* is reported on *Corymbia henryi* (SUMMERELL et al., 2006).

In contrast, the available studies on the *Apoharknessia* genus are focused on taxonomy and classification (LEE; GROENEWALD; CROUS, 2004; GRYZENHOUT et al., 2006; ROSMANN; FARR; CASTLEBURY, 2007; PINRUAN, et al., 2008; CROUS et al., 2012a;

MAHARACHCHIKUMBURA et al., 2015; CROUS et al., 2017; SENANAYAKE et al., 2017; MARIN-FELIX et al., 2018; FAN et al., 2018). As a consequence, information about pathogenicity of *Apoharknessia* is limited. Until 2017, when *A. eucalyptorum* was introduced, the genus *Apoharknessia* consisted of only *A. insueta* (CROUS et al., 2017), and a third species, *A. eucalypti*, was recently described (MARIN-FELIX et al., 2018). *Apoharknessia insueta* is considered pathogenic (CROUS; KNOX-DAVIES; WINGFIELD, 1989a; PARK et al., 2000; FAO, 2009), and isolates of the pathogen have been identified in Brazil, Colombia, Cuba, Mauritius (LEE; GROENEWALD; CROUS, 2004), United States (SUTTON, 1980) and Costa Rica (CROUS et al., 2012a). On the other hand, *A. eucalyptorum* and *A. eucalypti* are considered endophytic to *Eucalyptus pellita* (CROUS et al., 2017; MARIN-FELIX et al., 2018).

As highlighted by Wingfield et al. (2013), problems caused by new pests and pathogens are emerging frequently, which can threaten the productivity of plantations, and investment in research on pests and pathogens can help guarantee the success of forest enterprises. The concern with Apoharknessia should be considered, as the number of species is increasing. Eucalypt diseases are mainly introduced and dispersed through the circulation of infected material across different regions (HUNTER et al., 2011; AUER; SANTOS, 2009). Once dispersed, these diseases can infect plantations, and control of the pathogen can be unviable (CARNEGIE; ADES, 2002; MASSON et al., 2011). In this context, DNA analysis is another important approach to correctly identify pathogens, and is essential for breeding and selection of resistant genetic material.

In this study we present and discuss the first report of *A. eucalyptorum* on *E. dunnii*, and the first report of the fungus in South America, from a region in Southern Brazil. Moreover, conditions for *in vitro* cultivation of *A. eucalyptorum* are described, indicating optimal temperature and culture medium to incubate the fungus. Finally, the inoculation of *A. eucalyptorum* on leaves and trunks of *E. dunnii* and leaves of *C. citriodora* is discussed, demonstrating the pathogenicity of the fungus, its signs, symptoms, incidence, severity, and infection pattern by staining the fungal tissues on inoculated leaves.

OBJECTIVES OF THE STUDY

1. GENERAL OBJECTIVE

The general aim of this study is to show the occurrence of *Apoharknessia eucalyptorum* in Southern Brazil, infecting leaves of *Eucalyptus dunnii*, as well as demonstrate the *in vitro* cultivation, the pathogenicity, the symptoms, signs and infection pattern of the fungus.

2. SPECIFIC OBJECTIVES

The specific aims of this study are:

2.1. Report the occurrence of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* and the pathogenicity of the fungus (Chapter I): First report of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* in Brazil;

2.2. Demonstrate the better conditions of cultivation to promote growth and sporulation of *Apoharknessia eucalyptorum* on different culture media (Chapter II): Morphophysiological characterization of *Apoharknessia eucalyptorum* on different culture media;

2.3. Evaluate the aggressiveness of *Apoharknessia eucalyptorum* by the severity on inoculated leaves of *Corymbia citriodora* and *Eucalyptus dunnii*, demonstrating the symptomatology, signs and infection pattern of the fungus. Moreover, evaluate the pathogenicity, severity and symptoms of *Apoharknessia eucalyptorum* on trunks of *Eucalyptus dunnii* seedlings (Chapter III): Aggressiveness and symptomatology of *Apoharknessia eucalyptorum* on leaves and trunks of Eucalyptus and Corymbia.

REFERENCES

ALFENAS, A. C.; ZAUZA, E. A. V.; MAFIA, R. G.; ASSIS, T. F. de. **Clonagem e doenças do eucalipto**. 2. ed. Viçosa: Editora UFV, 2009. 500 p.

ANDERSON, P.K.; CUNNINGHAM, A.A.; PATEL, N.G.; MORALES, F.J.; EPSTEIN, P.R.; DASZAK, P. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. **Trends in Ecology & Evolution**, v. 19, n. 10, p. 535-544, 2004.

AUER, C. G.; SANTOS, Á. F. dos; Doenças em eucaliptos destinados à produção de energia na região Sul do Brasil. **Pesquisa Florestal Brasileira**. Colombo, v. 31, n. 68, p. 373-379, 2011.

AUER, C. G.; SANTOS, Á. F. dos. **Reconhecimento e Identificação dos Principais Patógenos de Importância Quarentenária Associados a Materiais de Propagação ou Madeira.** Colombo, PR: EMBRAPA Florestas, dez. 2009. 26 p.

ARNOLD, R.; LI, B.; LUO, J.; BAI, F.; BAKER, T. Selection of cold-tolerant Eucalyptus species and provenances for inland frost susceptible, humid subtropical regions of southern China. **Australian Forestry**, n. 78, v. 3, p. 180-193, 2015.

BALMELLI, G.; SIMETO, S.; MARRONI, V.; ALTIER, N.; DIEZ, J. J. Genetic variation for resistance to *Mycosphaerella* leaf disease and *Eucalyptus* rust on *Eucalyptus globulus* in Uruguay. **Australasian Plant Pathology**, n. 43, p. 97–107. 2014.

BARBER, P. A. Forest Pathology: The threat of disease to plantation forest in Indonesia. **Plant Pathology Journal**, n. 3, p. 97-104, 2004.

BERGAMIN FILHO, A.; AMORIN, L. Epidemiologia comparativa entre os patossistemas temperado e tropical: Conseqüências para a resistência a fungicidas. **Fitopatologia Brasileira**, v. 26, p. 119-127, 2001.

BOLAND D. J.; BROOKER, M. I. H.; CHIPPENDALE, G. M.; HALL, N.; HYLAND, B. P. M.; JOHNSTON, R. D.; KLEINIG, D. A.; HYLAND, B. P. M. **Forest trees of Australia**. 5. ed. Collingwood: CSIRO Publishing, 2006. 769 p.

BROOKER, M. I. H. A New Classification of the Genus *Eucalyptus* L'Hér. (Myrtaceae). **Australian Systematic Botany**, n. 13, p. 79–148. 2000.

BROWN, B. N. Diseases and Fungi of the reproductive structures of eucalypts. In: KEANE, P. J; KILE, G. A.; PODGER, F. D.; BROWN, B. N. **Diseases and pathogens of eucalypts**: Collingwood: CSIRO publishing. 2000. 153–239.

BURDON, J. J.; THRALL, P. H.; ERICSON, L. Plant Pathogens and Diseases: Newly Emerging Diseases. In: SCHAECHTER, M. **Encyclopedia of Microbiology**. New York: Elsevier, 2009. 647-654.

CARNEGIE, A. J. Forest health condition in New South Wales, Australia, 1996–2005. I. Fungi recorded from eucalypt plantations during forest health surveys. **Australasian Plant Pathology**, v. 36, n. 3, p. 213–224, 2007.

CARNEGIE, A. J.; STONE, C.; LAWSON, S.; MATSUKI, M.; Can we grow certified eucalypt plantations in subtropical Australia? An insect pest management perspective. **New Zealand Journal of Forestry Science**, v. 35, n. 2/3, p. 223–245, 2005.

CARNEGIE, A. J.; ADES, P. K. *Mycosphaerella* leaf disease reduces growth of plantation-grown *Eucalyptus globulus.* **Australian Forestry**, v. 66, n. 2, p. 113-119, 2002.

CHEEWANGKOON, R., CROUS, P. W., HYDE, K. D., GROENEWALD, J. Z., TO-ANAN, C. Species of *Mycosphaerella* and related anamorphs on *Eucalyptus* leaves from Thailand. **Persoonia**, v. 21, p. 77– 91, 2008.

CROUS, P. W.; WINGFIELD, M. J.; BURGESS, T. I. et al. Fungal Planet Description sheets: 558-624 - *Apoharknessia eucalyptorum*. **Persoonia - Molecular Phylogeny and Evolution of Fungi**, v. 38: 270-271, 2017.

CROUS, P. W.; SUMMERELL, B. A.; SHIVAS, R. G.; CARNEGIE, A. J.; GROENEWALD, J. Z. A re-appraisal of *Harknessia* (*Diaporthales*), and the introduction of *Harknessiaceae* fam. nov. **Persoonia**, v. 28, p. 49–65, 2012a.

CROUS, P. W.; SUMMERELL, B. A.; ALFENAS, A. C.; EDWARDS, J.; PASCOE, I. G.; PORTER, I. J.; GROENEWALD, J. Z. Genera of diaporthalean coelomycetes associated with leaf spots of tree hosts. **Persoonia**, v. 28, p. 66–75, 2012b.

CROUS, P.W.; MOHAMMED, C.; GLEN, M.; VERKLEY, G. J. M.; GROENEWALD, J. Z. *Eucalyptus* microfungi known from culture. 3. *Eucasphaeria* and *Sympoventuria* genera nova, and new species of *Furcaspora*, *Harknessia*, *Heteroconium* and *Phacidiella*. **Fungal Diversity**, v. 25, p. 19-36, 2007.

CROUS, P. W.; GROENEWALD, J. Z.; MANSILLA, P.; HUNTER, G. C.; WINGFIELD, M. J. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. S**tudies in Mycology**, n. 50, p. 195 – 214, 2004.

CROUS, P.W., WINGFIELD, M.J., NAG RAJ, T.R. *HARKNESSIA* SPECIES OCCURING IN SOUTH AFRICA. **Mycologia**, Lancaster, v. 85, n. 1, p. 108-118, 1993.

CROUS, P. W.; KNOX-DAVIES, P. S.; WINGFIELD, M. J. A List of Eucalyptus Leaf Fungi and their Potential Importance to South African Forestry. **Suid-Afrikaanse Bosboutydskrif**, v. 149, p. 17-29. 1989a.

CROUS, P. W.; ROGERS, J. D. Wuestneia molokaiensis and its anamorph *Harknessia molokaiensis* sp. nov. from Eucalyptus. **Sydowia**, v. 53, n. 1, p. 74-80, 2001.

DOBNER JR., M.; BATISTA, K. M.; SARTÓRIO, I. P.; ARCE, J. E.; QUADROS, D. S.

CRESCIMENTO E DESEMPENHO ECONÔMICO DE *Eucalyptus dunnii* EM DIFERENTES SÍTIOS NO PLANALTO SUL DO BRASIL. **FLORESTA**, Curitiba, PR, v. 47, n. 4, p. 397 - 406, 2017

EVANS, N.; BAIERL, A.; SEMENOV, M. A.; GLADDERS, P.; FITT, B. D. L. Range and severity of a plant disease increased by global warming. **Journal of the Royal Society Interface**, v. 5, p. 525–531, 2008.

FAN, X. L.; BEZERRA, J. D. P.; TIAN, C. M.; CROUS, P. W. Families and genera of diaporthalean fungi associated with canker and dieback of tree hosts. **Persoonia**, v. 40, p. 119 –134, 2018.

FARR, D. F.; ROSSMAN, A. Y. *Dwiroopa*, a coelomycetous genus with two species. **Mycoscience**, Tokyo, v. 44, p. 443–446, 2003.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. Global review of forest pests and diseases. **FAO Forestry Paper**, 156. Rome: FAO, 2009. Available at: <http://www.fao.org/3/a-i0640e.pdf>. Accessed on: 18 Apr. 2018.

FURLANETTO, C.; DIANESE, J. C. Some coelomycetes from Central Brazil, **Mycological Research**, Amsterdam, v. 102, n. 1, p. 19-29, 1998.

GARRETT, A. T. DE A.; CAMARGO, M. B. de; GARCIA, F. A. de O. Chemical Control of Mycosphaerella Leaf Disease on *Eucalyptus dunnii* in Southern Brazil. **Floresta e Ambiente**, v. 25, n. 2, 2018a.

GARRETT, A. T. de A.; IMANI, J.; TAMBARUSSI, E. V.; GARCIA, F. A. de O.; KOGEL, K. H.; FIGUEIREDO FILHO, A. First report of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* in Brazil. **Forest Pathology**, e12463, 2018b.

GRYZENHOUT, M.; MYBURG, H.; WINGFIELD, B. D.; WINGFIELD, M. J. Cryphonectriaceae (Diaporthales), a new family including *Cryphonectria*, *Chrysoporthe*, *Endothia* and allied genera. **Mycologia**, v. 98, n. 2, p. 239–249, 2006.

HIGA, R. C. V.; PEREIRA, J. C. D. **Usos Potenciais do *Eucalyptus benthamii* Maiden et Cambage**. Colombo: EMBRAPA Florestas, 2003. 4 p. (Comunicado Técnico, 100).

HUNTER, G. C.; CROUS, P. W.; CARNEGIE, A. J.; BURGESS, T. I.; WINGFIELD, M. J. *Mycosphaerella* and *Teratosphaeria* diseases of *Eucalyptus*; easily confused and with serious consequences. **Fungal Diversity**, n. 50, p. 145–166, 2011.

HUNTER, G. C.; CROUS, P. W.; CARNEGIE, A. J.; WINGFIELD, M. J. *Teratosphaeria nubilosa*, a serious leaf disease pathogen of *Eucalyptus* spp. in native and introduced areas. **Molecular Plant Pathology**, n. 10, p. 1-14. 2009.

IBÁ - INSTITUTO BRASILEIRO DE ÁRVORES. **REPORT 2017**. São Paulo, 2017. 80 p.

JIMU, L.; WINGFIELD, M. J.; MWENJE, E.; ROUX, J. Diseases on *Eucalyptus* species in

Zimbabwean plantations and woodlots. **Southern Forests**, v. 77, n. 3, p. 1-10, 2015.

KEANE, P. J.; KILE, G. A.; PODGER, F. D.; BROWN, B. N. **Disease and Pathogens of Eucalyptus**. Collingwood: CSIRO Publishing, 2000.

KOLEY, S.; MAHAPATRA, S. S. Evaluation of Culture Media for Growth Characteristics of *Alternaria solani*, Causing Early Blight of Tomato. **Journal of Plant Pathology & Microbiology**, Los Angeles, S1, p. 1-5, 2015.

LEE, S.; GROENEWALD, J. Z.; CROUS, P. W. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apoharknessia* gen. nov. **Studies in Mycology**, v. 50, p. 235–252, 2004.

MAHARACHCHIKUMBURA, S.; HYDE, K.; JONES, E.; MCKENZIE, E.; HUANG, S.; ABDEL-WAHAB, M.; DARANAGAMA, D.; DAYARATHNE, M.; D'SOUZA, M.; GOONASEKARA, I.; HONGSANAN, S.; JAYAWARDENA, R.; KIRK, P.; KONTA, S.; LIU, J.; LIU, Z.; NORPHANPHOUN, C.; PANG, K.; PERERA, R.; SENANAYAKE, I.; SHANG, Q.; SHENOY, B.; XIAO, Y.; BAHKALI, A.; KANG, J.; SOMROTHIPOL, S.; SUETRONG, S.; WEN, T.; XU, J. Towards a natural classification and backbone tree for *Sordariomycetes*. **Fungal Diversity**, v. 72, p. 199–301, 2015.

MARIN-FELIX, Y.; HERNÁNDEZ-RESTREPO, M.; WINGFIELD, M. J.; AKULOV, A.; CARNEGIE, A. J.; CHEEWANGKOON, R.; GRAMAJE, D.; GROENEWALD, J. Z.; GUARNACCIA, V.; HALLEEN, F.; LOMBARD, L.; LUANGSA-ARD, J.; MARINCOWITZ, S.; MOSLEMI, A.; MOSTERT, L.; QUAEDVLIEG, W.; SCHUMACHER, R. K.; SPIES, C. F. J.; THANGAVEL, R.; TAYLOR, P. W. J.; WILSON, A. M.; WINGFIELD, B. D.; WOOD, A. R.; CROUS, P. W. Genera of phytopathogenic fungi: GOPHY 2. **Studies in Mycology**, v. 92, p. 47-133, 2018.

MASCHIO, L. M. A.; AUER, C. G.; GRIGOLETTI JUNIOR, A. Fungos associados a *Eucalyptus* spp. no Paraná e em Santa Catarina. Colombo: EMBRAPA. **Pesquisa em andamento**, n. 5, p. 1-3, 1996.

MASSON, M.V.; MORAES, W.B.; MATOS, W.C.; ALVES, J.M.; FURTADO, E.L. Eficiência e viabilidade econômica do controle químico da ferrugem do eucalipto em condições de campo. **Summa Phytopathologica**, v.37, n.2, p.107-112, 2011.

MAÚSSE-SITOE, S. N. D.; CHEN, S.; WINGFIELD, M. J.; ROUX, J. Diseases of eucalypts in the central and northern provinces of Mozambique. **Southern Forests**, v. 78, n. 3, p. 1–15, 2016.

OLD, K. M.; WINGFIELD, M. J.; YUAN, Z. **A manual of diseases of eucalypts in South-East Asia**. Jakarta: Center for International Forestry Research (CIFOR), 2003. 98 p.

PALUDZYSZYN FILHO, E.; SANTOS, P. E. T.; FERREIRA, C. A. **Eucaliptos indicados**

para plantio no Estado do Paraná. Colombo: EMBRAPA Florestas, nov. 2006. 45 p.

PALUDZYSZYN FILHO, E.; SANTOS, P. E. T. **Considerações sobre o plantio de *Eucalyptus dunnii,* no estado do Paraná**. Colombo-PR: EMBRAPA Florestas, dec. 2005. 7p.

PARK, R. F.; KEANE, P. J.; WINGFIELD, M. J.; CROUS, P. W. Fungal diseases of eucalypt foliage. In: KEANE, P. J; KILE, G. A.; PODGER, F. D.; BROWN, B. N. **Diseases and pathogens of eucalypts**. Collingwood: CSIRO publishing, 2000. 153–239.

PASSADOR, M. M.; LIMA, P. R.; PIERI, C.; SIERRA-HAYER, J. F.; HARAKAVA, R.; FURTADO, E. L. Diversity of *Mycosphaerella* spp. and Teratosphaeria spp. in *Eucalyptus globulus* plantations in Brazil. **European Journal of Plant Pathology**, v. 137, p. 137–147, 2013.

PÉREZ, C. A.; REYNA, R.; MONTANARI, L.; TORRES-DINI, D.; NIKICHUK, N.; SIMETO, S. First Report of Rust Caused by *Puccinia psidii* on *Eucalyptus dunnii* in Uruguay. **Plant Disease**, v. 98, n. 10, p.1444-1444, 2014.

PINRUAN, U.; SAKAYAROJ, J.; HYDE, K.D.; JONES, E. B. G. *Thailandiomyces bisetulosus* gen. et sp. nov. (*Diaporthales*, *Sordariomycetidae*, *Sordariomycetes*) and its anamorph *Craspedodidymum*, is described based on nuclear SSU and LSU rDNA sequences. **Fungal Diversity** v. 29, p. 89-98, 2008.

RAYMOND, C. A.; THOMAS, D. S.; HENSON, M. Predicting pulp yield and pulp productivity of *Eucalyptus dunnii* using acoustic techniques. **Australian Forestry**, v. 73, n. 2, p. 91-97, 2010.

REIS, B. P.; LANNA FILHO, R.; ALFENAS, R. F.; ALFENAS A. C. First report of *Corynespora cassiicola* causing severe leaf blight on *Eucalyptus* in Brazil. **New Disease Reports**, v. 29, n. 7, p. 7, 2014.

REIS, C. A. F.; ASSIS, T. F. de; SANTOS, A. M.; PALUDZYSZYN FILHO, E. *Corymbia citriodora*: estado da arte de pesquisas no Brasil. Colombo, PR: EMBRAPA Florestas, 2013. 60 p.

ROSMANN, A. Y.; FARR, D. F.; CASTLEBURY, L. A. A review of the phylogeny and biology of the Diaporthales. **Mycoscience**, v. 48, n. 3, p. 135–144, 2007.

RIRDC - RURAL INDUSTRIES RESEARCH AND DEVELOPMENT CORPORATION. **Trees for Farm Forestry: 22 promising species**. Available at: < https://rirdc.infoservices.com.au/downloads/09-015.pdf> Accessed in: May, 10, 2017.

SANTOS, Á. F. dos; AUER, C. G.; GRIGOLETTI JÚNIOR, A. **Doenças do eucalipto no sul do Brasil: identificação e controle**. Colombo, PR: EMBRAPA Florestas, jun. 2001. 20 p.

SCHULTZ, B.; SBRAVATTI JÚNIOR, J. A.; AUER, C. G.; SANTOS, A. F. IMPACTO DA MANCHA FOLIAR CAUSADA POR *Cylindrocladium candelabrum* EM PLANTIOS

JOVENS DE *Eucalyptus benthamii* EM RIO NEGRINHO – SC. **Ciência Florestal**, v. 25, n. 2, p. 307-316, 2015.

SCHULTZ, B.; BORA, K. C.; NOGUEIRA, A. C.; AUER, C. G. Uso do silicato de potássio no controle de oídio em mudas de *Eucalyptus benthamii*. **Pesquisa Florestal Brasileira**, v. 32, n. 69, p. 93-99, 2012.

SENANAYAKE, I. C.; CROUS, P. W.; GROENEWALD, J. Z.; MAHARACHCHIKUMBURA, S. S. N.; JEEWON, R.; PHILLIPS, A. J. L.; BHAT, J. D.; PERERA, R. H.; LI, Q. R.; LI, W. J.; TANGTHIRASUNUN, N.; NORPHANPHOUN, C.; KARUNARATHNA, S.C.; CAMPORESI, E.; MANAWASIGHE, I. S.; AL-SADI, A. M.; HYDE, K. D. Families of Diaporthales based on morphological and phylogenetic evidence. **Studies in Mycology**, v. 86, p. 217–296, 2017.

SUMMERELL, B. A.; GROENEWALD, J. Z.; CARNEGIE, A.; SUMMERBELL, R. C.; CROUS, P.W. *Eucalyptus* microfungi known from culture. 2. *Alysidiella*, *Fusculina* and *Phlogicylindrium* genera nova, with notes on some other poorly known taxa. **Fungal Diversity**, v. 23, p. 323-350, 2006.

SUTTON, B. C.; PASCOE, I. G. Addenda to *Harknessia* (Coelomycetes). **Mycological Research**, v. 92, n. 4, p. 431-439, 1989.

SUTTON, B. C. **The Coelomycetes. Fungi imperfcti with pycnidia, acervuli and stromata**. Kew: Commonwealth Mycological Institute, 1980. 696 p.

SWART, H. J. AUSTRALIAN LEAF-INHABITING FUNGI III. OBSERVATIONS ON *HARKNESSIA*. **Transactions British Mycological Society**, v. 59, n. 2, p. 309- 311, 1972.

THOMAS, D.; HENSON, M.; JOE, B.; BOYTON, S.; DICKSON, R. Review of growth and wood quality of plantation-grown *Eucalyptus dunnii* Maiden. **Australian Forestry**, v. 72, n. 1, p. 3-11, 2009.

TEODORO, M. G.; FERREIRA, M. A.; GUIMARÃES, L. M. S.; MAFIA, R. G.; GRONEWALD, J. Z.; CROUS, P.; ALFENAS, A. C. *Mycosphaerella* and *Teratosphaeria* species associated with leaf diseases on *Eucalyptus globulus* in southern Brazil. **Phytopathologia Mediterranea**, v. 51, n. 2, p. 355−364, 2012.

YUAN, Z.; WARDLAW, T.; MOHAMMED, C. *Harknessia* species occurring on eucalypt leaves in Tasmania, Australia. **Mycological Research**, v. 104, n. 7, p. 888-892, 2000.

YUAN, Z.; MOHAMMED, C. Investigation of fungi associated with stem cankers of eucalypts in Tasmania, Australia. **Australasian Plant Pathology,** v. 26, p. 78-84, 1997.

WINGFIELD, J. W.; ROUX, J.; SLIPPERS, B.; HURLEY, B. P.; GARNAS, J.; MYBURG, A.; WINGFIELD, B. D. Established and new technologies reduce increasing pest and pathogen threats to Eucalypt plantations. **Forest Ecology and Management**. v. 301, p. 35–42, 2013.

GENERAL MATERIAL AND METHODS

The following material and methods were applied for all the three chapters presented herein.

Observation of leaf blight and isolation

In July 2014, we observed leaf blight on mature leaves of the lower crown of 5- to 7-year-old *E. dunnii* stands in Northern Santa Catarina State, southern Brazil (27°31.038'S, 49°54.441'W). The symptoms observed were irregular or circular leaf spots, with necrotic lesions of light brown color and pycnidia on the lesions. We collected diseased leaves to identify the organism associated with the leaf blight. After washing the leaves with water and liquid detergent, conidial masses were collected with a needle from conidiomata on diseased leaves and transferred directly under sterile conditions to Petri dishes containing malt extract agar (MEA) medium. The resulting cultures of the single isolate named APO1 were incubated at 25 °C.

DNA extraction and amplification

Monosporic fungal colonies, obtained by isolation and cultivation of a single spore of *A. eucalyptorum*, cultured on liquid malt extract medium incubated at 25 °C shacked at 60 RPM, and cultures on Petri dishes containing malt extract agar (MEA) medium were used for DNA extractions. As the cultures were of sufficient size, we extracted genomic DNA using the CTAB method and the *Quick*-DNA™ Universal Kit (Zymo Research Corp, Irvine, California, USA) from a sample of 1.0 g macerated with liquid nitrogen. The quantity of nucleic acid ranged from 10.4 to 20.3 $ng.\mu L^{-1}$, and the quality of the DNA extractions were performed in agarose gel 0.8%, evaluating the integrity of the resulting bands. We validated the taxonomic identity of the isolate by PCR amplification of the internal transcribed spacer 1 - 5.8S - internal transcribed spacer 2 (ITS) region of ribosomal RNA using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (CTCCGCTTATTGATATGCT) specific primers. Isolate identity was confirmed by PCR amplification of the ß-Tubulin gene with primers BT2b (ACCCTCAGTGTAGTGACCCTTGGC) and BT-T1F (AACATGCGTGAGATTGTAAGT), and amplification of the Calmodulin gene with primers CAL228F (GAGTTCAAGGAGGCCTTCTCCC) and CAL737 (CATCTTTCTGGCCATCATGG). PCR amplicons were sequenced at LGC Genomics GmbH (Berlin, Germany). The sequences were

deposited in GenBank under the following accession numbers: MG725682, MH257599, and MH257600.

Isolate identification and characterization

We compared the resulting ITS, ß-Tubulin, and Calmodulin sequences with available sequences in GenBank using BLAST to confirm pathogen identity. The isolate APO1 was deposited at the Forest Pathology Laboratory of the Universidade Estadual do Centro-Oeste (UNICENTRO), in Irati, Paraná, Brazil. The isolate was examined under a microscope to characterize and measure the length of 50 conidia.

CHAPTER 1 - First report of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* in Brazil

ABSTRACT

In 2014 leaf blight and leaf spots caused by a fungus were observed on leaves of the lower crown of *Eucalyptus dunnii* trees in Southern Brazil. The fungus was isolated and cultivated on malt extract agar medium (MEA) at 25 °C, and herein we present the identification of the causal agent, describing the conidia, phylogeny, the symptoms of infection on trees, and the inoculations to determine the pathogenicity of the fungus. The causal agent of leaf blight on *Eucalyptus dunnii* in Southern Brazil was identified by sequencing of ITS, ß-Tubulin and Calmodulin regions. The inoculations were on leaves of *E. dunnii* trees of 18 months. The inoculation methodologies were: inoculation of MEA discs colonized by *A. eucalyptorum*, and conidia suspension with concentration of $1x10^6$ conidia.mL^{-1}. Inoculations were on leaves punched with a needle, scraped with a needle, and leaves without lesions. The sequences analyzed showed 99% identity to the type isolate of *Apoharknessia eucalyptorum*, being this study the first report of *A. eucalyptorum* in Brazil, and on *Eucalyptus dunnii*. The morphology of the conidia, by the presence of appendages on conidia, color, and the size were also in the range described for the type isolate of *A. eucalyptorum*. Moreover, phylogenetic analysis confirmed the identity of the isolate from Southern Brazil. After inoculation the fungus caused disease mainly on wounded leaves, with irregular or circular spots with necrotic, light- or dark-brown lesions, and re-isolation of *A. eucalyptorum* from inoculated leaves confirmed the pathogenicity of the fungus, and that *E. dunnii* is a host for this leaf blight. Because the known distribution of *A. eucalyptorum* is limited, no analysis of the impacts of the disease on tree growth has been conducted, and more detailed studies are needed on the etiology and epidemiology of this leaf disease.

Keywords: pathogenicity; inoculation; leaf spots; leaf disease.

Primeiro relato de *Apoharknessia eucalyptorum* em *Eucalyptus dunnii* no Brasil

RESUMO

Em 2014 manchas foliares causadas por um fungo foram observadas em folhas da base da copa de árvores de *Eucalyptus dunnii* no Sul do Brasil. O fungo foi isolado e cultivo em meio extrato de malte agar (MEA) a 25 °C, e neste capítulo apresentamos a identificação do agente causal, descrevendo os conídios, a filogenia, os sintomas de infecção nas árvores, e as inoculações para determinar a patogenicidade do fungo. O agente causal das manchas foliares em *E. dunnii* no

Sul do Brasil foi identificado pelo sequencimaneto das regiões ITS, ß-Tubulina e Calmodulina. As inoculações foram em folhas de árvores de *E. dunnii* de 18 meses. As metodologias de inoculação foram: inoculação de discos de MEA colonizados por *A. eucalyptorum*, e suspensão de conídios com concentração de $1x10^6$ conidia.mL^{-1}. As inoculações foram em folhas perfurados com agulha, raspadas com agulha, e folhas sem lesões. As sequencias analisadas mostraram 99% de identidade com o isolado tipo de *Apoharknessia eucalyptorum*, sendo este estudo o primeiro relato de *A. eucalyptorum* no Brasil, e em *E. dunnii*. A morfologia dos conídios, pela presença de apêndices, coloração, e tamanho também estavam na amplitude descrita para o isolado tipo de *A. eucalyptorum*. Além disso, a análise filogenética confirmou a identidade do isolado do Sul do Brasil. Após a inoculação o fungo causou doença principalmente nas folhas com lesão, com manchas irregulares ou circulares com lesões necróticas, de coloração marrom clara ou escura, o reisolamento de *A. eucalyptorum* das folhas inoculadas confirmou a patogenicidade do fungo, e que *Eucalyptus dunnii* é hospedeiro dessa mancha foliar. Uma vez que a distribuição conhecida de *A. eucalyptorum* é limitada, nenhuma análise dos impactos da doença no crescimento das árvores foi conduzida e estudos mais detalhados são necessários sobre a etiologia e a epidemiologia desta doença foliar.
Palavras-chave: patogenicidade; inoculação; manchas foliares; doença foliar.

1. INTRODUCTION

Eucalyptus dunnii is recognized as a species that can grow well in relatively cold regions where frosts occur (ARNOLD et al., 2015) and, as such, it is currently grown in subtropical regions of Brazil for use in paper production. However, serious pests and diseases are becoming increasingly harmful to wood production in eucalypt (*Eucalyptus*) stands (WINGFIELD et al., 2013).

In South America, the number of recognized eucalypt diseases are increasing, including diseases caused by *Mycosphaerella* spp. and *Teratosphaeria* spp. (BERNREITER et al., 2016). Another disease caused by *Apoharknessia insueta* has been documented on eucalypts in South America and other continents (CROUS et al., 2012; LEE; GROENEWALD; CROUS, 2004), but limited information is available about the potential impacts of the disease. In 2017, a new species of the genus known as *Apoharknessia eucalyptorum* was described by Crous et al. (2017) as affecting eucalypts in Malaysia. The fungus was also isolated in association to leaf spots and leaf blight on old leaves of the lower crown of *Eucalytus dunnii* trees in 2014 in Southern Brazil.

The type isolate of *Apoharknessia eucalyptorum* was considered by Crous et al. (2017) as endophytic. However, the first species of the genus *Apoharknessia*, *A. insueta* described in 2004 (LEE; GROENEWALD; CROUS, 2004), is considered pathogenic (PARK et al., 2000; FAO, 2009). Moreover, the genus *Apoharknessia* is associated to the genus *Harknessia*, that is also composed by pathogenic species to *Eucalyptus* spp. (YUAN; MOHAMMED, 1997; PARK et al., 2000; CROUS et al., 2012). Although pathogenic, information about Harknessia species is also limited (CROUS et al., 2012), and the studies of Apoharknessia species are mainly focused on taxonomy (LEE; GROENEWALD; CROUS, 2004; ROSMANN; FARR; CASTLEBURY, 2007; CROUS et al., 2012; MAHARACHCHIKUMBURA et al., 2015; CROUS et al., 2017; SENANAYAKE et al., 2017; FAN et al., 2018). Thus, studies about the morphology, phylogeny, and pathogenicity of *A. eucalyptorum* are needed to resolve the association of the species with its hosts.

Herein, we provide the first identification and description of *A. eucalyptorum* as a causal agent of leaf blight in Brazil, and on *E. dunnii*. This fungal pathogen was isolated, morphologically and phylogenetically described, inoculated on leaves, and re-isolated from leaves that displayed leaf blight symptoms, proving the pathogenicity of *A. eucalyptourm*.

2. MATERIAL AND METHODS

2.1 Phylogenetic analysis

To confirm the identity of the isolate APO1 obtained in Southern Brazil a phylogenetic tree was performed. We constructed phylogenetic tree based on concatenated ITS, ß-Tubulin, and Calmodulin sequences of *A. eucalyptorum, A. insueta* and *Harknessia* species obtained from GenBank using the NCBI Genome Workbench (https://www.ncbi.nlm.nih.gov/projects/gbench/) (Table 1), constructing the tree using MEGA 7.0.26 (https://www.megasoftware.net/) according to the maximum parsimony tree method with 1000 bootstraps.

2.2 Inoculation and pathogenicity test

After isolate identification, we incubated the *A. eucalyptorum* monosporic isolate on MEA in Petri dishes at 25 °C with a 12-hour photoperiod for 2 weeks. After sporulation on MEA, we prepared a conidial suspension by adding 20 ml of sterilized water and scraping the surface of the colony with a sterile bent glass rod. The suspension was subsequently filtered with gauze and the conidial concentration determined in a Neubauer chamber, adjusting the conidial suspension to $1x10^6$ spores per ml (Appendix I). After preparing the conidial suspension, we tested two inoculation methods: 1) *A. eucalyptorum* conidial suspension; and 2) MEA discs (10 mm diameter) colonized by *A. eucalyptorum* (Appendix II). We applied both inoculation methods to: trees leaves without lesions; trees leaves punched with a needle; and trees leaves scraped with a needle. The control treatment included seedlings leaves scraped with a needle inoculated with sterile MEA discs (10 mm diameter) (Appendix III). Before inoculation, the leaf surface was disinfested with 70% ethanol and 1% sodium hypochlorite using a spray bottle and then washed twice with sterile water (Appendix IV). After inoculation, trees leaves were incubated for 48 hours. Each inoculation method and control were conducted on five leaves from three *E. dunnii* trees (18-months old) grown under outside nursery conditions in 5-liter pots, for a total of 15 inoculated leaves per plant. At 2-weeks post inoculation, we collected conidia from inoculated leaves and examined the samples under a microscope to compare morphological features with those of the cultures incubated *in vitro*, as well as determine the mean size of 50 conidia, and fulfill Koch's postulates (Appendix V).

Table 1. Accession numbers of the fungal isolates used for the phylogenetic analyses.

Species	Isolate	Accession numbers in GenBank		
		ITS	ß-Tubulin	Calmodulin
Apoharknessia insueta	CPC1451	JQ706083	-	-
Apoharknessia insueta	CPC11775	JQ706082	-	-
Apoharknessia insueta	LTL313	MF663566	-	-
Apoharknessia eucalyptorum	APO1	MG725682	MH257599	MH257600
Apoharknessia eucalyptorum	CBS142519	KY979752	KY979908	KY979866
Discula destructiva	CBS109771	EF512464	EU219092	EF512506
Harknessia eucalypti	CBS342.97	AY720745	AY720777	AY720808
Harknessia eucalypti	CPC13643	JQ706089	JQ706134	JQ706175
Harknessia eucalyptorum	CBS113620	AY720746	AY720778	AY720809
Harknessia eucalyptorum	CPC85	AY720747	AY720779	AY720810
Harknessia eucalyptorum	CPC11302	JQ706090	JQ706135	JQ706176
Harknessia eucalyptorum	CPC12697	JQ706091	JQ706136	JQ706177
Harknessia eucalyptorum	CPC14951	JQ706093	JQ706138	JQ706178
Harknessia fusiformis	CPC13649	JQ706097	JQ706140	JQ706180
Harknessia hawaiiensis	CBS114811	AY720723	AY720755	AY720786
Harknessia hawaiiensis	CBS111122	AY720726	AY720758	AY720789
Harknessia hawaiiensis	CBS110728	AY720725	AY720757	AY720788
Harknessia hawaiiensis	CBS115650	AY720724	AY720756	AY720787
Harknessia hawaiiensis	CPC15003	JQ706107	JQ706150	JQ706191
Harknessia hawaiiensis	CPC11013	JQ706106	JQ706149	JQ706190
Harknessia malayensis	CBS142544	KY979789	KY979941	KY979879
Harknessia pseudohawaiiensis	CPC13001	JQ706109	JQ706153	JQ706194
Harknessia pseudohawaiiensis	CPC17300	JQ706110	JQ706154	JQ706195
Harknessia pseudohawaiiensis	CPC17379	JQ706111	JQ706155	JQ706196
Wuestneia molokaiensis	CPC11127	JQ706123	JQ706166	JQ706206
Wuestneia molokaiensis	CPC12995	JQ706125	JQ706168	JQ706207
Wuestneia molokaiensis	CPC13859	JQ706126	JQ706169	JQ706208

3. RESULTS

Based on the NCBI-BLAST analysis of DNA sequences, the isolate APO1 obtained from *E. dunnii* leaves, the ITS sequence (deposited in GenBank under Accession Number MG725682) showed 99% identity with an *A. eucalyptorum* isolate [GenBank Accession Number KY979752; Identity = 627/631(99%), 2 gaps], compared to 93% identity with *A. insueta* isolates [GenBank Accession Number JQ706083; Identity = 572/616 (93%), 30 gaps; and GenBank Accession Number MF663566; Identity = 560/604 (93%), 30 gaps]. The ß-Tubulin sequence of the isolate APO1 (GenBank Accession Number MH257599) showed 99% identity to the *A. eucalyptorum* sequence [GenBank Accession Number KY979919; Identity = 619/626 (99%), 1 gap]. Similar results were found for the Calmodulin sequence (GenBank Accession Number MH257600), with 99% identity to the *A. eucalyptorum* sequence deposited in GenBank under Accession Number KY979867 [Identity = 495/498 (99%), 1 gap].

The phylogenetic analysis, based on concatenated ITS, ß-Tubulin and Calmodulin sequences, indicates that the 641 bp ITS sequence from isolate APO1 (GenBank Accession Number MG725682) resides in a clade containing *A. eucalyptorum* (GenBank Accession Number KY979752.1) with 100% bootstrap support (Figure 1). On MEA at 25 °C, *A. eucalyptorum* sporulated within 1 week, with pale white mycelium on the medium surface and dark pycnidioid conidiomata in the center. We observed pycnidioid conidiomata containing conidia that were smooth, brown, and globose in shape, as well as conidial masses on the leaf surface or erumpent, forming globose structures across the leaf surface (Figure 2 A and B) that are easily wiped off. Conidia of isolate APO1 were 7 µm to 12.5 µm in length and 4.5 µm to 7 µm in width, with a conical apiculus and a hyaline basal appendage that was tubular, smooth, and thin-walled, with a length of 2.5 µm to 3.5 µm (Figure 2 C).

After inoculation tests, we initially observed leaf blight symptoms as small, water-soaked spots that were sparse across the leaf surface. Over the time, the spots became irregular or circular with necrotic, light- or dark-brown lesions with soaked, pale-brown borders; these symptoms mainly occurred on inoculated leaves with lesions. Inoculation tests on leaves fulfilled the pathogenicity test and Koch’s postulates, in which symptoms similar to those observed on leaves from *E. dunnii* stands occurred 2- to 3-weeks post inoculation. From the inoculated leaves with leaf blight symptoms, we re-isolated the pathogen using the method described above.

4. DISCUSSION

DNA sequences from three regions (ITS, ß-Tubulin and Calmodulin) of isolate APO1, which was obtained from *E. dunnii* with leaf blight symptoms in Brazil, were 99% identical to the *A. eucalyptorum* sequences from *Eucalyptus pellita* in Malaysia, upon which Crous et al. (2017) based the species description. In contrast, ITS sequences of isolate APO1 were only 93% identical with *A. insueta*, a pathogen currently present in Brazil, as observed by Crous et al. (2017). *Apoharknessia insueta* and *A. eucalyptorum* are similar in appearance. *Apoharknessia insueta* was separated from *Harknessia insueta* based on the occurrence of conidia with a basal appendage (LEE; GROENEWALD; CROUS, 2004), whereas *A. eucalyptorum* was proposed by Crous et al. (2017) based on the host genus *Eucalyptus*, differences in conidial size, and ITS sequences (CROUS et al., 2017). The colony of isolate APO1 had the same characteristics as those described by both Crous et al. (2017) for *A. eucalyptorum* and Lee, Groenewald and Crous (2004) for *A. insueta*. The identification process requires detailed morphological characterization and DNA sequencing, thus illustrating the difficulty in distinguishing *A. eucalyptorum* from *A. insueta*. One of the main characteristics that is helpful in distinguishing these species is conidial size, as we observed conidia of 7 µm to 12.5 µm in length and 4.5 µm to 7 µm in width for isolate APO1, which is typical of *A. eucalyptorum* (CROUS et al., 2017). Another characteristic is the presence of the conical apiculus and a hyaline basal appendage.

Apoharknessia insueta has been identified in northern Brazil occurring on *Eucalyptus pellita* F. Muell, in Colombia and in Mauritius on other eucalypti species (LEE; GROENEWALD; CROUS, 2004), recently in southeastern Brazil (GenBank Accession Number: MF663566), and in Costa Rica on *Yucca elephantipes* (CROUS et al., 2012). However, our study is the first to isolate and describe *A. eucalyptorum* occurring on leaves of *E. dunnii* in Brazil, and more specifically in a subtropical region of southern Brazil. It is also the first study to reproduce symptoms on eucalypt leaves. Although *A. eucalyptorum* is currently limited to the mountainous and central regions of Santa Catarina State, there is little information about symptomatology and susceptibility or resistance of eucalypt species to this leaf blight, or about the etiology, epidemiology, exact distribution, and possible impacts of *A. eucalyptorum* on plantations. Similarly, knowledge about the pathogenicity of *Harknessia* and *Apoharknessia* species and their impacts on eucalypt species is limited (YUAN; WARDLAW; MOHAMMED, 2000). Due to this lack of information, further studies are necessary, particularly as new isolates become available, to better understand the morphological and genetic diversity associated with these leaf pathogens of eucalypt.

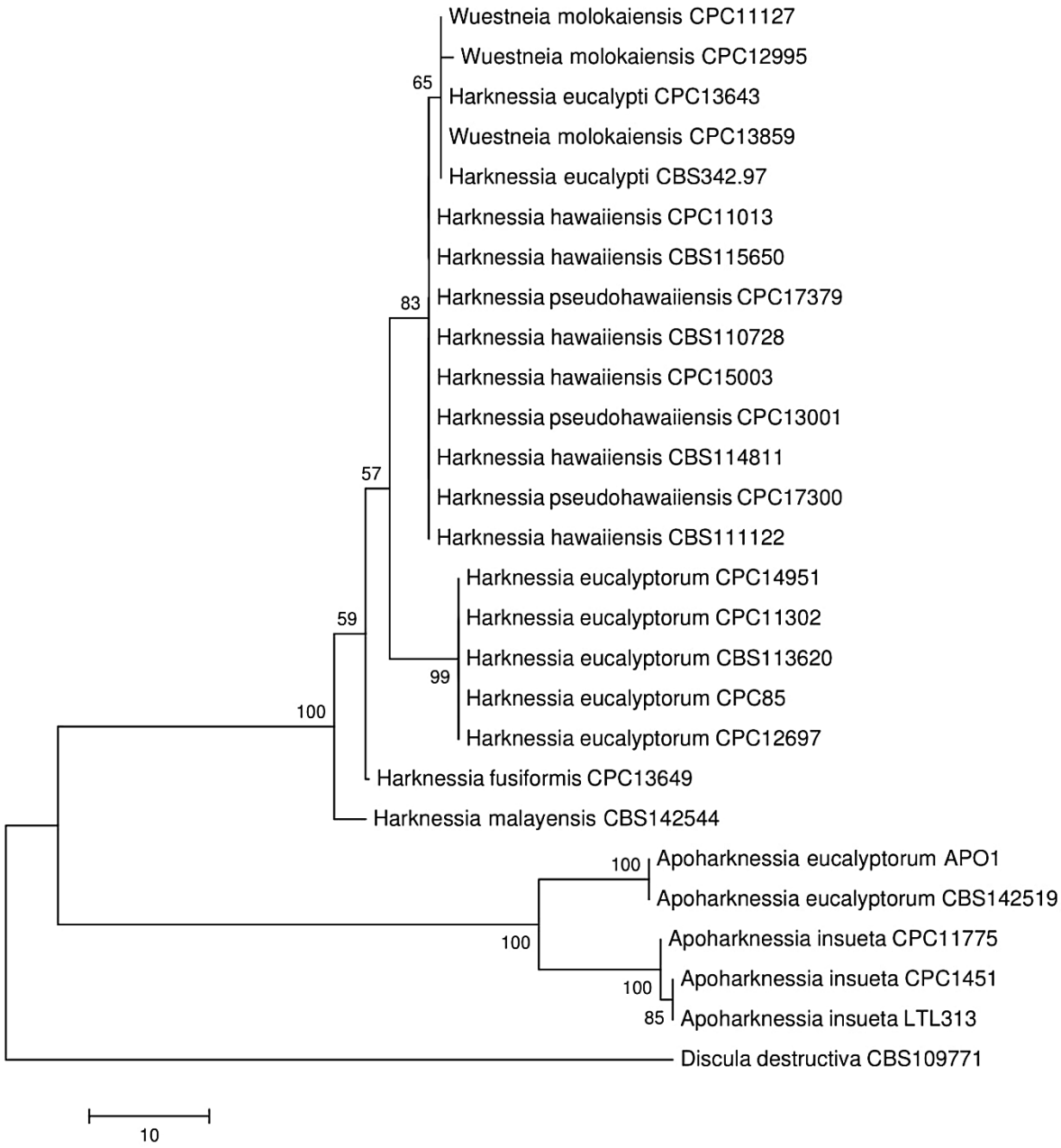


Figure 1. Concatenated maximum parsimony phylogenetic tree based on combined ITS, Calmodulin, and ß-Tubulin sequences of Harknessia and Apoharknessia species with bootstrap values. Outgroup: *Discula destructiva*. Source: The author.

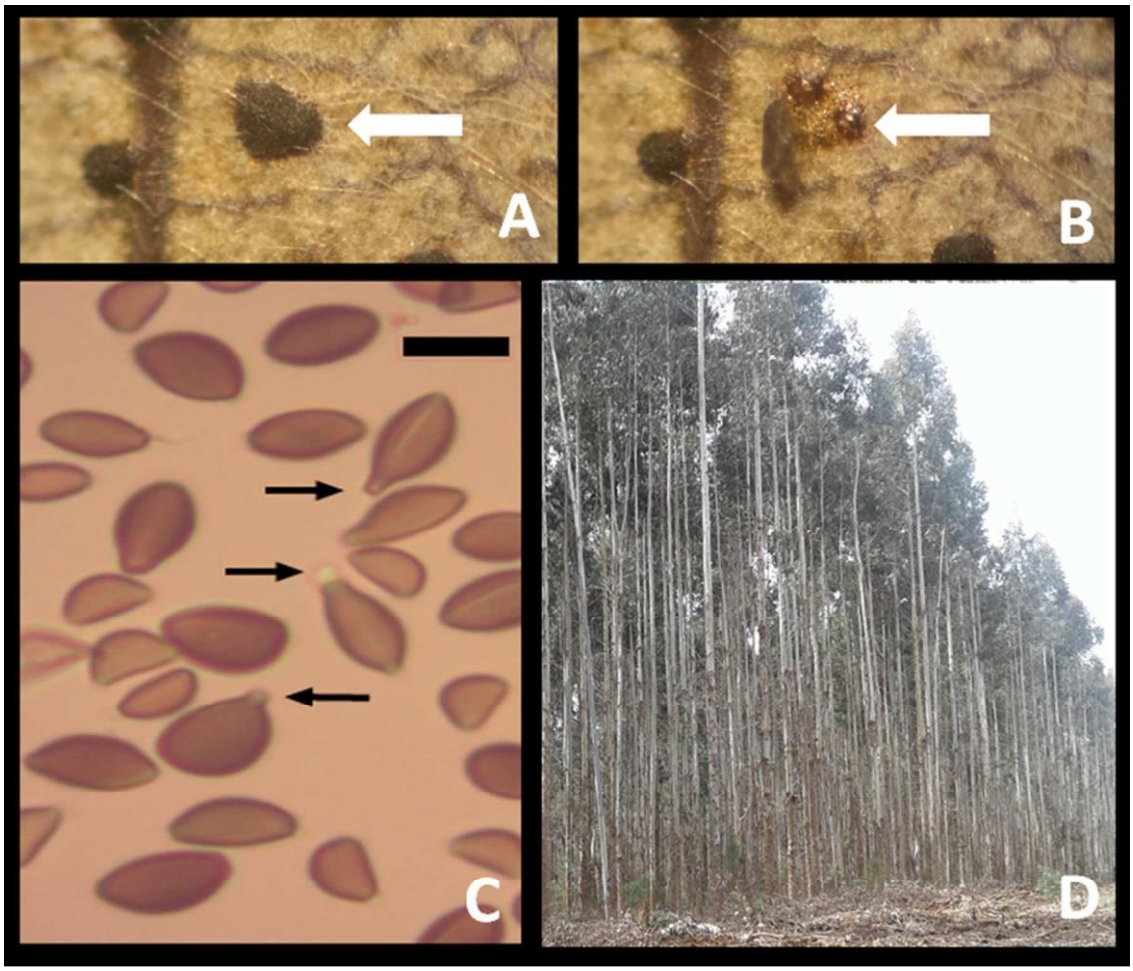


Figure 2. Structures of *Apoharknessia eucalyptorum* observed after inoculation. (A) Erumpent conidial masses over pycnidia on the leaf surface of *Eucalyptus dunnii*. (B) Detail of pycnidia behind the removed conidial masses. (C) Typical brown, globose conidia of *A. eucalyptorum* (Accession No MG725682) with hyaline basal appendage highlighted by arrows. Scale bar: 10 µm. (D) Eucalypt plantation (at 5 years of age) in the mountainous region of Santa Catarina where *A. eucalyptorum* was isolated. Source: The author.

5. REFERENCES

ARNOLD, R.; LI, B.; LUO, J.; BAI, F.; BAKER, T. Selection of cold-tolerant Eucalyptus species and provenances for inland frost susceptible, humid subtropical regions of southern China. **Australian Forestry**, n. 78, v. 3, p. 180-193, 2015.

BERNREITER, A.; TEIJEIRO, R. G.; GARRIDO, P.; RAMOS, L. *Mycosphaerella* and *Teratosphaeria* leaf spot diseases of *Eucalyptus globulus* in Ecuador. **Australasian Plant Disease Notes**, v. 11, n. 18, p. 1-3, 2016.

CROUS, P. W.; WINGFIELD, M. J.; BURGESS, T. I. et al. Fungal Planet Description sheets: 558-624 - *Apoharknessia eucalyptorum*. **Persoonia - Molecular Phylogeny and Evolution of Fungi**, v. 38: 270-271, 2017.

CROUS, P. W.; SUMMERELL, B. A.; SHIVAS, R. G.; CARNEGIE, A. J.; GROENEWALD, J. Z. A re-appraisal of *Harknessia* (*Diaporthales*), and the introduction of *Harknessiaceae* fam. nov. **Persoonia**, v. 28, p. 49–65, 2012.

FAN, X. L.; BEZERRA, J. D. P.; TIAN, C. M.; CROUS, P. W. Families and genera of diaporthalean fungi associated with canker and dieback of tree hosts. **Persoonia**, v. 40, p. 119 –134, 2018.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. Global review of forest pests and diseases. **FAO Forestry Paper**, 156. Rome: FAO, 2009. Available at: <http://www.fao.org/3/a-i0640e.pdf>. Accessed on: 18 Apr. 2018.

LEE, S.; GROENEWALD, J. Z.; CROUS, P. W. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apoharknessia* gen. nov. **Studies in Mycology**, v. 50, p. 235–252, 2004.

MAHARACHCHIKUMBURA, S.; HYDE, K.; JONES, E.; MCKENZIE, E.; HUANG, S.; ABDEL-WAHAB, M.; DARANAGAMA, D.; DAYARATHNE, M.; D'SOUZA, M.; GOONASEKARA, I.; HONGSANAN, S.; JAYAWARDENA, R.; KIRK, P.; KONTA, S.; LIU, J.; LIU, Z.; NORPHANPHOUN, C.; PANG, K.; PERERA, R.; SENANAYAKE, I.; SHANG, Q.; SHENOY, B.; XIAO, Y.; BAHKALI, A.; KANG, J.; SOMROTHIPOL, S.; SUETRONG, S.; WEN, T.; XU, J. Towards a natural classification and backbone tree for *Sordariomycetes*. **Fungal Diversity**, v. 72, p. 199–301, 2015.

PARK, R. F.; KEANE, P. J.; WINGFIELD, M. J.; CROUS, P. W. Fungal diseases of eucalypt foliage. In: KEANE, P. J; KILE, G. A.; PODGER, F. D.; BROWN, B. N. **Diseases and pathogens of eucalypts**. Collingwood: CSIRO publishing, 2000. 153–239.

ROSMANN, A. Y.; FARR, D. F.; CASTLEBURY, L. A. A review of the phylogeny and biology of the Diaporthales. **Mycoscience**, v. 48, n. 3, p. 135–144, 2007.

SENANAYAKE, I. C.; CROUS, P. W.; GROENEWALD, J. Z.; MAHARACHCHIKUMBURA, S. S. N.; JEEWON, R.; PHILLIPS, A. J. L.; BHAT, J. D.; PERERA, R. H.; LI, Q. R.; LI, W. J.; TANGTHIRASUNUN, N.; NORPHANPHOUN, C.; KARUNARATHNA, S.C.; CAMPORESI, E.; MANAWASIGHE, I. S.; AL-SADI, A. M.; HYDE, K. D. Families of Diaporthales based on morphological and phylogenetic evidence. **Studies in Mycology**, v. 86, p. 217–296, 2017.

WINGFIELD, M. J.; ROUX, J.; SLIPPERS, B.; HURLEY, B. P.; GARNAS, J. R.; MYBURG, A. A.; WINGFIELD, B. D. Established and new technologies reduce increasing pest and pathogen threats to eucalypt plantations. **Forest Ecology and Management**, 301, 35-42, 2013.

YUAN, Z.; WARDLAW, T.; MOHAMMED, C. *Harknessia* species occurring on eucalypt leaves in Tasmania, Australia. **Mycological Research**, v. 104, n. 7, p. 888-892, 2000.

YUAN, Z.; MOHAMMED, C. Investigation of fungi associated with stem cankers of eucalypts in Tasmania, Australia. **Australasian Plant Pathology,** v. 26, p. 78-84, 1997.

CHAPTER 2 – Morphophysiological characterization of *Apoharknessia eucalyptorum* on different culture media

ABSTRACT

Apoharknessia eucalyptorum was first described in 2017 and identified on leaves of *Eucalyptus dunnii* in Southern Brazil. However, information about *in vitro* cultivation for comparative analyses are lacking. In the present study, the growth and sporulation of *A. eucalyptorum* were evaluated at temperatures of 15, 20, and 25 °C on four culture media: malt extract agar (MEA); potato dextrose agar (PDA); V8 juice agar (V8); and bean dextrose agar (BEAN); under constant lighting. The best conditions for mycelial growth were at 25 °C on PDA, BEAN, and MEA. For sporulation, optimal conditions were 25 °C for all tested media, and 20 °C on PDA and BEAN. Colony characteristics changed with temperature; at 15 °C colonies formed a fluffy mycelium, whereas at 25 °C mycelium spread across the media forming dark margins lined by dirty-white mycelium and conidia. The conditions indicated for *in vitro* growth and sporulation of *A. eucalyptorum* are culture media MEA, PDA, and BEAN at 25 °C.

Keywords: colony characteristics; sporulation; growth; inoculation; temperature.

Caracterização morfofisiológica de *Apoharknessia eucalyptorum* em diferentes meios de cultura

RESUMO

Apoharknessia eucalyptorum foi inicialmente descrita em 2017 e identificada em folhas de *Eucalyptus dunnii* no Sul do Brasil. No entanto, faltam informações sobre o cultivo *in-vitro* para estudos complementares. No presente estudo o crescimento e esporulação *A. eucalyptorum* foram avaliados em temperaturas de 15 , 20 e 25 °C em quatro meios de cultura: extrato de malte-ágar (MEA), batata dextrose-ágar (PDA), suco V8-ágar (V8) e feijão dextrose-ágar (BEAN), sob luz constante. As melhores condições para o crescimento micelial foram em 25 °C em PDA, BEAN e MEA. Para a esporulação as melhores condições foram a 25 °C em todos os meios testados, e também a 20 °C em PDA e BEAN. As características das colônias mudaram com as temperaturas, em 15 °C as colônias formaram micélio cotonoso, enquanto que em 25 °C o micélio cresceu espalhado pelo meio de cultura formando margens escuras limitadas por micélio branco manchado e conídios. As condições indicadas para o crescimento e esporulação *in-vitro* de *A. eucalyptorum* são os meios MEA, PDA e BEAN em 25 °C.

Palavras-chave: características de colônia; esporulação; crescimento; inoculação; temperatura.

1. INTRODUCTION

Several pathogens can limit growth or reduce yield of eucalypt seedlings and plantations. *In vitro* cultivation is a key process in characterizing the size, color, and shape of phytopathogen structures and colonies for molecular studies and necessary to understand host-pathogen interactions. Thus, defining the best and most efficient (BATISTA; FERNANDES, 2015) culture media for a pathogen is the first step to further develop pathogenic studies (KOLEY; MAHAPATRA, 2015).

One pathogen that lacks information about *in-vitro* cultivation is *Apoharknessia eucalyptorum*. The genus *Apoharknessia* was separated from *Harknessia* by Lee, Groenewald and Crous (2004) and proposed due to phylogenetic clustering and hyaline conidia with apical appendage. The genus *Apoharknessia* consists of three species: *A. insueta* (LEE; GROENEWALD; CROUS, 2004), *A. eucalyptorum* (CROUS et al., 2017), and *A. eucalypti* (MARIN-FELIX et al., 2018). *Harknessia* spp., *A. insueta*, and *A. eucalyptorum* are eucalypt pathogens (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2012b; GARRETT et al., 2018; FAO, 2018) that cause leaf spots, leaf scorch, and cankers (YUAN; MOHAMMED, 1997; CROUS et al., 2012a). *Harknessia* species, some of which occur in Brazil (AUER; SANTOS, 2011), are better studied and considered economically significant as they have been shown to cause damage to plantations (RUPEREZ, A., MUÑOZ, 1980; LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2007). Although these species are better studied, there is still a lack of information about their pathogenicity (CROUS et al., 2012a), particularly for *Apoharknessia*. Moreover, some species are limited to specific regions and not aggressive, for example with only a few species occurring in Australia (YUAN; WARDLAW; MOHAMMED, 2000).

Currently, *A. insueta* is associated with leaf spots in the United States, Brazil, Colombia, Costa Rica, Cuba, Mauritius, and Zimbabwe (CROUS; KNOX-DAVIES; WINGFIELD, 1989; LEE; GROENEWALD; CROUS, 2004; MONTILLA; RODRIGUEZ, 2003; CROUS et al., 2012a), while *A. eucalyptorum* is present in Malaysia (CROUS et al., 2017) and Brazil (GARRETT et al., 2018), and *A. eucalypti* is restricted to Malaysia (MARIN-FELIX et al., 2018). Although Crous et al. (2017) consider that *Apoharknessia eucalyptorum* is endophytic, one isolated from lower crown leaves of *Eucalyptus dunnii* in Southern Brazil was demonstrated to be pathogenic (GARRETT et al., 2018).

Apoharknessia and Harknessia share similar morphophysiological characteristics. The similarity is observed for the conidia, mycelium, and sporulation (LEE; GROENEWALD; CROUS, 2004). *Harknessia* spp. are characterized by branched, septate, hyaline to pale brown

mycelium usually sporulating in one month (CROUS et al., 2012a), forming conidiomata (LEE; GROENEWALD; CROUS, 2004), while *Apoharknessia* species sporulate sooner than *Harknessia*, produce white mycelium, and sporulates on naked hyphae (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2017). Then, the morphophysiological characteristics of *A. eucalyptorum* must yet be determined.

To obtain pure *in vitro* cultures for further studies, microorganisms are cultivated on media to promote microorganism growth under laboratory conditions (MADIGAN et al., 2010). The main nutrients needed for microbiological growth are carbon, nitrogen, phosphorus, sulfur, potassium, magnesium, calcium, and sodium, and each microorganism has different metabolic requirements in terms of nutrients (MADIGAN et al., 2010). Physical factors must also be considered in promoting the growth of microorganisms, such as temperature, light regime, and pH (MADIGAN et al., 2010; DUBEY; MAHESHWARI, 2011; SU; QI; CAI, 2012).

Thus, due to the limited information about *in vitro* cultivation of *A. eucalyptorum*, this study characterized and evaluated mycelial growth and sporulation of *A. eucalyptorum* on four culture media at three temperatures to determine optimal conditions to cultivate the species.

2. MATERIAL AND METHODS

2.1 Culture media

Four culture media were tested to cultivate the isolate APO1 of *A. eucalyptorum*: Malt Extract Agar (MEA); Potato Dextrose Agar (PDA); V8 juice agar (V8) prepared according to Alfenas and Mafia (2007); and Bean dextrose agar (BEAN). The BEAN medium was prepared with 10 g of powdered beans, 20 g of dextrose, 20 g of agar, to 1 L of sterile water (REDA, 2017). The media were sterilized in an autoclave at 120 °C for 20 minutes, then 20 mL was poured onto 90 mm diameter Petri dishes under sterile conditions.

2.2 Experimental design

The experiment design was completely randomized as a factorial (culture medium x temperature) in a Bio-Oxygen Demand (BOD) incubator with controlled temperature and photoperiod. Petri dishes of all culture media were randomly placed in the BOD incubator, at three temperatures (15, 20, and 25 °C) with constant lighting. Each culture medium had five repetitions (Petri dishes) for each tested temperature.

2.3 Evaluation of mycelial growth and sporulation

One-centimeter discs from a sporulated monosporic culture of *A. eucalyptorum* cultured on MEA, were transferred to 90-mm Petri dishes containing each of the tested media. After seven days of incubation, mycelial growth was measured in length-wise, determining the growth rate (GR) in millimeters per day (mm/D). Sporulation was evaluated based on the number of spores per mL. To count the number of spores per mL, 20 mL of sterile water containing Tween 80 (0.05 %) was added to Petri dishes colonized by *A. eucalyptorum* and then scraped with a brush. The spore suspension was filtered with cheese cloth and counted in a Neubauer chamber.

2.4 Statistical analysis

Data on mycelial growth of *Apoharknessia eucalyptorum* and number of spores per mL in each medium were submitted to analysis of variance, and the averages compared by Tukey's

test at 5 % of significance ($\alpha \leq 0.05$). Data of mycelial growth was transformed by $\log_{10} X$, and sporulation data was transformed by $\log_{10} (X + 10)$.

3. RESULTS

The conidia of the APO1 isolate measured 7 to 12.5 µm in length and 4.5 to 7 µm in width, with a conical apiculus and a hyaline basal appendage that was tubular, smooth, and thin-walled, with a length of 2.5 to 3.5 µm (Figure 3 A and B).

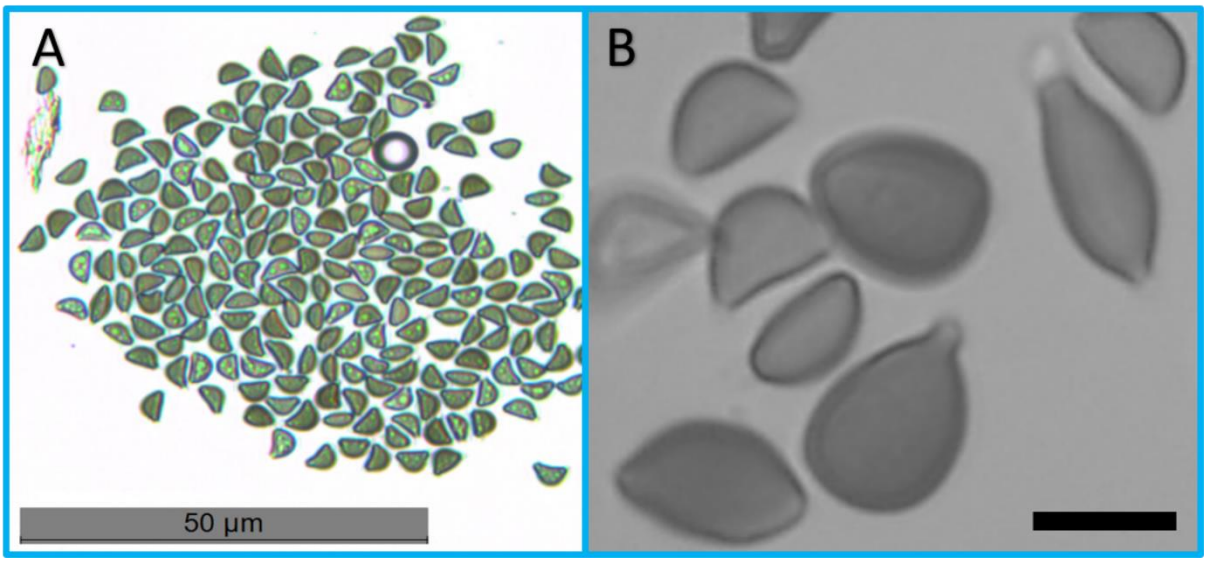


Figure 3. Spores of *Apoharknessia eucalyptorum*. (A) Conidia of *Apoharknessia eucalyptorum*. (B) Detailed conidia of *Apoharknessia eucalyptorum*, Scale: 10 µm. Source: The author.

For *A. eucalyptorum* mycelial growth, a significant interaction between media and tested temperature was observed (p = 0.0046). Mycelial growth increased with an increase temperature from 15 to 25 °C. Mycelial growth on each medium and at each temperature was greatest on PDA and lowest on V8 medium at 15 and 20 °C. However, mycelial growth at 25 °C was statistically the same for PDA, BEAN, and MEA (Table 2). In this study, the mycelia reached the edge of the Petri dishes on PDA and BEAN within six days.

Table 2. Average mycelial growth (millimeters) and growth rate of *Apoharknessia eucalyptorum* at temperatures of 15, 20, and 25 °C after seven days.

Culture Media	Diameter (mm) 15 °C	Diameter (mm) 20 °C	Diameter (mm) 25 °C	GR (mm/d) 15 °C	GR (mm/d) 20 °C	GR (mm/d) 25 °C
V8	26.06 Cc	37.05 Bc	47.65 Ab	3.72	5.29	6.81
MEA	44.02 Cb	68.48 Bb	89.16 Aa	6.29	9.78	12.74
PDA	50.69 Ca	77.00 Ba	90.00 Aa	7.24	11.00	15.00*
BEAN	40.04 Cb	63.17 Bb	90.00 Aa	5.72	9.02	15.00*
CV (%) 6.49						

Averages followed by the same uppercase letter in a row and lowercase letter in a column do not differ statistically according to Tukey's test ($\alpha \leq 0.05$). (CV) Coefficient of variation. (*) Growth Rate (GR) calculated six days after inoculation.

Interaction between medium and temperature was also significant ($p < 0.004$) for sporulation. Similarly to mycelial growth, sporulation of *A. eucalyptorum* increased as temperature increased. However, sporulation on V8 and MEA showed less conidia per ml than PDA and BEAN media at 15 or 20 °C. Sporulation at 20 and 25 °C sporulation was statistically the same for PDA and BEAN, and at 25 °C the number of conidia was the same for all tested media (Table 3). Sporulation was observed within four days for all media.

Table 3. Average sporulation (conidia.ml^{-1}) of *Apoharknessia eucalyptorum* at temperatures of 15, 20, and 25 °C after seven days.

Culture media	TEMPERATURE 15 °C	20 °C	25 °C
	Number of conidia x 10^5		
V8	0.56 Cc	2.95 Bb	11.28 Aa
MEA	1.45 Bb	2.30 Bb	10.40 Aa
PDA	2.68 Bab	15.35 Aa	9.65 Aa
BEAN	3.28 Ba	9.65 Aa	10.28 Aa
CV (%) 53.01			

Averages followed by the same uppercase letter in a row and lowercase letter in a column do not differ statistically according to Tukey's test ($\alpha \leq 0.05$). (CV) Coefficient of variation.

Figure 4 shows growth and sporulation characteristics in each tested medium. Mycelial growth and sporulation of *A. eucalyptorum* increased with greater temperatures, with growth and sporulation varying for the tested media. Mycelia were white and fluffy at 15 °C, becoming sparse, fine, and growing through the media with increased temperature.

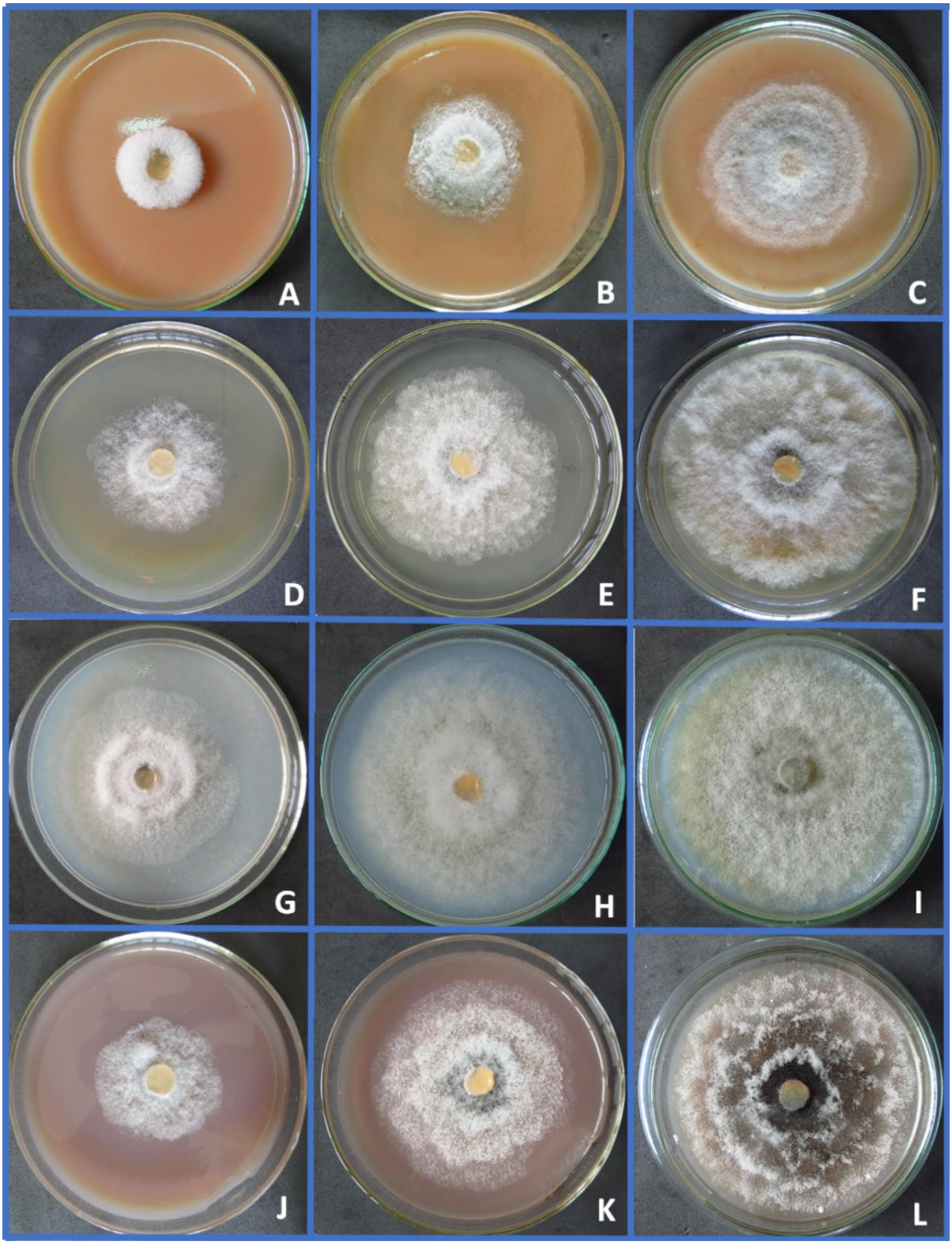


Figure 4. *In vitro* characteristics of mycelial growth and sporulation of *Apoharknessia eucalyptorum*. (A-D-G-J) 15 °C; (B-E-H-K) 20 °C; (C-F-I-L) 25 °C; (A, B, and C)

V8 medium; (D, E, and F) MEA medium; (G, H, and I) PDA medium; (J, K, and L) BEAN medium. Source: The author.

Viscous sporulation and conidia on naked hyphae were observed in the colonies (Figure 5 A and B), as seen in the dark area within the mycelia in the Figure 4 and Figure 5 D. Mycelial characteristics changed with an increase in temperature. At the beginning of growth, mycelium was filamentous (Figure 5 C), while at the end of the experiment, colonies formed halos of conidia lined by undulate to lobate dirty-white mycelium margins throughout the medium, with the presence of conidia on MEA and BEAN marked by dark areas (Figure 4). On MEA, the reverse side of the colonies showed olivaceous color (Figure 5 D); however, on V8, PDA, and BEAN this characteristic was less pronounced. The mycelium of *A. eucalyptorum* was brown and septate (Figure 6 A), and within the media tested was possible to observe the presence of conidiomata exuding and containing conidia of *A. eucalyptorum* (Figure 6 B).

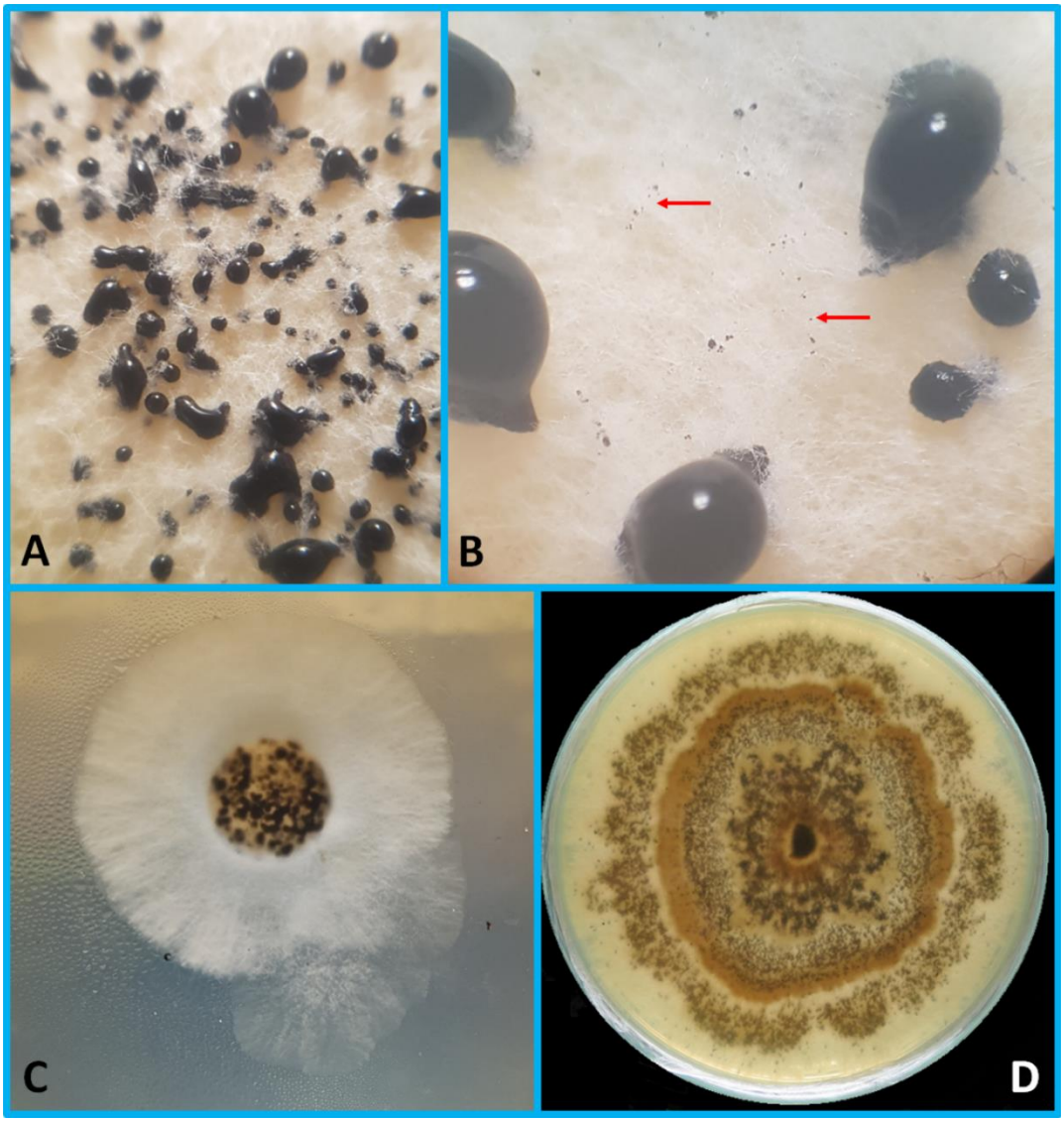


Figure 5. Characteristics of *Apoharknessia eucalyptorum* on MEA. (A) Viscous sporulation on

MEA. (B) Detail of viscous sporulation on MEA and conidia on naked hyphae (arrows). (C) Filamentous mycelia at the beginning of growth on MEA; (D) Reverse side of MEA colonized by *A. eucalyptorum* with olivaceous mycelia, and mycelia and sporulation forming borders on MEA. Source: The author.

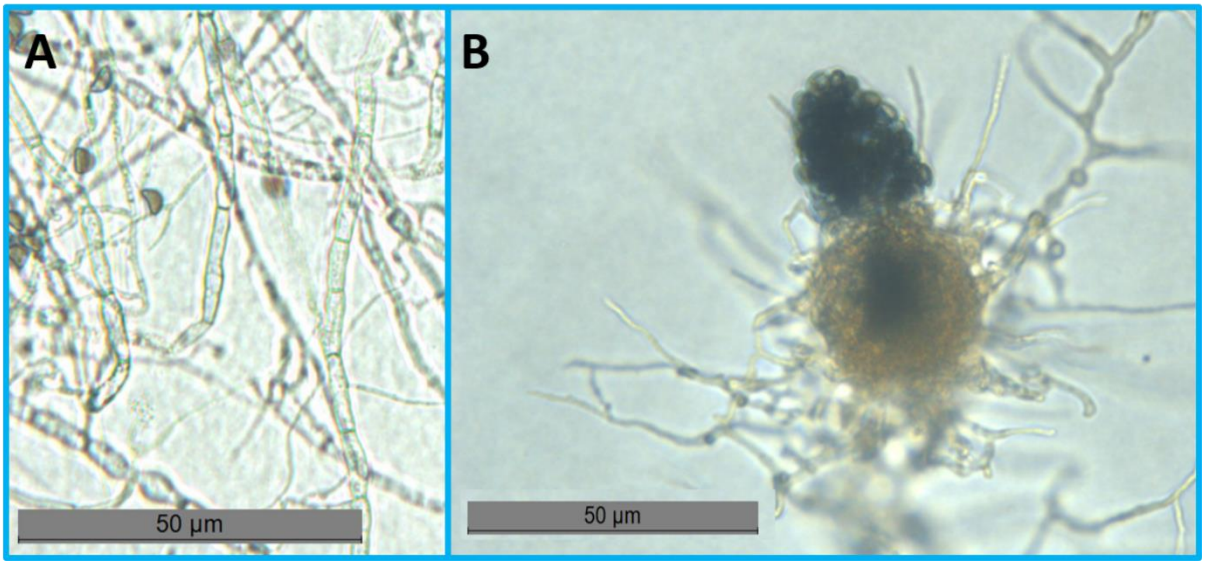


Figure 6. (A) Brown and septate mycelium of *Apoharknessia eucalyptorum* within the media tested. (B) Conidioma exuding and containing conidia of *Apoharknessia eucalyptorum*. Source: The author.

4. DISCUSSION

The ITS sequence confirmed the identity of the isolate APO1 as *A. eucalyptorum* when the resulting sequence was compared with the type isolate described by Crous et al. (2017) from Malaysia. The size of the conidia was in the range described by Crous et al. (2017) for the species, with the same morphology, showing the conical apiculus and hyaline basal appendage typical of the species. The discussion presented herein is based mainly on the related species of *Harknessia* and *A. insueta*, both of which considered pathogenic. Although pathogenic, there are few studies that discuss the ideal conditions for *in vitro* cultivation of Harknessia and Apoharknessia species, and such studies are important to obtain cultures, understand and manage host-pathogen interactions.

For different species of the related genus *Harknessia* greater growth rate is reported at different temperatures, for *Harknessia uromycoides* at 20 °C, and for *H. eucalyptorum* at 25 °C (CROUS; WINGFIELD; NAG RAJ, 1993), both cultured on MEA. Other *Harknessia* species have been cultured on Malt Extract Agar (MEA) at 25 °C for five to eight days to characterize the cultures (LEE; GROENEWALD; CROUS, 2004), as well as on Potato Dextrose Agar (PDA) (CROUS et al., 2012a) and tomato decoction (SWART, 1972). *Harknessia ipereneae* and *Harknessia gibbosa* grow better on PDA, and cover Petri dishes in two weeks with fluffy white mycelium (CROUS et al., 2007; CROUS et al., 2012a). Another isolate of *Harknessia* sp. from Venezuela was also cultured on PDA at 25 °C for 12 days (MONTILLA; RODRIGUEZ, 2003). Many *Harknessia* species have been cultured on MEA, PDA, and Oatmeal agar at 25 °C (CROUS et al., 2012a), while *H. renispora*, has been cultured on a medium based on a tomato or oatmeal decoction (SWART, 1972).

In this study, growth was greater at 25 °C for all tested media, with the greatest growth rate for MEA, PDA, and BEAN, the latter of which was developed recently for cultivation of *Pestalotiopsis* spp. (REDA, 2017). All the culture media tested in this study are natural media with uncertain nutrient contents, particularly in terms of nitrogen and carbon (ALFENAS et al., 2013). Nevertheless, the use of natural media is suitable due to ease of preparation and reduced cost (BASU et al., 2015). Moreover, while PDA has been shown to allow growth of different fungi (SHARMA; PANDEY, 2010), in this study the BEAN medium proved to be suitable for satisfactory growth and sporulation of *A. eucalyptorum*.

Production of spores *in vitro* is important for inoculation (ALFENAS et al., 2013) and taxonomic studies. *Harknessia* spp., such as *Harknessia eucalyptorum* (CROUS; WINGFIELD; NAG RAJ, 1993), usually sporulate after two weeks (CROUS et al., 2017), while *H. hawaiiensis* sporulates after one week and other species after two or more weeks

(CROUS; WINGFIELD; NAG RAJ, 1993). *Harknessia capensis* and *H. globispora* sporulate in a few days and have white mycelia with margins (LEE; GROENEWALD; CROUS, 2004). In comparison, the mycelial growth of *A. insueta* and *A. eucalyptorum* is quicker and sporulation occurs in one week, as described by Lee, Groenewald and Crous (2004) and Crous et al. (2017). Herein, sporulation was observed within four days for all tested media, and diameter reaching 90 mm within six days at 25 °C. Meanwhile, Crous et al. (2017) described growth to 70 mm in two weeks at 25 °C. This difference in growth is due to the light regime, since this study was done under constant lighting, which favored mycelial growth on all tested media. Even though sporulation occurred in a few days at 25 °C, mycelial growth on V8 did not reach the full diameter of the Petri dishes (90 mm). Growth on MEA across the entire dish was observed only after seven days, while it occurred within six days on PDA and BEAN, demonstrating that the tested media are suitable for *in vitro* cultivation of *A. eucalyptorum* with an intense light regime.

Another important aspect of sporulation *in vitro* is that the morphology of conidia can help to distinguish *Apoharknessia* from *Harknessia* and other fungal species (CROUS; WINGFIELD; NAG RAJ, 1993; FURLANETTO; DIANESE, 1998; FARR; ROSSMAN, 2003). According to Crous et al. (2012b), *Harknessia* species are characterized by stromatic or pycnidial conidiomata producing dark brown conidia with striations and tubular shaped basal appendages. In contrast, *Apoharknessia* species have brown conidia without striations, but with both basal and apical appendages, and *A. eucalyptorum* has a central guttule in conidia (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2012a; CROUS et al., 2017). Moreover, sporulation is also important as *A. eucalyptorum* differs from *A. insueta* in terms of the size of the conidia and the guttule in conidia (CROUS et al., 2017). *Apoharknessia eucalyptorum* also formed pycnidia, with conidial masses exuding from it (Appendix VI)

The morphology of the colonies was similar among the culture media with dirty-white mycelium growing sparsely within the medium, except on V8 and at 15 °C, *A. eucalyptorum* showed fluffy aerial mycelium. In contrast, the color of mycelium on *Harknessia* species vary and are usually fluffy (CROUS; WINGFIELD; NAG RAJ, 1993). On MEA, *H. rhabdosphaera* grows by spreading through the media, with fluffy dirty-cream mycelium, a surface color of buff to honey, and sporulation in black masses forming dark margins (SUMMERELL et al., 2006). These margins suggest similarity to *Harknessia* spp. and *A. eucalyptorum*, but variations in color and naked conidia on hyphae formed by *A. insueta* and *A. eucalyptorum* can help to distinguish these species.

According to Lee, Groenewald and Crous (2004), mycelium of *A. insueta* grows within the medium and sporulates on naked hyphae in one week, while *Harknessia* forms conidiomata

in vitro, and *A. eucalyptorum* has slimy sporulation on superficial mycelium according to Crous et al. (2017). Different culture media influence morphologic characteristics of fungi (SHARMA; PANDEY, 2010; KOLEY, S., MAHAPATRA, 2015), and such variations on different media can impede identification and distinction between species. For the different media tested growth pattern variation was observed only on V8 medium, and at the temperature of 15 °C. To distinguish *Harknessia* spp. from *Apoharknessia* spp., one of the characteristics used is the growth pattern and time of sporulation *in vitro* (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2017). Thus, cultivation and characterization of *in vitro* colonies are important to identify these related species.

In this study we present the first test of *in vitro* cultivation of *A. eucalyptorum* that is pathogenic to *E. dunnii* trees. However, there is a lack of information about its geographical distribution, inoculation methods, and potential impacts on plantations. Improving *in vitro* cultivation is a key step in molecular biology to understand the relation of a pathogen with different eucalypt hosts and for studies assessing the possible impacts of a pathogen on *Eucalyptus* spp. Based on our results, MEA, PDA, and BEAN are indicated for the cultivation of *Apoharknessia eucalyptorum* since they promoted better growth and sporulation of the species at 25 °C.

5. REFERENCES

ALFENAS, R. F.; PEREIRA, O. L.; FREITAS, R. G.; FREITAS, C. S.; DITA, M. A. D.; ALFENAS, A. C. Mass spore production and inoculation of *Calonectria pteridis* on Eucalyptus spp. under different environmental conditions. **Tropical Plant Pathology**, Brasília, v. 38, n. 5, p. 406-413, 2013.

ALFENAS, A. C.; MAFIA, R. G. **Métodos em fitopatologia**. Viçosa: Editora UFV, 2007. 382 p.

AUER, C. G.; SANTOS A. F. dos. Doenças em eucaliptos destinados à produção de energia na região Sul do Brasil. **Pesquisa Florestal Brasileira**, Colombo, v. 31, n. 68, p. 373-379, 2011.

BATISTA, K. A.; FERNANDES, K. F. Development and optimization of a new culture media using extruded bean as nitrogen source. **MethodsX**, Amsterdam, v. 2, p. 154–158, 2015.

BASU, S.; BOSE, C.; OJHA, N.; DAS, N.; DAS, J.; PAL, M.; KHURANA. S. Evolution of bacterial and fungal growth media. **Bioinformation**, Puducherry, v. 11, n. 4, p. 182–184, 2015.

CROUS, P. W.; WINGFIELD, M. J.; BURGESS, T. I. et al. Fungal Planet Description sheets: 558-624 - *Apoharknessia eucalyptorum*. **Persoonia - Molecular Phylogeny and Evolution of Fungi**, v. 38: 270-271, 2017.

CROUS, P. W.; SUMMERELL, B. A.; SHIVAS, R. G.; CARNEGIE, A. J.; GROENEWALD, J. Z. A. A re-appraisal of *Harknessia* (*Diaporthales*), and the introduction of *Harknessiaceae* fam. nov. **Persoonia**, Leiden, v. 28, p. 49–65, 2012a.

CROUS, P. W.; SUMMERELL, B. A.; ALFENAS, A. C.; EDWARDS, J.; PASCOE, I. G.; PORTER, I. J.; GROENEWALD, J. Z. Genera of diaporthalean coelomycetes associated with leaf spots of tree hosts. **Persoonia**, Leiden, v. 28, p. 66-75, 2012b.

CROUS, P. W.; MOHAMMED, C.; GLEN, M.; VERKLEY, G. J. M.; GROENEWALD, J. Z. *Eucalyptus* microfungi known from culture 3. *Eucasphaeria* and *Sympoventuria* genera nova, and new species of *Furcaspora*, *Harknessia*, *Heteroconium* and *Phacidiella*. **Fungal Diversity**, Dordrecht, v. 25, p. 19-36, 2007.

CROUS, P. W.; WINGFIELD, M. J.; NAG RAJ, T. R. *HARKNESSIA* SPECIES OCCURING IN SOUTH AFRICA. **Mycologia**, Lancaster, v. 85, n. 1, p. 108-118, 1993.

CROUS, P. W.; KNOX-DAVIES, P. S.; WINGFIELD, M. J. A List of Eucalyptus Leaf Fungi and their Potential Importance to South African Forestry. **Suid-Afrikaanse Bosboutydskrif**, Pretoria, v. 149, p. 17-29, 1989.

DUBEY, R. C.; MAHESHWARI, D. K. **Practical Microbiology**. 7. ed. Nova Delhi: S. Chand & Company Ltd, 2011. 413 p.

FARR, D. F.; ROSSMAN, A. Y. *Dwiroopa*, a coelomycetous genus with two species.

Mycoscience, Tokyo, v. 44, p. 443–446, 2003.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. Global review of forest pests and diseases. **FAO Forestry Paper**, 156. Rome: FAO, 2009. Available at: <http://www.fao.org/3/a-i0640e.pdf>. Accessed on: 18 Apr. 2018.

FURLANETTO, C.; DIANESE, J. C. Some coelomycetes from Central Brazil, **Mycological Research**, Amsterdam, v. 102, n. 1, p. 19-29, 1998.

GARRETT, A. T. de A.; IMANI, J.; TAMBARUSSI, E. V.; GARCIA, F. A. O.; KOGEL, K. H.; FIGUEIREDO FILHO, A. First report of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* in Brazil. **Forest Pathology**, e12463, 2018.

KOLEY, S.; MAHAPATRA, S. S. Evaluation of Culture Media for Growth Characteristics of *Alternaria solani*, Causing Early Blight of Tomato. **Journal of Plant Pathology & Microbiology**, Los Angeles, S1, p. 1-5, 2015.

LEE, S.; GROENEWALD, J. Z.; CROUS, P. W. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apoharknessia* gen. nov. **Studies in Mycology**, Utrecht, v. 50, p. 235–252, 2004.

MARIN-FELIX, Y.; HERNÁNDEZ-RESTREPO, M.; WINGFIELD, M. J.; AKULOV, A.; CARNEGIE, A. J.; CHEEWANGKOON, R.; GRAMAJE, D.; GROENEWALD, J. Z.; GUARNACCIA, V.; HALLEEN, F.; LOMBARD, L.; LUANGSA-ARD, J.; MARINCOWITZ, S.; MOSLEMI, A.; MOSTERT, L.; QUAEDVLIEG, W.; SCHUMACHER, R. K.; SPIES, C. F. J.; THANGAVEL, R.; TAYLOR, P. W. J.; WILSON, A. M.; WINGFIELD, B. D.; WOOD, A. R.; CROUS, P. W. Genera of phytopathogenic fungi: GOPHY 2. **Studies in Mycology**, v. 92, p. 47-133, 2018.

MADIGAN, M. T.; MARTINKO, J. M.; DUNLAP, P. V.; CLARK, D. P. **Microbiologia de Brock**. 12 ed. Porto Alegre: Artmed, 2010. 1160 p.

MONTILLA, J. O.; RODRIGUEZ, D. Comportamiento de cultivares de *Eucalyptus urophylla* S. T. Blake ante la necrosis foliar causada por *Harknessia* sp. **Revista de la Facultad de Agronomía**, Maracay, v. 20, p. 34-42, 2003.

REDA, F. R. **Caracterização morfofisológica e molecular, sintomatologia e severidade de *Pestalotiopsis* spp. STEYAERT em mudas de *Eucalyptus dunnii* MAIDEN**. Irati, 2017. 63 f. Dissertação (Mestrado em Ciências Florestais), Universidade Estadual do Centro-Oeste.

RUPEREZ, A.; MUÑOZ, C. Enfermedades de los eucaliptos en España. Boletín de sanidad vegetal. **Boletín de Sanidad Vegetal - Plagas**, Logroño, v. 6, p. 193-217, 1980.

SHARMA, G.; PANDEY R. R. Influence of culture media on growth, colony character and sporulation of fungi isolated from decaying vegetable wastes. **Journal of Yeast and Fungal Research**, Nairobi, v. 1, n. 8, p. 157–164, 2010.

SUMMERELL, B. A.; GROENEWALD, J. Z.; CARNEGIE, A.; SUMMERELL, R. C.; CROUS, P. W. *Eucalyptus* microfungi known from culture. 2. *Alysidiella*, *Fusculina* and *Phlogicylindrium* genera nova, with notes on some other poorly known taxa. **Fungal Diversity**, Dordrecht, v. 23, p. 323-350, 2006.

SU, Y-Y.; QI, Y-L.; CAI, L. Induction of sporulation in plant pathogenic fungi. **Mycology**, London, v. 3, n. 3, p. 195-200, 2012.

SWART, H. J. AUSTRALIAN LEAF-INHABITING FUNGI III. OBSERVATIONS ON *HARKNESSIA*. **Transactions of the British Mycological Society**, London, v. 59, n. 2, p. 309-311, 1972.

YUAN, Z.; WARDLAW, T.; MOHAMMED, C. *Harknessia* species occurring on eucalypt leaves in Tasmania, Australia. **Mycological Research**, Amsterdam, v. 104, n. 7, p. 888-892, 2000.

YUAN, Z.; MOHAMMED, C. Investigation of fungi associated with stem cankers of eucalypts in Tasmania, Australia. **Australasian Plant Pathology**, Berlin, v. 26, p. 78-84, 1997.

CHAPTER 3 – Aggressiveness and symptomatology of *Apoharknessia eucalyptorum* on leaves and trunks of Eucalyptus and Corymbia

ABSTRACT

Apoharknessia eucalyptorum has been associated with leaf spots on *Eucalyptus dunnii* in Southern Brazil. However, no data about its symptoms and severity are currently available. In this study, inoculation, symptomatology, and severity on leaves and trunks of *E. dunnii* and leaves of *Corymbia citriodora* are analyzed and discussed. Leaf and trunk samples with and without wounds were inoculated with *A. eucalyptorum* in two independent experiments. Severity on leaves was assessed based on coverage of the lesion area, whereas severity on trunks considered the size of the lesions. After inoculation, the following symptoms were observed on leaves with and without lesions: brown, circular or irregular lesions, occurring along the leaf margins, sparsely distributed, or coalescing throughout the leaf blade. The severity on leaves ranged from 13 to 63% on *E. dunnii* and from 30 to 38% on *C. citriodora*. Only inoculated trunks with wounds showed signs of the pathogen. The lesion area of the wounded trunks increased in only one of the experiments. This study confirms the pathogenicity of *A. eucalyptorum* on *E. dunnii* and *C. citriodora*, a new host for the disease, and concludes that the fungus can be considered a primary pathogen. In the region where *A. eucalyptorum* was isolated surveys were carried out to determine a possible wider presence of *A. eucalyptorum*, in the surveys two other species of *Harknessia* were isolated, but without determination to the species level. The dispersion of *A. eucalyptorum* and *Harknessia* spp. and the possible impacts of these pathogens on *Eucalyptus* spp. plantations should be monitored.

Keywords Inoculation; Symptoms; Pathogenicity; Stem canker; Leaf spot; Leaf blight.

Agressividade e sintomatologia de *Apoharknessia eucalyptorum* em folhas e caule de Eucalyptus e Corymbia

RESUMO

Apoharknessia eucalyptorum é associada com manchas foliares em *Eucalyptus dunnii* no Sul do Brasil. No entanto, nenhum dado sobre seus sintomas e severidade estão disponíveis atualmente. Neste estudo, a inoculação, sintomatologia, e a severidade em folhas e caule de mudas de *E. dunnii* e em folhas de *Corymbia citriodora* são analisados e discutidos. Amostras de folhas e caules com e sem ferimentos foram inoculadas com *A. eucalyptorum* em dois experimentos independentes. A severidade nas folhas foi avaliada com base na cobertura da

área das lesões, ao passo que a severidade nos caules considerou o tamanho das lesões. Após a inoculação os seguintes sintomas foram observados em folhas com e sem lesões: de cor marrom, de forma circular ou irregular, ocorrendo ao longo das margens das folhas, esparsamente distribuídos ou coalescendo por todo o limbo foliar. A severidade em *E. dunnii* variou de 13 a 63% e de 30 a 38% em *C. citriodora*. Somente os caules com ferimentos inoculados mostraram sinais do patógeno. A área da lesão dos caules com ferimentos aumentou em somente um dos experimentos. Este estudo confirma a patogenicidade de *A. eucalytorum* em *E. dunnii* e *C. citriodora*, um novo hospedeiro da doença, e conclui-se que o fungo pode ser considerado um patógeno primário. Na região onde *A. eucalyptorum* foi isolado pesquisas foram conduzidas para determinar uma possível maior presença de *A. eucalyptorum*, nas pesquisas duas outras espécies de Harknessia foram isoladas, mas sem determinação ao nível de espécie. A dispersão de *A. eucalyptorum* e *Harknessia* spp., e os possíveis impactos destes patógenos em plantios de *Eucalyptus* spp. devem ser monitorados.
Palavras-chave: Inoculação; Sintomatologia; Patogenicidade; Cancro; Mancha foliar; Queima das folhas.

1. INTRODUCTION

Diseases can limit growth and reduce production of agricultural and forest cultivars, with one plant being associated with as many as 100 potential pathogens (AGRIOS, 2009). These diseases can have an impact on human wellbeing, the economy, and the biodiversity (ANDERSON et al., 2004). According to the FAO (2018), there are more than 293 million hectares of planted forests in the world, with approximately 7.7 million in Brazil. As plantations, forest stands can be threatened by diseases, which can limit yield and affect economic return.

In Southern Brazil, the main eucalypt species grown in regions with the occurrence of frosts are *E. dunnii*, *E. benthamii*, and *E. viminalis*. All species are important for the economy of the region (SANTOS; AUER; GRIGOLETTI JUNIOR, 2001; AUER; SANTOS 2011; DOBNER JUNIOR et al., 2017; ANDREJOW et al., 2018) and planted for different end uses, including pulp and paper and wood paneling (IBÁ, 2017). In the region's eucalypt plantations, several diseases mainly caused by fungi have been reported (SANTOS; AUER; GRIGOLETTI JUNIOR, 2001), including *Mycosphaerella* sp., *Calonectria candelabrum*, *Chrysoporthe cubensis*, *Rhizoctonia* sp., *Austropuccinia psidii*, and *Pestalotiopsis* sp. (SANTOS; AUER; GRIGOLETTI JUNIOR, 2001; AUER; SANTOS, 2011; SOARES et al., 2017).

In 2014, a new disease was observed in Southern Brazil causing leaf spots or leaf blight on *E. dunnii* trees of five to seven years of age (GARRETT et al., 2018). The etiological agent was identified in 2017 as *Apoharknessia eucalyptorum*, belonging to the class Sordariomycetes, one of the largest groups of ascomycetes (MAHARACHCHIKUMBURA et al., 2015).

Eucalypt trees are common hosts of Harknessiaceae species (CROUS; WINGFIELD; NAG RAJ, 1993) which have been found on several continents, including Africa, Australia, Europe, and North and South America (LEE; GROENEWALD; CROUS, 2004). *Harknessia* spp. are associated with leaf spots, saprobes or endophytes (RUPEREZ; MUÑOZ, 1980; CROUS; KNOX-DAVIES; WINGFIELD, 1989a, 1989b; 1989c; CROUS et al., 2012b), and cankers (CROUS; KNOX-DAVIES; WINGFIELD, 1989b; YUAN; MOHAMMED, 1999), with about 60 species described to date.

The genus *Apoharknessia* is morphologically similar to *Harknessia* (LEE; GROENEWALD; CROUS, 2004; SENANAYAKE et al., 2017). Based on molecular data of *Harknessia insueta*, morphological and phylogenetic analysis enabled the separation of the genus *Apoharknessia* (LEE; GROENEWALD; CROUS, 2004). The morphological differences for the introduction of *Apoharknessia* are the hyaline apical apiculus on the conidia, and an absence of fluffy aerial mycelium on oatmeal or malt extract agar (MEA) when cultivated *in*

vitro, but growing within the medium and sporulating on naked hyphae (LEE; GROENEWALD; CROUS, 2004). The genus *Apoharknessia* now consists of three species: *Apoharknessia insueta* (LEE; GROENEWALD; CROUS, 2004), *A. eucalyptorum* (CROUS et al., 2017), and *A. eucalypti* (MARIN-FELIX et al., 2018). Molecular differences are also observed in the ITS sequences between *Harknessia* and *Apoharknessia* and also among *Apoharknessia* species (LEE; GROENEWALD; CROUS, 2004; CROUS et al., 2017; MARIN-FELIX et al., 2018).

Apoharknessia insueta is pathogenic to eucalypt trees in Brazil and Mauritius (CROUS; KNOX-DAVIES; WINGFIELD, 1989a), whereas *A. eucalyptorum* and *A. eucalypti* are considered endophytic to its host *Eucalyptus pellita*, where the species were first described (CROUS et al., 2017; MARIN-FELIX et al., 2018). However, the pathogenicity of *A. eucalyptorum* on *E. dunnii* has been confirmed in Brazil (GARRETT et al., 2018). Some *Harknessia* spp. are also pathogenic, however information is limited (CROUS et al., 2012b), and they are generally considered secondary or weak pathogens (CROUS; ROGERS, 2001; ALFENAS et al., 2009).

To date, the description and presentation of leaf necrosis symptoms caused by *Apoharknessia* spp. is limited. There is also a lack of information about inoculation, incidence, severity, signs of the pathogen, and infection pattern on any host. Moreover, the potential of *A. eucalyptorum* to damage *Eucalyptus* spp. in Brazil, as well its capacity to infect other hosts, is unknown. In this context, symptomatology is the first step to understanding a pathogen, including symptom characterization, which is imperative for correct diagnosis and evaluation of a disease (MAFIA; ALFENAS, 2003).

Thus, the aim of this study was to evaluate the inoculation, incidence, and severity of *A. eucalyptorum* on leaves and trunks of *E. dunnii* and leaves of *Corymbia citriodora*, and identify the signs, symptoms, and the infection pattern by staining fungal tissues on inoculated leaves of *Corymbia citriodora*.

2. MATERIAL AND METHODS

2.1 Plant material

Asymptomatic leaves of *E. dunnii* and *C. citriodora* were collected for the inoculation tests. The leaves of *E. dunnii* were collected in the field at the Universidade Estadual do Centro-Oeste (UNICENTRO), in the municipality of Irati, Paraná, Brazil, where the inoculations on *E. dunnii* leaves were performed. The leaves of *C. citriodora* were collected in a glass greenhouse, with controlled temperature (21 °C in summer and 15° in winter, with 50% filtered radiation, and with artificial daylight illumination) at the interdisciplinary Research Centre (iFZ) of the Justus Liebig University Giessen, Germany, where the inoculations on *C. citriodora* leaves were performed. For inoculation on trunks, seven-month-old seedlings of *E. dunnii* grown in pots were obtained from a commercial nursery.

2.2 Inoculation

Detached leaves were inoculated with a conidial suspension applied on the adaxial blade of the leaves. The conidial suspension was prepared by scraping *A. eucalyptorum* colonies (incubated at 25 °C on MEA medium) with a brush to remove the conidia and adding 20 ml of sterile water. The resulting conidial suspension was filtered with gauze, and the conidia concentration of the filtered suspension was determined with a Neubauer chamber, adjusting the volume of sterile water to reach a final concentration of $1x10^6$ conidia per ml. Trunks were inoculated with 2.5 mm diameter MEA discs colonized by *A. eucalyptorum*, the Petri dishes colonized by *A. eucalyptorum* were incubated for 10 days at 25 °C.

2.2.1 Inoculation on detached leaves

Before inoculation, the leaf surfaces were disinfested by immersion in 70% ethanol, sodium hypochlorite (1% active chlorine) for 45 seconds, followed by a sterile water wash. The inoculation of *A. eucalyptorum* suspension on leaves of *E. dunnii* and *C. citriodora* was performed using two treatments: 1) inoculation of conidial suspension on leaves with wounds; 2) inoculation of conidial suspension on leaves without wounds. A third treatment served as the control, with leaves with wounds sprayed with sterile water. Wounding of inoculated and control leaves was performed with a sterile needle, forming scratches parallel to the midrib on

the leaf blade. The conidial suspension and sterile water for the control were applied with 100 ml hand-held sprayers under sterile conditions. The sprayed leaves were incubated on water agar medium containing 40 $mg.L^{-1}$ of benzimidazole in 90 mm diameter Petri dishes at room temperature (Appendix VII). The incidence and severity of lesions on the leaves were evaluated at seven days after inoculation. The treatments consisted of six leaves of each species, for a total of 72 evaluated leaves. The experiment was conducted twice.

2.2.2 Inoculation on trunks of seedlings

The inoculation of *A. eucalyptorum* on trunks of *E. dunnii* was performed with two treatments: 1) inoculation with 2.5 mm diameter MEA discs colonized by *A. eucalyptorum* on trunks with wounds; 2) inoculation of MEA discs (2.5 mm diameter) colonized by *A. eucalyptorum* on trunks without wounds. A third treatment served as the control, with trunks with wounds treated with sterile MEA discs (2.5 mm diameter). Inoculated and control seedlings were wounded by removing the bark to the cambium and puncturing the trunk with a sterile stopper punch of 2.5 mm diameter at 2 centimeters above the root collar diameter. The MEA discs colonized with *A. eucalyptorum* and the sterile control MEA discs were attached to the trunks and covered with a plastic band (Appendix VIII). After inoculation, the seedlings were kept in shaded nursery conditions for six weeks, following the methodology of Wingfield, Crous and Coutinho (1996). The experiment was conducted twice.

2.3 Evaluation of incidence and severity and characterization of symptoms

The incidence and severity of the leaf blight were evaluated for the different inoculation treatments. Leaf blight severity was determined with the software QUANT 1.0.2 (VALE; FERNANDES FILHO; LIBERATO, 2003), considering the percentage of necrotic and asymptomatic area of the leaves. The symptoms were characterized by shape, color, and signs of *A. eucalyptorum*. Inoculation on trunks was evaluated by assessing the length and width of the lesions on the seedling trunks to determine the area of the lesions, based on MAFFIA et al., (2007) as follows:

$$S = \frac{\pi \; x \; W \; x \; L}{4}$$

where S is the lesioned area (mm^2); π is the constant 3.14159; W is the width of the lesion (mm); and L the length of the lesion (mm).

2.4 Staining of the fungus tissue on leaves

To identify the presence of mycelium, germinated conidia and infection pattern samples of inoculated *C. citriodora* leaves were fixed in a solution containing chloroform (20% v/v), ethanol (80% v/v), and trichloroacetic acid (0.15% w/v) for 24 hours. After fixation, leaf samples were incubated in 1x Phosphate-buffered saline (PBS buffer, pH 7.4) containing 10 $\mu g.ml^{-1}$ of WGA Alexa Fluor® 488 (Molecular Probes, Karlsruhe, Germany) and 0.02% Silwet L-77. During the incubation, leaf samples were stained by vacuum infiltration three times for 1 minute at 25 mm.Hg and kept in staining solution for 10 minutes. After, the samples were washed with 1x PBS buffer, and then were put on glass slides and WGA-Alexa Fluor® was detected using epifluorescence microscopy (Axioplan 2. Zeiss, Germany) at excitation of 470/20 nm and detection of 505-530 nm.

2.5 Survey to detect a wider presence of *A. eucalyptorum*

Surveys were conducted to detect a possible wider presence of *A. eucalyptorum* in the region near to where we isolated *A. eucalyptorum*. We conducted surveys to detect fungus dispersion. Leaves of *E. dunnii*, *E. benthamii*, and a hybrid of *E. dunnii* x *E. benthamii* with symptoms similar to those observed on the material infected by *A. eucalyptorum* were collected to isolate the fungus associated with these symptoms. The surveys were conducted in the Center-west and South of Paraná State and Northern Santa Catarina State.

2.6 Statistical analysis

The normality of the data was evaluated according to the Shapiro-Wilk test, with 1% significance. When needed, the severity data for *E. dunnii* leaves was transformed by $X = Observed\ severity^{0.7}$, whereas the severity data of *C. citriodora* was analyzed in its original scale. The length and area of the inoculated trunks of the second experiment were transformed by $X = Log(Observed\ value)$. The severity data for each species and experiment, including inoculated leaves with wounds, inoculated leaves without wounds, and control, were compared with the Tukey test. Inoculated trunks without wounds showed no signs of lesions; therefore, the width and length of the lesions on trunks was compared using the t-test between inoculated trunks with wounds and the control. The statistical comparisons assumed a significance level of 1%. The statistical analyses were performed with XLSTAT for Excel (www.xlstat.com).

3. RESULTS

Seven days after inoculation *A. eucalyptorum* caused necrotic lesions on all the inoculated leaves of *C. citriodora* and *E. dunnii*. On *C. citriodora* leaves with and without wounds, the disease severity was statistically similar in the two experiments. For *E. dunnii*, the severity was greater on non-wounded leaves in the first experiment (E1), while the severity was greater on wounded leaves in the second experiment (E2) (Table 4).

Table 4. Incidence (number of infected leaves) and mean severity (%) of leaf necrosis caused by *Apoharknessia eucalyptorum* on leaves of *Corymbia citriodora* and *Eucalyptus dunnii* seven days after inoculation.

Treatment	Incidence				Mean severity (%)			
	C. citriodora		*E. dunnii*		*C. citriodora*		*E. dunnii*	
	E1	E2	E1	E2	E1	E2	E1	E2
Inoculated (wounded)	100	100	100	100	30.32 a	31.06 a	35.15 a	35.32 b
Inoculated (non-wounded)	100	100	100	100	37.81 a	32.54 a	12.62 b	62.79 a
Control (wounded)	-	-	-	-	2.12 b	2.31 b	1.70 b	1.64 c
***p*-value**	-	-	-	-	<0.001	<0.001	< 0.001	< 0.001

E1: First Experiment. E2: Second Experiment. "-": Absent. Different letters in the same column for mean severity differ statistically according to the Tukey test with 1% significance.

The inoculated leaves of *C. citriodora* and *E. dunnii* with and without wounds showed symptoms and signs of *A. eucalyptorum*, whereas the control leaves showed no progression of necrosis, with the necrotic area limited to the wounds produced by the needle. The tissue of inoculated leaves with wounds was colonized by the pathogen beginning at the wounds and progressing through the leaf blade until coalescence. The inoculated leaves without wounds showed a sparse distribution of symptoms isolated throughout the leaf blade or occurring along the leaf margins. The symptoms were observed initially at three to four days after inoculation.

The symptoms on *C. citriodora* leaves were necrotic lesions, similar to soaked leaf blight, that were dark to light brown in color, circular or irregular, some with pale-brown borders, occurring isolated throughout the leaf blade, coalescing, or present along the leaf

margins (Figure 7). The symptoms on *E. dunnii* leaves were similar to those observed for *C. citriodora*, except for color which was light to pale brown (Figure 8). Signs of *A. eucalyptorum* were observed on both inoculated species as sporulation within lesions, formed by pycnidia and conidial masses arising from lesions, producing dark or black points throughout.

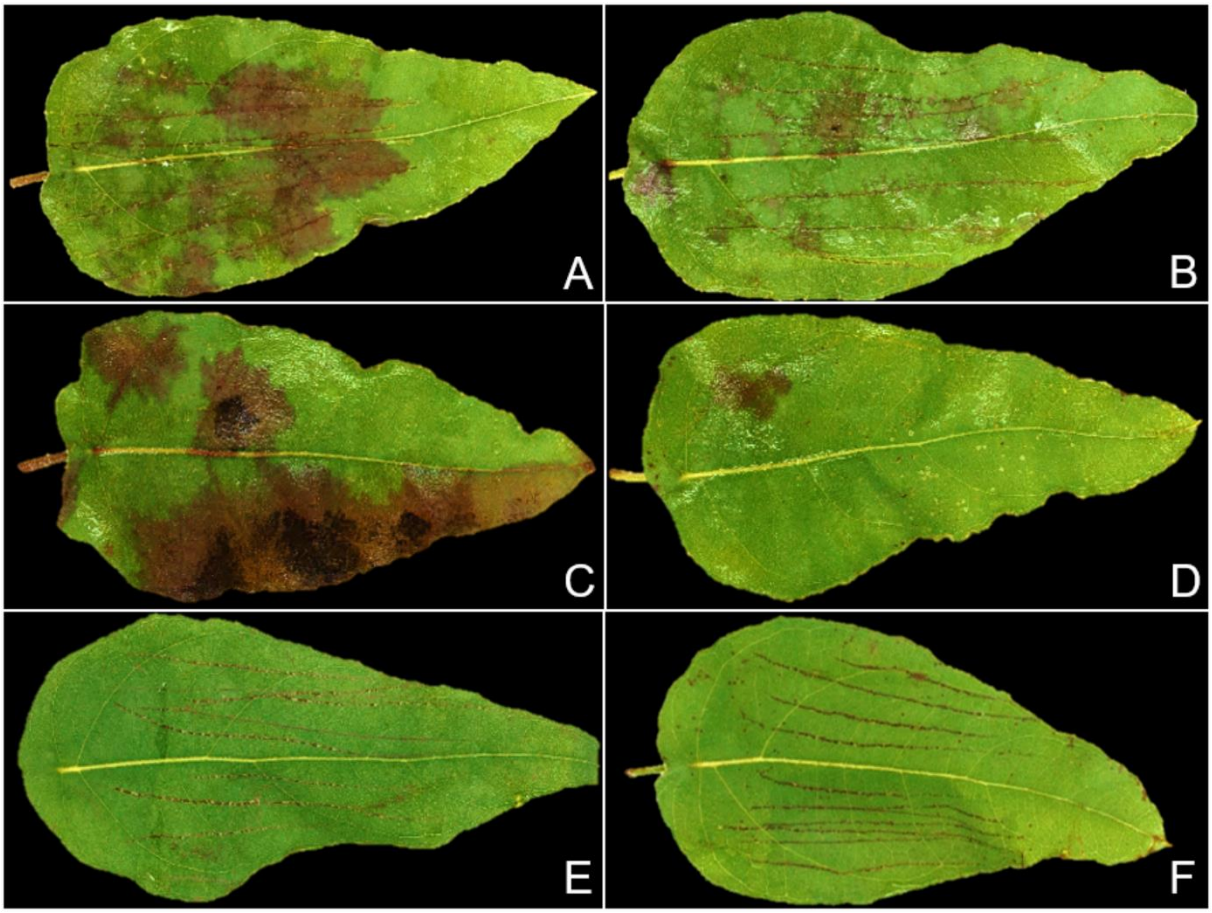


Figure 7. Symptoms of *Apoharknessia eucalyptorum* on *Corymbia citriodora* leaves seven days after inoculation. (A and B) Leaves with wounds inoculated with *A. eucalyptorum*. (C and D) Leaves without wounds inoculated with *A. eucalyptorum*. (E and F) Control leaves (wounded). Source: The author.

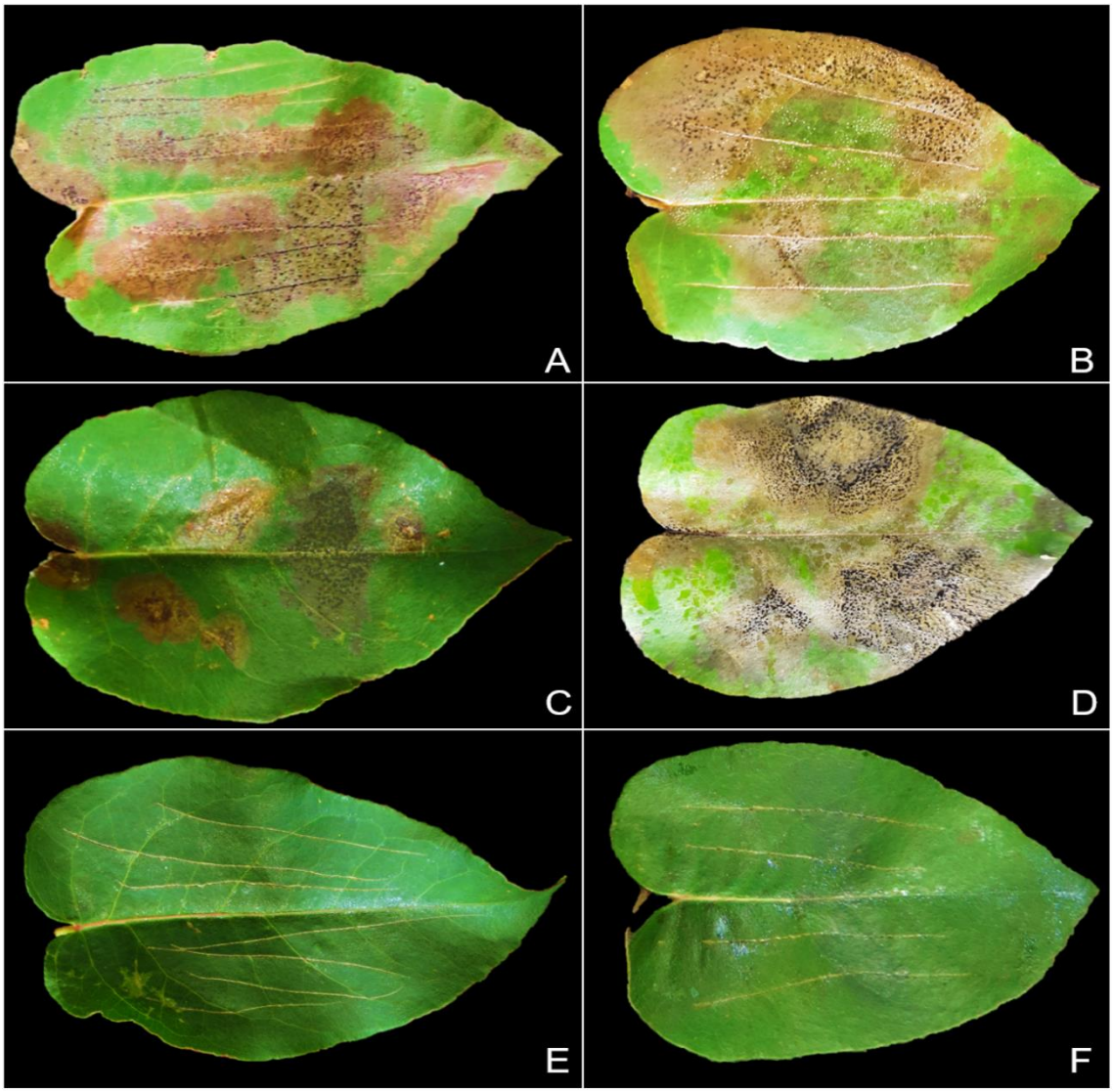


Figure 8. Symptoms of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* leaves seven days after inoculation. (A and B) Leaves with wounds inoculated with *A. eucalyptorum*. (C and D) Leaves without wounds inoculated with *A. eucalyptorum*. (E and F) Control leaves (wounded). Source: The author.

The signs of the pathogen were observed as dry conidial masses (Figure 9 A) or slimy conidial masses (Figure 9 B), forming erumpent pycnidia (Figure 9 C) exuding conidia through the ostioles (Figure 9 D). After evaluating for incidence and severity, *C. citriodora* leaves were stained to detect infection of *A. eucalyptorum*. On the inoculated leaves, we observed pycnidia throughout the leaf lesions (Figure 9 E and F), germinating conidia (Figure 10 A), mycelia developing in the direction of the stomata (Figure 10 B), appressoria formation (Figure 10 C) to infect and colonize, and pycnidia and mycelium development throughout the leaf tissue (Figure 10 D).

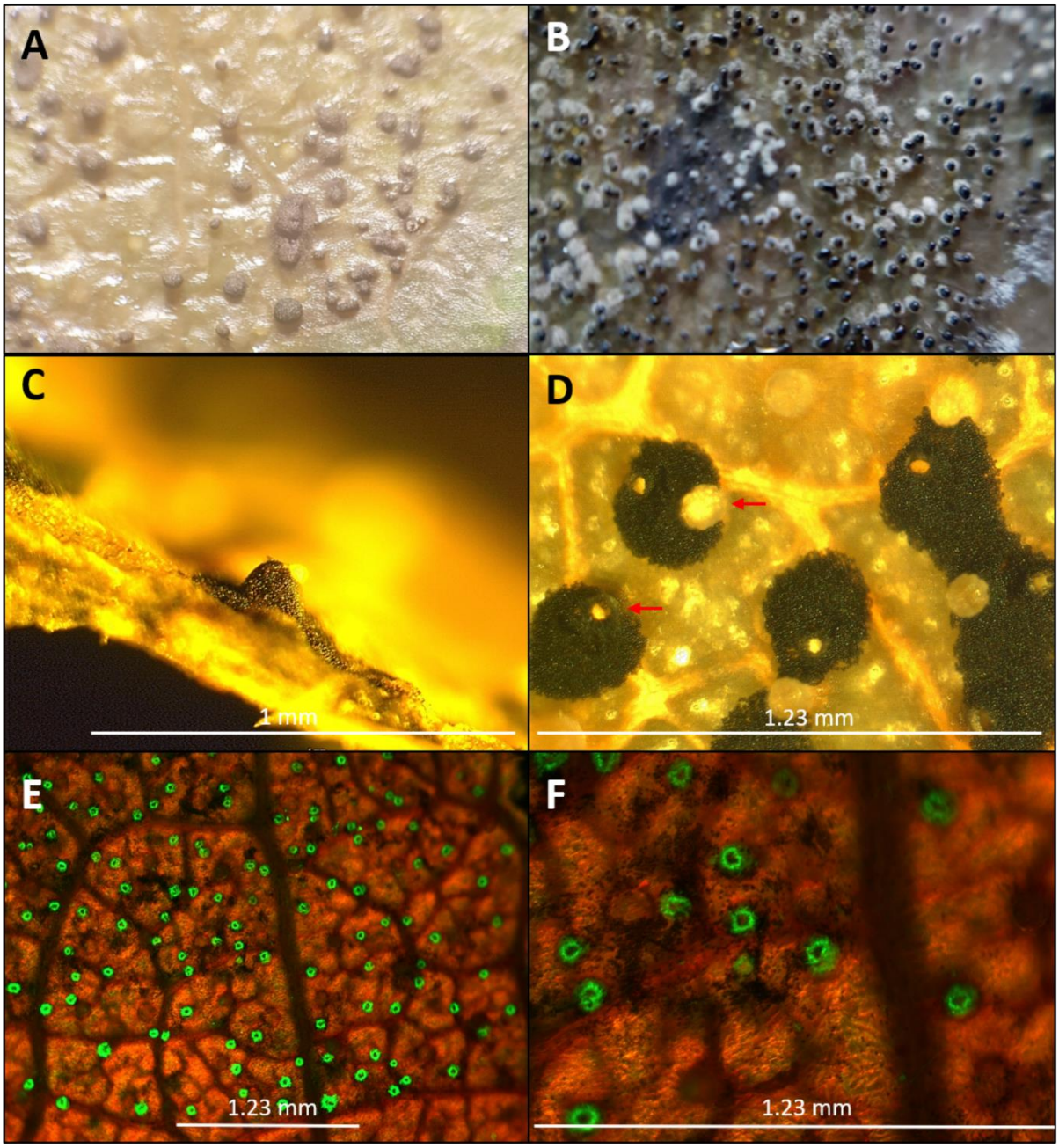


Figure 9. Signs of *Apoharknessia eucalyptorum* on inoculated leaves of *Eucalyptus dunnii* and *Corymbia citriodora*. (A) Pycnidia and dry signs of the pathogen on *E. dunnii*. (B) Pycnidia and slimy signs of the pathogen on *E. dunnii*. (C) Cross section of leaf tissue showing erumpent pycnidium. (D) Overview of pycnidia on the leaf blade with ostioles highlighted with arrows. (E and F) Stained pycnidia on the *C. citriodora* leaf blade. Source: The author.

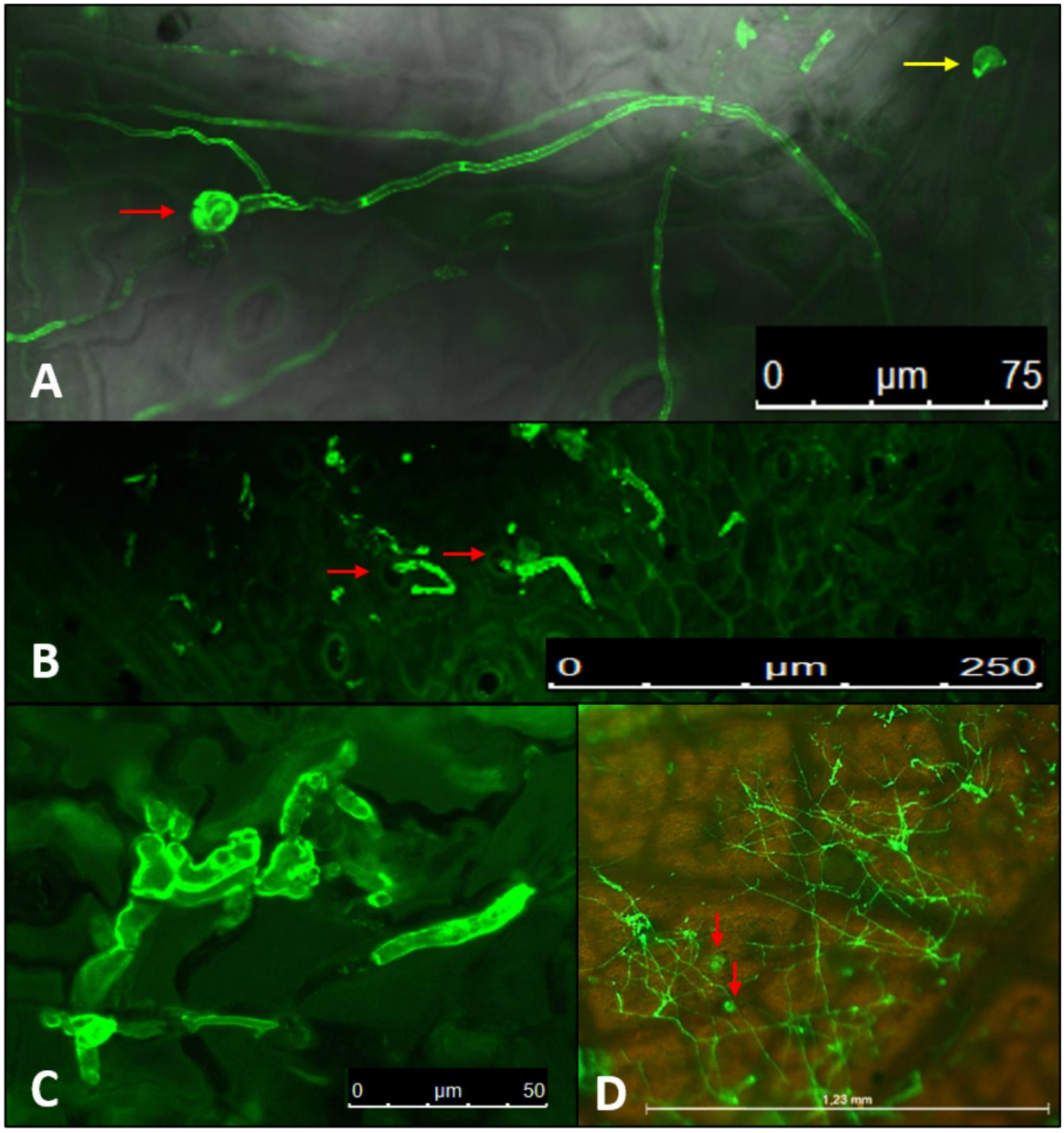


Figure 10. Stained leaves of *Corymbia citriodora* inoculated with *Apoharknessia eucalyptorum*. (A) Germinating conidia (red arrow) and non-germinated conidia (yellow arrow) of *A. eucalyptorum*. (B) Mycelium growing towards the stomata (red arrows). (C) Appressoria on the leaf blade. (D) Pycnidia (red arrows) and mycelium throughout the leaf tissue. Source: The author.

After inoculation on *E. dunnii* seedling trunks, the width and length were compared between the inoculated trunks with wounds and control plants as no lesions appeared on inoculated trunks without wounds. In E1, wounded trunks inoculated had greater necrotic area than control trunks, while in E2 no statistical difference was observed for necrotic area between treatments (Table 5). The control plants also showed no progression of lesions and cankers,

whereas the inoculated plants showed cankers and sporulation of *A. eucalyptorum* on the lesions (Figure 11 A). Although we observed cankers on wounded trunks, no severity was observed as the fungus did not induce xylem discoloration, and *A. eucalyptorum* growth and sporulation were limited to the wounds (Figure 11 B). However, the fungus was able to grow and sporulate on the bark above and below the lesioned area of the trunks (Figure 11 C). The sectioned tissues of inoculated trunks with wounds showed the presence of conidia within the xylem tissues (Figure 11 D and E).

Table 5. Width (mm) and length (mm) of the lesions caused by *Apoharknessia eucalyptorum* on inoculated trunks of *Eucalyptus dunnii* seedlings.

Treatment	Size and area of lesions on *E. dunnii* trunks					
	E1			E2		
	Width (mm)	Length (mm)	Area (mm²)	Width (mm)	Length (mm)	Area (mm²)
Inoculated (wounded)	2.69 a	4.26 a	9.00 a	2.76 a	3.89 a	8.52 a
Control (wounded)	2.49 a	3.40 a	6.63 b	2.59 a	3.69 a	7.50 a
***p*-value**	0.107	0.027	0.007	0.245	0.492	0.205

E1: First Experiment. E2: Second Experiment. Different letters in the same column for the means differ statistically according to the *t*-test with 5% significance.

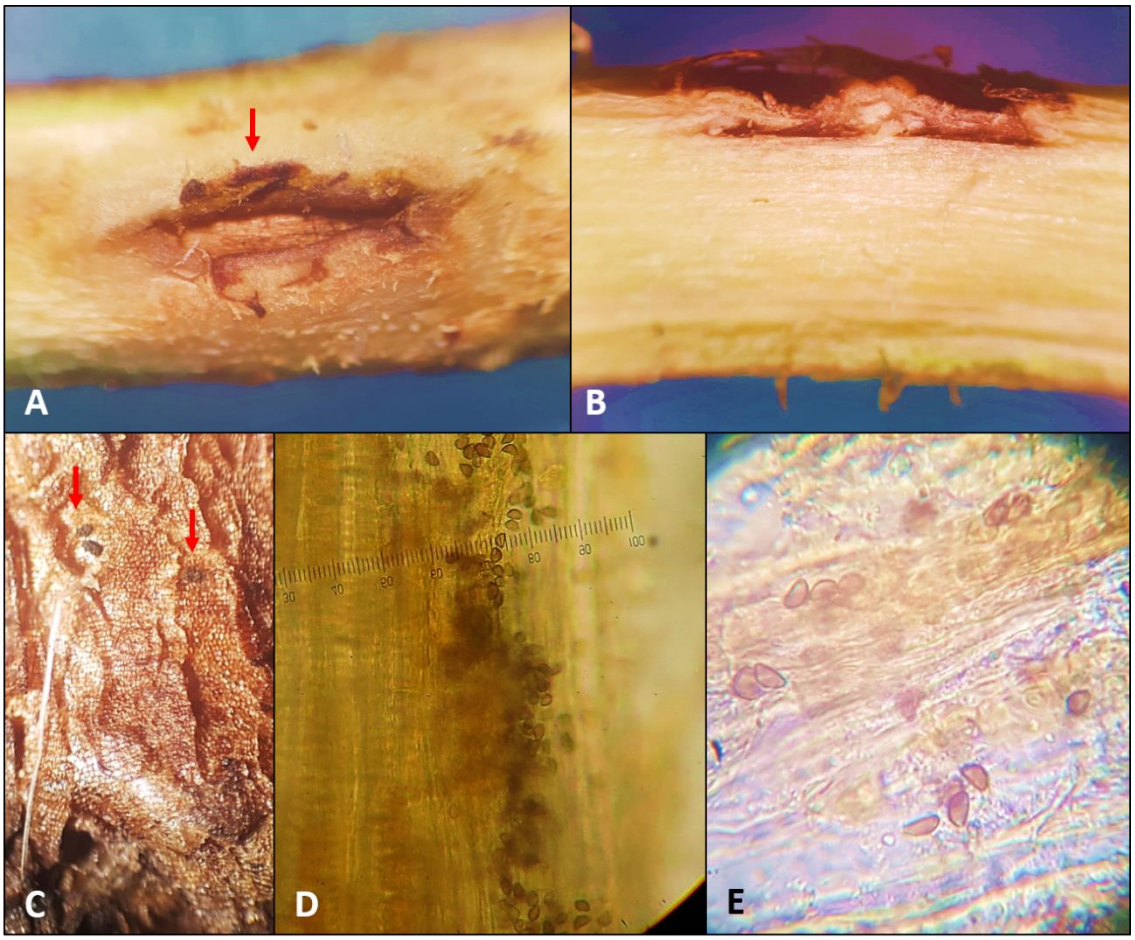


Figure 11. Trunks of *Eucalyptus dunnii* inoculated with *Apoharknessia eucalyptorum*. (A) Inoculated *E. dunnii* seedling with canker and sporulation on the lesion (red arrow). (B) Inoculated *E. dunnii* seedling with lesion and growth of the fungus limited to the lesion area. (C) Sporulation of *A. eucalyptorum* on the bark of inoculated *E. dunnii* trunk (red arrows). (D and E) Conidia of *A. eucalyptorum* within the xylem tissue of inoculated *E. dunnii* trunks. Source: The author.

4. DISCUSSION

The inoculated tissues showed symptoms on which signs of *Apoharknessia eucalyptorum* were observed. The fungal structures observed from the signs present on leaf tissues correspond to the morphology of *Apoharknessia* (LEE; GROENEWALD; CROUS, 2004). In comparison with descriptions by Crous et al. (2017) and Marin-Felix et al. (2018), the pycnidial conidiomata, conidia, and other structures observed are consistent with *A. eucalyptorum*. Although the isolate from Brazil has 99% identity to the type isolate for ITS, ß-Tubulin, and Calmodulin sequences (GARRETT et al., 2018), it is very important to confirm phenotypic similarity.

Until the description published by Garrett et al. (2018), the pathogenicity of *A. eucalyptorum* was uncertain, as Crous et al. (2017) described the species as an endophytic fungus of *E. pellita*. In the study by Marin-Felix et al. (2018) that introduced *A. eucalypti*, which is also considered endophytic, the authors described symptoms associated with *A. eucalyptorum* on *E. pellita* leaves, but no inoculation tests were performed to determine the association of *A. eucalyptorum* with the observed symptoms. Thus, the present work is the first description of the aggressiveness and symptomatology of *A. eucalyptorum* on leaves and trunks of *E. dunnii* and leaves of *C. citriodora*.

Previous research has shown that *A. insueta* (formerly *Harknessia insueta*) causes foliar disease on *Eucalyptus* in Northern Brazil, as well as in Mauritius (CROUS; KNOX-DAVIES; WINGFIELD, 1989a) and Colombia (LEE; GROENEWALD; CROUS, 2004). To date, *A. eucalypti* has not yet been found in South America, while *A. eucalyptorum* is currently restricted to a specific region in Southern Brazil. However, it is important to mention that *A. insueta* was recently identified in Southeastern Brazil on *Eucalyptus*, and *A. eucalyptorum* was reported on *Phyllanthus emblica* in Bangladesh (GenBank Accession Number: MH384891.1).

Species of the related genus *Harknessia* have been reported as infecting *E. dunnii*, such as *Harknessia hawaiiensis* (MASCHIO; AUER; GRIGOLETTI JUNIOR, 1996), *H. pseudohawaiiensis* (CROUS et al., 2012b), and *H. fumaginea* (CARNEGIE, 2007). For *Corymbia* spp., only *H. rhabdosphaera* isolated from *Corymbia henryi* has been identified in Australia (SUMMERELL et al., 2006). In Brazil, the reported *Harknessia* species are *H. hawaiiensis* and *H. fumaginea* (CROUS; KNOX-DAVIES; WINGFIELD, 1989a; MASCHIO; AUER; GRIGOLETTI JUNIOR, 1996).

Although *Harknessia* species are better understood, symptoms are not yet clearly defined (PARK et al., 2000), with Ruperez and Muñoz (1980), Crous, Knox-Davies, Wingfield (1989b; 1989c), and Crous et al. (2012b) describing symptoms of various tones of brown on

different hosts. *Harknessia* spp. symptoms are leaf spots that are clearly lined by borders or occur as leaf blight along the leaf margins, whereas the symptoms of *A. eucalyptorum* on *E. pellita*, as shown by Marin-Felix et al. (2018), are brown, irregular, or circular with borders, along the leaf margins or sparsely distributed throughout the leaf blade. The symptoms observed herein on *C. citriodora* and *E. dunnii* of brown leaf blight or leaf spots occurring along the leaf margins or sparsely across the leaf surface after inoculation with *A. eucalyptorum*, are similar to those shown for different species of *Harknessia* (RUPEREZ; MUNÕZ, 1980; CROUS et al., 2012b) and *A. eucalyptorum* on *E. pellita* (MARIN-FELIX et al., 2018).

Although the signs and symptoms are dependent on interactions among the pathogen, environment, and host, the symptomatology showed in this study is similar to the symptoms observed for *Harknessia* spp. and *A. eucalypti* on *Eucalyptus pellita*. For example, symptoms of *H. eucalyptorum* include light brown spots that are round to irregular in shape (PARK et al., 2000), while on other hosts the spots are brown surrounded by chlorotic borders, or along the leaf margins; *H. hawaiiensis* causes lesions with borders (CROUS; WINGFIELD; NAG RAJ, 1993); *Harknessia eucalypti* causes the spread of leaf spots along the leaf blade, limited by the nervures or irregular in shape, brown to dark brown at the beginning of the infection, becoming light brown as the disease develops (RUPEREZ; MUÑOZ, 1980). However, in this study we did not observe clearly defined borders surrounding the leaf spots, as described for several *Harknessia* species.

For *Harknessia*, the period of incubation is 3.3 days and the infectious period is six days, with sporulation reaching its height six days after the infectious period begins (COLMENÁREZ, 2000). For *A. eucalyptorum* we observed similar results to Colmenárez (2000). The incubation period was about four days and infectious period about 10 days. The infection is evident from the presence of black masses of conidia exuding from the conidiomata ostioles (Appendix IX and X), similar to the Harknessia conidiomata with acervular characteristics that erupt through the leaf surface (PARK et al., 2000). The sporulation on cultures incubated *in vitro* on MEA was also approximately four days for *A. eucalyptorum* (Chapter 2).

Data related to severity for leaf spots caused by *Harknessia* are currently limited. In Colombia, *H. venezuelensis* reached 25% severity on *E. urophylla* (COLMENÁREZ, 2000). While Montilla and Rodriguez (2003) observed different infection responses for different eucalypt cultivars after inoculating seedlings with *Harknessia* spp. spore suspension, with severity ranging from 5 to 18%, 75 days after inoculation. Moreover, a defoliation severity of 16% was associated with the disease. Severity data associated with leaf necrosis caused by *Apoharknessia* is not yet available. In this study, the severity for *C. citriodora* was the same for

inoculated leaves without and with wounds, ranging from about 30 to 38%. For *E. dunnii* leaves inoculated with *A. eucalyptorum* in the first experiment, the severity was statistically greater than the control only for inoculated leaves with wounds, with a mean severity of about 35%. In the second experiment, a higher severity level was observed for inoculated leaves without wounds, reaching 62.79%, and inoculated leaves with wounds showed a consistent severity level with E1, both of which are greater than the control. It is important to note that inoculation was done on detached leaves, thus it is possible that the severity could differ on leaves attached to plants.

Harknessia species are associated with pre-existing wounds (CROUS; ROGERS 2001; ALFENAS et al., 2009) caused by insects (CROUS et al., 2012b) or associated with other fungi (CROUS; WINGFIELD; NAG RAJ, 1993; YUAN; WARDLAW; MOHAMMED, 2000). Thus, we expected that the *Apoharknessia* species were also dependent on wounds for penetration. The severity results indicate that *A. eucalyptorum* can infect the leaf tissue without wounds. This was also demonstrated through analysis of the stained *C. citriodora* tissues showing that germinated conidia develop mycelium in the direction of the stomata and throughout the cells (Appendix XI). Colmenárez (2000) observed that *H. venezuelensis* infected *Eucalyptus* spp. leaves without wounds through the stomata and cell conjunctions; however, the author noted greater levels of severity on wounded leaves.

In the present study, we did not compare severity between the studied plant species. Nevertheless, we observed that the severity on *E. dunnii* leaves without wounds was greater than wounded leaves, suggesting that *A. eucalyptorum* is a primary pathogen that does not require pre-existing wounds or a vector for infection. Meanwhile, for *C. citriodora* there were no differences between the inoculation treatments, further suggesting the capacity of *A. eucalyptorum* to infect unwounded tissues.

Considering that several species of the related genus *Harknessia* have been isolated from stems and twigs, we inoculated *E. dunnii* seedling trunks with *A. eucalyptorum* to evaluate a possible association of the species with stem cankers. Examples of *Harknessia* spp. associated to lesions on stems and twigs are: *H. americana* from twigs of *Liquidambar* spp.; *H. caudata* from twigs and petioles of *Quercus* spp. (SUTTON, 1980); *H. ipereniae* from eucalypt stems (CROUS et al., 2007); *H. ravenstreetina* from leaves and twigs of Acacia in Australia (CROUS et al., 2012b); *H. molokaiensis* from stems of *Eucalyptus robusta* (CROUS; ROGERS, 2001); *H. eucalyptorum* from leaves and stems of different species of *Eucalyptus*; *H. uromycoides* from leaves, twigs, petioles and seeds of *Eucalyptus* (CROUS; WINGFIELD; NAG RAJ, 1993); and also the *Harknessia* teleomorph, *Wuestneia epispora*, that causes stem cankers in dead or dying branches of the lower crown (YUAN; MOHAMMED, 1997).

Crous, Knox-Davies, Wingfield (1989b) and Yuan and Mohammed (1999) reported that *H. eucalypti* causes stem necrosis and is associated with severe cankers. In the study of Yuan and Mohammed (1999), after inoculation of *H. eucalypti* on *E. nitens* and *E. globulus* seedling stems, the authors observed fruiting bodies on the lesions but considered the species non-pathogenic on stems. The conclusions of the authors were based on the size of the lesions and on the limited re-isolation of the fungus from the tissues. The same was observed herein for *A. eucalyptorum*, as the lesions on the wounded trunks were not statistically larger than the control plants in one of the experiments. However, in the first experiment, the area of the lesions was greater than the control. Despite a difference in lesion size, the root collar diameter and height of the seedlings were not affected (data not shown). The inoculated trunks without wounds did not show any lesion or progression of the fungus within the xylem nor on the bark.

After inoculation on *E. dunnii* seedlings, it was possible to isolate *A. eucalyptorum* from trunks, but mainly when the conidial masses on the bark were collected and transferred directly to Petri dishes containing MEA. Although conidia were observed within the xylem tissues, the isolation of *A. eucalyptorum* from the xylem tissues was limited. The width of the lesions was restricted by the root collar diameter of the seedlings, thus showing no difference between inoculated and control trunks. On the other hand, the length and area of the lesions showed variations in comparison to the control. The occurrence of frost, which is common in the region where *E. dunnii* is planted in Brazil, can damage bark and trunks tissues of seedlings and plants in the field, which favors the penetration of *A. eucalyptorum*. The results observed in this study demonstrate a novel characteristic that should be monitored in *E. dunnii*, as the pathogen has the capacity to infect not only the leaves, but also the trunks.

As observed by Crous, Knox-Davies, Wingfield (1989c) for the related *Harknessia* species, successful inoculation of *A. eucalyptorum* is the first step to determine the importance of the fungus as a pathogen. While the identification of coelomycetes based on morphology is limited (CROUS et al., 2012a), which is the case for both *Harknessia* and *Apoharknessia* species, the description of symptoms caused by *A. eucalyptorum* and its signs on different hosts can provide information to help detect the occurrence of the disease in other regions or hosts. It is also worth highlighting, that together with the description of *A. eucalyptorum*, Crous et al. (2017) described three new species of *Harknessia* with eucalypts as host: *H. platyphyllae* on *Eucalyptus platyphylla* in Australia; *H. pellitae* on *E. pellita* in Malaysia; and *H. malayensis* on *E. pellita* in Malaysia. Moreover, a new species of the genus *Apoharknessia* was introduced by Marin-Felix et al. (2018), demonstrating the importance of understanding the pathogenicity, symptomatology, geographical occurrence, hosts, and the possible impacts caused by *Apoharknessia*. In the survey in the region where *A. eucalyptorum* was isolated, we identified

two species of Harknessia that are still under study to identify the identity of the isolates. Identification of *Apoharknessia* spp. also should include DNA analysis, as the ITS sequences of *A. eucalyptorum* are 93% identical to *A. insueta* (CROUS et al., 2017; GARRETT et al., 2018), and *A. eucalypti* is 92% similar to the type species of the genus (MARIN-FELIX et al., 2018). The similarity between *A. eucalypti* and *A. eucalyptorum* is much higher, at 97% (MARIN-FELIX et al., 2018).

To date, the occurrence of *A. eucalyptorum* is limited to a specific region in Southern Brazil. However, a pathogen can be introduced into a region without causing disease, and incidence, geographical dispersion, and pathogenicity may increase only when a second factor is introduced (ANDERSON et al., 2004). According to the authors, until this second factor that favors the pathogen is present, the pathogen can remain restricted with reduced or no impact. Only time will tell if *A. eucalyptorum* will be harmful to eucalypt plantations in Southern Brazil or other regions and countries. As the number of species identified of the *Apoharknessia* and *Harknessia* genera is increasing, an understanding of these diseases or pathogen candidates is necessary. Their dispersion, hosts, possible impacts, and progression through eucalypt plantations must be monitored carefully. The inoculation methods adopted in this study proved to be suitable for *A. eucalyptorum* and can be adopted for other species of this genus, as well as for *Harknessia* spp. Moreover, the symptoms and signs showed in this study can guide the identification of the disease in other regions or hosts.

5. REFERENCES

AGRIOS, G. N. Plant Pathogens and Disease: General Introduction. In: SCHAECHTER, M. **Encyclopedia of Microbiology**. Oxford: Elsevier Inc., 2009. 613-646.

ALFENAS, A. C.; ZAUZA, E. A. V.; MAFIA, R. G.; ASSIS, T. F. de. **Clonagem e doenças do eucalipto**. 2. ed. Viçosa: Editora UFV, 2009. 500 p.

ANDERSON, P.K.; CUNNINGHAM, A.A.; PATEL, N.G.; MORALES, F.J.; EPSTEIN, P.R.; DASZAK, P. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. **Trends in Ecology & Evolution**, v. 19, n. 10, p. 535-544, 2004.

ANDREJOW, G. M. P.; PEDRASSANI, D.; TUSSULINI, F.; ANGELO, A. C.; TAMBARUSSI, E. V.; AUER, C. G. Planalto norte Catarinense: Considerações sobre o setor florestal e a eucaliptocultura. **Desenvolvimento Regional em debate**, v. 8, n. 2, p. 143-168, 2018.

AUER, C. G.; SANTOS, Á. F. dos; Doenças em eucaliptos destinados à produção de energia na região Sul do Brasil. **Pesquisa Florestal Brasileira**. Colombo, v. 31, n. 68, p. 373-379, 2011.

CARNEGIE, A. J. Forest health condition in New South Wales, Australia, 1996–2005. I. Fungi recorded from eucalypt plantations during forest health surveys. **Australasian Plant Pathology**, v. 36, n. 3, p. 213–224, 2007.

COLMENÁREZ, J. A. M. (2000). **Etiologia de la necrosis foliar de *Eucalyptus urophylla* S. T. Blake y resistencia de cultivares a la enfermedad**. Lara, 2000. 164 f. Degree work for Magister Scientiarum (Fitopathology). Universidad Centroccidental Lisandro Alvarado.

CROUS, P. W.; WINGFIELD, M. J.; BURGESS, T. I. et al. Fungal Planet Description sheets: 558-624 - *Apoharknessia eucalyptorum*. **Persoonia - Molecular Phylogeny and Evolution of Fungi**, v. 38: 270-271, 2017.

CROUS, P. W.; SUMMERELL, B. A.; ALFENAS, A. C.; EDWARDS, J.; PASCOE, I. G.; PORTER, I. J.; GROENEWALD, J. Z. Genera of diaporthalean coelomycetes associated with leaf spots of tree hosts. **Persoonia**, v. 28, p. 66–75, 2012a.

CROUS, P. W.; SUMMERELL, B. A.; SHIVAS, R. G.; CARNEGIE, A. J.; GROENEWALD, J. Z. A re-appraisal of *Harknessia* (*Diaporthales*), and the introduction of *Harknessiaceae* fam. nov. **Persoonia**, v. 28, p. 49–65, 2012b.

CROUS, P.W.; MOHAMMED, C.; GLEN, M.; VERKLEY, G. J. M.; GROENEWALD, J. Z. *Eucalyptus* microfungi known from culture. 3. *Eucasphaeria* and *Sympoventuria* genera nova, and new species of *Furcaspora*, *Harknessia*, *Heteroconium* and *Phacidiella*. **Fungal Diversity**, v. 25, p. 19-36, 2007.

CROUS, P. W.; ROGERS, J. D. *Wuestneia molokaiensis* and its anamorph *Harknessia molokaiensis* sp. nov. from Eucalyptus. **Sydowia**, v. 53, n. 1, p. 74-80, 2001.

CROUS, P.W., WINGFIELD, M.J., NAG RAJ, T.R. *HARKNESSIA* SPECIES OCCURING IN SOUTH AFRICA. **Mycologia**, Lancaster, v. 85, n. 1, p. 108-118, 1993.

CROUS, P. W.; KNOX-DAVIES, P. S.; WINGFIELD, M. J. A List of Eucalyptus Leaf Fungi and their Potential Importance to South African Forestry. **Suid-Afrikaanse Bosboutydskrif**, v. 149, p. 17-29. 1989a.

CROUS, P.W.; KNOX-DAVIES, P. S.; WINGFIELD, M. J. A Summary of Fungal Leaf Pathogens of *Eucalyptus* and the Diseases they Cause in South Africa. **Suid-Afrikaanse Bosboutydskrif**, v. 149, p. 9-16, 1989b.

CROUS, P.W.; KNOX-DAVIES, P. S.; WINGFIELD, M. J. Newly-recorded foliage fungi of *Eucalyptus* spp. in South Africa. **Phytophylactica**, v. 21, p. 85-88, 1989c.

DOBNER JR., M.; BATISTA, K. M.; SARTÓRIO, I. P.; ARCE, J. E.; QUADROS, D. S. CRESCIMENTO E DESEMPENHO ECONÔMICO DE *Eucalyptus dunnii* EM DIFERENTES SÍTIOS NO PLANALTO SUL DO BRASIL. **FLORESTA**, Curitiba, PR, v. 47, n. 4, p. 397 - 406, 2017.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. **Land Use – FAOSTAT**. Available at: <http://www.fao.org/faostat/en/#data/RL> Accessed in June, 28, 2018.

GARRETT, A. T. de A.; IMANI, J.; TAMBARUSSI, E. V.; GARCIA, F. A. de O.; KOGEL, K. H.; FIGUEIREDO FILHO, A. First report of *Apoharknessia eucalyptorum* on *Eucalyptus dunnii* in Brazil. **Forest Pathology**, e12463, 2018.

IBÁ - INSTITUTO BRASILEIRO DE ÁRVORES. **REPORT 2017**. São Paulo, 2017. 80 p.

LEE, S.; GROENEWALD, J. Z.; CROUS, P. W. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apoharknessia* gen. nov. **Studies in Mycology**, v. 50, p. 235–252, 2004.

MARIN-FELIX, Y.; HERNÁNDEZ-RESTREPO, M.; WINGFIELD, M. J.; AKULOV, A.; CARNEGIE, A. J.; CHEEWANGKOON, R.; GRAMAJE, D.; GROENEWALD, J. Z.; GUARNACCIA, V.; HALLEEN, F.; LOMBARD, L.; LUANGSA-ARD, J.; MARINCOWITZ, S.; MOSLEMI, A.; MOSTERT, L.; QUAEDVLIEG, W.; SCHUMACHER, R. K.; SPIES, C. F. J.; THANGAVEL, R.; TAYLOR, P. W. J.; WILSON, A. M.; WINGFIELD, B. D.; WOOD, A. R.; CROUS, P. W. Genera of phytopathogenic fungi: GOPHY 2. **Studies in Mycology**, v. 92, p. 47-133, 2018.

MAFFIA, L. A.; MIZUBUTI, E. S. G.; ALFENAS, A. C.; MAFIA, R. G. (2007). Quantificação de doenças em planta. In: ALFENAS, A. C.; MAFIA, R. G. **Métodos em Fitopatologia**. Viçosa: Editora UFV, 2007. 161-173.

MAFIA, R. G.; ALFENAS, A. C. Diferenciação sintomatológica de manchas foliares em Eucalyptus spp. causadas por patógenos fúngicos e bacterianos. **Fitopatologia Brasileira**, v. 28, n. 6, p. 688, 2003.

MAHARACHCHIKUMBURA, S.; HYDE, K.; JONES, E.; MCKENZIE, E.; HUANG, S.; ABDEL-WAHAB, M.; DARANAGAMA, D.; DAYARATHNE, M.; D'SOUZA, M.; GOONASEKARA, I.; HONGSANAN, S.; JAYAWARDENA, R.; KIRK, P.; KONTA, S.; LIU, J.; LIU, Z.; NORPHANPHOUN, C.; PANG, K.; PERERA, R.; SENANAYAKE, I.; SHANG, Q.; SHENOY, B.; XIAO, Y.; BAHKALI, A.; KANG, J.; SOMROTHIPOL, S.; SUETRONG, S.; WEN, T.; XU, J. Towards a natural classification and backbone tree for *Sordariomycetes*. **Fungal Diversity**, v. 72, p. 199–301, 2015.

MASCHIO, L. M. A.; AUER, C. G.; GRIGOLETTI JUNIOR, A. Fungos associados a *Eucalyptus* spp. no Paraná e em Santa Catarina. Colombo: EMBRAPA. **Pesquisa em andamento**, n. 5, p. 1-3, 1996.

MONTILLA, J. O.; RODRIGUEZ, D. Comportamiento de cultivares de *Eucalyptus urophylla* S. T. Blake ante la necrosis foliar causada por *Harknessia* sp. **Revista de la Facultad de Agronomía**, Maracay, v. 20, p. 34-42, 2003.

PARK, R. F.; KEANE, P. J.; WINGFIELD, M. J.; CROUS, P. W. Fungal diseases of eucalypt foliage. In: KEANE, P. J; KILE, G. A.; PODGER, F. D.; BROWN, B. N. **Diseases and pathogens of eucalypts**. Collingwood: CSIRO publishing, 2000. 153–239.

RUPEREZ, A.; MUÑOZ, C. Enfermedades de los eucaliptos en España. Boletín de sanidad vegetal. **Boletín de Sanidad Vegetal - Plagas**, Logroño, v. 6, p. 193-217, 1980.

SANTOS, Á. F. dos; AUER, C. G.; GRIGOLETTI JÚNIOR, A. **Doenças do eucalipto no sul do Brasil: identificação e controle**. Colombo, PR: EMBRAPA Florestas, jun. 2001. 20 p.

SENANAYAKE, I. C.; CROUS, P. W.; GROENEWALD, J. Z.; MAHARACHCHIKUMBURA, S. S. N.; JEEWON, R.; PHILLIPS, A. J. L.; BHAT, J. D.; PERERA, R. H.; LI, Q. R.; LI, W. J.; TANGTHIRASUNUN, N.; NORPHANPHOUN, C.; KARUNARATHNA, S.C.; CAMPORESI, E.; MANAWASIGHE, I. S.; AL-SADI, A. M.; HYDE, K. D. Families of Diaporthales based on morphological and phylogenetic evidence. **Studies in Mycology**, v. 86, p. 217–296, 2017.

SOARES, I. D.; AUER, C. G.; SANTOS, Á. F. dos; TAMBARUSSI, E. V.; REZENDE, E. H.; COELHO, T. A. V.; DUIN, I. M. Fungos associados à mancha foliar em *Eucalyptus benthamii*

Maiden et Cambage na região sul do Brasil. **BIOFIX Scientific Journal**, v. 2, n. 2, p. 32-37, 2017.

SUMMERELL, B. A.; GROENEWALD, J. Z.; CARNEGIE, A.; SUMMERBELL, R. C.; CROUS, P.W. *Eucalyptus* microfungi known from culture. 2. *Alysidiella, Fusculina* and *Phlogicylindrium* genera nova, with notes on some other poorly known taxa. **Fungal Diversity**, v. 23, p. 323-350, 2006.

SUTTON, B. C. **The Coelomycetes. Fungi imperfcti with pycnidia, acervuli and stromata**. Kew: Commonwealth Mycological Institute, 1980. 696 p.

YUAN, Z.; WARDLAW, T.; MOHAMMED, C. *Harknessia* species occurring on eucalypt leaves in Tasmania, Australia. **Mycological Research**, v. 104, n. 7, p. 888-892, 2000.

YUAN, Z. Q.; MOHAMMED, C. Pathogenicity of fungi associated with stem cankers of eucalypts in Tasmania, Australia. **Plant Disease**, v. 83, p. 1063-1069, 1999.

YUAN, Z.; MOHAMMED, C. Investigation of fungi associated with stem cankers of eucalypts in Tasmania, Australia. **Australasian Plant Pathology,** v. 26, p. 78-84, 1997.

VALE, F. X. R.; FERNANDES FILHO, E. I.; LIBERATO, J. R. QUANT – A software for plant disease severity assessment. In: Congress of Plant Pathology, 8., 2003, Christchurch, **Proceedings…** Christchurch, New Zealand: Plant Pathology Society, 2003. p. 105.

WINGFIELD, M. J.; CROUS, P. W.; COUTINHO, T. A. A serious canker disease *of Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. **Mycopathologia**, v. 136, p. 139-145, 1996.

FINAL CONSIDERATIONS

According to the results of this study, the potential of *Apoharknessia eucalyptorum* as a pathogen of Eucalyptus and Corymbia, that was so far unknown, is clarified. This study provides information for identification, *in vitro* cultivation, inoculation and symptomatology of *A. eucalyptorum*. Based on the information provided in this study, a possible wider dispersion of the fungus can be detected in the future, and further studies with the pathogen will be possible.

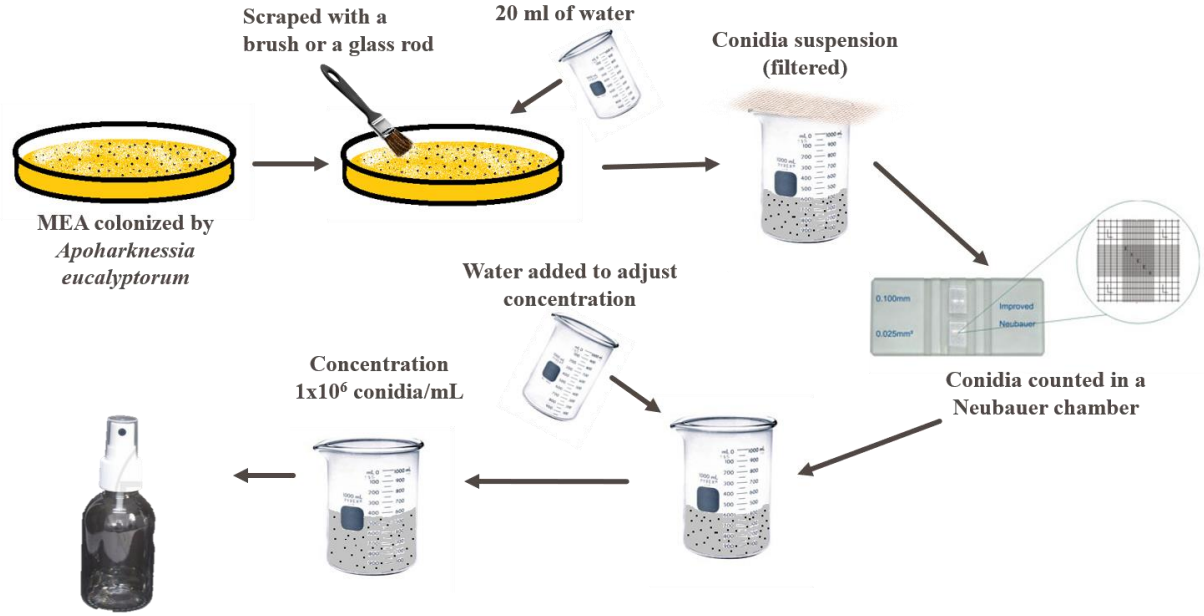
The fungus was identified in a restrict plantation area of *Eucalyptus dunnii*, and was not found in other regions of Southern Brazil, where *Harknessia* spp. were also identified on *Eucalyptus benthamii*, another important tree species for the forest sector in Southern Brazil. The restrict dispersion of *A. eucalyptorum* can be related to different factors, such the specific provenance of *E. dunnii* or the climate in the region. Thus, these factors must be considered, as the selection of clones and provenances can induce the pathogenicity of *A. eucalyptorum*, and the climate can favor the pathogen infection, what suggest a concern for all the eucalypti species in Southern Brazil. Moreover, *A. eucalyptorum* was able to infect a second genus, *Corymbia citriodora*, suggesting that the pathogen can be more aggressive than expected, as *A. eucalyptorum* was initially reported as endophytic. However, although *A. eucalyptorum* infected the wounded and non-wounded leaves of *E. dunnii* and *C. citriodora*, the impacts on growth and yield are still uncertain.

Finally, as no damage to plant growth or stands yield is associated with *A. eucalyptorum* so far, we expect and suggest that with the information provided in this study, researchers and forest sector agents monitor the *A. eucalyptorum* occurrence in Southern Brazil and other countries.

APPENDIX

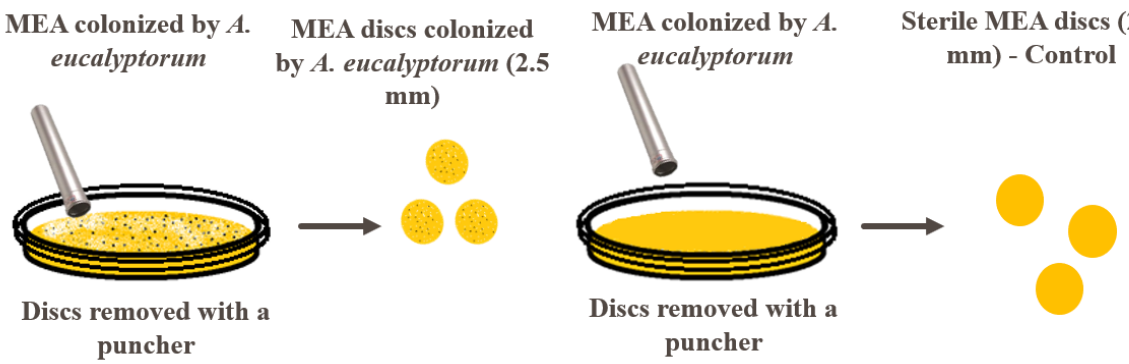
Appendix I. Preparation of conidia suspension for inoculation

Petri dishes containing MEA were incubated at 25 °C with a 12-hour photoperiod for 2 weeks to obtain pure cultures of *A. eucalyptorum*. After the two weeks, a conidial suspension was prepared by adding 20 ml of sterilized water on the Petri dishes, that were then surface scraped with a sterile bent glass rod or a brush. The suspension was subsequently filtered with gauze and the conidial concentration determined in a Neubauer chamber, adjusting the conidial suspension to $1x10^6$ spores per mL. The conidia suspension was then sprayed with a hand sprayer. Source: The author.



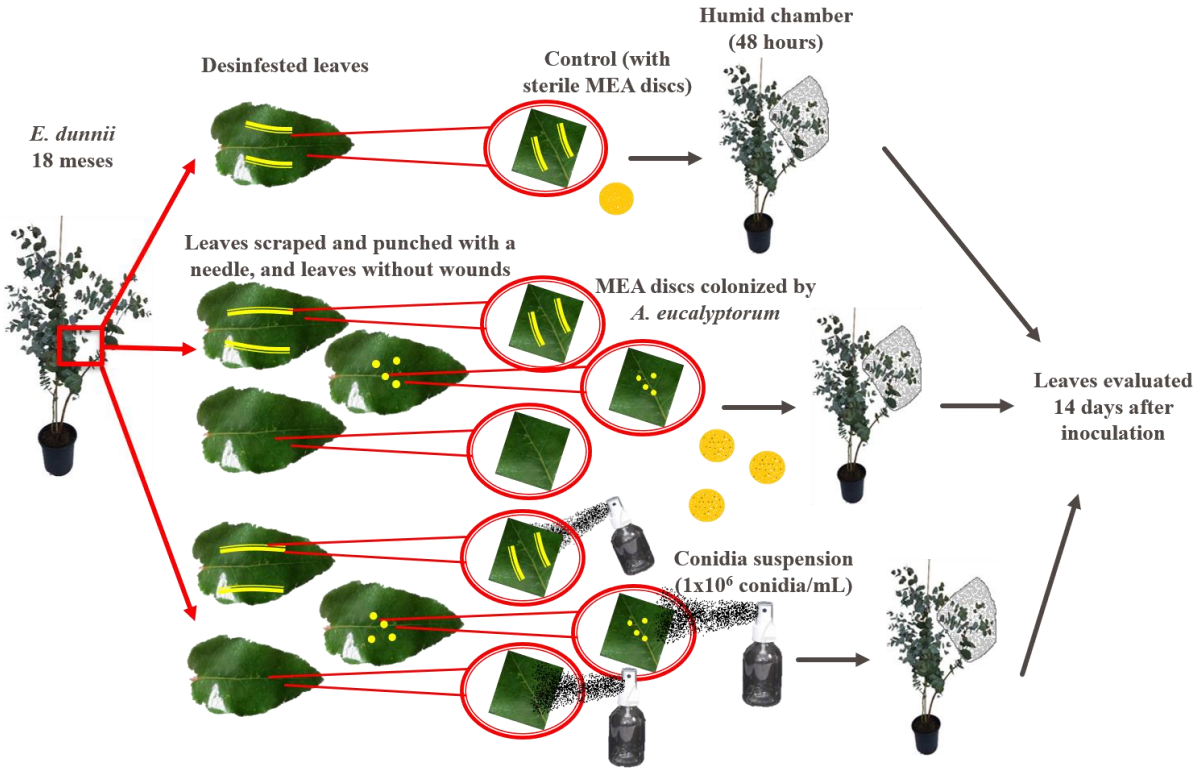
Appendix II. Preparation of MEA discs for inoculation

Petri dishes containing MEA were incubated at 25 °C with a 12-hour photoperiod for 2 weeks to obtain pure cultures of *A. eucalyptorum*. After the two weeks, discs of the colonized medium (10 mm diameter) were made with a puncher with 10 mm in diameter. From Petri dishes with sterile MEA, discs of the colonized medium (10 mm diameter and 2.5 mm diameter) were made with punchers with 10 mm and 2.5 mm in diameter. Source: The author.



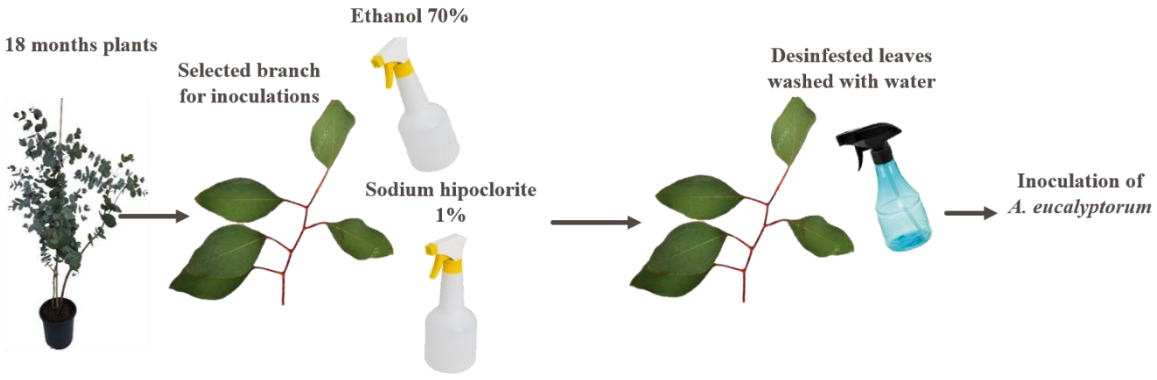
Appendix III. Inoculation treatments on inoculated leaves of *Eucalyptus dunnii*

The inoculation of leaves of *Eucalyptus dunnii* leaves with conidia suspension and MEA discs were applied to three treatments: leaves scraped with a needle; leaves punched with a needle; and leaves without lesions. The control treatment included leaves scraped with a needle inoculated with sterile MEA discs (10 mm diameter). After inoculation, the branches with the inoculated leaves were incubated with plastic bags for 48 hours. The infection and symptoms were evaluated 14 days after inoculation. Source: The author.



Appendix IV. Disinfestation of *Eucalyptus dunnii* leaves before inoculation

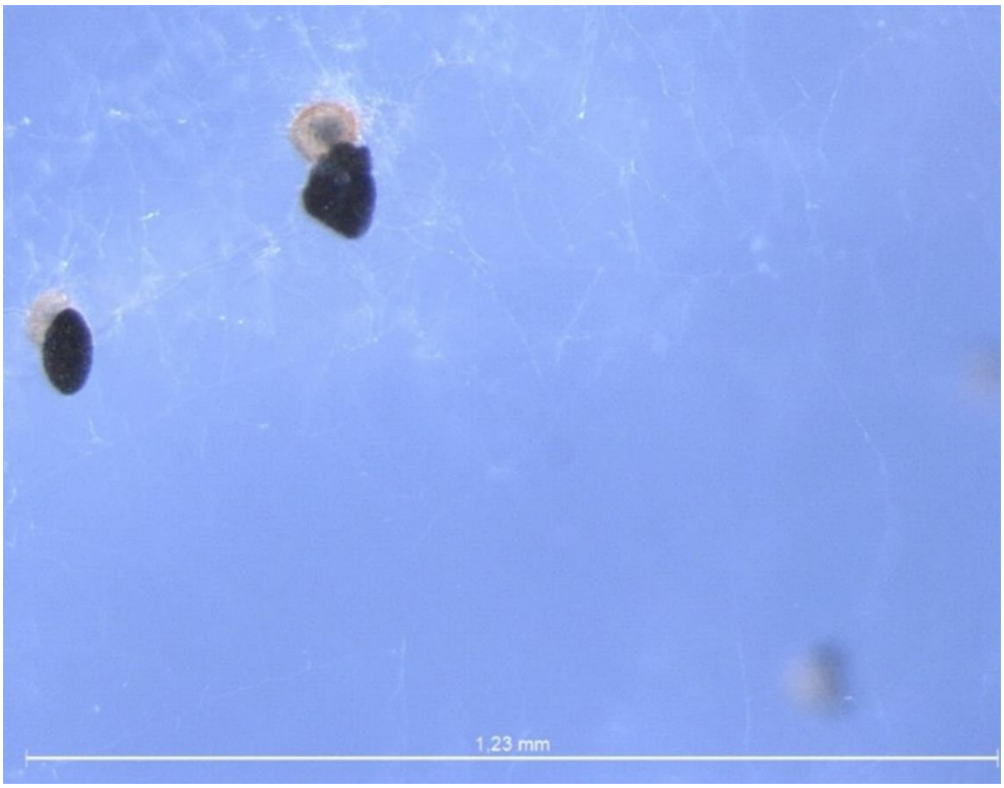
Before inoculation with conidia suspension and MEA discs, the leaf surface of the selected leaves was disinfested with 70% ethanol and 1% sodium hypochlorite using a spray bottle and then washed twice with sterile water. Source: The author.



Appendix V. Reisolation of *Apoharknessia eucalyptorum* from inoculated leaves and measurement of conidia

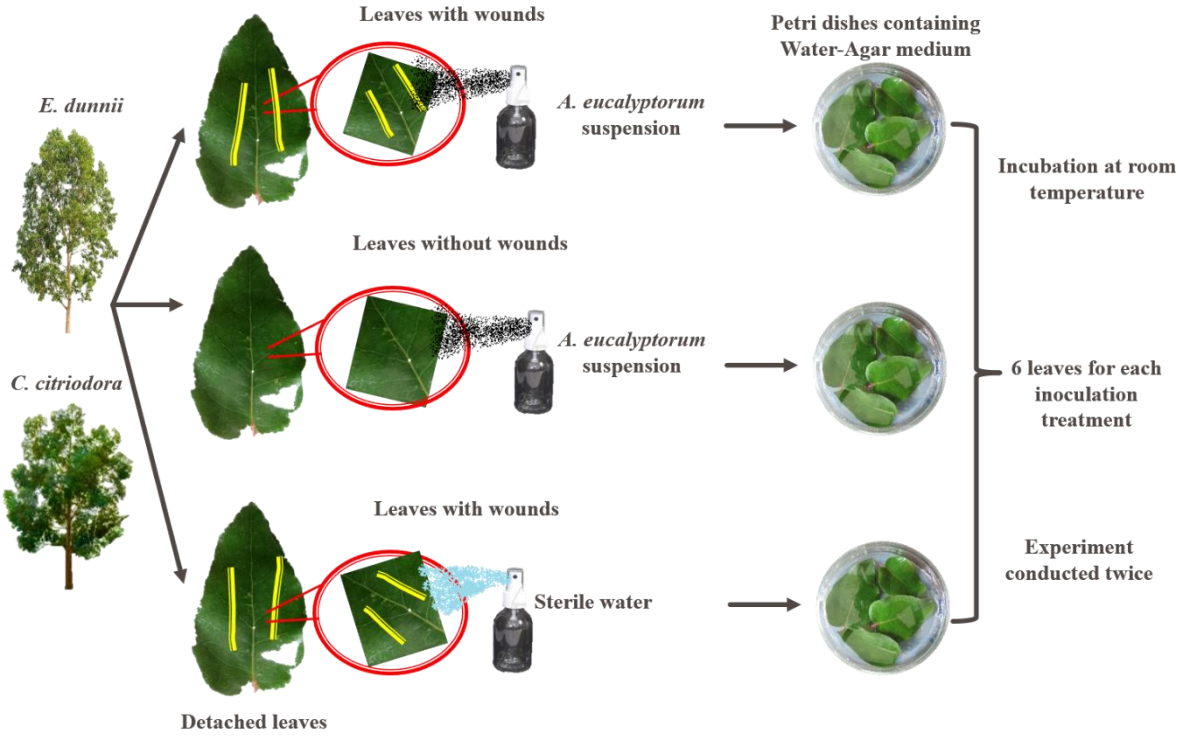
At 2-weeks post inoculation, conidia were collected from inoculated leaves and examined the samples under a microscope to compare morphological features with those of the cultures incubated *in vitro*, as well as determine the mean size of 50 conidia, and fulfill Koch's postulates. Source: The author.

Appendix VI. Pycnidia and conidial masses exuding from it on water-agar medium. Source: The author.



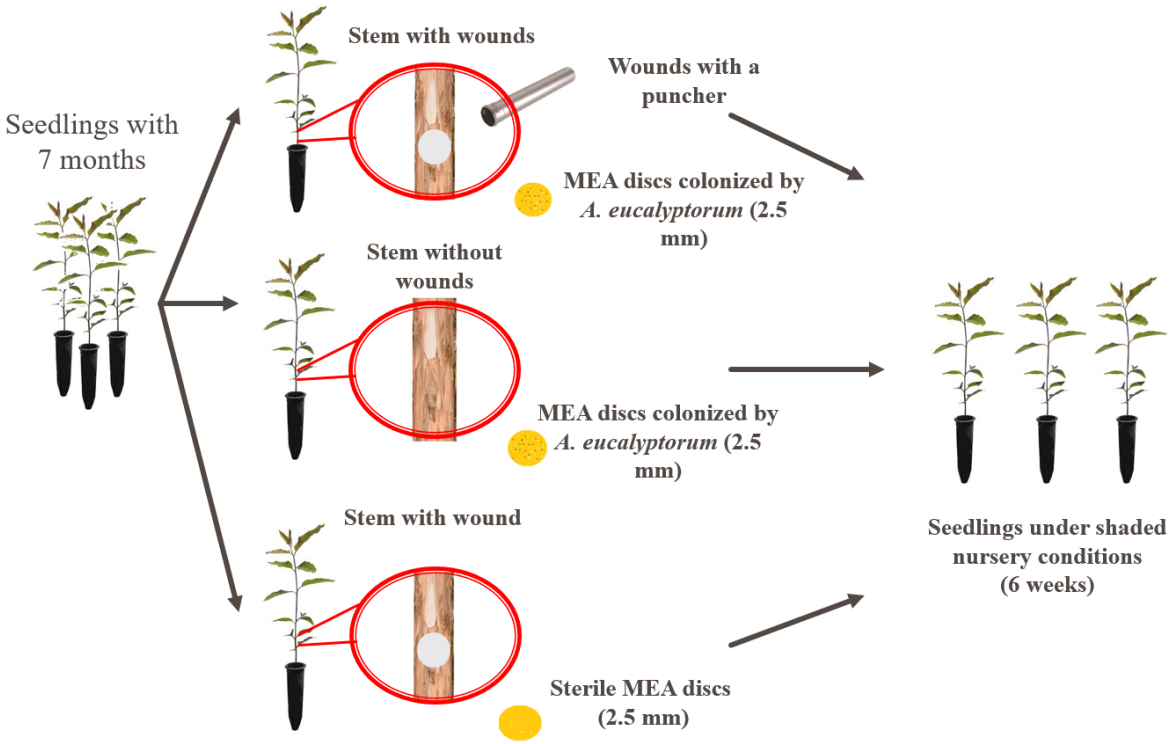
Appendix VII. Inoculation on leaves of *Eucalyptus dunnii* and *Corymbia citriodora* with conidial suspension

From asymptomatic leaves collected from *Eucalyptus dunnii* and *Corymbia citriodora* leaves. The detached leaves were inoculated with the conidia suspension ($1x10^6$ conidia/mL). Before inoculation, leaves were washed, and then under sterile conditions the leaf surface was sterilized for 30 to 45 seconds in ethanol 70% or 50%, 30 to 45 seconds in sodium hypochlorite 1.5% or 1%, and a final wash in sterile water. The sterilized leaves were then inoculated with the conidial suspension, avoiding runoff of the suspension from the leaf surface. The inoculation of *A. eucalyptorum* suspension on leaves of *E. dunnii* and *C. citriodora* was performed using two treatments: 1) inoculation of conidial suspension on leaves with wounds (performed with a needle); 2) inoculation of conidial suspension on leaves without wounds. A third treatment served as the control, with leaves with wounds (performed with a needle) sprayed with sterile water. The leaves were incubated for one week at room temperature on Petri dishes of 150 mm diameter containing sterile agar-agar medium. The inoculation was also performed on Petri dishes of 90 mm diameter containing water agar medium with 40 $mg.L^{-1}$ of benzimidazole at room temperature. Source: The author.

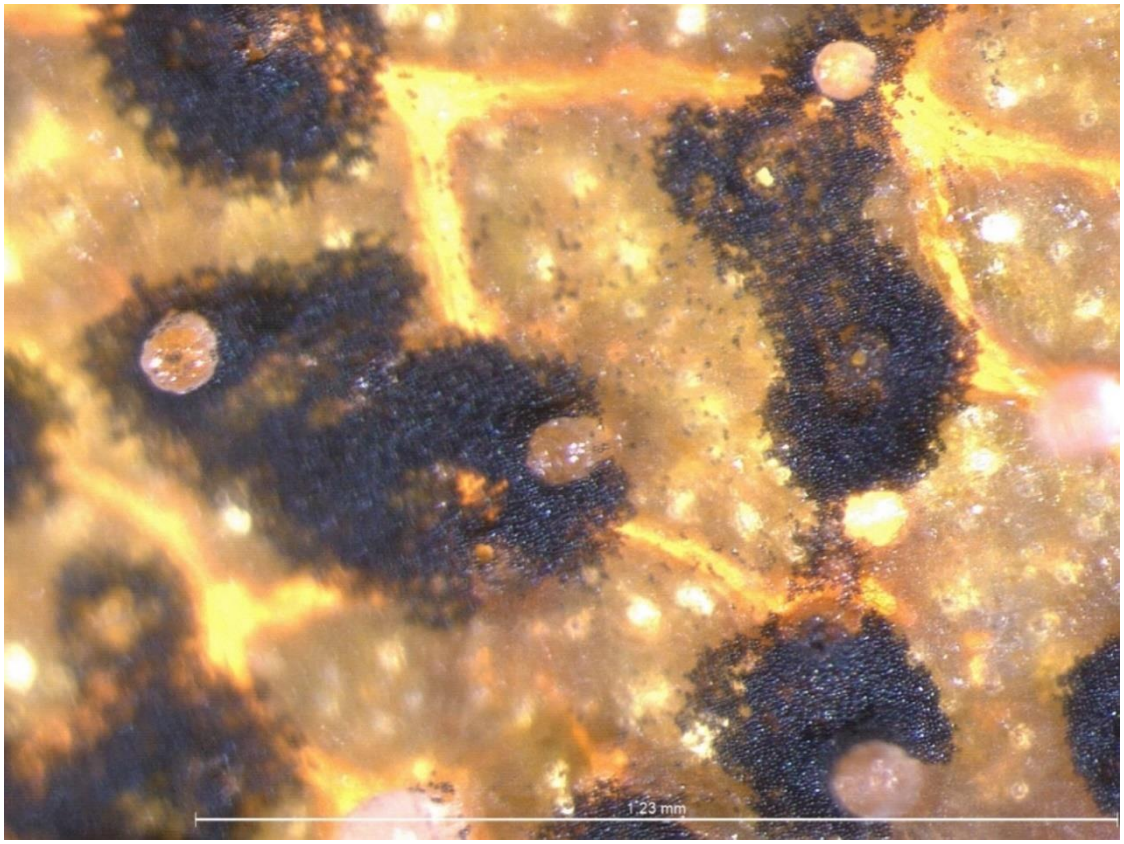


Appendix VIII. Inoculation of *Apoharknessia eucalyptorum* on trunks of *Eucalyptus dunnii*

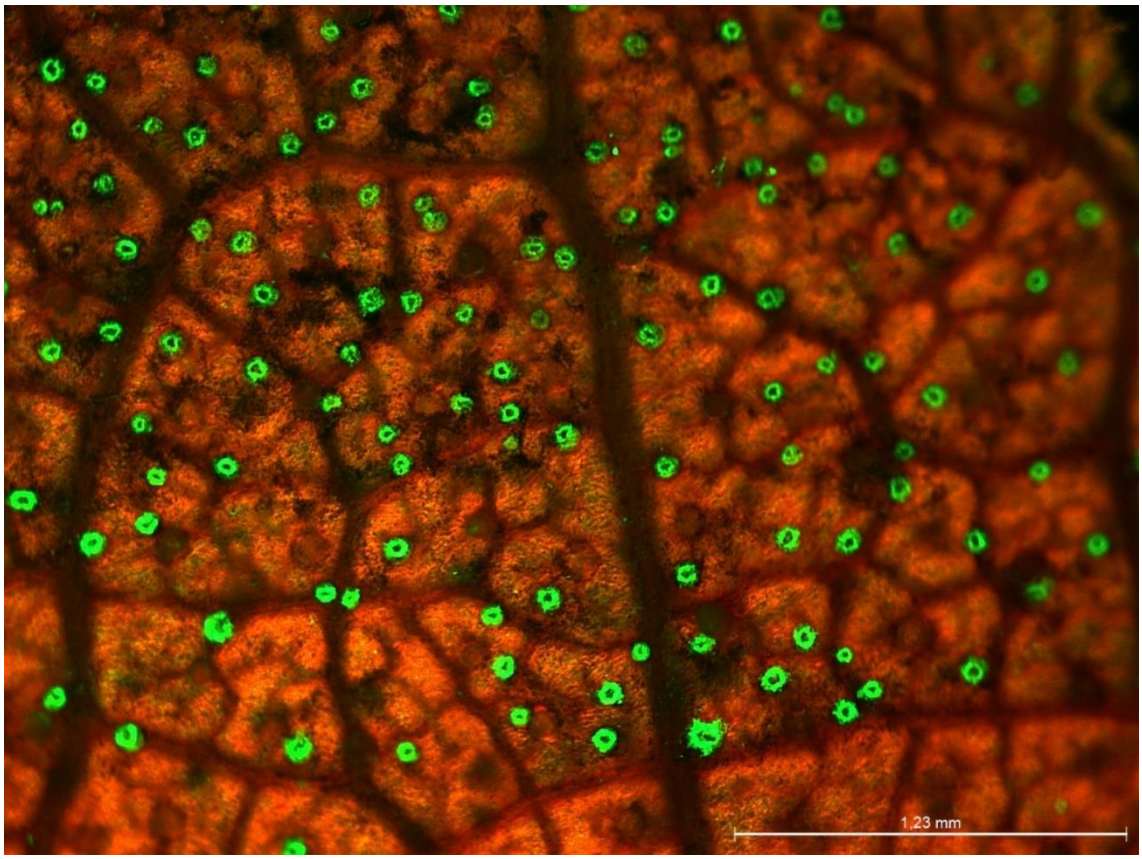
The inoculation of *A. eucalyptorum* on trunks of *E. dunnii* was performed with two treatments: 1) inoculation with 2.5 mm diameter MEA discs colonized by *A. eucalyptorum* on trunks with wounds; 2) inoculation of MEA discs (2.5 mm diameter) colonized by *A. eucalyptorum* on trunks without wounds. A third treatment served as the control, with trunks with wounds treated with sterile MEA discs (2.5 mm diameter). Inoculated and control seedlings were wounded by removing the bark to the cambium and puncturing the trunk with a sterile puncher of 2.5 mm diameter at 2 centimeters above the root collar diameter. The MEA discs colonized with *A. eucalyptorum* and the sterile control MEA discs were attached to the trunks and covered with a plastic band. After inoculation, the seedlings were kept in shaded nursery conditions for six weeks. Source: The author.



Appendix IX. Conidia exudates from pycnidia. Source: The author.



Appendix X. Pycnidia with exudated conidia on *Corymbia citriodora* leaves. Source: The author.



Appendix XI. Germinated conidia and appressorium

Stained structures of *Apoharknessia eucalyptorum* on *Corymbia citriodora* leaves. Red arrows: germinated conidia. Yellow arrows: appressoria. Source: The author.