

Mycelial growth and sporulation of *Apharknessia eucalyptorum* on different culture media

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ABSTRACT

Garrett, A.T.A.; Santos, A.F.; Figueiredo Filho, A.; Tambarussi, E.V.; Gonçalves, A.B.; Garcia, F.A.O. Mycelial growth and sporulation of *Apharknessia eucalyptorum* on different culture media. *Summa Phytopathologica*, v.45, n.3, p.272-278, 2019.

Apharknessia eucalyptorum was first described in 2017 and identified on leaves of *Eucalyptus dunnii* in Southern Brazil. However, information about *in vitro* cultivation for complementary studies is lacking. In the present study, leaves of *E. dunnii* were inoculated to demonstrate the pathogenicity of *A. eucalyptorum*, and growth and sporulation 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. *Apharknessia eucalyptorum* caused leaf blight on

the inoculated leaves. The best conditions for mycelial growth were at 25°C on PDA, BEAN and MEA. Considering sporulation, optimal conditions were 25°C for all tested media and 20°C for PDA and BEAN. Colony characteristics changed with temperature; at 15°C colonies formed a fluffy mycelium, whereas at 25°C mycelium spread across the medium forming dark margins lined by dirty-white mycelium and conidia. The conditions indicated for *in vitro* growth and sporulation of *A. eucalyptorum* are the culture media MEA, PDA and BEAN at 25°C

Keywords: colony characteristics; inoculation; temperature.

RESUMO

Garrett, A.T.A.; Santos, A.F.; Figueiredo Filho, A.; Tambarussi, E.V.; Gonçalves, A.B.; Garcia, F.A.O. Crescimento micelial e esporulação de *Apharknessia eucalyptorum* em diferentes meios de cultura. *Summa Phytopathologica*, v.45, n.3, p.272-278, 2019.

Apharknessia 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 folhas de *E. dunnii* foram inoculadas para demonstrar a patogenicidade de *A. eucalyptorum*, e o crescimento e esporulação 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. *Apharknessia eucalyptorum* causou manchas foliares nas

folhas inoculadas. 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; inoculação; temperatura.

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, providing colonies for molecular and host-pathogen interactions studies.

One pathogen that lacks information about *in-vitro* cultivation is *Apharknessia eucalyptorum* Crous & M.J. Wingfield. The genus *Apharknessia* was separated from *Harknessia* by Lee et al. (18) and proposed due to phylogenetic clustering and hyaline conidia with apical appendage. The genus consists of three species: *Apharknessia insueta* (18), *Apharknessia eucalyptorum* (6), and *Apharknessia eucalypti* (19). *Apharknessia eucalyptorum* was isolated from lower crown leaves of *Eucalyptus dunnii* that were planted in a cold region of Southern Brazil with the occurrence of frosts (GenBank Accession Number MG725682).

Species of the related genus *Harknessia* (7, 11) are dispersed around the world, causing leaf spots and cankers in eucalypts (18). Currently, *A. insueta* is associated with leaf spots in the United States, Brazil, Colombia, Costa Rica, Cuba, Mauritius, and Zimbabwe (7, 11, 18, 21), while *A. eucalyptorum* is restricted to Malaysia (6) and Brazil (16).

Harknessia spp., *A. insueta*, and *A. eucalyptorum* are eucalypt pathogens (8, 14, 16) that cause leaf spots, leaf scorch, and cankers (7, 29). *Harknessia* species, some of which occur in Brazil (3), are better studied and considered economically significant as they have been shown to cause damage to plantations (9, 18, 23). However, some species are limited to specific regions and not aggressive, for example with only a few species occurring in Australia (28). Although these species are better studied, there is still a lack of information about their pathogenicity (7), particularly for *Apharknessia*.

To obtain pure *in vitro* cultures for further studies, microorganisms

are cultivated on media to promote their growth under laboratory conditions (20). 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 (20). Physical factors must also be considered in promoting the growth of microorganisms, such as temperature, light regime, and pH (12, 20, 26).

Harknessia species have been cultured on Malt Extract Agar (MEA) at 25°C for five to eight days to characterize the cultures (18), as well as on Potato Dextrose Agar (PDA) (7) and tomato decoction (27). *Harknessia* spp. are characterized by branched, septate, hyaline to pale brown mycelium usually sporulating in one month (7), while *Apotharknessia* species sporulate sooner than *Harknessia* and produce white mycelium (6, 18).

Thus, due to the limited information about pathogenicity and *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.

MATERIAL AND METHODS

Apotharknessia eucalyptorum isolate

The isolate of *A. eucalyptorum* was obtained from *E. dunnii* plantations in Northern Santa Catarina State, Brazil. The pathogen was identified on old leaves of the crown, causing irregular or circular leaf spots, with necrotic lesions of light brown color and pycnidia on the lesions. Pycnidia were collected directly from the leaves and deposited on Petri dishes containing Malt Extract Agar (MEA 2%). After isolation, genomic DNA was extracted based on the CTAB method and using the *Quick-DNA*TM Universal Kit (Zymo Research Corp, Irvine, California, USA). Amplification of the internal transcribed spacer 1 - 5.8S - internal transcribed spacer 2 (ITS) region of ribosomal DNA, was used to confirm the isolate identity with the specific primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (CTCCGCTTATTGATATGCT). The PCR amplicon was sequenced at LGC Genomics GmbH, Germany. The ITS sequence was compared with available sequences in GenBank using BLAST to confirm pathogen identity. The isolate was examined under a microscope to characterize and measure the length of 50 conidia. The monosporic culture of *Apotharknessia eucalyptorum* APO1 (GENBANK Accession number MG725682) was used in the experiments and deposited in the Laboratório de Patologia Florestal of the Universidade Estadual do Centro-Oeste (UNICENTRO), in Irati, Paraná, Brazil.

Culture media

Four culture media were tested: Malt Extract Agar (MEA); Potato Dextrose Agar (PDA); V8 juice agar (V8) prepared according to Alfenas and Mafia (2); 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 (22). 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.

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.

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. Mycelial growth was measured length-wise, and sporulation was evaluated based on the number of spores per mL after seven days of incubation. Twenty millimeters of sterile water containing Tween 80 (0.05%) was added to Petri dishes colonized by *A. eucalyptorum* and then scraped with a brush to count the number of spores per mL. The spore suspension was filtered with cheese cloth and counted in a Neubauer chamber. After diameter growth evaluations, the growth rate (GR) was determined (mm D^{-1}).

Inoculation on leaves

Pathogenicity of *A. eucalyptorum* was confirmed by inoculating six leaves of *E. dunnii* with a conidial suspension. The conidial suspension was prepared from Petri dishes containing MEA on which *A. eucalyptorum* was incubated at 25°C with a 12-hour photoperiod for two weeks. After adding 20 mL of sterilized water, the colony was scraped with a glass rod to remove the conidia and the suspension was filtered with cheese cloth. Conidia concentration of the filtered suspension was determined in a Neubauer chamber and adjusted for a final suspension of 1×10^6 conidia per mL.

Leaves of *E. dunnii* collected in the field were inoculated with the suspension, avoiding the runoff of the suspension from the leaf surface. Before inoculation, leaves were washed and superficial sterilized under sterile conditions rising the leaves for 30 seconds in ethanol 50%, and in sodium hypochlorite 1.5% for 30 seconds, washing them in sterile water. The leaves were incubated for one week in 150-mm Petri dishes containing sterile agar-agar medium, at room temperature. After observing the symptoms and signs of the pathogen, conidia masses were collected from the leaves and examined microscopically to compare the morphology with cultures incubated *in vitro*.

Statistical analysis

Data on mycelial growth of *Apotharknessia 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% ($\alpha \leq 0.05$). Data of mycelial growth was transformed by $\log_{10} X$, and sporulation data was transformed by $\log_{10} (X + 10)$.

RESULTS AND DISCUSSION

The identification of the isolate was confirmed by BLAST in GenBank, with 99% identity to an isolate of *A. eucalyptorum* (Isolate KY979752; Identities = 627/631(99%), 2 gaps) obtained from Malaysia on *E. pellita*, and used by Crous et al. (6) to describe the species. 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 1-A and B). One week after inoculation, leaves inoculated with the conidia suspension showed symptoms and conidial masses of *A. eucalyptorum* (Figure 1-C). The average size of the conidia is in the range described by Crous et al. (6) for the species, showing the conical apiculus and hyaline basal appendage typical of the species.

Table 1. Average mycelial growth (millimeters) and growth rate of *Apharknessia eucalyptorum* at temperatures of 15, 20, and 25°C after seven days.

Culture Media	Diameter (mm)			GR (mm D ⁻¹)		
	15°C	20°C	25°C	15°C	20°C	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.

The discussion presented herein is based mainly on the related species of *Harknessia* and *A. insueta*, both of which are considered pathogenic. Although pathogenicity studies are important to understand and manage host-pathogen interactions, there are few studies that discuss the ideal conditions for *in vitro* cultivation.

Although Crous et al. (6) consider that *A. eucalyptorum* is endophytic, the pathogenic characteristic of the species is shown through the inoculation tests described herein and the previous study by Garrett et al. (16). The symptoms of *Harknessia* spp. are usually light to dark brown, of a round or irregularly shaped margins or occurring on the leaf edges (11, 21, 28). Species of *Harknessia* are also associated with symptoms of tip dieback and leaf scorch on *Eucalyptus* spp. (7). The symptoms of *A. eucalyptorum* found herein are similar to those described in the literature for *Harknessia* spp., including conidial masses on the lesions, similar to that observed for *H. eucalypti* on eucalypt plants (11).

A significant interaction between media and tested temperature was observed ($p = 0.0046$) for *A. eucalyptorum* mycelial growth. Mycelial

growth increased with an increasing 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 1). In this study, mycelia reached the edge of the Petri dishes on PDA and BEAN within six days.

Growth rate was greater for *Harknessia uromycoides* at 20°C and at 25°C, while for *H. eucalyptorum* it was observed at 25°C (10), both cultured on MEA. *Harknessia ipereneae* and *H. gibbosa* grow better on PDA, and covered Petri dishes in two weeks with fluffy white mycelium (7, 9). Another isolate of *Harknessia* sp. from Venezuela was also cultured on PDA at 25°C for 12 days (21). Many *Harknessia* species have been cultured on MEA, PDA, and Oatmeal agar at 25°C (7), while *H. renispora*, has been cultured on a medium based on a tomato or oatmeal decoction (27).

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.

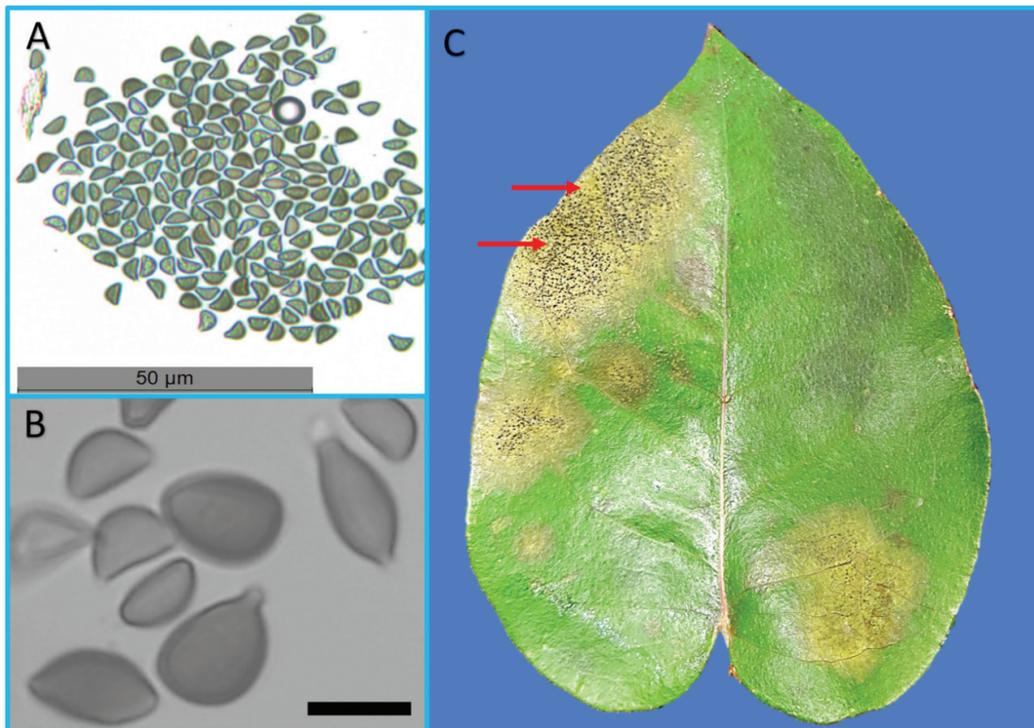


Figure 1. Spores and symptoms of *Apharknessia eucalyptorum* on wounded leaf. (A-B) Conidia. (C) Symptoms (brown area) and signs (black dots within brown area highlighted by red arrows) on *Eucalyptus dunnii* leaf inoculated with conidia suspension. Scale: 10 µm. Source: The authors.

Table 2. Average sporulation (conidia ml⁻¹) of *Apharknessia 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.

(22). All the culture media tested in this study are natural media with uncertain nutrient contents, particularly in terms of nitrogen and carbon (1). Nevertheless, the use of natural media is suitable due to ease of preparation and reduced cost (5). Moreover, while PDA has been shown to allow growth of different fungi (24), in this study the BEAN medium proved to be suitable for satisfactory growth and sporulation of *A. eucalyptorum*.

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 was statistically similar for PDA and BEAN, and at 25°C the number of conidia was the same for all tested media (Table 2). Sporulation was observed within

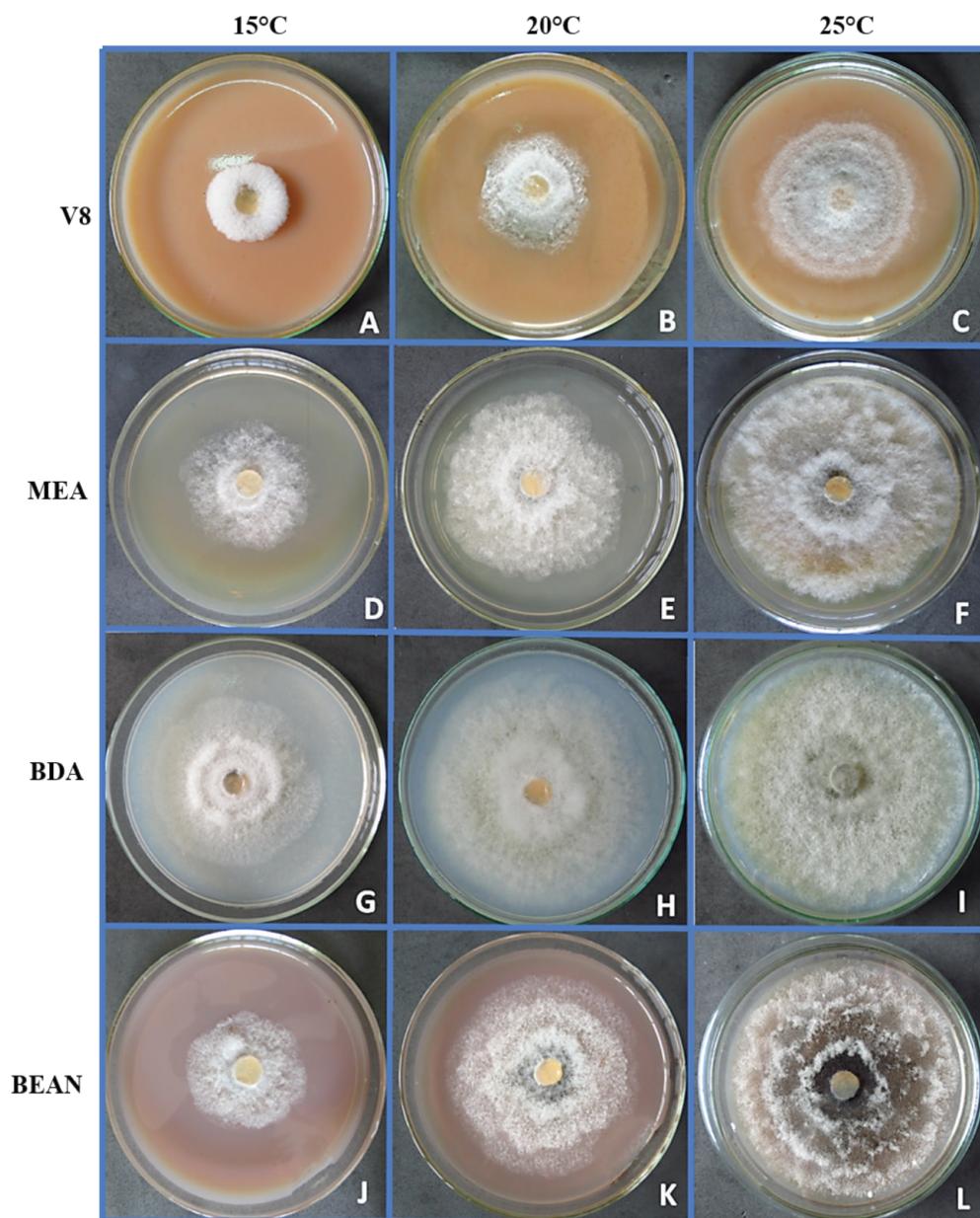


Figure 2. *In vitro* characteristics of mycelial growth and sporulation of *Apharknessia eucalyptorum*. (A-D-G-J) 15°C; (B-E-H-K) 20°C; (C-F-I-L) 25°C; (A, B, and C) V8 medium; (D, E, and F) MEA medium; (G, H, and I) PDA medium; (J, K, and L) BEAN medium. Source: The authors.

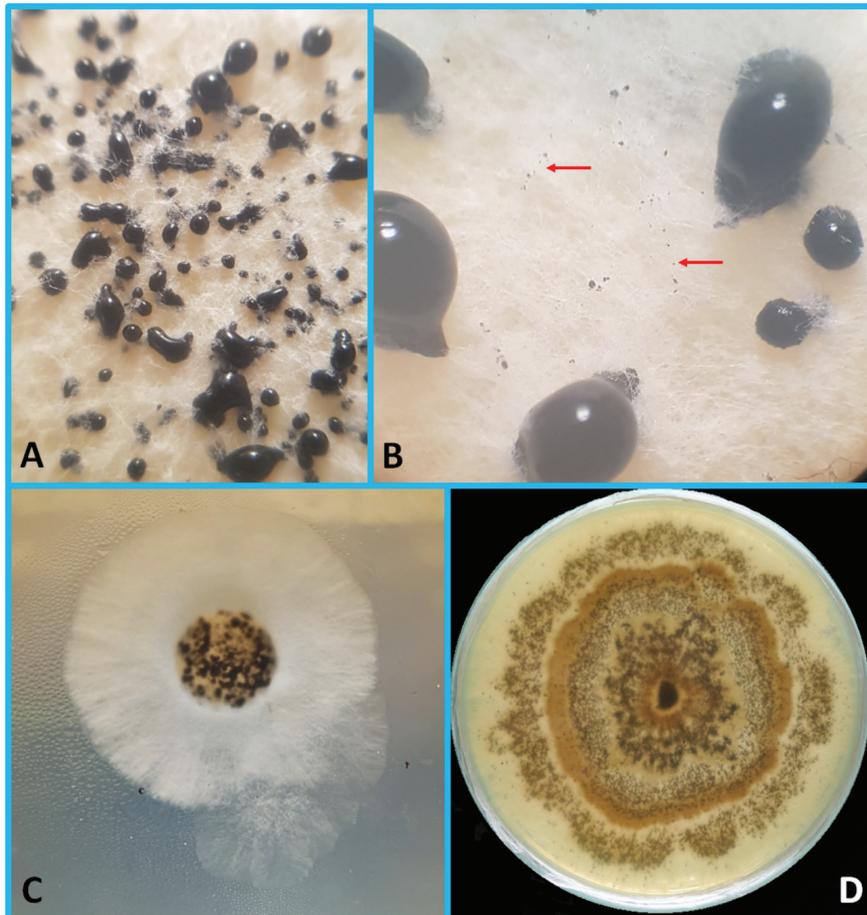


Figure 3. Characteristics of *Apoharknessia eucalyptorum* on MEA. (A) Viscous sporulation on MEA. (B) Detail of viscous sporulation on MEA and conidia on naked hyphae (arrows). (C) Filamentous mycelia at the beginning of growth on MEA; (D) Reverse side of MEA colonized by *A. eucalyptorum* with olivaceous mycelium, and mycelium and sporulation forming borders on MEA. Source: The authors.

four days for all media.

Production of spores *in vitro* is important for inoculation (1) and taxonomic studies. *Harknessia* spp., such as *Harknessia eucalyptorum* (10), usually sporulate after two weeks (6), while *H. hawaiiensis* sporulates after one week and other species after two or more weeks (10). *Harknessia capensis* and *H. globispora* sporulate in a few days and have white mycelia with margins (18). In comparison, the mycelial growth of *A. insueta* and *A. eucalyptorum* is faster and sporulation occurs in one week, as described by Lee et al. (18) and Crous et al. (6). 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. (6) described growth to 70 mm in two weeks at 25°C. This difference in growth could be 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.

Sporulation *in vitro* is necessary because the morphology of conidia can help to distinguish species of *Harknessia* from other fungal species, such as *A. insueta* and *A. eucalyptorum* (10, 13, 15). According

to Crous et al. (8), *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 (6, 7, 18).

Figure 2 shows growth and sporulation characteristics in each tested medium. Mycelial growth and sporulation of *A. eucalyptorum* increased with increasing 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 as temperature increased, with viscous sporulation and conidia on naked hyphae (Figure 3 A and B) as seen in the dark area within the mycelia. Mycelial characteristics changed with increasing temperatures. At the beginning of growth, mycelium was filamentous (Figure 3 - 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 3). On MEA, the reverse side of the colonies showed olivaceous color (Figure 3 - D); however, on V8, PDA, and BEAN this characteristic was less pronounced.

Except on V8 and at 15°C, *A. eucalyptorum* showed dirty-white mycelium growing sparsely within the medium that stimulated the

growth of fluffy aerial mycelium. The color of mycelium on *Harknessia* species vary and is usually fluffy (10). On MEA, *H. rhabdosphaera* grows by spreading through the medium, with fluffy dirty-cream mycelium, a surface color of buff to honey, with sporulation in black masses, forming dark margins (25). 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 et al. (18), 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. (6). Different culture media influence morphologic characteristics of fungi (17, 24), and such variations on different media can impede identification and distinction between species. One of the characteristics used to distinguish *Harknessia* spp. from *Aphoharknessia* spp. is growth pattern and time of sporulation *in vitro* (6, 18). Thus, cultivation and characterization of *in vitro* colonies are important to identify these related species. 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 (6).

The first test of *in vitro* cultivation of the pathogenic fungus *A. eucalyptorum* is presented in this study. 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 about the possible impacts of a pathogen on *Eucalyptus* spp. Based on these results, MEA, PDA, and BEAN are indicated for the cultivation of *Aphoharknessia eucalyptorum* since they promoted better growth and sporulation of the species at 25°C.

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