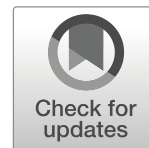
Tropical Plant Pathology (2019) 44:132–139
https://doi.org/10.1007/s40858-019-00277-0



Molecular, morphophysiological and pathogenic characterization of eucalypt *Pestalotiopsis grandis-urophylla* isolates, a new species

Daniel D. C. Carvalho[1] · Renata M. Oliveira[2] · Marina G. Marques[1] · Mariany D. Milan[1] · Danilo B. Pinho[3] · Érico C. Dianese[2]

Received: 13 March 2018 / Accepted: 11 February 2019 / Published online: 7 March 2019
© Sociedade Brasileira de Fitopatologia 2019

Abstract

Species of *Pestalotiopsis* have been reported to be pathogenic to eucalypt, yet few studies have addressed their real pathogenic potential or even their diagnosis. The objective of this study was to carry out the molecular, micromorphological, physiological and pathogenic characterization of four isolates of *Pestalotiopsis* sp. found in eucalypt leaf spots. DNA from the isolates was extracted and PCR amplified using primers for the internal transcribed spacer (ITS), partial β-tubulin (TUB) and translation elongation factor 1-alpha (EF1-α) gene regions. For morphophysiological characterization, the fungal structures were measured and isolates evaluated for mycelial growth and sporulation under different light regimes (0, 12, and 24 h). Pathogenicity tests were conducted on healthy eucalypt leaves. The results revealed that (a) the amplified ITS region is too conserved to be used for identification of *Pestalotiopsis* species, and thus, TUB and EF1-α sequences are recommended for this purpose; (b) based on micromorphological characteristics and DNA sequences, the four isolates were identified as the new species *Pestalotiopsis grandis-urophylla*; (c) *P. grandis-urophylla* presents faster mycelial growth when cultivated in the dark, but for mass production of inoculum the light regime does not have a strong influence; and (d) the pathogenic potential varied among the *P. grandis-urophylla* isolates.

Keywords *Eucalyptus* sp. · Fungal taxonomy · Koch's postulates · Sporulation

Introduction

The eucalypt (*Eucalyptus* sp.) plant is native to Australia, but also occurs naturally in Indonesia and adjacent islands (Carmo et al. 2013). The *Eucalyptus* genus belongs to the Plantae Realm in the Myrtaceae Family, which includes around 600 species and subspecies. These species are among the most important forest crops in the world, due to both their versatility, which allows cultivation in diverse regions, and their rapid growth, ascribing them great economic importance (Carmo et al. 2013). The favorable climatic conditions, soil types, and large crop areas make Brazil as one of the most promising markets for eucalypt worldwide. Moreover, this crop bears great economic value in Brazil due to its extensive use in the production of cellulose, paper, oils for the pharmaceutical industry, decoration objects, firewood, vegetable coal as well as in recovery of degraded areas, forest recomposing, sawmills, and wind breaks (Santos et al. 2001). Currently, the Brazilian forest sector accounts for about 5% of the gross national product and 8% exports of the country, generates 1.6 million direct and 5.6 million indirect jobs, and an annual revenue of $ 20 billion, which collects R$ 3 billion in taxes (Castro et al. 2015).

Nevertheless, the cultivation of *Eucalyptus* spp. may be hindered by phytosanitary problems, among which diseases caused by biotic components are considered important limiting factors to production (Carmo et al. 2013; Kimati et al. 2005). Eucalypt is attacked by several pathogens, mainly fungi, at different stages both in nurseries and during mature plant development. Besides, many phytosanitary problems are observed with several species in various locations and at different times of the year (Santos et al. 2001). For that reason,

Section Editor: Raul AllendeMolar

✉ Daniel D. C. Carvalho
daniel.carvalho@ueg.br

[1] Universidade Estadual de Goiás (UEG), 75780-000, Ipameri, Goiás, Brazil

[2] Universidade Federal de Goiás (UFG), 74001-970, Goiânia, Goiás, Brazil

[3] Universidade de Brasília (UnB), Brasília, Federal District 70910-900, Brazil

information and research on pathogen identification and disease control are often required in order to generate knowledge on biotic problems associated to eucalypt that enable the establishment of appropriate control strategies, help reduce losses, and increase productivity in the forest sector (Alonso et al. 2009; Kharwar et al. 2010). Leaf spots caused by fungal pathogens have acquired great importance recently, mainly in mature eucalypt plantations (Rodas et al. 2005; Lana et al. 2012).

With regard to the fungi that cause such spots, special attention has been given to *Pestalotiopsis* sp., reported to be responsible for spots on *E. globulus* (Alonso et al. 2009), associated with the fungus *Colletotrichum gloeosporioides* in leaf spots on *E. grandis*, a secondary pathogen in woody tissue of *E. viminalis* (Carmo et al. 2013), and an endophytic and epiphytic fungus in *E. citriodora* leaves (Kharwar et al. 2010). This is due to the high dissemination capacity of *Pestalotiopsis* sp. spores, which are capable of penetrating vegetable tissues through injuries or natural openings and infect a great variety of botanical species, particularly eucalypt (Carmo et al. 2013), causing damage that can reduce production by up to 60% (Royo 2012). Therefore, proper identification of the species is required as the first step in treating the disease it causes.

According to many taxonomists, the classification of species within the genus *Pestalotiopsis* is based on culture-related features, such as mycelial growth and sporulation, and particularly micromorphological traits, which include conidial length, width and length/width ratio as well as number and length of apical and basal appendages (Barber et al. 2011; Maharachchikumbura et al. 2011). Additionally, *Pestalotiopsis* isolates can be characterized through sequencing of conserved genome regions, such as the internal transcribed spacer (ITS), β-tubulin (TUB) and translation elongation factor 1-alpha (EF1-α), whose sequences are then compared to others deposited in databases such as the GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank/), and allocated to a species (Maharachchikumbura et al. 2011, 2014). There are few studies on the molecular, morphophysiological and pathogenic characterization of *Pestalotiopsis* sp. isolates found in eucalypt leaf spots in Brazil. Furthermore, there is not enough information on the *Pestalotiopsis* sp. found in leaf spots with respect to its actual aggressiveness levels worldwide. As a result, there is great need for research on these specific areas of the *Pestalotiopsis* associated with eucalypt. This study was carried out to (a) accomplish the molecular characterization by using combined ITS, TUB and EF1-α DNA sequences of four *Pestalotiopsis* sp. isolates; (b) accomplish the micromorphological characterization of these isolates; (c) evaluate the mycelial growth and sporulation of the isolates under different light regimes; and (d) evaluate the pathogenic potential of the *Pestalotiopsis* sp. isolates through pathogenicity tests with healthy leaves of mature *E. urograndis* 'GG 100' plants.

Materials and methods

Fungal isolates

In total, four *Pestalotiopsis* sp. isolates (E-72-02, E-72-03, E-72-04 and E-72-06) were obtained from spotted leaves of mature *E. urograndis* 'GG 100' plants aged 18 months, located at the Universidade Estadual de Goiás (UEG), Ipameri Campus (17°43′00.38"S, 48°08′40.96"W, 796 m). The isolates were purified and conserved in potato dextrose agar (PDA) medium at 5 °C. Monosporic cultures were deposited in the "Coleção de culturas de fungos fitopatogênicos Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil.

Molecular characterization and phylogenetic analysis

The DNA was extracted with a protocol modified from Dellaporta et al. (1983). For that, 200 μL of the Dellaporta extraction buffer (0.5 M NaCl, 0.1 M Tris, 0.05 M EDTA) were added to 100 mg of fungal mass obtained from a ten-day-old *in vitro* culture on PDA medium at 25 °C. The tissue was subsequently macerated in a 1.5-mL microfuge tube using a pistil, and then 33 μL of 20% SDS were added. The tubes were centrifuged for 2 min and then incubated at 65 °C for 10 min. 160 μL of 5 M potassium acetate were added to the tubes, which were vortexed for 2 min and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded, 500 μL of 75% ethanol added to the pellet, and the tube centrifuged at 14,000 rpm for 5 min. The supernatant was removed and the pellet resuspended in 50 μL of Milli-Q water.

The purified DNA was amplified by polymerase chain reaction (PCR) using 5 μL of EmeraldAmp® GT PCR Master Mix (Takara Bio Inc.); 0.2 μL of each primer; 2.6 μL of Milli-Q water and 1.0 μL of DNA in a final volume of 10 μL. Three primer sets were used: (a) ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') (White et al. 1990) for the ITS region, (b) BT2A (5'GGTAACCAAATCGGTGCTGCTTTC3') and BT2B (5'ACCCTCAGTGTAGTGACCCTTGGC3') (Glass and Donaldson 1995) for the TUB gene, and (c) EF1-526F (GTCGTYGTYATYGGHCAYGT) and EF1-1567R (ACHGTRCCRATACCACCRATCTT) (Rehner 2001) for a conserved region of EF1-α. For primer pairs ITS4/ITS5 the PCR program consisted of an initial denaturation step at 96 °C for 2 min, followed by 35 cycles at 96 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Amplification for primer pairs BT2A/BT2B, and EF1-526F/EF1-1567R was conducted by an initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The PCR products were visualized through electrophoresis in 1.0%

agarose gel in TBE and visualized under UV light. After observation of specific bands, the PCR products were precipitated with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.3), followed by a 12-h rest at −20 °C, and centrifugation at 14,000 rpm for 1 min. The pellet was rinsed with 70% ethanol and the DNA resuspended in 3 volumes of Milli-Q water. The DNA concentration was measured using a NanoDrop (ThermoFisher). DNA sequencing was carried out by ACTGene Molecular Analysis (Alvorada, RS, Brazil). The ITS, TUB and EF1-α sequences were deposited in GenBank under accession numbers KU926708, KU926716, KU926712 (for E-72-02), KU926709, KU926717, KU926713 (E-72-03), KU926710, KU926718, KU926714 (E-72-04) and KU926711, KU926719, KU926715 (E-72-06). The sequences were compared with other *Pestalotiopsis* sequences deposited in GeneBank using the Basic Local Alignment Search Tool (BLAST: Altschul et al. 1990) hosted at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment was constructed using CLUSTALX (Larkin et al. 2007) and BOXSHADE 3.21 (REFs). Phylogenetic analysis was constructed by the Neighbor-Joining method.

Micromorphological characterization

The slides for the study of micromorphological structures were prepared with lactoglycerol as the mounting medium by removing the fungal structures from 7-days old cultures on PDA. The images were captured under a Leica DM500 light microscope with an attached camera. Measurements of the fungal structures (conidia length and width, conidia median cell length and width, number and length of apical and basal appendages) of each isolate were carried out with the aid of the LAS EZ 2.0 (100×) software (LEICA). On average, 30 measurements were recorded for each structure.

Evaluation of mycelial growth and sporulation under different light regimes

PDA plugs (7 mm) with mycelia of the four E-72 *Pestalotiopsis* sp. isolates were extracted from colonies (approx. ten-days-old) and transferred to the center of Petri dishes containing PDA medium. Next, the dishes were kept in a BOD at 25 °C, under three light regimes: 0, 12 and 24 h. The radial mycelial growth was recorded based on the average between two opposite diameters, which were measured every 2 days from the second to the tenth day of growth. After the last reading of radial growth, the dishes were used to quantify spore production. To do so, 10 mL of sterile distilled water were added to each Petri dish, followed by the release of spores with a Drigalsky spatula. Then, spores were collected in a Becker and sieved through sterile gauze. Concentrates obtained from the suspensions were measured in a Neubauer chamber. The number of spores was counted five times for each culture. The experiment was set in a completely randomized design with five replications (Petri dishes) for each *Pestalotiopsis* sp. isolate. The experiments were carried out twice.

Evaluation of pathogenic potential

Healthy leaves of mature *E. urograndis* 'GG 100' plants (aged 18–24 months) were rinsed in running water and dried in a laminar flow hood for 10 min. For inoculation, five holes were needle-punctured in the center of the leaf surface, and a PDA plug (7 mm) containing the *Pestalotiopsis* sp. mycelium (from a 5-days culture) was placed on it (Serra and Coelho 2007). The leaves inoculated with the pathogen were placed inside a transparent Gerbox (11 × 11 × 3.5 cm) under high humidity by means of a germination paper constantly watered. Disease severity evaluations were performed by measuring lesion diameter around each inoculation point with a digital caliper rule at 4, 6, 8 and 10 days after inoculation (DAI). The experiments were conducted twice. Re-isolation of the fungus from infected leaves was attempted to fulfill Koch's postulates.

Statistical analysis

Data on mycelial growth and sporulation under different light regimes, pathogenicity tests, and micromorphological structures were submitted to analysis of variance. Scott-Knott test ($P < 0.05$) was used for mean comparisons and regression analysis to obtain representative models. Statistical analyses were performed with the SISVAR 5.3 software (Ferreira 2011).

Results

Molecular characterization and phylogenetic analysis

Four isolates of *Pestalotiopsis* sp. were obtained from spotted leaves of mature *E. urograndis* 'GG 100' plants. After purification and conservation of the cultures, the isolates were grown on PDA medium for DNA extraction. The DNA of the four isolates was amplified by PCR using primer sets ITS4/ITS5, BT2A/BT2B and EF1-526F/EF1-1567R for ITS, TUB and EF1-α, respectively. These primer sets amplified specific bands of approx. 600 bp for ITS, approx. 450 bp for TUB and approx. 500 bp for EF1-α. The sequences of these three genomic regions were used to obtain a combined data matrix that consisted of 76 sequences from 55 taxa originated from Maharachchikumbura et al. (2014), including the new four E-72 isolates as well as P. keteleeria (MFLUCC13–0915) and N. saprophytica (MFLUCC12–0282) as outgroups (Fig. 1). The combined alignment of the ITS, TUB and EF1-α contained 1913 characters, 395 of which were parsimony-informative, 572 were variable and 946 were

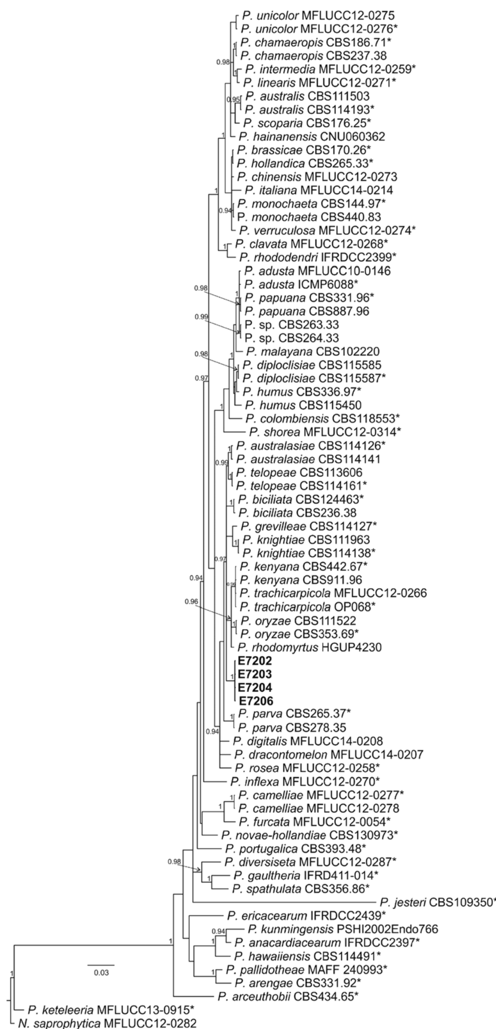


Fig. 1 Phylogenetic tree of *Pestalotiopsis* isolates based on the ITS, TUB and EF1-α sequences. The tree was constructed using the Neighbor-Joining method. A bootstrap analysis was performed with 100 repetitions. Scale bar indicates estimated 1% sequence divergence

conserved. Trees were sampled at every 1000 generations for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Isolates E-72 were genetically distant from the other species (Fig. 1).

Micromorphological characterization

All four isolates presented five-celled conidia, of which the apical and basal cells were hyaline and the three median cells were light and dark brown colored (Fig. 2). Measurements of the conidia did not reveal differences among the E-72 *Pestalotiopsis* isolates. Conidia had a length of 23.0–30.0 μm, a width of 5.0–6.5 μm and a length/width ratio varying from 4.0 to 5.3 (Table 1). The conidia median cells were light and dark brown colored with their lengths ranging from 5.0 to 7.0 μm and their width from 5.0 to 6.5 μm. The apical appendages were hyaline and amounted to 2–3 by conidium (Fig. 2), with length ranging from 9.5 to 19.5 μm. No lumps or branching were observed in the apical appendages, which had inserts at the top of the hyaline apical cell. One hyaline basal appendage whose length ranged from 3.5 to 8.0 μm was found in the conidia.

Evaluation of mycelial growth and sporulation under different light regimes

There were no statistical differences in mycelial growth among the isolates, which allowed regression models to be obtained for the entire set of isolates under each light regime. Each light regime produced a distinct mycelial growth pattern (Fig. 3). In the dark, a degree two polynomial was obtained ($y = -0.4379x^2 + 12.9791x - 14.2200$; $r^2 = 99.04\%$; $P \leq 0.01$). When subjected to 12 h of light, a linear model was obtained ($y = 7.2975x - 4.0350$; $r^2 = 99.92\%$; $P \leq 0.05$) and when subjected to 24 h of light, a degree three polynomial was produced ($y = 0.2348x^3 - 3.9758x^2 + 26.2086x - 29.0750$; $r^2 = 99.73\%$; $P \leq 0.05$). All models were significant and had high coefficients of determination (r^2).

There were no differences in sporulation either among isolates after 10 days of growth in PDA under the three light regimes or among different light regimes. Overall, the sporulation of the isolates was on average 22.9, 21.8 and 22.3 conidia mL^{-1} for the 0-, 12- and 24-h regimes, respectively.

Evaluation of pathogenic potential

Isolates E-72-04 and E-72-06 presented higher virulence than the others, producing spotted leaf areas of 27.6 and 26.8 mm^2, respectively (Table 2). Isolate E-72-02 caused the smallest spots to eucalypt leaves (12.0 mm^2), behaving differently as shown by its disease progress curve (Fig. 4), which was corrected into a simple linear model after regression analyses (Table 2). The other three isolates, with higher pathogenic potential than E-72-02, were corrected into a degree two polynomial. All models were significant and had high coefficient of determination. After inoculation, the typical symptoms of the disease caused by E-72 *Pestalotiopsis* were reproduced, which were characterized by dark brown necrotic spots after 10 DAI (Fig. 5). Each isolate was re-isolated from infected tissue and the same structures and growth behavior on PDA medium as those of the initial isolates were observed.

Discussion

This study was aimed to accomplish the molecular characterization of four E-72 *Pestalotiopsis* isolates through a fast procedure of DNA isolation and purification. The concentration

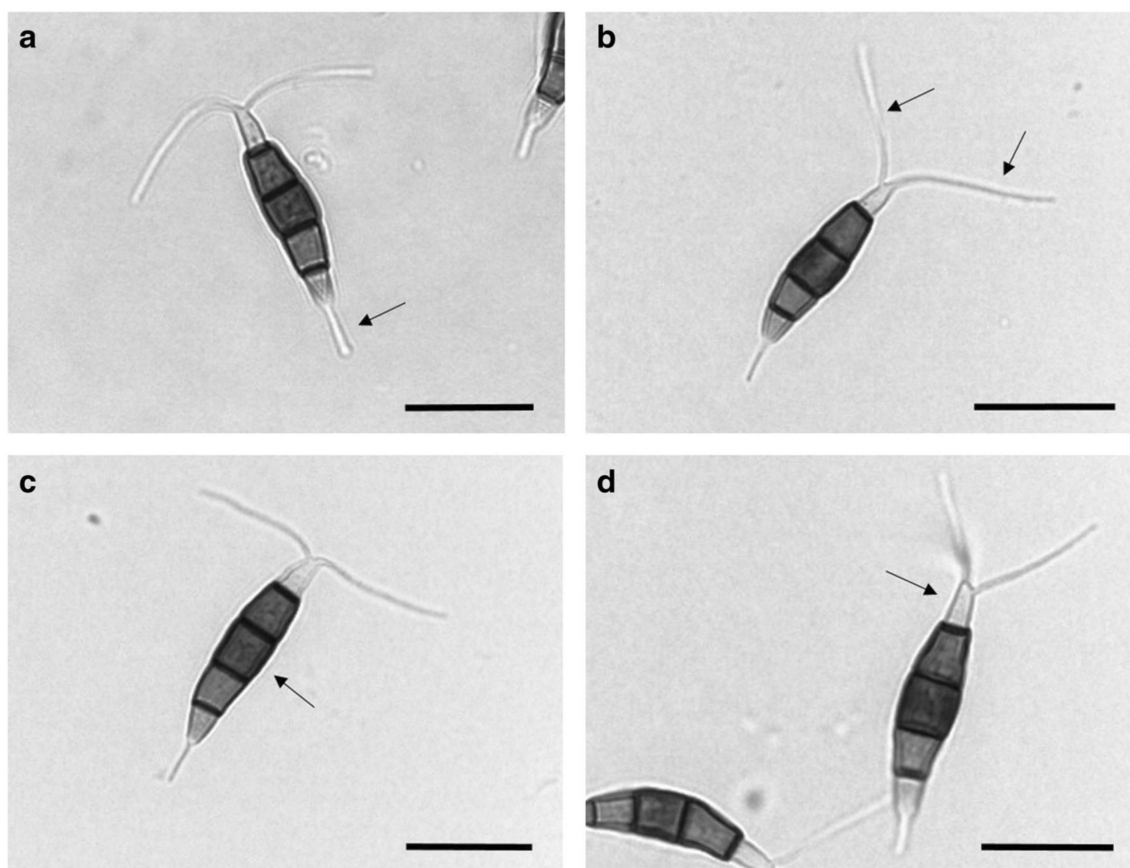


Fig. 2 *Pestalotiopsis grandis-urophylla* conidia obtained from spotted leaves of mature *Eucalyptus urograndis* 'GG 100' plants aged 18 months. **a** Isolate E-72-02 conidium with five cells, two of which are hyaline apical cells and the other three median cells presenting light and dark brown color. The arrow shows the conidium hyaline basal appendage. **b** Isolate E-72-03 conidium. Arrows show the conidium two hyaline apical appendages. **c** Isolate E-72-04 conidium. The arrow shows the dark brown median cell. **d** Isolate E-72-06 conidium. The arrow shows the conidium hyaline apical cell. The bars correspond to 15.5; 16.5; 16.0 and 14.5 μm for Figs. A, B, C and D, respectively

of obtained DNA was satisfactory since a total of 30–60 ng of DNA per sample was sufficient for the next steps. The concentrations obtained conform to Kruschewsky (2010), who obtained between 78.6 and 203.8 ng μL^{-1} of DNA per *Pestalotiopsis* sp. sample. Using the Dellaporta et al. (1983) extraction buffer contributed to the DNA sample integrity and to more efficient deproteination. After the subsequent extraction steps, the occurrence of the specific bands in a 1% agarose gel suggested that there was no DNA contamination by polysaccharides.

Hu et al. (2007), Liu et al. (2010) and Maharachchikumbura et al. (2012) suggested that a combined multigene

Table 1 Characteristics of *Pestalotiopsis grandis-urophylla* conidia obtained from mature *Eucalyptus urograndis* 'GG 100' plants

Isolates	Conidia[a]			Median cell[a]		Appendages[a]			
						Apical[c]		Basal[c]	
	Length (μm)	Width (μm)	L/W[b]	Length (μm)	Width (μm)	N	Length (μm)	N	Length (μm)
E-72-02	26.1 ± 2.0	5.9 ± 0.5	4.4 ± 0.4	6.0 ± 0.8	5.8 ± 0.4	2–3	15.8 ± 3.4	1	6.3 ± 1.5
E-72-03	26.1 ± 3.0	5.7 ± 0.5	4.6 ± 0.7	5.7 ± 1.1	5.7 ± 0.5	2–3	15.7 ± 3.8	1	5.1 ± 1.6
E-72-04	27.9 ± 2.3	6.0 ± 0.5	4.6 ± 0.5	6.4 ± 0.6	6.0 ± 0.5	2–3	16.6 ± 3.1	1	5.9 ± 1.1
E-72-06	25.8 ± 1.6	5.7 ± 0.6	4.6 ± 0.5	5.8 ± 0.5	5.7 ± 0.6	2–3	13.6 ± 3.9	1	5.4 ± 1.2

[a] Means of 30 conidia per isolate ± standard deviation

[b] L, length; W, width

[c] N, number

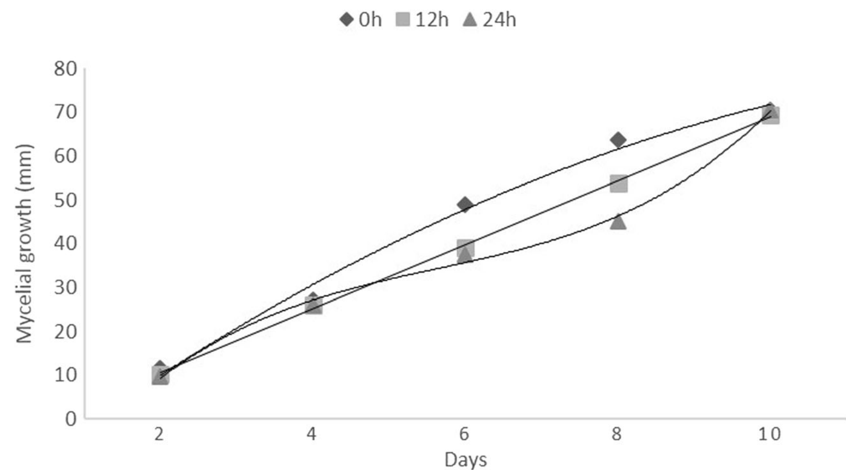


Fig. 3 Mycelial growth (mm) of *Pestalotiopsis grandis-urophylla* from 2 to 10 DAI after growing on PDA medium under different light regimes (0, 12 and 24 h of light)

dataset would better resolve the taxonomy of *Pestalotiopsis* and this conclusion is supported here. The ITS sequence was less informative than those of the TUB and EF1-α genes in distinguishing endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp. (Hu et al. 2007). Likewise, the present study shows that the sequence of the ITS region do not allow for *Pestalotiopsis* species separation since the alignment resulted in high levels of similarity (from 98 to 100%) among several different species indicating that this region is too conserved to be discriminating. According to Maharachchikumbura et al. (2014), the ITS sequence allied to those of the TUB and EF1-α genes provides better differentiation among *Pestalotiopsis* species.

The distinction of *Pestalotiopsis* species through the evaluation of individual morphological characters is more difficult given the occurrence of overlapping when broad ranges of values are obtained. According to Kruschewsky (2010), the micromorphological characters conidia length and width show little variance among isolates. However, most studies have not provided or even evaluated conidia length/width ratio. Such information should be provided in order to facilitate the distinction of species since the length/width ratio of E-72 isolates had a high variance (CV = 12.62%) when compared to that of conidia sizes (CV = 8.55%). Also, during the distinction of species, apical and basal appendages were taxonomically relevant in 80% of cases, since their lengths presented high variance. Such characters also presented high variance for E-72 isolates (CV = 23.35 and 24.46% for apical and basal appendages, respectively).

Isolates E-72 were named as *P. grandis-urophylla* because their host, the clone “GG 100”, is a *Eucalyptus grandis* × *Eucalyptus urophylla* hybrid. The mycelial growth of the *P. grandis-urophylla* isolates was aided by continuous darkness from the 4th to the 8th days after pricking (Fig. 3), which is very common for many Hyphomycetes fungi, as noted by Teramoto et al. (2013). Another characteristic reported for Hyphomycetes fungi is the induction of sporulation caused by the 24-h light regime (Carvalho et al. 2008). Since sporulation is dependent on other factors, such as the number of prickings during the sporulation peak and the broad variance in sporulation speed and intensity among isolates (Kruschewsky 2010), the impact of light regime was not determined for *Pestalotiopsis* spp. The relevance of such studies should be emphasized, especially with respect to the production of inoculum (Carvalho et al. 2008). Therefore, in order to achieve faster mycelial growth of *P. grandis-urophylla,* cultivation in the dark during 6–8 days is suggested, whereas for mass production of inoculum the light regime is not a significant factor.

Table 2 *Eucalyptus urograndis* ‘GG 100’ leaf area spotted by *Pestalotiopsis grandis-urophylla* at 10 DAI and regression models for leaf spot growth from 2 to 10 DAI

Isolate	Spotted leaf area at 10 DAI (mm^2)[a]	Regression model	r^2 (%)	($P \leq$ X)
E-72-02	12.0 c	$y = 0.84x + 5.04$	72.06	0.01
E-72-03	21.6 b	$y = -0.4285x^2 + 6.8228x - 4.32$	98.32	0.01
E-72-04	27.6 a	$y = -0.4928x^2 + 8.8542x - 11.52$	98.82	0.01
E-72-06	26.8 a	$y = -0.7371x^2 + 11.1977x - 12.86$	92.40	0.01
CV (%)	17.61	–	–	–

[a] Values in the column followed by the same letter are not significantly different according to Scott-Knott test ($P \leq 0.05$)

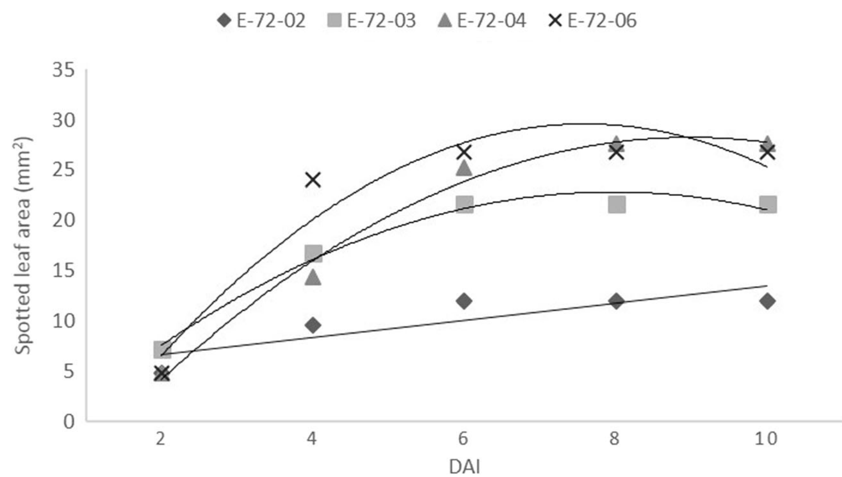


Fig. 4 *Eucalyptus urograndis* 'GG 100' leaf area spotted by *Pestalotiopsis grandis-urophylla* from 2 to 10 DAI

A total of 13 species of the *Pestalotiopsis* species on *Eucalyptus* sp. have already been reported (Farr and Rossman 2015). *Pestalotiopsis* spp. fungi have also been found in other plants, especially ornamentals. Jeon and Cheon (2014) found *Pestalotiopsis* causing brown spots on the leaf borders of *Taxus cuspidate*, an ornamental tree. The spots subsequently coalescence forming lesions similar to those shown in Fig. 5b. Wu et al. (2009) also reported *Pestalotiopsis* on *Reineckea carnea,* a medicinal herb, on which it caused 20 × 50-mm oval dark brown spots that coalesced on the leaf blade. In contrast to our study, Wu et al. (2009) found a yellowish halo around the spots, which is not very common for *Pestalotiopsis*. Zhang et al. (2010) studied the occurrence of *Pestalotiopsis* in the ornamental plant *Hypericum patulum*; the fungus attacked over 30% of the leaves, on which it initially caused light to dark brown spots sized 20–113 mm^2 that resulted in the formation of dark acervuli on their top.

The symptoms showed in Fig. 5 confirm the main observations previously reported in the literature. The pathogenicity of the *P. grandis-urophylla* isolates used in this study is believed to be due to the fact that they were obtained from spotted leaves, which rules them out as endophytic or weak secondary pathogens. Another important aspect to be mentioned here is the different pathogenic potential found among the E-72 isolates. In a study by McQuilken and Hopkins (2004), it was found that *Pestalotiopsis* isolates were non-specific and affected other plant species causing the typical dark brown spots. Likewise, Taguchi et al. (2001) suggested that *Pestalotiopsis* spp. isolates are not host specific. Since *Pestalotiopsis* spp. have been found in herbs and in other different hosts, such plants deserve attention, as they can serve as inoculum sources of this pathogen for eucalypt trees.

The conclusions of this study were: (a) Phylogeny based on combined ITS, TUB and EF1-α DNA sequences suggested the new species *P. grandis-urophylla* associated with *Eucalyptus grandis* × *Eucalyptus urophylla* hybrids; (b) the fungus *P. grandis-urophylla* has more rapid mycelial growth when cultivated in the dark, but for mass inoculum production the light regime is not so determinant; (c) the pathogenic potential varied among the *P. grandis-urophylla* isolates;

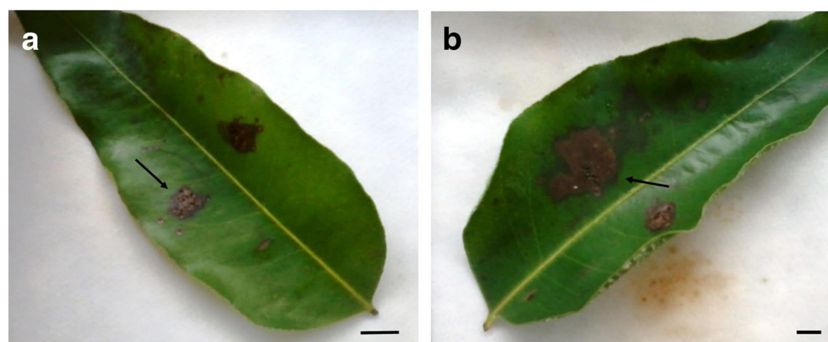


Fig. 5 *Eucalyptus urograndis* 'GG 100' leaves inoculated with *Pestalotiopsis grandis-urophylla* (E-72-06) showing spot symptoms at 10 DAI. **a** The arrow indicates a necrotic dark brown spot typically caused by *Pestalotiopsis*. **b** The arrow shows a 30-mm^2 lesion caused by the coalescence of smaller spots. Bars correspond to 8 and 6 mm for Figs. A and B, respectively

different significant models with high coefficients of determination for disease progress were obtained.

Acknowledgements The authors thank the Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the postdoctoral scholarship to Daniel D. C. Carvalho, and the Universidade Federal de Goiás (UFG) and Universidade Estadual de Goiás (UEG) for the institutional support.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Alonso R, Tiscornia S, Alfenas AC, Bettucci L (2009) Fungi associated to bark spots of *Eucalyptus globulus* stems in plantations from Uruguay. Rev Árvore 33:591–597

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J. Mol. Biol. 215:403–410

Barber PA, Crous PW, Groenewald JZ, Pascoe IG, Keane P (2011) Reassessing *Vermisporium* (*Amphisphaeriaceae*), a genus of foliar pathogens of eucalypts. Persoonia 27:90–118

Carmo ALM, Garcia FAO, Peres FSB (2013) Ocorrência de minicancro em *Eucalyptus viminalis* Labill. no Brasil. Enciclopédia Biosfera 9: 1634–1634

Carvalho DDC, Alves E, Batista TRS, Camargos RB, Lopes EAGL (2008) Comparison of methodologies for conidia production by *Alternaria alternata* from citrus. Braz J Microbiol 39:792–798

Castro AWV, Pedrozo EA, Quadros JL (2015) Cadeias produtivas do agronegócio florestal na região sul do Brasil. Available at: <http://cdn.fee.tche.br/jornadas/2/E13-06.pdf>. Accessed 5 Jan 2015

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation: version II. Plant Mol Biol Report 1:19–21

Farr DF, Rossman AY (2015) Fungal databases, systematic mycology and microbiology laboratory, ARS, USDA. Available at: <http://nt.ars-grin.gov/fungaldatabases/>. Accessed 5 Jan 2015

Ferreira DF (2011) Sisvar: a computer statistical analysis system. Ciênc Agrotecnol 35:1039–1042

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330

Hu HL, Jeewon R, Zhou DQ, Zhou TX, Hyde KD (2007) Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β-tubulin gene phylogenies. Fungal Divers 24:1–22

Jeon YH, Cheon W (2014) First report of leaf blight of Japanese yew caused by *Pestalotiopsis microspora* in Korea. Plant Dis 98:691

Kharwar RN, Gond SK, Kumar A, Mishra A (2010) A comparative study of endophytic and epiphytic fungal association with leaf of *Eucalyptus citriodora* hook and their antimicrobial activity. World J Microbiol Biotechnol 26:1941–1948

Kimati H, Amorim L, Rezende JAM, Bergamim Filho A, Camargo LEA (2005) Manual de Fitopatologia: Doenças das plantas cultivadas. 4th ed. v2. Agronômica Ceres, São Paulo, Brazil

Kruschewsky MC (2010) Taxonomia e ecologia do gênero *Pestalotiopsis* no Brasil, com ênfase para a mata atlântica do sul da Bahia. Universidade Estadual de Santa Cruz, Itabuna, Brazil. (Master's thesis in Plant Production)

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Lana VM, Mafia RG, Ferreira MA, Sartório RC, Zauza EAV, Mounteer AH, Alfenas AC (2012) Survival and dispersal of *Puccinia psidii* spores in eucalypt wood products. Australas Plant Pathol 41:229–238

Liu AR, Chen SC, Wu SY, Xu T, Guo LD, Jeewon R, Wei JG (2010) Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy. Mol Phylogenet Evol 57:528–535

Maharachchikumbura SSN, Guo LD, Chukeatirote E, Bahkali AH, Hyde KD (2011) *Pestalotiopsis* – morphology, phylogeny, biochemistry and diversity. Fungal Divers 50:167–187

Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E, Wu WP, Sun X, Crous PW, Bhat DJ, Mckenzie EHC, Bahkali AH, Hyde KD (2012) A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. Fungal Divers 56: 95–129

Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J, Crous PW (2014) *Pestalotiopsis* revisited. Stud Mycol 79:121–186

McQuilken MP, Hopkins K (2004) Biology and integrated control of *Pestalotiopsis* on container-grown ericaceous crops. Pest Manag Sci 60:135–142

Rehner S. (2001) Primers for elongation factor 1–α (EF1–α). Available at: <http://ocid.nacse.org/research/deephyphae/EF1primer.pdf>. Accessed 26 Sept 2016

Rodas CA, Lombard L, Gryzenhout M, Slippers B, Wingfield MJ (2005) *Cylindrocladium* blight of *Eucalyptus grandis* in Colombia. Australas Plant Pathol 34:143–149

Royo J (2012) Doenças reduzem em até 60% produção de eucalipto. Available at: <http://www.diadecampo.com.br/zpublisher/materias/Materia.asp?id=24158&secao=Pacotes%20Tecnol%F3gicos>. Accessed 5 Jan 2015

Santos AF, Auer CG, Grigoletti Junior A (2001) Doenças do eucalipto no sul do Brasil: identificação e controle. Circular Técnica, 45. Embrapa Florestas, Colombo, Brazil

Serra IMRS, Coelho RSB (2007) Mancha de *Pestalotiopsis* em helicônia: caracterização da doença e potenciais fontes de resistência. Fitopatol Bras 32:44–49

Taguchi Y, Watanabe H, Akita S, Hyakumachi M (2001) Occurrence and control of ripe rot symptoms of persimmon fruit. Jpn J Phytopathol 67:33–41

Teramoto A, Parisi MCM, Cunha MG (2013) Caracterização fisiológica de isolados de *Corynespora cassiicola*. Trop Plant Pathol 38:313–322

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press Inc, New York, pp 315–322

Wu MD, Li GQ, Jiang DH (2009) First report of *Pestalotiopsis microspora* causing leaf blight of *Reineckea carnea* in Central China. Plant Dis 93:667

Zhang M, Wu HY, Tsukiboshi T, Okabe I (2010) First report of *Pestalotiopsis microspora* causing leaf spot of Hidcote (*Hypericum patulum*) in Japan. Plant Dis 94:1064