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Stem rot of eucalyptus cuttings caused by *Neopestalotiopsis* spp. in Brazil

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Abstract

In Brazil, the fungus *Neopestalotiopsis* (= *Pestalotiopsis*) is known to cause disease in eucalyptus cuttings. However, although it occurs relatively frequently in cutting nurseries, the pathogenic species have yet to be identified. Thus, the aim of the present work was to perform a morphological and phylogenetic characterization to identify the aetiological agent. For this purpose, the isolates were subjected to a multilocus analysis using the two gene regions β-tubulin (TUB) and the translation elongation factor (TEF). Based on the genomic sequences, two known species and one new species of the pathogen were identified. After confirmation of their pathogenicity, *N. australis* was confirmed as a new report in eucalyptus. *Neopestalotiopsis rosae* failed to differ from the control, however, showed internal and external lesion in eucalyptus stem. In addition, in this study, a new species called *N. eucalypti* was described, causing disease in hybrids eucalyptus. Morphological characterization allowed for the confirmation of the *N. australis* and *N. rosae* isolates, primarily based on differences in the size and shape of the conidia. For *N. eucalypti*, no morphological marker was found that separated it from the other species within the genus. The results confirm the existence of at least three *Neopestalotiopsis* species as aetiological agents of leaf spot and stem rot in eucalyptus cuttings in Brazil.

KEYWORDS

eucalyptus, morphology, *Neopestalotiopsis eucalypti*, phylogeny

1 | INTRODUCTION

In Brazil, the fungus *Neopestalotiopsis* (= *Pestalotiopsis*) causes leaf spot and stem rot in eucalyptus plants. Although it is relatively common in nurseries that produce cuttings, the pathogen has not been identified yet. The disease usually occurs in weakened cuttings, and pathogen infections start from injuries caused by the vegetative propagation process. The environmental conditions required for the vegetative propagation of eucalyptus also favour the occurrence of the disease. Due to the combination of these specific conditions for the establishment of the disease, the pathogen has been considered secondary and opportunistic, although no more specific studies on the aetiology of the disease have been performed (Alfenas, Zauza, Mafia, & Assis, 2009).

Pestalotiopsis species are phytopathogenic, causing various diseases such as canker lesions, shoot dieback, needle blight, tip blight, scabby canker, leaf spots, grey blights, leaf blights and fruit rot as well as postharvest damage, sometimes resulting in

considerable economic loss. *Pestalotiopsis* species are common pathogens that cause a variety of diseases, reduce production and cause economic losses in apple, blueberry, coconut, chestnut, ginger, grapevine, guava, hazelnut, lychee, mango, orchid, peach, tea and wax apple due to disease. They also commonly occur as saprotrophs in the leaf litter of many plant species. *Pestalotiopsis* species have been recovered from soil, polluted stream water, wood, paper, fabrics and wool (Maharachchikumbura, Hyde, Groenewald, Xu, & Crous, 2014; Reddy, Murali, Suryanarayanan, Govindarajulu, & Thirunavukkarasu, 2016). Recent studies have shown that several species of *Pestalotiopsis* cause symptomless infections in plant tissues (Debbab, Aly, & Proksch, 2013) including the bark of trees (Murali, Thirunavukkarasu, Govindarajulu, & Suryanarayanan, 2013). Some species have been associated with human and animal infections, and others have also been isolated from extreme environments (Maharachchikumbura et al., 2014).

Pestalotiopsis is an anamorphic and monophyletic genus characterized by multicellular conidia bearing appendages, and it is widely distributed throughout tropical and temperate regions. The *Pestalotiopsis* genus has numerous species, with at least 253 represented as the asexual form. To date, only 13 sexual morphs have been recorded in the literature, and they were previously treated as species of *Pestalosphaeria* (Maharachchikumbura, Guo, Chukeatirote, Bahkali, & Hyde, 2011; Reddy et al., 2016). Recently, the *Pestalotiopsis* genus was reviewed, and two novel genera from *Pestalotiopsis*, namely, *Neopestalotiopsis* and *Pseudopestalotiopsis*, were proposed on the basis of morphological and DNA data (Maharachchikumbura et al., 2014).

Recently, the occurrence of rot in eucalyptus grafted plants with typical symptoms of the disease caused by *Pestalotiopsis* sp. was observed. The symptoms were observed in young plants at four months of age, which were used for grafting. Infections by the pathogen began at the sites where the lateral branches were pruned, and they progressed towards the primary stem of the plant, which often resulted in deterioration and death. Considering the conditions in which the disease occurred, which were less favourable than those normally observed during the clonal propagation of eucalyptus, the aim of this work was to study the disease aetiology and pathogenicity.

2 | MATERIAL AND METHODS

2.1 | Isolation

The disease was observed in 4-month-old eucalyptus plants used as rootstock, in July 2016, in a research area in Jacareí, state of São Paulo (SP), Brazil. The climate of the region is Cfa according to the Köppen classification, which is characterized as a humid climate with a hot summer, with an average annual temperature of 19.3°C, an altitude of 634 m and an annual rainfall of 1,396 mm (Alvares, Stape, Sentelhas, Moraes, & Sparovek, 2013). Twenty diseased cuttings exhibiting acervuli and conidia were collected and used to obtain the pathogen isolates. The fungus was isolated directly by diluting the conidia in 2% PDA (potato, dextrose and agar) culture medium to obtain monosporic cultures (Crous, Verkley, & Groenewald, 2009).

2.2 | Molecular characterization

Nine pure cultures (PA1 to PA7, PA9 and PA10) were grown on PDA medium at 25°C for 10 days to obtain sufficient fungal biomass for DNA extraction. Mycelium was scraped from the surfaces of actively growing cultures, placed in a frozen mortar and pestle containing liquid nitrogen and ground into a fine powder. DNA extractions were performed using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The DNA quality and quantity were checked using a NanoDrop® (Thermo Fisher Scientific). DNA samples were diluted to 50 ng/µl, and 1 µl was used in each PCR reaction.

The β-tubulin gene was amplified using the primers BT1 and BT-2b (TUB) (Glass & Donaldson, 1995; O'Donnell, Kistler, Cigelnik, & Ploetz, 1998). The elongation factor gene (TEF) was amplified using the primers EFE1-728F and EF2 1-alfa (TEF) (Carbone & Kohn, 1999; O'Donnell et al., 1998). All PCR reactions were prepared in a final volume of 25 µl using 2.5 µl of 10× Reaction Buffer, 2.5 µl of $MgCl_2$ (25 mM), 0.5µl of dNTPs (10 mM), 0.5 µl of each primer (10 µM), 1 µl of DNA solution and 0.3 µl of Taq polymerase using the GoTaq® DNA Polymerase (Promega), 18.1 µl of MiliQ water, and were performed in a thermocycler (My Cycler™ BIO-RAD), and the conditions were adjusted for each gene as previously described (Carbone & Kohn, 1999; Glass & Donaldson, 1995; O'Donnell et al., 1998). The consistency index (CI), retention index (RI) and composite index (CR) were calculated.

The purification of the PCR products and sequencing was performed by Macrogen Company (Korea). The generated electropherograms were edited using SeqAssem Software (Hepperle, 2004). Sequences from previous studies and sequences that were publically available in GenBank (Table 1) for species in the *Pestalotiopsis* and *Neopestalotiopsis* genera were used for the phylogenetic analysis. The sequences generated in this study were deposited in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Multiple alignments of nucleotide sequences were constructed using the CLUSTALW Program (Thompson et al., 1994) and MEGA 6.0 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The sequences were manually edited when needed. Phylogenetic trees were constructed using PAUP* software version 4.0 (Swofford, 2002) using Maximum parsimony (MP) and MRBAYES 3.2.1 (Ronquist et al., 2012). A bootstrap analysis (1,000 replicates) was also performed on the dataset to determine the confidence levels of the branches. Bayesian inference was used to generate posterior probabilities (PP) for the consensus nodes using MRBAYES (Huelsenbeck & Ronquist, 2001). The Monte Carlo Markov Chain (MCMC) (Larget & Simon, 1999) was run for 1,000,000 generations using the appropriate substitution evolution model determined by JMODELTEST. The trees were viewed and edited in FIGTREE 1.3.1. (http://tree.bio.ac.uk/software).

TABLE 1 Species, number of cultures, host/substrate, location and Genbank accession number of *Neopestalotiopsis* and *Pestalotiopsis* sequences included in this study

Species	Number of cultures	Host/substrate	Location	Genbank accession number	
				TEF	TUB
N. australis	PA1	*Eucalyptus* sp.	Brazil	MK253104	MK286940
N. rosae	PA2	*Eucalyptus* sp.	Brazil	MK253105	MK286941
N. eucalypti	PA3	*Eucalyptus* sp.	Brazil	MK253106	MK286942
N. eucalypti	PA4	*Eucalyptus* sp.	Brazil	MK253107	MK286943
N. australis	PA5	*Eucalyptus* sp.	Brazil	MK253109	MK286945
N. eucalypti	PA6	*Eucalyptus* sp.	Brazil	MK253108	MK286944
N. eucalypti	PA7	*Eucalyptus* sp.	Brazil	MK253110	MK286946
N. eucalypti	PA9	*Eucalyptus* sp.	Brazil	MK253111	MK286947
N. eucalypti	PA10	*Eucalyptus* sp.	Brazil	MK253112	MK286948
N. aotearoa	CBS36754	Canvas	New Zealand	KM199526	KM199454
N. brasiliensis	COAD 2,166	*Psidium guajava*	Brazil	MG692402	MG692400
N. asiatica	MFLUCC 12–0286	Unidentified tree	China	JX399049	JX399018
N. australis	CBS 114,159	*Telopea* sp.	Australia: New South Wales	KM199537	KM199432
N. chrysea	MFLUCC120261	Dead leaves	China	JX399051	JX399020
N. chrysea	MFLUCC120262	Dead plant	China	JX399052	JX399021
N. clavispora	CBS44773	Decaying wood	Sri Lanka	KM199539	KM199443
N. clavispora	MFLUCC120280	*Magnolia* sp.	China	JX399044	JX399013
N. clavispora	MFLUCC120281	*Magnolia* sp.	China	JX399045	JX399014
N. cubana	CBS60096	Leaf litter	Cuba	KM199521	KM199438
N. ellipsospora	CBS115113	Ardisia crenata	Hong Kong	KM199544	KM199450
N. ellipsospora	MFLUCC120283	Dead plant materials	China	JX399047	JX399016
N. ellipsospora	MFLUCC120284	Dead plant materials	Thailand	JX399046	JX399015
N. eucalypticola	CBS26437	*Eucalyptus globulus*	*	KM199551	KM199431
N. foedans	CGMCC39123	Mangrove plant	China	JX399053	JX399022
N. foedans	CGMCC39178	*Neodypsis decaryi*	China	JX399055	JX399024
N. foedans	CGMCC39202	*Calliandra haematocephala*	China	JX399054	JX399023
N. formicarum	CBS11583	Plant debris	Cuba	KM199519	KM199444
N. formicarum	CBS36272	Dead Formicidae (ant)	Ghana	KM199517	KM199455
N. honoluluana	CBS111535	*Telopea* sp.	USA: Hawaii	KM199546	KM199461
N. honoluluana	CBS114495	*Telopea* sp.	USA: Hawaii	KM199548	KM199457
N. javaensis	CBS 257.31	*Cocos nucifera*	Indonesia: Java	KM199543	KM199437
N. magna	MFLUCC12652	*Pteridium* sp.	France	KF582791	KF582793
N. mesopotâmica	CBS29974	*Eucalyptus* sp.	Turkey	KM199541	KM199435
N. mesopotâmica	CBS33686	*Pinus brutia*	Iraq	KM199555	KM199441
N. mesopotâmica	CBS46469	*Achras sapota*	India	*	KM199436
N macadamiae	BRIP63736a	*	NSW	KX186623	KX186651
N. macadamiae	BRIP63737b	*	NSW	KX186626	KX186653
N. macadamiae	BRIP63740a	*	NSW	KX186628	KX186656
N. natalensis	CBS13841	*Acacia mollissima*	South Africa	KM199552	KM199466
N. piceana	CBS22530	*Mangifera indica*	*	KM199535	KM199451
N. piceana	CBS25432	*Cocos nucifera*	Indonesia	KM199529	KM199452
N. piceana	CBS39448	*Picea* sp.	UK	KM199527	KM199453

(Continues)

TABLE 1 (Continued)

Species	Number of cultures	Host/substrate	Location	Genbank accession number	
				TEF	TUB
N. protearum	CBS 114,178	*Leucospermum cuneiforme* cv. 'Sunbird'	Zimbabwe	KM199542	KM199463
N. rosae	CBS101057	*Rosa* sp.	New Zealand	KM199523	KM199429
N. rosae	CBS124745	*Paeonia suffruticosa*	USA	KM199524	KM199430
N. samarangensis	CBS115451	Unidentified tree	Hong Kong	KM199556	KM199447
N. samarangensis	MFLUCC120233	*Syzygium samarangense*	Thailand	JQ968611	JQ968610
N. saprophytica	CBS115452	*Litsea rotundifolia*	Hong Kong	KM199538	KM199433
N. saprophytica	MFLUCC120282	*Magnolia* sp.	China	JX399048	JX399017
Neopestaoliopsis sp. clade 4	CBS23379	*Crotalaria juncea*	India	KM199528	KM199464
Neopestalotiopsis sp. Clade 10	CBS11020	*	*	KM199540	KM199442
Neopestalotiopsis sp. clade 15	CBS17725	*Dalbergia* sp.	*	KM199533	KM199445
Neopestalotiopsis sp. clade 15	CBS27429	*Cocos nucifera*	Indonesia: Java	KM199534	KM199448
Neopestalotiopsis sp. clade 15	CBS66494	*Cocos nucifera*	Netherlands	KM199525	KM199449
Neopestalotiopsis sp. clade 15	CBS32276	*Camellia* sp.	France	KM199536	KM199446
Neopestalotiopsis sp. clade 20	CBS16442	Dune sand	France	KM199520	KM199434
Neopestalotiopsis sp. clade 20	CBS36061	*Cinchona* sp.	Guinea	KM199522	KM199440
Neopestalotiopsis sp. clade 22	CBS11975	*Achras sapota*	India	KM199531	KM199439
Neopestalotiopsis sp. clade 26	CBS26637	*Erica* sp.	Germany	KM199547	KM199459
Neopestalotiopsis sp. clade 26	CBS32376	*Erica gracilis*	France	KM199550	KM199458
Neopestalotiopsis sp. clade 26	CBS36161	*Cissus* sp.	Netherlands	KM199549	KM199460
N. steyaertii	IMI192475	*Eucalyptus viminalis*	Australia	KF582792	KF582794
N. surinamensis	CBS45074	Soil under Elaeis guineensis	Suriname	KM199518	KM199465
N. surinamensis	CBS111494	*Protea eximia*	Zimbabwe	KM199530	KM199462
N. umbrinospora	MFLUCC120285	Unidentified plant	China	JX399050	JX399019
N. zimbabwana	CBS111495	*Leucospermum cunciforme* cv. 'Sunbird'	Zimbabwe	KM199545	KM199456
P. oryzae	CBS17126	*	Italy	KM199494	KM199397
P. oryzae	CBS111522	*Telopea* sp.	USA: Hawaii	KM199493	KM199394
P. biciliata	CBS23638	*Paeonia* sp.	Italy	KM199506	KM199401
P. biciliata	CBS124463	*Platanus* × *hispanica*	Slovakia	KM199505	KM199399
P. australis	CBS111503	*Protea neriifolia* × *susannae* cv. '*Pink Ice*'	South Africa	KM199557	KM199382
P. australis	CBS114474	*Protea neriifolia* × *susannae* cv. '*Pink Ice*'	South Africa	KM199477	KM199385
P. australasiae	CBS114141	Protea sp	Australia	KM199501	KM199410
P. australasiae	CBS114126	*Knightia* sp.	New Zealand	KM199499	KM199409

Abbreviations: CBS, Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC, China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; IMI, Culture collection of CABI Europe UK Centre, Egham, UK; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

The combination of the two genes was determined with a partition homogeneity test (PHT) (Farris, Kallersj, Kluge, & Bult, 1994).

The trees were constructed using 75 aligned sequences, including the nine sequences of *Eucalyptus* hybrid isolates and the *Xylaria hypoxylon* outgroup, according to Maharachchikumbura et al. (2014).

2.3 | Morphological characterization

The fungal structures and fruiting bodies that originated from herbarium material and plates containing the isolates were selected based on the phylogenetic analysis and used for the morphological characterization.

Morphological descriptions were made for isolates cultured in PDA medium at 25°C with a photoperiod of 12 hr. Eucalyptus stems containing acervuli were used to analyse these structures. The predominant colour of the colony grown on PDA was determined by comparing it with Rayner's colour chart (1970). Microscopic preparations were made in distilled water, with 30 measurements per structure, and were observed under an LX400 microscope with CAPTURE PRO software V2.8.8.5. Measurements of the conidiomata, conidiogenous cells, the conidial cells including the basal cell, three median cells, apical cell and apical and basal appendages were taken. The photos were captured on a Nikon Eclipse E-200 LED binocular microscope.

2.4 | Pathogenicity test

To test for pathogenicity, five isolates (PA1, PA2, PA3, PA4 and PA5) were inoculated in ten plants aged 90 days each. For the inoculations, mycelial discs were inoculated on the stem. For this purpose, the isolates used here were cultured in Petri dishes containing PDA medium and maintained for 7 days at 25°C, with a photoperiod of 12 hr. After incubation, 5-mm-diameter mycelial discs were placed at the site of injury of each plant, which also measured 5 mm in diameter. The fragment was covered with plastic film to maintain moisture and to protect the lesion from desiccation. The controls received only one disc containing PDA, without the fungus. After 18 days of inoculation, the lengths of the external and internal lesions caused by the pathogen were measured. The experiment was performed in a completely randomized design consisting of six treatments (five isolates and one control). The data were subjected to an analysis of variance, and the means were compared by Scott-Knott (1974) test ($p < .05$). After the pathogenicity was confirmed, the pathogen was again isolated from the inoculated plants and the morphological characteristics of the fungus were compared to fulfil Koch's postulates.

3 | RESULTS

3.1 | Isolation

Ten isolates were obtained from the acervuli and conidia associated with diseased *Eucalyptus* spp. cuttings. Only the asexual phase was found, and the primary symptoms were observed in young plants at four months of age, which were used for grafting. Pathogen infections began at sites where the lateral branches were pruned and then progressed towards the primary stem of the plant. Thus, symptoms under natural conditions of infection consisted of a stem rot with small grey to black punctuations, culminating in the death of the plant (Figure 1a).

3.2 | Molecular characterization

Phylogenetic trees were generated using maximum parsimony (MP) and Bayesian inference (BI) for the amplified regions (TUB and TEF). The phylogenetic analysis included 57 additional sequences of *Neopestalotiopsis* spp. and eight *Pestalotiopsis* spp., including the *Xylaria hypoxylon* as outgroup, as obtained from GenBank. Trees generated for the two gene regions presented similar topologies based on both parsimony and Bayesian methods. The partition homogeneity test ($p = .01$) showed that the data sets (TUB and TEF) had the same topology and could be combined. The concatenated DNA sequences of the two regions contain 1,355 characters of the 75 isolates resulting in 399 constant, 618 parsimony uninformative and 338 parsimony informative characters. One hundred trees with 1,355 steps were generated, with CI of 0.88, RI of 0.95 and CR of 0.84 for all the parsimony informative and uninformative sites.

The combined tree showed that the isolates sequenced in this study were grouped into different clades. It was possible to classify the PA1 and PA5 isolates as *Neopestalotiopsis australis* and the PA2 and PA9 isolates as *Neopestalotiopsis rosae* (Figure 2). The branch was supported by high bootstrap values (88% and 74%, respectively) and posterior probability (0.95 and 0.81, respectively). Isolates PA3, PA4, PA6, PA7 and PA10 were grouped into a new clade supported by high bootstrap and posterior probability values, at 96% and 0.98, respectively (Figure 2).

3.3 | Morphological characterization

Isolates PA1 (*N. australis*) and PA2 (*N. rosae*) were selected for morphological characterization based on the results of the phylogenetic analyses. There was no variation in the colour between the isolates when they were grown in PDA medium. The spores were characterized by conidia with five cells, three coloured medial cells (equally pigmented), hyaline terminal cells and appendages originating from the apex of the apical cell.

Neopestalotiopsis australis (Figure 3). Conidiomata in PDA, globose or clavate, aggregate or dispersed, semi or fully immersed, black, 200–500 μm in length; exuding globose conidial masses, dark brown to black; indistinct conidiophores, often reduced to conidiogenous cells. Discrete, hyaline, rough-walled conidiogenous cells, single, 2.5–4.0 μm in height and 8.5–14.0 μm in length. Conidium fusoid, ellipsoid, straight to slightly curved, 4 septa. Conical to obconical basal cell with a truncated, hyaline, highly verruculose and thin-walled base, 2.0–3.5 × 3.0–5.0 μm in length; three medial cells, brown, with one septum darker than the rest of the cell (second basal cell: 3.0–4.0 × 3.0–6.0 μm in length; third cell: 4.0–5.0 × 3.5–6.82 μm in length, fourth cell 3.5–5.0 × 5.0–5.5 μm in length); apical cell 2.0–3.0 × 3.0–5.00 μm in length, hyaline, cylindrical to sub-cylindrical, thin-walled, with 2–3 apical tubular appendages (mainly 3), arising from the apical crest, unbranched, filiform, 20 to 27 μm in length. Basal appendix single, tubular, unbranched, centred, 3.0–6.5 μm in length. Colonies on PDA reaching 55–60 mm in diameter after 10 days at 25°C, white, with dense aerial mycelium on the surface with black and concentric conidiomata, and reverse of similar colour.

Neopestalotiopsis rosae (Figure 4). Conidiomata, globose, solitary, semi-immersed, black, exuding masses of conidia and dark

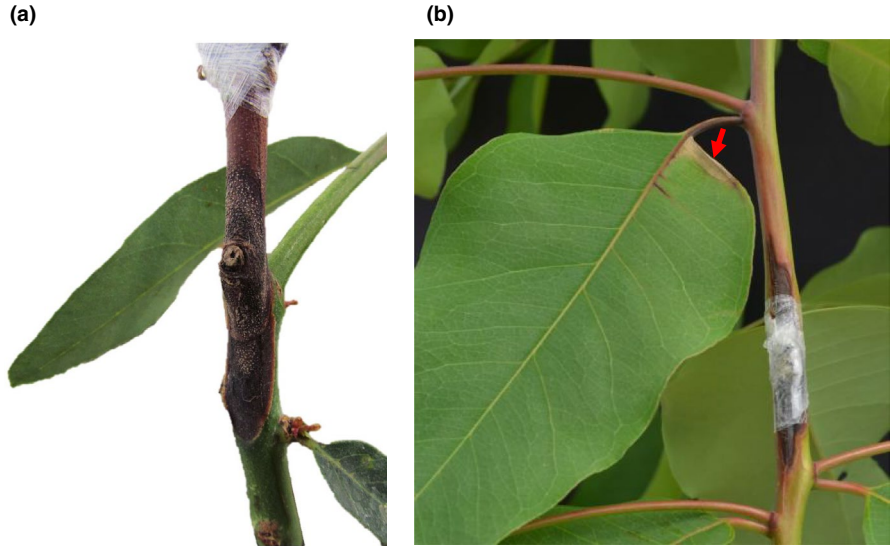

FIGURE 1 Symptoms of *Neopestalotiopsis* spp. in eucalyptus cuttings. (a) Four-month-old plants with disease under natural conditions of infection. (b) Reproduction of disease symptoms in artificially inoculated plants. Arrow: presence of the fungus in the petiole [Colour figure can be viewed at wileyonlinelibrary.com]

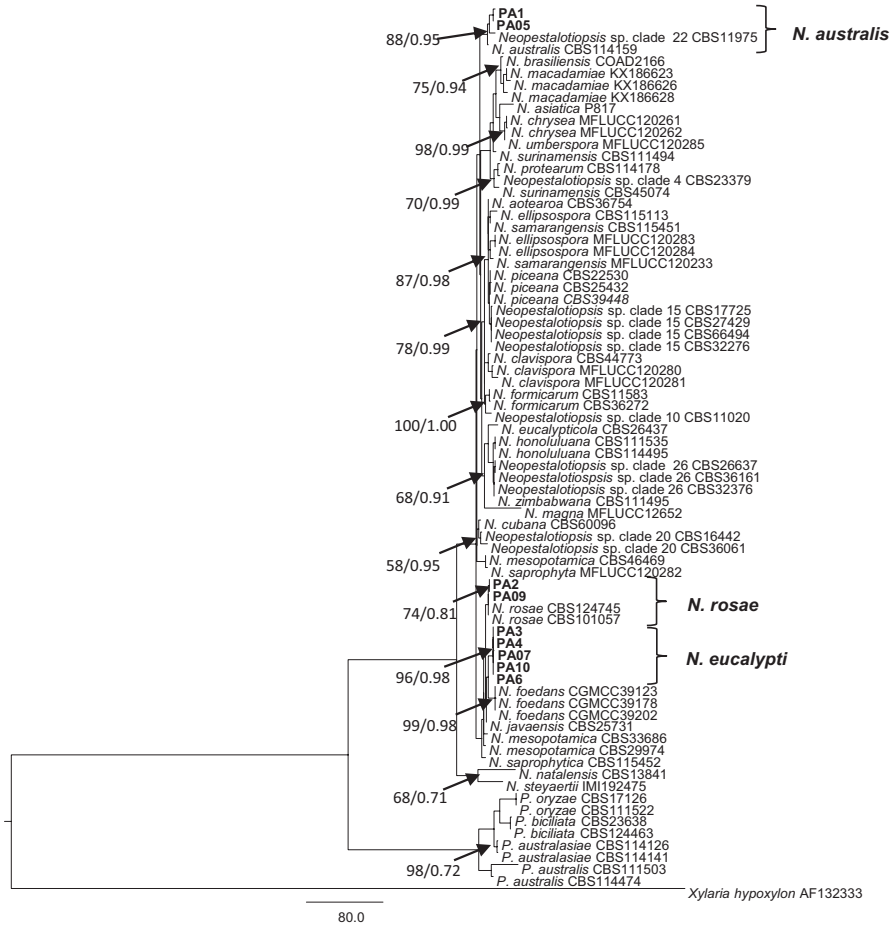

FIGURE 2 Bayesian phylogenetic tree of the combined alignment (TUB + TEF) for the analysed sequences of *Neopestalotiopsis* spp. and *Pestalotiopsis* spp. Bayesian posterior probabilities (PP) above 50%. The maximum parsimony bootstrap proportions are provided on the nodes (MP/PP). The tree was rooted with *Xylaria hypoxylon*

brown to black; indistinct conidiophores, often reduced to conidiogenous cells; discrete, cylindrical, hyaline, smooth-walled conidiogenous cells, single, with truncated apex with visible periclinal thickening, 2–5 × 5–18 μm in length; conidium fusoid, ellipsoid, straight to slightly curved, 4 septa; conical to obconical basal cell with a truncated base, hyaline, rough and thin-walled, 3.5–6.0 μm in length, often with a small basal appendix; three medial cells with light brown to dark pigmentation with a rough wall, and one septum darker than the rest of the cell (second cell from the base pale brown, 3.5–3.0 × 4.5–5.6 μm in length; third cell golden brown 3.5–5.0 × 4.5–7.0 μm in length; fourth cell brown, 3.5–4.5 × 4.5 to 7.0 μm in length); apical cell of 3.5– 3.0 × 4.5–5.5 μm in length, hyaline, cylindrical, thin and smooth-walled with 3–5 apical tubular appendages, not arising from the apical crest, but each inserted at

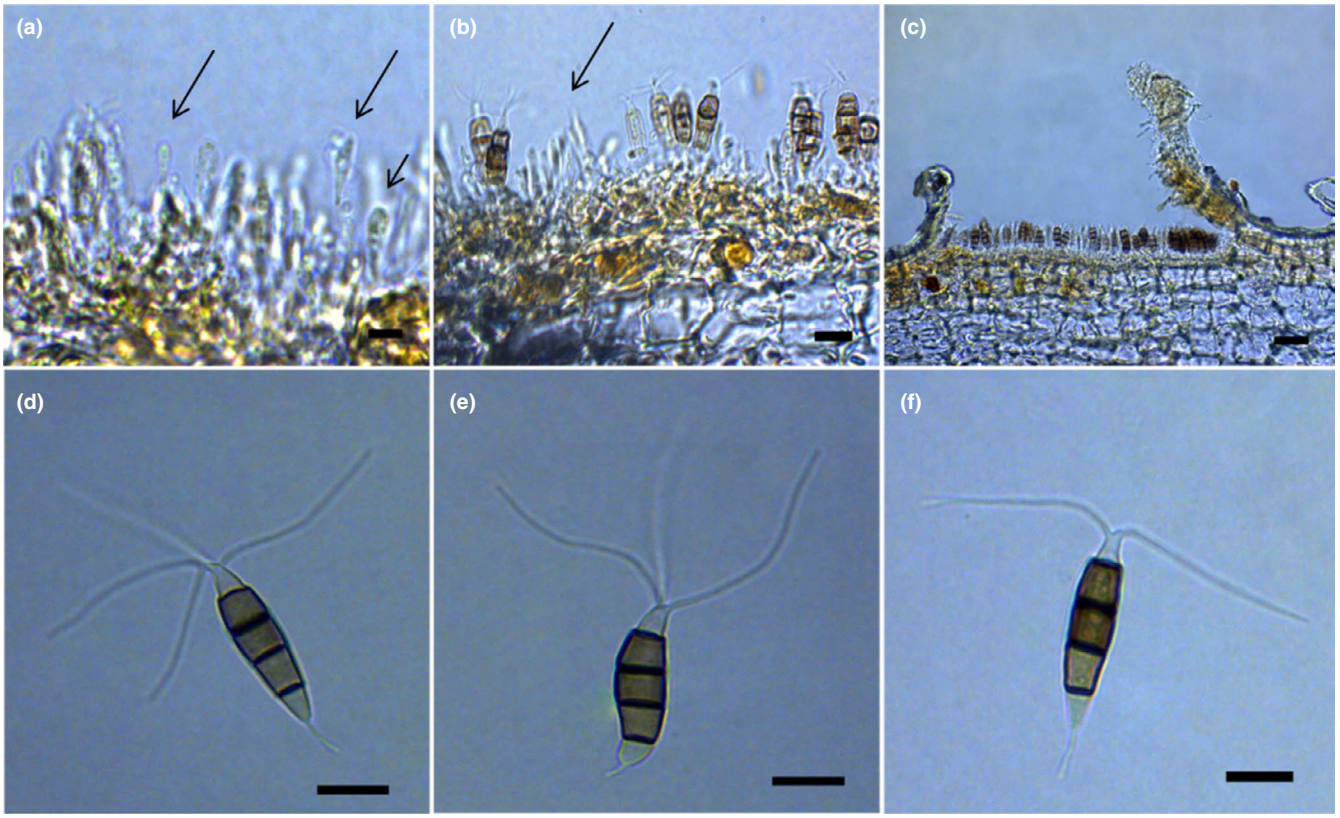

FIGURE 3 *Neopestalotiopsis australis*. (a, b) Conidiogenous cells (indicated by the arrows). (c) Acervuli. (d–f) Conidia. Scale bars = 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

a different locus in the upper half of the apical cell, unbranched, filiform, 20–31 μm in length; single basal appendix, tubular, unbranched, centred, 4.5–7.5 μm in length. A colony of the pathogen in the PDA culture medium showed light mycelium staining 50–60 mm after 10 days at 25°C.

3.4 | Taxonomy

Based on the results of the TUB and TEF sequence analyses together with an evaluation of the literature and morphology, a new species (PA3, PA4, PA6, PA7 and PA10) was proposed using the branch supported by high bootstrap (96%) and high posterior probability values. This result confirms the occurrence of this species in Brazil.

3.4.1 | Neopestalotiopsis eucalypti

R. G. Mafia, G. S. Santos, & M. A. Ferreira, sp. nov. Figure 5

Etymology: Related to the country where it was collected, Brazil.

Asexual morphology: Conidiomata acervular or picnidial in culture in PDA, globose, solitary or aggregate, semi or partially immersed and totally immersed, black, 100–500 μm in diameter; exuding globose, viscous and black conidial masses; indistinct conidiophores, often reduced to conidiogenous cells; conidiogenous cells discrete, hyaline, rough-walled, single, 1.5–4.5 × 9.0–20.5 μm; conidium fusoid, ellipsoid, straight to slightly curved, 5 cells (4 septa); basal cell with a truncated base, hyaline, rough and thin-walled, 2.0–3.5 × 1.5–6.0 μm in length; three medial cells light brown, septum darker than the rest of the cell (second cell from the base, 3.0–5.0 × 3.5–5.5 μm in length; third cell: 3.5–5.5 × 3.0–4.6 μm in length, fourth cell: 3.0–5.0 × 3.0–5.0 μm in length); apical cell 6.0–3.0 × 3.0–5.5 μm in length, hyaline, sub-cylindrical to obconical, thin-walled; with 2–4 apical tubular appendages, arising from the apical crest, unbranched, filiform, flexuose, 12–27 μm in length; basal appendix single, tubular, unbranched, centred, 3–7 μm in length.

Culture Characteristics: Colonies on PDA reaching 50–70 mm in diameter after 10 days at 25°C, mycelium white, odourless, exudate-free, with dense aerial mycelium on the surface with concentric and black conidiomata; Reverse smooth, pale cream. Mycelium hyaline, septate, neutral and smooth hyphae.

Habitat: *Eucalyptus* sp.

Distribution: Brazil.

Material examined: Brazil, July 2016, in a research area in the Jacareí region, in the Ribeira River valley-SP, 23°18'10"S and 45°17'31"W, observed in 6-month-old rootstocks in July 2016.

Notes: The proposed new species, *N. eucalypti*, differs from other species in terms of size, number of appendages and colour of medial cells. In this study, both the conidiomata and conidia that developed in PDA presented similar characters relative to the other described species. According to Maharachchikumbura et al. (2014), the conidia obtained from nature are generally more uniform in size and

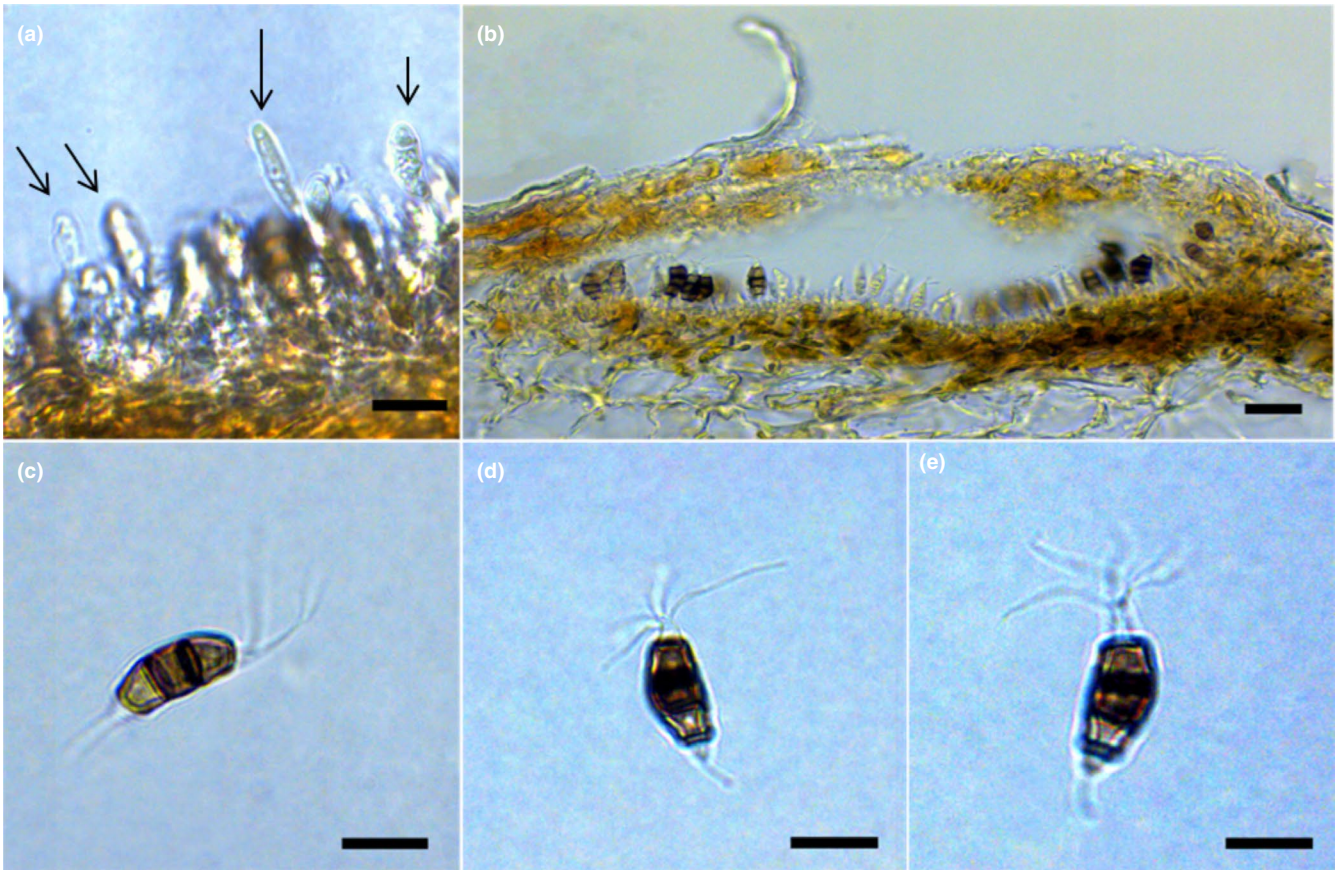

FIGURE 4 *Neopestalotiopsis rosae*. (a) Conidiogenous cells (indicated by the arrows). (b) Acervuli. (c–e) Conidia. Scale bars = 10 µm [Colour figure can be viewed at wileyonlinelibrary.com]

morphology than those obtained from artificial media. A comparison of morphological characteristics between *N. autralis*, *N. rosae* and *N. eucalypti* species is shown in Table 2.

3.5 | Pathogenicity test

The inoculations of PA3 and PA5 fungal isolates led to disease development. The isolates PA3 and PA5 failed to differ regarding disease severity, as expressed in terms of lesion length. The other isolates (PA1, PA2 and PA4) failed to differ from the control, however, showed internal and external lesion in eucalyptus stem (Figure 6). Koch's postulates were fulfilled through the isolation of the same fungus from the inoculated plants. The lesions present on inoculated plants were similar to those under natural conditions of infection. However, in addition to injury to the cutting stem (stem rot), a brownish-coloured lesion was noted in the plant petiole (Figure 1b).

4 | DISCUSSION

Based on the phylogenetic analyses of the combined TUB and TEF gene regions as well as the morphological characteristics, two species of *Neopestalotiopsis* (*N. australis* and *N. rosae*) were reported for the first time as aetiological agents of leaf spot and stem rot in eucalyptus cuttings. In addition, a new species, *N. eucalypti*, was described in the same host in Brazil. *Neopestalotiopsis rosae* was first reported in lesions in rose stems and *Paeonia suffruticosa*, whereas *N. australis* was first observed on the leaves and stem of *Vitis vinifera* and *Achras sapota* (Maharachchikumbura et al., 2014).

In Brazil, it was believed that these diseases in eucalyptus were caused by only one species belonging to the genus *Pestalotiopsis* (Alfenas et al., 2009). However, according to the results of this study, there is a complex of at least three species of this pathogen belonging to the genus *Neopestalotiopsis*. Maharachchikumbura et al. (2014) performed a phylogenetic reconstruction of the *Amphisphaeriaceae* family based on the morphology and phylogenetic analysis of rDNA (ITS) and 28S rRNA (LSU) sequences and of β-tubulin (BT) and elongation factor 1-alpha (TEF) genes. The alignment of the LSU region was used to determine where to place the genera of the *Pestalotiopsis* isolates within the family Amphisphaeriaceae. In a 2012 study, Maharachchikumbura et al. analysed ten genomic regions and found that the combined TUB and TEF regions have better resolution for species description. In 2014, Maharachchikumbura et al. used the ITS, TUB and TEF regions in combination to define the species. The authors proposed two new genera segregated from *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis*. In the present study, the ITS region did not present sufficient resolution to define the species; in addition, it did not

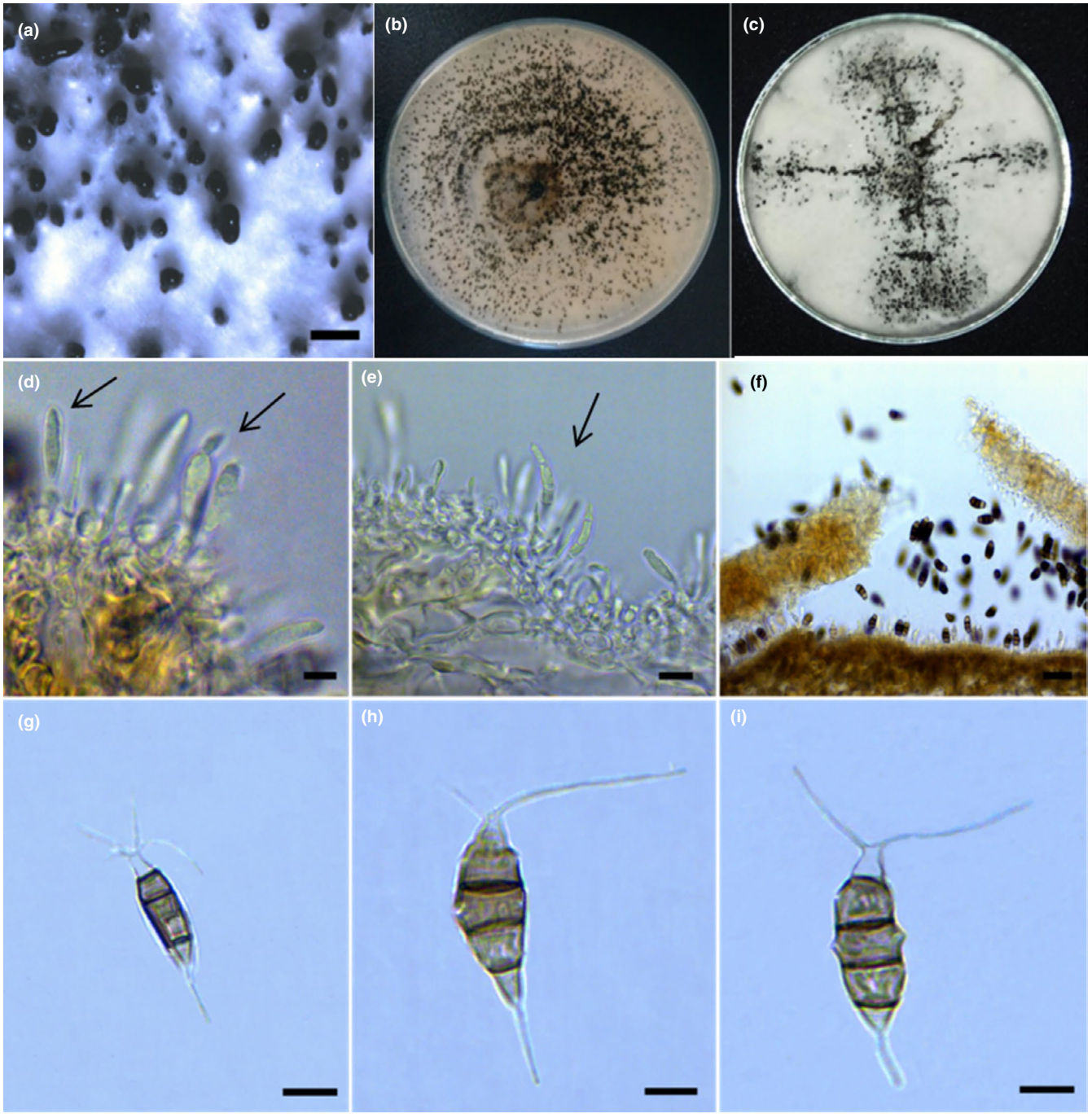

FIGURE 5 *Neopestalotiopsis eucalypti*. (a) Conidiomata in PDA. (b, c) Sporulation in Petri dishes containing Potato Dextrose Agar (front and back respectively) (d, e) conidiogenous cells (indicated by the arrows). (f) Acervuli. (g–i) Conidia. Scale bars = 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

present the same topology for the TUB and TEF trees. Therefore, it was removed from the study.

In this study, the sequences that clustered with clade 22 showed morphology similar to that of *N. australis*. As described in Maharachchikumbura et al. (2014), even though the species were separated phylogenetically and ecologically, they are morphologically similar. The morphology of *Neopestalotiopsis* sp. (clade 22) described in this study corroborates those observed by Maharachchikumbura et al. (2014) and Sangkyu et al. (2016). However, the morphological characters may vary based on the host and the environment.

The sequences of the isolates that clustered within the *N. rosae* clade described in this study presented one of the primary characteristics that differentiate it from other species, namely, the number of apical tubular appendages (3–5) that arise not from the apical crest but from different sites in the upper half of the apical cell. These

TABLE 2 Comparison of morphological characteristics between *N. autralis*, *N. rosae* and *N. eucalypti*

	Organisms		
Feature	***N. australis***	***N. rosae***	***N. eucalypti***
Colony diameter on PDA at 25°C, 10 days	55–60 mm	50–60 mm	50–70 mm
Colony colour	White	White	White
Conidiomata on PDA	Conidiomata, globose or clavate, aggregate or dispersed, semi or fully immersed, black, 200–500 μm in length; exuding globose conidial masses, dark brown to black	Conidiomata, globose, solitary, semi-immersed, black, exuding masses of conidia and dark brown to black	Conidiomata acervular or picnidial, globose, solitary or aggregate, semi or partially immersed and totally immersed, black, 100–500 μm in diameter; exuding globose, viscous and black conidial masses
Conidiophores	Indistinct conidiophores, often reduced to conidiogenous cells	Indistinct conidiophores, often reduced to conidiogenous cells	Indistinct conidiophores, often reduced to conidiogenous cells
Conidiogenous cells	Conidiogenous cells discrete, hyaline, rough-walled, single, 2.5–4.0 μm in height and 8.5–14.0 μm in length	Conidiogenous cells discrete, cylindrical, hyaline, smooth-walled, single, with truncated apex with visible periclinal thickening, 2–5 x 5–18 μm in length	Conidiogenous cells discrete, hyaline, rough-walled, single, 1.5–4.5 × 9.0–20.5 μm
Conidial shape	Conidium fusoid, ellipsoid, straight to slightly curved	Conidium fusoid, ellipsoid, straight to slightly curved	Conidium fusoid, ellipsoid, straight to slightly curved
Conidial septation	4 septa	4 septa	4 septa
Size of appendages	20–27 μm in length.	20–31 μm in length	12–27 μm in length
Number of appendages	2–3 (mainly 3)	3–5	2–4
Colour of medial cells	Brown	Light brown to dark	Light brown

results are consistent with those described by Maharachchikumbura et al. (2014).

The new species described in this study was named *N. eucalypti*. Phylogenetic analyses of the TUB and TEF sequences showed that *N. eucalypti* forms a distinct clade with bootstrap support of 96%.

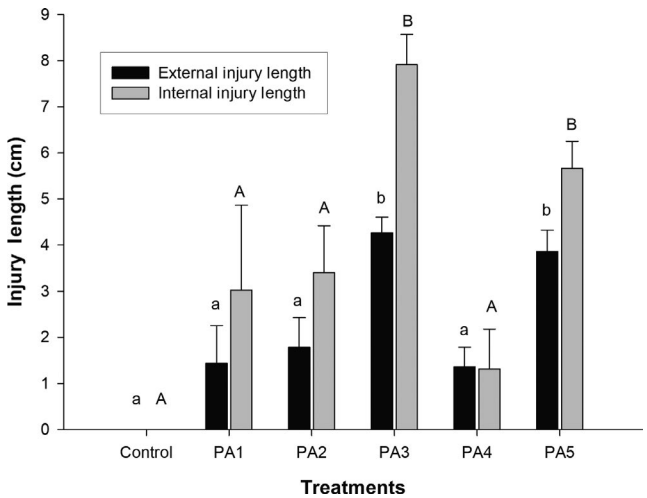

FIGURE 6 Lengths of lesions caused by *Neopestalotiopsis* spp. in *eucalypti* cuttings. Bars indicate the standard error of the mean. Mean values with the same lower-case (external injury length) and upper-case (internal injury length) letter do not differ significantly according to the Scott-Knott (1974) test ($p < .05$)

The versicolour morphology of the conidia, which is characteristic of the genus *Neopestalotiopsis*, corroborates the results of the molecular analyses. In this study, both the conidiomata and conidia developed in PDA presented similar characteristics compared to the other described species. However, no morphological marker was found that could differentiate it from the other species within the genus.

The species *N. australis*, *N. rosae* and *N. eucalypti* were pathogenic to eucalyptus. The isolates *N. eucalypti* (PA3) and *N. australis* (PA5) showed similar aggressiveness and superior to the others isolated in the pathogenicity test. The other isolates failed to differ from the control, however, showed internal and external lesion in eucalyptus stem, indicating that these isolates are less aggressive in eucalyptus. In addition to the injury to the cutting stem, the fungus was observed in the plant petiole, indicating that the fungus may migrate through the conductive vessels of the plant and cause lesions at distant points of inoculation. Due to these results, it would be interesting to conduct a more comprehensive evaluation of the pathogen population to determine if there are more pathogenic species and to evaluate the variability in virulence within this complex of species.

The symptoms observed in diseased plants were typical of diseases caused by opportunistic fungi. Lesions started in the sites where the lateral branches had been pruned, demonstrating that the penetration of the pathogen occurred indirectly through these injuries. This pattern of infection is similar to that observed in eucalyptus

mini-cutting rot (Alfenas et al., 2009). However, in the present study, the disease was observed in older cuttings after the clonal propagation process and under environment conditions less favourable to the pathogen. Thus, it is believed that this pathogen, which was initially considered opportunistic, should be further studied, together with the epidemiology of the disease.

The results obtained and presented in this study will help increase our knowledge about diseases caused by species in the *Neopestalotiopsis* genus in woody plants. In addition, there may be many more cryptic species within the family *Amphisphaeriaceae*. For eucalyptus, the disease has been shown to be caused by a complex of at least three species, and *N. australis* and *N. rosae* as well as a new species named *N. eucalypti* have been reported for the first time.

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