Contribución de especies de las familias *Peronosporaceae* y *Nectriaceae* al decaimiento y muerte de *Eucalyptus smithii* en Uruguay

Ing. Agr. Franco Michel De Benedetti Teske

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RESUMEN

En las últimas décadas, la forestación se posicionó como una de las principales actividades económicas del agro uruguayo. *Eucalyptus globulus* fue la especie preferida para la producción de pulpa de celulosa hasta que una enfermedad foliar afectó su productividad, lo que significó un riesgo al acceso a mercados internacionales. *Eucalyptus smithii* surgió como especie sustituta por poseer características similares a *E. globulus*. La expansión de *E. smithii* se vio afectada por una alta mortalidad de plantas, principalmente en los dos primeros años desde la plantación. Las plantas afectadas manifiestan podredumbre de raíces y cuello, reducción de masa radicular y decaimiento de la parte aérea, síntomas similares a los causados por patógenos de los géneros *Phytophthora* y *Calonectria*. Entre 2019 y 2021 se colectaron 132 plantas sintomáticas de 9 plantaciones comerciales y 60 plantines de 3 viveros, en el sur y sureste de Uruguay. De las 132 plantas se aislaron 32 cepas del género *Phytophthora* (*Peronosporaceae*) (24 %) y 17 cepas pertenecientes a la familia *Nectriaceae* (13 %), mientras que de los 60 plantines de vivero se aislaron 8 cepas de *Nectriaceae* (13 %). Dentro del género *Phytophthora*, *P. alticola* fue la especie predominante (n = 31), y la restante fue *P. boodjera* (n = 1). En cuanto a las cepas de la familia *Nectriaceae*, 15 correspondieron a *Calonectria pauciramosa* (9 de plantación y 6 de vivero), 2 a *Dactylonectria novozelandica* (de vivero) y las 8 restantes (de plantación) fueron descritas como *Ilyonectria charruensis* sp. nov. en este trabajo. Los estudios de patogenicidad confirmaron que todas las especies son capaces de infectar a *E. smithii* y reducir su masa radicular y aérea. Sin embargo, no se registraron muertes masivas similares a las observadas las plantaciones comerciales. Este hallazgo, junto a la baja frecuencia con que fueron aislados los patógenos, sugiere que los agentes bióticos no serían los únicos responsables de la mortalidad de *E. smithii*. Futuras investigaciones deberían explorar otros factores como el comportamiento según la procedencia de las semillas, los sistemas de producción y de plantación así como el manejo posterior al trasplante.

Palabras clave: análisis multigénico, ascomycetes, oomycetes, podredumbre radicular

Contribution of species of the *Peronosporaceae* and *Nectriaceae* families to the decline and mortality of *Eucalyptus smithii* in Uruguay

SUMMARY

In recent decades, forestry has emerged as a key economic activity in Uruguay. *Eucalyptus globulus* was the preferred species for cellulose pulp production until a foliar disease severely impacted its productivity, threatening the access to international markets. *Eucalyptus smithii* emerged as a substitute species, meeting the demands of quality standards. The rapid expansion of *E. smithii* coincided with significant plant mortality, particularly within the first two years after planting. Affected plants exhibited symptoms such as root and collar rot, reduced root mass and decline, symptoms similar to those caused by *Phytophthora* and *Calonectria* pathogens. Between 2019 and 2021, 132 symptomatic plants were collected from nine commercial plantations, along with 60 seedlings from three *E. smithii* nurseries in Uruguay. From the 132 plants, 32 strains of the *Phytophthora* genus (*Peronosporaceae*) (24%) and 17 belonging to the *Nectriaceae* family (13%) were isolated, whereas eight *Nectriaceae* strains (13%) were isolated from the 60 nursery plants. Within the *Phytophthora* genus, *P. alticola* was the predominant species (n = 31), with the remaining strain as *P. boodjera* (n = 1). Of the *Nectriaceae* strains, 15 were identified as *Calonectria pauciramosa* (nine from plantations and six from nurseries), two as *Dactylonectria novozelandica* (from nurseries), while the remaining eight (from plantations) did not match any known species, therefore, were here described as *Ilyonectria charruensis* sp. nov. Pathogenicity studies confirmed that all species can infect *E. smithii* roots, reducing root mass and aerial biomass compared to the control. However, massive deaths as observed in commercial plantations were recorded. This finding, along with the low frequency of pathogens isolation, suggests that biotic agents might not be the only cause of death of *E. smithii*. Future research should explore other factors, including physiological aspects, production and planting systems, post-transplant management, and behaviour based on seed provenances.

Keywords: ascomycetes, multi-gene analyses, oomycetes, root rot

1. INTRODUCCIÓN

El paisaje forestal mundial ha experimentado transformaciones notables a lo largo de las últimas décadas, evidenciando una disminución considerable de la superficie forestal total. Sin embargo, ha habido un incremento significativo en la cantidad de bosques plantados, con una alta proporción de plantaciones constituidas casi en su totalidad por especies introducidas (FAO, 2020). Uruguay, sin ser ajeno a esta tendencia, posee actualmente una superficie total de bosques de 1.900.000 hectáreas. De este total, 1.065.000 corresponden a bosques plantados con *Eucalyptus* como género dominante abarcando el 81 % de esa área y, en menor medida, especies del género *Pinus*. Respecto a las 835.000 hectáreas restantes, corresponden a bosques naturales con predominancia de especies nativas (MGAP-DIEA, 2022). En cuanto a la importancia económica de este rubro, la proyección para el año 2022 de las exportaciones del sector forestal en su conjunto fue de US$ 2.100 millones, lo que equivale aproximadamente a un 3,8 % del PBI nacional (MGAP-OPYPA, 2022).

Eucalyptus es un género perteneciente a la familia *Myrtaceae* e incluye más de 700 especies de árboles y arbustos originarios de Australia y zonas adyacentes. Su producción se ha extendido mundialmente gracias a su buena adaptabilidad para crecer en diferentes ambientes y a la versatilidad de sus productos derivados. Los usos van desde la producción de madera sólida para aserrío, madera destinada a fines energéticos y la producción de pulpa de celulosa, hasta la obtención de aceites esenciales y otros productos no madereros (Coppen, 2002, Doughty, 2000, FAO, 1981). De hecho, la plasticidad genética del eucalipto ha permitido su distribución en diferentes condiciones climáticas y tipos de suelo, convirtiéndose en un componente esencial de la silvicultura mundial (Richardson y Rejmanek, 2011, Coppen, 2002, FAO, 1981), incluido Uruguay (MGAP-DGF, 2022, Balmelli et al., 2016, Brussa, 1994).

En Uruguay, el eucalipto fue introducido a fines del siglo XIX, a partir de semillas de origen desconocido (Brussa, 1994, FAO, 1981), con diversos fines como elaboración

de ornamentos, producción energética y producción de cortinas rompevientos para diferentes cultivos y abrigo y sombra para animales (Brussa, 1994). Sin embargo, no fue hasta la promulgación de las leyes forestales de 1968 y, en especial, la de 1987 (ley pública n.º 15.939) que su importancia como rubro económico fue reconocida. Dicha ley impulsó un crecimiento explosivo de la forestación del país, mayormente con especies exóticas de los géneros *Eucalyptus* y *Pinus*, siendo *E. globulus* la especie más utilizada. *Eucalyptus globulus* se caracteriza por ser una especie cuya madera posee muchas propiedades de interés para la industria papelera, tales como un alto rendimiento celulósico por kilogramo de madera, alta densidad, bajo valor kappa (lo que facilita y abarata el blanqueamiento de la pasta) y excelente calidad de fibras, siendo esta última una característica muy deseada en la industria papelera (Carrillo et al., 2018, Gominho et al., 2015, Patt et al., 2006, Doughty, 2000,). Es de esta manera que, avalado por sus numerosas características de interés, la especie *E. globulus* es considerada la de mayor distribución geográfica dentro del género (Jacobs, 1981).

La consolidación de la forestación en Uruguay con *E. globulus* como especie predominante no estuvo exenta de problemas. En el año 2007, la detección del patógeno *Teratosphaeria nubilosa*, causante de la enfermedad foliar *Mycosphaerella leaf disease* (MLD) o *Teratosphaeria leaf disease* (TLD), afectó severamente a las plantaciones comerciales de *E. globulus* (Pérez et al., 2009). La introducción accidental de este patógeno provocó una caída del rendimiento y, consecuentemente, importantes pérdidas económicas (Balmelli et al., 2016). Esta problemática generó gran preocupación en el sector forestal uruguayo, que se vio obligado a implementar una serie de medidas para paliar la situación, dentro de las cuales consideró el uso de otras especies de eucalipto alternativas para producir pasta de celulosa (Alonso et al., 2013). La consigna era que las nuevas especies debían mantener el estándar de calidad maderera de *E. globulus* para, de esta manera, no perder los mercados internacionales. Es en este escenario que surge *E. smithii* como la especie alternativa a *E. globulus*, por lo que se comenzó a cultivar comercialmente a gran escala.

Eucalyptus smithii se introdujo en Uruguay hacia principios del siglo XX (Brussa, 1994) y demostró buena adaptación y significativa tolerancia a la ocurrencia de heladas (Krall, 1970). Esta especie es originaria del sureste de Australia y el norte de Tasmania (Krall, 1970) y se caracteriza por poseer una madera de alta calidad, con características pulpables muy similares a las de *E. globulus* (Carrillo et al., 2018). Por esta razón, la especie *E. smithii* se convirtió en una de las opciones más prometedoras para sustituir *E. globulus* y diversificar la silvicultura uruguaya, con una clara perspectiva de expansión a futuro.

De hecho, *E. smithii* alcanzó en el año 2022 el tercer lugar en cuanto a superficie efectiva plantada con aproximadamente 8000 hectáreas, y también se posicionó en el tercer lugar en cuanto a plantines producidos anualmente con 13.628.533, únicamente por detrás de *E. dunnii* y *E. grandis* (MGAP-DGF, 2022). En cuanto al método de producción de las plantas de eucalipto en Uruguay, hay una predominancia de la propagación clonal (57 %) respecto a la propagación a partir de semillas (43 %). Por ejemplo, *E. dunnii* es producido en un 52 % a partir de semillas, mientras que *E. grandis* lo es en un 33 %. En cambio, *E. smithii* es producido en su totalidad a partir de semillas que son importadas y cuya procedencia es Australia y Sudáfrica (MGAP-DGF, 2022).

Desafortunadamente, la expansión masiva de plantaciones con *E. smithii* coincidió con la ocurrencia de una alta mortalidad de árboles jóvenes, en su mayoría durante el primer y segundo año posterior a la plantación. Se ha estimado que la incidencia de muerte de árboles varía en un rango que va desde un 5 % a un 85 % (Rachid et al., 2021). Los árboles de *E. smithii* afectados desarrollan una sintomatología que consiste en una pérdida de turgencia de la parte aérea con una coloración atípica, que va desde tonalidades verde pálido a marrón rojizo u ocre y que finaliza con la muerte del árbol. Respecto a su distribución en el campo, es completamente azarosa, sin asociaciones con la topografía del lugar. Los árboles con decaimiento presentan en la base del tronco sectores necróticos, mientras que en las raíces se observan podredumbres y pérdida de masa radicular. Según la literatura, esta sintomatología podría ser ocasionada por

especies de la familia *Peronosporaceae*, en las que se destacan los géneros *Phytophthora* y *Pythium.* Estos patógenos, en particular *Phytophthora*, causan síntomas similares en una vasta gama de cultivos, incluyendo diversas especies forestales (Scott et al., 2013, Hardham, 2005, Erwin y Ribeiro, 1996).

Ambos grupos de patógenos han sido ampliamente estudiados en diversos cultivos, y es el género *Phytophthora* el que se ha encontrado con mayor frecuencia asociado a enfermedades radiculares de *Eucalyptus.* Diferentes especies de *Phytophthora* tienen la facultad de ocasionar enfermedad tanto en los viveros (Simamora et al., 2015) como en las plantaciones comerciales (Bose et al., 2017). Una de las especies más conocidas de este género es *P. cinnamomi*, la cual fue encontrada afectando a bosques de *E. marginata* en Australia, conocidos como "Jarrah", lo que ocasionó hasta un 75 % de pérdida de la masa forestal (Podger et al., 1965). Desde entonces, esta especie altamente polífaga y con gran capacidad destructiva se ha distribuido globalmente y afecta un amplio rango de cultivos (Scott et al., 2013, Hardham, 2005, Drenth y Guest, 2004), incluyendo diversas especies de eucaliptos, por ejemplo, en Sudáfrica (Linde et al., 1994), China (Keane, 2000) y Portugal (Diogo et al., 2022).

No obstante, patógenos pertenecientes a otros grupos taxonómicos también tienen la capacidad de generar en *Eucalyptus* sintomatologías similares a las descritas anteriormente, como es el caso de especies del género *Calonectria* (= *Cylindrocladium*), que pertenece a la familia *Nectriaceae* (Lombard et al., 2015a, Crous, 2002). Al igual que *Phytophthora*, especies del género *Calonectria* también son capaces de causar infecciones radiculares y del cuello de la planta en *Eucalyptus*, tanto en la etapa de vivero (Aiello et al., 2020, Lombard et al., 2010d, Alfenas et al., 2009, Crous et al., 2002, 1991) como en plantaciones comerciales (Alfenas et al., 2009, Crous, 2002). Sin embargo, especies de este género son más conocidas por causar enfermedades foliares en *Eucalyptus*, al que le ocasionan manchado y atizonado de hojas que conducen a defoliaciones severas y debilitamiento de plantas (Alfenas et al., 2015, 2009, Crous, 2002, Keane, 2000, Lombard et al., 2010c). En cuanto a las

enfermedades radiculares que causa este género, la información disponible es mucho más reducida.

En Uruguay, el primer estudio de identificación de especies de *Calonectria* se llevó a cabo en el año 2013 e implicó el estudio de cuatro viveros forestales de la zona norte del país, dónde en base a la secuenciación de la región ITS, se identificaron 18 cepas como *Ca. sulawesiensis*, siete como *Ca. pauciramosa* y dos como *Ca. humícola* (Gasparri et al., 2013). Subsecuentemente, Castro et al., (2015) llevó a cabo otro estudio de identificación de especies que partió de una colección de cepas de *Calonectria* obtenidas de plantas de *Eucalyptus* muertas en plantaciones comerciales, dónde se seleccionó una submuestra, se secuenciaron parcialmente tres regiones génicas; β-Tubulina (*tub2*), histona H3 (*his3*) y factor de elongación 1-alfa (*tef-1*) y como resultado se identificaron cuatro especies distintas, una de ellas presumiblemente *Ca. seminaria* y las otras tres no se agruparon con ninguna especie descrita pero se determinó que pertenecían al complejo de especies *Calonectria cylindrospora*.

Teniendo en cuenta que ambos grupos de patógenos (*Phytophthora* y *Calonectria*) pueden afectar a las plantas en la etapa de vivero y que la muerte de árboles de *E. smithii* en las plantaciones comerciales ocurre tempranamente (primer y segundo año) luego del establecimiento de la plantación, se sospecha que el origen del problema pueda estar en la sanidad de las plantas provenientes de los viveros. Es decir que al menos una parte de las infecciones ocasionadas por estos patógenos podría originarse en los viveros. Es en este contexto que surge este trabajo de tesis de maestría que consiste en determinar las causas de la muerte de árboles jóvenes de *E. smithii* en la zona sureste de Uruguay, donde se ubica la mayor parte de las plantaciones con esta especie.

Las hipótesis planteadas son:
— Especies de la familia *Peronosporaceae*, en particular del género *Phytophthora* y especies de la familia *Nectriaceae*, en especial del género *Calonectria*, son responsables de la muerte de árboles jóvenes de *E. smithii*.

— Al menos una parte de estas infecciones que llevan a la muerte de árboles jóvenes de *E. smithii* se origina en la etapa de producción de las plantas en los viveros.

El presente trabajo tiene por objetivo general: generar conocimientos sobre el rol que tienen especies de *Phytophthora* y de *Calonectria* en el decaimiento y muerte de árboles jóvenes de *E. smithii*.

En tanto, como objetivos específicos se propuso:

I) Aislar especies de *Phytophthora*, a partir de árboles jóvenes y plantas de vivero de *E. smithii* con síntomas de decaimiento, e identificar los aislamientos mediante análisis filogenéticos y fenotípicos.

II) Aislar especies de *Calonectria*, a partir de árboles jóvenes y plantas de vivero de *E. smithii* con síntomas de decaimiento, e identificar los aislados mediante análisis filogenéticos y fenotípicos.

III) Determinar la capacidad patogénica de los aislados de *Phytophthora* sp. y *Calonectria* sp. obtenidos de árboles jóvenes y plantas de vivero de *E. smithii* con síntomas de decaimiento, mediante su inoculación en plantas de vivero sanas de *E. smithii*.

La estructura de esta tesis se conforma de dos artículos. El primero corresponde a la investigación sobre el rol que tienen las especies del género *Phytophthora* en el decaimiento y muerte de árboles jóvenes de *E. smithii*. Este artículo fue publicado en la revista *Forest Pathology* (DOI: 10.1111/efp.12810). El segundo contiene el estudio de hongos pertenecientes a la familia *Nectriaceae* y su rol en el decaimiento y muerte de árboles jóvenes de *E. smithii*, donde además se presenta la descripción de una nueva especie dentro del género *Ilyonectria*. Este se pretende enviar a la revista *Mycological Progress* para su publicación.

2. *PHYTOPHTHORA ALTICOLA* AND *P. BOODJERA* ASSOCIATED WITH DECLINE OF YOUNG *EUCALYPTUS SMITHII* TREES IN URUGUAY

Artículo publicado en la revista *Forest Pathology*.

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Franco De Benedetti[1], Victoria Moreira[1], Pedro Mondino[1], Sandra Alaniz[1]

[1] Department of Plant Protection, Facultad de Agronomía, Universidad de la República, Av. Garzón 780, 12900, Montevideo, Uruguay.

Correspondence
Sandra Alaniz, Department of Plant Protection, Facultad de Agronomía, Universidad de la República, Av. Garzón 780, 12900, Montevideo, Uruguay.
Email: salaniz@fagro.edu.uy

***Phytophthora alticola* y *P. boodjera* asociadas con el decaimiento de árboles jóvenes de *Eucalyptus smithii* en Uruguay**

2.1 RESUMEN

La producción de eucaliptos destinada principalmente a la producción de pulpa de celulosa ha experimentado un fuerte crecimiento en los últimos treinta años en Uruguay. *Eucalyptus smithii* surgió recientemente como una especie prometedora para la producción de pulpa de celulosa. Sin embargo, en promedio el 40% de los árboles jóvenes mueren durante el primer y segundo verano después de plantados. En este estudio se obtuvieron 32 aislamientos de *Phytophthora* a partir de 132 árboles jóvenes de *E. smithii* con síntomas de pudrición de raíz y cuello, confirmando la asociación de *Phytophthora* con el decaimiento de *E. smithii*. En base al análisis filogenético de secuencias parciales de las regiones ITS, TUB2, COX1 y HSP90, y a características fenotípicas, se identificaron dos especies pertenecientes al clado 4 del género *Phytophthora*, *P. alticola* (96%) y *P. boodjera* (4%). Los estudios de patogenicidad demostraron que aislados de ambas especies inoculados en plantínes de *E. smithii* redujeron significativamente el peso seco de sus brotes y raíces, en comparación a los plantínes sin inocular. Hasta donde sabemos, esta es la primera vez que se aísla *P. alticola* y *P. boodjera* de árboles jóvenes sintomáticos de *E. smithii* en plantaciones comerciales, así como la primera vez que se encuentran estas especies en el continente Americano.

Palabras clave: Podredumbre de cuello, podredumbre de raíz, *Eucalyptus globulus*, filogenia, análisis multigénicos, oomycetes, *Phytophthora arenaria*

2.2 ABSTRACT

Eucalyptus production mainly destined to cellulose pulp production has expanded strongly in the last thirty years in Uruguay. *Eucalyptus smithii* has recently emerged as a promising species for cellulose pulp production. However, an average of 40% of young trees die during the first and second summer of post-planting. In this study, 32 *Phytophthora* isolates were obtained from 132 *E. smithii* young trees with root and collar rot symptoms, confirming the association of *Phytophthora* to *E. smithii* decline. Based on phylogenetic analysis of ITS, TUB2, cox1 and HSP90 gene regions and phenotypical characteristics, two species belonging to the genera *Phytophthora* clade 4 were identified, *P. alticola* (96%) and *P. boodjera* (4%). Tested isolates of both species significantly reduced both shoot and root dry weights of inoculated *E. smithii* seedlings compared to control plants. To our best knowledge, this is the first time that *P. alticola* and *P. boodjera* are recovered from young symptomatic *E. smithii* trees in commercial plantations as well as the first time these species are found in the Americas.

Keywords: Collar rot, *Eucalyptus globulus*, multi-gene phylogeny, Oomycetes, *Phytophthora arenaria*, Root rot

2.3 INTRODUCTION

Since the approval of the last forestry law in 1987, commercial forestry activities in Uruguay had a great expansion. Planted surface currently reaches 1.053.693 hectares and covers 6.10% of the productive land area (MGAP-DIEA, 2021). Commercial forestry relies mostly on monocultural exotic tree plantations with *Eucalyptus* as the most widely planted genera (81%). *Eucalyptus* wood is mainly destined to cellulose pulp production, as round-wood and chips. Other destinations are solid wood for saw timber, panels and veneers production and wood for fuel (Boscana & Boragno, 2022).

Until 2007, *E. globulus* was the most widely planted *Eucalyptus* species in Uruguay for pulp production, due its several advantages in Kraft pulp manufacturing, including high pulp yields, high wood density, excellent fibre quality and desirable hand-sheet properties (Doughty 2000; Patt et al., 2006; Gominho et al., 2015; Carrillo et al., 2018). Unfortunately, in 2007 *Teratosphaeria nubilosa*, one of the causal agents of *Mycosphaerella* leaf disease (MLD), severely affected young plantations of *E. globulus* and *E. maidenii* (Pérez et al., 2009). Owing to the great damage caused by this epidemic outbreak, 5000 ha of young *E. globulus* plantations were destroyed. This event forced the forestry companies to re-plant using alternative *Eucalyptus* species (Ansuberro et al., 2015).

Eucalyptus smithii emerged as a promising species to replace *E. globulus*. This species is grown for commercial essential oil, but also for pulp production due to its excellent wood properties as high pulp and fibre yields, high wood density, brightness, low kappa number and alkali consumption, amongst other 'pulpability' characteristics, similar to those of *E. globulus* (Clarke et al., 1999; Carrillo et al., 2018). Furthermore, *E. smithii* proved to be significatively less susceptible to leaf spot caused by *Mycosphaerella* spp. and *Teratosphaeria* spp., causal agents of MLD and *Teratosphaeria* leaf blight (TLB) (Carnagie et al., 1998). In Uruguay, *E. smithii* is produced by seeds from Australia and South Africa. However, since *E. smithii* plantations expanded massively in Uruguay, an average of 40% (ranging from 5 to

85%) of young dead trees has been recorded during the first and second summer after plantation (Rachid et al., 2021). The symptoms include leaf chlorosis, root and collar rot with eventual gum exudation, decline and death of trees. These symptoms are typical of those caused by *Phytophthora* spp.

Phytophthora root and collar rot is a widespread destructive disease affecting nurseries, forests, urban environments, and natural ecosystems around the world, and is associated with several plant hosts including *Eucalyptus* spp**.** (Kroon et al., 2004; Shearer et al., 2004; Burgess et al., 2009; Moralejo et al., 2009; Simamora et al., 2015; 2018). *Phytophthora cinnamomi* was first described as the causal agent of die back disease of the `jarrah´ (*E. marginata*) forest in Western Australia (Podger et al., 1965). Moreover, other *Phytophthora* species including *P. cinnamomi*, *P. boehmeriae*, *P. nicotianae, P. alticola* and *P. frigida* were associated with root and collar rot of various cold-tolerant *Eucalyptus* spp. in South Africa such as *E. dunni*, *E. badjensis*, *E. macarthurii* and *E. smithii* (Linde et al., 1994; Wingfield et al., 1994; Maseko et al., 2001; Bose et al., 2017).

In addition, a study conducted in South Africa revealed that *P. alticola*, *P. cinnamomi*, *P. frigida*, *P. multivora* amongst other species, were commonly isolated from roots and soil of native and non-native tree species (Bose et al., 2018). More recently in time, *P. alticola* and *P. cinnamomi* were found associated with root rot and dieback of *E. globulus* in Portugal (Diogo et al. 2022).

To better understand *E. smithii* establishment in Uruguay and its relationship with *Phytophthora* spp., the aims of this study were i) to determine the presence of *Phytophthora* spp. associated to decline of *E. smithii* young trees, ii) to identify and characterize the isolates based on morphological features and molecular data and iii) to verify their pathogenicity on *E. smithii* by inoculating nursery-produced seedlings.

2.4 MATERIALS AND METHODS

2.4.1 Field symptoms, sampling and isolation

Between 2019 and 2021, nine *Eucalyptus smithii* commercial plantations of 1 year-old including natural soil and re-established sites (sites with previous rotations of *Eucalyptus* spp.) (Fig. 1) were surveyed. The plantations were located in the south-eastern of Uruguay (Canelones, Lavalleja and Florida departments), the main region where *Eucalyptus smithii* is planted. In each plantation two surveys were conducted, in early and late summer.

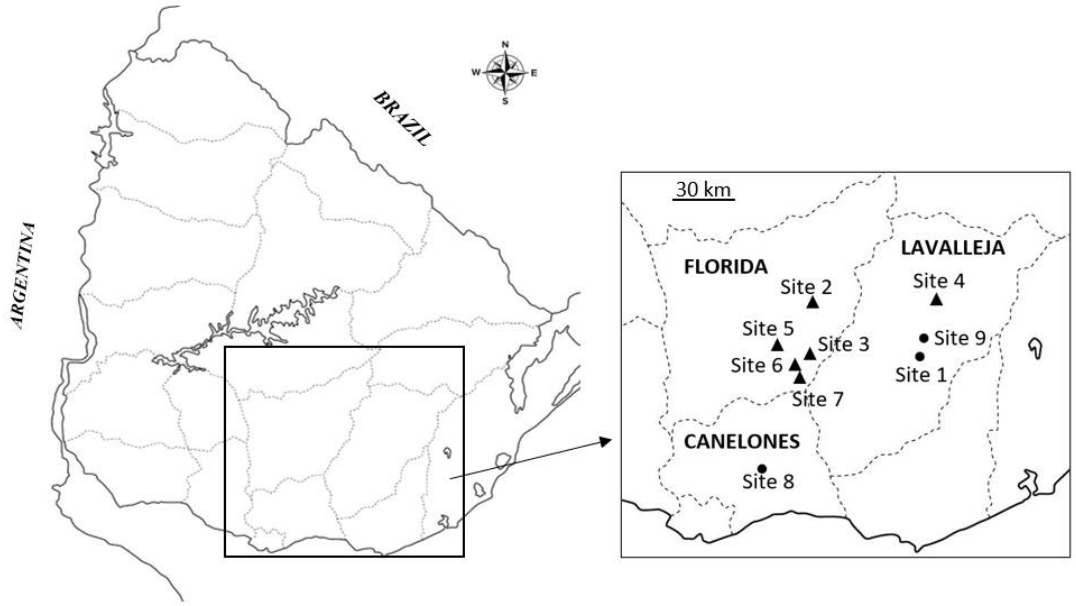

FIGURE 1 Geographical localization of the nine survey sites in the south and south-east of Uruguay. Round points indicate natural soil sites; triangles indicate re-established sites (sites that had previous rotation with Eucalypts). Sites 1 to 5 were planted in spring 2019; site 6 in autumn 2020 and sites 7 to 9 in spring 2020.

In each *E. smithii* plantation, 10 to 15 trees with initial symptoms of decline were randomly collected. The whole tree root system was removed and placed into a single

plastic bag, moistened with water, and sealed to prevent desiccation. A total of 132 declined trees were collected and evaluated for the presence of *Phytophthora* spp. Isolation from each individually diseased tree was performed within 24-36 h of collection. For this, roots were rinsed gently with abundant water and the bark of roots and collar was removed with a scalpel. Small wood pieces (2 × 5 mm) were aseptically taken from the internal margin of the necrotic lesions and placed directly on selective NARPH medium (Hüberli et al., 2000) with modifications; [(CMA) 60 g corn seeds boiled for 1 h with 1 L distilled water, let cool and filter. Add distilled water to the filtrate up to 1 L final volume and 17 g/L of agar (Oxoid Ltd., Hampshire, England), sterilize. After cooling, amend with 50 µg/mL nystatin, 200 µg/mL ampicillin, 10 µg/mL rifampicin, 25 µg/mL pentachloronitrobenzene (PCNB) and 50 µg/mL hymexazol 3-hydroxy-5-methylisoxazole, Sigma-Aldrich, St. Louis]. Petri dishes were incubated at 20 °C in the dark and were examined within 2-7 d using a microscope.

Developed colonies with coenocytic mycelia were transferred to V8 agar medium [(V8A) Campbell's V8 juice 200 mL, 3 g CaCO3, 15 g agar and 800 mL distilled water)] (Tsao, 1983) and incubated at 20 °C. To verify the presence of *Phytophthora* spp., sporangia development was induced by submerging three to five 1 x 1 cm colonized V8A squares taken from active growing edges of 5 d-old cultures, into clean Petri dishes containing 30 mL of sterile soil extract water (100 g soil flooded with 1 L distilled water for 24 h, mix 50 ml of this supernatant with 950 ml of distilled water, autoclave twice) (Pérez-Sierra et al., 2010).

Pure cultures of the unknown *Phytophthora* were established by hyphal tips taken from the edge of growing colonies and sub-cultured onto V8A for further studies. Maintenance of isolates was carried out in 90 mm petri dishes containing V8A, and in 5 mm colonized V8A discs placed into 30 mL vial glass bottles containing up to 20 mL of sterile soil extract water. Plates and tubes were stored at 15°C. Isolates used in the present study were deposited in the fungal culture collection at the Plant Protection Department, Faculty of Agronomy, University of the Republic, Uruguay.

2.4.2 DNA isolation, sequencing and phylogenetic analysis

Total DNA was extracted from 32 unknown *Phytophthora* isolates obtained in this study. The isolates were grown in V8A for 1 wk. at 20 °C in the dark and a commercial kit ZR Fungal/ Bacterial Quick-DNA™ Miniprep Kit (Zymo Research, USA) was used according to the manufacturer's instructions. For a primary identification, internal transcribed spacer region of the ribosomal DNA (ITS1-5.8S-ITS2) was amplified from the 32 isolates requiring identification, using the primers ITS6/ITS4 (Cooke et al., 2000; White et al., 1990). Additional gene regions, β-tubulin (TUB2), cytochrome c oxidase subunit I (cox1), and heat shock protein 90 (HSP90), were amplified from a sub-set of 16 unknown *Phytophthora* isolates using the primer pairs BtubF1A/BtubR1 (Blair et al., 2008; Kroon et al., 2004), FM84/FM83 (Martin & Tooley 2003) and HSP90F1/HSP90R2(Blair et al., 2008), respectively.

Individual PCR reactions were carried out using 1x PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.4 µM of each primer, 1 U of DNA polymerase (Bioron, Germany) and 1 µL of template DNA. The PCR reaction was adjusted to a final volume of 20 µL with PCR grade water. Amplifications were performed on a MultiGene™ Mini (Labnet International, Inc., USA) and the amplification program used for ITS/TUB2/cox1/HSP90 consisted in an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, annealing temperature at 55/60/56/62 °C and time 30/30/60/30 sec respectively, 72 °C for 1 min and a final extension cycle of 72 °C for 10 min. PCR products were visualized on a 1.5% agarose gel stained with GelRed™ in a transilluminator under UV light, using GeneRuler 100-bp DNA ladder plus as a molecular weight marker (Thermo, Lithuania). PCR products were purified and sequenced in Macrogen Inc., Seoul, South Korea.

Sequences of each gene region were aligned using Clustal W program, available within MEGA 11.0.11 program (Tamura et al., 2021) and were manually edited when necessary. Related sequences as well as sequences of the phylogenetically closest species from *Phytophthora* clade 4 (including type, ex-type and selected specimens)

were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and *IDphy* online resource (http://idtools.org/id/phytophthora/index.php.) (Abad et al., 2022) and incorporated in the alignments (Table 2).

The ITS sequences obtained from the 32 isolates were compared with those deposited in GenBank using the BLAST search tool (blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses of the 16 selected isolates were performed separately using the four amplified gene regions and a concatenated dataset alignment was built using Sequence Matrix v.1.8 (http://www.ggvaidya.com/taxondna/). Phylogenetic trees were constructed using Bayesian inference (BI) and Maximum likelihood (ML) methods. BI and ML analyses were inferred with MrBayes 3.2.7 (Ronquist et al., 2012) and RAxML 8.2.12 (Stamatakis, 2014) programs respectively, implemented in CIPRES Science Gateway v 3.3 (http://www.phylo.org/). For BI phylogenetic analysis, the best-fit model of each locus was selected according to Akaike information criterion corrected (AICc) in MEGA 11.0.11 program. Four Markov Chain Monte Carlo (MCMC) chains were run simultaneously starting from a random tree to 10 million of generations. Trees were sampled every 1000 generations, and the first 2500 were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7500 trees. For the ML analysis, generalized time-reversible with gamma correction (GTR +GAMMA) nucleotide substitution model and 1000 bootstrap iterations were used. The additional parameters were used as default settings. Sequences generated in this study were deposited in the GenBank (Table 1).

2.4.3 Phenotypic characteristics

Colony morphology was described according to Erwin & Ribeiro, (1996). For this, mycelial plugs of 5 mm diameter were taken from the margin of 7 d-old cultures of the 16 selected *Phytophthora* strains growing on V8A at 20 °C in the dark and were placed at the centre of 90 mm Petri dishes containing four growth media; 10% Carrot Agar [CA: 0.1 L filtered carrot juice, 17 g agar (Oxoid Ltd., Hampshire, England) and

0.9 L distilled water], 2% Malt Extract Agar [MEA: 20 g Malt Extract (Biolab, Merck, South Africa), 15 g agar and 1 L distilled water], V8A (as indicated above) and Potato Dextrose Agar [PDA: 39 g (Oxoid Ltd., Hampshire, England) and 1 L distilled water].

For temperature growth studies, each of the 16 *Phytophthora* strains was sub-cultured onto V8A and CA plates and incubated for 24 h at 20 °C for growth stimulation. Thereafter, three replicate plates per isolate were moved to incubators fixed between 5 to 40 °C with intervals of 5 °C. Plates were monitored daily to ensure the colonies did not reach the edge of Petri dishes. Reverse colony diameter measurements were carried out within 5-7 days using an electronic digital calliper (Kamasa®, USA) along two perpendicular lines and mean growth rates (mm-per-day) were registered. After 7 days those plates with no colony growth were returned to 20 °C for 72 h to check the isolate viability.

TABLE 1 Identity, host information, locations and GenBank accessions for *Phytophthora* spp. considered in this study

Species	Isolate identity	Host association	Country/Survey site	Isolation date	GenBank Accession No.			
					ITS	BTUB	COX I	HSP90
P. alticola	**PHY 1**	*Eucalyptus smithii*	Uruguay/ site 2	2020	OP484867	OP495738	OP495754	OP495770
	PHY 2	*E. smithii*	Uruguay/ site 2	2020	OQ437244	--	--	--
	PHY 3	*E. smithii*	Uruguay/ site 2	2020	OP484868	OP495739	OP495755	OP495771
	PHY 4	*E. smithii*	Uruguay/ site 2	2020	OQ437245	--	--	--
	PHY 5	*E. smithii*	Uruguay/ site 2	2020	OP484869	OP495740	OP495756	OP495772
	PHY 6	*E. smithii*	Uruguay/ site 3	2020	OQ437246	--	--	--
	PHY 7	*E. smithii*	Uruguay/ site 3	2020	OP484870	OP495741	OP495757	OP495773
	PHY 8	*E. smithii*	Uruguay/ site 3	2020	OQ437247	--	--	--
	PHY 9	*E. smithii*	Uruguay/ site 3	2020	OP484871	OP495742	OP495758	OP495774
	PHY 10	*E. smithii*	Uruguay/ site 3	2020	OP484872	OP495743	OP495759	OP495775
	PHY 11	*E. smithii*	Uruguay/ site 4	2020	OQ437248	--	--	--
	PHY 12	*E. smithii*	Uruguay/ site 4	2020	OP484873	OP495744	OP495760	OP495776
	PHY 13	*E. smithii*	Uruguay/ site 4	2020	OP484874	OP495745	OP495761	OP495777
	PHY 14	*E. smithii*	Uruguay/ site 5	2020	OP484875	OP495746	OP495762	OP495778
	PHY 15	*E. smithii*	Uruguay/ site 6	2020	OQ437249	--	--	--
	PHY 17	*E. smithii*	Uruguay/ site 6	2020	OQ437250	--	--	--
	PHY 18	*E. smithii*	Uruguay/ site 5	2021	OQ437251	--	--	--
	PHY 19	*E. smithii*	Uruguay/ site 5	2021	OP484876	OP495747	OP495763	OP495779
	PHY 20	*E. smithii*	Uruguay/ site 5	2021	OQ437252	--	--	--
	PHY 21	*E. smithii*	Uruguay/ site 5	2021	OQ437253	--	--	--
	PHY 22	*E. smithii*	Uruguay/ site 6	2021	OP484877	OP495748	OP495764	OP495780
	PHY 23	*E. smithii*	Uruguay/ site 7	2021	OP484878	OP495749	OP495765	OP495781
	PHY 24	*E. smithii*	Uruguay/ site 8	2021	OQ437254	--	--	--
	PHY 25	*E. smithii*	Uruguay/ site 8	2021	OQ437255	--	--	--
	PHY 26	*E. smithii*	Uruguay/ site 8	2021	OP484879	OP495750	OP495766	OP495782

	PHY 27	*E. smithii*	Uruguay/ site 8	2021	OQ437256	--	--	--
	PHY 28	*E. smithii*	Uruguay/ site 8	2021	OQ437257	--	--	--
	PHY 29	*E. smithii*	Uruguay/ site 8	2021	OP484880	OP495751	OP495767	OP495783
	PHY 30	*E. smithii*	Uruguay/ site 8	2021	OQ437258	--	--	--
	PHY 31	*E. smithii*	Uruguay/ site 9	2021	OP484881	OP495752	OP495768	OP495784
	PHY 32	*E. smithii*	Uruguay/ site 9	2021	OQ437259	--	--	--
	CBS 141718*	*E. grandis* soil	Commondale, KwaZulu-Natal, South Africa (SA)	2014	KX247599	KX247592	KX247585	KX247578
	CBS 141719	Natural forest soil	Melmoth, KwaZulu-Natal, SA	2015	KX247600	KX247593	KX247586	KX247579
	CBS 141721	*E. grandis* soil	Melmoth, KwaZulu-Natal, SA	2016	KX247602	KX247595	KX247588	KX247581
	CBS 141723	*Acacia mearnsii* soil	Commondale, KwaZulu-Natal, SA	2016	KX247604	KX247597	KX247590	KX247583
P. boodjera	PAB 11.67	*E. marginata*	Dalkeith, Perth, WA	2011	KC748461	KJ372276	KJ396682	KJ396704
	VHS 26806*	Soil dump	Tincurrin, WA	2012	KJ372244	KJ372283	KJ396688	KJ396710
	VHS 26631	*Eucalyptus sp.*	Kensington, WA	2012	KJ372240	KJ372277	KJ396683	KJ396705
	PHY 16	*E. smithii*	Uruguay/ site 6	2020	OP484882	OP495753	OP495769	OP495785
P. arenaria	CPHST BL 78*	*E. drummondii*	Eneabba, WA	2009	MG783377	MH493906	MH136848	n.a
	VHS 20537	*Banksia attenuata*	Eneabba, WA	2008	KJ372253	KJ372299	KJ396698	KJ396727
P. castaneae	CPHST BL 47G*	*Castanea crenata*	Japan	1971	MG865470	MH493918	MH136866	n.a
P. heveae	CPHST BL 67*	Soil	United States	2003	MG865505	MH493947	MH136899	MK020314
P. litchii	CPHST BL 145*	*Litchi chinensis*	Taiwan	1978	MG865524	OL466902	MH136919	MK020333
	CPHST BL 48G*	*L. chinensis*	Taiwan	1978	MG865525	MH493966	MH136920	MK020332
P. megakarya	CPHST BL 22*	*Theobroma cacao*	Nigeria, Africa	1974	MG865533	n.a	MH136928	MK020339
P. palmivora	CPHST BL 105*	*Areca catechu*	India	1956	MG865559	MH493992	MH136949	MK020363
	CPHST BL 106*	*Cocos nucifera*	Indonesia	n.a	MG865561	MH493993	MH136951	MK020364
P. quercetorum	CBS 121119*	*Quercus rubra* soil	United States	2004	KX759518	KX759519	KX759520	KX759521

2.4.4 Morphology of reproductive structures

Reproductive structures induction was achieved by submerging three to five 1 x 1 cm colonized V8A blocks cut from active growing edges of 5 d-old cultures, into clean Petri dishes containing 30 mL of sterile soil extract water. Inductive plates were maintained at room temperature (22-25°C) and sterile soil extract water was replaced after 8 and 24 h. After 24-72 h, length and breadth measurements of 30 randomly selected mature sporangia were made for each isolate and rated according to their shape.

After 15-21 days, measurements of 10 randomly selected mature oogonia, oospores and antheridia per isolate was registered. Observation and measurement of all the structures were made under microscope at 400X magnification using a digital camera (Microscope-eye-piece-camera, AM-4023X, Taiwan) incorporated to the microscope and complemented with Dino-eye® 2.0 software. The oospore wall index was calculated according to Dick, (1990) as the ratio between the volume of the oospore wall and the volume of the whole oospore.

2.4.5 Pathogenicity tests

Phytophthora alticola isolates PHY 1, PHY 5, PHY 10 and *P. boodjera* isolate PHY 16, were selected for pathogenicity tests on three months old *E. smithii* (nursery-produced) seedlings using two inoculation methods.

Inoculation method 1: *Phytophthora* isolates were grown on V8A for 7 d at 20 °C in dark. Then, each colony was cut into 1 x 1 cm squares using a sterile scalpel and placed into single clean plastic boxes (10 x 7 x 5 cm) and flooded with 100 mL of sterile soil extract water to induce sporangia production. Boxes were incubated at 20 °C in dark for 3 d and sterile soil extract water was replaced daily. Development of sporangia in each box was checked under microscope.

For seedling inoculation, first a thick peat basal layer was placed at the bottom of 1 L sturdy plastic pots and then the inducted squares of the corresponding isolate, one colony per pot. Thereafter, root tips of *E. smithii* seedlings were slightly cut and immediately planted. Finally, the pots were filled with peat substrate and the sterile soil extract water of the corresponding isolate box was used to watering each pot. Six seedlings were inoculated per isolate, plus other six seedlings inoculated with water-agar squares serving as controls. All the pots were flooded for 24 h after inoculation and then once a month.

Inoculation method 2: The inoculum was prepared according to Belhaj et al., (2018) with slightly modifications. Briefly, a compound substrate was made in 1 L glass flasks by adding 500 mL of vermiculite, 10 g of *Panicum miliaceum* (millet) seeds, and 500 mL of sterile V8 juice broth (200 mL V8 juice, 2 g CaCO3 and 800 mL distilled water). Flasks were autoclaved two times at 121 °C for 40 min with one hour of cooling time within cycles. Once the substrate had completely cooled, each flask was inoculated with 1 x 1 cm squares of *Phytophthora* isolates grown on V8A for 7 d at 20 °C in dark, one isolate per flask. Flasks were gently shaken and incubated at 20 °C in dark. For inoculum mixture, flasks were shaken every 3 d during the first 2 wk. After 6 wk. inoculum was rinsed with sterile distilled water to remove V8A nutrients excess (Matheron & Mircetich, 1985) and was immediately used. Inoculum viability was confirmed by plating subsamples directly on NARPH medium.

For seedling inoculation, a thick peat basal layer was placed at the bottom of 1 L sturdy plastic pots followed by a 6 g inoculum layer (approximately 2% of peat weight in a spot) added above. Thereafter, *E. smithii* seedlings root tips were slightly cut and immediately planted over both layers and covered with peat. Six seedlings per isolate were inoculated, and six seedlings inoculated with sterile vermiculite and V8 juice broth served as control. All the pots were flooded for 24 h after inoculation and then once a month.

Maintenance, assessments and re-isolation. Inoculated seedlings were maintained in a greenhouse at natural temperatures (20 ± 5°C), fertilized with a foliar fertilizer (Foliar plus® Finonsur S.A., NPK 15.8.6 and micronutrients) and watered when necessary. After ten months, all seedlings were removed from the pots and the roots were thoroughly washed with tap water until free of soil. Then, each root system was separated from the aerial part by cutting at the crown zone and both seedling parts were put by separate into paper bags. Bags were placed into a dry-heat oven set at 45 ± 2 °C and 20 ± 2 % relative-humidity for 2 wk. until they reached a constant weight. Then, dry weight of root and shoot was measured. Re-isolations were made from plants of each treatment including control plants, by plating small and affected root tips directly onto NARPH media to confirming Koch's postulates. To determine the effect of *Phytophthora* spp. inoculations on *E. smithii* seedlings, analyses of variance (ANOVA) were conducted with root and shoot dry weights data. Mean values were separated by Tukey test ($P < 0.05$) using InfoStat software version 2016 (http://www.infostat.com.ar).

2.5 RESULTS

2.5.1 Field symptoms and isolation

In the field, symptoms displayed by young and affected *E. smithii* trees were observed from early summer (Dec) to early fall (Mar-Apr) but mainly in late summer, in all the surveyed sites except in site 1 (Fig. 2). The observed symptoms were typical of those caused by *Phytophthora* spp. including an initial mild chlorosis and decline that progressed to a pronounced chlorosis turning reddish to dark brown (Fig. 2a-d), stem cankers with eventual gum exudation in the lower part of the stem, root rot and collar lesions (Fig. 2e-g). Moreover, in all the surveyed sites diseased trees were randomly distributed across the field (Fig. 2a). Mortality values were approximately 20 to 50 % with a higher incidence in re-established sites. Thirty-two *Phytophthora* isolates were obtained from 132 diseased trees (one isolate per tree) collected along the surveyed

sites. These results confirm the association of *Phytophthora* with *E. smithii* decline in Uruguay. The percentage of isolation was 24% in average ranging from 10 % (site 7) to 70% (site 8).

2.5.2 Sequencing and phylogenetic analysis

The ITS alignment showed that the 32 *Phytophthora* isolates were identical and had 100% homology with *P. alticola* ex-type CBS 141718 (Bose et al., 2017) a species belonging to clade 4 (Abad et al., 2022).

Based on the analyzed DNA sequence data of four gene regions, two haplotypes of *P. alticola* were found in this study; haplotype 1 composed by 13 isolates and haplotype 2 composed by two isolates (PHY 26 and PHY 29) that differ each other on 1 bp in the B-tub gene region. Moreover, isolates from haplotype 1 differed by 1 bp in B-tub, 1 bp in cox1 and 1 bp in HSP90 with *P. alticola* ex-type CBS 141718 (Bose et al., 2017), while isolates from haplotype 2 differed by 1 bp in B-tub and 1 bp in cox1 gene regions respectively (Fig. 3). Furthermore, the single isolate PHY 16 representing *P. boodjera* differed by 1 bp in ITS and 1 bp in HSP90 with *P. boodjera* ex-type VHS26806 (Simamora et al., 2015) (Fig. 3).

2.5.3 Phenotypic characteristics

The *P. alticola* and *P. boodjera* isolates produced appressed, cottony colonies, with smooth and entire margins with no distinctive growth patterns on the four different culture media (CA, V8A, MEA, PDA) after 7 d of incubation at 20 °C in the dark (Figs. 4 and 5). Colonies produced on CA were less cottony than those produced on the other three media.

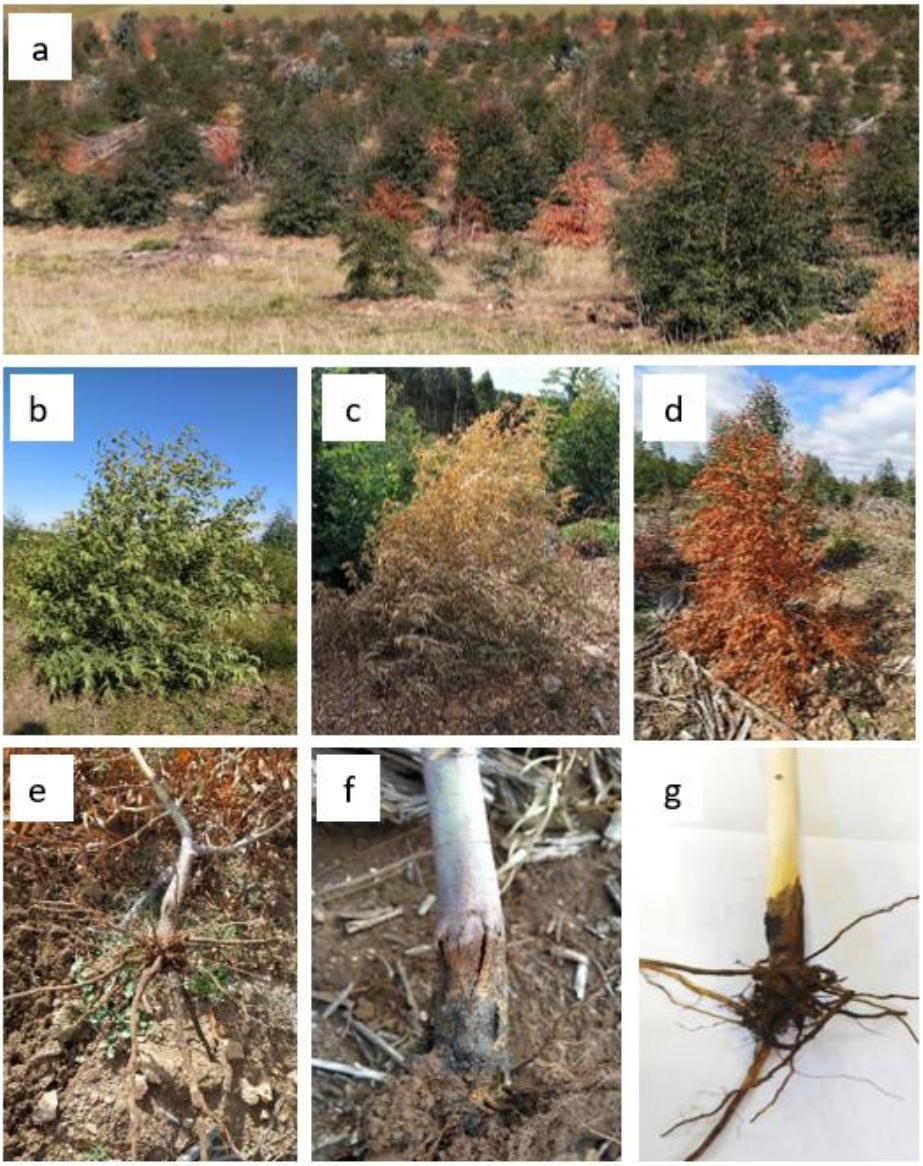

FIGURE 2 Field symptoms on affected young *Eucalyptus smithii* trees; (a) randomly distribution of affected trees across the field; (b-d) initial to advanced symptoms of leaf chlorosis and wilting; (e) radicular system with rot roots; (f) collar lesions with cankers and eventual gum exudation; (g) under-bark root and collar lesions showing healthy to necrotic transition zone.

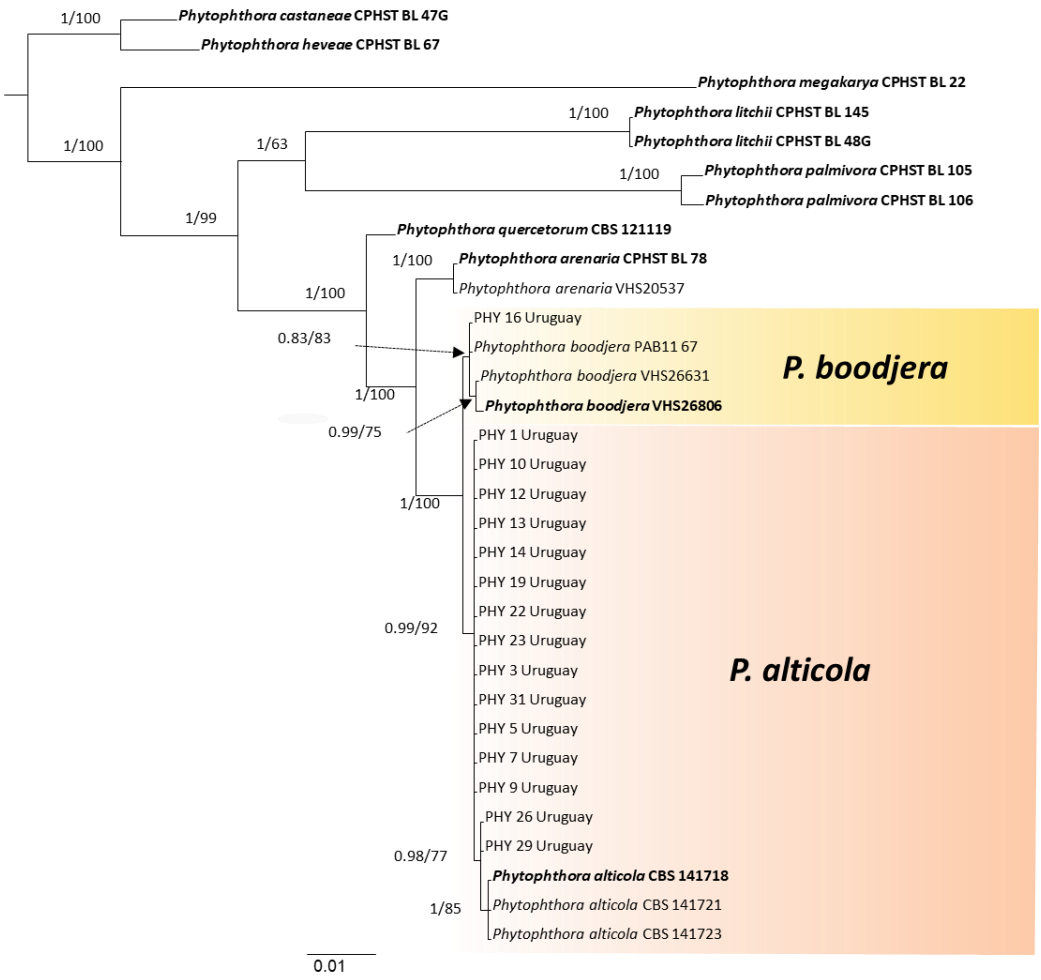

FIGURE 3 Bayesian inference phylogenetic tree of *Phytophthora* spp. Uruguayan isolates obtained from *Eucalyptus smithii* samples with root and collar rot symptoms. The tree was built based on concatenated sequence data from ITS, β-tubulin, cox1 and HSP90 gene regions. Posterior probabilities (PPs) >0.90 and boostrap support (BS) values >70 are shown on branches (PP/BS). Phylogeny is rooted with *P. castaneae* and *P. heveae* as outgroups. The remaining taxa are used as reference and were retrieved from National Centre for Biotechnology Information and IDphy online resource, with ex-type cultures in bold. Scale bar represents the estimated number of substitutions per site. Double hash marks indicate branch lengths were shortened at least twofold to facilitate visualization.

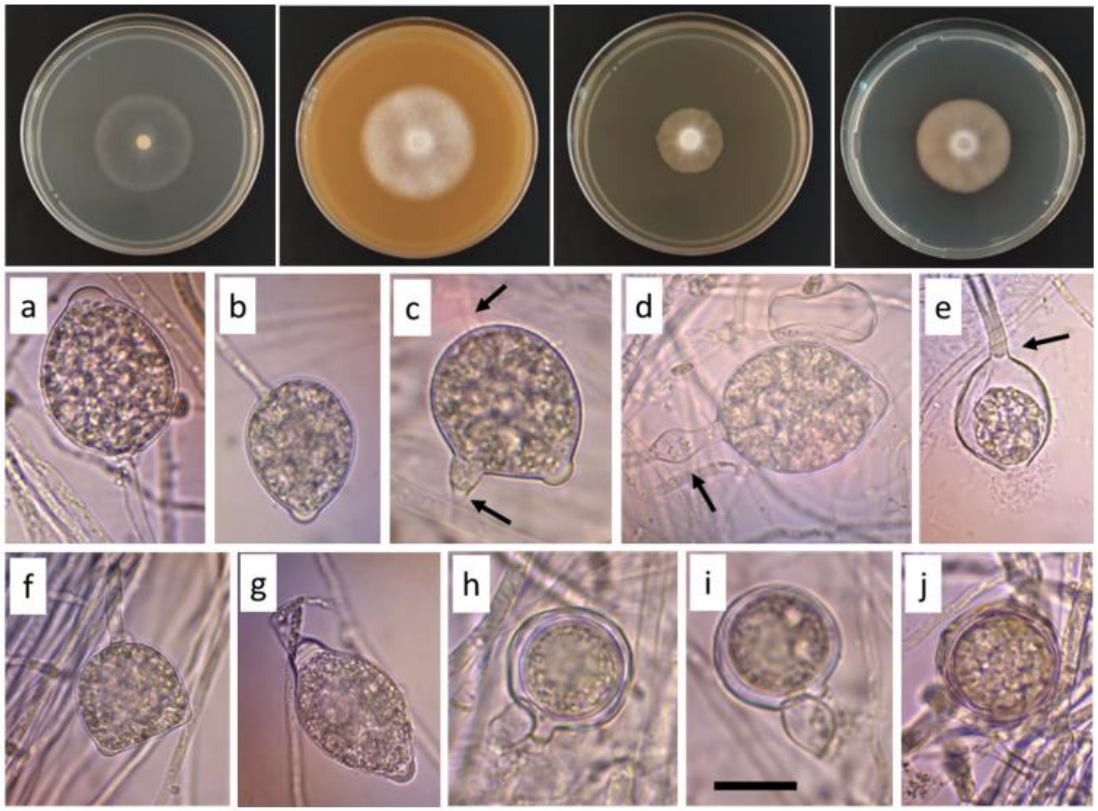

FIGURE 4 Colony morphology and morphological structures of *Phytophthora alticola*. Top: Colony morphology of isolate PHY 5 of *P. alticola* after 7 days growth at 20 °C on carrot agar, V8 agar, malt extract agar and potato dextrose agar (from left to right). (a-g) Papillate sporangia formed on V8 agar squares flooded with sterile soil extract water; (a-e) ovoid to broadly ovoid; (b, c) sporangiophore laterally attached; (c) broadly ovoid with conspicuous basal plug and swelling (down arrow), hyphal extension (top arrow) and vacuole; (d) broadly ovoid sporangia with bulbous ovoid-shaped sporangiophore (arrow); (e) sporangium releasing zoospores with conspicuous basal plug (arrow) and narrow exit pore; (f) bipapilliate, distorted or turbinate-shaped; (g) limoniform, with large vacuole and prominent swelling base, occasional sporangiophore constriction near sporangia base; (h-i) Oogonia with aplerotic oospores and paragynous antheridia, containing ooplasts; (j) mature oogonia with slightly wavy outer wall, golden to dark brown walls and thick-walled oospore. Scale bar 30 µm.

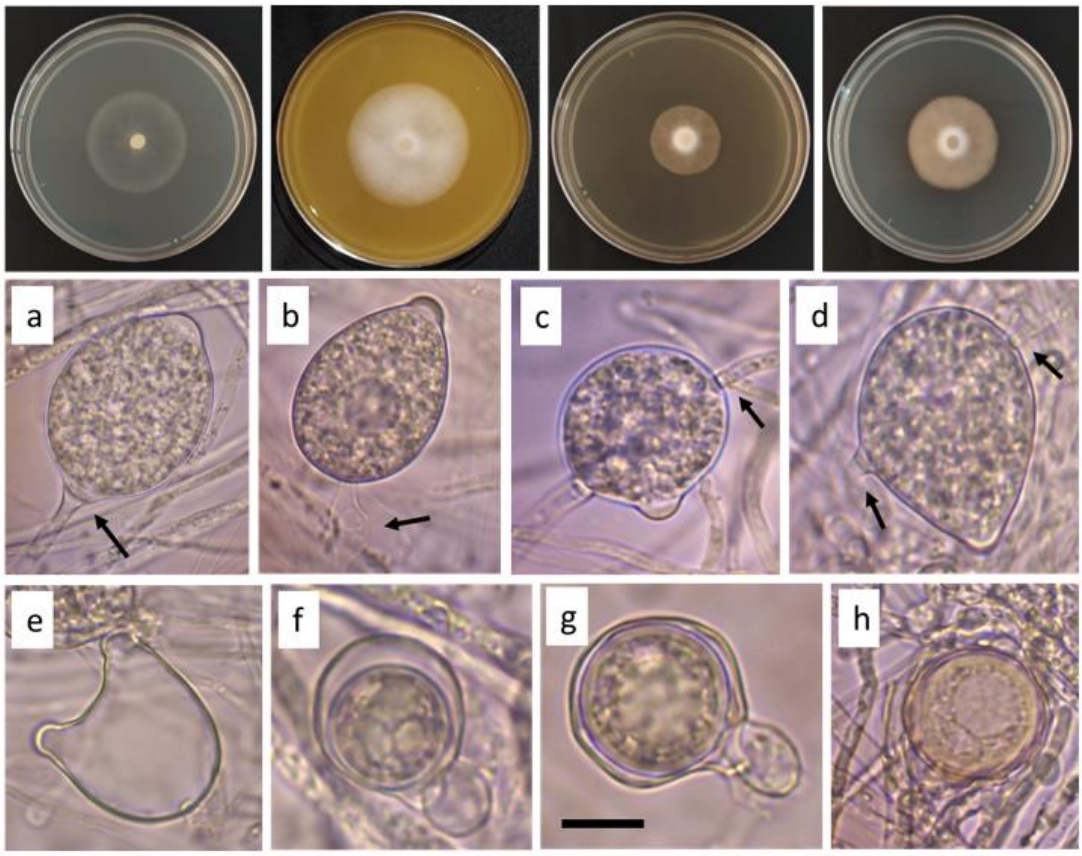

FIGURE 5 Colony morphology and morphological structures of *Phytophthora boodjera*. Top: Colony morphology of isolate PHY 16 of *P. boodjera* after 7 days growth at 20 °C on carrot agar, V8 agar, malt extract agar and potato dextrose agar (from left to right); (a-e) papillate sporangia formed on V8 agar squares flooded with sterile soil extract water; (a-d) ovoid to broadly ovoid; (a) broadly ovoid with conspicuous basal plug (arrow); (b-d) sporangiophore laterally attached; (b) sporangia with large vacuole and bulbous sporangiophore with distorted swelling (arrow); (c, d) broadly ovoid with hyphal extension (c arrow, d top arrow); (d) sporangia with conspicuous basal plug and swelling base (down arrow); (e) empty bipapilliate. (f, g) Oogonia with aplerotic oospores and paragynous antheridia, containing ooplasts; (h) mature oogonia with slightly wavy outer wall, golden to dark brown walls and thick-walled oospore containing ooplasts. Scale bar 20 µm.

The *P. alticola* and *P. boodjera* strains grew at temperatures between 15 and 35 °C on both CA and V8A culture media, and no growth was registered at 5, 10 and 40 °C. The viability assessment of the isolates indicated that the growth resumption occurred only on the plates incubated at 10 °C. The maximum growth rate registered for *P. alticola* was at 25 °C on both CA (9.33 mm/day) and V8A (9.57 mm/day) media, while for *P. boodjera* the maximum growth rate was registered at 30 °C on CA (10.08 mm/day) and on V8A (9.87 mm/day).

The minimum growth rate recorded for *P. alticola* and *P. boodjera* was at 15 °C on CA (0.89 and 0.82 mm/day, respectively) and slightly higher on V8A (1.39 and 1.38 mm/day, respectively) (Table 2).

2.5.4 Morphology of reproductive structures

All the isolates of *P. alticola* and *P. boodjera* species produced asexual and sexual structures in sterile soil extract water. Morphological features of asexual structures including persistent papillate sporangia (Figs. 4a-g and 5a-e), produced on simple sporangiophores, sometimes bulbous with globose, ovoid or distorted swellings (Figs. 4d and 5b) and occasional constriction near the sporangia base (Fig. 4g). Although predominantly ovoid (around 70% for both species), (Figs. 4a-e and 5a-d), a range of sporangial shapes was observed including broadly ovoid (Figs. 4a, c, d and 5a-d), elongated ovoid, peanut-shaped (2-3%), obpyriform (3-6%), pyriform (2%), limoniform (6-10%, Fig. 4g), distorted often turbinate-shaped and bipapilliate (10-15%, Figs. 4f and 5e), tripapilliate rarely observed. Sporangia special features were often observed, including laterally displaced apices (Figs. 4c), swellings close to the sporangial base (Figs. 4g and 5d), very often with a conspicuous basal plug (Figs. 4c, e and 5a, d). Sporangium lateral attachments (Figs. 4b, and 5b, c, d), vacuoles (Figs. 4c, d, f, g and 5b-d) and hyphal extensions (Figs. 4c and 5c-d) were commonly observed, while hyphal coils were rarely observed.

Sporangia of *P. alticola* isolates averaged 41.08 ± 1.43 µm in length and in 30.92 ± 0.79 µm in width, (18.43-68.06 x 19.54-42.3 µm), with narrow exit pores (Fig. 4e) of 6.64 ± 0.27 µm width (4.98-8.94 µm). The single isolate of *P. boodjera* averaged 41.52 ± 6.47 µm in length and 31.25 ± 2.55 µm in width (29.77-53.73 x 25.2-41.86 µm), with narrow exit pores of 6.84 ± 0.83 µm in width (5.32-8.22 µm) (Table 2). No chlamydospores production was observed in either species.

Both *Phytophthora* species proved to be homothallic and produced abundant gametangia. Oogonia of both species contained oospores which matured within 14-21 d. Oogonia diameter of *P. alticola* isolates averaged 28.26 ± 1.60 µm (21.42-32.66 µm) while those produced by *P. boodjera* isolate averaged 27.67 ± 1.84 µm (24.13-30.28 µm) (Table 2). A slightly wavy oogonia outer wall was often observed and turned golden-brown to dark-brown (Figs. 4j and 5h) whit maturity. Oospores for all isolates were aplerotic and very often contained ooplasts when semi-mature to mature (Figs. 4h-j and 5f-h). *P. alticola* oospores averaged 25.95 ± 1.74 µm (19.15-30.54 µm), while *P. boodjera* oospores averaged 25.43 ± 1.80 µm (22.17-27.98 µm). Oospore wall thickness of the species averaged 2.53 ± 0.11 µm and 2.51 ± 0.18 µm, while the oospore wall index averaged 0.50 ± 0.09 and 0.49 ± 0.07 µm respectively (Table 2). Antheridia for both species were paragynous (Figs. 4h, i and 5f, g) averaging 10.42 ± 0.48 x 8.70 ± 0.45 µm (9.55-11.34 x 8.06-9.82 µm) and 10.42 ± 0.06 x 8.43 ± 0.06 µm (9.01-12.02 x 6.88-9.85 µm) (Table 2). Antheridia were attached to the oogonia close to the basal stalk (Figs. 4h, i and 5f, g). Antheridia with finger-like projections were occasionally to rarely observed.

TABLE 2 Comparison of reproductive structures, morphology, and phenotypic characteristics between Uruguayan *Phytophthora alticola* and *P. boodjera* isolates and the original species description (including the species ex-type)

Morphological characters	Species	Species	Species	Species
	***P. alticola* (n= 15)** (present study)	***P. alticola* (n= 7)** (Bose et al. 2017)	***P. boodjera* (n= 1)** (present study)	***P. boodjera* (n= 12)** (Simamora et al. 2015)
Sporangia (µm)				
LxB mean	41.08 ± 1.43 x 30.92 ± 0.79	37.6 ± 3.2 x 28.8 ± 4.5	41.52 ± 6.47 x 31.25 ± 2.55	39.2 ± 4.4 x 29.7 ± 3.4
Range	18.43-68.06 x 19.54-42.3	20.8-45.3 x 18.4-33.7	29.77-53.73 x 25.2-41.86	15.2-64.5 x 13.9-42.5
Range of isolates means	38.49-44.36 x 29.23-31.88	37.9 ± 4.1 x 27.2 ± 4.5	n.a	32.6-44.6 x 24.7-33.3
L/B ratio	1.33 ± 0.03	1.28 ± 0.05	1.33 ± 0.16	1.27 ± 0.16
Range of isolates means	1.27-1.39	1.16-1.33	n.a	1.19-1.35
Sporangial characteristics	Papillate, frequently bipapillate, rarely bilobed	Papillate, frequently bipapillate, rarely bilobed	Papillate, frequently bipapillate, rarely bilobed	Papillate, rarely bipapillate or bilobed
Persistence	Persistent	Persistent	Persistent	Persistent
Sporangiophores	Simple or branched sympodia often with bulbous base, very often laterally attached	Simple or branched sympodia often with bulbous base, very often laterally attached	Simple or branched sympodia often with bulbous base, very often laterally attached	Simple or branched sympodia often with bulbous base, very often laterally attached
Sporangia shape	Ovoid 72.8%, distorted 10.2%, limoniform 6.2%, obpyriform 5.6%, peanut-shaped 3.3%, pyriform 1.8%	Ovoid 87%, obpyriform 9%, distorted 4%	Ovoid 70%, limoniform 10%, distorted 14.7%, obpyriform 3.3%, peanut-shaped 2%	Ovoid 64%, Limoniform 20%, peanut-shaped 10%, distorted 6%

Proliferation	Absent	Absent	Absent	Absent
Exit pores (µm)				
Width	6.64 ± 0.27	6.53 ± 1.27	6.84 ± 0.83	6.09 ± 1.02
Width range	4.98-8.94	6.07-8.7	5.32-8.22	4.85-8.89
Chlamydospores	Absent	Absent	Absent	Absent
Breeding system	Homotallic	Homotallic	Homotallic	Homotallic
Ogonia (µm)				
Mean diameter	28.26 ± 1.60	27.6 ± 1.7	27.67 ± 1.84	29.4 ± 2.3
Diameter range	21.42-32.66	22.4-30.3	24.13-30.28	24.3-33.9
Range of isolates means	24.63-30.21	20.4-32.3	n.a	24.6-33.4
Oospores (µm)				
Mean diameter	25.95 ± 1.74	24.7 ± 1.9	25.43 ± 1.80	25.5 ± 1.9
Diameter range	19.15-30.54	19.1-29.2	22.17-27.98	20.92-29.3
Range of isolates means	21.60-27.83	23.03 ± 2.47	n.a	21.3-29.5
Wall thickness	2.53 ± 0.11	2.48 ± 0.14	2.51 ± 0.18	2.47 ± 0.33
Oospore wall index	0.50 ± 0.09	0.51 ± 0.07	0.49 ± 0.07	0.47 ± 0.05
Oogonial characteristics	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour	Aplerotic oospores, mature oogonia with a slightly wavy surface and golden-brown in colour
Antheridia (µm)				
Position	Paragynous, occasionally to rarely finger-like projections	Paragynous, often with finger-like projections	Paragynous, occasionally to rarely finger-like projections	Paragynous
LxB mean	10.42 ± 0.48 x 8.70 ± 0.45	10.2 ± 1.2 x 8.2 ± 1.7	10.42 ± 0.06 x 8.43 ± 0.06	10.4 ± 1.9 x 8.3 ± 1.5
LxB range	9.55-11.34 x 8.06-9.82	6.2-12.8 x 5.7-10.7	9.01-12.02 x 6.88-9.85	7.9-16.4 x 6.0-10.5
Growth characteristics				

Max temp (°C)	35	30	35	35
Opt temp (°C)	25 (CA), (V8A)	25	30 (CA), (V8A)	25-30
Min temp (°C)	>10<15	>10<15	>10<15	>10<15
Lethal temp (°C)	>35<40	35	>35<40	>37.5
Growth rate at 20°C (mm/day)	6.61 (CA), 5.88 (V8A)	3.75 (CA), 2.33 (V8A)	6.05 (CA), 6.08 (V8A)	6.12 (V8A)
Growth rate at Opt temp (mm/day)	9.33 (CA), 9.01 (V8A)	7.42 (CA), 3.50 (V8A)	10.08 (CA), 9.87 (V8A)	9.18 (V8A)
Colony morphology	Appressed and cottony with no distinctive growth pattern and regular smooth margins on CA, V8A, PDA and MEA	Appressed and cottony with no distinctive growth pattern and regular smooth margins on CA, V8A and PDA; sparse, slow growth on MEA	Appressed and cottony with no distinctive growth pattern and regular smooth margins on CA, V8A, PDA and MEA	Appressed and cottony with no distinctive growth pattern and regular smooth margins on CA, V8A, MEA and PDA

2.5.5 Pathogenicity tests

Inoculated seedlings with *P. alticola* and *P. boodjera* isolates displayed root growth stunt in contrast to control seedlings. However, no typical leaf chlorosis turning reddish to dark brown, nor decline like those observed in the fields was observed, and none of the inoculated seedlings died in the two pathogenicity trials.

In *inoculation method 1*, ten months after inoculation the ANOVA *P* values did not show a significant effect in shoot dry weight ($P = 0.07$) but in contrast, it was significative for root dry weights ($P = 0.014$). All *Phytophthora* isolates significantly decreased root dry weights of inoculated seedlings compared to control plants (Fig. 6). In *inoculation method 2*, ten months after inoculation the ANOVA *P* values showed a significant effect in both shoots dry weight ($P = 0.0001$) and roots dry weights ($P = 0.0001$). In this essay, all *Phytophthora* isolates significantly reduced both shoots and roots dry weights of inoculated seedlings compared to control seedlings (Fig. 6). Moreover, inoculated roots displayed different brown shades, from light to dark brown and black, while control roots were predominantly light to slightly dark brown.

Re-isolations were made from three random plants of each treatment, including control plants. *P. alticola* re-isolation for *inoculation method 1* was 33 % and for *inoculation method 2* was 42 %, while *P. boodjera* re-isolation was 33% and 66 % in inoculation method 1 and 2, respectively. From control plants, no *Phytophthora* was re-isolated.

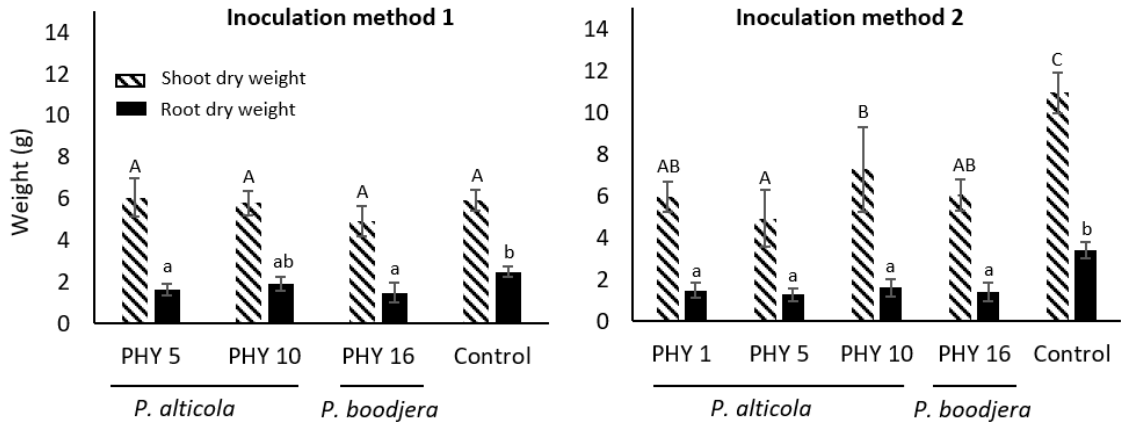

FIGURE 6 Pathogenicity test results of Uruguayan *Phytophthora* spp. inoculated on *Eucalyptus smithii* seedlings ten months after inoculation. Values represent the mean of six replications of each isolate. Different letters indicate significant differences according to Tukey-test ($P \leq 0.05$). Vertical bars indicate Standard deviations.

2.6 DISCUSSION

In the present study we investigated the decline and sudden death of *E. smithii*, an important commercial *Eucalyptus* species cultivated for cellulose pulp production in Uruguay. Plants with symptoms of decline observed on the field did not follow a pattern of distribution but were scattered throughout the field. Moreover, decline symptoms on young trees included chlorosis of leaves that evolved to reddish and dark brown, root and collar rot, gum exudation from collar cankers and death of trees, which corresponds to those symptoms described by Erwin & Ribeiro (1996); Maseko et al., (2001, 2010); Pérez-Sierra et al., (2010) to be attributable to *Phytophthora* spp. Nevertheless, only 32 *Phytophthora* strains were isolated from the 132 symptomatic trees collected (24 % of recovery rate). Additionally, three *E. smithii* nurseries were visited to know the sanitary status of seedlings. Nevertheless, no symptoms like those observed in young *E. smithii* commercial plantations were observed nor was possible to isolate *Phytophthora* spp. from randomly selected seedlings (data not shown).

E. smithii is a tree species known for having high levels of post-establishment mortality within the first year of plantation and for having specific planting requirements (Swain et al., 2000). Furthermore, poor survival of this species is particularly higher when is planted on re-established sites (Jarvel, 1998) as we observed in the present study. In accordance with the observations of Maseko et al. (2010) affected trees appeared mainly during the summer but more pronounced towards the end of the summer, when soil conditions are usually drier.

Previous results indicate that at least four *Phytophthora* species such as *P. cinammomi, P. nicotianae, P. boehmeriae* and *P. frigida* are pathogenic to *E. smithii* (Linde et al., 1994; Wingfield & Kemp 1994; Linde et al., 1999; Linde et al., 2001; Maseko et al., 2010). In this study we confirm the presence of two new *Phytophthora* species namely *P. alticola* and *P. boodjera* associated to root and collar rot of *E. smithii* plants. Based on morphological data and molecular analysis of three nuclear and one mitochondrial gene region, *P. alticola* was found as the predominant species (94%) and the other species identified was *P. boodjera* (6%).

P. alticola was first isolated from cold-tolerant *Eucalyptus* species (*E. dunnii, E. badjensis, E. macarthurii*) in high-altitude plantations in South Africa (Maseko et al., 2007). Until few years ago, this species used to be known for having a confused identity due to the original description of the species was based on a data set including three different taxa (Maseko et al., 2007). This situation was discussed in detail by Simamora et al., (2015) where they concluded that *P. alticola* should be treated as *nomen dubium*. In addition, Bose et al., (2017) isolated *P. alticola* from *E. grandis* and *A. mearnsii* soil samples and designated a neotype. Thus, the original description of this species was emended based on this new collection. On the other hand, *P. boodjera* was described as the causal agent of damping-off and seedling mortality of *Eucalyptus* spp. in Western Australia. This species was isolated mainly from dead and dying *Eucalyptus* spp. seedlings in nurseries and declined trees from urban and natural landscapes in WA (Simamora et al., 2015).

To the date, *P. alticola* has been found in South Africa (Bose et al., 2017) and recently in Portugal causing root rot and dieback in *E. globulus* (Diogo et al., 2022). In this way, Bose et al., (2017) suggested this species could be native to South Africa, while Diogo et al., (2022) considered it as an introduction of a new species in Portugal. Regarding to *P. boodjera*, this species was thought to have a limited distribution to nurseries and urban gardens (Simamora et al., 2018), but it has also been isolated from the remote region of Forrestania in the Great Western Woodlands of WA and from New South Wales, suggesting that *P. boodjera* could be native to Australia (Burgess et al., 2021).

Phytophthora alticola, *P. boodjera* and *P. arenaria* a species isolated from Kwongan vegetation and mainly from *Banksia* species in south-west Australia (Rea et al., 2011), are close relatives species (Simamora et al., 2015; Bose et al., 2017). Due to their high similarities on morphology and phylogenetic proximity, it was proposed that these three species could form a species complex (Simamora et al., 2015; Abad et al., 2022). Moreover, in a recent update of Australian *Phytophthora* species, it was concluded that *P. alticola* and *P. boodjera* could represent lineages within a single species rather than separate species (Burgess et al., 2021). Thus, further studies are needed to clarify the taxonomic status of these three *Phytophthora* related species.

According to the morphological results of this study, morphological features of *P. alticola* and *P. boodjera* overlap in size, but in accordance with previous results *P. boodjera* proved to have higher optimum growth temperature than *P. alticola* (Simamora et al., 2015; Bose et al., 2017). Furthermore, in contrast to Bose et al., (2017) results, Uruguayan *P. alticola* isolates have a higher growth rate at 20, 25 and 30 °C. Also, the Uruguayan isolates grew at 35 °C, which was lethal to South African *P. alticola* isolates. Moreover, colonies growing on CA were less cottony than those produced on the other three media. Probably, this variation could be due to the CA is a home-made growth media.

Pathogenicity tests results proved that both *Phytophthora* species are pathogenic to *E. smithii*. Symptoms of inoculated trees included high root losses, roots with dark brown to black shades and a significant reduction in growth, resulting in a substantially smaller dry weight. Despite of this, none of the inoculated seedlings died nor wilted, probably because they were frequently watered and did not suffer drought stress at any time. These results are congruent with those found by Bose et al. (2019) who concluded that *P. alticola* was less aggressive when inoculated in *E. grandis*. Regarding the low recovery rates of these species in our pathogenicity trials (33 % to 66 %), these results are consistent with those of Bose et al. (2019) where *P. alticola* recovery rate was 30 %.

Finally, considering the low percentage of *Phytophthora* retrieved from field symptomatic plants, the well-known poor establishment of *E. smithii* and the fact that no plant death occurred during the pathogenicity tests, probably the poor survival of *E. smithii* in commercial plantations involved other unknown factors besides *Phytophthora* spp. Further research should be focused on the study of factors such as soil properties specially on re-established sites, land preparation, fertilization management, water requirements, *E. smithii* genotype behaviour and seeds origin. To obtain this knowledge is especially important in order to contribute with the challenge of minimizing the mortality levels observed in the fields, and thus, ensure the viability of the *E. smithii* production in Uruguay. Finally, to our best knowledge, this is the first time that *P. alticola* and *P. boodjera* are recovered from young symptomatic *E. smithii* trees in commercial plantations. Furthermore, it is the first time these species are found in the Americas.

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2.8 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.9 ORCID

Franco De Benedetti https://orcid.org/0000-0003-1982-0618

Victoria Moreira https://orcid.org/0000-0001-5784-5918

Pedro Mondino https://orcid.org/0000-0002-4494-5271

Sandra Alaniz https://orcid.org/0000-0002-6530-7279

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3. *NECTRIACEAE* SPECIES ASSOCIATED TO ROOT ROT OF NURSERY AND YOUNG *EUCALYPTUS SMITHII* TREES IN URUGUAY WITH *ILYONECTRIA CHARRUENSIS* AS NOVEL SPECIES

Artículo que se pretende publicar en la revista *Mycological Progress*.

***Nectriaceae* species associated to root rot of nursery and young *Eucalyptus smithii* trees in Uruguay with *Ilyonectria charruensis* as novel species**

Franco De Benedetti[1], María Julia Carbone[1], Pedro Mondino[1], Sandra Alaniz[1]

[1]Department of Plant Protection, Facultad de Agronomía, Universidad de la República, Av. Garzón 780, 12900, Montevideo, Uruguay.
Correspondence: salaniz@fagro.edu.uy

Especies de *Nectriaceae* asociadas a la pudrición de raíces en plantas de vivero y árboles jóvenes de *Eucalyptus smithii* en Uruguay con *Ilyonectria charruensis* como nueva especie

3.1 RESUMEN

La silvicultura constituye una actividad agronómica importante en Uruguay con el cultivo de árboles exóticos, en su mayoría especies del género *Eucalyptus*. Su principal destino es la producción de pulpa de celulosa. En la última década, *E. smithii* surgió como una especie de interés para producir pulpa de celulosa. Sin embargo, su rápida expansión ha coincidido con altas tasas de mortalidad de árboles jóvenes, que varían del 5 % al 85 %, especialmente durante el primer y segundo verano después de la plantación. A partir de muestreos realizados en nueve campos comerciales de *E. smithii* y tres viveros en el sur y este de Uruguay, se obtuvo una colección de 25 cepas aisladas a partir de podredumbre de raíz de *E. smithii* que pertenecen a la familia *Nectriaceae*. El objetivo de este estudio fue identificar y caracterizar estos aislamientos mediante estudios fenotípicos y moleculares, y evaluar su patogenicidad en plantines de *E. smithii*. Basándose en características morfológicas, los aislamientos de *Nectriaceae* se dividieron en dos grupos, uno con características de *Calonectria* (n = 15) y otro del tipo *Cylindrocarpon* (n = 10). Para los estudios filogenéticos de los aislados de *Calonectria*, se amplificaron y secuenciaron seis regiones génicas (*his3*, *act*, *cmdA*, *rpb2*, *tef1*, *tub2*), mientras que para los aislados del tipo *Cylindrocarpon* se utilizaron cuatro (*his3*, *ITS*, *tef1* y *tub2*). De acuerdo a los análisis filogenéticos y a las características fenotípicas de las cepas obtenidas en este estudio, se identificaron tres especies, *Calonectria pauciramosa* (n = 15), *Dactylonectria novozelandica* (n = 2) y un nuevo taxón que describimos aquí como *Ilyonectria charruensis* sp. nov (n = 8). Los ensayos de patogenicidad revelaron que aislamientos de las tres especies redujeron significativamente tanto el peso seco de las raíces como la parte aérea de los plantínes de *E. smithii* inoculados, en comparación con las plantas de control.

Palabras clave: análisis filogenético multigénico, *Calonectria pauciramosa*, *Cylindrocladium*, *Dactylonectria novozelandica*, taxonomía

3.2 ABSTRACT

Forestry constitutes an important agronomical activity in Uruguay, involving the cultivation of exotic trees mainly for cellulose pulp production with *Eucalyptus* species. Over the last decade, *E. smithii* emerged as a species of interest for cellulose pulping. However, its rapid expansion has coincided with high mortality rates among young trees ranging from 5 to 85%, especially during the first and second summer after plantation. Disease surveys conducted on nine *E. smithii* commercial fields and three nurseries in southern and eastern Uruguay yielded a collection of 25 isolates from *E. smithii* root rot belonging to the *Nectriaceae* family. In this study, we aimed to identify and characterize these isolates employing phenotypical and molecular studies and to assess their pathogenicity on *E. smithii* seedlings. Based on morphological features, the *Nectriaceae* isolates were subdivided into two groups, one resembling *Calonectria* (n = 15) and another *Cylindrocarpon*-like (n = 10). For phylogenetic studies, six gene regions (*his3, act, cmdA, rpb2, tef1, tub2*) and four gene regions (*his3, ITS, tef1* and *tub2*) were amplified for *Calonectria* and *Cylindrocarpon*-like groups, respectively. Based on phylogenetic analysis and phenotypical features three species were identified, *Calonectria pauciramosa* (n = 15), *Dactylonectria novozelandica* (n = 2) and a novel taxon which we describe here as *Ilyonectria charruensis* sp. nov (n = 8). The pathogenicity trials revealed that isolates from the three species significantly reduced both root and shoots dry weights of inoculated *E. smithii* seedlings compared to control plants.

Keywords: *Calonectria pauciramosa*, *Cylindrocladium*, *Dactylonectria novozelandica*, multigene phylogeny, taxonomy

3.3 INTRODUCTION

Commercial forestry in Uruguay covers 1.065 million hectares and relies on the growing of exotic trees primarily to produce raw wood destined to cellulose pulp production, with *Eucalyptus* as the most widely planted genus (MGAP-DIEA 2022). *Eucalyptus smithii*, commonly known as 'gully gum', is native to Victoria and New South Whales, Australia. This species is cultivated in different subtropical and template world regions for pulpwood or essential oil production, such as China (Arnold et al. 2004) or Africa (Jacovelli 2003, Gardner et al. 2016). In Uruguay, *E. smithii* was introduced in the earliest 1900s showing good adaptation and moderate cold tolerance (Brussa 1994).

In 2007, *E. globulus*, the most planted *Eucalyptus* species destined to pulpwood production in Uruguay, was severely affected by *Mycosphaerella* leaf disease caused by *Teratosphaeria nubilosa* (Pérez et al. 2009). Thereafter, *E. smithii* gained prominence as an alternative species for commercial cultivation due to its desirable wood properties in the papermaking industry, including high pulp yields, high fibre yields and fibre length, high wood density and alpha cellulose contents, low lignin and extracting contents as well as other characteristics (Clarke et al. 1997; Carrillo et al. 2018; Carrillo-Varela et al. 2019). Additionally, *E. smithii* proved to have substantially less susceptibility to leaf spotting caused by *Mycosphaerella* sp. and *Teratosphaeria* sp. (Carnegie et al. 1998; Swain et al. 2000).

Since *E. smithii* plantations expanded massively in Uruguay, a range of 5 to 85% of tree mortality has been reported during the two first years after plantation, mainly during summer periods (Rachid et al. 2021). Symptoms observed in the fields include leaf chlorosis and root rot and collar rot, both of which lead to decline and death of trees.

From 2019 to 2021, disease surveys were conducted on *E. smithii* commercial plantations in Uruguay to investigate the mortality of young *E. smithii* trees. Subsequently, two *Phytophthora* species, namely *P. alticola* and *P. boodjera*, were

isolated from root rot and collar rot of symptomatic *E. smithii* trees (De Benedetti et al. 2023). Furthermore, unknown species with *Nectriaceous* characteristics, displaying typical structures of the *Calonectria* genus and *Cylindrocarpon*-like anamorphs, were also isolated from root rot of declining young *E. smithii* trees. This finding suggests that, in addition to *Phytophthora* species, *Calonectria* and *Cylindrocarpon*-like genera could also play a role in the mortality of young *E. smithii* trees.

The *Nectriaceae* family involves several plant pathogens of a wide range of crops, including forestry, horticulture, and ornamental crops (Lombard et al. 2015). In forestry, it is widely known that *Calonectria* species are the causal agents of important diseases of *Eucalyptus* spp. in commercial plantations and nurseries. The reported symptomatology includes seedling rot and damping-off (Crous et al. 1991; Crous 2002; Lombard et al. 2010a, 2011; Aiello et al. 2020) cutting rot, stem lesions, root and crown rot (Crous 2002; Lombard et al. 2010d, 2011) and leaf and shoot blight (Crous 2002; Crous et al. 2004b; Chen et al. 2011; Lombard et al. 2010a, 2015b; Alfenas et al. 2015). However, associations between *Cylindrocarpon*-like anamorphs and *Eucalyptus* spp. trees are scarce. For example, Iles et al. (2010) reported *Cylindrocarpon destructans* (=*Ilyonectria radicicola*) affecting *Eucalyptus regnans* natural regeneration in Australia. *Cylindrocarpon*-like anamorphs include five phylogenetically distinct lineages, namely *Dactylonectria*, *Ilyonectria, Thelonectria, Rugonectria* and *Neonectria*, divided based on comprehensive DNA sequences analyses and morphological features (Chaverri et al. 2011; Lombard et al. 2014).

In order to understand the contribution of the *Nectriaceae* family members in the decline of young *E. smithii* trees in Uruguay, this study aims to: I) identify and characterize the *Nectriaceae*-like isolates obtained from root rot of young *E. smithii* and nursery plants based on morphological features and phylogenetic analyses, and II) assess their pathogenicity on *E. smithii* by inoculating nursery-produced seedlings.

3.4 MATERIALS AND METHODS

3.4.1 Field symptoms and sampling

Between 2019 and 2021, disease surveys were conducted in nine commercial plantations of *E. smithii* up to two years of age and three nurseries located in the southern and southeastern regions of Uruguay (Fig. 1). The commercial plantations comprised both natural soil as well as sites where *Eucalyptus* trees had been previously planted (re-established sites). The plantations were surveyed twice, once in early summer and again in late summer (Fig. 1).

In each plantation, 10 to 15 trees exhibiting symptoms of decline were randomly selected and excavated. The root system of each diseased tree was separated from the aerial part and placed in individual sturdy plastic bags for transportation. Three commercial nurseries were surveyed at the beginning of spring, just prior to the shipment of seedlings to the fields. In each nursery, 20 seedlings with noticeable poor growth were randomly collected and enclosed in individual plastic bags. All the bags were sealed to prevent desiccation and were processed separately in the laboratory within 24 h of collection.

3.4.2 Fungal isolations

For fungal isolations, roots were gently rinsed with abundant water before bark removal using a scalpel. Small tissue pieces of approximately 2 × 5 mm in size, were excised from the margin of necrotic root lesions, disinfected with a 2% NaClO solution for 1 minute and rinsed with distilled sterile water. Excess water was removed from the tissue pieces by blotting with sterile paper towels. These samples were placed into 9 cm Petri plates containing potato dextrose agar (PDA) (Oxoid Ltd., Hampshire, England) amended with 0.40 g/L of streptomycin sulphate (Sigma-Aldrich

Laboratories, St. Louis, MO, USA). Plates were incubated at 25 °C and monitored daily up to 2 weeks.

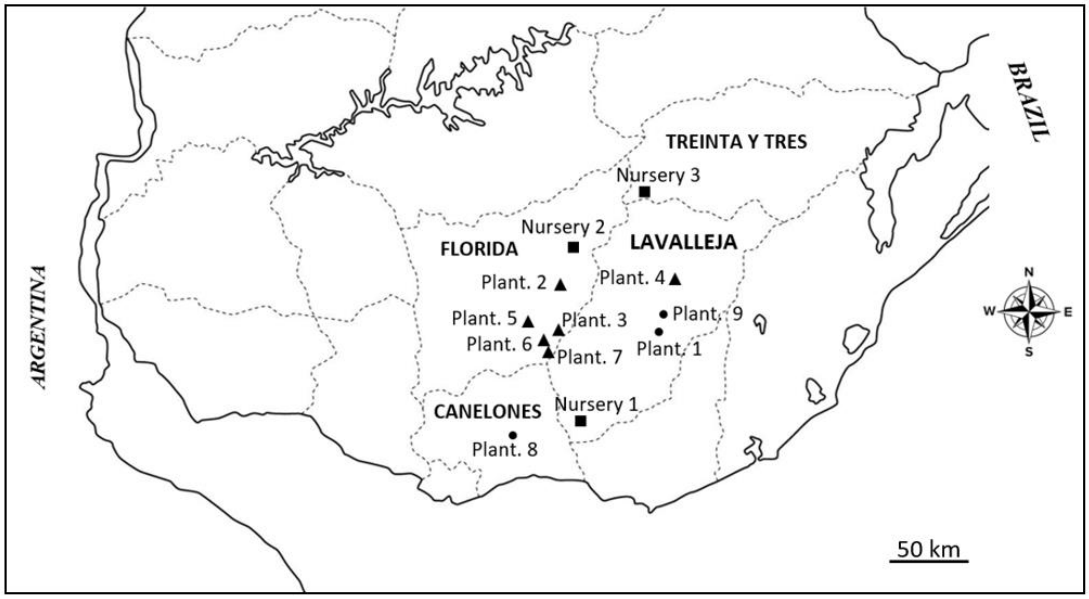

Fig. 1 Geographic location of the nine commercial plantations (Plant.) and three nurseries of *Eucalyptus smithii* sampled in the south and south-east of Uruguay. Solid points indicate plantations over natural soil and triangles indicate plantations over re-established sites (sites where *Eucalyptus* trees had been previously planted). *Notes:* plantation dates; Plant. 1 to 5, spring 2019; Plant. 6, autumn 2020; Plant. 7 to 9, spring 2020.

Developed fungal colonies exhibiting morphological features characteristic of the *Nectriaceae* family, such as distinct shades of brown, ochreous or dark amber colours (Crous 2002; Chaverri et al. 2011; Lombard et al. 2010a; Cabral et al. 2012a, b; Lombard et al. 2014, 2015a; Mora-Sala et al. 2018) were transferred to Petri dishes containing 2% (w/v) malt extract agar (MEA) (20 g malt extract, Biolab, Midland, South Africa; 20 g Difco agar, Becton Dickinson, Maryland, USA) and incubated at 25 °C under a near-UV light with a 12-hour photoperiod to encourage the development of reproductive structures. To establish pure cultures, the isolates were single-spored and preserved on sterile filter papers at -20 °C. Monosporic isolates were deposited in

the fungal culture collection at the Plant Protection Department, Faculty of Agronomy, University of the Republic, Uruguay.

3.4.3 DNA isolation, PCR amplification and sequencing

Total DNA was extracted from the unknown *Nectriaceae*-like isolates grown on MEA for 10 days at 25 °C in the dark, using the commercial kit ZR Fungal/Bacterial Quick-DNA™ Miniprep Kit (Zymo Research, USA) following the manufacturer's instructions. For preliminary identification, the partial sequence of the histone H3 (*his3*) gene region was amplified and sequenced on the twenty-five *Nectriaceae* isolates of this study. Additionally, five gene regions including actin (*act*), calmodulin (*cmdA*), RNA polymerase II second largest subunit (*rpb2*), translation elongation factor 1-alpha (*tef1*) and β-tubulin (*tub2*) were amplified on the 15 isolates having *Calonectria* features (Liu et al. 2020). Regarding the 10 *Cylindrocarpon*-like isolates, three additional partial gene regions including *tef1*, *tub2* and internal-transcribed spacer and intervening 5.8S gene (ITS) region were sequenced (Cabral et al. 2012a; Mora-Sala et al. 2018).

The primer pairs used for amplifying the partial *his3* gene region for all *Nectriaceae*-like isolates were CYLH3F and CYLH3R (Crous et al. 2004b). Additional primer pairs ACT-512F and ACT-783R (Carbone and Kohn 1999) for *act*; CAL-228F (Carbone and Kohn 1999) and CAL-2Rd (Quaedvlieg et al. 2011) for *cmdA;* fRpb2-5F and fRpb2-7cR (Liu et al. 1999) for *rpb2;* EF1-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998) for *tef1*; and T1 (O'Donnell and Cigelnik 1997) and CYLTUB1R (Crous et al. 2004b) for *tub2* gene regions, respectively, were used to amplify the *Calonectria* isolates, and ITS1 and ITS4 (White et al. 1990) for ITS regions, T1 and Bt2b (Glass and Donaldson 1995) for *tub2*; CylEF-1 (J. Z. Groenwald, unpublished) and CylEF-R2 (Crous et al. 2004b) for *tef1* were used to amplify the *Cylindrocarpon*-like isolates.

Individual PCR reactions for each gene region were performed using a standardized mixture and under uniform thermocycling conditions. The PCR mix included 1x PCR buffer, 2.5 mM MgCl2, 0.4 mM of each dNTP, 0.4 µM of each primer, 1 U of DNA polymerase (Bioron, Germany) and 1 µL of template DNA, in a 20 µL final volume with PCR water. The thermocycling protocol started with an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 seconds, specific annealing temperatures for each gene region for 30 seconds, extension at 72 °C for 1 minute and a final extension at 72 °C for 10 min. The annealing temperatures used to amplify the *his3*/*act*/*cmdA*/*tef1*/*tub2* gene regions in *Calonectria* isolates were 55/60/55/52/57 °C, respectively, whereas the annealing temperatures utilized to amplify *his3*/*tef1*/*tub2*/ITS on *Cylindrocarpon*-like isolates were 56/58/58/59 °C, respectively. To amplify the *rpb2* region on *Calonectria* isolates, the following modified program was used, an initial denaturation step at 94 °C for 4 min, followed by 10 cycles of 94 °C for 30 sec, annealing temperature of 58 °C for 48 sec, 72 °C for 45 sec, and 25 cycles of 94 °C for 30 sec, annealing temperature of 56 °C for 55 sec, 72 °C for 1 min and a final extension of 72 °C for 10 min.

Amplifications were conducted using a PCRmax Alpha AC196 (Bibby Scientific Limited, UK) thermocycler. All PCR products were visualized on a 1.5% agarose gel stained with GelRed™, observed under UV-light in a transilluminator, with GeneRuler 100-bp DNA ladder plus as a molecular weight marker (Thermofisher, Lithuania). Subsequently, the PCR products were purified and sequenced by Macrogen Inc., Seoul, South Korea.

3.4.4 Phylogenetic analyses

Sequences obtained from the 25 *Nectriaceae*-like isolates of the present study were compared with those deposited in GenBank using the BLAST search tool (blast.ncbi.nlm.nih.gov/Blast.cgi). Raw sequences of *Calonectria*, *Ilyonectria* and *Dactylonectria* were individually aligned per genus using Clustal W program within MEGA 11.0.11 and manually edited when necessary (Tamura et al. 2021). In addition,

sequences of closest phylogenetically related species, including ex-type isolates, were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and incorporated into the corresponding alignment for each genus (Table 1).

To determine phylogenetic relationships among the species, analyses based on Bayesian inference (BI) and maximum likelihood (ML) methods were conducted on each gene region and the multi-locus alignment. BI and ML analyses were performed with MrBayes v.3.2.7 (Ronquist et al. 2012) and RAxML v.8.2.12 (Stamatakis 2014), respectively, both implemented on the CIPRES Science Gateway v.3.3 (http://www.phylo.org/). Multi-locus alignments were conducted using Sequence Matrix v.1.8 (http://www.ggvaidya.com/taxondna/).

The best evolutionary model of nucleotide substitution for each gene region was determined using jModelTest2 (Darriba et al. 2012) on the XSEDE tool in the CIPRES portal, selected according to the Akaike Information Criterion (AIC). The following BI models were applied: K80 (*act*), GTR + G (*his3*, *rpb2*, *tub2*), GTR + I + G (*cmdA*, *tef1*) for *Calonectria* isolates; GTR + G (*tef1*, ITS, *tub2*), GTR + I + G (*his3*) for *Dactylonectria* isolates and GTR + G (*tef1*, *his3*), HKY + G (*tub2*), GTR + I + G (ITS) for *Ilyonectria* sp. isolates.

Table 1. Identity, host association, country and GenBank accession numbers of DNA sequences of *Nectriaceae* species used in this study.

Species	Isolate identity	Host association	Location/Country	GenBank Accession No.						
				ITS	*his3*	*tub2*	*tef1*	*cmdA*	*act*	*rpb2*
Calonectria brasiliana	CBS 111484 T	Soil	Brazil	--	MT335438	MT412951	MT412729	MT335198	MT334968	MT412502
	CBS 111485	Soil	Brazil	--	MT335439	MT412952	MT412730	MT335199	MT334969	MT412503
Ca. brassiana	CBS 134855 T	Soil of *Eucalyptus brassiana* plantation	Piauí, Brazil	--	KM396139	KM395969	KM395882	KM396056	--	--
	CBS 134856	Soil of *E. brassiana* plantation	Piauí, Brazil	--	KM396140	KM395970	KM395883	KM396057	--	--
Ca. brevistipitata	CBS 115671 T	Soil	Mexico	--	MT335443	MT412956	MT412734	MT335203	MT334973	MT412507
	CBS 110928	Soil	Mexico	--	MT335444	MT412957	MT412735	MT335204	MT334974	MT412508
Ca. candelabra	CMW 31000 T	*Eucalyptus* sp.	Amazonas, Brazil	--	MT335447	MT412959	MT412738	MT335207	MT334977	MT412511
	CMW 31001	*Eucalyptus* sp.	Amazonas, Brazil	--	MT335448	MT412960	MT412739	MT335208	MT334978	MT412512
Ca. colombiana	CBS 115127 T	Soil	La Selva, Colombia	--	FJ972442	FJ972423	FJ972492	GQ267455	GQ280538	--
	CBS 115638	Soil	La Selva, Colombia	--	FJ972441	FJ972422	FJ972491	GQ267456	GQ280539	--
Ca. eucalypticola	CBS 134847 T	*Eucalyptus* sp. seedling	Minas Gerais, Brazil	--	KM396134	KM395964	KM395877	KM396051	--	--
	CBS 134846	*Eucalyptus* sp. leaf	Bahía, Brazil	--	KM396133	KM395963	KM395876	KM396050	--	--
Ca. exiguispora	CMW 49752 T	Soil of *Eucalyptus* sp. plantation	Quinchía, Colombia	--	OP822382	OP822596	OP822168	OP822275	OP796405	OP822489
	CMW 49753	Soil of *Eucalyptus* sp. plantation	Quinchía, Colombia	--	OP822383	OP822597	OP822169	OP822276	OP796406	OP822490
Ca. fragariae	CBS 133607 T	*Fragaria* × *ananassa*	Espírito Santo, Brazil	--	KM998964	KM998965	KM998963	KM998966	--	--
	LPF141.1	*Fragaria* × *ananassa*	Espírito Santo, Brazil	--	KX500194	KX500195	KX500197	KX500191	--	--
Ca. glaebicola	CBS 134852 T	Soil of *Eucalyptus* sp. plantation	Minas Gerais, Brazil	--	KM396136	KM395966	KM395879	KM396053	--	--
	CBS 134853	*Eucalyptus* sp. leaf	Bico do Papagaio, Brazil	--	KM396137	KM395967	KM395880	KM396054	--	--
Ca. gracilipes	CBS 115674 T	Soil	La Selva, Colombia	--	MT335492	MT413001	MT412783	MT335252	MT335022	MT412554
	CBS 111141	Soil	La Selva, Colombia	--	MT335493	MT413002	MT412784	MT335253	MT335023	MT412555
Ca. hemileiae	COAD 2544 T	*Hemileia vastatrix*	Brazil	--	MK006026	MK037391	MK006027	MK037392	--	--
Ca. imperata	CCDCA 11649 T	*E. urophylla*	Brazil	--	OM974339	OM974366	OM974357	OM974330	ON009351	OM974348
	PFC7	*E. urophylla*	Brazil	--	OM974340	OM974367	OM974358	OM974331	ON009352	OM974349
Ca. matogrossensis	GFP006 T	*E. urophylla*	Brazil	--	MH837648	MH837664	MH837659	MH837653	--	--

	GFP018	*E. urophylla*	Brazil	--	MH837652	MH837668	MH837663	MH837657	--	--
Ca. metrosideri	CBS 133603 T	*Metrosideros polymorpha*	Minas Gerais, Brazil	--	KC294307	KC294313	KC294310	KC294304	--	--
	CBS 133604	*M. polymorpha*	Minas Gerais, Brazil	--	MT335528	MT413033	MT412819	MT335288	MT335056	MT412585
Ca. nemoricola	CBS 134837 T	Soil of tropical rainforest	Minas Gerais, Brazil	--	KM396149	KM395979	KM395892	KM396066	--	--
	CBS 134838	Soil of tropical rainforest	Minas Gerais, Brazil	--	KM396150	KM395980	KM395893	KM396067	--	--
Ca. pauciramosa	CBS 138824 T	Soil	Knysna, South Africa	--	MT335565	MT413068	MT412856	MT335325	MT335093	MT412618
	CMW 31474	*E. urophylla* × *E. grandis*	China	--	MT335576	MT413079	MT412867	MT335336	MT335104	MT412629
	CMW 7592	*E. grandis*	Uruguay	--	FJ972447	FJ972380	FJ972497	--	--	--
	CMW 7597	*E. grandis*	Uruguay	--	FJ972474	FJ972406	FJ972523	--	--	--
	CMW 1786	*E. smithii*	South Africa	--	FJ972445	FJ972378	FJ972495	--	--	--
	ES 1	*E. smithii*/ nursery	Florida, Uruguay	--	OR258718	OR258763	OR258748	OR258703	OR258688	OR258733
	ES 2	*E. smithii*/ nursery	Florida, Uruguay	--	OR258719	OR258764	OR258749	OR258704	OR258689	OR258734
	ES 3	*E. smithii*/ nursery	Florida, Uruguay	--	OR258720	OR258765	OR258750	OR258705	OR258690	OR258735
	ES 4	*E. smithii*/ nursery	Florida, Uruguay	--	OR258721	OR258766	OR258751	OR258706	OR258691	OR258736
	ES 5	*E. smithii*/ nursery	Lavalleja, Uruguay	--	OR258722	OR258767	OR258752	OR258707	OR258692	OR258737
	ES 6	*E. smithii*/ nursery	Lavalleja, Uruguay	--	OR258723	OR258768	OR258753	OR258708	OR258693	OR258738
	ES 7	*E. smithii*/ plantation	Florida, Uruguay	--	OR258724	OR258769	OR258754	OR258709	OR258694	OR258739
	ES 8	*E. smithii*/ plantation	Florida, Uruguay	--	OR258725	OR258770	OR258755	OR258710	OR258695	OR258740
	ES 9	*E. smithii*/ plantation	Florida, Uruguay	--	OR258726	OR258771	OR258756	OR258711	OR258696	OR258741
	ES 10	*E. smithii*/ plantation	Florida, Uruguay	--	OR258727	OR258772	OR258757	OR258712	OR258697	OR258742
	ES 11	*E. smithii*/ plantation	Florida, Uruguay	--	OR258728	OR258773	OR258758	OR258713	OR258698	OR258743
	ES 12	*E. smithii*/ plantation	Florida, Uruguay	--	OR258729	OR258774	OR258759	OR258714	OR258699	OR258744
	ES 13	*E. smithii*/ plantation	Florida, Uruguay	--	OR258730	OR258775	OR258760	OR258715	OR258700	OR258745
	ES 14	*E. smithii*/ plantation	Florida, Uruguay	--	OR258731	OR258776	OR258761	OR258716	OR258701	OR258746
	ES 15	*E. smithii*/ plantation	Florida, Uruguay	--	OR258732	OR258777	OR258762	OR258717	OR258702	OR258747
Ca. piauiensis	CBS 134850 T	Soil of *Eucalyptus* sp. plantation	Piauí, Brazil	--	KM396143	KM395973	KM395886	KM396060	--	--
	CBS 134851	Soil of tropical rainforest	Piauí, Brazil	--	KM396144	KM395974	KM395887	KM396061	--	--
Ca. pseudometrosideri	CBS 134845 T	Soil of *Eucalyptus* sp. plantation	Alagoas, Brazil	--	KM396083	KM395909	KM395821	KM395995	--	--

	CBS 134843	*M. polymorpha*	Minas Gerais, Brazil	--	KM396081	KM395907	KM395819	KM395993	--	--
Ca. pseudospathulata	CBS 134841 T	Soil of tropical rainforest	Minas Gerais, Brazil	--	KM396153	KM395983	KM395896	KM396070	--	--
	CBS 134840	Soil of tropical rainforest	Minas Gerais, Brazil	--	KM396152	KM395982	KM395895	KM396069	--	--
Ca. putriramosa	CBS 111449 T	*Eucalyptus* sp. cutting	Brazil	--	MT335604	MT413105	MT412895	MT335364	MT335129	MT412657
	CBS 111470	Soil	Brazil	--	MT335605	MT413106	MT412896	MT335365	MT335130	MT412658
Ca. silvicola	CBS 135237 T	Soil of tropical rainforest	Bahía, Brazil	--	KM396148	KM395978	KM395891	KM396065	--	--
	CBS 134836	Soil of tropical rainforest	Minas Gerais, Brazil	--	KM396145	KM395975	KM395888	KM396062	--	--
Ca. spathulata	CMW 16744 T	*E. viminalis*	Brazil	--	MT335616	MT413117	MT412907	MT335376	MT335139	MT412668
	CBS 112513	*Eucalyptus* sp.	Colombia	--	MT335617	MT413118	MT412908	MT335377	MT335140	MT412669
Ca. venezuelana	CBS 111052 T	Soil	Acarigua, Venezuela	--	MT335634	MT413132	MT412925	MT335394	MT335155	MT412685
Campylocarpon fasciculare	CBS 112613 T	*Vitis vinifera*	South Africa	AY677301	JF735502	AY677221	JF735691	--	--	--
C. pseudofasciculare	CBS112679 T	*V. vinifera*	South Africa	AY677306	JF735503	AY677214	JF735692	--	--	--
Dactylonectria alcacerensis	CBS 129087 T	*V. vinifera*	Portugal	JF735333	JF735630	AM419111	JF735819	--	--	--
	Cy134	*V. vinifera*	Spain	JF735332	JF735629	AM419104	JF735818	--	--	--
D. amazonica	MUCL55430 T	*Piper* sp.	Ecuador	MF683706	MF683685	MF683643	MF683664	--	--	--
	MUCL55433	*Piper* sp.	Ecuador	MF683707	MF683686	MF683644	MF683665	--	--	--
D. anthuriicola	CBS 564.95 T	*Anthurium* sp.	The Netherlands	JF735302	JF735579	JF735430	JF735768	--	--	--
D. ecuadoriensis	MUCL55424 T	*Piper* sp.	Ecuador	MF683704	MF683683	MF683641	MF683662	--	--	--
	MUCL55425	*Piper* sp.	Ecuador	MF683705	MF683684	MF683642	MF683663	--	--	--
D. estremocensis	CBS 129085 T	*V. vinifera*	Portugal	JF735320	JF735617	JF735448	JF735806	--	--	--
	CPC 13539	*Picea glauca*	Canada	JF735330	JF735627	JF735458	JF735816	--	--	--
D. hispanica	CBS 142827 T	*Pinus halepensis*	Spain	KY676882	KY676864	KY676876	KY676870	--	--	--
	Cy228	*Ficus* sp.	Portugal	JF735301	JF735578	JF735429	JF735767	--	--	--
D. hordeicola	CBS 162.89 T	*Hordeum vulgare*	The Netherlands	AM419060	JF735610	AM419084	JF735799	--	--	--
D. macrodidyma	CBS 112615 T	*V. vinifera*	South Africa	AY677290	JF735647	AY677233	JF735836	--	--	--
	CBS 112601	*V. vinifera*	South Africa	AY677284	JF735644	AY677229	JF735833	--	--	--
D. novozelandica	CBS 113552 T	*Vitis sp.*	New Zeland	JF735334	JF735633	AY677237	JF735822	--	--	--
	CBS 112608	*V. vinifera*	South Africa	AY677288	JF735632	AY677235	JF735821	--	--	--

	BV-0760	*Vitis berlandieri* x *Vitis rupestris*	Navarra, Spain	MK602785	MK579247	MK602800	MK602815	--	--	--
	ES 17	*E. smithii*/ nursery	Treinta y Tres, Uruguay	OR237200	OR258686	OR258684	OR258682	--	--	--
	ES 18	*E. smithii*/ nursery	Treinta y Tres, Uruguay	OR237201	OR258687	OR258685	OR258683	--	--	--
D. palmicola	MUCL55426 T	*Euterpe precatoria*	Ecuador	MF683708	MF683687	MF683645	MF683666	--	--	--
D. pauciseptata	CBS 120171 T	*Vitis sp.*	Slovenia	EF607089	JF735587	EF607066	JF735776	--	--	--
	BV-1354	*V. berlandieri* x *V. rupestris*	Galicia, Spain	MK602783	MK579256	MK602798	MK602813	--	--	--
D. pinicola	CBS 173.37 T	*Pinus laricio*	U.K.	JF735319	JF735614	JF735447	JF735803	--	--	--
	CBS 159.34	n.a	Germany	JF735318	JF735613	JF735446	JF735802	--	--	--
D. polyphaga	MUCL55209 T	*Costus* sp.	Ecuador	MF683689	MF683668	MF683626	MF683647	--	--	--
	MUCL55208	*Costus* sp.	Ecuador	MF683699	MF683678	MF683636	MF683657	--	--	--
D. riojana	BV-1396 T	*V. berlandieri* x *V. rupestris*	Navarra, Spain	MK602796	MK602831	MK602811	MK602826	--	--	--
	BV-1397	*V. berlandieri* x *V. rupestris*	Navarra, Spain	MK602797	MK602832	MK602812	MK602827	--	--	--
D. torresensis	CBS 129086 T	*V. vinifera*	Portugal	JF735362	JF735681	JF735492	JF735870	--	--	--
	CBS 119.41	*Fragaria* sp.	The Netherlands	JF735349	JF735657	JF735478	JF735846	--	--	--
D. valentina	CBS 142826 T	*Ilex aquifolium*	Spain	KY676881	KY676863	KY676875	KY676869	--	--	--
D. vitis	CBS 129082 T	*V. vinifera*	Portugal	JF735303	JF735580	JF735431	JF735769	--	--	--
Ilyonectria capensis	CBS 132815 T	*Protea* sp.	South Africa	JX231151	JX231135	JX231103	JX231119	--	--	--
	CBS 132816	*Protea* sp.	South Africa	JX231160	JX231144	JX231112	JX231128	--	--	--
I. changbaiensis	4404 T	*Panax ginseng*	China	MF350464	MF350437	MF350410	MF350491	--	--	--
	72R2	*Pa. ginseng*	China	MF350465	MF350438	MF350411	MF350492	--	--	--
***I. charruensis* sp. nov.**	**ES 19**	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242484	OR258778	OR258794	OR258786	--	--	--
	ES 20	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242485	OR258779	OR258795	OR258787	--	--	--
	ES 21	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242486	OR258780	OR258796	OR258788	--	--	--
	ES 22	*E. smithii*/ plantation	Florida, Uruguay	OR242487	OR258781	OR258797	OR258789	--	--	--
	ES 23	*E. smithii*/ plantation	Florida, Uruguay	OR242488	OR258782	OR258798	OR258790	--	--	--
	ES 25	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242489	OR258783	OR258799	OR258791	--	--	--
	ES 26	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242490	OR258784	OR258800	OR258792	--	--	--
	ES 27	*E. smithii*/ plantation	Lavalleja, Uruguay	OR242491	OR258785	OR258801	OR258793	--	--	--

I. communis	1512 T	*Pa. ginseng*	China	MF350456	MF350429	MF350402	MF350483	--	--	--
	J410	*Pa. ginseng*	China	MF350457	MF350430	MF350403	MF350484	--	--	--
I. coprosmae	CBS 119606 T	*Metrosideros* sp.	Canada	JF735260	JF735505	JF735373	JF735694	--	--	--
I. crassa	CBS 158.31 T	*Narcissus* sp.	The Netherlands	JF735276	JF735535	JF735394	JF735724	--	--	--
	CBS 129083	*Panax quinquefolium*	Canada	AY295311	JF735536	JF735395	JF735725	--	--	--
I. cyclaminicola	CBS 302.93 T	*Cyclamen* sp.	The Netherlands	JF735304	JF735581	JF735432	JF735770	--	--	--
I. radicicola	CBS 264.65 T	*Cyclamen persicum*	Sweden	AY677273	JF735506	AY677256	JF735695	--	--	--
I. europaea	CBS 129078 T	*V. vinifera*	Portugal	JF735294	JF735567	JF735421	JF735756	--	--	--
	CBS 102892	*Phragmites australis*	Germany	JF735295	JF735569	JF735422	JF735758	--	--	--
I. gamsii	CBS 940.97 T	Soil	The Netherlands	AM419065	JF735577	AM419089	JF735766	--	--	--
I. ilicicola	CBS 142828 T	*Ilex* sp.	Spain	KY676884	KY676866	KY676878	KY676872	--	--	--
	Cy-FO-226	*Ilex* sp.	Spain	KY676885	KY676867	KY676879	KY676873	--	--	--
I. leucospermi	CBS 132809 T	*Leucospermum* sp.	South Africa	JX231161	JX231145	JX231113	JX231129	--	--	--
	CBS 132810	*Protea* sp.	South Africa	JX231162	JX231146	JX231114	JX231130	--	--	--
I. liliigena	CBS 189.49 T	*Lilium regale*	The Netherlands	JF735297	JF735573	JF735425	JF735762	--	--	--
	CBS 732.74	*Lilium* sp.	The Netherlands	JF735298	JF735574	JF735426	JF735763	--	--	--
I. liriodendri	CBS 110.81 T	*Liriodendron tulipifera*	USA	DQ178163	JF735507	DQ178170	JF735696	--	--	--
	CBS 117527	*V. vinifera*	Portugal	DQ178165	JF735509	DQ178172	JF735698	--	--	--
I. lusitanica	CBS 129080 T	*V. vinifera*	Portugal	JF735296	JF735570	JF735423	JF735759	--	--	--
I. mors-panacis	CBS 306.35 T	*Pa. quinquefolium*	Canada	JF735288	JF735557	JF735414	JF735746	--	--	--
	CBS 124662	*Pa. ginseng*	Japan	JF735290	JF735559	JF735416	JF735748	--	--	--
I. palmarum	CBS 135754 T	*Howea forsteriana*	Italy	HF937431	HF922620	HF922608	HF922614	--	--	--
	CBS 135753	*H. forsteriana*	Italy	HF937432	HF922621	HF922609	HF922615	--	--	--
I. panacis	CBS 129079 T	*Pa. quinquefolium*	Canada	AY295316	JF735572	JF735424	JF735761	--	--	--
I. protearum	CBS 132811 T	*Protea* sp.	South Africa	JX231157	JX231141	JX231109	JX231125	--	--	--
	CBS 132812	*Protea* sp.	South Africa	JX231165	JX231149	JX231117	JX231133	--	--	--
I. pseudodestructans	CBS 129081 T	*V.vinifera*	Portugal	AJ875330	JF735563	AM419091	JF735752	--	--	--
	CBS 117824	*Quercus* sp.	Austria	JF735292	JF735562	JF735419	JF735751	--	--	--
I. qitaiheensis	H309 T	*Pa. ginseng*	China	MF350472	MF350445	MF350418	MF350499	--	--	--
	J919	*Pa. ginseng*	China	MF350473	MF350446	MF350419	MF350500	--	--	--
I. robusta	CBS 308.35 T	*Pa. quinquefolium*	Canada	JF735264	JF735518	JF735377	JF735707	--	--	--
	CBS 129084	*V. vinifera*	Portugal	JF735273	JF735532	JF735391	JF735721	--	--	--
I. rufa	CBS 153.37 T	Dune sand	France	AY677271	JF735540	AY677251	JF735729	--	--	--
	CBS 640.77	*Abies alba*	France	JF735277	JF735542	JF735399	JF735731	--	--	--

I. venezuelensis	CBS 102032 T	Bark	Venezuela	AM419059	JF735571	AY677255	JF735760	--	--	--
I. vivaria	BV-2305 T	*V. berlandieri* x *V. rupestris*	Navarra, Spain	MK602795	MK602830	MK602810	MK602825	--	--	--
	BV-1924	*V. berlandieri* x *V. rupestris*	Navarra, Spain	MK602793	MK602828	MK602808	MK602823	--	--	--
I. vredehoekensis	CBS 132807 T	*Protea* sp.	South Africa	JX231155	JX231139	JX231107	JX231123	--	--	--
	CBS 132814	*Protea* sp.	South Africa	JX231158	JX231142	JX231110	JX231126	--	--	--
I. zarorii	CPC 37835 T	Soil of *Maytenus boaria*	Valdivia, Chile	MW114893	MW119259	MW119263	MW119261	--	--	--
	CPC 37837	Soil of *M. boaria*	Valdivia, Chile	MW114894	MW119260	MW119264	MW119262	--	--	--

The Markov Chain Monte Carlo (MCMC) sampling analysis of four chains was executed simultaneously, starting from a random tree topology for 10 million generations (Rodríguez et al. 1990). Trees were sampled every 1000 generations, with the first 2500 generations discarded as burn-in for each analysis. Posterior probabilities were calculated from a majority-rule consensus tree of the remaining 7500 trees. For the ML analysis, the generalized time-reversible with gamma correction (GTR + G) nucleotide substitution model and 1000 bootstrap replicates were used, with the additional parameters set to default. The sequences generated in this study were deposited in the GenBank (Table 1). Trees were visualized using FIGTREE v. 1.4.4 (Rambaut et al. 2009), and multi-gene trees were edited with INKSCAPE v.1.2.2 (https://inkscape.org).

3.4.5 Cultural and morphological characterization

Monosporic isolates were cultured on synthetic nutrient-poor agar (SNA) (Nirenberg 1981) supplemented with 1 cm^2 blocks of sterile filter paper on the media surface to induce the production of asexual structures (Crous 2002; Cabral et al. 2012a). Plates were maintained under a n-UV-light with a 12-h photoperiod at room temperature (22-25 °C). Reproductive structures were scraped off, mounted in a drop of 85% lactic acid or water on a glass slide, and examined at 400x magnification using Olympus CX23 or Nikon Eclipse Ci microscopes.

For each unknown *Calonectria* isolate, dimensions, shape and septation of 30 macroconidia, the width and shape of 10 vesicles, and the presence of chlamydospores were measured (Crous 2002; Lombard et al. 2010a; Liu et al. 2020). For unknown *Cylindrocarpon*-like isolates, dimensions, shape and septation of 30 macroconidia (50 for the novel taxon) and microconidia, as well as the appearance and size of chlamydospores, were recorded (Cabral et al. 2012a; Lombard et al. 2014, 2015a). Images and measurements were captured using a digital camera (Microscope-eye-piece-camera, AM-4023X, Taiwan) attached to the microscope and analysed with Dino-eye® 2.0 software. Measurement values for length and width of

conidia, and width of terminal vesicles are presented as (minimum) lower limit of a 95% confidence interval – upper limit of a 95% confidence interval (maximum). For other measurements, only the extreme values are given.

Colony characteristics such as texture, density, transparency, coloration, zonation, and growth were described for 10-day-old colonies growing on MEA at 25 °C in darkness, and additionally on PDA for the novel taxon. Optimal growth temperatures for each isolate were determined by incubating a 5 mm mycelial plug at the centre of MEA plates, in triplicate, at temperatures ranging from 5 to 35 °C with intervals of 5 °C. After 7 days, colony diameters were measured along two perpendicular axes on the reverse side of each plate using an electronic digital calliper (Kamasa®, USA), and growth rates (mm/day) at each temperature were calculated. Plates showing no growth after 7 days were returned to 20 °C to confirm the colony viability.

The formation of perithecia (sexual morph.) for the putative new species was attempted by pairing the monosporic isolates in all possible combinations, including self-crosses. Pairings were conducted in duplicate on minimum salt agar media (MSA) (Cabral et al. 2012a), with sterilized pine needles on the surface, and incubated under n-UV light with a 12-hour photoperiod at room temperature (22-25 °C) for up to three months.

Additional descriptive data for the new species is available in MycoBank (XXXX) http://www.mycobank.org (Crous et al. 2004a).

3.4.6 Pathogenicity tests and re-isolations

Pathogenicity tests for *Nectriaceae* species were conducted using selected isolates: ES 5, ES 10, and ES 15 from *Calonectria;* ES 17 and ES 18 from *Dactylonectria*; and ES 21 and ES 26 from *Ilyonectria*. Each isolate was inoculated into eight 3-month-old *E. smithii* nursery-produced seedlings. Inoculum was prepared by culturing eight replicates of each isolate on MEA plates at 25 °C under continuous

n-UV-light for 14 days. For each colony, 100 mL of distilled water was added before being liquefied individually in a blender. After trimming the root tips to induce stress, seedlings were submerged in the prepared inoculum of the corresponding isolate and planted in 1 L sturdy plastic pots filled with peat substrate, and then watered with the remaining liquid inoculum. Eight seedlings with trimmed root tips, were watered with distilled water to serve as control plants.

The seedlings were maintained in a greenhouse at natural temperatures (20 ± 5 °C) and watered regularly. After three months, they were removed from the pots, and their root systems were carefully rinsed with tap water over a fine plastic mesh until soil-free. The root systems, separated from the stems and shoots, were placed into paper bags and dried in a dry-heat oven set at 45 ± 2 °C and 20 ± 2% relative humidity until a constant weight (approximately 2 weeks). To fulfill Koch's postulates, re-isolations from both the inoculated and control plants were carried out by plating disinfected root pieces on PDA and incubating them at 25 °C. The morphology of the recovered colonies was compared with the original inoculum for identification.

The effect of inoculation with selected *Nectriaceae* isolates on *E. smithii* seedlings was assessed through a non-parametric Kruskal-Wallis test with 5% significance level, as the homogeneity of variance assumption was not met for root dry weight data according to Levene´s test. The analyses were performed with InfoStat software v.2020 (http://www.infostat.com.ar).

3.5 RESULTS

3.5.1 Field symptoms and fungal isolation

Eucalyptus smithii trees from which *Nectriaceae* isolates were recovered were scattered across the plantations and displayed a generalized chlorosis and decline, with abnormal colourations ranging from pale green to yellowish (Fig. 2A). The root systems of affected trees showed dark brown to black discolorations, root rot and

root loss (Fig. 2B, C), with internal dark-coloured streaks (Fig. 2D). In the nurseries, although symptoms typical of those in the commercial fields were not observed, some scattered seedlings exhibited a noticeable poor growth.

A total of 25 isolates were obtained from the root rot of *E. smithii*: 17 from commercial plantations and 8 from nurseries (Table 1). Isolates were differentiated into two primary groups based on colony characteristics. The first group consisted of 15 isolates (9 from plantations and 6 from nurseries) with morphology reminiscent of *Calonectria*, featuring stipe extensions with terminal vesicles and cylindrical conidia (Crous 2002; Lombard et al. 2010a; Liu et al. 2020). The second group included 10 isolates (8 from plantations and 2 from nurseries) exhibiting *Cylindrocarpon*-like morphology, characterized by large, straight or slightly curved cylindrical macro- and microconidia, and chlamydospores produced on the mycelium (Chaverri et al. 2011; Cabral et al. 2012a; Lombard et al. 2014, 2015a).

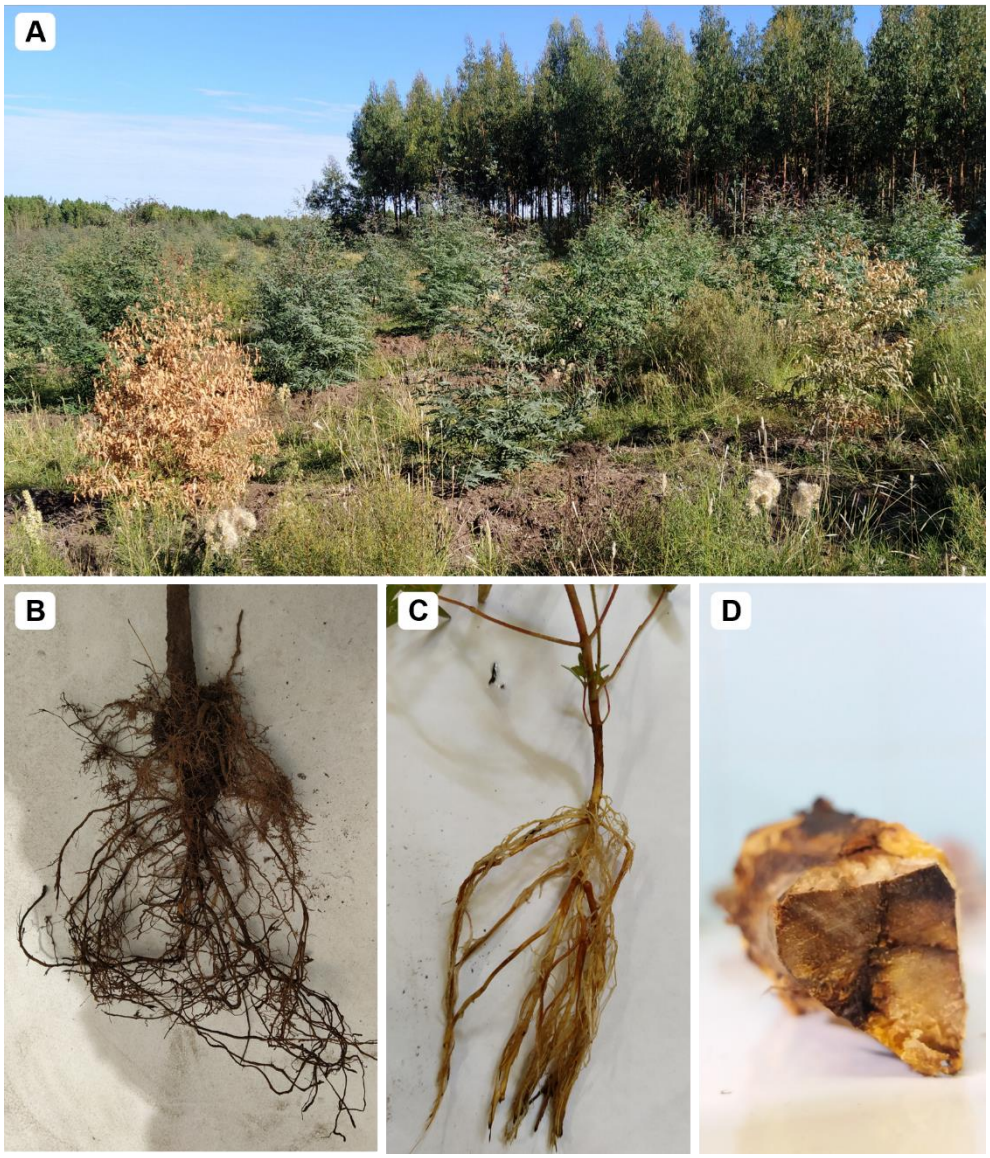

Fig. 2 *Eucalyptus smithii* symptoms, **(A)** declined trees with chlorosis scattered across the fields, **(B)** dark-brown to black coloured root systems, root rot and feeder root loss, **(C)** necrotic sections through seedling roots, **(D)** wood exhibiting internal necrotic lesions with dark brown to black streaks.

3.5.2 Molecular characterization and phylogenetic analyses

In an initial BLAST search with the *his3* gene region, the 25 *Nectriaceae*-like isolates were divided into three major clades belonging to the genera *Calonectria* (*n=15*: 9

from plantation and 6 from nursery), *Dactylonectria* (*n=2*, both from nurseries) and *Ilyonectria* (*n=8*, all from plantations). The 15 unknown *Calonectria* isolates showed 100% homology with the ex-type isolate CBS 138824 of *Ca. pauciramosa* from South Africa. The two *Dactylonectria* isolates, clustered together with the ex-type isolate CBS 113552 of *D. novozelandica* from New Zealand with 100% homology. However, the 8 unknown *Ilyonectria* isolates exhibited 97.83% of homology with the ex-type isolate CBS 308.35 of *I. robusta* from Canada, suggesting that they may represent a novel taxon.

Sequence data sets from individual gene regions were congruent with each other and with the final concatenated phylogenetic tree for each fungal group, revealing no significant conflicts in tree topologies. This concordance suggests that the genes regions could be combined for multi-gene phylogenetic analysis. The multi-gene data set for *Calonectria* included 62 taxa (15 from this study) and encompassed 3125 characters including gaps, corresponding to six loci (*act*: 1-215, *cmdA*: 216-862, *his3*: 863-1271, *rpb2*: 1272-2128, *tef1*: 2129-2581, *tub2*: 2582-3125). The *Dactylonectria* multi-gene data set contained 34 taxa (2 from this study) and encompassed 2331 characters including gaps, corresponding to four loci (ITS: 1- 503, *tef1*: 504-1319, *tub2*: 1320-1826, *his3*: 1827-2331). The multi-gene data set for *Ilyonectria* contained 55 taxa (8 from this study) and encompassed 2439 characters including gaps, corresponding to four loci (*his3*: 1-478, *tef1*: 479-1288, *tub2*: 1289-1877, ITS: 1878-2439). The Bayesian inference (BI) trees, supported by both BI and maximum likelihood (ML) node values (BI/ML), are presented (Figs. 3, 4 and 5).

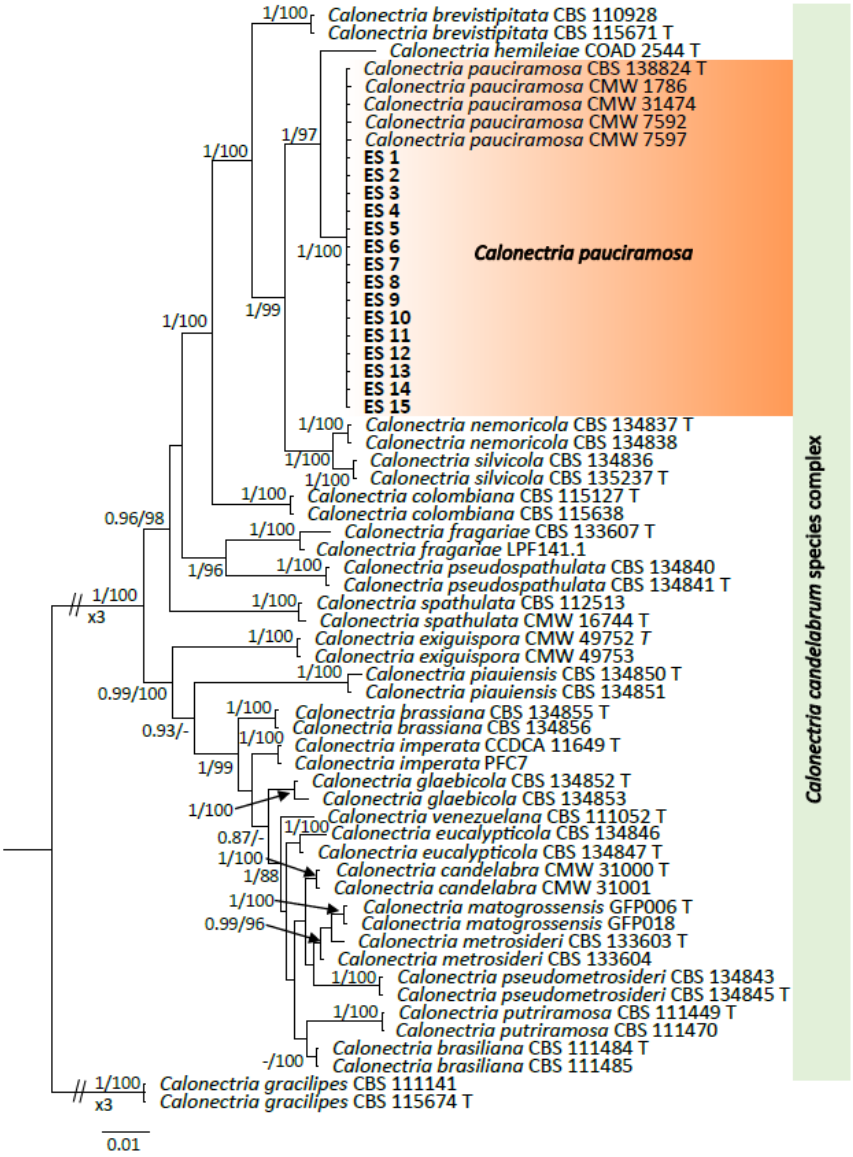

Fig. 3 Bayesian inference phylogenetic tree based on the analyses of concatenated *act*, *cmdA*, *his3*, *rpb2*, *tef1* and *tub2* gene regions. Bayesian posterior probability values ≥ 0.80 and RAxML bootstrap values ≥ 70% are presented at the nodes (BI/ML). Posterior probabilities values below 0.80 and bootstrap below 70% are presented as "-". The tree was rooted to *Calonectria gracilipes* (CBS 115674, CBS 111141). Ex-type isolates are indicated with a "T", isolates from this study are indicated in **bold**. The scale bar indicates 0.01 expected nucleotide substitutions per site.

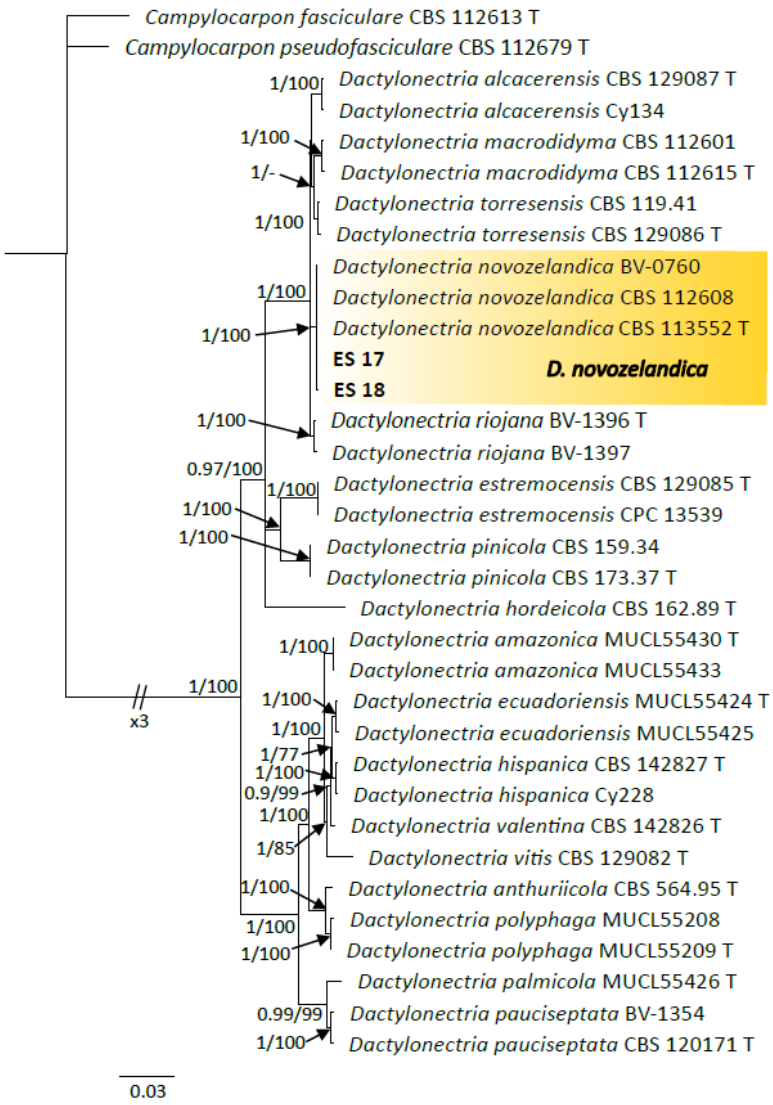

Fig. 4 Bayesian inference phylogenetic tree based on the analyses of concatenated *ITS*, *tef1*, *tub2* and *his3* gene dataset. Bayesian posterior probability values ≥ 0.80 and RAxML bootstrap values ≥ 70% are presented at the nodes (BI/ML). Posterior probabilities values below 0.80 and bootstrap below 70% are presented as "-". The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *C. pseudofasciculare* (CBS 112679). Ex-type isolates are indicated with a "T", isolates from this study are indicated in **bold**. The scale bar indicates 0.03 expected nucleotide substitutions per site.

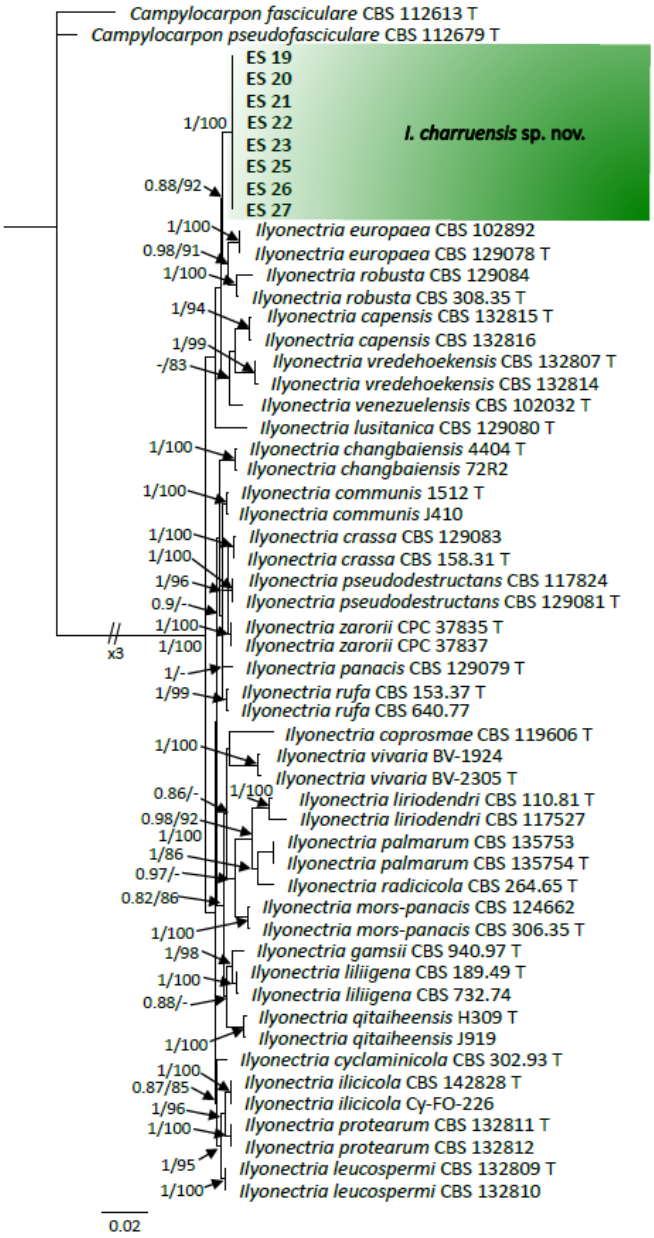

Fig. 5 Bayesian inference phylogenetic tree based on the analyses of concatenated *his3, tef1, tub2* and *ITS* gene dataset. Bayesian posterior probability values ≥ 0.80 and RAxML bootstrap values ≥ 70% are presented at the nodes (BI/ML). Posterior probabilities values below 0.80 and bootstrap below 70% are presented as "-". The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *C. pseudofasciculare* (CBS 112679). Ex-type isolates are indicated with a "T", isolates from this study are indicated in **bold**. The scale bar indicates 0.02 expected nucleotide substitutions per site.

In these trees, the 15 *Calonectria* isolates formed a robust clade (BS/PP: 1/100) with *Ca. pauciramosa* including the ex-type strain of this species confirming the identity of this Uruguayan strains as *Ca. pauciramosa* (Fig. 3). The two *Dactylonectria* isolates were also confirmed as *D. novozelandica*, clustering in a strong clade (BS/PP: 1/100) together with the ex-type of this species validating the identity of these two isolates (Fig. 4). Nevertheless, the eight *Ilyonectria* isolates formed a distinct and well-supported clade (BS/PP: 1/100), separate from any known species, indicative of a new taxon. Consequently, the multigene phylogenetic analysis supports the identification of a novel taxon within the *Ilyonectria* genus obtained from *E. smithii* root rot, hereby named *Ilyonectria charruensis* (Fig. 5).

3.5.3 Cultural and morphological characterization

Calonectria pauciramosa

On SNA. *Macroconidiophores* consisted of a stipe bearing clusters of fertile branches in a penicillate arrangement, and stipe extensions with terminal vesicles (Fig. 6C). *Stipes* were hyaline and septate. *Stipe extensions* hyaline, up to six-septate, straight to flexuous, ending in an obpyriform to ellipsoidal vesicle (2.3)3.7 – 4.7 (6.2) µm (av. 4.2 µm) (Fig. 6D, F). *Conidiogenous apparatus* with up to three aseptate branches, producing up to six doliiform, reniform or ellipsoidal phialides 5.2–10.4 µm long × 2.1–3.7 µm wide (Fig. 6G). *Macroconidia* were hyaline, straight cylindrical with both ends rounded, lacking a visible hilum, 1-septate (32.6)38.5 – 40.3(49.3) × (3.1)4.2 – 4.4(5.39) µm (av. 39.3 × 4.28 µm), held in clusters by a colourless slime (Fig. 6H, I). Mega and microconidia were not observed. *Chlamydospores*, globose to sub-globose or ovoid, abundantly produced in clumps or chains, frequently surrounded by a yellowish-orange or burnt-orange coloured slime (Fig. 6J). Extensive microsclerotia was formed throughout the media after 30 days.

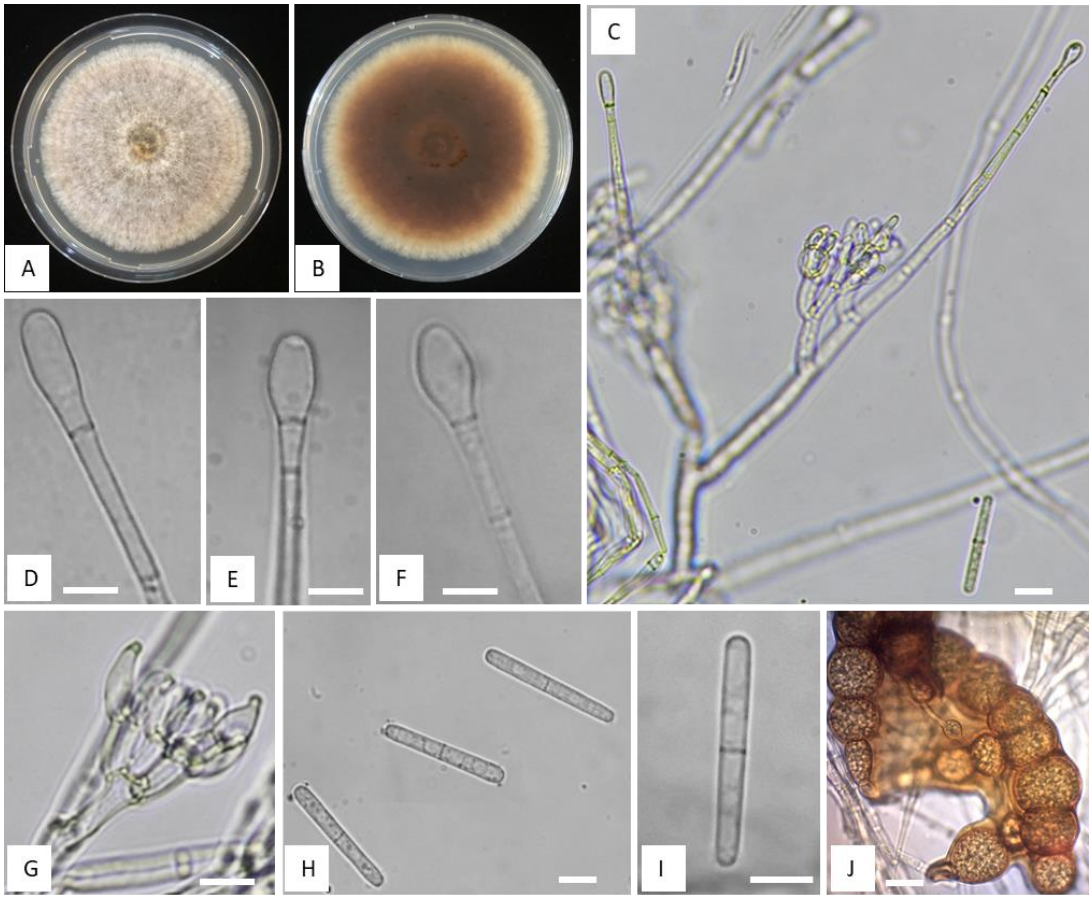

Fig. 6 *Calonectria pauciramosa*, **(A-B)** surface and reverse on MEA plates after 10 days grown at 25 °C in darkness, **(C)** macroconidiophores with stipe extensions and terminal vesicles, **(D-F)** obpyriform to ellipsoidal vesicles, **(G)** doliiform to reniform phialides, **(H-I)** 1-septate macroconidia, **(J)** chlamydospores on growth media. Scale bars = C, H, I, J 10 µm; D-G 5 µm.

Culture characteristics: on MEA after 10 days colonies produced abundant white felty aerial mycelium, turning light-brown towards the centre, with even margins (Fig. 6A). Reverse, whitish outer margins, ochre to sepia, and sienna towards the centre (Fig. 6B). *Growth characteristics*: on MEA no growth was registered at 5 °C. Isolates ES 5, ES 7, ES 8, ES 9, ES 10 and ES 14 grew at 35 °C after 7 days. Optimal temperature of growth between 20-25 °C. Growth rates at 10/15/20/25/30/35 °C were 1.98/3.48/5.41/7.19/4.79/0.92 mm/day, respectively. Isolates incubated at 5 °C

resumed their growth when transferred at 25 °C, whereas isolates with no growth incubated at 35 °C did not. *Isolates studied*: ES 1 to ES 15.

Dactylonectria novozelandica

On SNA. *Simple conidiophores*, arising from aerial mycelium single or sparsely branched, solitary or loosely aggregated, bearing up to three-septate phialides, 19.3–38.5 µm long, with a tapering base towards the apex (Fig. 7C, D). *Complex conidiophores*, not observed. *Macroconidia*, hyaline, straight, cylindrical to slightly curved, hilum frequently visible centrally or laterally displaced, 1-3 septate: 1-septate (23.1)26.1 – 29.1(31.7) × (4.1)4.5 – 5.1(5.9) µm (av. 27.6 × 4.8 µm), length:width ratio 5.3–6.5 µm; 2-septate (24.9)30.8 – 35.1(38.9) × (4.9)5.7 – 6.3(6.8) µm (av. 33.0 × 6.1 µm), L:W ratio 4.4–6.3 µm; 3-septate (31.9)35.4 – 38.6(44.5) × (5.1)6.5 – 7.1(7.6) µm (av. 37 × 6.8 µm), L:W ratio 4.9–6.3 µm (Fig. 7E- G). *Microconidia*, hyaline, ovoid to ellipsoid or broadly ellipsoid, 0-1 septate: 0-septate (4.3)8.2 – 9.9(14.3) × (2.6)3.5 – 4.2(5.9) µm (av. 9 × 3.8 µm), L:W ratio 0.9–4 µm; 1-septate (11.3)12.9 – 14.7(16.9) × (5.0)5.2 – 5.9(6.6) µm (av. 13.8 × 5.6 µm), L:W ratio 2.4–4.4 µm (Fig. 7E- G). *Chlamydospores*, rarely observed, hyaline or slightly yellow-brown coloured, globose to sub-globose, 8.5–13.6 × 8.0–10.5 µm, frequently distorted, smooth but often appearing rough due to deposits, thick-walled, occurring in chains (Fig. 7H).

Culture characteristics: on MEA after 10 days colonies produced felty, chestnut to sienna aerial mycelium, cottony and buff towards the centre, with regular margins (Fig. 7A). Reverse, outer margins dark buff to sepia towards the centre (Fig. 7B). *Zonation*, conspicuous, white fluffy to cottony mycelium. *Growth characteristics*, on MEA no growth was registered at 5 and 35 °C after 7 days. Optimal temperature of growth between 20-25 °C. Growth rates at 10/15/20/25/30 °C were 1.27/2.52/4.01/5.08/0.74 mm/day, respectively. Isolates incubated at 5 °C resumed their growth when transferred at 25 °C, whereas isolates incubated at 35 °C did not. *Isolates studied*: ES 17 and ES 18.

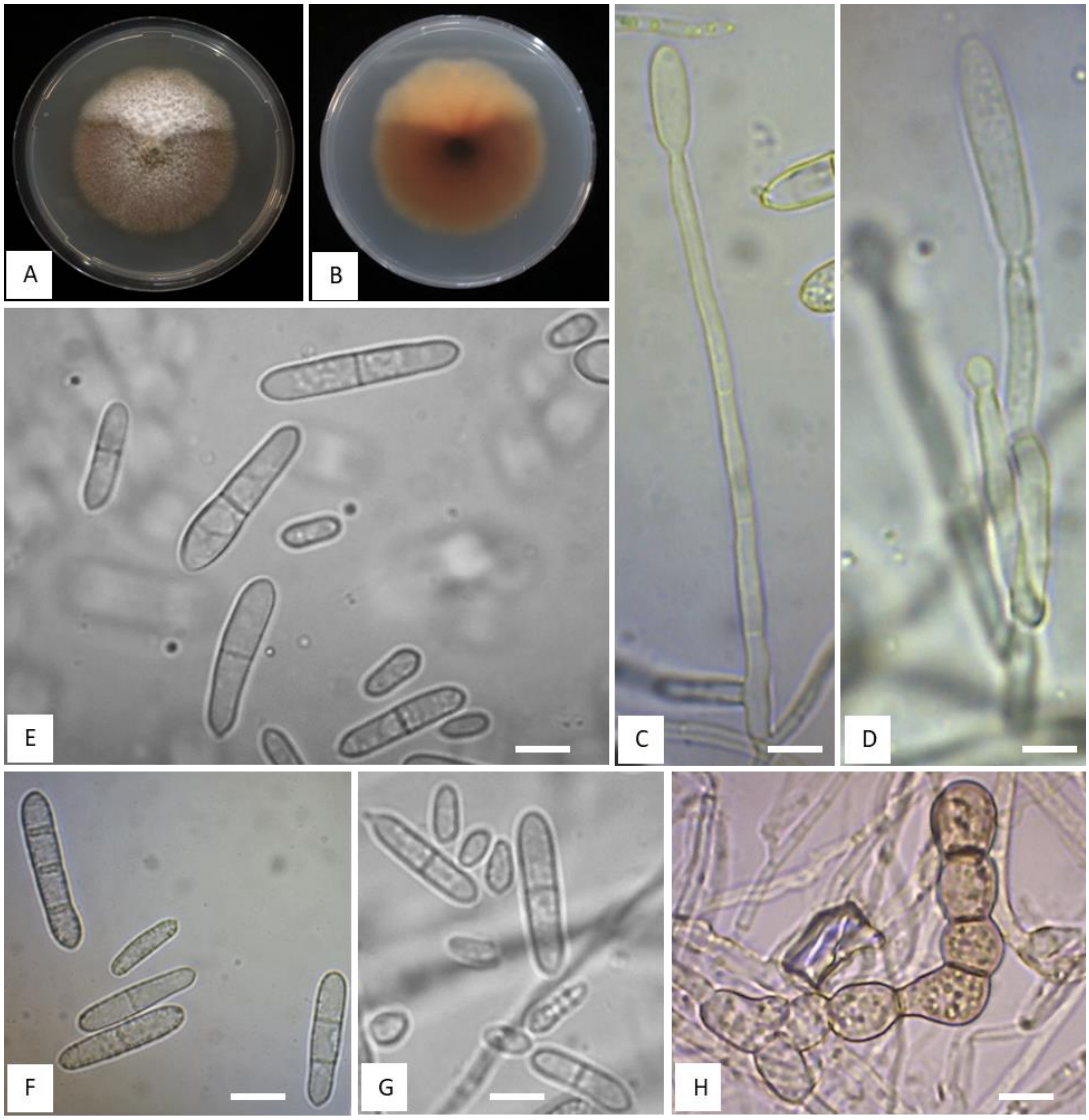

Fig. 7 *Dacylonectria novozelandica*, **(A-B)** surface and reverse on MEA plates after 10 days grown at 25 °C in dark, **(C-D)** simple and sparsely branched conidiophores of the aerial mycelium, **(E-G)** micro and macroconidia, **(H)** chlamydospores on mycelium. Scale bars = 10 µm.

3.5.4 Taxonomy

Based on phylogenetic analyses and phenotypical characters, a new species of *Ilyonectria* is here described. No perithecia were observed in the performed crosses on MSA after 2 months.

Ilyonectria charruensis. F. De Benedetti, M. J. Carbone, P. Mondino & S. Alaniz, sp. nov. MycoBank (number) (Fig. 8).

Etymology: named honouring the indigenous group *Charrúas* that inhabited the lands of present-day Uruguay and represents an emblem of the country's national identity. *Diagnosis*: Morphologically, *Ilyonectria charruensis* can be distinguished from *I. robusta* and *I. europaea* by having slightly longer and narrower macroconidia, and by having longer simple conidiophore than *I. europaea* and slightly shorter than *I. robusta*. This novel taxon is well distinguished by *his3*, *tub2* and *tef1* gene regions. *Type*: Uruguay: Mariscala, Lavalleja, on *Eucalyptus smithii* roots over a natural-soil plantation site, November 2019, F. De Benedetti (collection and number of collection) (CBS X-XXXX) – holotype; CBS XXXX = ES 21 – ex-type culture).

Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to loosely aggregated, unbranched or sparsely branched bearing up to three phialides, 1 to 3- septate, 57–143 µm long; phialides monophialidic, cylindrical, tapering towards the apex, 24 to 61 µm long, 2.2 to 3.8 µm wide at the base, 2.1 to 4.5 µm at the widest point, 1.3 to 2.5 µm near the apex (Fig. 8E, F). *Complex conidiophores* or sporodochial, not observed. *Macroconidia* predominating, formed on simple conidiophores, on SNA formed in flat domes of slimy masses, hyaline, relative straight, cylindrical, with obtusely rounded ends, frequently clavated towards one end, sometimes base with a visible hilum centrally located or one-side laterally displaced, 1-3-(4) septate: 1-septate predominant, (16.7)23.2 – 26.2(31.1) × (3.9)4.5 – 4.9(6.0) µm (av. 24.7 × 4.7 µm), length:width ratio 3.6–6.5 µm; 2-septate (27.9)31.1 – 35.0(39.8) × (4.2)4.9 – 5.6(6.0) µm (av. 33.0 × 5.3 µm), L:W ratio 5.3–

7.0 µm; 3-septate (33.3)35.6 – 41.1(48.4) × (4.9)5.3 – 5.9(6.2) µm (av. 38.3 × 5.6 µm) L:W ratio 6.3–7.9 µm; 4-septate rarely observed (44.1)44.5 – 49.0(50.0) × (5.9)6.0 – 6.9 (7.1) µm (av. 46.8 × 6.5 µm), L:W ratio 7.0–7.4 µm (Fig. 8E- H). Other macroconidia shapes observed include obclavate, peanut-shape, asymmetrical or distorted shapes. *Microconidia*, hyaline, ellipsoidal to ovoid, relatively straight, 0-1 septate: 0-septate (4.2)7.9 – 9.0(13.2) × (2.7)3.4 – 3.9(4.7) µm (av. 8.5 × 3.7 µm), L:W ratio 1.5–3.2 µm; 1-septate (11.9)13.2 – 14.1(15.5) × (4.0)4.8 – 5.4(6.4) µm (av. 13.7 × 4.3 µm), L:W ratio 2.4–3.3 µm (Fig. 8E- H). *Chlamydospores*, globose to sub-globose, or ovoid, 6.8–18.9 × 5.1–15.4 µm, occurring single, intercalary, or terminal on short lateral branches, in clumps or chains, smooth but often appearing rough due to deposits, thick-walled, some surrounded by a colourless to dark-orange or orange-brown coloured slime, observed in mycelium on PDA, MEA and SNA media (Fig. 8I- L).

Culture characteristics: mycelium felty with average density. Surface on PDA fawn to pale brown, with aerial mycelium sparse, dark buff, with buff margin. Surface on MEA sepia to sienna, with aerial mycelium cottony, dense, fawn to cinnamon buff, with buff margins. Zonation absent, with relative homogeneous transparency and even margins. Reverse on PDA, similar to surface, dark pale brown with wide, buff outer margins. On MEA, cinnamon to dark sienna towards the centre, with thick buff margins. *Growth characteristics*: on MEA no growth was registered at 5 and 35 °C after 7 days. Optimal temperature of growth between 20-25 °C. Growth rates at 10/15/20/25/30 °C were 1.67/3.40/4.16/6.44/2.23 mm/day, respectively. Isolates incubated at 5 °C resumed their growth when transferred at 25 °C, whereas isolates incubated at 35 °C did not.

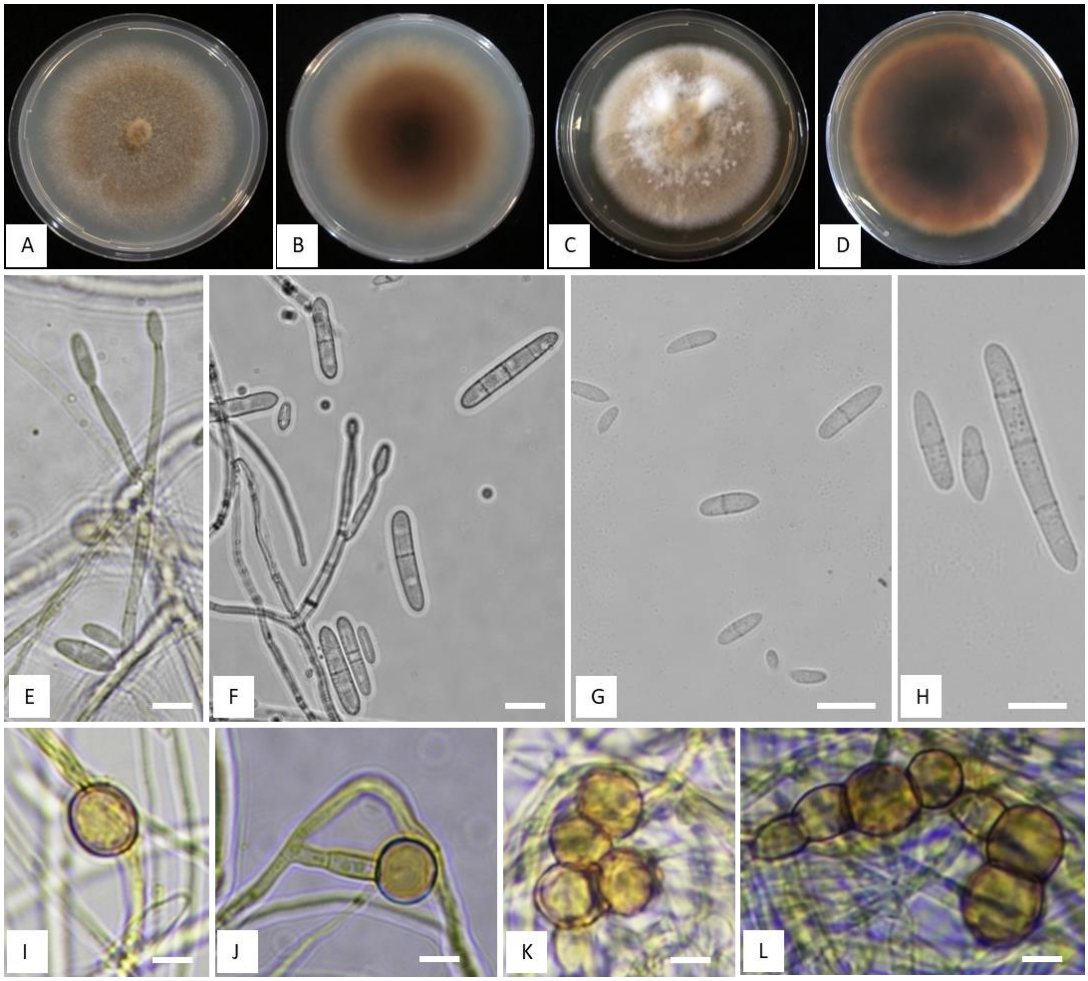

Fig. 8 *Ilyonectria charruensis* sp. nov. **(A-B)** surface and reverse on PDA plates and **(C-D)** on MEA plates after 10 days grown at 25 °C in dark, **(E-F)** simple conidiophores of the aerial mycelium, **(G-H)** micro and macroconidia, **(I-L)** chlamydospores on mycelium. Scale bars = 10 µm.

Notes: I. charruensis exhibits morphological distinctions from its closest relatives *I. europaea* and *I. robusta*, such as slightly longer and narrower macroconidia, and the uncommon production of 4-septate macroconidia, a feature not observed in the latter two species. Additionally, *I. charruensis* differs in its temperature growth range, capable of growing at 30 °C, unlike *I. europaea*, and unable to growth at 4 °C, unlike both, *I. europaea* and *I. robusta*. Phylogenetically, *I. charruensis* can be distinguished from *I. europaea*/ *I.robusta* in ITS by 6/5 polymorphisms, in *his3* by

20/10 polymorphisms, in *tub2* by 14/13 polymorphisms and in *tef1* by 12/15 polymorphisms, respectively.

These morphological and phenotypic variances, along with distinct polymorphisms across four loci, clearly delineate this novel taxon. *Isolates studied:* ES 21 (type), with ES 19, ES20, ES22, ES23, ES25, ES26, and ES27 also contributing to the characterization.

3.5.5 Pathogenicity tests and re-isolations

The pathogenicity test revealed that all the *Nectriaceae* isolates inoculated successfully infected the roots of *E. smithii* seedlings. After three months, the aerial parts of the inoculated seedlings showed stunted growth, decline and, in some cases, death of plants. Declined seedlings displayed a generalized chlorosis with yellowish colours, while dead plants progressively turned darker in colour, including defoliation. The root systems of inoculated seedlings displayed dark-brown to blackish colourations, contrasting to the whitish to light-brown colourations of control seedlings roots, and were significantly smaller in size.

Significant differences were observed in both root ($P = 0.0001$) and shoot weight ($P = 0.0001$). Compared to the control plants, isolates of *Ca. pauciramosa* were the most aggressive, causing an average reduction of 60% in root dry weight and 49% in shoot dry weight. *Da. novozelandica* isolates were the less aggressive, resulting in an average reduction of 32% in root dry weight, and 17% in shoot weight; however, the latter reduction was not statistically significant when compared with the control plants. Isolates of *Ilyonectria charruensis* sp. nov. demonstrated intermediate aggressiveness, causing average reductions of 51% and 25% in root and shoot dry weight, respectively. All the inoculated isolates were successfully re-isolated from infected roots (100%), while no *Nectriaceae* were re-isolated from the control plants (Table 2).

Table 2. Pathogenicity results of selected isolates of *Calonectria pauciramosa*, *Ilyonectria charruensis* sp. nov. and *Dactylonectria novozelandica* inoculated on *Eucalyptus smithii* seedlings

Species	Isolate	Roots dry weight (g)		Shoots dry weights (g)		Re-isolation (%)
Ca. pauciramosa	ES 5	1.11	a	2.64	a	100
	ES 10	1.10	a	2.11	ab	100
	ES 15	1.39	ab	2.95	ab	100
I. charruensis sp. nov.	ES 21	1.48	ab	3.87	cd	100
	ES 26	1.42	ab	3.29	bcd	100
D. novozelandica	ES 17	1.99	b	4.05	de	100
	ES 18	2.12	b	4.20	de	100
	Control	2.96	c	4.99	e	--

Means with different letters are significantly different ($P < 0.05$)

Data was analysed using a non-parametric Kruskal-Wallis test at 5% significance level

3.6 DISCUSSION

In this study, we identified twenty-five isolates residing in three genera belonging to the clades III (*Calonectria*) and VI (*Dactylonectria* and *Ilyonectria*) of the *Nectriaceae* family (Lombard et al. 2015). These isolates were associated with root rot of nursery and young *E. smithii* trees on commercial fields, in the southern and southeastern regions of Uruguay where this *Eucalyptus* species is extensively planted. Fifteen isolates were identified as *Ca. pauciramosa*, two as *Da. novozelandica* and eight represent a new species described in this study, named *Ilyonectria charruensis* sp. nov. Interestingly, the aerial part of the affected *E. smithii* trees on commercial plantations where the majority of the *Nectriaceae* isolates were obtained, displayed a generalized chlorosis and decline; however, they did not show the typical reddish shades presented in trees that proved to be infected by *Phytophthora* spp. (De Benedetti et al. 2023). On this way, nurseries seedlings only displayed noticeable poor growth, with slightly to no anormal foliage colorations.

Calonectria pauciramosa, the predominant species in this study was found in association with root rot of both young trees in plantations and nursery seedlings. Our pathogenicity tests confirmed its high aggressiveness, demonstrated by substantial reductions in both root and aerial biomass of the inoculated *E. smithii* seedlings. Recognized as a soil-borne pathogen, *Ca. pauciramosa* affects multiple hosts globally, including forestry crops (Schoch et al. 2001a, b; Crous 2002; Lombard et al. 2010b; Li et al. 2021). This species is part of the *Calonectria candelabrum* species complex and belongs to the Prolate group in which species are characterized by having clavate, pyriform or ellipsoidal vesicles and mostly 1-septate macroconidia (Liu et al. 2020).

Calonectria pauciramosa was first reported in Uruguay in 2010, associated with *E. grandis* (Lombard et al. 2010b). However, that study did not provide information regarding the symptomatology association of the isolates, nor cultural or pathogenicity data. The identification of these *Ca. pauciramosa* Uruguayan isolates (CMW 7592, CMW 7597 and CMW 7600) was based on the multigene analysis of three gene regions: *his3*, *tub2* and *tef1*, as well as their mating type (Mat-1). Additionally, in 2013, four forest nurseries in the north of Uruguay were sampled in search of *Calonectria* spp. This led to a collection of strains, some of which were identified as "*Ca. pauciramosa*" based on the ITS region analysis (Gasparri et al. 2013). However, it has been found that the ITS region is less informative and not a reliable marker for identifying *Calonectria* spp. (Schoch et al. 1999b, Lombard et al. 2010c).

The 15 *Calonectria* isolates of this study were identified based on the multigene analysis of six gene regions including those used by Lombard et al. (2010b), plus *act*, *cmdA* and *rpb2* gene regions. Therefore, this study enriches the existing knowledge about Uruguayan *Ca. pauciramosa* isolates, offering a comprehensive update on their

molecular and phenotypical characterization, and providing new insights into their pathogenicity.

Variation within isolates of *Ca. pauciramosa* have been previously observed in several studies (Schoch et al. 2001a, b; Crous 2002; Lombard et al. 2010b, c; Li et al. 2021). The morphological characterization of *Ca. pauciramosa* isolates from this study showed smaller macroconidia dimensions (av. 39 x 4 µm) compared to the type species (av. 50 x 4.5 µm), however, were molecularly identical to the type species (CBS 138824) in six gene regions. Recently, eight *Calonectria* species were reduced as synonymy of '*Ca. pauciramosa*', exposing the intraspecific differences between isolates of *Ca. pauciramosa* from different regions of the world (Liu et al. 2020).

To our best knowledge, the discovery of two *D. novozelandica* isolates from *E. smithii* seedlings in a nursery represents a novel association between this species and the *Eucalyptus* genus. Despite their low isolation frequency, we demonstrated that this species could cause significant reductions in the root systems of *E. smithii* seedlings, as evidenced in the pathogenicity test results. Primarily, this species has been linked to grapevines black-foot disease (Cabral et al. 2012a). Nowadays, it is known that this species can cause disease in a broad range of woody plants, including species of *Juniperus*, *Pinus*, *Crataegus*, *Quercus* (Mora-Sala et al. 2018), *Prunus*, *Pistacia* (Lawrence et al. 2019), *Panax* (Zhang et al. 2019) and *Fragaria* (Chen et al. 2021), and now, extending its host range to *Eucalyptus*. In Uruguay, this species was found in association with grapevine nursery plants (Carbone et al. 2022) and strawberry plants (Viglieca et al. 2022).

A similar scenario involves the eight isolates of *Ilyonectria charruensis* sp. nov. obtained from root rot of declined *E. smithii* from young commercial plantations. The association of the *Ilyonectria* genus damaging the roots of many crops is well-documented. For example, *I. capensis* was found causing black foot disease of *Proteaceae* (Lombard et al. 2013) and *Prunus* (Lawrence et al. 2019), *I. europaea* in *Vitis* and *Phragmites, I. robusta* associated with *Quercus*, *Panax, Tilia*, (Cabral et al.

2012b) and *Olea* (Lawrence et al. 2019), *I. mors-panacis* affecting *Panax* (Cabral et al. 2012b), *I. ilicicola* causing disease in *Ilex* (Mora-Sala et al. 2018). However, to our best knowledge, this is the first report of an *Ilyonectria* species infecting the *Eucalyptus* genus. Furthermore, given its absence elsewhere, we propose that *I. charruensis* might be endemic to this South American region.

In summary, this work provides new insights into the decline of *E. smithii*, elucidating the contribution of pathogens from the *Nectriaceae* family in the decline of young and nursery *E. smithii* trees. In this way, we demonstrated that, in addition to *P. alticola* and *P. boodjera* (De Benedetti et al. 2023), species of *Calonectria*, *Dactylonectria* and *Ilyonectria* genera contribute to the decline and death of young *E. smithii* trees. However, despite of the occurrence of pathogens infecting *E. smithii* roots, further research is needed to fully understand the origin of the decline and mortality of *E. smithii* trees. Thus, future studies should focus on plant-physiology, seed provenance, optimal site selection for planting, silvicultural requirements and exploration of root development enhancement techniques. These studies will provide valuable information to better understand the contribution of abiotic factors in the high mortality of *E. smithii* trees.

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3.8 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.9 ORCID

Franco De Benedetti https://orcid.org/0000-0003-1982-0618
María Julia Carbone https://orcid.org/0000-0003-1845-9277
Pedro Mondino https://orcid.org/0000-0002-4494-5271
Sandra Alaniz https://orcid.org/0000-0002-6530-7279

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4. CONCLUSIONES Y PERSEPECTIVAS GENERALES

El sector forestal uruguayo ha recurrido a *Eucalyptus smithii* para la producción de pulpa de celulosa de alta calidad, destinada a mercados internacionales. En plena expansión de esta especie surgió un problema, la alta mortalidad de árboles jóvenes en las plantaciones comerciales, lo que cuestiona su viabilidad. La mortalidad de plantas alcanza promedios en el entorno del 40 % durante sus primeros dos años después de plantados. Los síntomas que manifiestan las plantas afectadas incluyen podredumbre de raíces y cuello, reducción de masa radicular y decaimiento de la parte aérea, indicando que factores bióticos podrían ser los responsables del decaimiento de esta especie de eucalipto.

Este trabajo se destaca por constituir el primer abordaje a la problemática de la mortalidad de árboles jóvenes de *E. smithii* en la producción forestal uruguaya, procurando determinar qué factores bióticos son responsables. Solo se pudieron aislar patógenos en un bajo porcentaje de las plantas con síntomas (24 % de aislamiento de cepas de *Peronosporaceae* y 13 % de aislamiento de cepas de *Nectriaceae*). Se confirmó la asociación de especies de *Phytophthora*, *Calonectria*, *Dactylonectria* e *Ilyonectria* con el decaimiento de *E. smithii*. Las pruebas de patogenicidad evidenciaron que estos patógenos tienen la capacidad de infectar las raíces y reducir la masa radicular de *E. smithii*; sin embargo, no se observaron en estos ensayos muertes masivas similares a las observadas en las plantaciones comerciales.

El bajo porcentaje de aislamiento de patógenos a partir de plantas sintomáticas y el hecho de que no se registraron muertes generalizadas en las pruebas de patogenicidad sugieren que los factores bióticos estudiados no serían los únicos responsables de las muertes de *E. smithii*. Aunque se ha demostrado el impacto negativo que tiene en *E. smithii* la presencia de ciertos organismos patógenos, la situación actual de *E. smithii* en Uruguay es posiblemente el resultado de una interacción compleja de factores, lo

que indica que nuevas investigaciones deberán ser abordadas para elucidar el papel que juegan en la mortalidad de *E. smithii.*

En cuanto a las infecciones causadas por patógenos, estas podrían suponer un estado de estrés y consecuente debilitamiento de las plantas, aumentando la probabilidad de predisponerse a efectos del ambiente como el calor extremo del verano y la falta de precipitaciones, como también estar más expuesta a un deterioro radicular más rápido por parte de la acción natural de microorganismos descomponedores habitantes del suelo. El mismo razonamiento podría darse a la inversa: el estrés al que están sometidas las plantas debido a efectos del ambiente, métodos de plantación, tipos de suelo en el que se desarrollan, entre otros, podrían predisponerlas a ser infectadas por patógenos.

Tomando en consideración el estado del conocimiento actual, es esencial optimizar el manejo silvicultural de *E. smithii,* desde la etapa inicial de producción hasta el momento de implantación de los plantínes en el campo, buscando minimizar el estrés y el consecuente decaimiento de los árboles. En la etapa de vivero, sería conveniente evaluar diferentes sistemas de producción de plantas, que favorezcan un mejor desarrollo de las raíces. Por ejemplo, utilizar contenedores de plantínes (tubetes) de mayor tamaño, diferentes tipos de sustrato o el uso de promotores del desarrollo radicular que, en combinación con un riego óptimo, podrían contribuir en la producción de plantas con mayor tolerancia a estrés tanto biótico como abiótico.

Estas recomendaciones no se contraponen con la necesidad de desarrollar nuevas investigaciones en el área de fisiología vegetal, por ejemplo, conocer qué factores están incidiendo en el desarrollo radicular que presenta esta especie. Además, se sugiere evaluar las diferentes procedencias de semillas de *E. smithii*, con el objetivo de identificar y seleccionar aquellas con mejor adaptabilidad y rendimiento.

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