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Basidiospore infection of Austropuccinia psidii and resistance of open-pollinated Eucalyptus globulus families to rust and Teratosphaeria leaf disease

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Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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ABSTRACT

ALMEIDA, Rosiane Fátima de, D.Sc., Universidade Federal de Viçosa, November, 2024. **Basidiospore infection of Austropuccinia psidii and resistance of open-pollinated Eucalyptus globulus families to rust and Teratosphaeria leaf disease**. Adviser: Acelino Couto Alfenas. Co-adviser: Rafael Ferreira Alfenas.

Eucalyptus globulus can produce high-density wood with high cellulose and low lignin content. However, plantations can be affected by Myrtle rust, caused by *Austropuccinia psidii* and/or Teratosphaeria Leaf Disease (TLD), caused by *Teratosphaeria nubilosa*. Planting resistant materials is the most efficient strategy to control both diseases. Successful control requires the availability of resistance sources, a comprehensive understanding of pathogen population diversity, and the selection of resistant genotypes through inoculations under controlled environmental conditions. This study aimed to assess the resistance of native families of *E. globulus* to *A. psidii* and *T. nubilosa* (Brazilian populations) compared to the pathogen populations in Australia. Additionally, it aimed to elucidate the sexual cycle of *A. psidii* in *Eucalyptus* and its potential role in the genetic diversity observed among populations. The results of *A. psidii* inoculations showed that families of the subrace Southern Tasmania exhibited broad-spectrum resistance to both rust biotypes: the Brazilian biotype infecting Eucalyptus spp. and *Syzygium jambos*, and the pandemic biotype that occurs in Australia. However, the Brazilian biotype appears to be more virulent. Although physiological differences between the two biotypes of *A. psidii* were confirmed, sexual reproduction could not be conclusively identified as the source of the genotypic variation observed between these populations through inoculation tests with basidiospores of *A. psidii* race 1 (biotype Brazilian) in *Eucalyptus*. A single multilocus genotype (MLG) of *T. nubilosa* predominates in Brazil and Spain - an important *E. globulus*-producing country. However, this MLG differs from a representative isolate from Australia. Although this, the results of *T. nubilosa* inoculations under controlled environmental conditions carried out in Brazil were consistent with field trial outcomes in Australia using the same *E. globulus* families. The most TLD-resistant populations of *E. globulus* are native to Northern Tasmania, regions of the Bass Strait, and extend northward into mainland Australia. These families can be targeted in genetic breeding programs, with selected plants being cloned and cultivated in countries such as Spain.

Keywords: genetic control; genetical diversity; myrtaceae

RESUMO

ALMEIDA, Rosiane Fátima de, D.Sc., Universidade Federal de Viçosa, novembro de 2024. **Infecção de basidiósporos de Austropuccinia psidii e resistência de famílias de polinização aberta de Eucalyptus globulus á Ferrugem e Teratosphaeria Leaf Disease**. Orientador: Acelino Couto Alfenas. Coorientador: Rafael Ferreira Alfenas.

Eucalyptus globulus pode produzir madeira de alta densidade com alto teor de celulose e baixo teor de lignina. No entanto, as plantações podem ser afetadas pela ferrugem do eucalipto, causada por *Austropuccinia psidii* e/ou Teratosphaeria Leaf Disease (TLD), causada por *Teratosphaeria nubilosa*. O plantio de materiais resistentes é a estratégia mais eficiente para controlar ambas as doenças. O controle bem-sucedido requer a disponibilidade de fontes de resistência, conhecimento sobre a diversidade populacional do patógeno e a seleção de genótipos resistentes por meio de inoculações sob condições ambientais controladas. Este estudo teve como objetivo avaliar a resistência de famílias nativas de *E. globulus* à *A. psidii* e *T. nubilosa* (populações brasileiras) em comparação com as populações do patógeno na Austrália. Além disso, teve como objetivo elucidar o ciclo sexual de *A. psidii* em *Eucalyptus* e seu papel potencial na diversidade genética observada entre as populações. Os resultados das inoculações de *A. psidii* mostraram que famílias da sub-raça ‘Southern Tasmania’ exibiram resistência de amplo espectro a ambos os biótipos de ferrugem: o biótipo brasileiro que infecta *Eucalyptus* spp. e *Syzygium jambos*, e o biótipo pandêmico que ocorre na Austrália. No entanto, o biótipo brasileiro parece ser mais virulento. A reprodução sexuada de *A. psidii* raça 1 (biótipo brasileiro) em *Eucalyptus* e *S. jambos* não pôde ser confirmada, por meio de inoculações com basidiósporos, como sendo fonte da variação genotípica observada entre populações do patógeno. Um único genótipo multilocus (MLG) de *T. nubilosa* predomina no Brasil e na Espanha. No entanto, esse MLG difere de um isolado representativo da Austrália. Apesar disso, os resultados das inoculações de *T. nubilosa* realizadas no Brasil foram consistentes com os resultados de ensaios de campo na Austrália usando as mesmas famílias de *E. globulus.* As populações mais resistentes a TLD de *E. globulus* são nativas do norte da Tasmânia, Estreito de Bass e sul da Austrália continental. Essas famílias podem ser alvos de programas de melhoramento genético, com plantas selecionadas sendo clonadas e cultivadas em países como a Espanha.

Palavras-chave: controle genético; diversidade genética; myrtaceae

SUMARY

GENERAL INTRODUCTION

The eucalypt (which includes the genera *Angophora, Corymbia* and *Eucalyptus*) is predominantly native to Australia but has been widely planted in different regions of the world (Hager & Benson, 2010). *Eucalyptus globulus* Labill. was the first *Eucalyptu*s species to be widely cultivated outside the native areas (Potts et al. 2004), leading to the establishment of large plantations particularly in Chile, Portugal, and Spain. The good performance of this species can be attributed to its rapid growth, high coppice capacity, high density and pulp yield, and low lignin content in the wood (Eldridge et al. 1994; Potts et al. 2004). In Brazil, *E. globulus* has been planted in hybrid combinations with *Eucalyptus* species adapted to edaphon-climatic conditions (Alfenas et al. 2009; Xavier et al. 2007).

As a result of the interest in *E. globulu*s production, genetic studies were developed across different countries, employing seed collections sourced from native populations. The largest collection of *E. globulus* seeds was obtained by the Australian Tree Seed Center (ATSC) of CSIRO (Commonwealth Scientific and Industrial Research Organization) between 1987 and 1988 (Dutkowski & Potts 1999; Potts et al. 2008). Research developed from this collection provides important information about the intraspecific genetic variability of *E. globulus* (Potts et al. 2004; Potts et al. 2008). For example, it was based on the study of open-pollinated families maintained in ATSC/CSIRO seed orchards that Dutkowski & Potts (1999) proposed a system of 13 races and 20 subraces of *E. globulus*, distinct by geographic patterns of quantitative characteristics and evolutionary relationships (Steane et al. 2006) (Figure 1).

Many of these native races and subraces of *E. globulu*s form the basis of breeding programs in many countries where the species is being genetically improved to increase the production of cellulose and paper (Potts et al. 2004). However, as diseases can limit the productivity of elite eucalypt genotypes, it is crucial for breeding programs to analyze the genetic variability of these races and subraces of *E. globulus* for resistance against pathogens. Two important pathogens that affect *E. globulus* are *Austropuccinia psidii* (G. Winter) Beenken, the causal agent of Myrtle rust, and *Teratosphaeria nubilosa* (Cooke) Crous & U. Braun, which causes Teratosphaeria leaf disease (TLD)

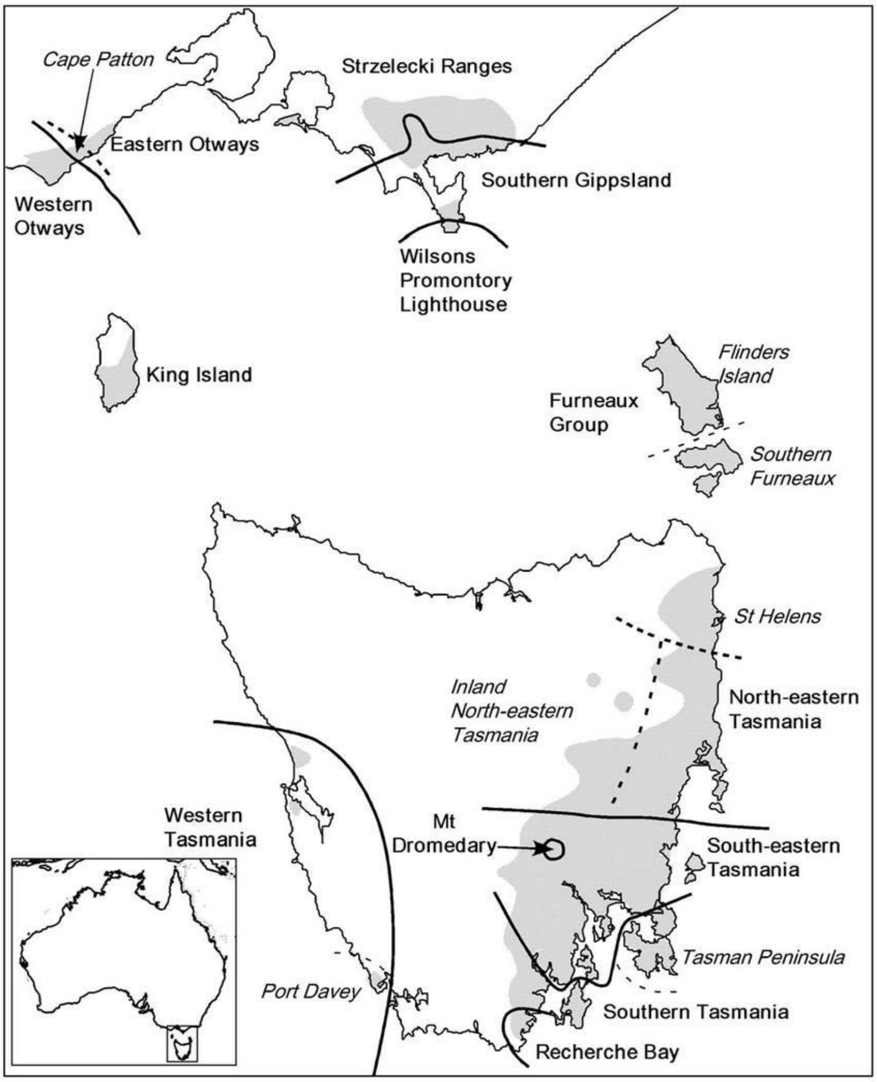

Fig. 1 Map of Tasmania and Southern Victoria (Australia) showing the distribution of the 13 races and 20 sub-races of *Eucalyptus globulus* proposed by Dutkowski and Potts (1999) and revised by Lopez et al. (2001).

Austropuccinia psidii (Basidiomycota) is a biotrophic pathogen originally described by Winter (1884) as *Puccinia psidii* on guava (*Psidium guajava* L.), in São Francisco do Sul, Santa Catarina, Brazil. Currently, it has wide distribution in South America and North America, as well as in countries in Asia, Africa, and Oceania. New Zealand, Australia, and Brazil are the countries with the highest occurrence of *A. psidii* in the world (GBIF, 2024). There was an abrupt increase in reports of rust occurrences following the bushfires in Australia in 2019 and 2020. Since then, the pathogen has been causing severe damage to native Myrtaceae species regenerating in this country (Pegg et al. 2020). Important to note that in both Australia and New Zealand, a biotype of *A. psidii* prevails, which has not been documented in Brazil. This biotype is known as the 'pandemic biotype' due to its wide geographic distribution and greater virulence

(Berthon et al. 2019; Hardstaff et al. 2022; Ross-Davis et al. 2013; Stewart et al. 2018) in comparison with three other biotypes described: a South African biotype (Roux et al. 2016) and two biotypes that occur in Brazil (Stewart et al. 2018). In Brazil, one biotype was associated with *Eucalyptus* spp. and *Syzygium jambos* the other with *P. guava* (Ross-Davis et al. 2013; Stewart et al. 2018).

On the other hand, *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*) (Ascomycota) was described in 1891 as *Sphaerella nubilosa* in leaves of *Eucalyptus* sp. in Victoria, Australia (Cooke, 1891). Later, Crous et al. (2004) described the epitype of the species based on infected leaves of *E. globulu*s. Subsequently, other records of the pathogen occurred in other regions of Australia, including Tasmania, New South Wales, South Australia, and Western Australia (Hunter et al. 2008; Maxwell et al. 2001; Milgate et al. 2001). Outside Australia, *T. nubilosa* was reported in the North Island of New Zealand (Dick, 1982) and South Africa (Wingfield et al. 2020). The pathogen was accidentally introduced into commercial *E. globulus* plantations in Spain and Portugal, where it caused significant damage (Crous et al. 2004). In 2007, *T. nubilosa* was reported for the first time in South America, causing serious damage to *E. globulus* plantations in Uruguay and Brazil (Teodoro et al. 2012). Interestingly, in Portugal, Spain, and Uruguay non-native populations of *T. nubilosa* are constituted by single clones (Hunter et al. 2008; Hunter et al. 2009; Pérez et al. 2009). Recent studies also show that the *T. nubilosa* isolates infecting eucalypt in Brazil are genotypically identical (A. C. Alfenas, personal communication, 2024).

Since the genetic variability of pathogens can result in variations in the ability to adapt to different environmental conditions and hosts, understanding this variability and applying it to the selection of resistant genotypes is an essential measure for the success of eucalypt breeding programs.

The present study is structured into three chapters. Chapter 1 aims to (i) evaluate the resistance of open-pollinated families of *E. globulus* to Brazilian biotype of *A. psidii* (biotype associated with *Eucalyptus* spp. and *S. jambos,* represented by *A. psidii* race 1), previously evaluated for resistance to the pandemic biotype in Australia; (ii) determine the correlation for resistance between Brazilian biotype versus pandemic biotype of *A. psidii* to the family- and subrace-level of *E. globulus*; and (iii) know potential sources of rust resistance to be cloned and tested under field conditions. Chapter 2 aims to investigate the infectivity of *A. psidii* basidiospores in eucalypt and its potential role in the sexual cycle and genotypic diversity of the pathogen. Finally, Chapter 3 aims to (i) determine the current genetic variability among *T. nubilosa* populations from different geographic regions, especially Brazil and Spain; (ii) determine the genetic variability among native *E. globulus* populations for resistance to TLD through inoculations under controlled environmental conditions; (iii) Determine whether there is a positive correlation among the resistance of *E. globulus* families evaluated in field trials in native areas (Hamilton et al. 2013) and the resistance of seedlings to TLD evaluated by artificial inoculation in Brazil; and iv) (iii) know potential sources of TLD resistance to be cloned and tested under field conditions.

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CHAPER 1 - RESISTANCE OF OPEN-POLLINATED FAMILIES OF *Eucalyptus globulus* TO TWO BIOTYPES OF *Austropuccinia psidii*

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ABSTRACT

Myrtle rust caused by *Austropuccinia psidii* (G. Winter) Beenken is an important disease of Myrtaceae species in tropical and subtropical regions of the world. The pathogen is of concern to the forest plantation industry in Brazil which is primarily based on introduced eucalypts from Australia. *A. psidii* has multiple biotypes, including a native Brazilian biotype, that causes damage to *Eucalyptus* spp. and *Syzygium jambos*, and a pandemic biotype which as an invader affects numerous Myrtaceae hosts in countries around the world, including those in Australia. Although *A. psidii* has had little impact on eucalypt plantations in Australia to date, this may change if the Brazilian or other biotypes invade, or if there is genetic change in the pandemic biotype in Australia. In Australian seedling inoculation studies, families from range-wide native stand seed collections of Australia's main plantation eucalypt, *Eucalyptus globulus*, have shown considerable genetic variation in resistance to the pandemic biotype of *A. psidii*. In a seedling inoculation study undertaken in Brazil with a subset of these families, we similarly show significant genetic variation in host resistance to the Brazilian biotype, represented by race 1 of *A. psidii* at the family and provenance (i.e., subraces) levels. The disease severity in the

Brazilian and Australian studies was significantly correlated at the family level, ranging between 0.49 and 0.57, due mainly to genetic-based covariation in the immune response. Overall, the *E. globulus* families exhibited less resistance to the Brazilian than the pandemic biotype, but it is unclear whether this is due to greater pathogen virulence or different inoculation and growing conditions. Regardless, genetic variation in susceptibility to both *A. psidii* biotypes, selection of *E. globulus* for reduced susceptibility to one biotype is expected to make positive gains in resistance to the other, and some families exhibited relatively 'stable resistance' with low susceptibility to both biotypes.

Keywords: Eucalypt rust, Guava rust, Heritability, Inoculation, Myrtle rust, Tasmanian blue gum.

INTRODUCTION

Austropuccinia psidii (G. Winter) Beenken (syn. *Puccinia psidii*), causal agent of the disease called 'myrtle rust' but also 'guava rust' or 'eucalypt rust', is a biotrophic plant pathogen native to Central and Southern America, which has now been recorded from at least 27 countries on four continents (Berthon *et al.* 2019; Carnegie and Pegg 2018). The widespread dispersion of the pathogen is facilitated by its dry spores and ability to infect many host species in the Myrtaceae family, including at least 480 species (Soewarto *et al.* 2019). The Myrtaceae is a large, predominantly southern hemisphere, plant family comprising many species of ecological and economic significance (Grattapaglia *et al.* 2012), hence *A. psidii* outbreaks have deleterious consequences for natural ecosystems as well as forestry and other industries (Carnegie and Pegg 2018). The Myrtaceae family includes the eucalypts (comprising the genera *Angophora, Corymbia* and *Eucalyptus*), which are native to Australia and islands to its north but are grown globally for forestry purposes (Grattapaglia *et al.* 2012).

The first serious myrtle rust outbreak on eucalypts occurred in 1973 in the Espírito Santo state, Brazil, where about 400,000 seedlings of *E. grandis* were refused for planting due to high rust infection. Subsequently, several rust outbreaks have occurred each year in this country (Alfenas *et al.* 2009) especially in plantations most susceptible during the first year after planting and after wood harvesting and in the sprouting of the stump when trees of susceptible clones are between 0.5 to 3.0 m in height (Zauza *et al.* 2010). In recent decades, Brazilian forestry companies have selected for rust resistance traits in their eucalypt breeding programs and planted resistant clones as an economical and effective strategy for management the disease

(Alfenas *et al.* 2009). However, *A. psidii* variants are able to overcome the resistance of eucalypt clones. Five physiological races of *A. psidii* are currently known in Brazil (Almeida *et al.* 2021; Graça *et al.* 2011). Race 5 has the ability to infect a greater number of eucalypt clones than the other. However, *A. psidii* race 1 is the most widespread in the country and it has been widely used for selection of rust-resistant eucalypt clones (Almeida *et al.* 2021). According to genetic diversity studies by Stewart *et al.* (2018), two biotypes of *A. psidii* occur in Brazil: one associated with *Psidium guajava* (genetic cluster C6) and the other with *Eucalyptus* spp. and *S. jambos* (genetic clusters C2/C3). Race 1 of *A. psidii* belongs to the Brazilian biotype associated with eucalypt and rose apple (Stewart *et al.* 2018).

The existence of different biotypes of *A. psidii* challenges the commonly accepted view that the risks of invasive pathogens can be assessed at the species level (Graça *et al.* 2013). Indeed, the invasive biotype prevalent in areas of the Pacific, including Australia, California, China, Hawaii, Indonesia, Japan, and New Caledonia is not the Brazilian biotype but belongs to the ‘C1/C4 genetic cluster (Stewart *et al.* 2018). This pandemic *A. psidii* biotype is having a significant ecological impact on native biodiversity in Australia (Fensham and Radford-Smith, 2021; Winze *et al.* 2019) and New Zealand (Beresford *et al.* 2019). However, there has been little impact of myrtle rust on eucalypt plantations in Australia (Carnegie and Pegg, 2018), but this could change under climate change (Berthon *et al.* 2018) or if a more virulent biotype becomes established. Similarly, the pandemic biotype may pose a threat to Brazil, where it has not been reported as far as we know but has been reported in bordering Columbia (Granados *et al.* 2017). Given that host genetic resistance to a pathogen can be isolate- or biotype-specific (Gill *et al.* 2015, Schulze-Lefert *et al.* 2011), a key question in disease management for biodiversity conservation and forestry is whether selections based on the different biotypes/strains of myrtle rust exhibit non-specific resistance. Isolate specific resistance has been suggested for *A. psidii* from Brazil (Almeida et al. 2021; Graça *et al.* 2011). We here address this question by comparing the susceptibility of *E. globulus* germplasm to isolates of the pandemic biotype of myrtle rust and the most widespread biotype in Brazil (*A. psidii* race 1).

Eucalyptus globulus Labill, native to southeastern Australia (Dutkowski and Potts 1999), has great commercial value to the pulp and paper industry due to its high cellulose and low lignin content and has been widely cultivated in temperate regions of the world (Xavier *et al.* 2007), including Australia, Portugal, Spain and Chile (Eldridge *et al.* 1994; Potts *et al.* 2004, 2014). In Brazil, *E. globulus* is crossed with other species, more adapted to its edaphic-climatic conditions (Xavier *et al.* 2007). In native stands, there is high genetic variability among *E.*

globulus provenances for quantitative traits which has resulted in the partitioning of the species gene pool for breeding purposes into multiple geographic races and subraces (Dutkowski and Potts 1999). Open-pollinated seeds collected from these native races form the basis of breeding programs in many countries where *E. globulus* is being genetically improved mainly for pulp and paper production (Potts *et al.* 2004). However, as disease can limit the cultivation of high-yielding genotypes (Milgate *et al.* 2005; Simeto *et al.* 2020), understanding genetic variability for disease resistance is important for selection of resistant genotypes and successful plantation improvement. *E. globulus* is the major plantation eucalypt in Australia (Potts *et al.* 2014), and while plantations are currently not impacted by myrtle rust, extensive pre-emptive screening of base population and advanced generation families has been undertaken in Australia by controlled inoculation with the pandemic biotype (Butler *et al.* 2016; Freeman *et al.* 2019; Potts *et al.* 2016; Yong *et al.* 2019a). This work has revealed significant family and provenance differences in susceptibility to myrtle rust within *E. globulus*, with highly stable rankings across different screenings (Butler *et al.* 2016; Freeman *et al.* 2019; B. Potts unpublished data). In South America, *A. psidii* has been recorded from commercial plantations of *E. globulus* as early as 2000 in Brazil (region of Guaíba, Rio Grande do Sul, Alfenas *et al.* 2003) and 2003 in Uruguay (Telechea 2003). Later, Xavier *et al.* (2007) evaluating the resistance of seedlings of *E. globulus* (from Chilean landrace provenances) to *A. psidii* infection through artificial inoculations found intraspecific genetic variability in *E. globulus* for resistance to *A. psidii* race 1.

We here use families derived from open-pollinated seed lots collected from native stands in Australia and previously evaluated for seedling susceptibility to the pandemic biotype (Freeman *et al.* 2019) to: (i) evaluate if there is genetic variation in susceptibility among *E. globulus* families and subraces to the common Brazilian race 1 of *A. psidii*; (ii) determine whether resistance to *A. psidii* race 1 and the pandemic biotype found in Australia is positively associated at the family and *E. globulus* subrace levels with previous evaluations of resistance to the pandemic biotype; and (iii) identify new potential sources of *E. globulus* resistance to *A. psidii*.

MATERIAL AND METHODS

Plant material

A total of 56 single-tree, open-pollinated seed lots (here after referred to as ‘families’) from 11 *E. globulus* sub-races from native stands in Australia were analyzed for resistance to race 1 of *A. psidii* (Table 1) at the ‘Clonar Resistencia a Doenças Florestais’ company in Cajuri - Minas Gerais, Brazil. Fifty-four of these families had previously been screened by Dr. Geoff Pegg (Department of Agriculture, Fisheries and Forestry, Queensland, Australia) for resistance to the pandemic biotype of *A. psidii* in Brisbane as part of a larger screening reported in Freeman *et al.* (2019). This is a rare and quite representative seed bank of *E. globulus*. The families were sown in pots (430 mL) containing substrate Tropstrato supplemented with 3 $Kg.m^{-3}$ of simple superphosphate and 3 $Kg.m^{-3}$ of Osmocote (15-09-12) and kept in a greenhouse at 25 °C ± 5 °C and natural light (1 seed lot per pot; Figure 1). Thirty days after sowing, seedlings from the 56 families were pricked out into individual pots of 55 cm^3 with the same substrate and fertilization and kept in the same conditions after sowing. After 120 days from sowing the plants were transplanted into plastic tubes of 290 cm^3 as previously described. Seedlings from the same family were kept together in a single pot or set of tubes. The family positions (whether in pots or in a set of tubes) remained randomized until inoculation.

Inoculum production, inoculation, and evaluation tests

For the fungus multiplication, an inoculum suspension (2 x 10^4 urediniospores/mL) of the isolate UFV 2 (*A. psidii* race 1), containing 0.05% Tween, was sprayed in young leaves of *S. jambos* (rose apple) and the inoculated plants were incubated in a mist-irrigation chamber in the dark for 24 h, and then transferred to a growth chamber at 22°C with a 12- h photoperiod and a light intensity of 130 µmol photons s^{-1} m^{-2} (Ruiz *et al.*, 1989). The newly produced urediniospores were collected in 1.5 mL microtubes and stored at −80 °C, in the mycological collection of the Forest Pathology Laboratory/Bioagro, Department of Plant Pathology, ‘Universidade Federal de Viçosa’, Viçosa, Minas Gerais, Brazil, until inoculation (30 days, approximately). For the resistance screening of *E. globulus* seed lots, the inoculation procedure was performed as previously described for inoculum production in rose apple. The UFV-2 isolate was chosen for inoculations since it represents the predominant *A. psidii* race in Brazil (Almeida et al. 2021).

Two screenings were undertaken (A and B) with different plants after 60 and 75 days of growth, respectively, to minimize the difference in height (cm) between plants of the same seedling lot (mean height ± s.d. - Screening A 19.0 ± 3.3, Screening B 16.6 ± 3.4). Each screening comprised 10 blocks, with the 56 families and two clones - CLR 371 (*E. urophylla*) and CLR 239 (*E. urophylla*) - used as a positive and negative control of inoculation process, respectively - represented once per block and randomly arranged. This meant that a maximum of 20 seedlings of each family and 20 ramets of each clone were screened. Block assembly and plant height measurements were performed on the same day as the first inoculation and the same experimental design was maintained throughout the experiment. In addition, three inoculated plants of *S. jambos* highly susceptible were randomly distributed among the blocks to ensure inoculation efficiency.

Two inoculations were performed (Inoculation 1 and 2) (Supplementary material S1). Resprouted plants (after pruning plants close to the stem base, keeping one branch) from the first inoculation were re-inoculated after 60 days from pruning, but there were less plants assessed as some plants were infected and killed by *Botrytis cinerea*. Following each inoculation, two assessments were undertaken (at 14 days and 21 days post-inoculation). The timing of the last assessment was similar to that undertaken in the Australian screening of the same *E. globulus* families by Freeman *et al.* (2019) which occurred 25 days post-inoculation.

Rust severity was scored on a four-class (0-3) ordered severity scale: 0 = immunity or hypersensitive reaction (HR) type “fleck” or necrotic; 1 = pustules < 0.8 mm in diameter; 2 = pustules from 0.8 to 1.6 mm in diameter; and 3 = pustules >1.6 mm in diameter (Junghans *et al.* 2003b) (Figure S1B). In the present case, plants with immune or HR phenotype were considered resistant (R) with the HR phenotype signaling an incompatible interaction; plants scored as 1 which are considered moderately resistant (MR); plants scored as 2 are considered susceptible (S); and plants scored as 3 are considered highly susceptible (HS). This scoring system is the scale commonly used in Brazil but was slightly different to the five-class numeric system (1-5) used by Pegg (2014) for many Australian studies (Figure S1A), including those with *E. globulus* (Butler *et al.* 2016; Freeman *et al.* 2019). The resistant category (class 0) of Junghans et al. (2003b) system mainly reflects the no reaction category of Butler *et al.* (2016) (their class 1), but with an HR component of their class 2.

Data analysis

The two inoculations on the same plants in Brazil were treated as separate response variables, due to the imbalanced data. For each inoculation, the following traits were studied: i) rust severity score (0-3) averaged across the 14 and 21-day scorings (score_1421); ii) the presence/absence of resistance (R) based on the 21-day scoring, where a severity score of 0 is coded as 1 and others as 0 (pa_res_21); and iii) presence/absence of *B. cinerea* damage (pa_*Botrytis*), where presence (coded as 1) was derived from the absence of records of myrtle rust severity scores in the second inoculation.

Genetic differences in disease susceptibility

The testing of subrace and family within subrace effects was undertaken using PROC GLIMMIX of SAS® and fitting a fully random model with the terms screening (A or B), block within screening (1-10), subrace (n = 11) and family within subrace (n = 56). In the fully random model the variances attributable to screening, block, subrace, family within subrace, and residual were estimated and the variances from the fitted terms tested from zero using a one-tailed likelihood ratio test. These analyses were undertaken for the average rust severity score for each plant (score_1421) and the binary rust immunity (pa_res_21), and binary *B. cinerea* damage (pa_*Botrytis*). The binary analyses were undertaken using a probit link function and BLUPs (Best Linear Unbiased Predictions) obtained on the probit scale.

The narrow-sense heritability (h^2_{op}) of the phenotypic variation within sub-races (that is the proportion due to additive genetic variation) assuming an outcrossing rate of 70% in the open-pollinated families (hence the OP subscript) was calculated as equation 1, following Freeman *et al.* (2019):

$$\hat{h}^2_{op} = \frac{\hat{\sigma}^2_a}{\hat{\sigma}^2_{phen}} = \frac{2.5 * \hat{\sigma}^2_{fam(subrace)}}{\hat{\sigma}^2_{fam(subrace)} + \hat{\sigma}^2_{residual}} \qquad \text{(Eq. 1)}$$

The variance component estimation and subrace BLUPs were obtained from the above fully random model fitted to data without control samples. The BLUPs are presented as deviations from the grand mean. As families were uniquely coded, overall family BLUPs were obtained from a similar model but with the exclusion of the subrace term. These overall family BLUPs were calculated from analyses without control samples but for comparative purposes analyses were also undertaken including the controls for plotting.

Association of susceptibility to the pandemic biotype (Australian screening) versus Brazil biotype (*A. psidii* race 1) (Brazil screening)

For comparison of the Brazil and Australian inoculation data, the data from Freeman *et al.* (2019) were re-analyzed using the same random model(s) fitted to the Brazil data but with the inclusion of an incomplete block term. Analyses were undertaken using just the subset of 54 families from 11 subraces which were in common with the Brazil screening. Variance components were estimated and subrace BLUPs obtained from these analyses for comparison with those obtained from the Brazil data. The Pearsons correlation coefficients were estimated among BLUPs derived from the Brazil screening with BLUPs from the Australian myrtle rust screening and tested from zero using Proc CORR of SAS. Regression analyses were similarly undertaken with the Australian BLUPs used as the predictor for the Brazilian BLUPs using Proc REG and plots produced using Proc SGSCATTER of SAS®

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RESULTS

Screening with *Austropuccinia psidii* race 1 (Brazilian biotype from cluster C2/C3)

A total of 983 seedlings were assessed for rust severity following the first inoculation, but after plants were pruned only 641 seedlings were assessed following the second inoculation due to 34,8% mortality, mainly due to infection on shoots by *B. cinerea*. The average rust severity score increased, and the proportion of resistant seedlings (class = 0) decreased slightly between the 14- and 21-day severity scores (Table 2). Similarly, average severity scores were higher, and the proportion of resistant plants lower in the second than the first inoculation (Table 2), and this trend was still evident even when the same cohort of 641 survivors were compared across inoculations (data not shown). The proportion of plants classified as resistant at day 21 was 0.25 for the first inoculation and 0.16 in the second inoculation, but the proportion of survivors in the second inoculation which also classified as resistant in the first inoculation was only 0.07, which compares with a proportion of 1 for the resistant control clone CLR239R and 0 for the susceptible control clone CLR371S.

In both inoculations, there were highly significant ($P < 0.001$) differences between the *E. globulus* families within subraces for the average severity score (score_1421) and the probit transformed presence of resistance (probit pa_res_21) (Table 3). For both rust response traits, the within subrace heritability estimates were high (0.45 to 0.68), but higher in the first compared with the second inoculation. Significant ($P<0.05$) subrace differences in both traits were detected following the first but not the second inoculation (Table 3). The genetic variation detected in the average rust severity score in the first inoculation was not solely driven by genetic variation in the presence of resistance (pa_res_21 = 1), as significant subrace ($P = 0.003$)

and family within subrace ($P < 0.001$; $h_{op}^2 = 0.39$) variation was also detected for the average severity score (score_1421) in just the cohort of plants with pustules (i.e. those where pa_res_21 = 0). The general reduction in genetic variation at both subrace and family levels following the second inoculation likely reflected differential susceptibility to *B. cinerea* at the subrace ($P<0.05$) and family within subrace ($P<0.001$; $h_{op}^2 = 0.34$) levels (probit pa_*Botrytis*, Table 3). The overall family BLUPs for *B. cinerea* damage (probit pa_*Botrytis*) were significantly negatively associated with those for height prior to inoculation (regression, adjusted $R^2 = 0.14$, $P=0.003$) and positively associated with those for average rust severity in the first inoculation (score_1421 - adjusted $R^2 = 0.10$, $P = 0.011$; Supplementary Figure S2). These were independent effects contributing to *B. cinerea* damage as both predictors were still statistically significant when jointly modeled (adjusted $R^2 = 0.19$; height, $P = 0.010$; score_1421, $P = 0.042$).

Given the confounding associations, coupled with the greater sample size and better expression of genetic variation at the subrace and family levels, we focused on the myrtle rust susceptibility scoring from the first inoculation. As the subrace variance was small, we also focused on the overall family BLUPs for susceptibility to *A. psidii* race 1. These overall family BLUPs for the average severity score (score_1421) and presence of resistance (probit pa_res_21) were highly negatively correlated ($r = -0.94$, $P < 0.001$). Further, despite the confounding factors the overall family BLUPs estimated from the surviving plants after the second inoculation were significantly positively associated with those from the first inoculation for both the average severity score (score_1421; adjusted $R^2 = 0.57$, $P < 0.001$) and the presence of resistance (probit pa_res_21; adjusted $R^2 = 0.44$, $P <0 .001$) (Figure 2).

Based on the overall BLUPs for the average severity score (score_1421), the families least susceptible to race 1 of *A. psidii* in the first inoculation were 7_11 and 7_9 from the Southern Tasmania and 8_1 from St Helens subraces (low values on the x-axis in Figure 3A). In the analysis of the presence of resistance at 21 days after inoculation (probit pa_res_21), the top three families were all from Southern Tasmania (7_11, 7_9, and 7_2; high values on the x-axis in Figure 3B). Family 7_11 was the most resistant family tested as supported by its performance in both the first and second inoculation (Figure 3), but it did not reach the levels exhibited by the resistant control (CLR239R) (Figure 2). In contrast, many *E. globulus* families were as susceptible as the susceptible control (CLR371S) (Figure 2). There was a slight positive association between the overall family BLUPs for rust resistance in inoculation 1 and initial height, but the regression was not statistically significant (i.e. regression coefficient is negative with score_1421 [adjusted $R^2 = 0.03$, $P = 0.090$] and positive with probit pa_res_21 [adjusted $R^2 = 0.01$, $P = 0.202$]). The more resistant *E. globulus* families (7_11 and 8_1) were vigorous and above average in their initial height (Supplementary Figure S3). However consistent with the regression results even within the Southern Tasmania subrace, families of above-average height varied markedly in their rust susceptibility (e.g. families 7_11, 7_2, 7_4, 7_3 to 7_15; Supplementary Materials Figure S3).

Association of susceptibility to the pandemic biotype (Australian screening) versus Brazil biotype race 1 (Brazil screening)

As the scoring systems used in Brazil and Australia were not the same, the comparison of the level of damage in each screening was undertaken using the presence of resistance (Table 4). The comparison between the Australian and Brazilian screening based on the same set of open-pollinated families revealed that regardless as to whether class 2 is included with class 1 as resistant in the Australian screening, there is a higher percentage of plants classified as resistant compared to the Brazilian screening. The percentage of plants recorded as resistant in the Australian screening was 42%, or 58% if class 2 is also included as resistant, compared with 25% in the Brazilian screening (Table 4).

The correlation between overall family BLUPS for *A. psidii* severity scores from a common set of 54 families in response to inoculation 1 with the Brazil race 1 and the pandemic biotype of *A. psidii* was positive and highly significant ($P < 0.001$). The correlation was 0.54 between (i) BLUPs calculated from our average of the 14- and 21-day assessments based on the 4-class severity scale [score_1421] and (ii) the BLUPs for the same families based on the 5-class severity scale scored 25 days post-inoculation in Australia. The correlation based on the BLUPs for presence of resistance on the probit scale was 0.57 if only class 1 [no symptoms] of the Australian data is considered resistant (i.e. our pa_res_21 [immunity and HR] versus class 1 in the Australian data). The correlation was 0.49 when both classes 1 and 2 [no symptoms + HR] of the Australian data are considered resistant. Partialling out the influence of the symptomless response (i.e. Australian presence of resistance = class 1, Fig. S1 A1) resulted in the overall family BLUP correlation for the severity scores dropping from 0.54 to a nonsignificant 0.07 ($P = 0.674$), suggesting the correlations between the two screenings was mainly driven by family variation in the symptomless response (i.e. as shown in A1 of Fig S1). Regardless, selection on family BLUPs for resistance to the pandemic biotype in Australia based on the Freeman *et al.* (2019) data would translate to improvement of family resistance to the Brazil race 1 biotype as assessed in the present study (severity scores - adjusted $R^2 = 0.28$, $P < 0.001$; presence of resistance (Australian class 1), adjusted $R^2 = 0.31$, $P < 0.001$) (Figure 3).

While there is wide scatter in the overall family BLUPs, there are a subset of families predicted to exhibit above average resistance to the Brazil race 1 and the pandemic biotype regardless of susceptibility trait (e.g. 7_11, 7_9, 8_1, 7_2, 7_4, 3_3 and 3_1; Figure 3).

The likelihood ratio test indicated that the subrace variance for the severity score was significant in the Brazil screening (inoculation 1, Table 2) and in the Australian screening, regardless of whether all families or just the common families were used in the analysis (data not shown). The correlation between subrace BLUPs from the Brazil and Australian screenings were positive, for both the average severity score and the presence of resistance. Based on the same set of 54 common families, the highest positive subrace correlation occurred for the presence of resistance (Brazil class 0 and Australian class 1; r = 0.72, P = 0.012). Accordingly, selection of subraces based on the presence of resistance to the pandemic biotype of *A. psidii* in Australia would be expected to result in significant improvement in subrace resistance to *A. psidii* race 1 in Brazil (adjusted $R^2 = 0.47$, P = 0.012; Figure 4). Of the subraces represented by five or more families, the highest resistance to both the pandemic biotype and Brazil race 1 of *A. psidii* occurred in the Southern Tasmania and Flinders Island subraces. However, it was in the Southern Tasmania subrace where the more resistant families were identified (see above).

DISCUSSION

Eucalyptus globulus is clearly genetically variable in seedling resistance to the most widespread race (race 1) of the Brazil biotype of *A. psidii* at the family and subrace levels, with most genetic variation residing within subraces.

Our narrow-sense heritability (h_{op}^2) estimates of the variation in resistance are relatively high (0.45 to 0.68), consistent with results from inoculations with the pandemic biotype in *E. globulus* seedlings (0.63-0.70, Freeman *et al.* 2019; 0.53 Runa 2023). There is a large variation in h_{op}^2 for others eucalypt species for which similar range-wide screenings as such as *Corymbia calophylla* (0.34, Duong *et al.* 2022), *C. citriodora* (0.18 to 0.54, Pegg *et al.* 2014); *C. henryi* (0.43-0.47, Pegg *et al.* 2014); *E. argophloia* (0.24 to 0.63, Lee *et al.* 2015) and *E. cloeziana* (0.24 to 0.63, Lee *et al.* 2015). Taken together, studies to date suggest that the natural gene pools of most eucalypt species are likely to contain genetic variation for resistance to the pandemic biotype of *A. psidii*, which would allow genetic improvement in resistance. Results from our screening suggest this is also likely the case for other *A. psidii* biotypes. Further, the moderate to high heritabilities of disease severity phenotypes also suggest that this genetic variation is readily detectable at the seedling stage of eucalypts using suitably designed artificial inoculation trials.

Our inoculation with Brazil race 1 of *A. psidii* provides evidence for genetic variation in *E. globulus* for the binary expression of resistance (class 0 vs classes 1-3, Fig. S1) as well as pustule development within the susceptible class (classes 1 vs 2 vs 3; Fig. S1). The genetic control of eucalypt resistance to *A. psidii* is complex and likely involves interactions between multiple genes (Butler *et al.* 2016; Mamani *et al.* 2010; Yong *et al.* 2021). Different loci have been implicated in the symptomatic, HR and pustulation responses (Butler *et al.* 2016; Yong *et al.* 2021). A major rust resistance locus, *Ppr1*, was identified in *E. grandis* and validated in partially related or unrelated pedigrees (Junghans *et al.* 2003a; Mamani *et al.* 2010), as well as *E. obliqua* (Yong *et al.* 2021). However, in *E. globulus* Butler *et al.* (2016) reported that four other loci are related to rust resistance: *Ppr2*, *Ppr3*, *Ppr4* and *Ppr5*. These loci mapped to four different linkage groups, none of which overlap with *Ppr1*, with two affecting the binary symptomatic response and two the hypersensitive response (HR) (Butler *et al.* 2016). While Yong *et al.* (2021) did detect some SNP-trait associations for the hypersensitive and pustulation responses in a genome-wide association study of the responses of *E. obliqua* to *A. psidii* inoculation, 79% of the associations detected involved the binary symptomatic response. This binary symptomatic response, which is likely under relatively strong polygenic control, appears to be the primary driver of our positive overall *E. globulus* family BLUP correlation between inoculation responses to the pandemic biotype and Brazil race 1, suggesting a common mechanism of genetic resistance to both biotypes at this level.

As the correlated response we observed between the pandemic and Brazil race 1 biotype of *A. psidii* involves genetic covariation for the presence or absence of a symptomatic response, a nonhost mechanism (*sensu*) may be involved which acts early in the infection cycle (type I mechanism - *sensu* Mysore and Ryu 2004). This mechanism could involve pre-formed physical or chemical barriers that broadly affect the adhesion, germination and appressoria formation of *A. psidii* (Chock 2020), including cuticular wax characteristics and composition (Potts *et al.* 2016; dos Santos *et al.* 2019; Xavier *et al.* 2015) and leaf essential oils (Hsieh *et al.* 2021; Potts *et al.* 2016; Yong *et al.* 2019c). Following inoculation of resistant and susceptible phenotypes of *E. obliqua* and *E. globulus* with the pandemic biotype of *A. psidii*, Yong *et al.* (2019b) found little difference between phenotypes in the number of *A. psidii* germ tubes on the leaf surface. *A. psidii* mainly penetrates the leaf through the cuticle rather than through the stomata (Xavier *et al.* 2015; Yong *et al.* 2019b). However, the greater thickness and amount of cuticular wax in older leaves of a susceptible *E. grandis* clone have been shown to reduce not only fungal germination but also appressorium formation, penetration, and sporulation, suggesting that resistance to *A. psidii* infection may be related to cuticular wax (Xavier *et al.* 2015). While another pre-formed chemical or physical barrier affecting both pathogen biotypes cannot be dismissed, an alternative explanation for the correlated response to infection may be a rapid induced response triggered after penetration of the cuticle, caused by a general pathogen recognition mechanism (pathogen-associated molecular pattern triggered-immunity – PTI, Dodds and Rathjen 2010). Indeed, Butler *et al* (2016) suggest that early activation of such non-host resistance may be a possible mechanism underlying the two loci (*Ppr2* and *Ppr3*) detected in *E. globulus* which affect the presence or absence of a symptomatic response. Nevertheless, the correlated response associated with the presence or absence of a symptomatic response only explains 28 to 31% of the variation in overall family BLUPs following inoculation with the Brazil race 1, raising the possibility that pathogen-specific induced responses involving host variation in recognition mechanisms such as the plant resistance (R) proteins (Naidoo *et al.* 2014), may also contribute to differences among the *E. globulus* families. Such pathogen-specific responses are evident in other eucalypt species, *E. grandis* and *E. urophylla*, from the defeat of the *Ppr1* resistance to *A. psidii* by the Brazil race 5, but not the common race 1 (Almeida *et al.* 2021).

The lower levels of resistance observed following inoculation of *E. globulus* with the Brazilian race 1 compared to the pandemic biotype of *A. psidii* could be due to inherent genetic differences in their virulence on *E. globulus*. Interestingly, Carnegie and Pegg (2018) reported that the pandemic biotype of *A. psidii* has little impact on eucalypt plantations in Australia, which is consistent with the hypothesis that the Brazilian biotype race 1 has a wider spectrum of virulence on *E. globulus* than the pandemic biotype. However, this hypothesis cannot be confirmed unless the experimental conditions (host genotypes and environment conditions) are the same for both pathogen biotypes and host germplasm. Within pathogen variants, genotypic differences in virulence on the same host species is common (Duong *et al.* 2022; van Heerden *et al.* 2005), as exemplified by the different Brazilian races of *A. psidii* (Almeida *et al.* 2021). Environmental differences between the experiments undertaken in Australia and Brazil may also affect the pathogen performance as well as host susceptibility (Barrett *et al.* 2009).

While we did not specifically monitor *B. cinerea* disease severity in our experiment we did assess the consequences of its infection through host mortality after plants were pruned for resprouting and re-inoculation with *A. psidii*. *B. cinerea*-mortality was genetically based and at the family level was to some extent associated with increasing severity of the initial *A. psidii* infection. However, while the *B. cinerea* infection would appear to accentuate the adverse impacts of *A. psidii* infection on host fitness, the exact cause of this effect is unclear. It could be due to a combination of factors including (i) weakened host growth, (ii) more available dead tissue remaining on the pruned host plants, or (iii) the slower resprouting of susceptible families resulting in shorter plants, which are subdominant and normally show more pronounced leaf wetness and thus more favorable to *B. cinerea* infection. Natural coinfection of a host with multiple pathogens (Balmelli *et al.* 2014) for plantation-grown *E. globulus* in Uruguay, is likely a common phenomenon in such environments which are generally favorable for infection (e.g. warm and moist). In these cases, the initial pathogen-host interactions may have positive or negative effects on the subsequent infection depending upon whether systemic susceptibility or defense responses are induced, respectively (Barrett *et al.* 2009). Growth of the generalist, necrotrophic pathogen *Botrytis cinerea*, for example, is better in hosts infected with *Pseudomonas* spp. (Gavrin and Levine 2000). Humidity, relatively lower temperatures, and the presence of rust lesions are optimal microclimatic conditions for the development of *B. cinerea* (Alfenas *et al.* 2009; Romanazzi and Feliziani 2014).

From a breeding perspective, our study argues that genetic improvement of *E. globulus* resistance to the most common *A. psidii* race in Brazil (race 1) is possible, although complete resistance may only be achievable through selection of low-frequency genotypes. With relatively strong genetic control of resistance evident to both biotypes, our results suggest some improvement of resistance to Brazilian race 1 would be achieved by selection based on screenings undertaken with the pandemic biotype, but direct selection based on screenings with the Brazilian race 1 would provide greater genetic gains. The reverse argument applies for the improvement of *E. globulus* for resistance to the pandemic biotype. The severity of *A. psidii* (race 1) infection at the family level was not significantly associated with the initial seedling growth prior to inoculation, suggesting that such selection for resistance would not have an adverse correlated effect on seedling vigor. Further, while there is a general positive trend in the susceptibility of the *E. globulus* families to each biotype/race of *A. psidii*, the relationship is relatively noisy and families which would be classified as the more resistant to the pandemic biotype in Australia exhibit the full range of variation in their susceptibility to race 1 in the Brazilian screening. Of note are the families identified as showing a stable low susceptibility and are partially resistant to both rust biotypes ("stable resistance"). The most resistant *E. globulus* individuals, preferably from the more rust-resistant families, could be vegetatively propagated and further tested in clonal field trials, although we note that genotypes may be lost at this stage, particularly from the Southern Tasmanian subrace (Cañas *et al.* 2004), as well as in the subsequent development of operationally suitable clones (Griffin 2001). The development of rust-resistant seed orchards is another possibility, and likely better suited to Australian propagation systems (Potts *et al.* 2008). In Brazil, intensive eucalypt production forestry has a focus on hybrid breeding and clonal propagation (Mamani *et al.* 2010). While less well-adapted than more subtropical eucalypt species such as *E. grandis*, the temperate *E. globulus* is used in hybrid combination with better adapted species due to its superior pulping characteristics (Assis 2000). However, it is highly susceptible to infection by Brazil race 1 of *A. psidii* (Alfenas *et al.* 2009), emphasizing the importance of our study in indicating sources of myrtle rust resistance in *E. globulus* for possible integration into hybridisation programs. While the present study was based on only a limited selection of native open-pollinated families, the variation among subraces detected provides a guide for subraces which may warrant more intensive sampling for increasing the diversity of *E. globulus* genotypes with enhanced *A. psidii* resistance. Our Brazilian race 1 screening suggests that the southern Tasmanian subrace is one such target. This subrace has performed above average in several screenings with the pandemic biotype (e.g. Freeman *et al.* 2019, Fig. 2a; Yong *et al.* 2019a, Fig. 2a), but is highly diverse containing families that near the extremes of *A. psidii* resistance (e.g. 7_11 versus 7_15), emphasizing the high genetic diversity which may reside within subraces and the importance of family- and individual-level screening. However, the myrtle rust screening of advanced generation material from *E. globulus* breeding programs (Potts *et al.* 2014), would be advantageous to avoid losing gains in traditional breeding objective traits that would occur if base population germplasm were utilized directly.

AUTHORS’ CONTRIBUTIONS

R. F. Almeida produced and managed the seedlings, evaluated the resistance of *Eucalyptus globulus* to *Austropuccinia psidii* (race 1), and collaborated on writing the manuscript. J. V. P. V. Ferreira produced the pathogen inoculum for inoculations. R. F. Alfenas reviewed the manuscript. B. Potts and J. Freeman provided materials, analyzed the data, and participated in interpreting the results. F. R. Fernández provided materials. A. C. Alfenas supervised the experiment and reviewed the manuscript. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study (Brazil screenings) are not publicly available due to confidentiality and privacy constraints but are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

No conflicts of interest are declared by the authors.

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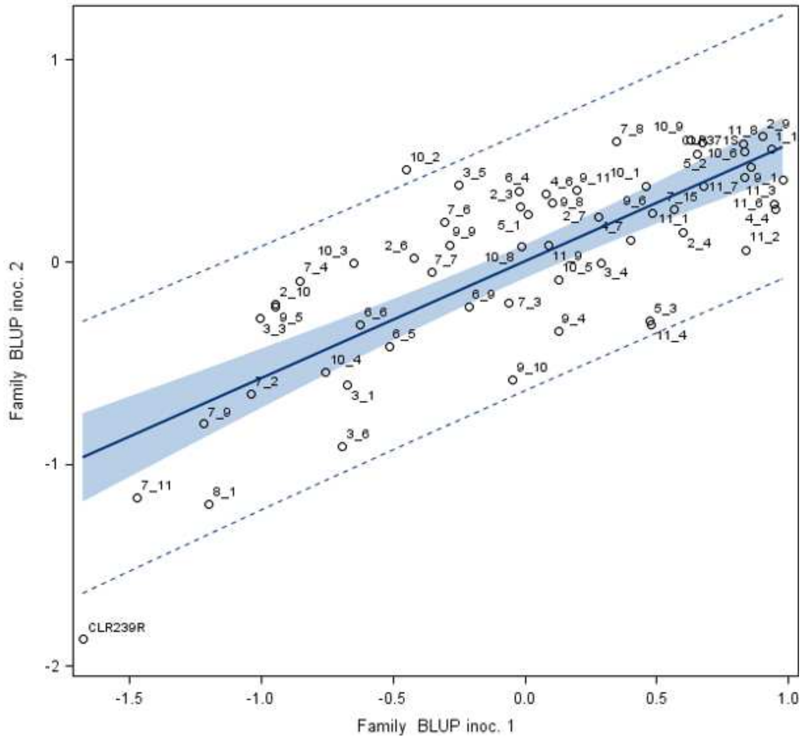
FIGURES AND TABLES

Fig. 1. Seedling development stages, inoculum production, inoculation and severity range of rust reactions for evaluation of *Eucalyptus globulus* resistance to *Austropuccinia psidii*. A- Seedlings of *E. globulus* in pots. B- Inoculum production of *A. psidii* race 1 in *Syzygium jambos*. C- Seedlings of *E. globulus* in tubes. D- Blocks of *E. globulus* inoculated with *A. psidii*. E-

Inoculation. F-G- Susceptible individuals. H- Levels of *A. psidii* severity observed in *E. globulus* seedlings and eucalypt clones used as controls.

A. Average severity score (Brazil race 1)



B. Presence of resistance (Brazil race 1)

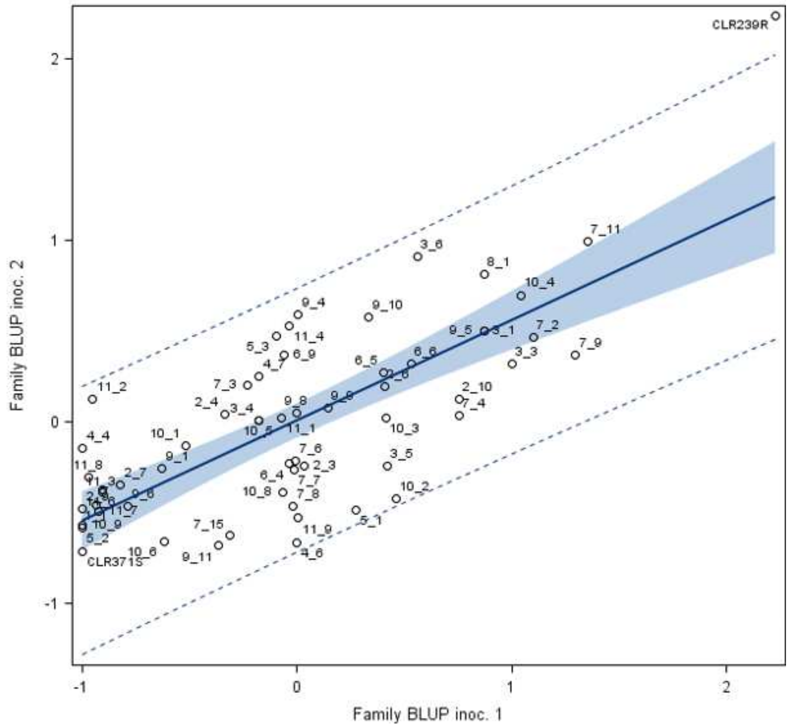
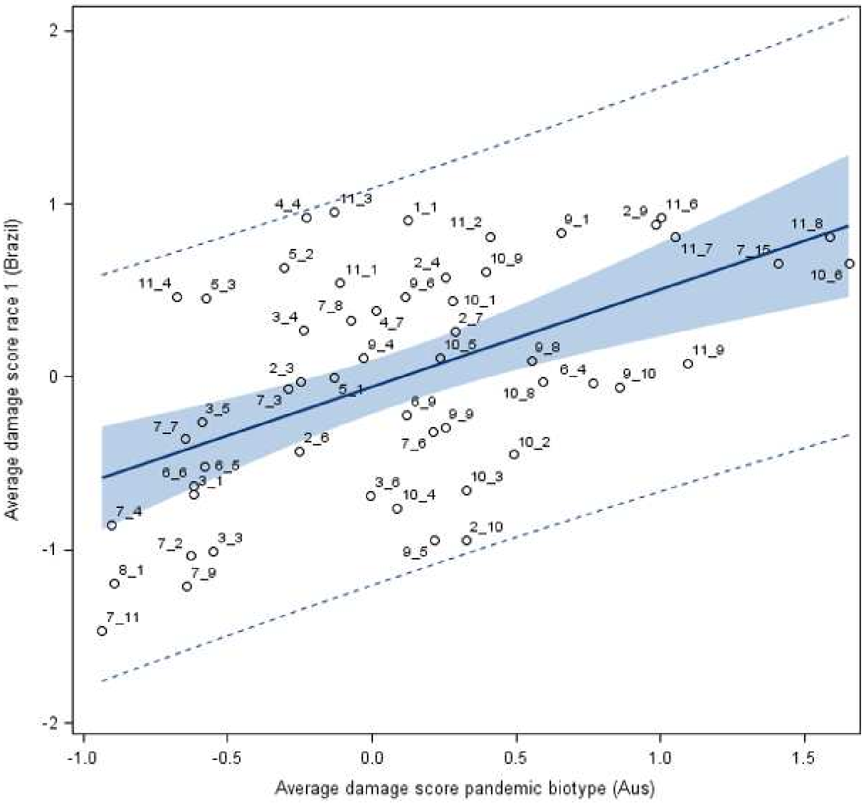


Fig. 2 Regression of the BLUPs for the family myrtle rust severity score in inoculation 2 against the BLUPs from Inoculation 1. Given the randomization of families in the experiment, these family differences are expected to reflect genetic differences. A - Average severity score (score_1421); B- Presence of rust resistance (probit pa_res_21) after inoculation with the Brazil race 1 of *A. psidii*. The family codes are shown with the subrace number indicated in Table 1

as the first number and the second number referring to the specific family within the subrace. BLUPs were derived from the analysis including the resistant (CLR239R) and susceptible (CLR371S) control clones which have been included in the plot.

A. Severity score



B. Presence of resistance

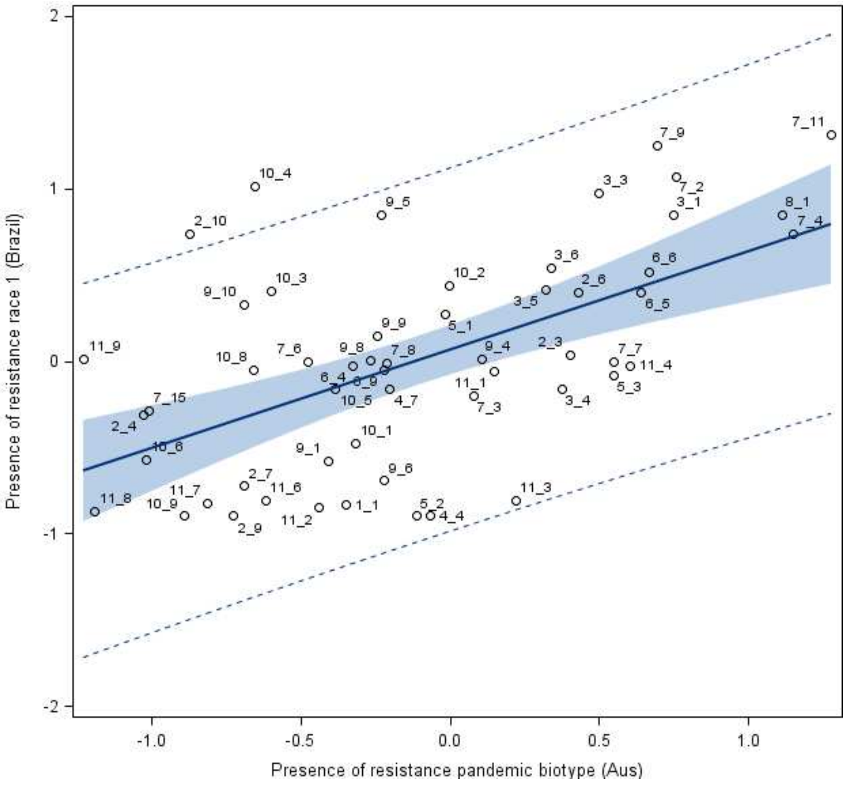


Fig. 3 The regression of the overall family BLUPs for the average severity score in the Brazil inoculation 1 with Brazil rust race 1 plotted against BLUPs from the Australian inoculation

using the pandemic strain of *A. psidii* (Freeman *et al.* 2019). BLUPs derived are drived from A) *A. psidii* severity scores and B) the binary presence of resistance (Brazil scoring class 0 = 1, Australian data class 1 = 1; Fig. S1). For both axes for the severity scores in A low BLUP values are resistant and high values are susceptible, but the reverse is the case for the presence of resistance in B. The family codes are shown with the subrace number indicated in Table 1 as the first number and the second number referring to the specific family within the subrace. The dotted line and shaded shaded band represent the 95% confidence interval for individual and mean predicted values for each observation, respectively.

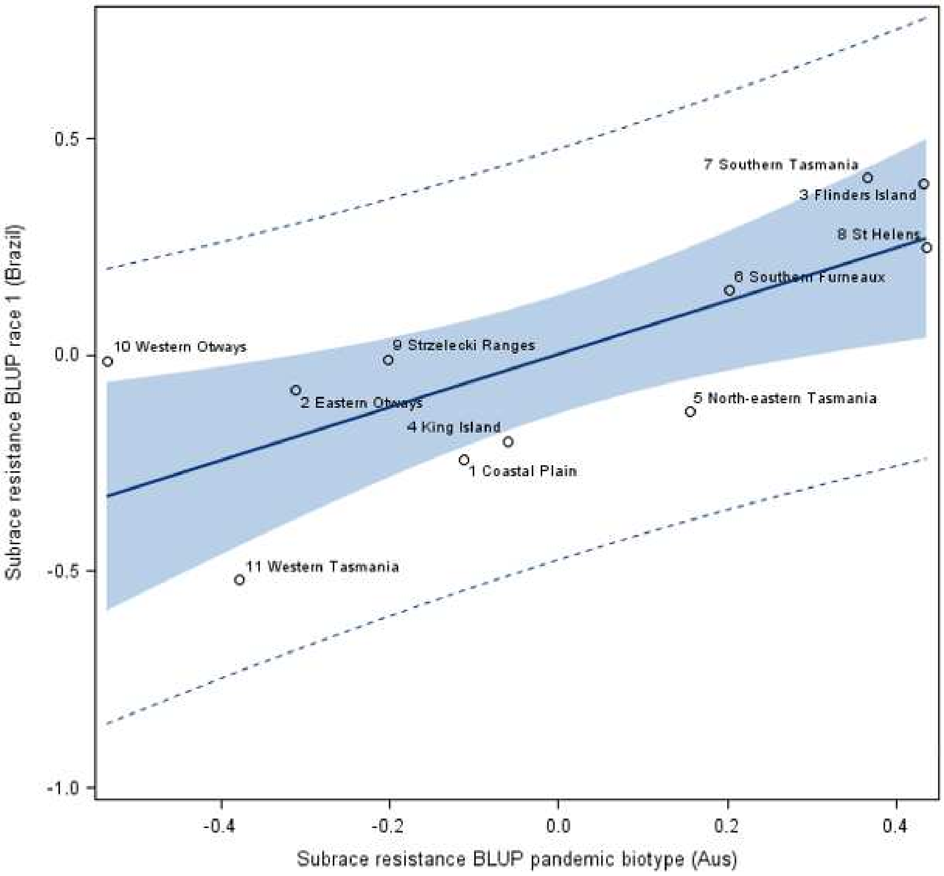


Fig. 4 Subrace BLUPs from the Brazil screening (pa_res_21) regressed against the subrace BLUPs from the Australian screening calculated using a common set of 54 families in each case. Higher values on both axes represent increased *A. psidii* resistance. The dotted line and shaded band represent the 95% confidence interval for individual and mean predicted values for each observation, respectively. The subrace labels and codes correspond to those in Table 1.

Table 1. Number of native open-pollinated seed lots of *Eucalyptus globulus* per subrace inoculated with *Austropuccinia psidii* Brazil race 1 (Brazilian biotype) and compared to the pandemic biotype (Freeman *et al.* 2019)

Subrace	Subrace code	Brazil race 1 (Bra)	Pandemic biotype (Aus)
Mainland			
Strzelecki Ranges	9	8	7
Coastal Plain	1	1	1
Eastern Otways	2	6	6
Western Otways	10	8	8
Bass Strait Islands			
Flinders Island	3	5	5
Southern Furneaux	6	4	4
King Island	4	3	2
Tasmania			
Western Tasmania	11	8	8
North-eastern Tasmania	5	3	3
St Helens	8	1	1
Southern Tasmania	7	9	9
Grand Total		56	54

Table 2. The grand mean severity scores and their standard deviation (s.d.) on the Brazilian 0-3 scale and proportion of plants classified as resistant (pa_res; class = 0) as scored on day 14 and 21 for the 56 *Eucalyptus globulus* families inoculated with myrtle rust, *Austropuccinia psidii* Brazil race 1. Statistics are shown for the plants in the first inoculation and the survivors following the second inoculation undertaken after the previously inoculated plants were pruned and resprouted. Score_1421 is the average of the 14 and 21 day severity scores for each plant

Scoring day/trait	Inoculation 1 (n=983)			Inoculation 2 (n=641)		
	Mean	s.d.	pa_res	Mean	s.d.	pa_res
14	1.70	1.30	0.30	1.95	1.14	0.21
21	1.94	1.28	0.25	2.26	1.12	0.16
average1421	1.82	1.25	0.24	2.10	1.10	0.16

Table 3. Variance components, their standard errors (s.e.), and likelihood ratio test (LRT chisq) and significance (Prob) from zero for each inoculation with Brazil race 1 of *A. psidii*. The estimated narrow-sense heritability of the traits (h_{op}^2) is also shown

rust_score_1421days (mean of day 14 and 21 severity scores)								
	Inoculation 1				Inoculation 2			
Factors	Variance component	s.e.	LRT chisq	Prob	Variance component	s.e.	LRT chisq	Prob
Screening	0.00	0.01	0.4	0.261	0.00	.	0.0	1.000
Block	0.01	0.01	1.5	0.113	0.04	0.02	8.4	0.004
Subrace	0.20	0.14	8.4	0.002	0.04	0.06	0.9	0.348
Family (Subrace)	0.32	0.08	127.9	<0.001	0.20	0.06	37.5	<0.001
Residual	1.06	0.05			0.93	0.06		
h_{op}^2	0.57				0.45			
pa_res_21 (Event='1') probit								
	Inoculation 1				Inoculation 2			
Screening	0.02	0.04	1.2	0.138	0.00	.	0.0	1.000
Block	0.03	0.02	2.2	0.069	0.00	.	0.0	1.000
Subrace	0.15	0.12	4.6	0.016	0.04	0.09	0.4	0.541
Family (Subrace)	0.37	0.11	63.3	<0.001	0.27	0.11	18.1	<0.001
Residual	1				1			
h_{op}^2	0.68				0.54			
pa_Botrytis(infected='1') probit								
	Inoculation 1				Inoculation 2			
Screening					0.19	0.28	16.93	<0.001
Block					0.02	0.02	1.61	0.102
Subrace					0.06	0.05	3.18	0.037
Family (Subrace)					0.16	0.05	29.99	<0.001
Residual					1			
h_{op}^2					0.34			

Table 4. Statistics for the common set of 54 *Eucalyptus globulus* families evaluated in the Australia and Brazil inoculations with myrtle rust, *Austropuccinia psidii*. The Australian inoculation was done with the pandemic biotype and scored on a 1 to 5 scale, 25 days post-inoculation (Freeman *et al*. 2019) The Brazilian inoculation was done with the Brazil race 1 and scored on a 0 to 3 scale 14 and 21 days post-inoculation (Figure S1) Due to the different scoring scales it is the presence of resistance (pa_res) which is most comparable across studies, with the Brazilian resistance severity class 0 expected to include a component of the Australian class 2 response.

Variable	Mean/proportion	s.d.
Australian inoculation (pandemic biotype)		
score_25	2.32	1.35
pa_res (class 1=1)	0.42	
pa_res (class1&2=1)	0.58	
Brazilian inoculation (race 1)		
score_14	1.70	1.30
score_21	1.94	1.28
score_1421	1.82	1.25
pa_res_14 (class 0=1)	0.31	
pa_res_21 (class 0=1)	0.25	

SUPPLEMENTARY MATERIAL

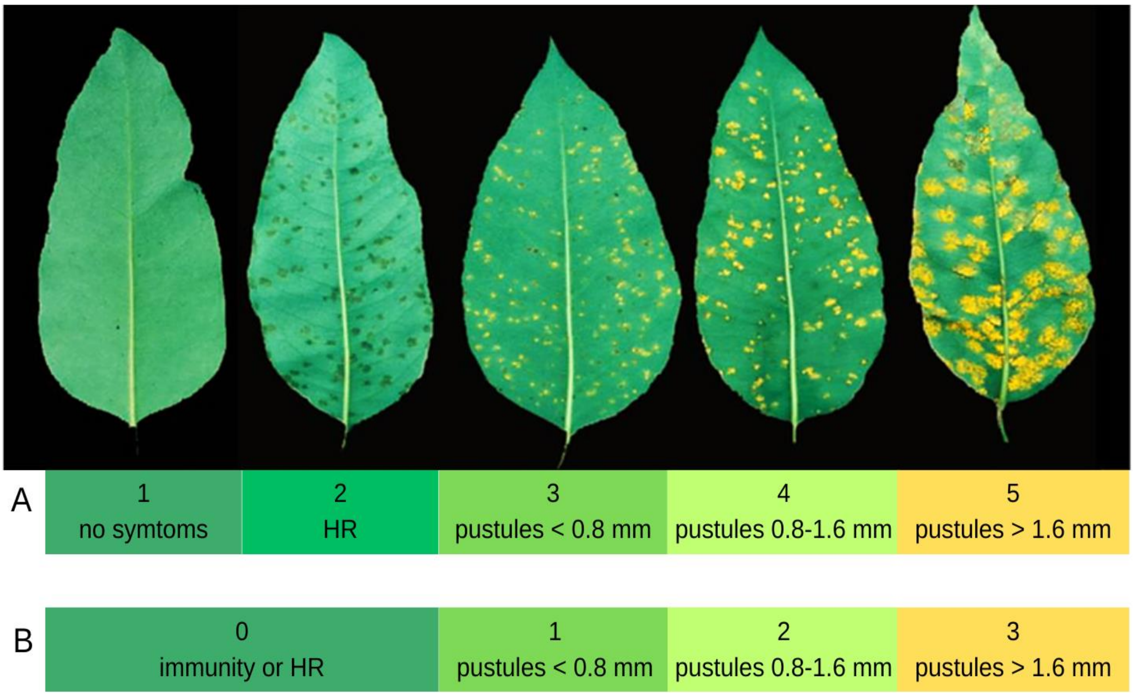


Fig. S1 Correspondence of classes between the Myrtle rust severity scale adapted by Pegg *et al*. (2014) (A) and the original scale of Junghans *et al*. (2003) (B) This correspondence is based on pustule diameter. Class 1 is the asymptomatic class and includes plants with leaves showing no symptoms or the presence of yellow flecking (Pegg *et al*. 2014). Adapted by the senior author.

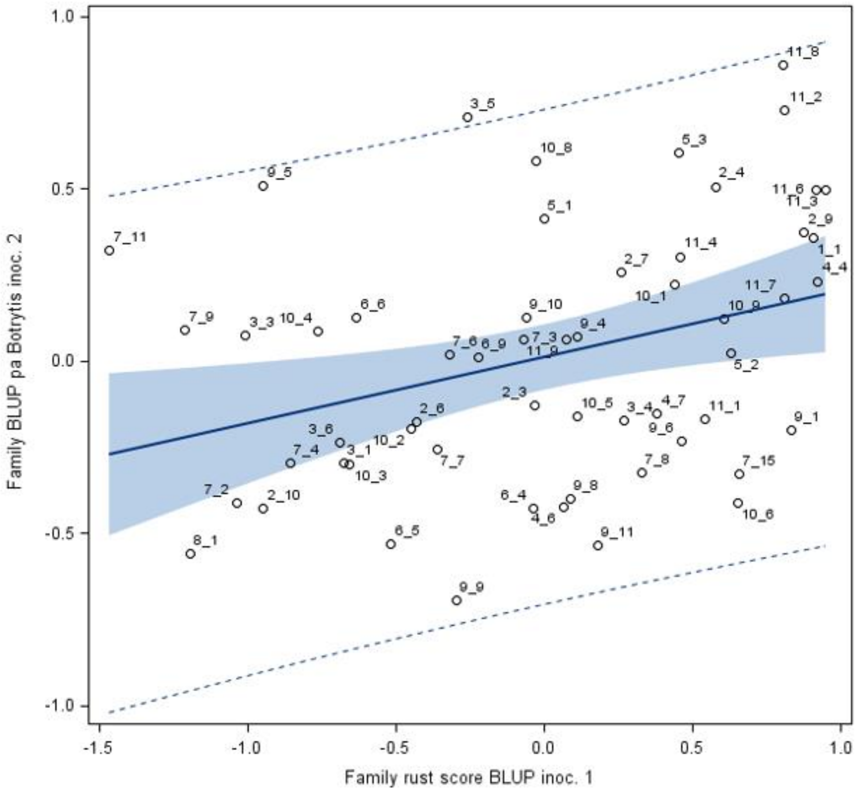


Fig. S2 The regression of the overall family BLUPs for the presence of *Botrytis cinerea* damage as observed in the second inoculation (probit pa_Botrytis) against the family BLUPs for the average *Austropuccinia psidii* severity score (score_1421) in the first inoculation. The family codes are shown with the subrace number indicated in Table 1 as the first number and the second number referring to the specific family within the subrace.

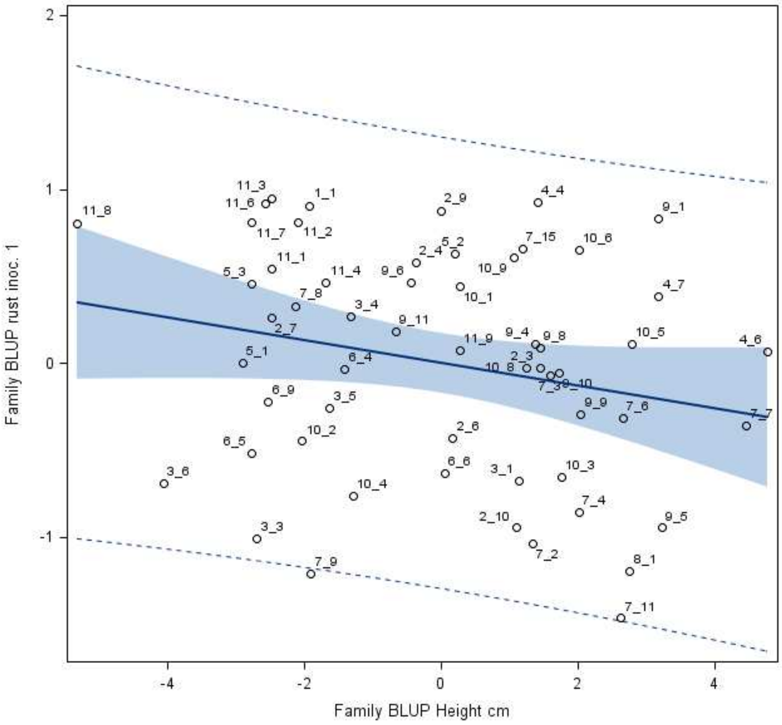


Fig. S3 Regression of overall family BLUPs for the average severity score (score_1421) in the first inoculation on the overall family BLUPs for plant height (cm) just prior to inoculation. The family codes are shown with the subrace number indicated in Table 1 as the first number and the second number referring to the specific family within the subrace.

CHAPER 2 - NO DIRECT OBSERVATIONAL EVIDENCE FOR BASIDIOSPORE-DERIVED INFECTION OF EUCALYPT AND ROSE APPLE (*Syzygium jambos*) BY THE MYRTLE RUST PATHOGEN, *Austropuccinia psidii*, FROM BRAZIL

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ABSTRACT

Myrtle rust caused by *Austropuccinia psidii* is one of the most important diseases affecting eucalypts (*Eucalyptus* spp. and *Corymbia* spp.) in Brazil. Asexual spores of the myrtle pathogen, urediniospores, give rise to infection of young tissues (e.g., leaf, stem, flower, and fruit) of the myrtaceous hosts, frequently in the nursery, during the first year after planting in the field, or following coppice. Symptoms of myrtle rust disease are necrosis and leaf deformation, and death of the apical shoots, while the primary diagnostic signs of myrtle rust disease are yellow urediniospores, which are formed by the myrtle rust pathogen on the infected organs. Occasionally, teliospores are formed in brown pustules, usually at higher temperatures on more mature leaves of susceptible hosts, and these teliospores can geminate to produce basidia that produce basidiospores. Previous studies have demonstrated that genetic recombination in *A. psidii* was associated with basidiospores of the South African and Pandemic biotypes of *A. psidii* on rose apple (*Syzygium jambos*). However, it remains unconfirmed whether basidospore-derived infection and genetic recombination also occurs within the most damaging biotype of *A. psidii* in Brazil. Furthermore, direct observation of basidiospore-associated infection by *A. psidii* has not been previously reported. In the present work, no evidence of basidiospore germination and/or germ tube penetration into the host tissue of eucalypt (*E. urophylla*) and rose apple was found using scanning electron microscopic observations, raising questions about the role of basidiospores in life cycle of *A. psidii* in Brazil.

Keywords: Life cycle, Rust, Sexual reproduction.

INTRODUCTION

Three life cycle stages of the myrtle rust pathogen, *Austropuccinia psidii* (G. Winter) Beenken, are commonly observed: uredinia (II), telia (III), and basidia (IV). The aecial stage (I) was only reported by Figueiredo et al. (1984) following apparent basidiospore-derived infection of rose apple (*Syzygium jambos*) – a host species that is highly susceptible to myrtle rust under controlled environmental conditions. The pycnial/spermagonial stage (0) has not yet been observed for *A. psidii* (Coutinho et al. 1998; Glen et al. 2007). Urediniospores of *A. psidii* are asexual spores formed from uredinia, by simple segmentation of an unbranched dikaryotic hypha that serves as a pedicel (Figueiredo & Passador, 2008). The urediniospore is unicellular, dikaryotic, and generally spherical. Urediniospores (10–20 x 15–25 µm) are slightly echinulated on the external wall, which is believed to help the pathogen adhere to the host surface (Coutinho et al. 1998; Ferreira, 1989). Teliospores, formed on telia, have dark-brown pigmentation, which is possibly due to the accumulation of substances that autoinhibit germination and/or may also be related to the pathogen survival in older tissues of the host under high temperatures or ultraviolet light (Aparecido et al. 2003). Teliospores of *A. psidii* are pedicellate, bicellular, and clavate, may have an apical papilla on the posterior cell wall, with an overall size of 15–18 x 30–60 µm (Coutinho et al. 1998; Ferreira, 1989). During teliospore germination, one or both diploid cells begin to grow. The promycelium that emerges, known as the basidium, is typically ca. 40-70 µm in length. From the basidium, sterigmata are formed that support four hyaline basidiospores, 8-11 µm in diameter (McTaggart et al. 2018), with each basidiospore containing one or two haploid nuclei (Aparecido et al. 2003; Aparecido & Passador, 2014; Coutinho et al. 1998; Ferreira, 1989), which can be heterokaryotic or homokaryotic (McTaggart et al. 2018; Morin et al. 2014).

The biological role of *A. psidii* basidiospores has been investigated since the 1980s. Figueiredo et al. (1984) reported that *A. psidii* basidiospores appear to infect rose apple, with subsequent formation of aeciospores. However, the aforementioned results have since come under question because of potential flaws in the method used ('germinatelio') and the fact that aeciospores are morphologically similar to urediniospores (Hiratsuka, 1973; Morin et al. 2014).

Following basidiospore inoculations in detached leaves of *Agonis flexuosa* and rose apple, Morin et al. (2014) did not observe pycnium formation, and the resulting formation of sori was attributed to contamination by urediniospores because no evidence of sexual recombination as found. More recently, McTaggart et al. (2018) used a suspension *A. psidii* basidiospores (South Africa biotype) separated by vacuum filtration with an 8-µm membrane for inoculations of rose apple that resulted in infections. These authors further noted that the spores resulting from basidiospore infection were genetic recombinants. Based on this evidence, the authors proposed that the genotypic diversity observed in *A. psidii* populations may be attributed to sexual recombination resulting from basidiospore infection.

In previous studies, other authors (Stewart et al. 2018; Graça et al. 2013; Roux et al. 2016) found considerable genotypic diversity within *A. psidii*. Differences in host range and geographic distribution were detected among *A. psidii* populations in different countries, leading to the determination of at least four biotypes of the pathogen: a biotype in Brazil/Uruguay which infects eucalypts (*Eucalyptus*) and rose apple (genetic clusters C2/C3); a biotype Brazil that infects guava (*Psidium guava*) and Brazilian guava (*P. guineense*) (genetic cluster C6); a pandemic biotype with a wide host range and broad geographic distribution (genetic clusters C1/C4), and the other biotype that occurs only in South Africa (Roux et al. 2016; Stewart et al. 2018). It is important to note that the biotype that infects eucalypt/rose apple in Brazil contains the UFV-2 isolate (race 1) of *A. psidii*, predominant in Brazil (Almeida et al. 2021). Additional biological and physiological behaviors of each biotype have not been well characterized.

McTaggart et al. (2018) previously confirmed that *A. psidii* can complete its life cycle in a single host (autoecious), but with a hypothetical life cycle involving only basidiospore-derived hyphae anastomosing within host tissue to form dikaryotic hymenium that produces uredinia and/or telia, depending on the environmental conditions. In this situation, aecial and pycnial/spermagonial stages do not occur according to the aforementioned authors. More recently, McTaggart et al. (2020) and Ferrarezi et al. (2022) further demonstrated the role of basidiospores in sexual reproduction of *A. psidii* infecting hosts of the Myrtaceae family.

Despite previous work, the role of basidiospores in infection by *A. psidii* has not yet been characterized for the dominant *A. psidii* biotype that causes myrtle rust of eucalypts and rose apple in Brazil. For example: a) Do basidiospores of a Brazilian biotype of *A. psidii* infect eucalypts?; b) How does dikaryotization occur in *A. psidii*?; c) Does *A. psidii* form pycnia/spermagonia and/or aecia in eucalypt tissues?; and d) What is the role of *A. psidii* basidiospore-derived hyphal anastomosis within eucalypt tissues? To better understand the potential role basidiospores of in the sexual cycle, genetic recombination, and genotypic diversity of *A. psidii*, this work uses scanning electron microscopy to examine potential basidiospore-associated infectivity of *A. psidii* in eucalypt and rose apple.

MATERIAL AND METHODS

Inoculum multiplication

The UFV-2 isolate of *A. psidii* (race 1, a representative of the biotype from eucalypt/rose apple in Brazil/Uruguay, C2/C3 genetic cluster), which was collected from young plantations of *E. grandis* in Itapetininga, SP, Brazil (Junghans et al. 2003; Xavier, 2002), was multiplied on young leaves of rose apple at 22° C (Ruiz et al. 1989). Subsequently, newly produced urediniospores were collected and stored in 1.5-mL microtubes at -80 °C for later use, within 30 days after multiplication (Graça et al. 2011).

Teliospore production

Young, rooted cuttings of *E. urophylla* (clone CLR371, susceptible to myrtle rust) (Figure 1A) were grown in 2-L, plastic bags, containing Tropstrato® (VidaVerde, Mogi Mirim, SP, Brazil) substrate supplemented with 3 kg m^{-3} of simple superphosphate and 3 kg m^{-3} of Osmocote® (15-09-12) (Bloomington Brands, LLC, Bloomington, IN, USA). The cuttings maintained in a greenhouse at 25 °C (± 5 °C) until they reached an optimal growth stage for inoculations. Plants were inoculated with urediniospores to allow subsequent production of teliospores (Ruiz et al. 1989).

Five to 10 rooted cuttings of *E. urophylla* clone CLR371 were inoculated with a suspension (2×10^4 spores mL^{-1}) of *A. psidii* urediniospores prepared with autoclaved distilled water and 0.05% Tween 20®. Young leaves were uniformly atomized with an inoculum suspension on both (adaxial and abaxial) leaf sides, using an electric compressor-driven atomizer. After inoculation, the plants were incubated in a mist chamber, in the dark at 25 ± 2 °C, for 24 h. After incubation, inoculated plants were transferred to a growth chamber at 25 ± 2 °C and a 12-h photoperiod with an intensity of 110 μmol photons s^{-1} m^{-2} (Ruiz et al. 1989) until teliospores were produced (ca. 20 days after inoculation; dai) (Figure 1A-B).

Basidiospore production

In the first assay, pieces of eucalypt leaves, containing predominantly the teliospore stage of myrtle rust (Figure 1B), were attached with adhesive tape onto the upper, inside lid of a Petri dish (100 x 20 mm) (Figure 1C). A volume of 2 mL of autoclaved, distilled water was added into bottom of the Petri dish to collect the basidiospores produced from the germinated teliospores that were attached to the underside of the dish lid (Figure 1C). The set was sealed with Parafilm® and maintained at 22 °C for 48 h in the dark. After the incubation period, 5 µL Tween 20® (0.05%) was added and homogenously mixed into the basidiospore-containing suspension (Figure 1D). The suspension was vacuum filtered at maximum pressure (695 mm Hg) using an 8-µm Millipore® membrane (Figure 1E) (McTaggart et al. 2018). An aliquot of the basidiospore suspension was examined in a light microscope to confirm the purity of the basidiospore inoculum, and this assay was replicated. In the purity assays, only one immature urediniospores was observed in the post-filtration, basidiospore suspension. Therefore, the first assay was repeated using a 10 µm Millipore® membrane in a low-pressure vacuum system (27.3 mm Hg).

A second assay was performed due to the low concentration of basidiospores obtained after filtering the suspension in the vacuum system. Eucalypt leaves containing predominantly the teliospore stage of myrtle rust (Figure 1B) were detached and brushed with a sterile, soft bristle brush to facilitate the collection of basidiospores into the Petri dishes (150 x 25 mm) (Figure 2A). Subsequently, 5 mL of distilled water was added into each Petri dish. Open Petri dishes were placed inside a Gerbox® and sealed with Parafilm® to form a moist chamber. The set was kept in the dark at 22 °C for 48 h for teliospore germination to facilitate production of a higher concentration of basidiospores. After incubation (Figure 2B), the suspension, containing a mixture of spores (urediniospores, teliospores, and basidiospores) was mixed with Tween 20® (0.05%) and used for inoculation. This test was repeated once.

A third assay was performed to analyze *in vivo* basidiospore production (Figure 2C). Plants containing teliospores (*E. urophylla*, clone CLR371) were individually placed within plastic bags that were moistened inside with water to form a moist chamber (Figure 2D). The plants were incubated in the dark for 48 h at 22 °C. After incubation, the leaves of each plant were analyzed under stereo light and scanning electron microscopes. The plants were returned to the growth chamber at 22 °C under a 12-h photoperiod with 110 µmol photons s^{-1} m^{-2} light intensity to monitor basidiospore germination directly on the leaf surface.

Inoculation of *Austropuccinia psidii* basidiospores in *E. urophylla* and *S. jambos*

In first inoculation assay, ca. 10 µL of the basidiospore suspension was deposited on pre-designated sites on young leaves of eucalypt (*E. urophylla,* clone CLR371) and rose apple (Figure 3A-B). In addition, 10 µL of the urediniospore suspension was deposited on pre-designated sites on at least seven young leaves of each of the two hosts as a control. The inoculated plants were kept in the dark within two separate mist-irrigation chambers equipped with a portable atomizer (Figure 3C) for 24 h at 25 ± 2 °C. After incubation, the plants were transferred to two separate growth chambers at 22 °C and a 12-h photoperiod with 110 µmol photons s^{-1} m^{-2} light intensity (Ruiz et al. 1989).

For the second inoculation assay, approximately 10 µL of the spore mixture (containing basidiospores, urediniospores and teliospores) was inoculated onto young leaves of both hosts: *E. urophylla* (clone CLR371) and rose apple.

Analysis of inoculation with *Austropuccinia psidii* basidiospores

To obtain evidence for adhesion and germination of *A. psidii* basidiospores and subsequent basidiospore-derived infection, observations and micrography were conducted on leaf samples using a scanning electron microscope (SEM; LEO, model 1430VP). For these observations, samples of the inoculated leaves from both eucalypt and rose apple plants were collected at 1, 6, 12, 18, 24, and 48 h after inoculation (hai). The leaf samples collected from each time point were dried and kept in a desiccator, containing silica gel until analysis within 30 days. The samples were mounted on aluminum stubs and gold-plated using a sputter coater coupled with a freeze-drying unit (Balzers, FDU010). Subsequently, the samples were examined and photographed under a SEM operating at 12-15 kV. A stub with two leaf samples - one from eucalypt and rose apple - was examined for each sampling time.

In addition, at 1, 12, 18, 24, 36, and 48 hai, ca. 50 µL of the remaining inoculation suspension was collected from the Petri dish, which was incubated under the same conditions as the seedlings. The aliquot was then examined under a light microscope to assess the germination of basidiospores *in vitro*, submerged in water.

RESULTS

Inoculation with filtered basidiospore suspension

In the first assay, a low concentration of *A. psidii* basidiospores was obtained after filtration of the spore suspension that was collected in the Petri dish under incubated teliospores (Figure 4A). At 14 dai, a pustule containing *A. psidii* urediniospores and teliospores was detected within the demarcated area that was inoculated on a rose apple leaf (Figure 4B-C). However, even after 21 dai, no evidence was observed for basidiospore-derived infection in the inoculated eucalypt leaves.

When the assay was repeated, the presence of an immature urediniospores was observed in the basidiospore suspension, even after filtering the suspension through an 8-µm membrane. It is important to note that the collection of basidiospores through the 8-µm membrane was achievable only under the maximum vacuum system pressure (695 mm Hg). This elevated pressure likely compromised the integrity of the membrane and/or facilitated the passage of spores slightly larger than the basidiospores. This situation also suggests that the rust symptoms observed previously (Figure 4B) may have occurred due to urediniospore-derived infection. Consequently, for subsequent trials, the vacuum pressure was reduced and a 10-µm membrane was utilized to ensure the controlled filtration to allow passage of basidiospores.

No contamination by immature urediniospores was detected in the initial basidiospore suspension, nor in the aliquots periodically collected post-inoculation to monitor basidiospore germination in water (Figure 4D). Basidiospore germination was not observed in water, even after 48 hours of incubation, as confirmed by light microscopy analysis (Figure 4D). Ten basidiospores were recorded at each time point to verify the presence or absence of germination. Furthermore, no rust symptoms (Figure 4E-F) and no germinated basidiospores (Figure 5A-B) were detected on the inoculated young leaves of eucalypt and rose apple, as confirmed by subsequent scanning electron microscopy analysis. In contrast, urediniospore-derived infections were observed in the control conditions (Figure 4G-H).

Inoculation of a spore mixture

In the second assay, *A. psidii* pustules were formed on eucalypt leaves (Figure 6A) but not on rose apple leaves (Figure 6B-C), even 21 dai. However, no germinated basidiospores (out of five identified) were observed on any leaves sampled over time in both hosts (Figure 7A and 7C). In contrast, germination of urediniospores and subsequent germ tube penetration was observed by scanning electron microscopy (Figure 7B and 7D).

When the assay was repeated, *A. psidii* infection was observed in the delimited inoculation areas on leaves of both eucalypt and rose apple at 9 dai (Figure 6D-G). However, again no germination was observed for any of the 26 basidiospores observed on the leaf surface of both hosts (six basidiospores on rose apple and 20 basidiospores on eucalypt) (Figure 7E-H).

For this reason, *A. psidii* infections in this assay were attributed to urediniospores, but not basidiospores, for which germination was not observed.

***In vivo* basidiospore production**

Observation of eucalypt leaves with teliospores incubated for 48 h in Petri dishes (first assay) under a stereoscopic light microscope revealed germination of basidiospores and formation of hyphal connections between pustules (Figure 8A-B). These connections were much more evident on plants with teliospores, incubated in a moist chamber. Analysis of infected tissues under a scanning electron microscope revealed germination of basidiospores and fusion between germinating hyphae of adjacent pustules (Figure 9A-C). The plants were maintained in a growth chamber at 22 °C, but after 5 to 10 days, eucalypt leaves containing teliospores and basidiospores were intensely colonized by hyperparasites and the leaves became necrotic, which prevented continued observations (Figure 8C-D).

DISCUSSION

In our assays, no evidence of basidiospore-derived infection by *A. psidii* was observed on epidermal cells of eucalypt and rose apple. Morin et al. (2014) also did not observe evidence of basidiospore penetration into leaf cells of *Agonis flexuosa* and rose apple during several tests with an Australian biotype of *A. psidii* (presumed to be the Pandemic biotype). Morin et al. (2014) also inoculated a teliospore suspension onto *A. flexuosa,* but, for both cases, the resulting uredinial sori were attributed to urediniospores contaminating in the inoculum suspension.

To resolve the problem of inoculum contamination with urediniospores, McTaggart et al. (2018) filtered the spore suspension of a South African biotype of *A. psidii* using a membrane with 8-µm pores coupled to a vacuum system, before subsequently inoculating rose apple. Following the methodology of McTaggart et al. (2018), we conducted an experiment to investigate the success of pure basidiospore infection in rose apple and eucalypt. However, scanning electron microscopy observations did not yield any evidence to support the basidiospore-derived infection by *A. psidii*.

If there is no germination and penetration of basidiospores in the tested myrtaceous hosts, what is the role of potentially recombinant basidiospores in the genotypic diversity reported for *A. psidii*? Genotypic analysis of sori that resulted from basidiospore inoculation of rose apple (McTaggart et al. 2018) revealed 11 multi-locus genotypes that differed from the parental rust genotype. McTaggart et al. (2020 identified numerous recombinant multi-locus genotypes of *A. psidii* with unlinked loci, suggesting possible sexual reproduction of the myrtle rust pathogen from the observed teliospores and subsequent basidiospores (McTaggart et al. 2020). Using only microsatellite analyses, Stewart et al. (2018) identified 23 unique multi-locus genotypes among *A. psidii* isolates collected in Brazil, Costa Rica, Jamaica, Mexico, Puerto Rico, Uruguay, and the USA (Graça et al. 2013). These unique multi-locus genotypes were categorized into nine genetic clusters of *A. psidii* (Stewart et al. 2018). The C1/C4 genetic clusters was defined as the 'pandemic biotype' of rust, which has a wide host range and broad geographic distribution. The C2/C3 genetic clusters constitute the biotype that infects eucalypt and rose apple in Brazil with genotypes of C2 also occurring on eucalypt in Uruguay, and the C6 genetic cluster that infects guava and Brazilian guava in Brazil. In addition to the observed genetic diversity, differences in host associations and climatic conditions favorable to the occurrence of each biotype were also categorized and mapped (Stewart et al. 2018).

Despite the observed diversity among *A. psidii* populations, the source of this variation is not well characterized. How much of the genetic variation in *A. psidii* is attributed to sexual reproduction and recombination events, as suggested by the previous studies with rose apple (McTaggart et al. 2018; McTaggart et al. 2020)? New genotypes of *A. psidii* formed by sexual reproduction could contribute to evolutionary processes in the myrtle rust pathogen that allow adaptions that facilitate establishment in new geographic areas and/or overcoming the constitutive or induced resistance of a host, as observed in recent years (Graça et al. 2011; Almeida et al. 2021). In this study, we found no direct evidence of sexual reproduction via basidiospore-derived infection by *A. psidii* race 1 (Brazilian biotype) in eucalypt and rose apple. For this reason, we cannot confirm the role of basidiospore-derived infection and of sexual reproduction of *A. psidii* in creating the genotypic, biological, and physiological variation observed among local populations of *A. psidii* (Almeida et al. 2021; Graça et al. 2011; Stewart et al. 2018; Xavier, 2002). However, the apparent anastomosis of basidiospore-derived germtubes of *A. psidii* from separate pustules raises the possibility of a mechanism for sexual recombination on rare occasions before leaf infection, but this mechanism was not confirmed.

One consideration is that the environmental conditions evaluated in this study were based on the optimal parameters for infection by the pathogen's urediniospores in eucalypt and rose apple, which may not align with the optimal conditions for basidiospore infection in these same hosts. Additionally, although eucalypt and rose applegenotypes are recognized as suitable hosts for *A. psidii* (Alfenas et al., 2009; Ruiz et al., 1989), the possibility that other hosts are involved in a heteroecious life cycle of the pathogen cannot be excluded. From a pathogen-centric perspective, if sexual reproduction of *A. psidii* occurs, it appears to be a rare event.

As recently reported (Tobias et al. 2021), the haploid genome of *A. psidii* is substantial in size (ca. 1 Gb) and is comprised of 90% transposable elements (TE), which suggests a limitation in the pathogen's capacity to defend against detrimental effects of TEs through recombination during meiosis and sexual reproduction (Tobias et al. 2021). In addition, Ferrarezi et al. (2022) suggested that the *A. psidii* mating system is tetrapolar which could result in more complex and less frequent sexual reproduction. In the tetrapolar mating system, sexual reproduction depends on two distinct levels of compatibility: the initial level determined by pheromones and pheromone receptors that mediate cell-to-cell recognition (hyphal fusion), and the subsequent level dependent on a heterodimeric homeodomain transcription factor (HD1/HD2) that controls post-mating sexual development, active exclusively in the dikaryon stage (Bakkeren & Kronstad, 1994; Coelho et al. 2008).

Consistent with the hypothesis of McTaggart et al. (2018), our results indicate that *A. psidii* basidiospores can germinate (Figure 8) and that fusion appears to occur between different basidiospore-derived hyphae (Figure 9), surpassing the first level of sexual compatibility. However, contrary to the findings of Morin et al. (2014), germinated basidiospores were observed exclusively in incubation systems involving plant tissue. Evaluating the post-germination process of basidiospores in plant tissues using scanning electron microscopy presents challenges due to the overwhelming colonization by rust mycoparasites and leaf necrosis that commonly occurs at the end of the myrtle rust disease cycle on host tissue (Figure 8). For this reason, we could not follow teliospore/basidiospore production and subsequent development at later stages of myrtle rust to further confirm that basidiospore-derived infections do not play a major role in sexual recombination of *A. psidii* on eucalypt and rose apple in Brazil.

In conclusion, analysis by scanning electron microscopy shows no evidence of basidiospore-derived infection by *A. psidii* race 1 (biotype that infect eucalypt/rose apple in Brazil) in eucalypt and rose apple. Based on these observations, our results provide no support for basidiospore-derived sexual reproduction/genetic recombination of *A. psidii* as a major source of the genotypic variation observed among myrtle rust pathogen populations in Brazil.

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DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to confidentiality and privacy constraints but are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

No conflicts of interest are declared by the authors.

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FIGURES

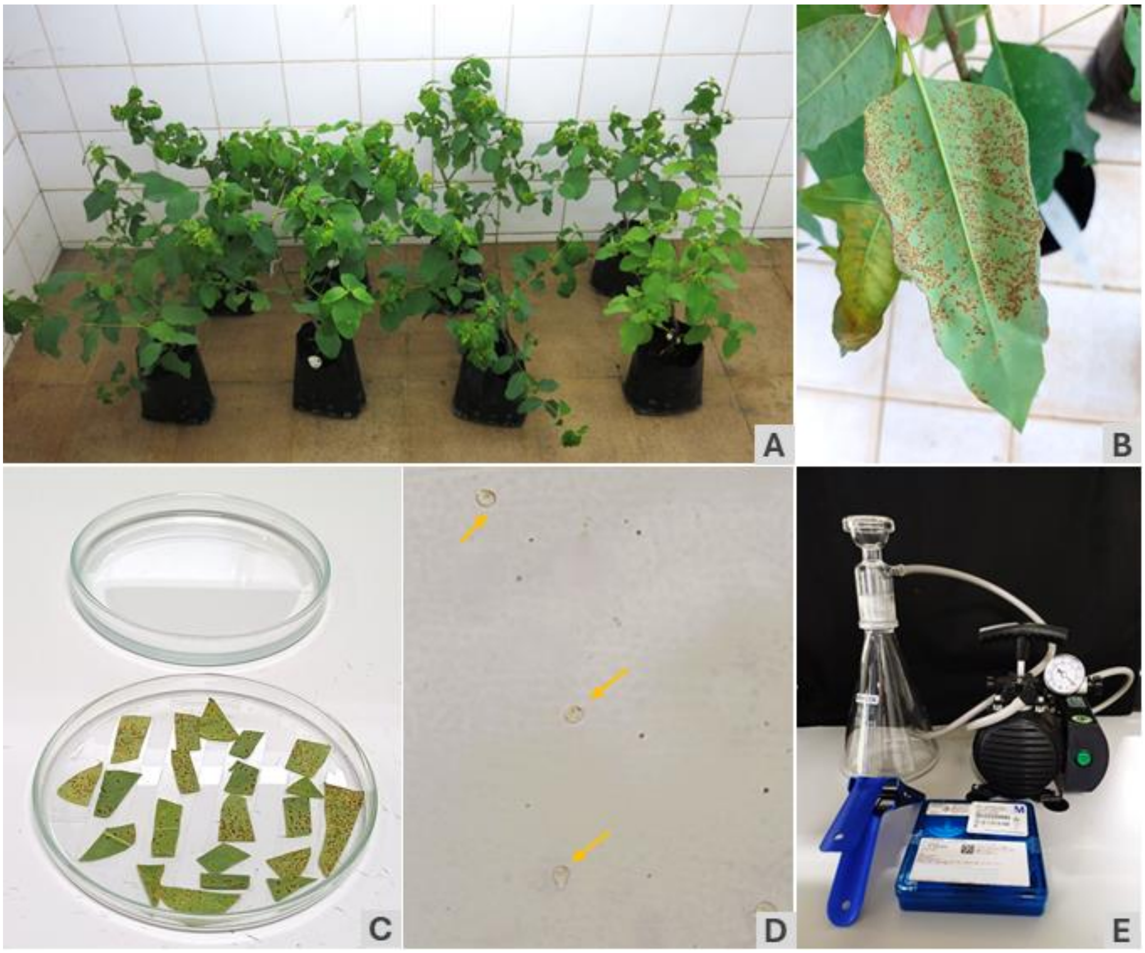

Figure 1. Teliospore production and incubation with basidiospore filtration system used for *Austropuccinia psidii* (race 1) inoculations of eucalypt (*Eucalyptus urophylla* clone CLR371) and *Syzygium jambos*. A-B- Teliospore production on eucalypt; C-E- First assay; C- Incubation of eucalypt leaf fragments in a Petri dish containing distilled water; D- Basidiospores of *A. psidii* in suspension before filtration, observed under the optical microscope; E- Vacuum filtration system with a Millipore® membrane (8-µm pore).

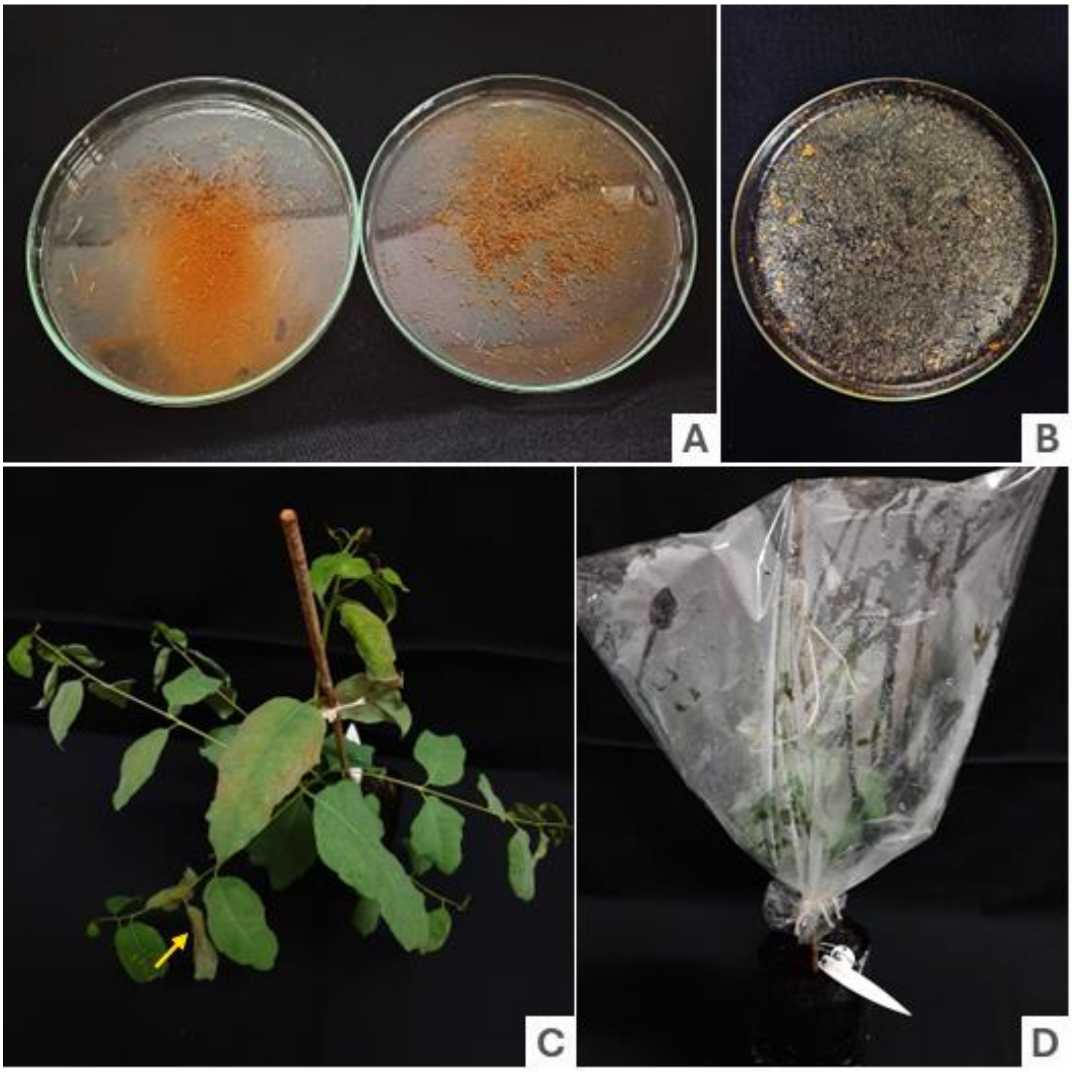

Figure 2. *In vitro* and *in vivo* incubation of *Austropuccinia psidii* (race 1) teliospores. A-B- Second assay. A- Teliospores freshly collected from eucalypt (*Eucalyptus urophylla* clone CLR371) leaves; B- Petri dishes with spores after incubation for 48 hours, in the dark; C-D. Third assay; C- Plants with teliospores; D- Plant with teliospores into a humid chamber system made with a moistened plastic bag.

Figure 3. Inoculation of eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) with a suspension containing basidiospores of *Austropuccinia psidii* (race 1). A- Young eucalypt leaves inoculated in previously demarcated sites; B- Young rose apple leaves inoculated in previously demarcated sites; C- Eucalypt plant in an incubation chamber with leaves covered by a fine mist with evidence of the spore suspension mixed with Tween 20® placed at the inoculation site; D- Plants inoculated in a growth chamber at 22 °C.

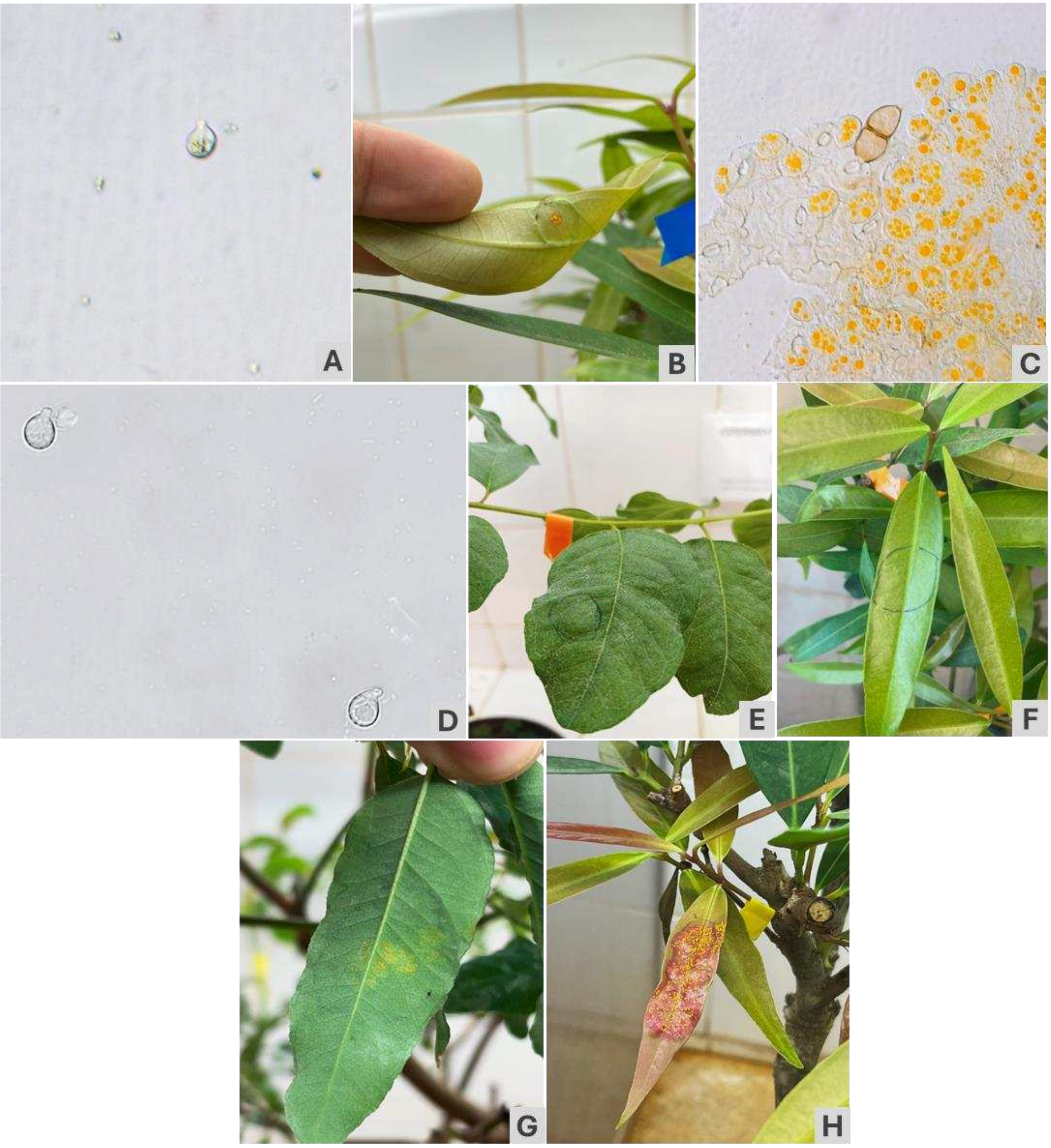

Figure 4. Results of inoculations with *Austropuccinia psidii* (race 1) basidiospores on eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) in first assay. A- Basidiospore from inoculation suspension observed under an optical microscope; B- Sori resulting from inoculation of rose apple leaves; C- Urediniospores and teliospores resulting from inoculation, observed under an optical microscope; D- Basidiospores in purified suspension used to inoculation, under an optical microscope; E-F- Absence of rust symptoms after inoculation of basidiospores in eucalypt leaves and rose apple leaves, respectively; G-H- Presence of rust symptoms after inoculation of urediniospores in eucalypt leaves and rose apple leaves, respectively

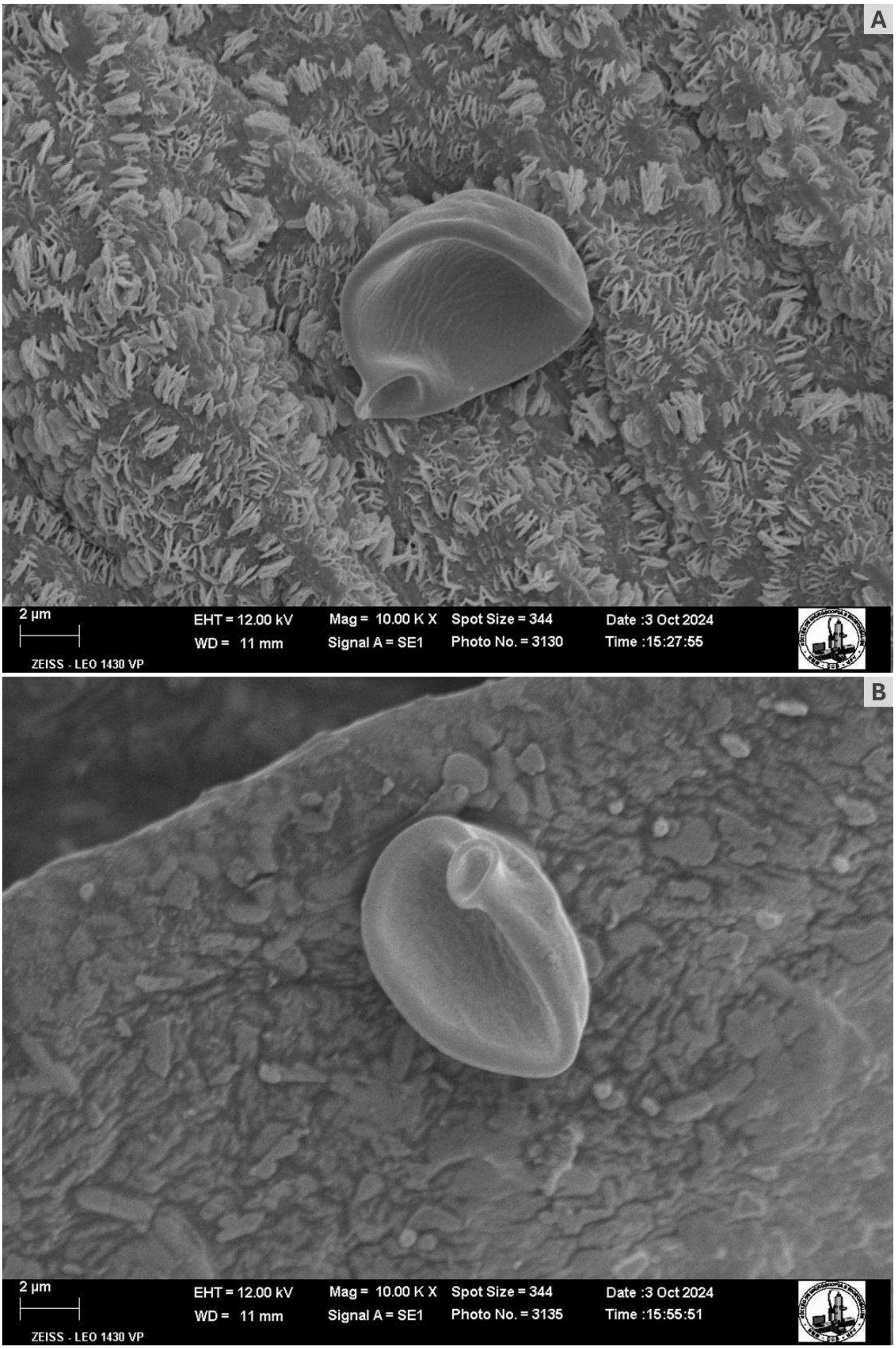

Figure 5. Scanning electron microscopy of eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) leaves inoculated with basidiospores of *Austropuccinia psidii* (race 1). A- Basidiospore on eucalypt leaf; B- Basidiospore in rose apple leaf.

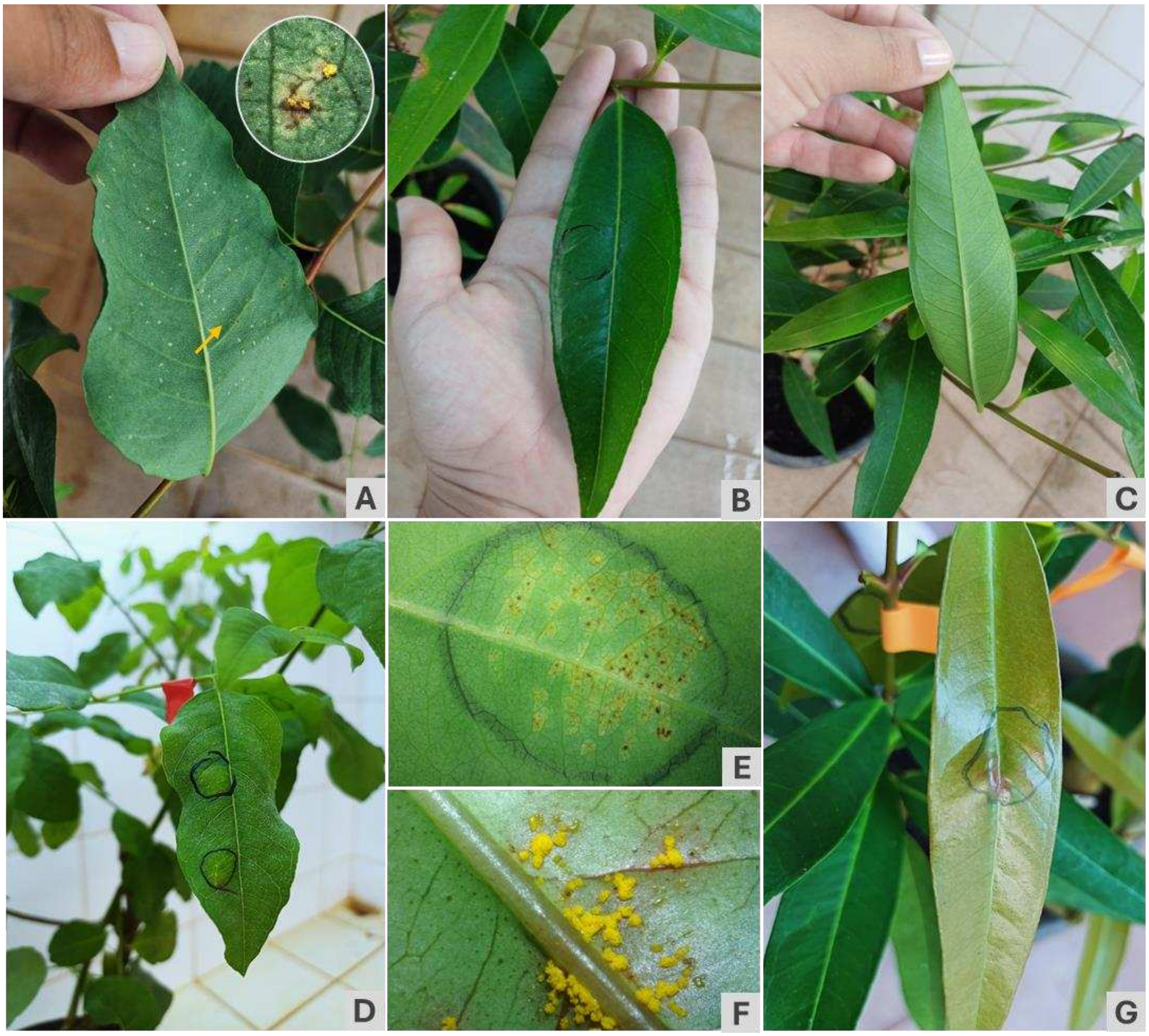

Figure 6. Results of inoculations with a mixture of *Austropuccinia psidii* (race 1) spores on eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) in second assay. A- Developing myrtle rust observed at the eucalypt inoculation site (first repetition, highlight with arrow and magnification); B-C- Absence of signs of myrtle rust on the inoculated rose apple leaf (first repetition); D-E- Rust infection observed at the inoculation site on eucalypt (second repetition); and F-G- Myrtle rust on rose apple (second test, second repetition). Black circle on the leaves indicating the inoculation site.

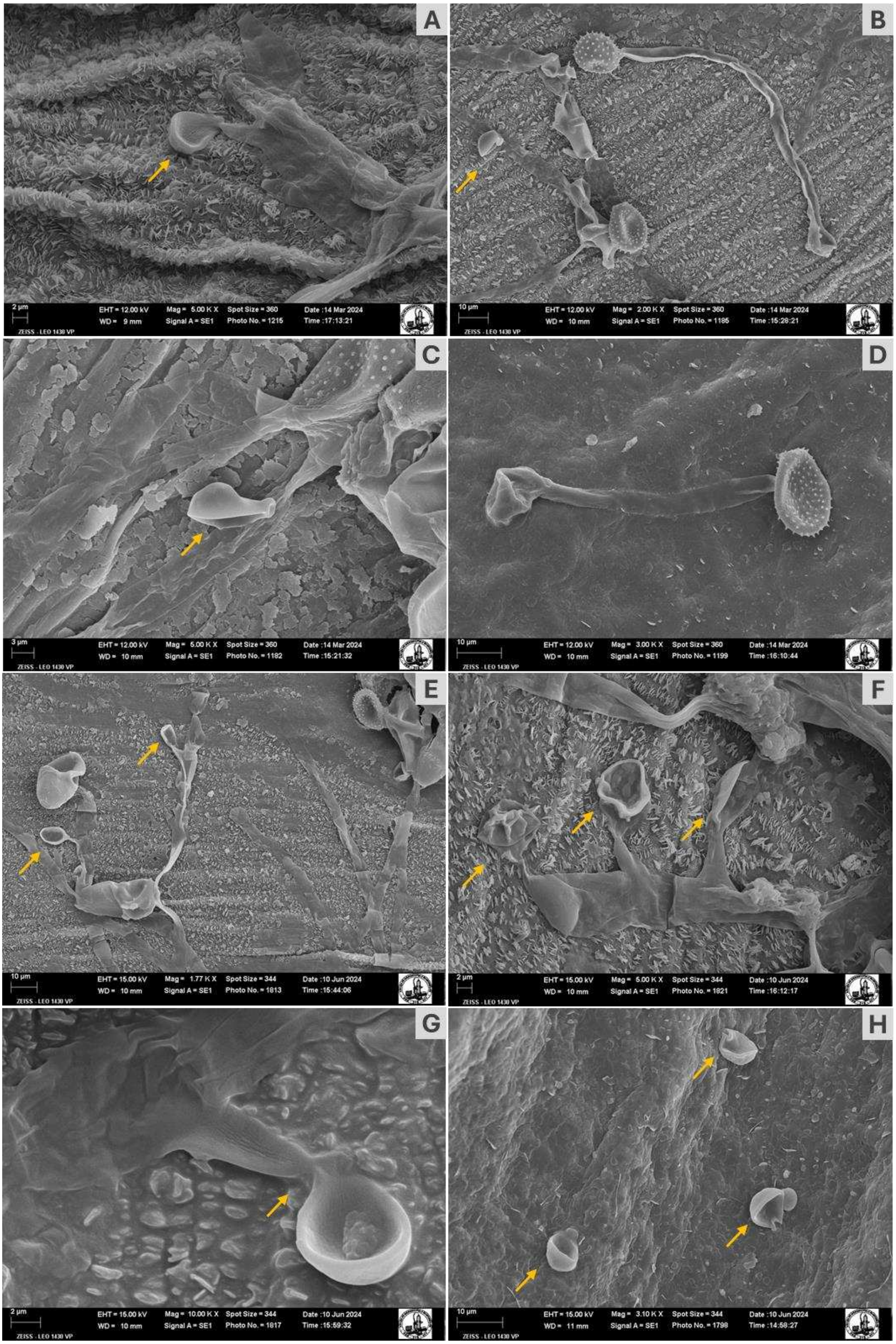

Figure 7. Scanning electron microscopy of eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) leaves inoculated with spore suspension of *Austropuccinia psidii*

(race 1). Figures A to D show examples from the first repetition of the second assay. Figures E to F show examples from the repetition of the second assay. A- Non-germinated basidiospore under sterigma, 24 hours after inoculation (hai) on eucalypt; B- Urediniospore with germ tube and appressorium next to non-germinated teliospores and basidiospore, 12 hai on eucalypt; C- Non-germinated basidiospore, 24 hai on rose apple; D- Urediniospore with germ tube and appressorium, 24 hai on rose apple; E-F- Non-germinated basidiospores under sterigma, at 24 and 48 hai on eucalypt, respectively; G-H- Non-germinated basidiospores on the leaf surface of rose apple, at 6 and 48 hai, respectively. Arrows point to basidiospores.

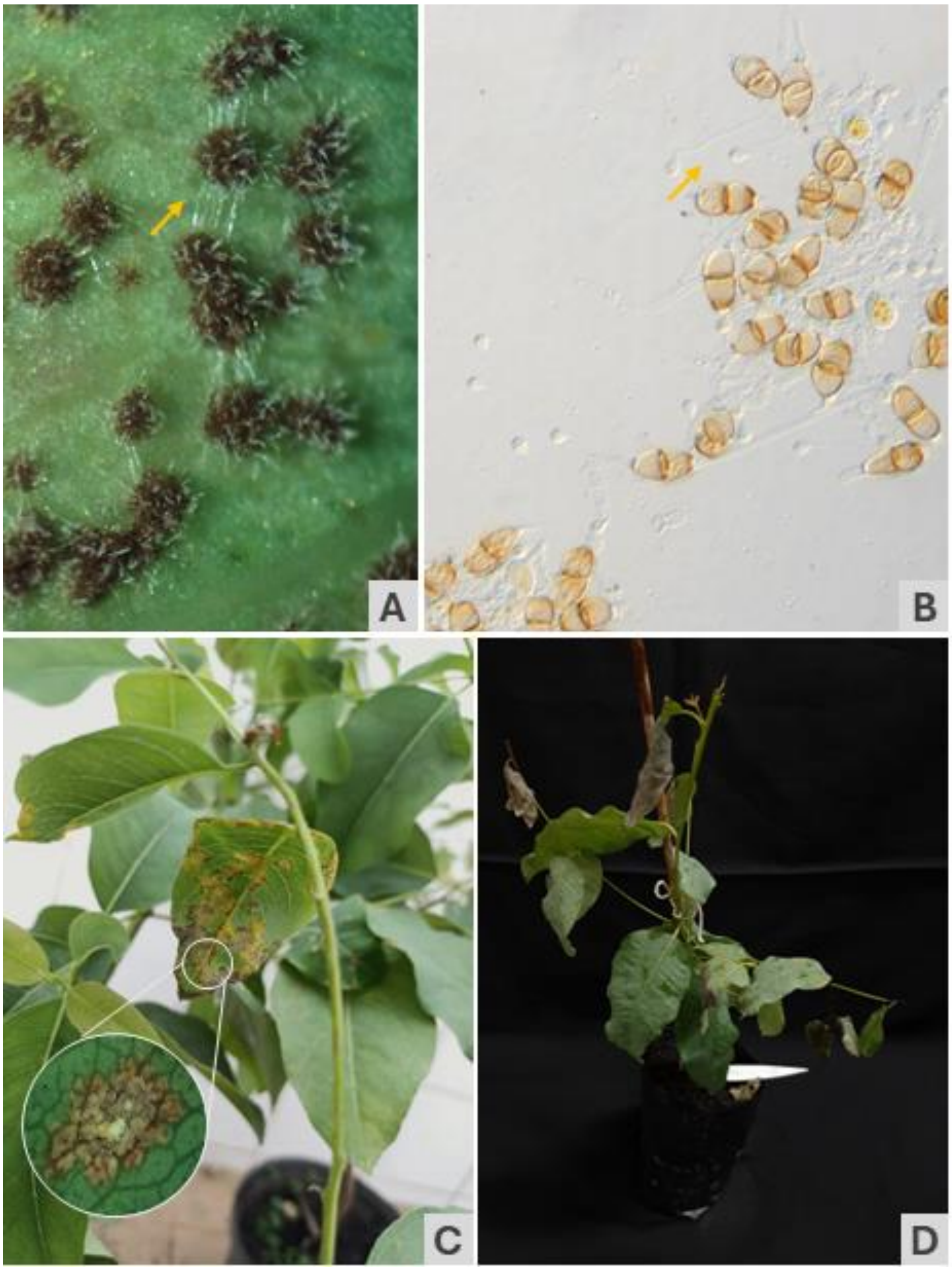

Figure 8. Teliospores of *Austropuccinia psidii* (race 1) produced on eucalypt (*Eucalyptus urophylla* clone CLR371) after incubation in a humid chamber. A- Apparent connection of hyphae among teliospore pustules, at 48 h after incubation; B- Basidiospores germinated after 48 h of incubation on leaf tissue with teliospores; C- Colonization of teliospore-containing pustules by mycoparasites (denoted by enlarged view), 5 days after incubation; D-Necrosis of leaves with myrtle rust, at 17 days after inoculation.

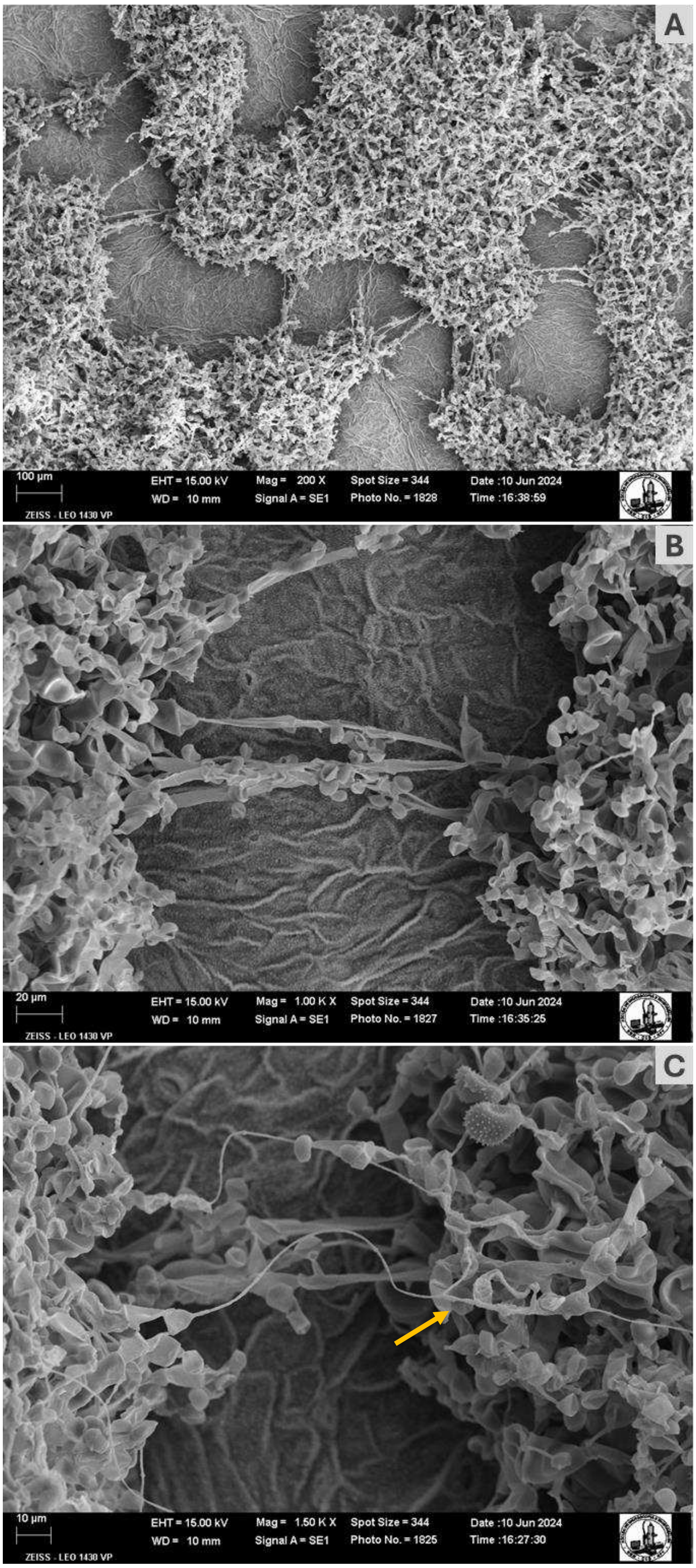

Figure 9. Apparent connection of hyphae among basidiospores-containing pustules of *Austropuccinia psidii* (race 1), at 48 h after inoculation of eucalypt (*Eucalyptus urophyll*a clone CLR371).

CHAPTER 3 - RESISTANCE OF OPEN-POLLINATED FAMILIES OF *Eucalyptus globulus* TO TERATOSPHAERIA LEAF DISEASE BY *Teratosphaeria nubilosa*

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ABSTRACT

Eucalyptus globulus Labill. it is native to south-eastern Australia and is cultivated in temperate regions world-wide. *Teratosphaeria nubilosa* (Cooke) Crous & U. Braun, is the main pathogen contributing to Teratosphaeria Leaf Disease (TLD) in *E. globulus.* This pathogen causes significant damage to *E. globulus* plantations in Spain, one of the leading countries in the production of *E. globulus* for cellulose and paper manufacturing. A predominant multilocus genotype of *T. nubilosa* has been identified in experimental *E. globulus* fields in Brazil. An isolate of this genotype was used to inoculate 99 open-pollinated families of *E. globulus* from an Australian collection, aiming to identify genotypes resistant to TLD. The study compared these results with natural *T. nubilosa* infection assessments in the same *E. globulus* families grown in Tasmania, Australia. Our findings indicate high genetic variability for TLD resistance within *E. globulus* subraces (i. e., provenance). However, variations among subraces were influenced by plant height. A positive correlation was observed between the resistance of *E.*

globulus subraces to TLD in Tasmanian field trials, where *E. globulus* plantations have historically experienced severe TLD outbreaks, and the results of inoculation experiments conducted in Brazil.

Keywords: Eucalypt. Genetic control. Leaf spot. Tasmanian blue gum

INTRODUCTION

Eucalyptus globulus Labill. is native to south-eastern Australia (Dutkowski and Potts 1999) and has been cultivated in temperate regions of the world due to its high commercial value to the pulp and paper industry (Eldridge *et al.* 1994; Potts *et al.* 2004; Potts *et al.* 2014; Xavier *et al.* 2007).

One of the most important biotic diseases affecting *E. globulus* plantations worldwide is Teratosphaeria leaf spot, or Teratosphaeria leaf disease (TLD), caused by a complex of species of the genus *Teratosphaeria*. The main TLD-causing species is *Teratosphaeria nubilosa*, previously classified as *Mycosphaerella nubilosa* (Smith et al. 2018). Since its initial identification in south-eastern Australia, the pathogen has posed a significant challenge to the successive cultivation of cold-tolerant eucalypt species in native areas and in regions from Chile, Portugal, and Spain (Hunter et al. 2009, Pérez et al. 2009a).

In eucalypt, TLD by *T. nubilosa* is characterized by numerous lesions, initially rounded and straw-colored that coalesce to irregular large blight on the leaf surface, on which numerous black pseudothecia, containing ascospores, are typical. High humidity, and temperatures of approximately 24°C are key conditions for the active ejection of ascospores. Spore dispersion occurs mainly by wind, which upon germination penetrates young and mature eucalypt leaves via stomata (Hunter et al. 2009; Freitas et al. 2024). As the infection progresses in the plant, necrosis can affect a significant portion of foliage and ultimately culminates in rapid leaf defoliation (Hunter et al. 2009).

Crown damage from TLD in young plantations of *E. globulus* can range from <10% to 80% which reduces the growth and survival of plants (Balmelli et al. 2014; Balmelli et al. 2016). Additionally, the disease can induce changes in stem morphology and wood quality, as well as delays in harvesting attributable to reduced growth rates (Smith et al., 2017). The use of fungicides to control TLD is expensive and impractical (Carnegie and Ades, 2001). However, since resistance in *E. globulus* to the disease is under relatively strong genetic control (Balmelli et al. 2014; Costa Silva et al. 2013; Freeman et al. 2008; Hamilton et al. 2013; Milgate et al.

2005; Quezada et al. 2022), the selection and planting of resistant genotypes (pure or hybrid) can constitute an effective strategy to control the disease in the field to avoid significant economic losses. However, the restricted availability of resistant genotypes to *T. nubilosa* limits the cultivation of *E. globulus* in areas where the pathogen is present and environmental conditions for the occurrence of TLD are favorable (Eldridge et al. 1994; Potts et al. 2004).

In native stands, *E. globulus* exhibits significant genetic variability among provenances for quantitative traits, leading to the division of the species gene pool into multiple geographic races and subraces (Dutkowski and Potts 1999). Open-pollinated seeds obtained from these native races and subraces constitute the foundation of breeding programs in numerous countries (Potts et al. 2004).

We here use families of *E. globulus* derived from open-pollinated seed lots collected from native stands in Australia and previously evaluated in field for seedling resistance to the *T. nubilosa* (Hamilton et al. 2013) to: (i) determine the genetic variability among native *E. globulus* populations for resistance to an isolate of TLD which is prevalent in Brazil and Spain through inoculations under controlled environmental conditions; (ii) Determine whether there is a positive correlation among the resistance of *E. globulus* families evaluated in field trials in native areas (Hamilton et al. 2013) and the resistance of seedlings to TLD evaluated by artificial inoculation of the widespread isolate in experiments in Brazil; (iii) identify and clone, by cuttings, genotypes of *E. globulus* resistant to *T. nubilosa* in Brazil for cultivation in Spain.

MATERIAL AND METHODS

Phylogeny analysis and genotyping of *Teratosphaeria* isolates

A multilocus sequence analysis (MLSA) and genotyping of *Teratosphaeria* isolates were performed to identify the species used in the present study and analyze the genotypic diversity, mainly between isolates from Brazil and Spain.

Initially, the monosporic cultures of *Teratosphaeria* isolates were grown in potato dextrose agar (PDA) (Sigma–Aldrich) medium for 30 days. Genomic DNA was extracted as previously described (Specht et al., 1982), with modifications (Teixeira et al., 2011).

For phylogeny of two *Teratosphaeria* isolates (LPF2392 from Brazil [the source of inoculation used in the present study] and LPF2543 from Spain), the internal transcribed spacer region (ITS), β-tubulin gene (Btub), and translation elongation factor1-alpha (EF-1α) were amplified by PCR using primer pairs (Table 1, Supplementary Material 1).The PCR reaction was prepared for a final volume of 15 μl, containing 2.0 μl of DNA (15 ng/μl), 1.5 μl of each primer (10 mM); 7.5 μl of GoTaq Master Mix® (Promega), and 2.5 μl of ultrapure distilled water. PCRs were performed in a Veriti 96® Well Thermal Cycler Thermocycler. The conditions for PCR amplification were performed according to Quaedvlieg et al. (2014). To confirm the fragment amplification, PCR products were separated by electrophoresis on the 2 % agarose gels, stained with ethidium bromide, and visualized under UV light. The size of the amplified fragments was verified by comparison to a 1 Kb plus DNA Ladder (Invitrogen). PCR products were purified and sequenced. The same primers used for DNA amplification were used for sequencing on the ABI Prism 3500-Avant Genetic Analyzer (Applied Biosystems). For phylogenetic analyses, besides the multilocus DNA sequence dataset generated in this study, sequences of reference isolates of ITS, Btub, and EF-1α regions were retrieved from the *Teratosphaeria* species database compiled by Quaedvlieg et al. (2014). Sixty-seven reference isolates were selected, representing 38 recognized species of *Teratosphaeria* (including isolates of the ex-epitype of *T. nubilosa* from Australia, CBS 116005) and one outgroup (*Staninwardia suttonii*) (Table 2 from Supplementary Material 1). A data set for each gene was constructed. The alignments were generated using the Clustal W tool (Thompson et al. 1994) implemented in the software MEGA 7 (Kumar et al. 2016). A combined data matrix (ITS + Btub + EF-1α) was constructed using the software Sequence Matrix 1.8 (Vaidya et al. 2011). Phylogenetic analyses by Maximum Likelihood (ML) were performed for a combined data matrix. Maximum Likelihood (ML) analyses were performed using the software RAxML-HPC v.8 on XSEDE (8.2.12) implemented in CIPRES Science Gateway (Miller et al., 2010), starting with a randomized stepwise addition parsimony tree under a GTR CAT model. The branch support values were calculated using 1000 bootstraps under the same model.

A total of 29 isolates of *Teratosphaeria* were genotyped with microsatellite markers, including: the isolates LPF2392 and LPF2543; other isolates obtained in this study from the inoculum source and inoculated plants with TLD symptoms; isolates obtained from *E. globulus* from Rio Grande do Sul in Brazil; as well as Spain; Australia and South Africa. Identification of the isolates was confirmed by PCR using the *T. nubilosa* MNF/MNR specific primer pair (199pb) (Table 1, Supplementary Material 1) in agarose gel. The LPF2392 and LPF2543 isolates were used as positive controls and the *T. pseudoeucalypti* isolate LPF2760 as a negative control.

Microsatellite genotyping was performed two times with six polymorphic markers (Hunter et al. 2006). The forward primers were labeled at the 5′ ends with one of the following fluorescent dyes: 6-FAM or NED. To optimize the reaction, the primer pairs was divided into four pools (Table 3, Supplementary Material 1). Amplifications of loci were conducted for a final volume of 15 μl as follows: Pool 1: 2.0 μl of DNA (10 ng/μl), 0.75μl of each primer MN-1, 0.5μl of each primer MN-10, 0.25μl of each primer MN-14 at 10 mM, 7.5 μl of GoTaq Master Mix® (Promega) and 2.5 μl of ultrapure distilled water. Pool 2: 2.0 μl of DNA (10 ng/μl), 0.25μl of each primer MN-2, 0.5μl of each primer MN-3, 0.5μl of each primer MN-9 at 10 mM, 7.5 μl of GoTaq and 2.75 μl of ultrapure distilled water. Pool 3: 2.0 μl of DNA (10 ng/μl), 0.5μl of each primer MN-4, MN-7, and MN-11 at 10 mM, 7.5 μl of GoTaq and 2.5 μl of ultrapure distilled water. Pool 4: MN-8 marker, performed with 2.0 μl of DNA (10 ng/μl), 1μl of each primer, 7.5 μl of GoTaq, and 3.5 μl of ultrapure distilled water. PCR products were diluted in ultrapure water to a final volume of 50 μl (or 40 μl to MN-8), from which 2 μl were mixed with 7.8 μl HiDi formamide and 0.2 μl GeneScan 600 Liz inner band size standard. The mix was denatured at 95 °C for 5 min and then subjected to capillary electrophoresis on an ABI Prism 3500-Avant Genetic Analyzer. The amplicon size of PCR products was analyzed in GeneMapper v. 4.1 Software. The reagents and equipment's used were from Applied Biosystems. Each estimated product length was rounded to the nearest whole number, and alleles were assigned based on a comparison to 99 isolates from a large dataset from Hunter et al. (2008). Data from markers MN-2, MN-3, MN-4 and MN-10 were not used in the genotypic analyses presented.

For determining the minimum number of loci necessary to discriminate between individuals in a population, genotype accumulation curves with 1,000 bootstrap replications were created for each population. Populations were defined based on their geographical origin. Nei's gene diversity (H) index (Nei, 1973) was calculated with a clone correction in order to avoid bias in subsequent analyses because of repeated genotypes. Genotypic diversity was estimated using Stoddart and Taylor's (G) index (Stoddart & Taylor, 1988) with rarefaction. All analysis were performed using the POPPR package (Kamvar et al. 2014) for software RStudio.

Screening of *Teratosphaeria nubilosa* resistance

Seeds of 99 native open-pollinated families (seedlots) collected in Australia, representing 11 subraces of *E. globulus* (Table 1), were sown into 290 cm^3 tubes containing substrate Tropstrato® supplemented with 3 Kg.m^{-3} of simple superphosphate and 3 Kg.m^{-3} of Osmocote® (15-09-12). Five seeds were sown in each tube. At 45 days after sowing, one seedling was left in each tube and the remaining seedlings were transplanted to tubes from the same seedlot that did not germinate. Individual seedlings from the same seedlot were kept close together in their respective tubes. The seedlot positions remained randomized until inoculation. The plants were grown in a greenhouse (Figure S5) (25 °C ± 5 °C; RH = 44 % (± 30%). Irrigation was performed 3-4 times a day, as needed.

At 80 days from sowing, the height (cm) of plants of each seed lot was measured and the plants were arranged in a modified random block. The inoculation experiment comprised 20 blocks, with each block represented by two trays of 54 cells, and with each seedlot represented by one plant in each block where possible. Since seedling germination was variable and the replacement of tubes with remaining seedlings was random, e.g., if seedlot 1.1, tube 6, did not contain seedling, the position of 1.1 R6 in block 6 was empty. *E. globulus* CLR381 clone and CLR172 clone (*E. grandis x E. urophylla*) were used as susceptibility and resistance controls, respectively, and were each represented once per block. The assembly of the blocks was performed on the same day of inoculation. To minimize the occurrence of the "umbrella" effect (taller plants buffering smaller plants from the ascospore rain), each plant was randomly placed within the block except with the constraint they were stratification by height such that a taller plant stayed closer to other taller plants, while smaller plants were positioned nearer to other smaller plants.

Teratosphaeria nubilosa **inoculum production, inoculation, and evaluation**

To promote the spread and natural infection of *T. nubilosa*, artificially infected plants of *E. globulus*, inoculated with the LPF2392 Brazilian isolate were grown in the field or in plastic pots and maintained under natural conditions. Infected leaves, containing black pseudothecia were collected from these plants and used as the inoculum source for resistance screening (Figure S1).

The eucalypt plants were inoculated by the ascospore ejection method from diseased leaves of *E. globulus* in inoculation chambers (Figure S2) (Freitas et al. 2024). Each inoculation chamber consisted of a rectangular (2.52 × 1.25 × 1.00 m) metallic structure, fixed to the ground, containing a galvanized steel screen at the top and the sides covered with translucent plastic. The infected leaves used as inoculum were collected and kept immersed in water for 2 h, to favor the release and ascospore ejection. Subsequently, they were uniformly distributed (550 leaves/m^2) over the galvanized steel of each inoculation chamber, with the abaxial surface - which naturally presents a higher number of pseudothecia - facing the plants to be inoculated. The height (cm) of plants at an ideal stage for inoculation was measured, and these plants were placed inside the inoculation chambers. A nylon screen was used to protect the infected leaves used as inoculum. An air humidifier (Fresh Air Model 3.5 liters) was installed in each chamber to maintain moisture saturation and leaf wetness, continuously for 72 h inoculation. In each inoculation chamber, four optical microscope slides, evenly covered with water-agar, were kept in four Petri® dishes positioned between the galvanized screen and the plants to monitor ascospore ejection (Figure S3). These Petri® dishes were replaced 24h after inoculation. The inoculated plants were moved on top of the galvanized steel screens after 72 h of inoculation and maintained in the greenhouse (temperature of 25 °C (± 5 °C), average relative humidity of 44% (± 30%)). Irrigation was performed as needed, three times a day.

At 45 days after inoculation, TLD severity, expressed by the percentage of lesioned leaf area, was evaluated according to a diagrammatic scale (Figure S4) (Passador et al. (2013). Five leaves with the highest levels of disease severity were evaluated for each plant. The final seedling score was calculated as the average and maximum TLD severity among the five leaves evaluated. Plants with less than or equal to 12% of average infected leaf area were selected for repeat inoculation by pruning close to the base of the stem, keeping a branch, to be re-inoculated after resprouting to confirm their resistance level. After re-inoculation, only plants with a maximum TLD severity of less than or equal to 12% of infected leaf area were selected as resistant plants for planting in the field (Federico Ruiz Fernández, Ence, personal information 2023).

Testing for sub-race and family within-subrace effects was performed using PROC GLIMMIX of SAS®. Regression and correlation analyses were used to assess the relationship between TLD severity and plant height, as well as for TLD severity data obtained under controlled conditions versus data obtained under natural conditions (Hamilton et al. 2013).

RESULTS

Phylogeny analysis and genotyping of *Teratosphaeria*

Multilocus phylogenetic analysis (ITS, Btub, and EF-1α) (Figure S6) and specific PCR (MNF/MNR, *T. nubilosa* specific primer pair) (Figure S7) confirmed that the isolates (Table 2) studied are *T. nubilosa*.

Genotyping analysis of 29 isolates using six polymorphic microsatellite markers and a UPGMA dendrogram including more than 97 isolates from Spain and Portugal (Figure 1) provides strong evidence that most of the isolates analyzed represent a single clonal complex, except the *T. nubilosa* epitype (CBS116005) and one isolate from South Africa (CBS114708) used for the development of the microsatellite primers (Hunter et al. 2006). In addition, two isolates (LPF2836 and LPF2838 or ID 15 and ID 17, respectively) are genotypically different from the others Spanish isolates by the SSR marker MN8 - the most polymorphic marker within the set used (Table 2, Supplementary Material 1). Isolate LPF2836 was obtained from *E. globulus* in the Chouza do Vinõ farm, and isolate LPF2838 from *E. nitens* in Monte Cenardas farm, both in Northern Spain.

Therefore, a total of five multilocus genotypes were identified among the 126 *T. nubilosa* isolates using microsatellite markers (Table 3). Nei's gene diversity (H) was calculated for each of the 5 populations of *T. nubilosa* from Spain, Portugal, Brazil, Australia, and South Africa. Relatively low levels of gene diversity were found among isolates from Brazil, Spain, and Portugal. A gene diversity value of 0.000 was found in the Brazil and Portugal populations because they were a single-genotype population. However, a gene diversity value of 0.005 was found in Spain. A second calculation of gene diversity was made using unique genotypes from each population (clone corrected for population) (Table 3). Australia and South Africa had only one isolate and therefore it was not possible to perform an analysis of gene diversity for these countries. The highest Stoddart & Taylor's genotypic diversity (G) index with rarefaction was found in populations from Spain (G = 1.6). The remaining populations had genotypic diversity values G = 1.0 (Table 3). The genotype accumulation curves indicated that 100% of the genotypes were detected by three markers.

Resistance of *Eucalyptus globulus* after two consecutive inoculations with *Teratosphaeria nubilosa*

First inoculation

In the first inoculation, the average ascospore ejection rate was 169.7 ascospores/cm². A profuse ejection and germination of ascospores were relatively high up to 72 h of inoculation. Out of the 1698 germinated seedlings, 548 died by *B. cinerea* infection immediately after inoculation with *T. nubilosa* due to favorable environmental conditions. Therefore, only 1150 remained for evaluation of resistance to *T. nubilosa*. Out of 1150 plants, 780 plants with average % TLD ≤ 12% were selected, evidencing the occurrence of escape. These plants were re-conducted for re-screening in order to confirm either complete or partial resistance to the pathogen. The remaining 370 plants that presented more than 12% infected leaf area on average, as the susceptible control (Figure 2D-F) were discarded.

There was a significant positive association between plant height (cm) of *E. globulus* and TLD (transformed data, log10(%TLD +1)) at the individual plant ($F_{1,1146} = 361$, p=<0.001, adjusted R^2 of 23.9%) and family mean level. For the family means, linear regression fitted with SAS® PROC REG was highly significant and has an adjusted R^2 of 32.6% (Figure 3A). Based on Figure 3B, after accounting for height, the most resistant families of *E. globulus* (i.e. seedlots), on average, were 2_9, 1_3, and 10_5 from native areas Eastern Otways, Coastal Plain, and Western Otways, respectively. The arithmetic means log10(%TLD+1) for the resistant and susceptible controls was 0.011 and 1.270, respectively. Therefore, family averages fall between these resistance extremes.

Fitting a mixed model with PROC GLIMMIX OF SAS® to the transformed data (log10(%TLD +1) which fitted a spline term to account for the effect of height (P<0.001) showed there was a significant fixed effect of subrace (P=0.0200), whereas the subrace effect was not significant if the height of the plant is not accounted for. After accounting for plant height as described, the subrace with the lowest least-square means TLD damage was Flinders Island followed by Southern Furneaux and then Southern Tasmania (Table 4), but after accounting for multiple comparisons using the Tukey-Kramer test there was no significant difference among the subraces. The random variation between families within subraces was significant (P<0.001) regardless of whether or not height was included as a covariate.

The subraces were fitted as a random term to obtain BLUPs (Best Unbiased Linear Predictions) and a fixed term to obtain LSMeans (least-square means) in models with and without plant height included as a covariate. When the height adjusted BLUPs and LSMeans of the experiment in this study were compared with the BLUPs of Hamilton et al. (2013) – i.e. % TLD data adjusted for juvenile foliage height in 1-year-old plants, Tasmania, trial 3 [TEM06]) - the correlation was positive and significant (BLUPs R= 0.64; P= 0.034; LSMeans R = 0.66; P = 0.029) (Figure 4).

Second inoculation

In the second inoculation (i.e., in the inoculation of resprouted plants with %TLD scores ≤ 12% in the first inoculation) a total of 305 plants (out the 780 selected in the first inoculation) from 84 (out of 99) *E. globulus* families (Table 1) were re-inoculated and evaluated. Among the 305 plants, 147 plants had average % TLD ≤ 12% and 119 had highest severity class per plant less than or equal to 12% of TLD and were considered resistant. The individuals 8.3R18

(St Helens) and 9.4R19 (Strzelecki Ranges) had no disease symptoms in two consecutive inoculations and are therefore potentially immune.

However, there was a significant positive relationship (non-linear Generalized Additive Model – GAM in R Studio®) between plant height and %TLD (transformed data, log10(%TLD) severity (Figure 5). For this reason, selected resistant plants (based on two inoculations) were re-conducted and transplanted in pots 40L to be clonally multiplied, and new inoculation with *T. nubilosa* to confirm resistance. A total of 53 out 119 plants survived after pruning transplanting, even after an application of the Nativo® fungicide (trifloxistrobin + tebuconazole, 2mL/L) and an application of the Rovral® fungicide (iprodione, 1 mL/L) to control of *A. psidii* and *B. cinerea,* respectively. Also, some plants died due to root malformation, e.g plants of the seedlot CG812 from subrace King Island (Figure S8).

DISCUSSION

A unique clonal complex of *T. nubilosa* prevails in regions where *E. globulus/E. nitens* are cultivated in Spain and Brazil, and this isolate was used for inoculations in the current study. It is important to highlight that the clonal complexes were defined here as groups of isolates that do not differ at any microsatellite loci employed. Previous studies (Hunter et al. 2008; Hunter et al. 2009; Pérez et al. 2009b; Pérez et al. 2010; Pérez et al. 2012) also showed low genetic diversity among populations of *T. nubilosa* from Spain, Portugal, and Brazil, as well as Uruguay and Tanzania. One hypothesis posits that this unique multilocus genotype originated in Australia, disseminated to South Africa, and subsequently spread to other countries in Africa and Europe (Hunter et al. 2008). The transfer of *E. globulus* plants between forestry companies from Europe to Uruguay may have facilitated the dissemination of the unique multilocus genotype of *T. nubilosa* to South America. From Uruguay, this strain reached Brazil. Although high genetic diversity has been reported among populations of the pathogen from Australia and South Africa, only a fraction of this diversity appears to have reached and established in Europe, and in turn, South America due to 'founder effects' (Hunter et al. 2008; Hunter et al. 2009; Pérez et al. 2009; Pérez et al. 2010; Pérez et al. 2012). Additional sampling carried out in our study suggests that the unique multilocus genotype of *T. nubilosa* has been maintained in the production areas of *E. globulus* and/or *E. nitens* in Spain and Brazil. Pérez et al. (2010) proposed that *T. nubilosa* has mixed mating strategies, i.e., it can reproduce by self-fertilization or by crossing, however, self-fertilization prevails over outcrossing and maintains the dominance of some genotypes in populations in South Africa (Pérez et al. 2010). This also appears to be occurring in Spain and Brazil.

While isolate LPF2392 was prevailent two other multilocus genotypes were identified in Spain in the study (Chouza do Vinõ and Monte Cenardas). Other isolates of the same origin can be obtained and genotyped to further investigate the genotypic diversity and distribution in these regions. Although it is important to note that genetic variability that is evident in microsatellites may not correspond with the variability in virulence and aggressiveness of the pathogen populations; however, *E. globulus* clones selected with some level of resistance to the Brazilian *T. nubilosa* isolate may also confer resistance to the predominant phenotype in Spain.

In the first inoculation with *T. nubilosa*, *E. globulus* subraces exhibited different degrees of TLD damage (adjusted for plant height), with Flinders Island showing the least damage, followed by Southern Furneaux and Southern Tasmania. Interestingly, Southern Tasmania is outside the latitudes where more TLD-resistant *E. globulus* populations have been identified, i.e. approximately between 41°S and 38°S, increasing northward into mainland Australia (Hamilton et al. 2013). Hamilton et al. (2013) and Freeman et al. (2019), suggested that populations originating from warmer and more humid environments are less susceptible to TLD (e.g. Flinders Island and Southern Furneaux) as a result of historical selection imposed by pathogens. Such genetic variation in host susceptibility is common in native pathosystems in which spatial and temporal fluctuations in selection pressure from pathogens are likely to maintain variation in host resistance (Burdon et al. 2014; Laine et al. 2011).

In the second inoculation, a significant proportion of resistant individuals were observed in the St Helens and Strzelecki Ranges subraces (selection imposed by pathogens). Individuals from these subraces showed greater survival and shoot formation throughout the experiments. In contrast, substantial losses to *B. cinerea* were noted in plants from the Southern Furneaux and Southern Tasmania subraces, potentially disrupting the proportion of resistant genotypes. Furthermore, the persistent influence of plant height on TLD resistance warrants further investigation. It is noteworthy that families originating from the Western Otways subraces may exhibit resistance to both *T. nubilosa* (e.g., family ID 10_5) and *A. psidii* race 1 (e.g., family ID 10_4) (A. C. Alfenas, unpublished data). However, Freeman et al. (2019) reported that *E. globulus* susceptibility to *A. psidii* was not genetically correlated with susceptibility to *Teratosphaeria* sp., suggesting that eucalypt resistance mechanisms to these pathogens are pathogen-specific rather than general mechanisms.

The positive relationship between the controlled inoculation and field results for the *E. globulus* subraces is encouraging given the many factors that can cause noise such as discrepancies between families represented in the Brazil screening and the Tasmanian field screening; the impact of *B. cinerea* on the nursery in Brazil; the effect of plant size; and the relatively poor discrimination between sub-races in the nursery inoculation compared to field trial.

In conclusion, all isolates obtained and/or investigated were phylogenetically identified as *Teratosphaeria nubilosa*. A total of five multilocus genotypes of *T. nubilosa* were identified, with the genotype most prevalent in Spain being identical to the genotype that occurs in Brazil. There is significant genetic variation within *E. globulus* subraces for resistance of the juvenile foliage to TLD. It is worth highlighting two families from the Western Otways subrace that may present resistance to *T. nubilosa* and *A. psidii*, although it appears to be a rare event. There is a significant positive relationship between the *E. globulus* subrace TLD resistance observed in the field trials in Tasmania and inoculation results in Brazil. The immediate and systematic cloning of the resistant genotypes selected in this study will be essential for the establishment of subsequent field trials in genetic breeding programs.

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DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study (Brazil screenings) are not publicly available due to confidentiality and privacy constraints but are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

No conflicts of interest are declared by the authors.

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FIGURES E TABLES

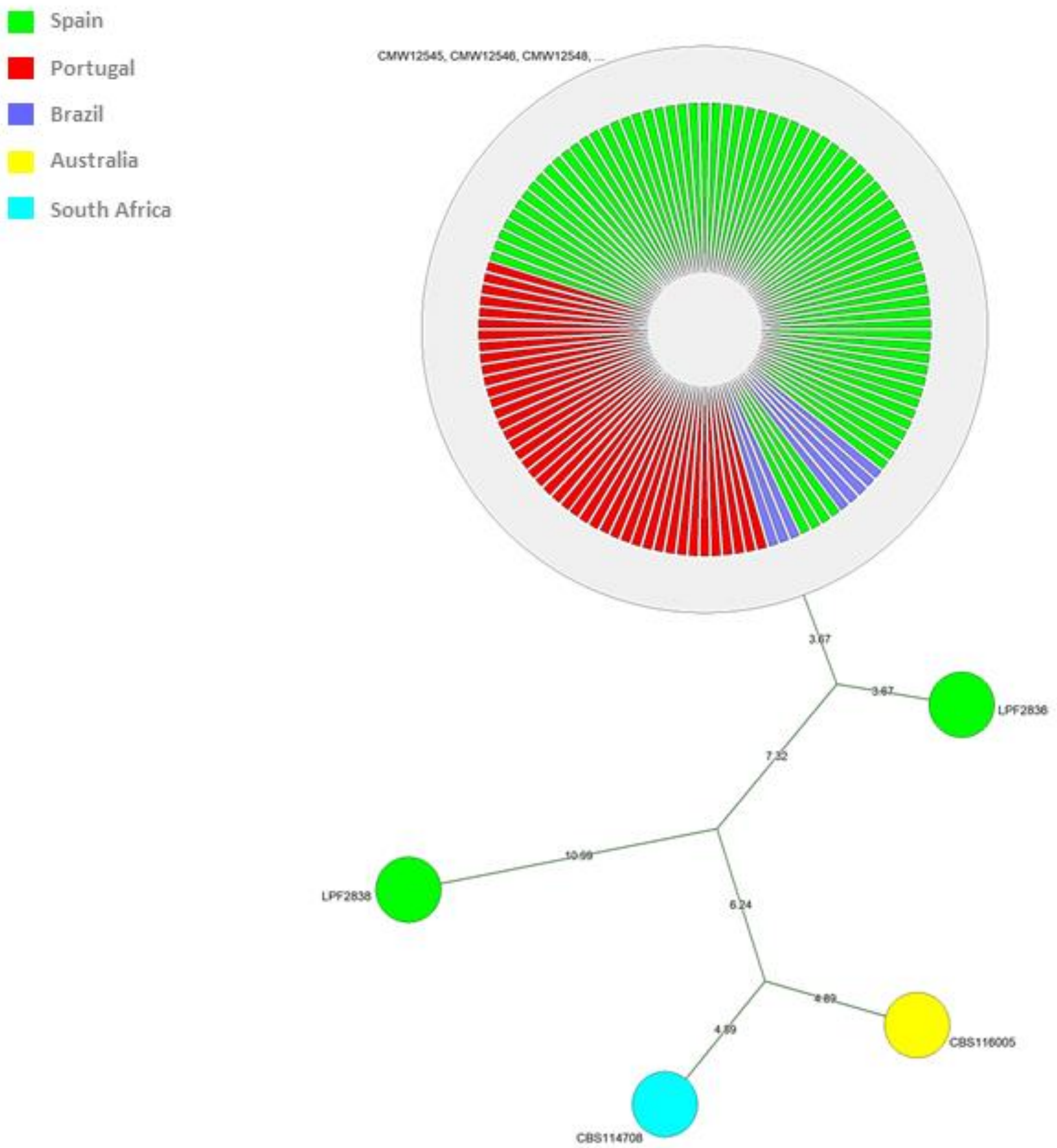

Figure 1. UPGMA tree generated based on the alleles of six polymorphic SSR loci from 126 isolates of *Teratosphaeria nubilosa* from Spain, Portugal, Brazil, Australia, and South Africa. The tree was constructed using BioNumerics software version 6.6 and analyzed through Euclidean distance. Color codes indicate the geographic region of origin. The number of sections within each circle represents the number of strains sharing the same haplotype.

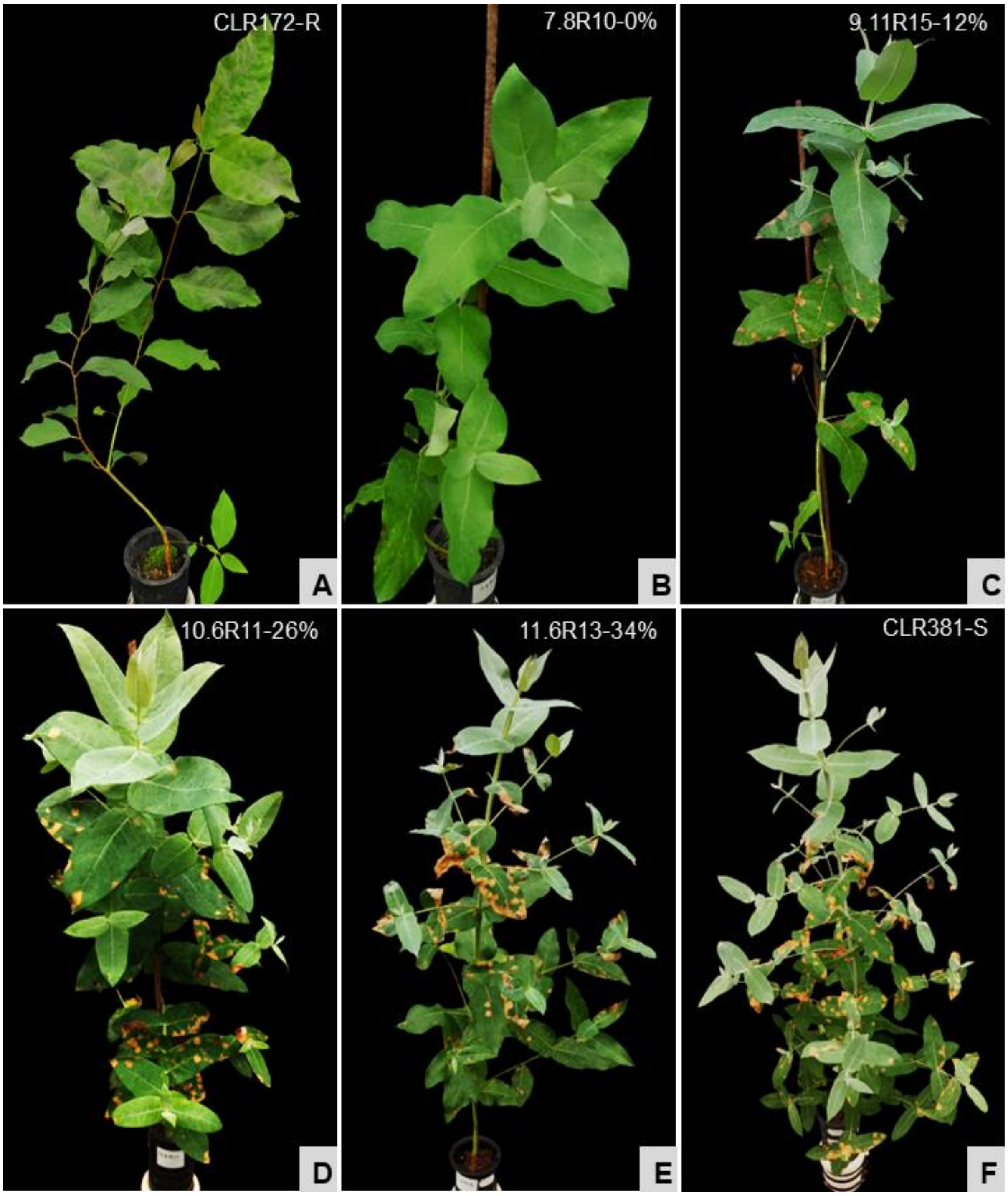

Figure 2. *Eucalyptus* plants inoculated with *Teratosphaeria nubilosa:* A- Eucalypt clone CLR172 (*E. grandis x E. urophylla*), used as the resistance control, exhibiting immunity to Teratosphaeria-Leaf-Disease (TLD); B-C- Plants exhibited resistance to TLD; D-E- Seedling exhibiting susceptibility; F- *Eucalyptus globulus* clone CLR381, used as the susceptibility control. Unaffected new foliage at the top was formed after the inoculation (first inoculation).

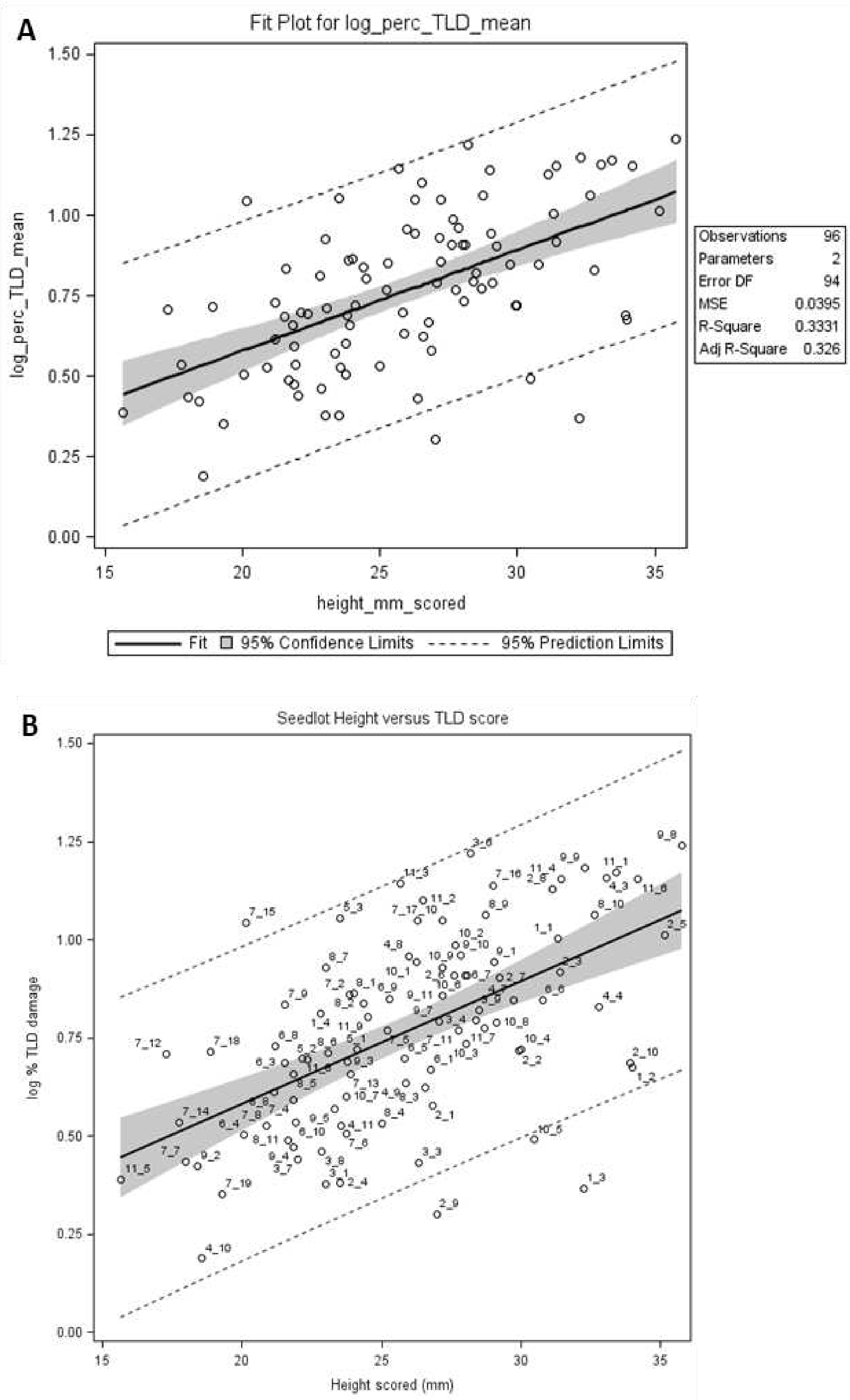

Figure 3. Relationship between plant height of *Eucalyptus globulus* families measured on the day of inoculation and the severity of TLD - Teratosphaeria Leaf Disease (transformed data, log10(%TLD +1)), 45 days after inoculation. The subrace family codes are indicated in figure 3B. Data of first inoculation.

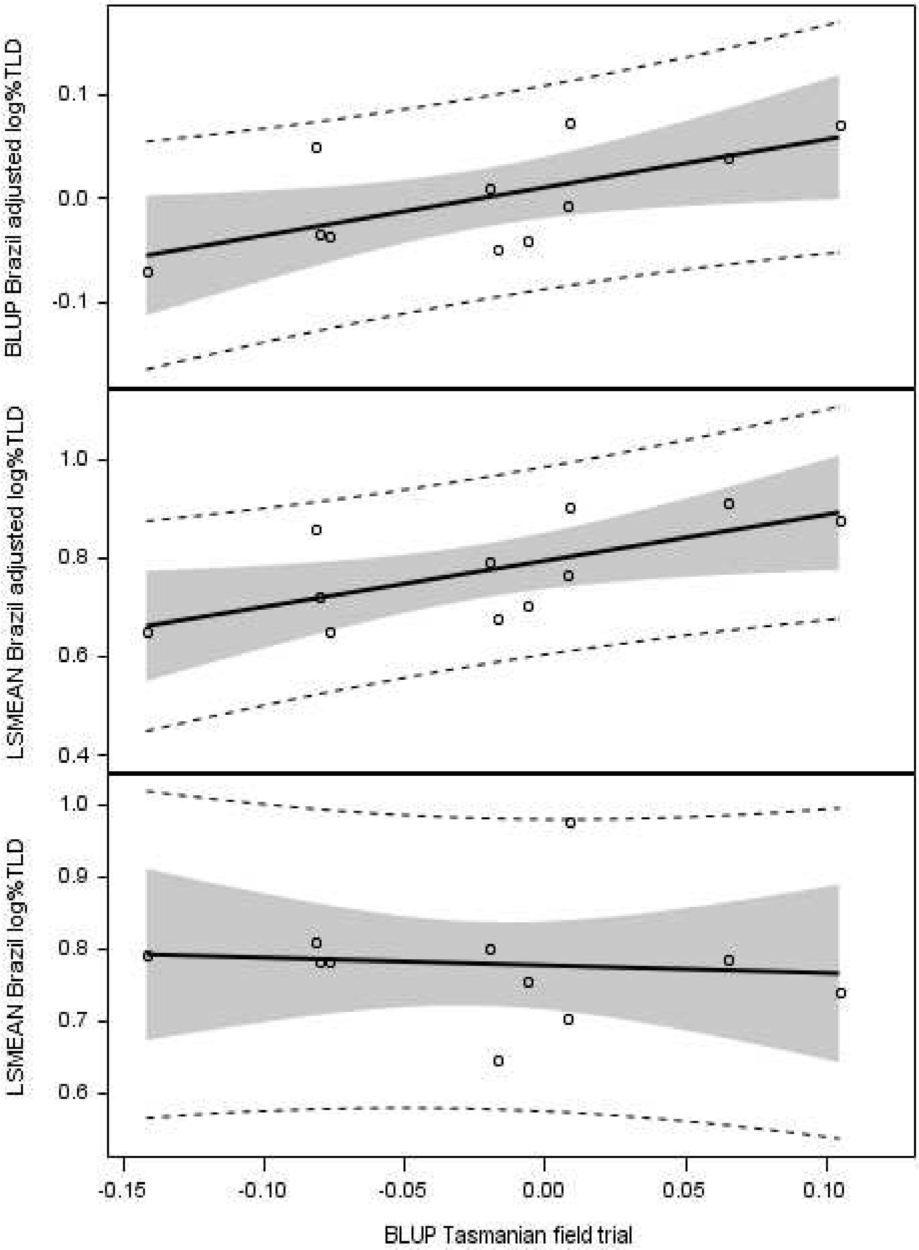

Figure 4. Association between sub-race differences of *Eucalyptus globulus* in the Tasmanian field trial and the first inoculation in Brazil. Data adjusted for height. Data not adjusted for height have no significant effect.

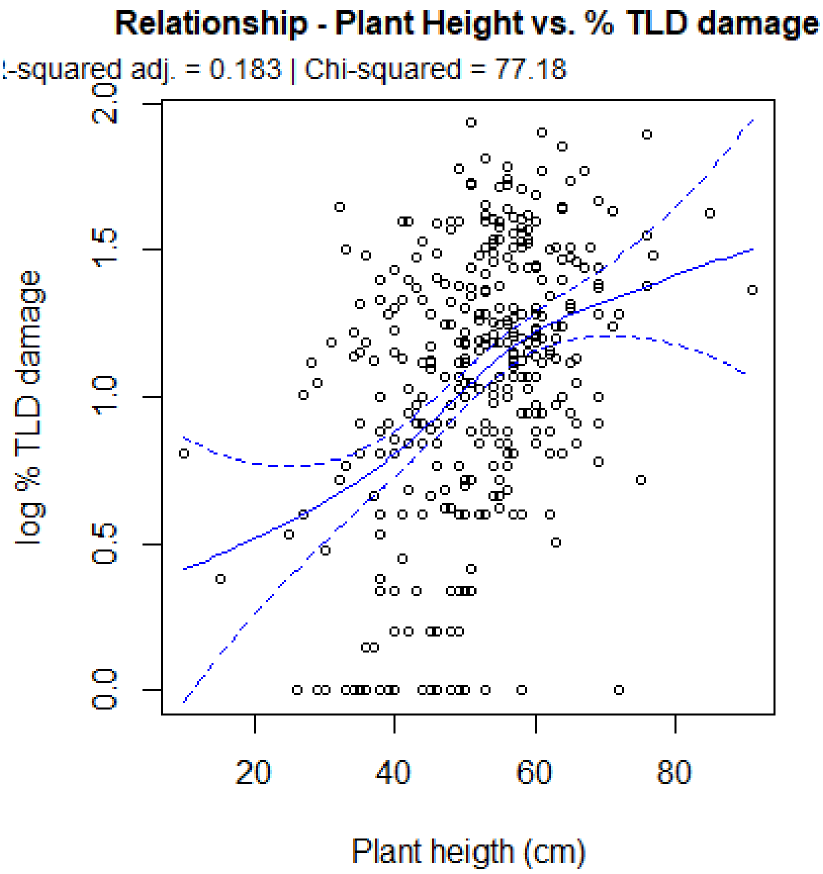

Figure 5. Positive non-linear relationship between plant height, measured on the day of inoculation, and severity by TLD - Teratosphaeria leaf disease (transformed data, log10(%TLD) 45 days after inoculation.

Table 1. Native open-pollinated families of *Eucalyptus globulus* per subrace screened for resistance to *Teratosphaeria nubilosa*

ID	*E. globulus* subrace[1]	Seedlots screened in first inoculation	Inoculated plants in first inoculation	Resistant plants by average severity score[2]	Seedlots screened in second inoculation	Inoculated plants in second inoculation	Resistant plants by average score[1]	Resistant plants by maximum severity score[3]
1_	Coastal Plain	4	60	18	2	6	3	3
2_	Eastern Otways	10	174	57	9	34	11	8
3_	Flinders Island	7	136	50	6	23	11	9
4_	King Island	7	134	75	6	20	14	12
5_	North-eastern Tasmania	3	50	21	3	11	8	7
6_	Southern Furneaux	10	180	87	9	34	12	10
7_	Southern Tasmania	19	291	142	13	35	17	10
8_	St Helens	11	148	81	11	42	21	19
9_	Strzelecki Ranges	10	199	113	10	58	25	22
10_	Western Otways	9	165	90	9	35	19	16
11_	Western Tasmania	9	161	46	6	7	6	3
Total	11	99	1698	780	84	305	147	119

[1] Subrace classification (Dutkowski & Potts, 1999).

[2] Plants presumably resistant with % TLD (Teratosphaeria Leaf Disease) less than or equal to 12%, based on the average of 5 leaves.

[3] Resistant plants (% TLD $\leq$ 12%) based on the highest severity class recorded on any of the 5 leaves.

Table 2. Estimated sizes (bp) of alleles of six microsatellite (SSR) loci in *Teratosphaeria nubilosa* populations from Spain, Portugal, Brazil, Australia, and South Africa.

ID	Code	Country	Origin	Host	Allele (bp)						Reference
					MN1	MN7	MN8	MN9	MN11	MN14	This study
1	LPF2543	Spain	-	*E. globulus*	267	380	319	215	224	100	This study
2	LPF 2545	Spain	-	*E. globulus*	267	380	319	215	224	100	This study
3	LPF 2546	Spain	-	*E. globulus*	267	380	319	215	224	100	This study
4	LPF 2547	Spain	-	*E. globulus*	267	380	319	215	224	100	This study
5	LPF 2392	Brazil[1]	Cajuri, MG (Clonar)	*E. globulus*	267	380	319	215	224	100	This study
6	LPF 2393	Brazil[2]	Cajuri, MG (Clonar)	*E. globulus*	267	380	319	215	224	100	This study
7	LPF 2541	Brazil	Cajuri, MG (Clonar)	*E. globulus*	267	380	319	215	224	100	This study
8	LPF 2683	Brazil	Guaíba, RS (CMPC)	*E. globulus*	267	380	319	215	224	100	This study
9	LPF 2686	Brazil	Guaíba, RS (CMPC)	*E. globulus*	267	380	319	215	224	100	This study
10	LPF 2689	Brazil	Guaíba, RS (CMPC)	*E. globulus*	267	380	319	215	224	100	This study

11	LPF 2693	Brazil	Guaíba, RS (CMPC)	*E. globulus*	267	380	319	215	224	100	This study
12	LPF 2697	Brazil	Guaíba, RS (CMPC)	*E. globulus*	267	380	319	215	224	100	This study
13	LPF2834	Spain	Monte Arrayada	*E. globulus*	267	380	319	215	224	100	This study
14	LPF2835	Spain	Monte Arrayada	*E. globulus*	267	380	319	215	224	100	This study
15	LPF2836	Spain	Chouza do Vinõ	*E. globulus*	267	380	310	215	224	100	This study
16	LPF2837	Spain	Monte Cenardas	*E. globulus*	267	380	319	215	224	100	This study
17	LPF2838	Spain	Monte Cenardas	*E. nitens*	267	380	292	215	224	100	This study
18	LPF2839	Spain	Monte Carballinõ	*E. viminalis*	267	380	319	215	224	100	This study
19	LPF2840	Spain	Monte Mata	*E. nitens*	267	380	319	215	224	100	This study
20	LPF2841	Spain	Monte Carballinõ	*E. nitens*	267	380	319	215	224	100	This study
21	LPF2842	Spain	Monte Carballinõ	*E. globulus*	267	380	319	215	224	100	This study
22	LPF2843	Spain	Monte Axelán	*E. globulus*	267	380	319	215	224	100	This study
23	LPF2844	Spain	Monte Axelán	*E. globulus*	267	380	319	215	224	100	This study
24	LPF2845	Spain	Monte Axelán	*E. globulus*	267	380	319	215	224	100	This study
25	LPF2846	Spain	Monte Axelán	*E. globulus*	267	380	319	215	224	100	This study
26	LPF2847	Spain	Monte Axelán	*E. benthamii*	267	380	319	215	224	100	This study
27	LPF2849	Spain	Monte Santa Cruz	*E. globulus*	267	380	319	215	224	100	This study
28	CBS116005	Australia[3]	Victoria	*E. globulus*	267	380	319	216	203	100	This study
29	CBS114708	South Africa	-	*E. nitens*	267	380	319	216	191	100	This study

30	CMW12545	Spain	-	*E. globulus*	267	*380*	319	216	224	100	Hunter et al. 2008
31	CMW12546	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
32	CMW12548	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
33	CMW12549	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
34	CMW12550	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
35	CMW12551	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
36	CMW12552	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
37	CMW12553	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
38	CMW12554	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
39	CMW12555	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
40	CMW12556	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
41	CMW12557	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
42	CMW12558	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
43	CMW12559	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
44	CMW12560	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
45	CMW12561	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
46	CMW12562	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
47	CMW12563	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
48	CMW12564	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
49	CMW12565	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
50	CMW12566	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008

51	CMW12568	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
52	CMW12570	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
53	CMW12571	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
54	CMW12572	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
55	CMW12573	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
56	CMW12574	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
57	CMW12575	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
58	CMW12576	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
59	CMW12577	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
60	CMW12578	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
61	CMW12579	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
62	CMW12580	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
63	CMW12581	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
64	CMW12582	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
65	CMW12583	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
66	CMW12584	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
67	CMW12585	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
68	CMW12586	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
69	CMW12587	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
70	CMW12588	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
71	CMW12589	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008

72	CMW12590	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
73	CMW12591	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
74	CMW12592	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
75	CMW12593	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
76	CMW12594	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
77	CMW12595	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
78	CMW12596	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
79	CMW12597	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
80	CMW12598	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
81	CMW12599	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
82	CMW12600	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
83	CMW12601	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
84	CMW12602	Spain	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
85	CMW18796	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
86	CMW18797	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
87	CMW18798	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
88	CMW18799	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
89	CMW18800	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
90	CMW18801	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
91	CMW18802	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
92	CMW18803	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008

93	CMW18804	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
94	CMW18805	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
95	CMW18806	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
96	CMW18807	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
97	CMW18808	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
98	CMW18809	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
99	CMW18810	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
100	CMW18811	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
101	CMW18812	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
102	CMW18813	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
103	CMW18814	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
104	CMW18815	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
105	CMW18817	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
106	CMW18818	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
107	CMW18819	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
108	CMW18820	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
109	CMW18821	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
110	CMW18822	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
111	CMW18823	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
112	CMW18824	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
113	CMW18825	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008

114	CMW18826	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
115	CMW18827	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
116	CMW18828	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
117	CMW18829	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
118	CMW18830	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
119	CMW18831	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
120	CMW18832	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
121	CMW18833	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
122	CMW18834	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
123	CMW18835	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
124	CMW18836	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
125	CMW18837	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008
126	CMW18838	Portugal	-	*E. globulus*	267	380	319	216	224	100	Hunter et al. 2008

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[1] Isolate from the inoculum source used in this study; [2] Isolate from plant inoculated with *T. nubilosa* in this study; 3 ex-epitype of *T. nubilosa*.

Table 3. Genetic diversity of *Teratosphaeria nubilosa* populations from Spain, Portugal, Brazil, Australia, and South Africa based on six polymorphic microsatellite loci

Populations	N° of isolates	N° multilocus genotypes	Nei's gene diversity (H) index		[1]Stoddart & Taylor's genotypic diversity (G) index[1]	
			All isolates	Clone corrected[2]	All isolates	Clone corrected
Spain	74	3	0.00537	0.10	1.06	3
Portugal	42	1	0.00000	-	1.00	-
Brazil	8	1	0.00000	-	1.00	-
Australia	1	1	Na[3]	Na	1.00	1
South Africa	1	1	Na	Na	1.00	1
Total	126	5	0.01107	5	1.07	0.25

[1] Stoddart & Taylor's genotypic diversity (G) index with rarefaction.

[2] Clone correction removed isolates that had genotypes identical to other isolates from the same site.

[3] Not available

Table 4. Subrace Least-Square Mean (lsmeans) log transformed % TLD damage and their standard errors as estimated from mixed model accounting for covariation with height using a spline term and fitting block and family within subrace as random terms. The ranking is shown with the most resistant being one (first inoculation).

Subrace	lsmeans	s.e.	Rank
Flinders Island	0.6452	0.0962	1
Southern Furneaux	0.7011	0.0778	2
Southern Tasmania	0.7376	0.0586	3
King Island	0.7540	0.0847	4
Coastal Plain	0.7794	0.1370	5
Western Otways	0.7813	0.0764	6
North-eastern Tasmania	0.7835	0.1433	7
Eastern Otways	0.7892	0.0796	8
Strzelecki Ranges	0.7977	0.0698	9
St Helens	0.8083	0.0764	10
Western Tasmania	0.9756	0.0816	11

SUPPLEMENTARY MATERIAL

Figure S1. Production of *Teratosphaeria nubilosa* (LPF2392 Brazilian isolate) inoculum in natural conditions; A-B- Inoculum bank of *Teratosphaeria nubilosa* using *Eucalyptus globulus*; C- Symptom of *Teratosphaeria* leaf disease (TLD), emphasizing the black pseudothecia.

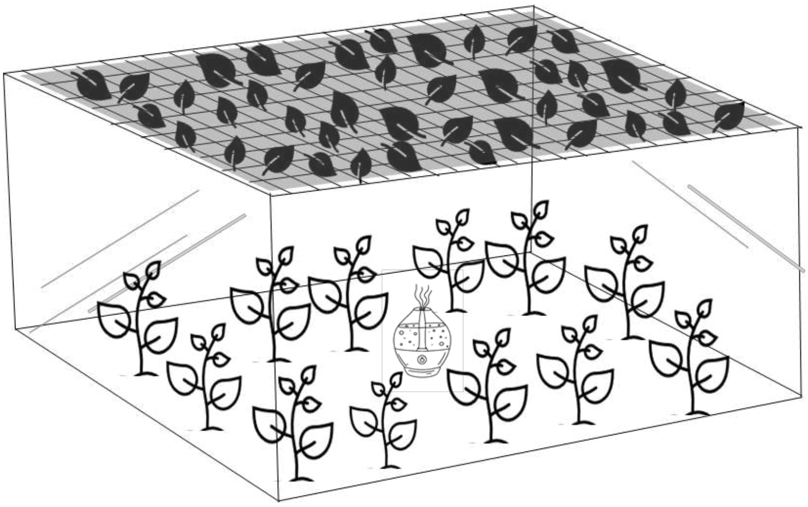

Figure S2. Design of the chamber used for inoculation of *Eucalyptus* spp. with *Teratosphaeria nubilosa* by ascospore ejection.

Figure S3. Inoculation process of *Eucalyptus* spp. with *Teratosphaeria nubilosa*. A- Distribution of *T. nubilosa* infected leaves on the steel screen over the top of the inoculation chamber; B-C-System for the inoculation of eucalypt plants with *T. nubilosa* by the ascospores ejection method; D- Petri dishes with water-agar medium, placed between the inoculation source and the eucalypt plants; E- Germinated ascospores, 12 h after inoculation. F- Seedlings of *E. globulus* 72 h after inoculation.

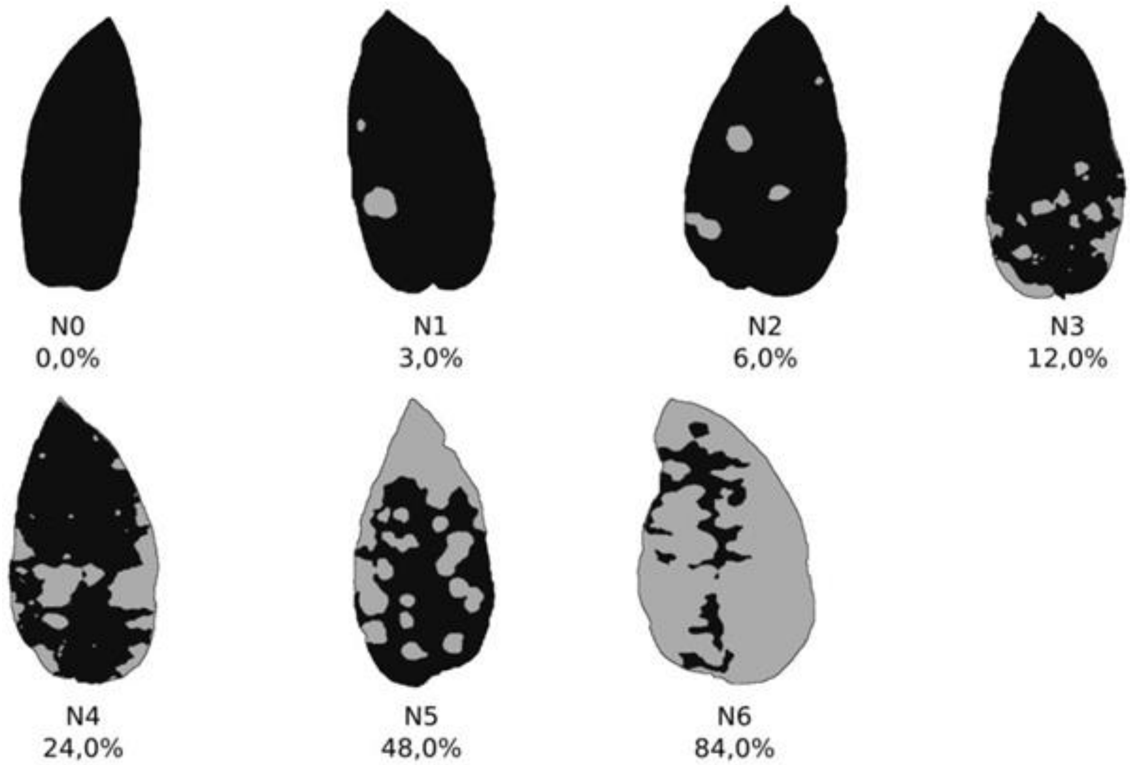

Figure S4. Diagrammatic scale for assessing the severity of Teratosphaeria Leaf Disease (TLD) in *Eucalyptus*. Values in percentage of leaf area with symptoms of leaf spot (shown as grey). Adapted from Passador et al. (2013).

Figure S5. Seedling growth of *Eucalyptus globulus* in the greenhouse: A- Seedlings 15 days old; B- Plants 50 days old; C- Plants 80 days old; D- Reconducted plant; E- Resprouted plants.

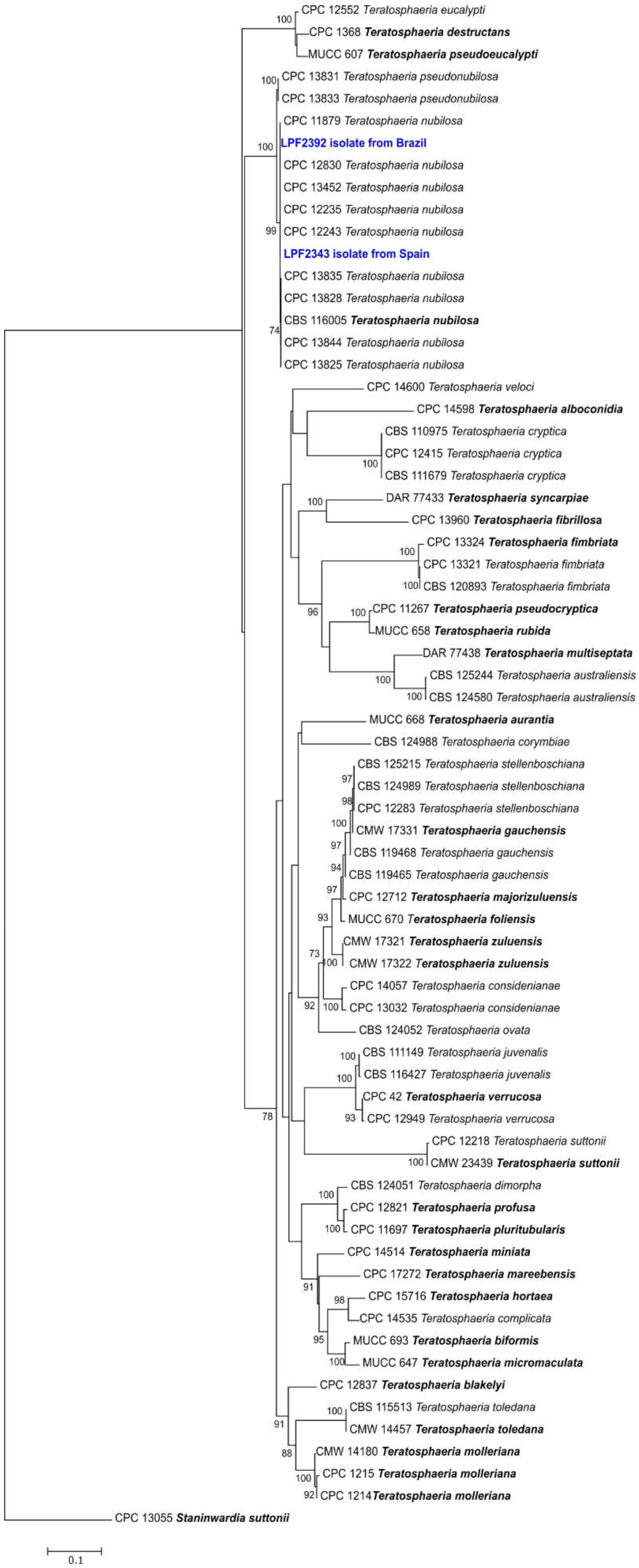

Figure S6. Consensus tree constructed by Maximum Likelihood based on a combined ITS, EF-1α, and Btub alignment, containing isolates associated with Teratosphaeria leaf disease (TLD) of *Eucalyptus*. Posterior probabilities (PP) ≥ 0.70 are above the nodes. The tree was rooted to *Staninwardia suttonii*. The scale in the tree base shows the length of the branches. Brazilian and Spanish isolates are in blue. The bold highlight represents the ex-epitype or ex-type.

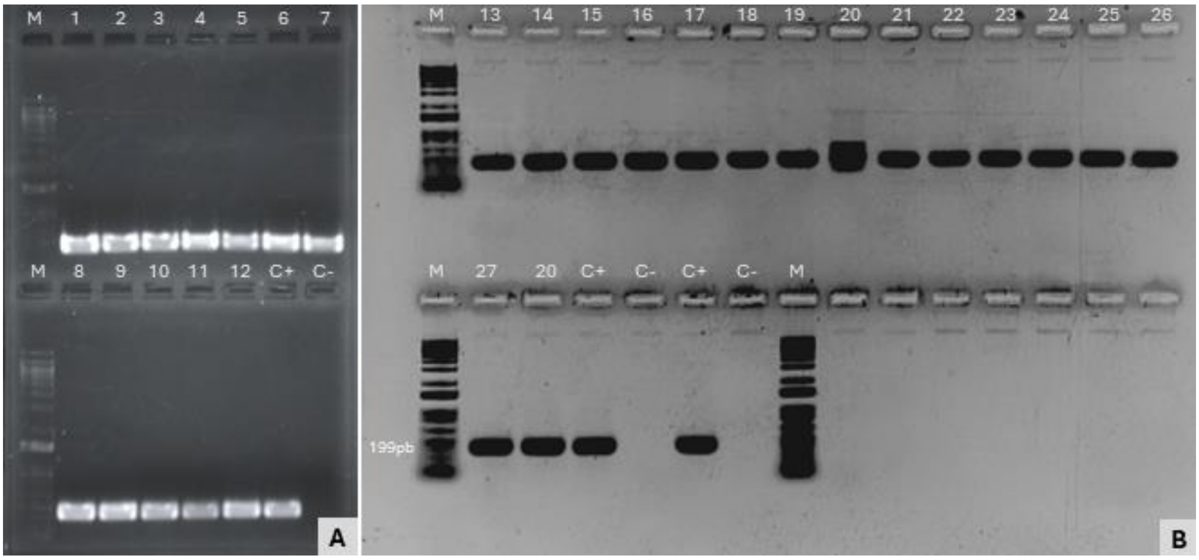

Figure S7. Bands (199 bp) amplified by PCR from DNA samples of *Teratosphaeria nubilosa* isolates using the species-specific primer pair MNF/MNR. The LPF2392 and LPF2543 isolates were used as positive controls (C+) and the T. *pseudoeucalypti* isolate LPF2760 as a negative control (C-).

Figure S8. Dead plants due to root malformation.