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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. Symptoms of myrtle rust disease are necrosis and leaf deformation, and death of the apical shoots, whereas 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 germinate 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 occur 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 this study, we found no evidence of basidiospore germination and/or germ tube penetration into the host tissue of eucalypt (*E. urophylla*) and rose apple (*S. jambos*) using scanning electron microscopic observations, raising questions about the role of basidiospores in the life cycle of *A. psidii* in Brazil.

1 | 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. 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 and Passador 2008). The urediniospore is unicellular, dikaryotic and generally

spherical. Urediniospores (10–20 × 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 × 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 and 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 was found. More recently, McTaggart et al. (2018) used a suspension of *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.

Other authors (Stewart et al. 2018; Graça et al. 2013; Roux et al. 2016) have also reported 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 that infects eucalypts (*Eucalyptus*) and rose apple (genetic clusters C2/C3); a biotype in 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 another 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 eucalypts/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 traits of each biotype of *A. psidii* are being progressively characterised. In Brazil, Ferreira (1981) demonstrated that the myrtle rust pathogen from guava was incapable of infecting eucalypts or rose apple. Graça et al. (2013), utilising microsatellite markers, demonstrated that Brazilian populations of *A. psidii* from guava are evolutionarily independent and exhibit significant genetic differentiation from Brazilian populations associated with eucalypts and rose apple. More recently, Morales et al. (2024) demonstrated through microscopic analyses that a Brazilian isolate of *A. psidii* obtained from guava was able to infect and colonise rose apple leaf tissues, but sporulation did not occur. Similarly, the *A. psidii* isolate from rose apple infected guava plants but did not sporulate.

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 anastomosis of basidiospore-derived hyphae within host tissue to form a 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 the 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 characterised 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 of basidiospores in the sexual cycle, genetic recombination and genotypic diversity of the predominate *A. psidii* biotype in Brazil, this work uses scanning electron microscopy to examine potential basidiospore-associated infectivity of *A. psidii* in eucalypt and rose apple.

2 | Materials and Methods

2.1 | 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 according to the methodology described by 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).

2.2 | Teliospore Production

Young, rooted cuttings of *Eucalyptus urophylla* (clone CLR371, susceptible to myrtle rust) were grown in 2-L plastic bags, containing Tropstrato (Vida Verde, 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 were maintained in a greenhouse at 25°C (±5°C) with manual irrigation twice a day 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°C ± 2°C, for 24 h. After incubation, inoculated plants were transferred to a growth chamber at 25°C ± 2°C and a 12-h photoperiod with an intensity of 110 μmol photons s^{-1} m^{-2}. At this temperature, teliospores are produced in greater abundance than urediniospores (Ruiz et al. 1989). Teliospores were produced 20 days after inoculation (dai) (Figure 1A).

2.3 | Basidiospore Production

In the first assay, pieces of eucalypt leaves, containing predominantly the teliospore stage of myrtle rust (Figure 1A), were attached with adhesive tape onto the upper inside lid of a Petri dish (100 × 20 mm) (Figure 1B). A volume of 2 mL of autoclaved, distilled water was added into the 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 1B), and these Petri dishes were 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. The suspension was vacuum filtered at maximum pressure (695 mmHg) using an 8-μm Millipore membrane (Figure 1C) (McTaggart et al. 2018). An aliquot of the basidiospore suspension was examined with light microscopy to confirm the purity of the basidiospore inoculum, and this assay was replicated. In the purity assays, only one immature urediniospore was observed in the post-filtration basidiospore suspension. Consequently, the first assay was repeated using a 10-μm Millipore membrane in a low pressure vacuum system (27.3 mmHg).

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 1A) were detached and brushed with a sterile, soft bristle brush into the Petri dishes (150 × 25 mm) (Figure 2A). Subsequently, 5 mL of distilled water was added to 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 to allow teliospore germination and facilitate the 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 analyse 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 analysed under stereo light and scanning electron microscopes. The plants were returned to the growth chamber at

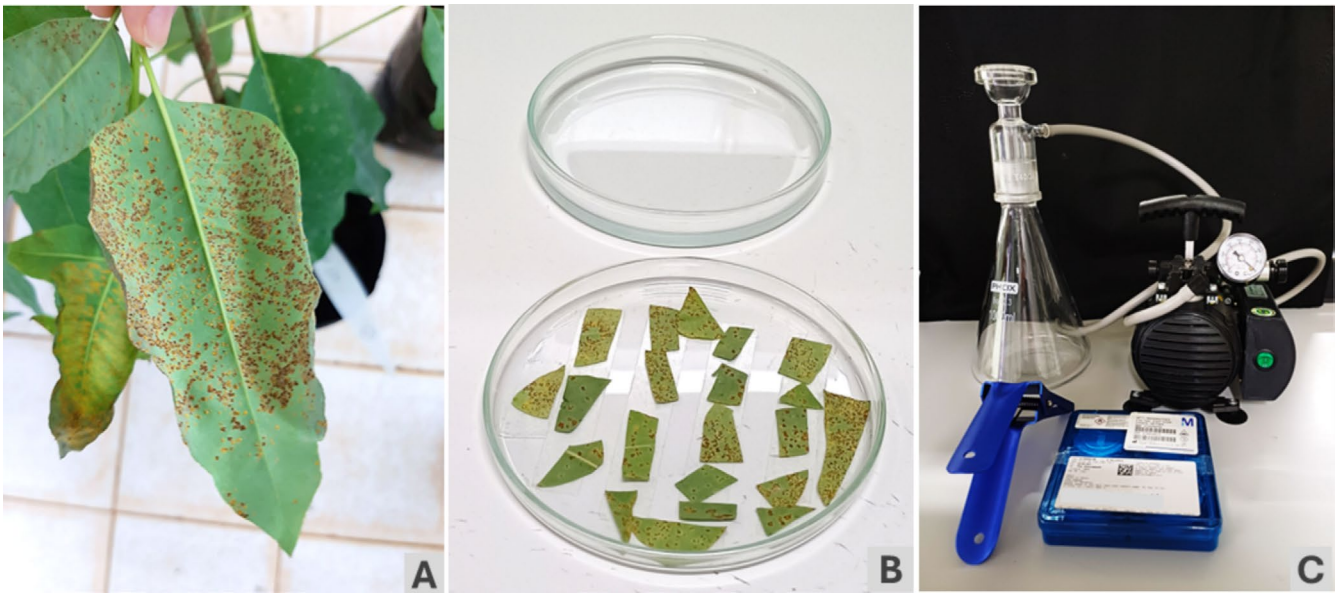

FIGURE 1 | Teliospore production and incubation with basidiospore filtration system used for *Austropuccinia psidii* (race 1) inoculations of eucalypt (*Eucalyptus urophylla* clone CLR371) and *Syzygium jambos*. (A) Teliospore production on eucalypt; (B) incubation of eucalypt leaf fragments in a Petri dish containing distilled water; (C) 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 h, in the dark; (C, D) third assay; (C) plants with teliospores; (D) plant with teliospores in a humid chamber system made with a moistened plastic bag.

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.

2.4 | Inoculation of *A. psidii* Basidiospores in *E. urophylla* and *S. jambos*

In the 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°C ± 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.

2.5 | Analysis of Inoculation With *A. 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 aluminium 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 one from rose apple—was examined for each sampling time.

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.

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.

3 | Results

3.1 | Inoculation With Filtered Basidiospore Suspension Produced No Infection

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 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 mmHg). 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 utilised to ensure 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 h of incubation, as

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.

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).

3.2 | Inoculation of a Spore Mixture Produced No Basidiospore Germination

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,C). In contrast, germination of urediniospores and subsequent germ tube penetration was observed by scanning electron microscopy (Figure 7B,D).

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.

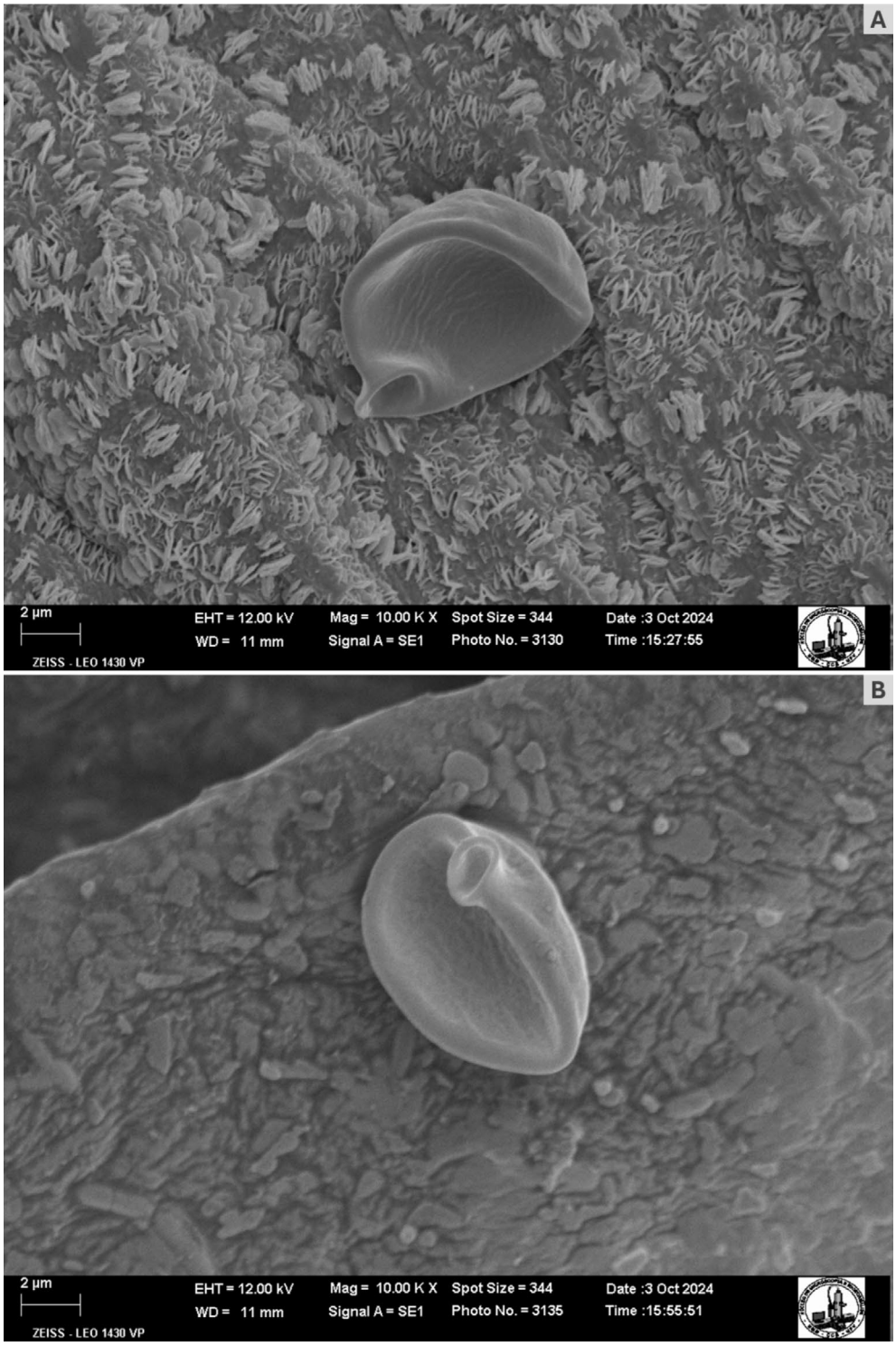

FIGURE 5 | Scanning electron microscopy of eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) leaves inoculated with basidiospores of *Austropuccinia psidii* (race 1). (A) Basidiospore on eucalypt leaf; (B) basidiospore in rose apple leaf.

3.3 | In Vivo Basidiospores Germinated and Formed Hyphal Connections

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 5–10 days, eucalypt leaves containing teliospores and basidiospores were intensely colonised by hyperparasites and the leaves became necrotic, which prevented continued observations (Figure 8C,D).

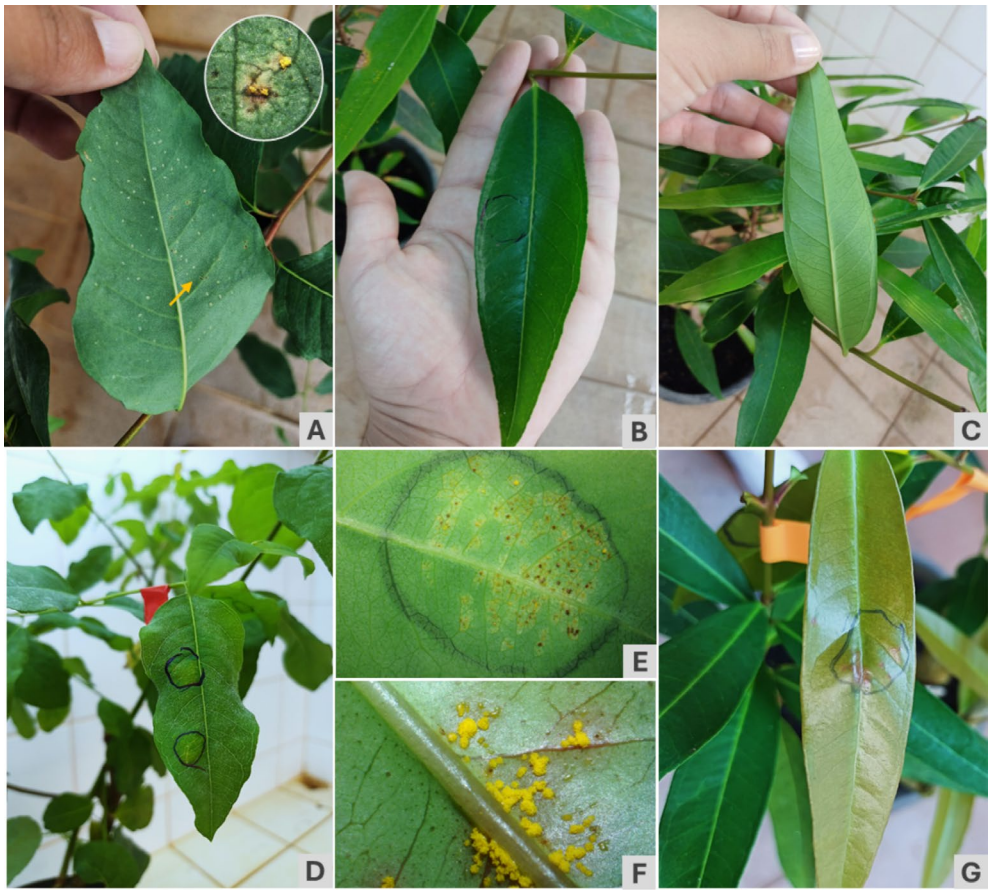

FIGURE 6 | Results of inoculations with a mixture of *Austropuccinia psidii* (race 1) spores on eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) in second assay. (A) Developing myrtle rust observed at the eucalypt inoculation site (first repetition, highlight with arrow 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.

4 | 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 urediniospore contamination 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 multilocus genotypes that differed from the parental rust genotype. McTaggart et al. (2020) identified numerous recombinant multilocus genotypes of *A. psidii* with unlinked loci, suggesting possible sexual reproduction of the myrtle rust pathogen from the observed teliospores and subsequent basidiospores. Using only microsatellite analyses, Stewart et al. (2018) identified 23 unique multilocus 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 categorised into nine genetic clusters of *A. psidii* (Stewart et al. 2018). The C1/C4 genetic clusters were 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 favourable to the occurrence of each biotype were also categorised and mapped (Stewart et al. 2018).

Despite the observed diversity among *A. psidii* populations, the source of this variation is not well characterised. How much of the genetic variation in *A. psidii* is attributed to sexual

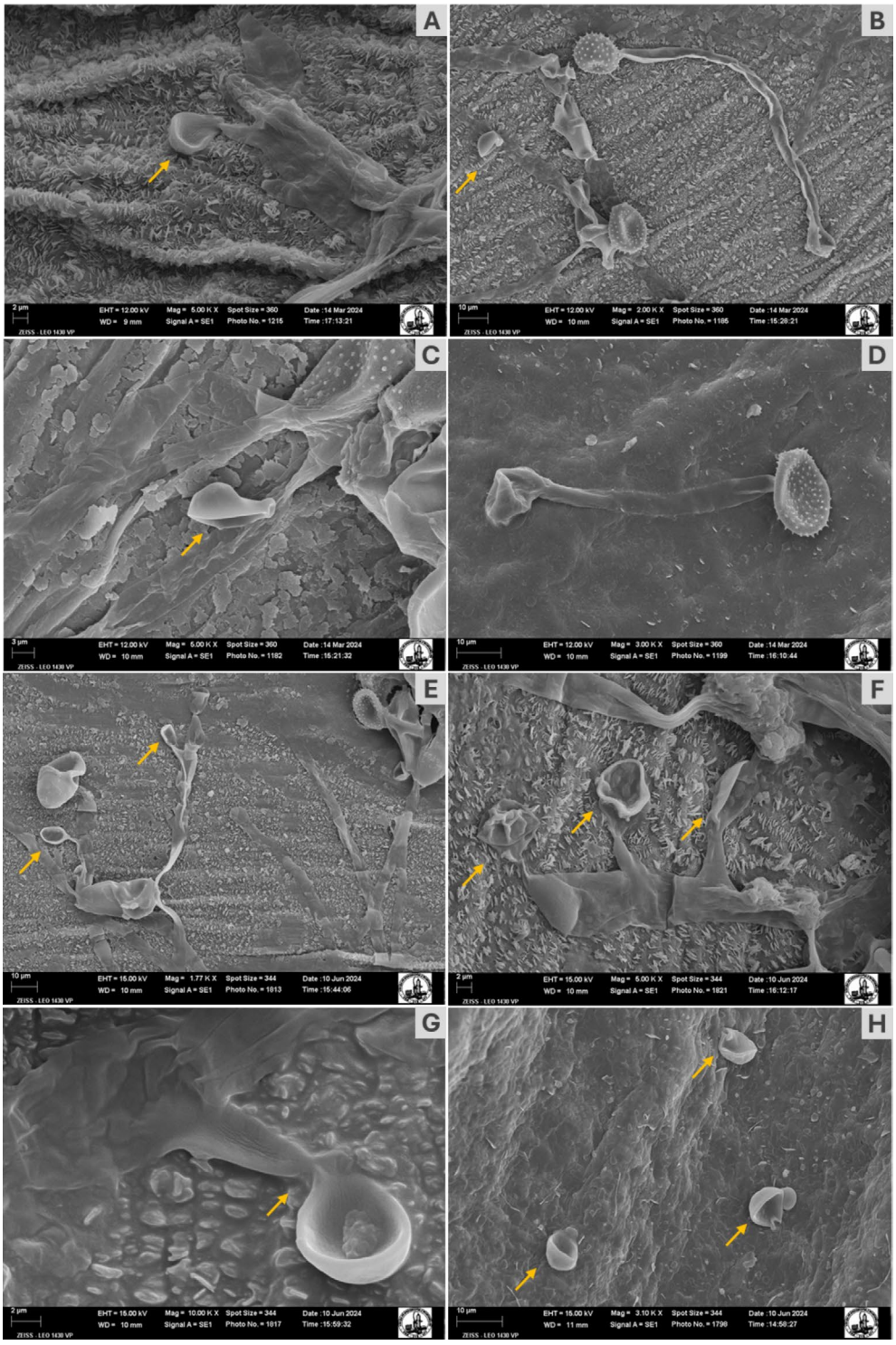

FIGURE 7 | Scanning electron microscopy of eucalypt (*Eucalyptus urophylla* clone CLR371) and rose apple (*Syzygium jambos*) leaves inoculated with spore suspension of *Austropuccinia psidii* (race 1). (A–D) Show examples from the first repetition of the second assay. (E, F) Show examples from the repetition of the second assay. (A) Non-germinated basidiospore under sterigma, 24 h 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.

reproduction and recombination events, as suggested by the previous studies with rose apple (McTaggart et al. 2018, 2020)? New genotypes of *A. psidii* formed by sexual reproduction could contribute to evolutionary processes in the myrtle rust pathogen that allow adaptations 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

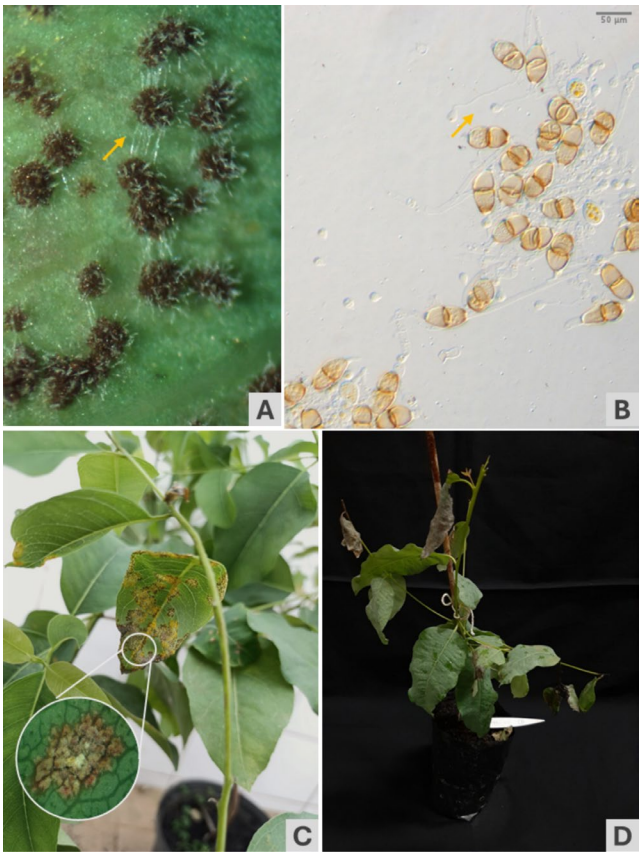

FIGURE 8 | Teliospores of *Austropuccinia psidii* (race 1) produced on eucalypt (*Eucalyptus urophylla* clone CLR371) after incubation in a humid chamber. (A) Apparent connection of hyphae among teliospore pustules, at 48 h after incubation; (B) basidiospores germinated after 48 h of incubation on leaf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

of sexual reproduction via basidiospore-derived infection by *A. psidii* race 1 (Brazilian eucalypt/rose apple 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 germ tubes of *A. psidii* from separate pustules raises the possibility of a mechanism for sexual recombination 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 apple genotypes are recognised 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.

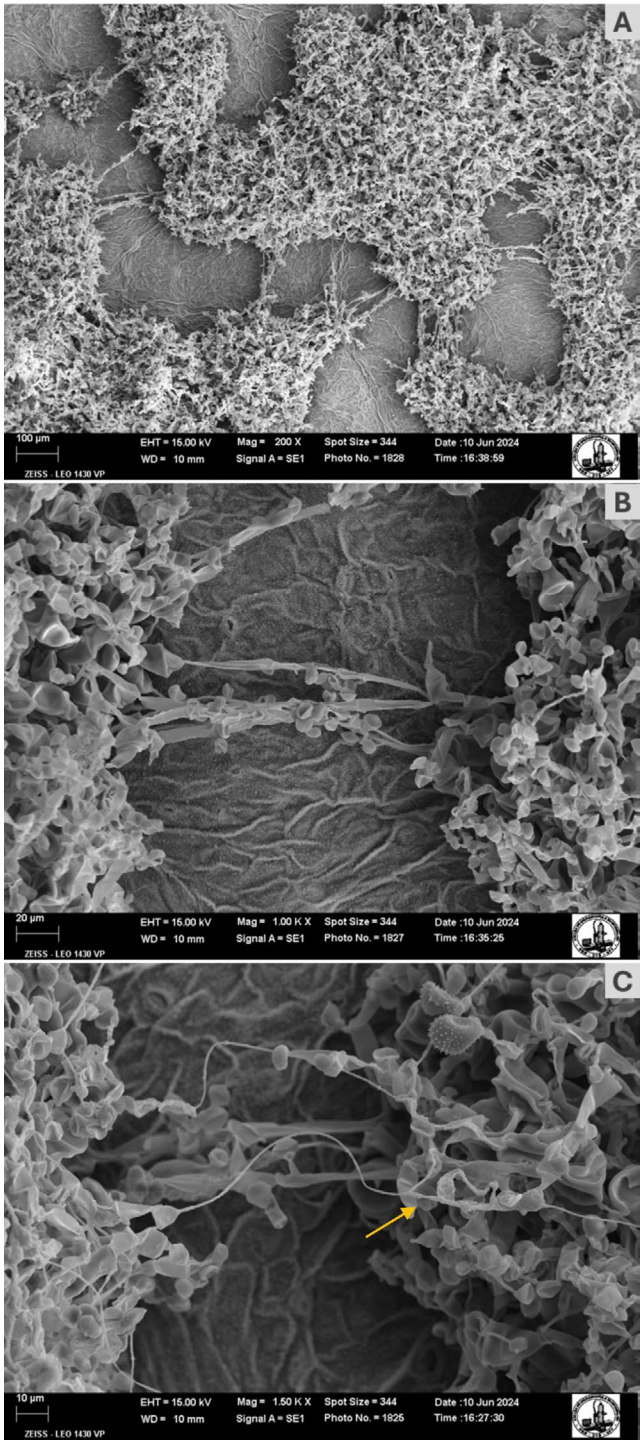

FIGURE 9 | Apparent connection of hyphae among basidiospores-containing pustules of *Austropuccinia psidii* (race 1), at 48 h after inoculation of eucalypt (*Eucalyptus urophyll*a clone CLR371).

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 the 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 and 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 colonisation 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 of later stages of myrtle rust infection 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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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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