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A new race of *Puccinia psidii* defeats rust resistance in eucalypt

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Abstract Rust caused by *Puccinia psidii* is one the most destructive diseases of *Eucalyptus*. Management of the disease is achieved through selection of resistant host genotypes. Recently, eucalypt plants from clone BA6021, resistant to *P. psidii* isolate race-1, were infected by rust in Brazil. Microsatellite profiles of infected plants confirmed that the host was indeed clone BA6021. In pathogenicity tests, the resistant clones BA6021 and G21 (which carry the resistance gene *Ppr-1*) were found susceptible to the newly discovered isolate EUBA-1, indicating a new biotype of the pathogen. These results show that the isolate EUBA-1 and other potentially unrecognized pathogen races should be given strong consideration for eucalypt breeding programs aimed rust resistance.

Keywords *Eucalyptus* · Rust · Resistance gene · Variability · Race

Introduction

Rust caused by *Puccinia psidii* Winter is a limiting factor to eucalypt production in Brazil, where the disease has caused up to 41% wood-volume losses in some plantations (Takahashi 2002). This disease can cause growth reduction, apical death, and even kill young plants intended for planting (Alfenas et al. 2009). Besides eucalypt, this pathogen also infects at least 70 other myrtaceous species (Farr and Rossman 2010). Because this rust pathogen poses a high potential for damage to the Myrtaceae family and its capacity to be dispersed over long distances, *P. psidii* is considered a serious threat to the native flora of Australia and surrounding islands and to commercial eucalypt plantations in South Africa (Coutinho et al. 1998; Glen et al. 2007).

The high inter- and intra-specific genetic variability for rust resistance in *Eucalyptus* species has allowed disease control through selection and planting of cuttings, seedlings, or species with resistance to *P. psidii* (Alfenas et al. 1997; Carvalho et al. 1998; Dianese et al. 1984; Tommerup et al. 2003). In a particular *E. grandis* family, the rust resistance is controlled by a single major gene, *Ppr-1* (*Puccinia psidii* resistance gene 1), with variable expression depending on the host genetic background (Junghans et al. 2003a). According to this pattern of inheritance, slight changes in the genetic structure of the pathogen could be enough to overcome this resistance gene.

Because urediniospores are easily dispersed over long distances and have the capacity to form large effective populations, as observed with other rusts, *P. psidii* is expected to have a high genetic variability and consequently a high evolutionary potential. Several cross-inoculation studies using Myrtaceae species have indicated the physiologic differences among *P. psidii* populations (Maclachlan 1938; Joffily 1944; Ferreira 1981; Castro et al. 1983; Coutinho and Figueiredo 1984; Coelho et al. 2001; Aparecido et al. 2003),. Considering that *P. psidii* is a biotrophic pathogen that is capable of infecting different Myrtaceae species across a wide geographic range, it appears reasonable that host-specific virulence genes could have been selected within the pathogen populations.

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In April 2008, yellow uredinial pustules of *P. psidii* were observed on clone BA6021 (*E. grandis* hybrid of Rio Claro, SP) plants in Southern Bahia, Brazil. In previous studies (data not shown), this clone had been previously classified as resistant to the isolate UFV-2 (race 1), which was commonly used to select rust-resistant eucalypt genotypes in Brazil (Junghans et al. 2003a; Xavier 2002). Two hypotheses were tested to explain the incidence of rust on the clone BA6021: 1) clonal admixture, plants infected with rust in South Bahia were not truly representative of clone BA6021; and 2) genetic variability within/among *P. psidii* populations, exemplified by the emergence of a new pathogen race with the capability to overcome the resistance mechanisms of the clone BA6021. An additional objective of the present study was to verify if the potentially new *P. psidii* race was capable of overcoming resistance conferred by the *Ppr-1* gene in *E. grandis*.

Material and methods

Clonal admixture

To evaluate the hypothesis of clonal admixture, cuttings from the clone BA6021 were collected from four separate forestry nurseries located in different Brazilian states. DNA was extracted from each cutting, and cuttings were genotyped using 15 microsatellite (SSR) markers (Brondani et al. 1998). PCR amplifications were performed in 15-μL reaction volumes with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C for primer annealing and 1 min at 72°C for DNA extension; and a final extension at 72°C for 15 min. Final reagent concentrations were 30 ng template DNA, 2.5 mM dNTPs, 0.25 units of Taq DNA polymerase (Applied Biosystems), 10 μM of each primer, 1.5 μL of 10× PCR buffer and 6.15 μL of MiliQ water. The amplified DNA fragments were separated on a 10% (w/v) polyacrylamide gel (29:1 acrylamide/bis-acrylamide). A 10-bp DNA ladder (Life Technologies) was used to score the allele sizes.

Puccinia psidii isolates

Two single-uredinial isolates were used in this study: isolate UFV-2, collected from young *E. grandis* plantations in São Paulo, Brazil (Junghans et al. 2003a; Xavier 2002), and the isolate EUBA-1, collected on cuttings of clone BA6021 (hybrid *E. grandis* × *E. urophylla*) from a forest nursery in southern Bahia, Brazil. Single-pustule-derived urediniospores of each isolate were multiplied on the young leaves of *Syzygium jambos* cuttings (Ruiz et al. 1989). After 12 days, the newly produced urediniospores were collected and stored in 1.5-mL Eppendorf® tubes at −80°C. The isolates were used for tests within 30 days of multiplication on *S. jambos*. To assure isolate purity, isolates were inoculated on eucalypt clones on different days. The inoculated plants were physically separated under controlled conditions until the end of the experiment (Ruiz et al. 1989).

Plant material

Seven eucalypt clones (G-21, G-26, BA-6021, 1183, 847, 3918, and 1205) from different species were inoculated with *P. psidii* (Table 1). Of these, *E. grandis* clones G21, G26, and G38 were used in a previous study of rust inheritance (Junghans et al. 2003a), and clone BA6021 was previously classified as resistant to the UFV-2 isolate (data not shown), but recently found to be infected with *P. psidii* in southern Bahia. Ten cuttings of each clone were transplanted to 2-L pots containing the substrate MecPlant® supplemented with 6 $Kg.m^{-3}$ of simple super phosphate and 3 $Kg.m^{-3}$ of Osmocote® (19N-6P-10K). The plants were inoculated after 30-days growth in the pots.

Physiologic difference between *Puccinia psidii* isolates

Cuttings from the *E. grandis* clone BA6021 (clonal identity was confirmed by microsatellite markers, results not shown) were inoculated with the isolate UFV-2 or EUBA-1. Both isolates were also used to inoculate two clones (*E. urophylla* clone 1183 and *E. grandis* clone 3918) that were previously classified as susceptible to the isolate UFV-2, and four clones (*E. grandis* clone G21, *E. grandis* clone G26, *E. grandis* clone 1205, and *E. urophylla* clone 847) that were previously classified as resistant to the isolate UFV-2. The clone G21 is heterozygous for the rust-resistance gene *Ppr-1* (Junghans et al. 2003a). The eucalypt

Table 1 *Eucalyptus* spp. clones rated based on rust resistance, according to the disease rating system proposed by Junghans et al. (2003b), 12 and 20 days after inoculation with the *Puccinia psidii* isolates UFV-2 and EUBA-1

Clones	12 Days		20 Days	
	UFV-2	EUBA-1	UFV-2	EUBA-1
G-21	HR	S1	HR	S2
G-26	HR	S1	HR	S1
BA-6021	HR	S3	HR	S3
1183	S3	S3	S3	S3
847	HR	HR	HR	HR
3918	S2	S3	S3	S3
1205	HR	HR	HR	HR

*HR, S0 and S1 = resistant; and S2 and S3 = Susceptible

plants were inoculated with a suspension of 2×10^4 urediniospores mL^{-1} (Ruiz et al. 1989). Ten plants of each clone were inoculated with the isolate UFV-2, and ten plants of each clone were inoculated with the isolate EUBA-1. To avoid cross-contamination of isolates, the inoculations were conducted on different days, and the plants were kept physically separated in mist and growth chambers. The inoculum suspension of each isolate was uniformly sprayed on both surfaces of young leaves, using a n° 15 De Vilbss, with an electrical compressor at 0.8 kgf cm^{-2}. After inoculation, plants were kept for 24 h in a mist chamber at 25±2°C in the dark, and then they were transferred to a growth chamber at 22±2°C with a 12-h light cycle (Ruiz et al. 1989). To assure uniform inoculation and validate inoculum viability, five *S. jambos* cuttings were utilized as susceptible controls for each isolate.

At 20 days post-inoculation, rust severity was evaluated using a disease rating system with four severity scores (Junghans et al. 2003b): S0 = immunity or hypersensitive reaction (HR); S1 = punctiform pustules, <0.8 mm; S2 = medium pustules, from 0.8 to 1.6 mm; and S3 = large pustules, >1.6 mm, and in some cases with pustules on the leaf petioles and young branch. Plants classified as S0 or S1 were considered resistant, and plants classified as S2 or S3 were considered susceptible. Two independent experiments were conducted at different times.

Puccinia psidii isolates aggressiveness on eucalypt

The aggressiveness of *Puccinia psidii* isolates, UFV-2 and EUBA-1, was evaluated through inoculation on the same seven clones used in the physiologic difference study (Table 1). The same experimental design was used, including the same number of replications. The aggressiveness of both isolates was assessed within 12 and 20 days post-inoculation. Disease severity was evaluated on the second leaf pair, counted from the plant apices to the base, using the disease rating system of Junghans et al. (2003b). Digital pictures were analyzed using the software Quant® to determine the percentage of leaf area with lesions (Vale et al. 2003). To count the number of urediniospores produced in 1 cm^2 of leaf area (Ruiz et al. 1989), three circular segments (1.2 cm) removed from the central part of the first or second leaf were placed in glass vials with 3 mL distilled water plus 2% Tween 20. The vials containing the leaf segments were mixed in a vortex for 1.5 min and the number of urediniospores was determined using a Neubauer chamber (hemacytometer). Two evaluations were made for each vial. Data analyses were performed with the software Statistica® 7.0. Two independent experiments were conducted at different times.

Results and discussion

Clonal admixture

The hypothesis of clonal mixture was rejected based on the multilocus genetic profiles in polyacrylamide gel of the four sources of the clone BA6021, which showed an identical genetic profile for all. It was concluded that the eucalypt genotype infected by *P. psidii* in southern Bahia was indeed the clone BA6021, which was previously classified as resistant to the isolate UFV-2 (race 1).

Physiologic difference between *P. psidii* isolates

Of seven clones inoculated with *P. psidii,* three (847, 1205, and G26) were classified as resistant, and two (1183 and 3918) were classified as susceptible to both isolates, UFV-2 and EUBA-1 (Table 1). However, two clones (BA6021 and G21) were found to be resistant to isolate UFV-2, but susceptible to isolate EUBA-1 (Table 1). This result further confirms the existence of physiologic difference within *P. psidii* populations (Aparecido et al. 2003; Castro et al. 1983; Coelho et al. 2001; Coutinho and Figueiredo 1984; Ferreira 1981; Joffily 1944; Maclachlan 1938). Three *P. psidii* races have been previously reported in Brazil (Xavier 2002); however, the isolate EUBA-1 represents a fourth pathogen race in Brazil, based on our results. Clones resistant to the isolate EUBA-1 and susceptible to UFV-2 were not observed. Therefore, isolate EUBA-1 was virulent on a greater number of eucalypt genotypes, of the seven clones tested here.

Based on previously studies, the genotypes G21 and G26 were previously classified as resistant to 21 *P. psidii* isolates collected from different locations in Brazil, which included the isolate UFV-2. In those studies, the G21 and G26 clones were rated as S0, showing hypersensitive reactions, and it was further demonstrated that the rust resistance on clone G21 is controlled by a single dominant gene, *Ppr-1*, *Puccinia psidii* resistance gene-1, which is heterozygous in clone G21 (Junghans et al. 2003a). In the current study, clone G21 was still resistant to the isolate UFV-2, and exhibited a hypersensitive response. However, clone G21 was susceptible to the isolate EUBA-1, with a rust rating of S2. This is the first report of the break-down of rust resistance linked to gene *Ppr-1*in eucalypts. The breakdown of resistance in clone G21 has been demonstrated only through artificial inoculations, and a similar breakdown of resistance in clone G21 in the field has not been reported to date. It should be noted that eucalypt clone G21 is only planted in Sao Paulo state, and the new rust race (represented by EUBA-1) was found in a forest nursery in southern Bahia, where the clone G21 is not commercially planted. Based on this information, it appears that urediniospores of *P. psidii* EUBA-1 isolate have not yet migrated from South Bahia to Sao Paulo.

Table 2 Analysis of variance on percentage area with lesions, 12 and 20 days post-inoculation with the *Puccinia psidii* isolates UFV-2 and EUBA-1 on different eucalypt clones

Source	df	*F* value	*P*
12 days post-inoculation			
Isolate	1	32.95	0.0001
Clone	6	0.85	0.3689
Isolate × Clone	6	9.73	0.0066
20 days post-inoculation			
Isolate	1	31.48	0.0001
Clone	6	16.69	0.0001
Isolate × Clone	6	0.31	0.5869

Within the clones resistant to both isolates, clone G26, previously classified as resistant to the isolate UFV-2 (Junghans et al. 2003a), showed small pustules (S1 score) surrounded by a chlorotic halo after inoculation with the EUBA-1 isolate. Therefore, the EUBA-1 isolate showed a capacity to partially overcome the initial defense mechanisms of clone G26. Clones 847 and 1205 were classified as resistant (S0 score) when inoculated with both isolates; clone 847 showed a strong hypersensitive response, and clone 1205 exhibited tiny chlorotic spots or "flecks".

Puccinia psidii isolates aggressiveness on eucalypt

Analysis of variance showed significant effects ($P \leq 0.01$) of isolates and isolate × clone on the percentage of leaf area with lesions at 12 days post-inoculation (Table 2), indicating that different rust isolates produced different responses when inoculated on the same eucalypt clone. No significant differences in disease severity were observed among clones BA6021, 1183, and 3918 when inoculated with the EUBA-1 isolate; these three clones were all susceptible to the EUBA-1 isolate (Fig. 1). However, only the clones 1183 and 3918 were susceptible to inoculations with the UFV-2 isolate, showing 6.7% and 7.8% leaf area with lesions, respectively. Clone BA6021 was classified as resistant to the UFV-2 isolate (Fig. 1). Significant effects ($P \leq 0.01$) on leaf area with lesions were observed at 12 days post-inoculation for *P. psidii* isolates and eucalypt clones. However, no significant effects on clone × isolate were observed at 20 days post-inoculation (Table 2).

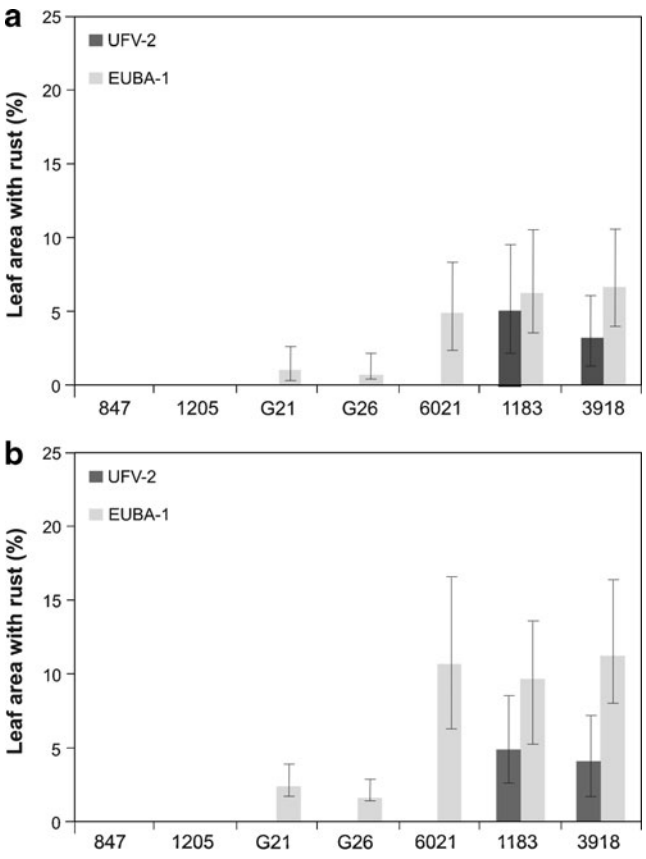

Fig. 1 Leaf area with rust 12 (**a**) and 20 days (**b**) after inoculation with *Puccinia psidii*. *Error bars* correspond to ± confidence interval

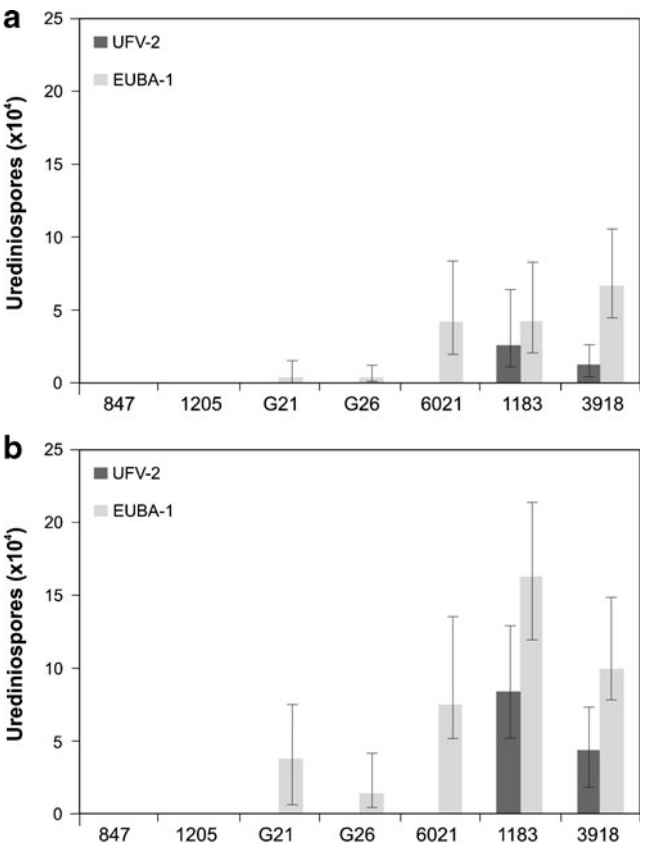

Fig. 2 Number of urediniospores ($\times 10^4$) produced for 1 cm^2 of leaf area, 12 (**a**) and 20 (**b**) days after inoculation with *Puccinia psidii*. *Error bars* correspond to ± confidence interval

Puccinia psidii urediniospores were observed on the clones G21, G26, 6021, 3918, and 1183, at 12 and 20 days post-inoculation with the EUBA-1 isolate. However, when inoculated with the UFV-2 isolate, *P. psidii* urediniospores were observed only on the clones 3918 and 1183, at 12 and 20 days post-inoculation (Fig. 2). At 12 days post-inoculation, a larger number of urediniospores (6.7×10^4 urediniospores.cm^{-2} of leaf area) were produced on the clone 3918 when inoculated with the EUBA-1 isolate, compared to urediniospores (1.25×10^4 urediniospores.cm^{-2} of leaf area) produced by the UFV-2 isolate (Fig. 2). Thus, the EUBA-1 isolate appears to be more aggressive than the UFV-2 isolate on clone 3918. The fact that the EUBA-1 isolate was more aggressive on the eucalypt clones tested and virulent on a larger number of clones, indicates that the EUBA-1 isolate, and possibly other undiscovered races, are important factors for consideration in eucalypt breeding programs to increase rust resistance. However, despite the observed difference in virulence and aggressiveness between the isolates EUBA-1 and UFV-2, based on ten *P. psidii* microsatellite markers (Zhong et al. 2008) this two isolates had identical multilocus genotypes (unpublished data). The discovery of a new pathogen race, more aggressive and virulent to larger number of eucalypt clones, reinforces the need to include as broad a range as possible of *P. psidii* isolates in the selection of resistant eucalypt genotypes. Furthermore, population genetic studies aimed at understanding the distribution of genetic variability within pathogen populations from different hosts and geographic locations are essential for any programs directed toward managing or better understanding eucalypt rust disease.

Conclusions

The *P. psidii* isolate EUBA-1 was able to overcome the rust-resistance gene *Ppr-1* in *E. grandis* (clone G21), therefore this isolate belongs to a new pathogen race, named here as race 4.

The EUBA-1 isolate (race 4) was also able to infect plants from the clone BA6021, which was previously classified as resistant to the isolate UFV-2 (race 1).

The isolates EUBA-1 (race 4) and UFV-2 (race 1) differ in virulence, and EUBA-1 displays virulence on a larger number of eucalypt clones.

The isolate EUBA-1 (race 4) is more aggressive than the isolate UFV-2 on the clone 3918.

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References

Alfenas AC, Valle LAC, Xavier AA, Brommonschenkel SH, Grattapaglia D, Silva CC, Bertolucci FL (1997) Penchel Rok. Eucalyptus rust: genetic variability of elite clones and histological characterization of the resistance reaction. Proceedings IUFRO Conference on Silviculture and Improvement of Eucalypt, Salvador BA, 2: 60–64

Alfenas AC, Zauza EAV, Mafia RG, Assis TF (2009) Clonagem e doenças do eucalipto. Editora UFV, 2nd edn. 2009. 500p

Aparecido CC, Figueiredo MB, Furtado EL (2003) Groups of physiological variability in *Puccinia psidii* populations. Summa Phytopathologica 29:234–238

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998) Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. Theor Appl Genet 97:816–827

Carvalho ADO, Alfenas AC, Maffia LA, Carmo MGF (1998) Resistance of *Eucalyptus* species, progenies and provenances to *Puccinia psidii*. Pesquisa Agropecuária Brasileira 33:139–147

Castro HA, Krugner TL, Iderilha CHF, Capello MS, Marchi AB (1983) Inoculação cruzada de *Eucalyptus*, goiaba (*Psidium guajava*) e jambeiro (*Syzygium jambos*) com *Puccinia psidii*. Fitopatologia Brasileira, Brasília 8:491–497

Coelho L, Alfenas AC, Ferreira FA (2001) Physiologic variability of *Puccinia psidii*—the rust of *Eucalyptus*. Summa Phytopathologica 27:295–300

Coutinho LN, Figueiredo MB (1984) Estudos sobre especializações fisiológicas em *Puccinia psidii*. Summa Phytopathologica 10:56–57

Coutinho TA, Wingfield MJ, Alfenas AC, Crous PW (1998) Eucalyptus rust: a disease with the potential for serious international implications. Plant Dis 82:819–825

Dianese JC, Moraes TSD, Silva AR (1984) Reponse of *Eucalytus* species to field infection by *Puccinia psidii*. Plant Dis 68:314–316

Farr DF, Rossman AY (2010) Fungal databases, systematic mycology and microbiology laboratory, ARS, USDA. Retrieved May 4, 2010, from http://nt.ars-grin.gov/fungaldatabases/

Ferreira FA (1981) Ferrugem do eucalipto—ocorrências, temperatura para germinação de uredosporos, produção de teliosporos, hospedeiro alternativo e resistência. Fitopatologia Brasileira 6:603–604

Glen M, Alfenas AC, Zauza EAV, Wingfield MJ, Mohammed C (2007) *Puccinia psidii*: a threat to the Australian environment and economy—a review. Australas Plant Pathol 36:1–16

Joffily J (1944) Ferrugem do eucalipto. Bragantia, Campinas 4 (8):475–487

Junghans DT, Alfenas AC, Brommonshenckel SH, Oda S, Mello EJ, Grattapaglia D (2003a) Resistance to rust (*Puccinia psidii* Winter) in Eucalyptus: mode of inheritance and mapping of a major gene with RAPD markers. Theor Appl Genet 108:175–180

Junghans DT, Alfenas AC, Maffia LA (2003b) Escala de notas para quantificação da ferrugem em *Eucalyptus*. Fitopatologia Brasileira 28:184–188

MacLachlan JD (1938) A rust of pimento tree in Jamaica, B.W.I. Phytopathology 28:157–170

Ruiz RAR, Alfenas AC, Ferreira FA (1989) Influência da temperatura, do tempo de molhamento foliar, fotoperíodo e intensidade de luz sobre a infecção de *Puccinia psidii* em eucalipto. Fitopatologia Brasileira 14:55–61

Takahashi SS (2002) Ferrugem do eucalipto: índice de infecção, análise temporal e estimativas de danos relacionados a intensi-

dade da doença no campo. Dissertação de mestrado apresentada à Faculdade de Ciências Agronômicas da UNESP—Campus de Botucatu

Tommerup IC, Alfenas AC, Old KM (2003) Guava rust in Brazil—a threat to *Eucalyptus* and other Myrtaceae. New Zeal J Forest Sci 33:420–428

Vale FXR, Fernandes Filho EI, Liberato JR (2003) QUANT. A software plant disease severity assessment. In: Proceedings of the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 105

Xavier AA (2002) Histopatologia da interação *Puccinia psidii* e virulência de isolados do patógeno em espécies de Myrtaceae. PhD thesis, Federal University of Viçosa, Brazil

Zhong S, Yang B, Alfenas AC (2008) Development of microsatellite markers for the guava rust fungus, *Puccinia psidii*. Mol Ecol Notes 8:348–350