CLAUDIA NOHEMY MONTOYA ESTRADA

***Erwinia psidii* - *Eucalyptus* spp.: COLONIZATION, GENETIC VARIABILITY, AGGRESSIVENESS AND IMMUNOLOGICAL TEST FOR PATHOGEN DETECTION**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia para obtenção do título de *Doctor Scientiae.*

VIÇOSA
MINAS GERAIS-BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

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2018

Montoya Estrada, Claudia Nohemy, 1984-
Erwinia psidii - Eucalyptus spp. : colonization, genetic variability, aggressiveness and immunological test for pathogen detection / Claudia Nohemy Montoya Estrada. – Viçosa, MG, 2018.
xiv, 82 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Acelino Couto Alfenas.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Doenças bacterianas das plantas. 2. Anticorpos policlonais. 3. Bactérias - Detecção. *4*. Morte descendente. 5. Virulência (Microbiologia). 6. Seca-de- ponteiros. 7. *Psidium guajava*. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-Graduação em Fitopatologia. II. Título.

CDD 22. ed. 579.3

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APROVADA: 26 de fevereiro de 2018.

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Aos meus pais, Octavio (*In Memoriam*) e Nohemí

Ao meu esposo, Gustavo

As minhas filhas Gabriela e Martina (*In Memoriam*)

Aos meus familiares e amigos

que sempre acreditaram em mim, dedico.

AGRADECIMENTOS

Primeiramente agradeço a Deus, por ser minha fortaleza, por ter me concedido tantas oportunidades e por me dar coragem para seguir na caminhada.

Aos meus pais Octavio (*In Memoriam*) e Nohemí, por todas as palavras de amor, apoio, motivação, e companhia durante toda minha vida.

Ao meu esposo Gustavo, por todo o apoio, pela imensa paciência e amor incondicional, essencial para que eu chegasse até aqui.

As minhas filhas Gabriela e Martina (*In Memoriam*), por tantos ensinamentos, pela valentia e coragem e por me ensinar que existe o verdadeiro amor. Sempre em meu coração.

Aos meus colegas e amigos da Universidade Católica de Manizales (Colômbia), em especial ao Programa de Bacteriologia, por todo companheirismo, amizade e pela motivação para iniciar esta caminhada longe de casa.

Ao professor, Jairo Castaño Zapata, pelos ensinamentos durante meu mestrado e por despertar em mim a paixão pela Fitopatologia.

Ao professor, Acelino Couto Alfenas, pela orientação, ensinamentos e pela oportunidade de trabalhar com uma equipe maravilhosa e por despertar em mim a paixão pela Patologia Florestal.

Aos Professores, Jorge Luis Badel Pacheco, Leandro Licursi de Oliveira e ao Pós-Doc /dr. Lúcio M. S. Guimarães, pela orientação, dedicação e atenção com o trabalho desenvolvido.

À Organização dos Estados Americanos (OEA) e o Grupo de Coimbra de Universidades Brasileiras (GCUB) pela bolsa de estudos, por me permitir fazer um sonho realidade.

À Clonar Resistência a Doenças Florestais pela estrutura de inoculação disponibilizada e pela propagação do material vegetal utilizado nos experimentos

À Universidade Federal de Viçosa, pelo acolhimento e infraestrutura, importantes para a minha formação.

Ao Núcleo de Microscopia e microanálise, pelo apoio durante o desenvolvimento desta pesquisa.

Ao Departamento de Fitopatologia, em especial, a todos os professores e colegas que me acompanharam durante esta caminhada, pelos conselhos e orientações agradeço.

Aos Laboratórios de Biotecnologia do Cafeeiro, Imunoquímica e Glicobiologia, Interação Planta-Patógeno, Biologia de Populações e Genética e Genômica das Interações Planta-Patógeno, pelo apoio e empréstimos de equipamentos importantes para o desenvolvimento da pesquisa.

Aos amigos do Laboratório de Patologia Florestal/Bioagro – PATOMOL, pelo carinho, pelas conversas e convivência e por tornarem os dias de trabalho mais felizes. Em especial, agradeço à Ana Cristina Miranda, Camila Ribeiro, Mara Cândido, Paulo Sinei e Elenice Martins pela amizade, ajuda constante e apoio durante este tempo.

Enfim, a todos que fizeram parte desta jornada, muito obrigada!

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RESUMO

MONTOYA, CLAUDIA NOHEMY ESTRADA. D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. ***Erwinia psidii* - *Eucalyptus* spp.: colonização, variabilidade genética, agressividade e teste imunológico para a detecção do patógeno.** Orientador: Acelino Couto Alfenas. Coorientadores: Lúcio Mauro da Silva Guimarães, Jorge Luis Badel Pacheco e Leandro Licursi de Oliveira.

Seca de ponteiros, causada por *Erwinia psidii* é atualmente uma das doenças emergentes mais severas na cultura do eucalipto. Apesar de sua importância econômica, em virtude dos riscos que pode causar na eucaliptocultura, pouco se sabe sobre este patossistema. Assim, neste trabalho, a partir de cortes histológicos, estudou-se o processo de colonização em plantas de eucalipto usando um isolado de *E. psidii* transformado com *gfp*, estimou-se a variabilidade genética das populações do patógeno por meio de marcadores moleculares rep–PCR (ERIC, REP e BOX) e desenvolveu-se um imunoteste para detecção rápida de *E. psidii* usando colônias puras isoladas de plantas sintomáticas e assintomáticas. Neste estudo, conseguiu-se transformar *E. psidii* com pGreen-TIR e demonstrar que o plasmídeo é estável na ausência de seleção com antibióticos *in vitro* e *in vivo*. Usando microscopia de fluorescência, demonstrou-se que a colonização de *E. psidii* nos tecidos não está restrita ao ponto de inoculação (axila foliar). *E. psidii* coloniza os vasos do xilema, esclerênquima e parênquima das folhas e caule do eucalipto. Aos 35 dias após a inoculação (dai), a bactéria encontrava-se a 5 cm acima do ponto de inoculação, indicando que foi capaz de colonizar a planta acropetalmente, acompanhando o fluxo da água e nutrientes. Análises por microscopia confocal de amostras de plantas inoculadas via sistema raicular revelaram que *E. psidii* penetra e coloniza raízes primárias e secundárias e atinge os vasos do xilema. No entanto, nos tempos após inoculação avaliados neste estudo, a bactéria ficou restrita nas raízes e não atingiou o caule da planta. Estudos adicionais avaliando tempos após inoculação maiores devem ser realizados para confirmar esses resultados. Acredita-se que *E. psidii* seja disseminada a partir de mudas infectadas assintomáticas. Análises moleculares (rep-PCR) de 101 isolados de *E. psidii* obtidos de *Eucalyptus* spp. e cinco isolados de goiabeira (*Psidium guajava*) indicam que as populações de *E. psidii* do Brasil apresentam baixa variabilidade genética. Observou-se, contudo, o agrupamento dos isolados por espécie hospedeira, embora dois isolados de goiaba (LPF681 e LPF682) agruparam-se com os de eucalipto (LPF609). Testes de Wilcoxon e ANOVA dos dados de severidade e AACPD para diferentes isolados da bactéria inoculados em dois clones de eucalipto indicaram que há interação isolado × clone. A AACPD e a severidade da doença variaram significativamente entre isolados e nos dois clones testados. A variabilidade em agressividade entre isolados de *E. psidii* demonstraram a importância do emprego de isolados mais agressivos para a seleção de genótipos de eucalipto resistentes para plantio em escala comercial. O anti-soro contra o isolado LPF534 de *E. psidii* (Anti-Ep), obtido neste estudo, reagiu positivamente contra todos os isolados de *E. psidii* testados, incluindo alguns isolados de goiaba. O teste de aglutinação desenvolvido com este anti-soro é importante para o diagnóstico de *E. psidii*, por representar uma alternativa rápida e de baixo custo, em comparação com métodos baseados em PCR para a detecção do patógeno em plantas assintomáticas.

ABSTRACT

MONTOYA, CLAUDIA NOHEMY ESTRADA. D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. ***Erwinia psidii* - *Eucalyptus* spp.: colonization, genetic variability, aggressiveness and immunological test for pathogen detection.** Adviser: Acelino Couto Alfenas. Co-advisers: Lúcio Mauro da Silva Guimarães, Jorge Luis Badel Pacheco and Leandro Licursi de Oliveira.

Dieback, caused by *Erwinia psidii* is currently one of the most severe emerging diseases in eucalypt plantations. Despite its economic importance, due to the risks posed to eucalypt production, little is known about this pathosystem. In this work, we studied the plant colonization by a *gfp*-transformed *E. psidii* isolate using histological sections, estimated the genetic variability of pathogen populations using molecular rep–PCR markers (ERIC, REP and BOX) and developed an immunoassay for the rapid detection of *E. psidii* in symptomatic and asymptomatic plants. In this study, we were able to transform *E. psidii* with pGreen-TIR and to demonstrate that the plasmid is stable in the absence of antibiotic selection both *in vitro* and *in vivo*. Using fluorescence microscopy, it was possible to demonstrate that tissue colonization by *E. psidii* is not restricted to the inoculation point (leaf axil) and that it colonizes the xylem vessels, sclerenchyma and parenchyma of the leaves and stem of eucalypt. At 35 days after inoculation (dai), the bacterium was found at 5 cm above the inoculation point, indicating that it was able to colonize the plant acropetally, following the water flow. Confocal microscopy analysis of plant samples inoculated via radicular system revealed that *E. psidii* penetrates and colonizes primary and secondary roots and reaches the xylem vessels. However, at the times after inoculation evaluated in this study, the bacterium was restricted to the roots and did not reach the stem of the plant. Further studies with longer times after inoculation must be performed to confirm these results. It is believed that *E. psidii* is disseminated from infected asymptomatic cuttings. Molecular analyzes (rep-PCR) of 101 *E. psidii* isolates obtained from eucalypt (*Eucalyptus* spp.) and five guava (*Psidium guajava*) demonstrated that the populations in Brazil have low genetic variability. Nonetheless, grouping of isolates by host plant was observed, although two guava isolates (LPF681 and LPF682) grouped with eucalypt isolates (LPF609). The Wilcoxon and ANOVA tests on disease severity and AUDPC data indicated an isolate × clone interaction. AUDPC and disease severity varied significantly among isolates and between the two clones tested. The variability in aggressiveness among isolates of *E. psidii* demonstrated the importance of using the most aggressive isolates in the selection of resistant eucalypt genotypes for commercial scale planting. An antiserum against *E. psidii* isolate LPF534 (Anti-Ep) obtained in this study reacted positively against all strains of *E. psidii* tested, including some isolated from guava. The agglutination test designed using this antiserum can be considered important for the diagnosis of *E. psidii*, as it represents a rapid and low-cost alternative compared to PCR-based methods for detecting the pathogen in asymptomatic plants.

INTRODUCTION

Eucalypt trees (*Eucalyptus* spp. and *Corymbia* spp.), native to Australia and neighboring islands, are currently the largest source of wood products and derivatives from forest plantations worldwide, mainly in tropical and subtropical regions. Adaptability, rapid growth, and high productivity led to the expansion of eucalypt plantations in Brazil. Nowadays, Brazil is the world's leader of pulp, paper, and wood panels production, with exports that undeniably contribute to its trade balance and generate many jobs and significant income in all regions of the country. In 2016, eucalypt plantations covered 7.5 million hectares in the country, mostly located in the Southeast Region (3.1 million hectares), folloed by the Central-West Region (1.4 million hectares), Northeast Region (908. 832 hectares) and North Region (602.403 hectares) (IBGE, 2016). However, the climatic instability and the emergence of new diseases, such as dieback and wilt, caused by *Erwinia psidii* Rodrigues Neto, Robbs & Yamashiro (Rodrigues Neto et al., 1987; Alfenas et al., 2009) threaten eucalypt production.

Erwinia psidii was first described in *Psidium guava* L. in 1987 (Rodrigues Neto et al., 1987). Twenty-four years later, it was reported in *Eucalyptus* spp. in Argentina and Uruguay (Coutinho et al., 2011) and subsequently in Brazil, where, up to now, it was confirmed in the states of Mato Grosso do Sul, Rio Grande do Sul, and São Paulo (Arriel et al., 2014). Recently, *E. psidii* was recorded causing dieback in *Carica papaya* L. in Malaysia (Chai et al., 2017). Dieback and wilting, caused by *E. psidii* is currently one of the most severe emergent bacterial diseases of *Eucalyptus* spp. in South America (Arriel et al., 2014). To date, it has been observed in stands of *E. saligna, E. dunnii, E. grandis* and *E. urophylla* × *E. grandis, E. urophylla* × *E. maidenii* hybrids (Arriel et al., 2014). Although, the disease has not been observed in wild native and exotic myrtaceous species, recent inoculation studies, under controlled conditions, showed that *E. psidii* is pathogenic to a large number of species within *Myrtaceae.* It is possible that the disease has not been found under natural conditions because those species are not planted on a large scale (Caires, 2017). Currently, *E. psidii* is one of the most important pathogens of guava orchards in Central Brazil (Teixeira et al., 2009).

Erwinia psidii kills the plant's leader and lateral shoots and consequently breaks down the apical dominance of the tree (Coutinho et al., 2011; Arriel et al., 2014). Disease symptoms are characterized by dieback and water-soaked leaf lesions at the sides and along the main rib in the apical third of the plant. As the disease progresses, superficial or deep mini-cankers develop along the apical and lateral branches and, finally, the plants may wilt and die (Ferraz et al., 2016). On some highly susceptible eucalypt genotypes, the disease can be detected in almost 100% of the plants, consequently leading to growth reduction and productivity. Currently, diagnosis of this disease relies on the use of phenotypic methods (i.e., culture and biochemical tests) and detection of bacterial DNA by polymerase chain reaction (PCR), which is time-consuming and relatively expensive. In the field, the disease is diagnosed based on the symptoms that sometimes can be confused with other diseases, such as the wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi. Before reporting *E. psidii* in eucalypt, the etiology of bacterial wilt was attributed to *R. solanacearum* based solely on the bacterial oozing, and in many cases, the disease was incorrectly diagnosed (Ferraz et al., 2016). Due to the losses resulting from the disease, it is important to develop highly sensitive, accurate, inexpensive and rapid methods for diagnosis to prevent dissemination of the bacterium through symptomatic or asymptomatic plants both in nurseries and in the field.

Little is known about the biological cycle, survival, mechanisms of pathogenicity and infection process of *E. psidii*, knowledge considered essential for the establishment of effective management strategies. In this regard, a clear understanding of the plant infection and colonization by the bacterial pathogen is of fundamental importance to tackle other important aspects of the pathosystem. The use of green fluorescent protein (GFP) as reporter to track down the movement of bacterial cells within host tissues has facilitated studies on plant infection and colonization. Bacteria transformed with *gfp* have been proved to be highly effective to elucidate these biological processes in interactions such as *Xylella fastidiosa*-*Vitis vinifera* (Newman et al., 2003), *Pseudomonas syringae* pv. *tomato*-*Arabidopsis thaliana* (Melotto et al., 2006) and *Dickeya* sp.-*Solanum tuberosum* (Czajkowski et al., 2010).

Inoculations of *Erwinia psidii* in *Eucalyptus* spp. and *Corymbia* spp., under controlled conditions suggest that there is genetic variability for host resistance, which may vary according to plant species (Caires, 2017). These observations suggest the possibility to effectively use plant resistance against the pathogen in breeding or biotechnological programs. However, in addition to the availability of resistant genotypes, a good understanding of the genetic diversity and aggressiveness in pathogen populations is necessary to assist in the creation of novel and durable strategies for resistance deployment.

In this work, we studied the bacterium colonization in the host tissues, its genetic variability and aggressiveness on eucalypt and developed an immunological test for rapid detection of *E. psidii* in symptomatic and asymptomatic plants.

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ARTICLE 1: Root infection and aerial colonization of eucalypt host plants by *Erwinia psidii*

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ABSTRACT

Dieback, caused by *Erwinia psidii* is currently one of the most severe emerging diseases on *Eucalyptus* spp. in Brazil. However, because of its recent report, little is known about the mechanisms underlying bacterial infection. In this work, we studied the colonization and movement of *E. psidii* in host tissue using a strain labeled with green fluorescent protein. We were able to transform *E. psidii* with pGreen-TIR and to demonstrate that the plasmid is stable in the absence of antibiotic selection both *in vitro* and *in vivo*. Using fluorescence microscopy, we demonstrate that tissue colonization by *E. psidii* is not restricted to the inoculation point (leaf axil). Our results show that *E. psidii* colonizes the xylem vessels, sclerenchyma and parenchyma of the leaves and stem of eucalypt. At 35 days after inoculation, the bacterium was found at 5 cm above the inoculation point, indicating that it was able to colonize the plant acropetally, following the water flow. Confocal microscopy analysis revealed that when root-inoculated the bacterium penetrates the primary and secondary roots and reaches the xylem. However, the bacterium was never found in the plant crown or stem, irrespective of the evaluation time. Our results provide insights into the biology of *E. psidii*-eucalypt interaction, a pathosystem recently described that necessitates further studies aimed at filling knowledge gaps required to design and stablish better strategies for pathogen control and disease management.

Keywords: *Eucalyptus* spp., bacterial infection, fluorescence microscopy.

INTRODUCTION

Over the past few years, new diseases have emerged in eucalypt plantations, leading to a greater risk of loss of productivity in the crop. Among them, it is worth considering the dieback and wilt, caused by the bacterium *Erwinia psidii* Rodrigues Neto, Robbs &

Yamashiro (Coutinho et al., 2011; Arriel et al., 2014). *Erwinia psidii* was first reported in Brazil in guava (*Psidium guajava* L.) causing dieback in the1980s (Rodrigues Neto et al., 1987). In eucalypt (*Eucalyptus* spp.), *E. psidii* was described in 2014 in Brazil, occurring on young plants, in the states of São Paulo, Mato Grosso do Sul and Rio Grande do Sul (Arriel et al., 2014). In addition to Brazil, *E. psidii* was also reported causing disease in eucalypt in Argentina and Uruguay (Coutinho et al., 2011) and it was recently recorded in *Carica papaya* L. in Malaysia (Chai et al., 2017). Recent studies suggest that *E. psidii* can infect a wide range of plant species of the *Myrtaceae* family; however, the symptoms on such plants are not readily noticed probably they are not cultivated on a commercial scale (Caires, 2017).

Erwinia psidii is currently one of the most severe emerging diseases on *Eucalyptus* spp. in Brazil. However, because of its recent report, little is known about the mechanisms underlying bacterial infection. In the field, the characteristic symptoms of the disease appear mainly in the aerial part. However, the bacterium has been isolated from infected shoots and roots but not from the stems of samples collected in the field. To understand the bacterial colonization and movement in the plant tissues, we used *gfp* as a tool to observe the route of bacteriial infection and colonization in the plant. The use of *gfp* (green fluorescent protein)-transformed bacteria is a highly effective method to observe pathogen colonization of host tissue, as previously found in other pathosystems, such as *Xylella fastidiosa*-*Vitis vinifera* (Newman et al., 2003), *Pseudomonas syringae* pv. *tomato*-*Arabidopsis thaliana* (Melotto et al., 2006) and *Dickeya* sp.-*Solanum tuberosum* (Czajkowski et al., 2010).

In this work, a *gfp*-transformed strain of *E. psidii* was used to study the pathogen colonization of eucalypt cuttings inoculated in the roots and in the leaf axil and to establish a possible correlation between the symptoms developed in inoculated plants under controlled conditions with those observed in the field.

MATERIAL AND METHODS

Bacterial isolates

The isolate LPF534 of *E. psidii* used in this study was obtained from *E. dunnii* in Guaíba (RS, Brazil) and belongs to the Collection of Plant Pathogenic Bacteria of the Forest Pathology Laboratory/Bioagro of the Universidade Federal de Viçosa (CPBFPL-UFV). The identity of the bacterium was confirmed through biochemical tests and PCR amplification of conserved regions of the *E. psidii* genome (Arriel et al., 2014). Rifampicin resistance was induced in LPF534 by successive transfers in liquid LB medium containing increasing concentrations (1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL) of the antibiotic, according to the method described by Miller (1972). The identity of the rifampicin resistant strain was confirmed by PCR with specific primers for *E. psidii* (Silva et al., 2015) and deposited in the CPBFPL-UFV collection under accession number LPF534R. *Erwinia psidii* and *Escherichia coli* strains were routinely grown at 28 °C and 37 °C, respectively, for 24 h on solid LB medium amended with antibiotics (rifampicin, 100 µg/mL; gentamycin, 25 µg/mL), as needed.

Triparental mating

Plasmid pGreen-TIR, containing the gene encoding the GFP protein and conferring gentamycin resistance, was used for transformation (Miller & Lindow, 1997) by triparental mating. Individual colonies of *E. coli* HB101, *E. coli*(pGreen-TIR) and *E. psidii* LPF534R were transferred separately into Falcon tubes, containing 50 mL of LB medium and incubated at 30 °C with shaking at 280 rpm. After 24 h of incubation, the bacterial suspensions were centrifuged at 2,300 rcf and 4 °C for 5 min. The supernatant was discarded and the bacterial cells were suspended in 25 mL of sterile distilled water. The absorbance of the bacterial cell suspension of each strain was adjusted to an $OD_{600} = 0.5$ (approx. $1x10^6$ colony forming units (CFU)/mL). Then, 350 µL of the *E. coli* (pGreen-TIR) suspension, 350 µL of the *E. coli* HB101 suspension and 700 µL of the LPF534R suspension were mixed together and the mixture centrifuged at 13,400 rcf for 1 min. The supernatant was discarded, the pellet resuspended in 900 µL of water and 100 µL spotted on LB medium amended with gentamycin and rifampicin. Transformation of rifampicin and gentamycin resistant strains was confirmed by emission of fluorescence. For that, transconjugant and wild-type strains were grown on solid LB medium at 28 °C for 24 h, bacterial suspensions prepared, and the cells observed under a fluorescence microscope (Olympus BX51) using light excitation at 488 nm and emission at 515 nm. The *E. psidii* transconjugant expressing GFP was stored in 30% glycerol at -80 °C and deposited in the CPBFPL-UFV under accession number LPF534T.

Plasmid stability

To determine the stability of the pGreen-TIR plasmid in *E. psidii*, the LPF534T strain was grown on solid LB medium at 28 ºC. After 24 h of incubation, three individual colonies were separately transferred into test tubes containing 10 mL of liquid LB medium amended with rifampicin 100 µg/mL and gentamycin, 25 µg/mL. After 24 h of incubation at 28 ºC, 500 µL of the original culture were transferred into 4,5 mL of LB medium with rifampicin but without gentamycin and the tubes incubated as before. Five successive transfers into liquid medium without gentamycin were made for each of the three individual LPF534T colonies. At each transfer, the rest of the original culture was centrifquged at 11,200 rcf for 3 min, the pellet resuspended in 10 mM $MgCl_2$ and the bacterial suspension adjusted to an OD_{600} = 0.5 (approx. $1x10^6$ colony forming units (CFU)/mL). Then, serial dilutions were prepared, 100 µL of each dilution were spotted on solid LB medium containing rifampicin with or without gentamycin and the plates incubated at 28 ºC. Each serial dilution was spotted on LB medium in two independent plates to obtain technical replicates. The stability of the plasmid was determined based on the ratio between the number of CFU on medium amended with both antibiotics and the number of CFU on medium without gentamycin. Subsequently, the plates were observed under an UV-lamp to confirm emission of fluorescence by the bacterial cells.

Pathogenicity tests

In order to confirm the pathogenicity of the GFP-expressing strain, 60-day-old cuttings of the susceptible clone 37350 (*E. urophylla* x *E. globulus*) were inoculated with LPF534T and LPF534. For inoculation, bacterial mass taken from a 24 h culture on solid 523 medium was impregnated on the tip of sterile toothpicks and deposited in the first three axillary buds from the plant apex, previously wounded with a sterile hypodermic needle (Ferraz et al., 2016). Ten plants were inoculated with each strain and the disease symptoms rated at 30 days after inoculation (dai). Wounded plants treated with autoclaved distilled water served as control. Inoculated and control plants were maintained in a growth chamber at 26 ± 2 °C (12 h photoperiod; 165 µmol/s/m^2 light intensity).

Host colonization

Two inoculation methods were used to investigate the colonization of eucalypt tissue by *E. psidii*: *i*) root inoculation and *ii*) leaf axil inoculation (Ferraz et al., 2016). An LPF534T cell suspension adjusted to an $OD_{600} = 0.5$ (approx. $1x10^6$ colony forming units (CFU)/mL) was prepared in 10 mM $MgCl_2$ using a 24 h culture of LPF534T on solid medium. The root system of 60-day-old cuttings of the eucalypt clone 37350 was carefully washed with tap water and immersed in the bacterial suspension. The control plants were immersed in sterile distilled water. After 30 min at room temperature, the plants were transplanted into 2 L plastic bags, filled with Carolina Soil substrate® (70% sphagnum peat, 20% carbonized rice husks, 10% perlite), amended with single superphosphate (6 kg m^{-3}) and Osmocote® (19:06:10 (N:P:K) at 1.5 kg m^{-3}) and maintained at 28 ± 5 °C in a growth chamber with a 12 h photoperiod 165 µmol/s/m^2 light intensity. For leaf axil inoculation, the procedure and plant growth conditions were essentially as described above for the pathogenicity tests. Twelve and eight plants were inoculated for the leaf axil and root immersion methods, respectively. The same number of non-inoculated plants served as control.

Plant tissue was sampled every seven days during 42 and 28 days, for the leaf axil and root immersion methods, respectively. Two plants of each inoculation method as well as non-inoculated control plants were sampled. Transverse and longitudinal sections (20 to 25 µm thick) were obtained with a LEICA SM 2000 R sliding microtome (Leica Biosystems, Germany). The transverse sections were made at every one centimeter from the inoculation point until reaching the plant apex. In the inoculation of the root, the transverse sections were obtained until reaching the collect. The sections were fixed in paraformaldehyde (4%) for 1.5 h, washed three times with sterile distilled water and stored at 4 °C until analyzed. Tissue samples were observed on either a Zeiss LSM 510 META confocal laser microscope (Zeiss, Germany) with 470 to 490 nm filters and transmission peak between 510 and 560 nm or an Olympus BX51 fluorescence microscope (Olympus, Japan).

RESULTS

Erwinia psidii expressing GFP retains full virulence

Green-fluorescent, 1-2 µm length bacilli were observed in bacterial suspensions prepared with the rifampicin and gentamycin resistant transconjugants under the fluorescent microscope (Figure S1.1). After four consecutive transfers of bacteria to liquid medium, *E. psidii* cells showed a 100% retention rate of the pGreen-TIR plasmid as assessed by their gentamycin resistance, when evaluating 7 to 28 generations in the three biological replicates (Figure 1.1a). Furthermore, fluorescence excited by the UV-lamp was still emitted by the cells in culture, indicating that the pGreen-TIR plasmid is highly stable in *E. psidii* in the absence of antibiotic selection (Figure 1.1b).

The first disease symptoms (purplish lesions in the petiole and leaf midrib, and water-soak lesions in the leaf midrib) caused by the LPF534T strain were observed at 14 dai. Later, the leaf lesions became brown and evolved to necrosis along the midrib and secondary veins. As the disease progressed, foliar wilting and blighting were also observed in all inoculated plants. There were no differences in disease incidence (percent of inoculated plants showing symptoms) or severity between the transformed (LPF534T) and control (LPF534) strains. Under our experimental conditions, both strains caused typical disease symptoms (Figure S1.2).

Aerial infection and colonization of eucalypt host plants

When bacteria were inoculated into the leaf axil of eucalypt cuttings, typical disease symptoms were observed at 14 dai. At around 35 dai, all of the inoculated plants showed loss of apical dominance, dieback as well as necrosis on the stem below the inoculation point. The leaves below and nearby the inoculation point also exhibited typical disease symptoms. At 42 dai, the main stem was completely necrotized and brittled and showed evidence of saprophytic fungal growth (Figure 1.2).

Observations of plant tissue sections under the fluorescence microscope at different evaluation times always revealed the presence of GFP-labelled bacteria in the inoculation site. From 14 dai onwards, *E. psidii* was seen in the leaves at 4 cm below the inoculation point with bacterial population increasing up to the 35 dai. At 35 dai, the bacterium was observed in the plant apex, approximately 5 cm above the inoculation point (Figure 1.3). Microscopic observation of longitudinal leaf sections showed darkening and necrosis of the leaf midrib and *E. psidii* localized to the xylem, sclerenchyma and parenchyma (Figure 1.4). *Erwinia psidii* was also observed in the parenchyma cells in the margins of symptomless leaves, indicating that the bacteria moved toward adjacent and asymptomatic tissues (data not shown). At 42 dai, the bacterium was found 2 cm below the inoculation point inside the stem medulla. We were not able to detect the bacterium at 5 cm or below the inoculation point or in the root during the evaluation period when using the leaf axil inoculation method (Figure 1.3).

Root infection and colonization of eucalypt host plants

In the root inoculations, samples of plant tissue were assessed using both fluorescence (data not shown) and confocal microscopy. In both cases, we were able to observe GFP-labelled bacteria inside the plant tissue regardless of the evaluation time, there were no symptoms of necrosis or darkening of the root due to bacterial infection (Figure S1.3). *Erwinia psidii* was observed in the primary root until the 14 dai. Later (21 and 28 dai), *E. psidii* was no longer detected. In secondary roots, the bacterium was detected since the first day until the 28 dai. Irrespective of the evaluation time, the bacterium was not found in the crown or stem of the plant. However, the bacterium was clearly seen in the xylem vessels at 28 dai by using fluorescence microcopy (Figure 1.5).

DISCUSSION

In this study, we were able to transform *E. psidii* with pGreenTIR and to demonstrate that the plasmid is stable in the absence of antibiotic selection both *in vitro* and *in vivo*. Gentamycin resistance and colony fluorescence of bacterial cells obtained from *in vitro* and *in vivo* samples indicated a 100% plasmid retention in *E. psidii*. The availability of the GFP-labelled strain will facilitate conducting experiments aimed at understanding the mechanisms underlying diverse aspects of the interaction of *E. psidii* with its host plants.

Using fluorescence microscopy, it was possible to demonstrate that tissue colonization by *E. psidii* is not restricted to the inoculation point (leaf axil). In this study, it is demonstrated that the bacterium has the ability to move in the plant stem and leaves acropetally, following the water flow. These observations are in agreement with the progression of disease symptoms under field conditions, where stem darkening progresses ascropetally until reaching the leaves of the pointer. These results are also consistent with the observed movement of *Erwinia amylovora* in *Malus domestica* plants, in which the negative hydraulic pressure of the xylem should facilitate pathogen invasion (Bogs et al., 1998).

Our study also shows that *E. psidii* colonizes the sclerenchyma, parenchyma and xylem tissues as well as the margins of leaves that have not yet shown any macroscopic disease symptom. The speed at which the bacterium spreads in the xylem likely allows it to rapidly colonize different plant tissues (Bogs et al., 1998). This is probably due to the diameter of the vessel pore of the xylem, which is larger than that of the phloem vessels (Barak et al., 2002). This could explain the quick onset of disease symptoms, followed by meristem death of the *E. psidii*-infected plants.

Although different nutrients, including nitrate, sulfate, phosphate, magnesium, manganese, carbohydrates such as glucose, fructose, sucrose, raffinose, trehalose and ribose, amino acids and organic acids can be present in the xylem, they are found at low concentrations and vary according to the plant growth stage. Nonetheless, even with this nutrient shortage in the xylem, pathogens meet their nutritional requirements through enzymatic degradation of plant cell walls, colonizing neighboring cells or inducing nutrient leakage (Yadeta & Thomma, 2013). Amino acids found in the xylem, such as asparagine and glutamine, have been demonstrated to be chemoattractants for *E. amylovora*, allowing fast bacterial colonization (Bogs et al., 1998). Rapid bacterial colonization of the xylem has also been shown for other pathosystems, including *Xylella fastidiosa-Vitis vinifera* (Newman et al., 2003), *Clavibacter michiganensis* subsp. *michiganensis-Solanum lycopersicum* (Chalupowicz et al., 2012) and *Xanthomonas campestris* pv. *vitians-Lactuca sativa* (Barak et al., 2002). Although it is currently unknown if some compounds serve as chemoattractants for *E. psidii* in the xylem, our studies show that its movement and colonization occur mainly through the xylem vessels.

The finding that *E. psidii* can be detected at 2 cm below the inoculation point in eucalypt plants, without colonizing the phloem tissue, is in agreement with results obtained by Caires et al. (2017), who concluded that the bacterium movement occurs via the xylem vessels, both acropetally and basipetally. The same pattern of movement was observed for *E. amylovora* in apple shoots, being the bacterium found in the roots as well (Bogs et al., 1998). Hypothetically, such a downward bacterial movement could be explained by the cohesion-tension theory of xylem fluid movement, when a reduction in water uptake by the roots and a low leaf transpiration rate, accompanied by a rupture in the xylem elements, for example by an injury in the plant, allow fluid movement in different directions (upward, downward or both), according to the forces inside the xylem elements at the moment of the injury (Tattar &Tattar, 1999, Bogs et al., 1998). This may explain the fact that in some disease eucalypt plants the bacterium isfound in the apex and roots despite not being detected in the stem.

The downward movement of *E. psidii* in the xylem could be compared to that of some bacteria that possess hydrolytic enzymes that can degrade the xylem vessels, thus creating an embolism of the vascular system resulting in a reverse transport of water in the xylem. For instance, *E. amylovora* has an arsenal of enzymes that degrade cell walls and are important for virulence (Zhao et al., 2005), whereas *X. fastidiosa* is able to degrade the xylem vessels due to the presence of a gene encoding an endopolygalacturonase (Warren et al., 2015). In any case, the mechanisms (physical, physiological and biochemical) underlying the bi-directional movement of *E. psidii* in the xylem vessels of eucalypt cannot be explained solely based on the results of our study and remain to be investigated.

Our results from the root inoculation experiments, demonstrate that *E. psidii* is able to penetrate the primary and secondary roots and to reach the xylem. However, the bacterium was never found in the plant crown or stem, irrespective of evaluation time. For vascular phytopathogenic bacteria that cause wilt symptoms, such as *E. amylovora*, *P. stewartii*, *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *actinidae*, *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, *X. fastidiosa* and *R. solanacearum*, diverse routes of colonization have been described in different plant organs, including the roots (Bae et al., 2015). One of the most common routes for those pathogens is their movement upward to reach the zone of root elongation, where high amounts of exudates can be found, as well as the axils, where secondary roots begin to form. Then, bacteria begin to move to the cortex, and later, cross the endoderm and reach the xylem vessels (Vasse et al., 1995).

When root-inoculated, *E. psidii* was not seen in tissues other than in the roots, ruling out the possibility to assume that colonization takes place through the root system under natural conditions. However, further studies must be conducted in order to confirm or reject this hypothesis. Currently, the most widely accepted hypothesis is that *E. psidii* is transmitted by contaminated cuttings and asymptomatic plants where the bacterium has the ability to survive, which is supported by the low genetic variability found in the *E. psidi*i population (Teixeira et al., 2009).

In summary, our study shows that *E. psidii* colonizes the xylem vessels as well as the sclerenchyma and parenchyma of the host stem and leaves. Its movement in the xylem is faster acropetally than basipetally, and there is no evidence of upward movement from the root, although some colonization of the root system can be observed. Therefore, it is tempting to speculate that it is unlikely that infections in the field and nurseries originate from soil inoculum. Nonetheless, additional experiments using different inoculation methods, including wounding the roots, and performing evaluations for a longer incubation period, must be performed in order to better elucidate the routes of bacterial entry into the plant. As the bacterium is frequently detected in symptomatic and asymptomatic plants in the field and in nurseries, it is advisable to carry out detection tests on different parts of the plant before establishing new plantations to avoid losses due to this disease.

Our results provide insights into the biology of the *E. psidii*-eucalypt interaction, a pathosystem recently described that necessitates further studies aimed at filling knowledge gaps required to design and stablish better strategies for pathogen control and disease management.

ACKNOWLEDGEMENTS

The authors thank Doctor Alan Collmer, Department of Plant Pathology, Cornell University, for providing the pGreen-TIR plasmid; Nucleus of Microscopy and Microanalysis at the Universidade Federal de Viçosa; CMPC Celulose Riograndense and Clonar Resistência a Doenças Florestais for the propagation of the plant material used in the inoculation experiments; the Organization of American States (OAS) and Coimbra Group of Brazilian Universities (GCUB) for the Doctoral Scholarship.

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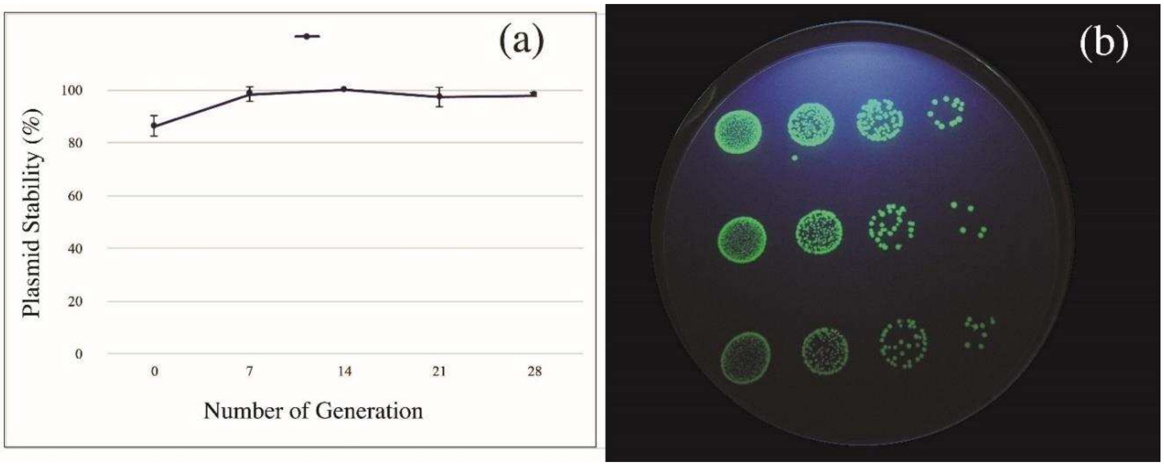

Figure 1. 1. Stability of the pGreen-TIR plasmid in *Erwinia psidii* LPF534R. (a) Stability (%) of the pGreen-TIR plasmid in *Erwinia psidii* LPF534R determined by the ratio between the number of colony forming units (CFU) on culture medium containing gentamycin and the number of CFU on culture medium without the antibiotic. The experiment was carried out with three biological and two technical replicates. (b) Fluorescence of LPF534 *E. psidii* transformed with pGreen-TIR on solid LB medium amended with gentamicin, visualized with a bench UV-lamp.

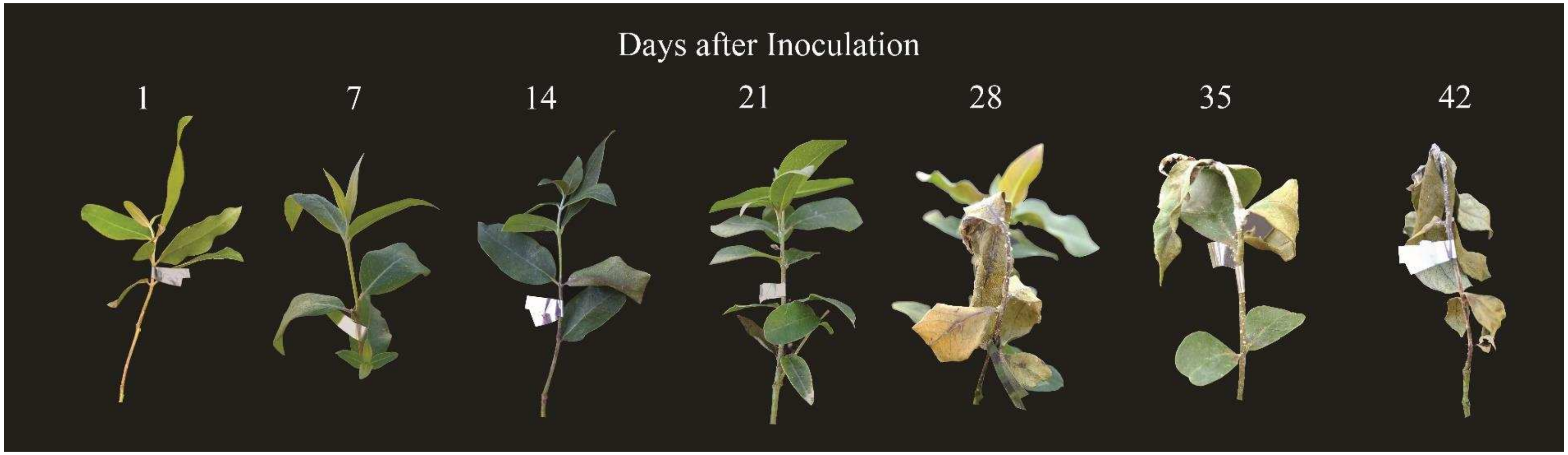

Figure 1.2. Symptoms caused by *Erwinia psidii* expressing the pGreen-TIR plasmid in cuttings of eucalypt hybrid clone 37350 (*Eucalyptus urophylla* × *E. globulus*) inoculated in the leaf axil at different days after inoculation.

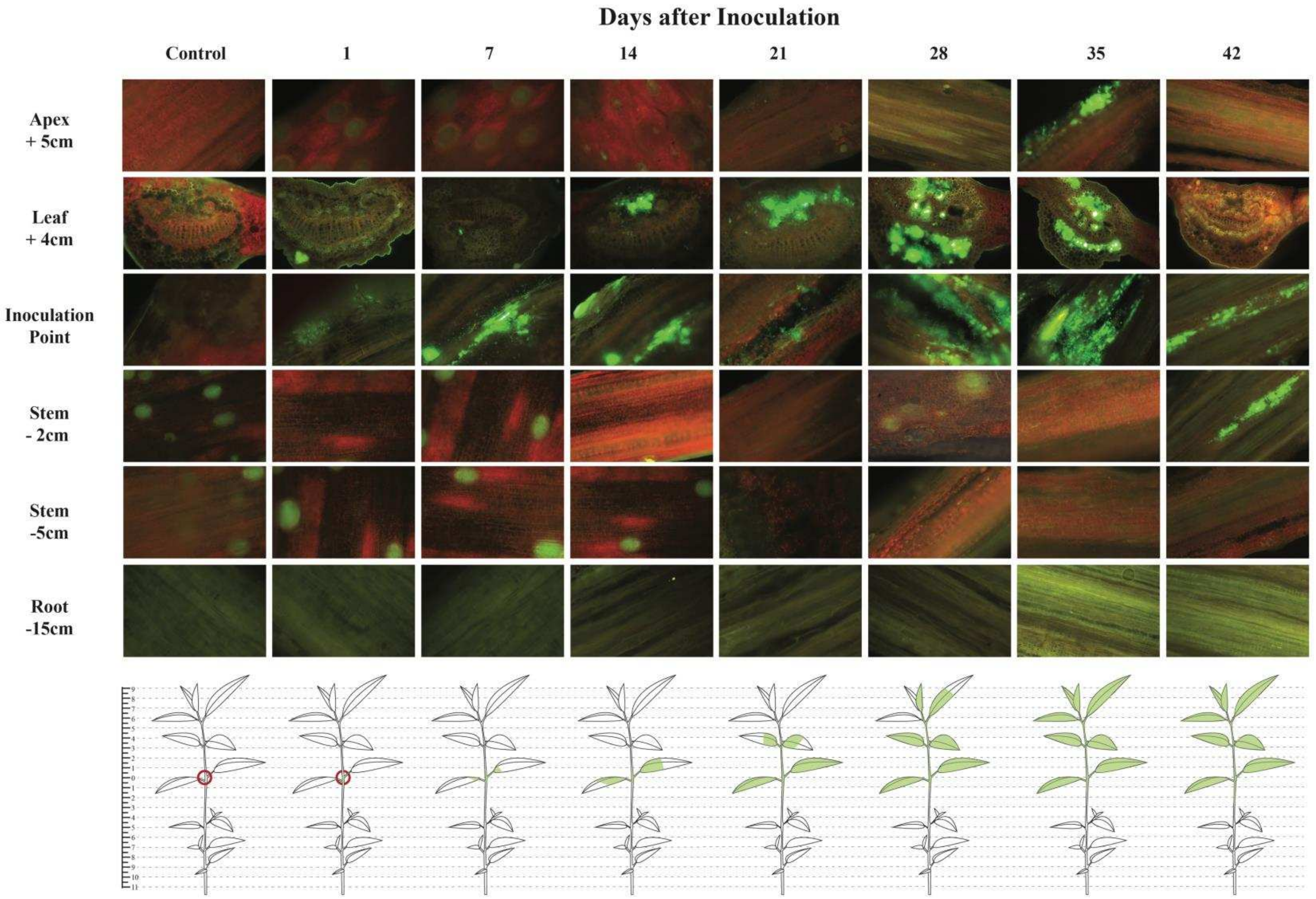

Figure 1. 3. Colonization of *Erwinia psidii* in cuttings of eucalypt hybrid clone 37350 up to 42 days after inoculation in the leaf axil. Either a transverse or a longitudinal section of approximately 1 cm length is shown for each evaluation time. The bottom panel shows illustrations depicting bacterial colonization over the evaluation period. The red circle indicates the inoculation point, whereas bacterial colonization is represented in green. Images were obtained by fluorescence microscopy at 10x magnification.

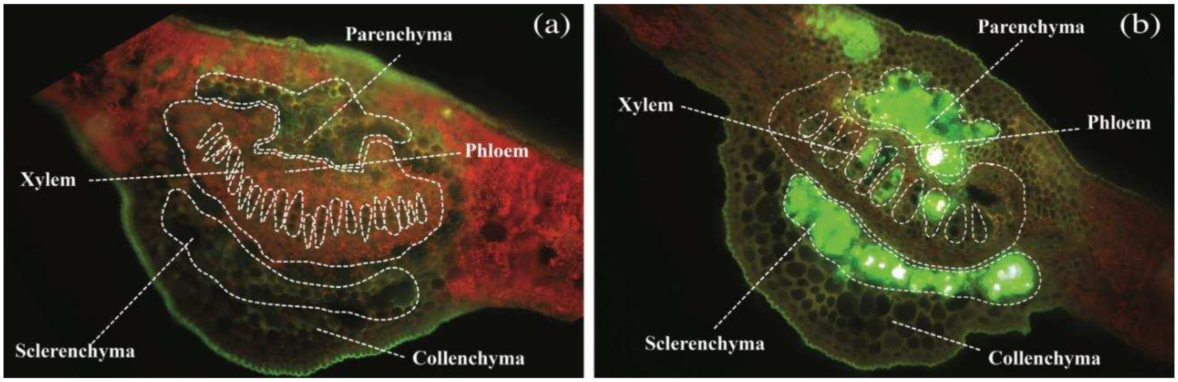

Figure 1. 4. Leaf tissue colonization in eucalypt hybrid clone 37350 by *Erwinia psidii* LPF534T. (a) Non-inoculated eucalypt leaf. (b) Inoculated eucalypt leaf at 35 days of incubation. Morphologically distinct leaf tissues were delimited with a white line to facilitate visualization. Images of transverse sections of eucalypt leaves were taken by fluorescence microscopy at 10x magnification.

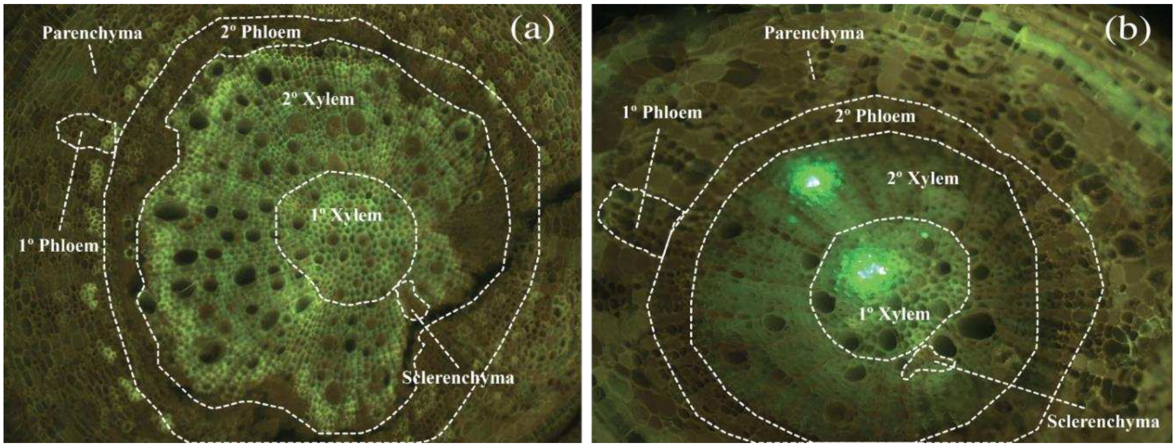

Figure 1. 5. Colonization of root tissues in cuttings of eucalypt clone 37350 inoculated with *Erwinia psidii* by root immersion. Transverse sections of root tissue were observed under the fluorescence microscope at a 10x magnification. (a) Non-inoculated root. (b) Root inoculated at 28 days of incubation. Morphologically distinct root tissues were delimited with a white line to facilitate visualization.

SUPPLEMENTARY FIGURES

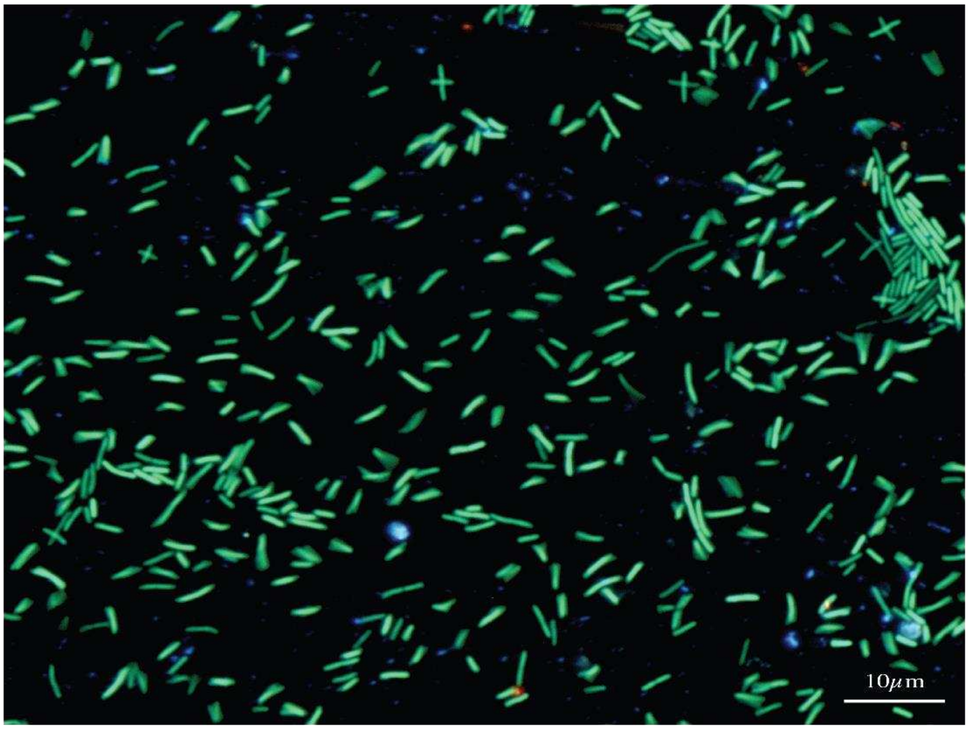

Figure S1. 1. Fluorescence of *Erwinia psidii* LPF534R expressing the pGreen-TIR plasmid. Bacterial cells were observed by fluorescence microscopy (100x) with filter of 470 to 490 nm.

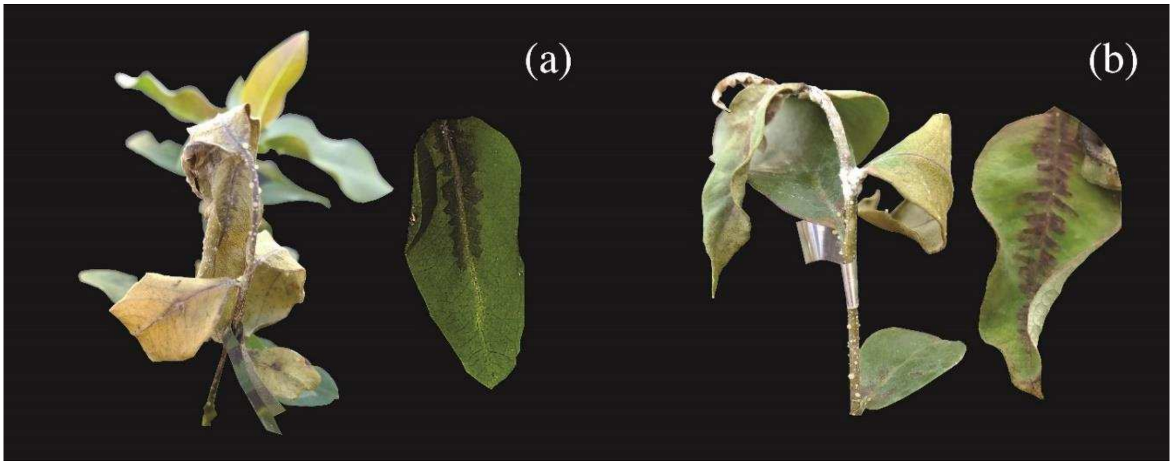

Figure S1. 2. Cuttings of eucalypt clone 37350 showing leaf midrib necrosis and apical dieback and necrosis after inoculation with *Erwinia psidii*. (a) Cutting inoculated with LPF534T. (b) Cutting inoculated untransformed strain LPF534.

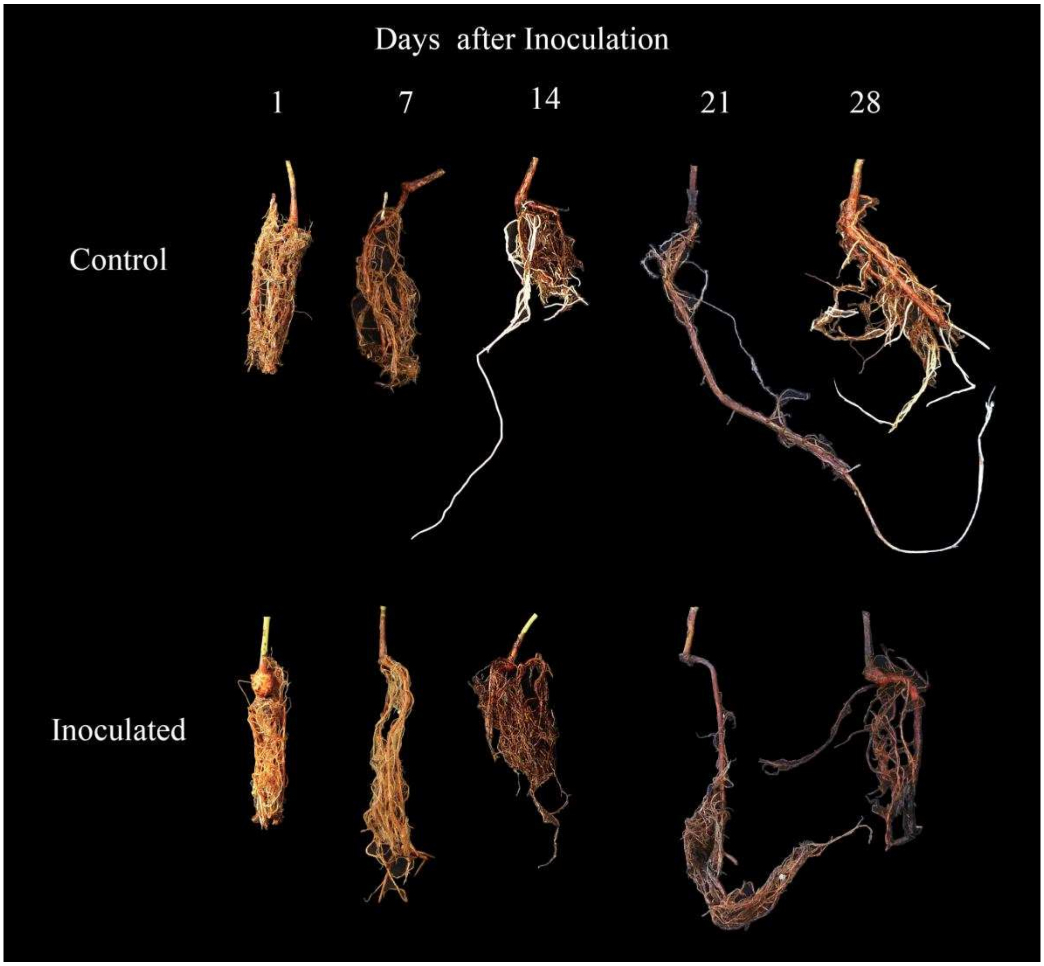

Figure S1. 3. Roots of eucalypt clone 37350 inoculated with *Erwinia psidii* LPF534T by root immersion.

ARTICLE 2: Genetic diversity and aggressiveness of *Erwinia psidii* on *Eucalyptus* spp. in Brazil

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ABSTRACT

We studied the genetic variability and aggressiveness of *Erwinia psidii* isolates from eucalypt. Rep-PCR markers of 101 isolates from *Eucalyptus* spp. and five from *Psidium guajava*. showed that the populations of *E. psidii* display a relatively low genetic variability In general the isolates grouped by the host of origin, except two isolates from guava (LPF681 and LPF682) that grouped with one isolate (LPF609) from eucalypt, indicating that a strong differentiation by host plant cannot be conclusively established. Wilcoxon and ANOVA tests of disease severity data indicated the existence of isolate × clone interaction. The area under disease progress curve (AUDPC) and disease severity for some isolates were significantly different between the two clones tested. The variability in aggressiveness among isolates of *E. psidii* is important to select highly aggressive isolates in screening for disease resistance

Keywords: *Psidium guajava*, variability, bacterium, virulence.

INTRODUCTION

Eucalypt (*Eucalyptus* spp.) trees, native to Australia and neighboring islands, are currently the largest source of wood products and derivatives from forest plantations worldwide, mainly in tropical and subtropical regions. In Brazil, traits such as adaptability, rapid growth, and high productivity led to the expansion of eucalypt plantations. Nowadays, Brazil is the world's leader of pulp, paper, and wood panels production, with exports that undeniably contribute to its trade balance and generate many jobs and significant income in all regions of the country. In 2016, eucalypt plantations covered 7.5 million hectares in the country, mostly located in Southeast Region (3.1 million hectares), folloed by the Central-West Region (1.4 million hectares), Northeast Region (908.832 hectares), North Region (602.403 hectares) and South Region (1.7 million hectares) (IBGE, 2016). However, the climatic instability and the emergence of new diseases, such as dieback and wilt, caused by *Erwinia psidii* Neto, Robbs & Yamashiro (Rodrigues Neto et al., 1987; Alfenas et al., 2009) threaten eucalypt production.

Erwinia psidii was first described causing dieback on *Psidium guajava* L. in Brazil in 1987 (Rodrigues Neto et al., 1987) and is currently one of the most severe bacterial diseases of *Eucalyptus* spp. in Argentina, Uruguay (Coutinho et al., 2011) and Brazil (Arriel et al., 2014). It has been recorded on *E. saligna, E. dunnii, E. grandis,* and *E. urophylla* × *E. grandis* and *E. urophylla* × *E. maidenii* hybrids and has already been detected in commercial nurseries and in asymptomatic rooted cuttings. Control measures for this disease, once the plant is infected, have been proved ineffective, which can cause significant losses in productivity and serious risks for pathogen dissemination. Up to now, eucalypt dieback and wilt of eucalypt caused by *E. psidii* has only been recorded in Argentina, Brazil and Uruguay (Coutinho et al., 2011; Arriel et al., 2014), but it may have been spread to other eucalypt growing areas in the world with favorable conditions for bacterial infection. Currently, little is known about the genetic composition and diversity of *E. psidii* (Teixeira et al., 2009).

Several DNA-based techniques are useful for detecting diversity in bacterial populations (Louws et al., 1999). Among them, rep-PCR based on repetitive sequence–based polymerase chain reaction (REP-PCR), ERIC (Enterobacterial Repetitive Intergenic Consensus) and BOX (Box A, B and C subunits) use primers to amplify certain repetitive DNA sequences in Gram-negative bacteria. In addition to unveiling interspecific diversity in bacteria, these repetitive elements, which are located in the intergenic regions of many bacterial genomes, are useful for elucidating relationships within bacterial species, since their fingerprints are conserved at the pathovar and strain infraspecific levels (Katawczik et al., 2016; Zulperi et al., 2016).

Knowledge of the variability in pathogen aggressiveness is important for disease epidemiology and management since it provides information on the evolutionary potential of the pathogen and the risk of plant resistance breakdown (Cabrefiga & Montesinos, 2005). Pathogenicity involves aggressiveness and virulence (Bos & Parlevliet, 1995). Arguably, aggressiveness refers to the intensity of disease or attack produced by a pathogen (Andrivon, 1993; Bos & Parlevliet, 1995), while virulence is the capacity of a pathogen to cause disease due to expression of genetic determinants or virulence factors in the host-pathogen interaction (e.g., virulent or nonvirulent) (Shaner et al., 1992). It is currently accepted that the outcome of the plant-pathogen interaction is determined by whether or not at least one avirulence protein of the pathogen is recognized by a resistance protein in the host plant (Jones & Dangl, 2006). Aggressiveness can be measured in terms of the progression of infections, migration of pathogen cells from the point of inoculation, amount of pathogen inoculum needed to produce a given effect on the host, or rate of disease progression (Cabrefiga & Montesinos, 2005).

Field observations and results of inoculations under controlled environmental conditions have shown that planting of resistant eucalypt clones is one of the best strategies for disease control (Oliveira et al., 2015). However, knowledge of the genetic structure of pathogen populations and its variability in aggressiveness is important to establish appropriate programs for resistance deployment and to obtain long-term disease resistance. Thus, in this study we evaluated the genetic diversity of *E. psidii* isolates from eucalypt and guava using Rep-PCR and their aggressiveness by inoculating clones of *Eucalyptus urophylla* × *E. grandis* and *E. urophylla*, known to be susceptible under field conditions.

MATERIALS AND METHODS

Plant sampling and bacterial isolation

A search for diseased trees was conducted in eucalypt plantations in the states of Mato Grosso do Sul (MS), Rio Grande do Sul (RS) and São Paulo (SP) (Figure 2. 1). Infected tissue samples were collected in the field, placed inside paper bags and taken to the Laboratory of Forest Pathology/Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil, where they were processed. The tissue samples were collected between 2014 and 2016.

For isolation of bacteria, leaf, branch and root samples of plants with dieback symptoms were washed with soap and tap water, and the excess water was removed by wiping with a paper towel. Small tissue fragments, taken from the edges of the lesions were disinfested by successive transfer in 50% ethanol for 30 s and 1% NaClO for 1.5 s, rinsed in sterile distilled water for 2 min and then macerated. The macerate was plated on solid 523 culture medium (Kado & Heskett, 1970) and the plates incubated at 28 °C. After 48 h of incubation, individual colonies were transferred to the same medium to obtain pure cultures. All isolates confirmed as *E. psidii* were stored in 30% glycerol at -80 °C and deposited in the Collection of Plant Pathogenic Bacteria of the Forest Pathology Laboratory/Bioagro of the Universidade Federal de Viçosa (CPBFP-UFV). A total of 103 bacterial isolates obtained from infected plant tissue and three isolates from the Collection of the Instituto Biológico, Campinas, SP, Brazil (IBSBF) were included in this study (Table S2.1).

Total DNA isolation and species-specific PCR

DNA was extracted from bacteria grown in liquid 523 medium at 28 °C for 48 h. One-mL of bacterial culture was centrifuged at 20,790 *g* for 3 min and the genomic DNA was then extracted and purified from the cells using the Wizard kit (Promega) according to the manufacturer's protocol for Gram-negative bacteria. DNA concentration was quantified using a Nanodrop$^{®}$ spectrophotometer (Thermo Scientific), and the final concentration was adjusted to 15 ng/µL to conduct the PCR. To confirm the identity of isolates as *E. psidii*, sequencing of the 16S-rDNA region and PCR using oligonucleotides fd2 and rP1 (Weisburg et al., 1991) and conditions reported by Arriel et al. (2014) were used. The reaction products were sequenced in a DNA analyzer (Applied Biosystems 3500/3500XL) and the DNA sequences compared to other known sequences deposited in the GenBank using Blast (Altschul et al., 1990) searches hosted at National Institute of Biotechnology Information (NCBI) server. The DNA sequences were then analyzed and edited using Sequence Scanner software 2 and aligned in Mega. 7.0.

Rep-PCR

The rep-PCR was conducted using the primers REP 1R-I (5'-IIIICGICGICATCIGGC-3') and REP 2–I (5'–ICGITTATCIGGCCTAC–3') for REP-PCR, ERIC 1R (5' ATGTAAGCTCCTGGGGATTCA–3') and ERIC 2 (5'–AAGTAAGTGACTGGGGTGAGCG–3') for ERIC-PCR, and BOXA 1R (5'–CTACGGCAAGGCGACGCTGAC–3') for BOX-PCR (Louws et al., 1994). The ERIC and BOX primers were synthesized by Integrated DNA Technologies (IDT) and the REP primers were synthesized by Sigma-Aldrich. All rep-PCR was performed in 15 µL of reaction mix containing 0.5 U/µL GoTaqDNA polymerase (Promega), 0.75 mM $MgCl_2$, 1 mM each dNTP, 1 mM each primer and 2.66 ng DNA. The REP-PCR was performed with cycling conditions as follows: initial denaturation at 95 °C for 7 min followed by 30 cycles of 94 °C for 1 min, 44 °C for 8 min, 65 °C for 8 min and final extension at 65 °C for 8 min (Louws et al., 1994). The ERIC-PCR was performed in 15 µL of reaction mix with the following cycling conditions: initial denaturation at 95 °C for 7 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 65 °C for 8 min and final extension at 65 °C for 8 min (Louws et al., 1994). The amplification using the BOX primer was performed in 20 µL of reaction mix with cycling conditions of 95 °C for 7 min, followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min, 65 °C for 8 min, and a final step at 65 °C for 8 min (Louws et al., 1994). The reactions were performed on a Veriti^{TM} 96 Thermal Cycler (Life Technologies, USA). Amplification bands were separated by electrophoresis on a 1.5% agarose gel, visualized by staining with ethidium bromide, and the gels photographed with a L.PIX camera (Loccus Biotechnology, Brazil). The sizes of the amplified fragments were estimated by comparison with the molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

The ERIC-, REP- and BOX-PCR fingerprints obtained from the digital images were used to estimate the genetic similarity among isolates. For that, presence and absence of bands were scored in a binary model and first analyzed for each repetitive sequence (BOX, ERIC, REP) separately, and then, for all sequences combined (BOX+ERIC+REP) with the PAST 3.06 program (Hammer et al., 2001) using the Jaccard´s similarity coefficient and the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) clustering method.

Genetic diversity

The number of genotypes for each population was calculated using Mobyle SNAP Workbench (Monacell & Carbone, 2014) and the genotype network was constructed using EDENetwork (Kivelä et al., 2015). Gene diversity and analysis of molecular variance (AMOVA) were conducted using the package poppr for R (Kamvar et al., 2014). Depending on the frequency of genotypes in each population, the diversity was estimated by Hill numbers, or else, the effective numbers of genotypes was calculated. Accordingly, Hill's numbers 0, 1 or 2 correspond to estimates of the genotype richness, Shannon's index, and Simpson's index, respectively (Chao et al., 2014; Jost, 2007). Integrated curves that allow rarefaction and extrapolation were used to compare these numbers from samples of different sizes using the non-asymptotic approach (Chao & Jost, 2012). For each curve, the 95% confidence interval (95% CI) was generated and plotted. Diversity in different populations was compared based on the overlapping of the 95% CIs (Chao & Jost, 2012). The diversity analyzes were conducted using the package iNEXT for R (Hsieh & Chao, 2016). Genotype accumulation curves were created for each population using 1,000 bootstrap replicates with the package poppr for R (Kamvar et al., 2014).

Aggressiveness

Fourteen representative isolates were selected to investigate possible differences in aggressiveness. The selection of the isolates was based on molecular diversity and geographic origin. The isolates were inoculated on 30-day-old cuttings of two clones: CLR375 (*E. urophylla* × *E. grandis*) and CLR440 (*E. urophylla*) grown in 2 L plastic bags, filled with Carolina Soil substrate® (70% sphagnum peat, 20% carbonized rice husks, 10%

perlite), amended with single superphosphate (6 kg m-3) and Osmocote® (19:06:10 (N:P:K) at 1.5 kg m-3). These two clones are known to be susceptible to *E. psidii* in the field. For inoculation, bacterial mass taken from a 24 h solid culture was impregnated on the tip of sterile toothpicks and deposited in the first three axillary buds from the plant apex, previously wounded with a sterile hypodermic needle (Ferraz et al., 2016). Wounded plants treated with autoclaved distilled water served as control. Inoculated and control plants were maintained in a growth chamber (12 h photoperiod; 165 µmol/s/m^2 light intensity). Five plants of each clone were inoculated with each isolate and arranged in a completely randomized design. Each experimental unit consisted of a single plant.

After 30 min at room temperature, the plants were transplanted into 2 L plastic bags, filled with Carolina Soil substrate® (70% sphagnum peat, 20% carbonized rice husks, 10% perlite), amended with single superphosphate (6 kg m^{-3}) and Osmocote® (19:06:10 (N:P:K) at 1.5 kg m^{-3}) and maintained at 28 ± 5 °C in a growth chamber with 12 h photoperiod and 165 µmol/s/m^2 light intensity.

Disease symptoms were evaluated at every seven days for up to 35 days using a rating scale (Ferraz et al., 2016). The experiment was performed twice, between October and November of 2017 (maximum and minimum temperatures of 37 ± 3 °C and 16 ± 3 °C, respectively, RH of 78.2%) and between December 2017 and January 2018 (maximum and minimum temperatures of 39.1 ± 3 °C and 15.8 ± 3 °C, respectively, RH 79%), at Clonar Resistência a Doenças Florestais, Cajuri, Minas Gerais, Brazil. Disease severity data were used to calculate the area under the disease progress curve (AUDPC).

Because similar results were obtained in both experiments, statistical analysis on disease severity and AUDPC was conducted on the combined data. Significant differences among isolates and between clones were determined by the nonparametric statistical tests Kruskal-Wallis and Wilcoxon, respectively. Isolate x clone interaction was determined with the Wilcoxon test and ANOVA on square-root transformed values. All statistical tests were performed using the package agricolae for R (Kamvar et al., 2014). In order to assign isolates into different aggressiveness categories, the following criteria were used: (1) if the AUDPC was above 100 and there was no significant difference with the maximum value, the isolate was classified as highly aggressive; (2) if the AUDPC was bellow 30 and there was not significant difference with the lowest value, the isolate was classified as weakly aggressive; (3) all other isolates were classified as aggressive, except for isolates that did not cause disease on the eucalypt clone, in which case, it was classified as nonvirulent.

RESULTS

Genetic diversity

Among 350 field samples collected from infected plant tissues, 103 bacterial isolates were obtained (101 from eucalypt and 2 from guava plants) (Table S2.1). Partial 16S-rDNA sequences of approximately 1500 bp were obtained for all isolates. Blast searches at NCBI showed that the 16S rDNA sequence of all isolates exhibited higher than 98% identity with that of *E. psidii*.

Amplification with ERIC-PCR primers produced 14 distinct bands, varying from 300 to 1,000 bp (Table S2.2, Figure S2.1), nine of which were polymorphic. ERIC profiles separated the isolates in four main groups, the largest of which (Group 1) showed 100% similarity among isolates. The majority of isolates in this group were collected from eucalypt, but three isolates from guava (LPF681and LPF682) were also included. Group 2, contained three isolates (LPF632, LPF635 and LPF670). In Group 2, isolate LPF670 was the most divergent. Group 3, contained two isolates (LPF549 and LPF547) from eucalypt collected in Mato Grosso do Sul (MS) and three isolates from guava (IBSBF435, IBSBF454 and IBSBF493) that shared 65% similarity. Group 4 contained two isolates (LPF 630 and LPF633) from MS sharing 37.5% similarity (Figure S2.2).

The banding patterns generated by REP-PCR was composed of 17 distinct bands, varying from 250 to 5,000 bp (Table S2.2, Figure S2.3), nine of which were polymorphic. Four distinct groups were formed in the dendrogram, with the largest group (Group 1) containing most eucalypt isolates from MS, Rio Grande do Sul (RS) and São Paulo (SP) and four isolates from guava (IBSBF454, IBSBF493 and IBSBF435), including the type strain. Isolates allocated in this group share 100% similarity. Group 2, sharing 80% similarity, contained one isolate from eucalypt (LPF609) and two isolates from guava (LPF681 and

LPF682). Group 3, sharing 70% similarity, contained four isolates from eucalypt and Group 4, with 20% similarity, was comprised of one eucalypt isolate from MS (LPF610) (Figure S2.4).

The BOX-PCR primer produced 15 distinct bands from 400 to 3000 pb (Table S2.2, Figure S2.5) with ten of them being polymorphic. The majority of eucalypt isolates were clustered in the same group (Group 1), in which isolates shared 100% similarity, except for LPF609, which was divergent from the others. Group 2, with 55% similarity, was comprised of one eucalypt isolate from MS (LPF554). Group 3, with 50% similarity, contains four isolates (IBSBF454, IBSBF493 and IBSBF435) from guava, including the type strain, and one isolate from eucalypt (LPF556) (Figure S2.6).

The dendrogram, obtained combining the three markers together (BOX+ERIC+REP), indicated the existence of seven groups, with no correlation to their geographic origin. One larger group (Group 1), with 92-100 % similarity, was formed by the majority of the eucalypt isolates, regardless of the state of origin. The second largest cluster (Group 2), with 90% similarity, contained two guava isolates (LPF681, LPF682) and one eucalypt isolate (LPF609). The guava isolates from the IBSBF, clustered in a different group (Group 5). The LPF610 isolate from MS was the most divergent (Group 7) (Figure 2.2).

Sixteen genotypes were observed in the *E. psidii* populations from eucalypt and guava sampled in RS, MS and SP. These genotypes differ according to the source host but not according to the geographic origin (Figures 2.1 and 2.3, Table S2.3). When the eucalypt populations are compared separately, a total of 13 different genotypes were observed, with 12 genotypes recovered from MS, four from RS, and one from SP (Figure 2.3, Table S2.4).

When the genetic diversity is assessed using Hill numbers, MS presents the greatest evenness and richness, 12 (95% CI = 19.671 - 317.849), compared to the states of RS, 4 (95% CI = 4.177-25.775), and SP, 1 (95% CI = 1.000 - 1.005). According to the Shannon´s index by Hill numbers (1), isolates from MS, 4.712 (CI = 4.712 - 17.176, are more diverse than those from RS, 1.387 (CI = 1.387 - 1.869), and SP 1.000 (CI = 1.000 - 1.000). According to the Simpson´s Index (2) no significant difference in effective number of genotypes based on Hill numbers was observed between MS (2.473), RS (1.154) and SP (1.000). Therefore, gene diversity was not significantly different between RS, MS and SP (Table 2.1, Figure

S2.7). AMOVA did not show significant differences among the populations (data not shown). The genotype accumulation curves indicated that 100% of the genotypes were detected by 22 markers (Figure 2.4).

Aggressiveness

Differences in aggressiveness were observed among a group of *E. psidii* isolates selected from the large collection recovered from eucalypt and guava plants (Figure 2.5). The inoculated plants showed typical wilting and dieback symptoms that resembled those observed under conditions of natural infection.

According to the disease severity based on a rating scale, the isolates belonging to the Group 1 (92-100% similarity) of the combined rep-PCR were more aggressive when inoculated on the hybrid *E. urophylla* × *E. grandis* clone than on the *E. urophylla* clone. The most genetically divergent isolate (LPF610) was aggressive on the both clones (Figure 2.5). The aggressiveness of some isolates varied according to the eucalypt clone, for example, LPF534, LPF615, LPF624, LPF633, LPF640, LPF680, LPF681 and LPF683 were notoriously more aggressive on *E. urophylla* than on *E. urophylla* × *E. grandis*, demonstrating an isolate × clone interaction (Figure 2.5). This isolate x clone interaction was confirmed when analysis of variance was separately applied on each of the two independent experiments (Table S2.5).

The *E. urophylla* × *E. grandis* clone displayed high mean disease severity values for most isolates (scale rate not significantly different from 4), and therefore, it was considered susceptible to most isolates, except for LPF681, which was the least aggressive isolate on both clones. In contrast, the *E. urophylla* clone exhibited moderate-to-high resistance to half of the isolates. The *E. urophylla* clone also exhibited high mean disease severity values for several isolates, nonetheless, several of them did not cause symptoms rated above 2.0 (LPF615, LPF534, LPF640, LPF680 and LPF681) (Figure 2.5).

Analysis of the area under the disease progress curve (AUDPC) with the Kruskal-Wallis test, revealed that IBSBF435 (guava) and LPF615 (eucalypt from RS) were significantly more aggressive on the *E. urophylla* × *E. grandis* clone than most of the other isolates, achieving AUDPC values above 100, and accordingly, were classified as highly aggressive. On the other hand, LPF681 (guava) was the least aggressive on the same clone with an AUDPC of 18.3 (Table 2.2) and was classified as weakly aggressive. In the *E. urophylla* clone, five isolates were highly aggressive (IBSBF435, LPF556, LPF610, LPF553 and LPF609) whereas two were weakly aggressive (LPF680 and LPF640). The LPF681 isolate did not cause disease on the *E. urophylla* clone in neither experiment, and therefore, was considered nonvirulent (Table 2.2). Both, the nonparametric Wilcoxon test and ANOVA on square-root transformed data indicated an isolate × clone interaction. These tests revealed that the AUDPC and disease severity caused by isolates LPF534, LPF615, LPF624, LPF633, LPF640, LPF680, LPF681 and LPF683 were significantly different between the two clones (Table 2. 2, Figure 2.5).

DISCUSSION

In this study, the combination of ERIC-, REP- and BOX-PCR molecular markers showed that the *Erwinia psidii* population from Brazil clustered in seven distinct groups, with no obvious correlation with their state of origin, although the bacterial populations from eucalypt seemed to differ from that of guava. Similarly, when studying guava isolates from different Brazilian regions, Teixeira et al. (2009) concluded that *E. psidii* isolates from guava from the DF are genetically similar to those from São Paulo, Espírito Santo and Paraná. Nonetheless, although we observed separation of isolates by host plant, two guava isolates (LPF681 and LPF682) grouped with one eucalypt isolate (LPF609), indicating that a strong differentiation by host plant cannot be conclusively established. This observation was supported by the results obtained from plant inoculation which showed that the guava isolate IBSBF435 was very aggressive on susceptible eucalypt clones. Furthermore, Arriel et al. (2014) demonstrated that the eucalypt LPF534 and the guava IBSBF435 isolates were pathogenic to both host plants.

The population of *E. psidii* from Mato Grosso do Sul presented a greater genotypic richness compared to those from RS and SP. These results indicate that in MS there is the highest number of genotypes and that they are distributed more evenly within the population. The greater variability observed in the MS population may be explained by a wider sampling (a distance difference between regions above 200 km) since the number of genotypes (genotypic richness) of a population may vary with the size of the sample (Chao et al., 2014). Although sampling has an important effect on genotypic diversity, it is worthy to mention that MS has played an outstanding role in the expansion of forest plantations in Brazil. Over the last five years, the area planted with eucalypt has grown 2.4%; MS has led this expansion, with an increase of 400,000 hectares over this period and an average growth of 13% per year (IBA, 2017). In order to reach this expansion rate, it was necessary to implement new nurseries with cuttings from different states of the country, which could have favored the spread of the pathogen and the introduction of some genotypes to MS, resulting in a greater genotypic richness.

Although MS has greater richness and evenness, based on genetic diversity by Hill numbers (Shannon´s and Simpson´s index), the pathogen populations displayed relatively low genetic diversity, most likely because *E. psidii* recently adapted to eucalypt. Similar results using Rep-PCR were found by McManus & Jones, (1995) who found genetic homogeneity in the *E. amylovora* population collected from apple and pear trees and different geographic regions. According to Teixeira et al. (2009), the genetic homogeneity of *E. psidii* from guava suggests a low frequency of recombination in the pathogen population. Alternatively, it may also suggest a recent adaptation of a local population originally associated with an indigenous *Psidium* species, such as *P. cattleyanum* (known as red *araçá*) to common guava (*P. guajava*), which is native to tropical America.

Differences in aggressiveness were observed among *E. psidii* isolates, even though most of them were originally obtained from *Eucalyptus* spp. plants. Aggressiveness variation among isolates have already been reported for other bacterial phytopathogens. For instance, Maciel et al. (2017) found variation in aggressiveness among *Pseudomonas syringae.* pv. *garcae* isolates obtained from *Coffee arabica*. All isolates included in their study were pathogenic to coffee seedlings but were divided into three different aggressiveness classes: highly, moderate and weakly aggressive. Rodrigues et al. (2017) not only observed differences in aggressiveness among *P. syringae* pv. *garcae* isolates, but also differences in the expression of aggressiveness according to the temperature. Some *P. syringae* pv. *garcae* isolates were more aggressive at mild weather conditions, while others were more aggressive at higher temperatures. Our inoculation experiments were conducted under controlled conditions, ruling out a possible influence of the temperature on our results.

Despite the *E. psidii* populations studied displaying a relatively low genetic diversity, differences in aggressiveness were observed among isolates. These results emphasize the importance of understanding the phenotypic aspects of the interaction between pythopathogens and their hosts. Differences in aggressiveness among *E. psidii* isolates in a given population may challenge the establishment of effective methods for disease management. For example, when comparing among the isolates included in our inoculation experiments, it would not be advisable to screen plant genotypes for resistance inoculating with isolate LPF633 since it is moderately aggressive on the *E. urophylla* clone, but is highly aggressive on the hybrid clone. Furthermore, isolate LPF681 caused disease symptoms on the hybrid clone, but not on the *E. urophylla* clone. Using inappropriate *E. psidii* isolates in germoplasm screening could lead to the selection of plant genotypes whose resistance may not be effective under field conditions.

Overall, our results showed that the populations of *E. psidii* in Brazil display a relatively low genetic variability, which leads us to hypothesize that the spread of the bacterium in the country probably occurred through asymptomatic rooted cuttings. The observed isolate × eucalypt clone interaction demonstrates the importance of gaining knowledge on the aggressiveness variability among isolates of the *E. psidii* populations in order to select the most aggressive ones for inoculation on plant genotypes with different genetic backgrounds aiming at selecting effective plant resistance. Our results represent a foundation for establishing approaches to improve current control strategies for dieback and wilting of eucalypt caused by *E. psidii*.

ACKNOWLEDGEMENTS

The authors thank CMPC Celulose Riograndense, Eldorado Brasil, Fibria, Suzano Papel e Celulose for the support in the collection of samples in plantations and Clonar Resistência a Doenças Florestais for the propagation of the plant material and for providing facilities for the inoculation experiments; the Organization of American States (OAS) and Coimbra Group of Brazilian Universities (GCUB) for the Doctoral Scholarship.

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Table 2. 1. Estimates of genetic diversity for the *Erwinia psidii* populations on *Eucalyptus* spp. from Mato Grosso do Sul, Rio Grande do Sul and São Paulo, Brazil.

	Population (Host/local)			
Parameters	***Eucalyptus* spp. / Rio Grande do Sul**	***Eucalyptus* spp. / Mato Grosso do Sul**	***Eucalyptus* spp. / São Paulo**	**Total**
Sample size (n)	57	32	12	101
Genotypes richness	4 (4.177-25.775)	12 (19.671- 317.849)	1 (1.000-1.005)	13
Shannon´s Index	1.387 (1.387 - 1.869)	4.712 (4.712 -17.176)	1.000 (1.000 - 1.000)	-
Simpson´s Index	1.154 (1.154 -1.335)	2.473 (2.473- 4.395)	1.000 (1.000 - 1.000)	-
Stoddart & Taylor (G)	0,134	0,596	0,00	-
Evenness index (H$_E$)	1,15	2,47	1,00	-

Table 2. 2. Aggressiveness of *Erwinia psidii* isolates on *Eucalyptus grandis* × *E. urophylla* and *E. urophylla* clones, estimated by the area under the disease progress curve (AUDPC). Similar results were obtained in two independent experiments, statistical analysis was conducted on the combined data.

Strains	**Host**	**Area under the disease progress curve (AUDPC)**	**Aggressiveness category**	**Area under the disease progress curve (AUDPC)**	**Aggressiveness category**	**Between-clone difference p-value ****
		Clone *E. urophylla* × *E. grandis*		**Clone *E. urophylla***		
IBSBF435	*Psidium guajava*	126.7 a	Highly Aggressive	111.7 a	Highly Aggressive	0.0002137
LPF615	*Eucalyptus* sp.	117.0 ab	Highly Aggressive	51.0 cd	Aggressive	0.0002847
LPF556	*Eucalyptus* sp.	100.0 bc	Aggressive	100.8 a	Highly aggressive	0.01468
LPF610	*Eucalyptus* sp.	94.6 cd	Aggressive	110.6 a	Highly aggressive	0.6418
LPF534	*Eucalyptus* sp.	89.0 cde	Aggressive	49.3 cd	Aggressive	0.0003235
LPF683	*Eucalyptus* sp.	80.1 de	Aggressive	70.2 b	Aggressive	0.000343
LPF624	*Eucalyptus* sp.	78.3 de	Aggressive	64.8 bc	Aggressive	0.001279
LPF553	*Eucalyptus* sp.	70.1 ef	Aggressive	109.2 a	Highly aggressive	1
LPF680	*Eucalyptus* sp.	56.0 fg	Aggressive	24.2 ef	Weakly aggressive	0.002518
LPF609	*Eucalyptus* sp.	52.5 fg	Aggressive	101.8 a	Highly aggressive	0.5882
LPF640	*Eucalyptus* sp.	44.8 g	Aggressive	38.2 de	Weakly aggressive	0.0009739
LPF554	*Eucalyptus* sp.	42.1 g	Aggressive	97.0 a	Aggressive	0.05207
LPF633	*Eucalyptus* sp.	41.8 g	Aggressive	59.5 bc	Aggressive	0.0001405
LPF681	*P. guajava*	18.3 h	Weakly aggressive	0.0 f	Non virulent	0.005283
CONTROL		0.0 i		0.0 f		

* Means followed by the same letter are not significantly different ($p \leq 0.05$; Kruskal-Wallis test).

****** p-values lower than 0.05 indicate significant differences (Wilcoxon test).

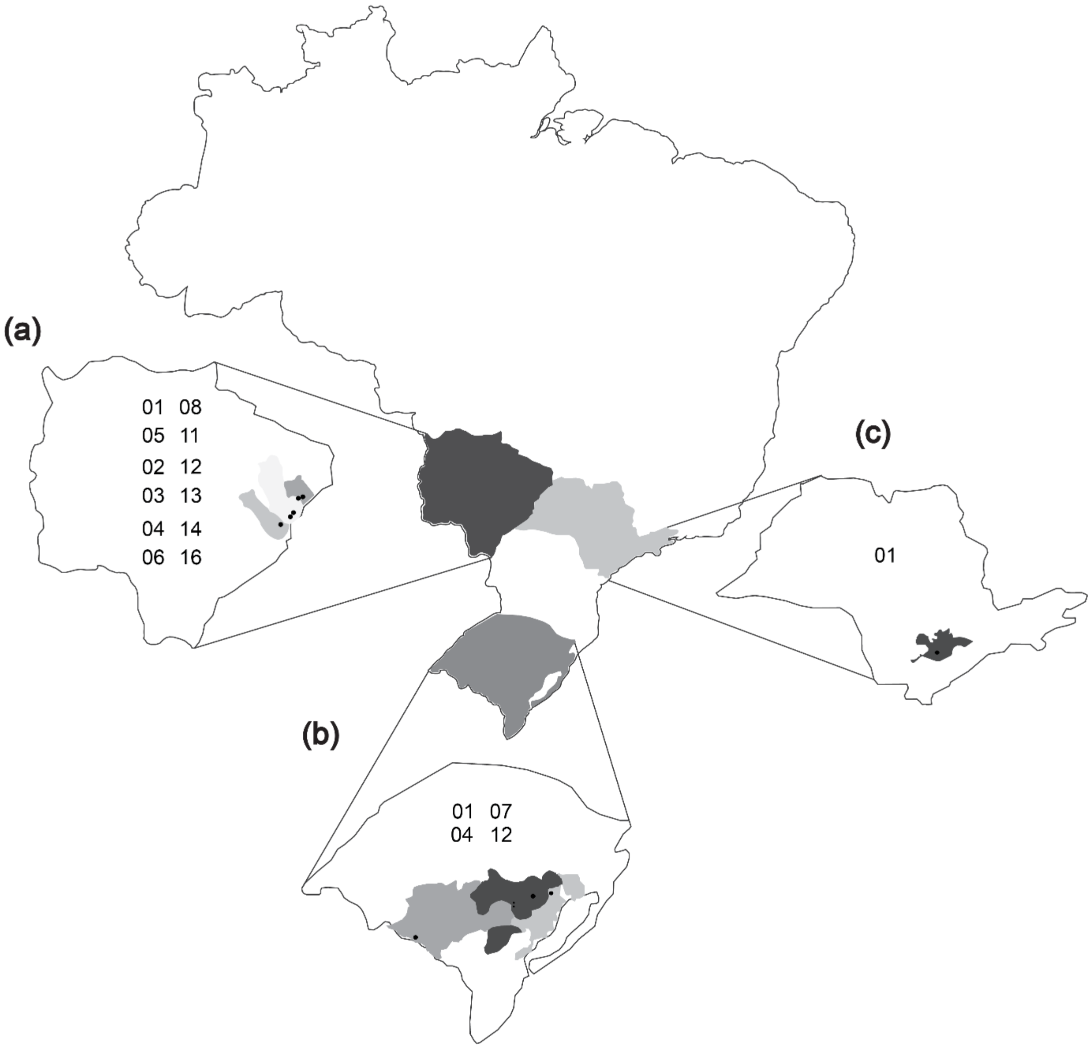

Figure 2. 1. Map of the Brazilian localities sampled with the number of observed genotypes indicated for each state. Gray and dark areas represent the sampled localities, (a) Mato Grosso do Sul (MS), three sampled localities and twelve genotypes (b) Rio Grande do Sul (RS), four sampled localities and four genotypes and (c) São Paulo (SP), one sampled locality and one genotype.

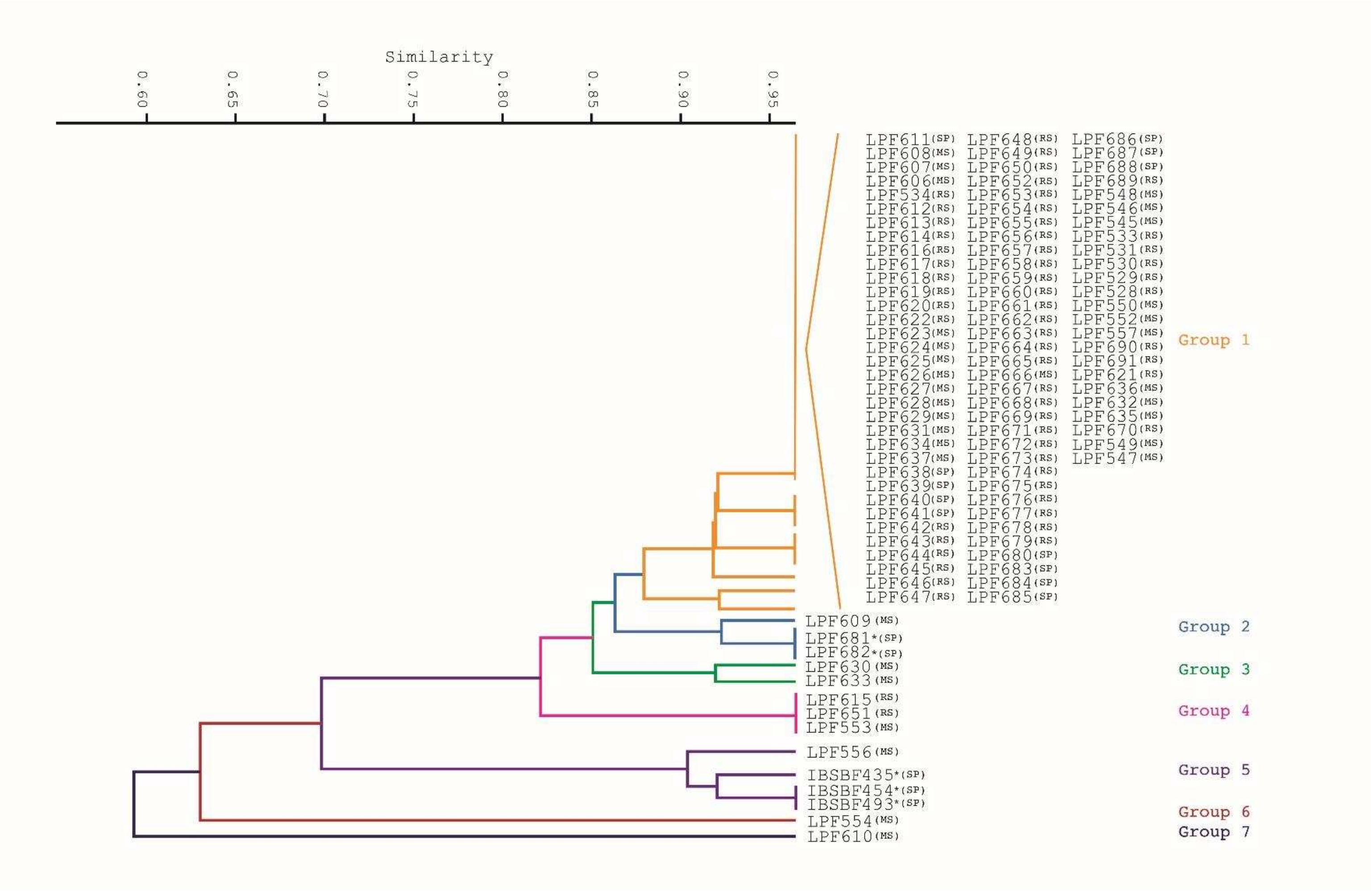

Figure 2. 2. Rep-PCR dendrogram of *Erwinia psidii* populations from Brazil. The tree was generated with combined ERIC-, REP- and BOX-PCR band profiles of isolates obtained from *Eucalyptus* spp. and *Psidium guajava* in the states of Mato Grosso do Sul, Rio Grande do Sul and São Paulo. The differences among profiles are indicated by the percentage of similarity. The dendrogram was constructed using the Jaccard's similarity coefficient and the UPGMA clustering method. Asterisks indicate isolates obtained from *P. guajava.*

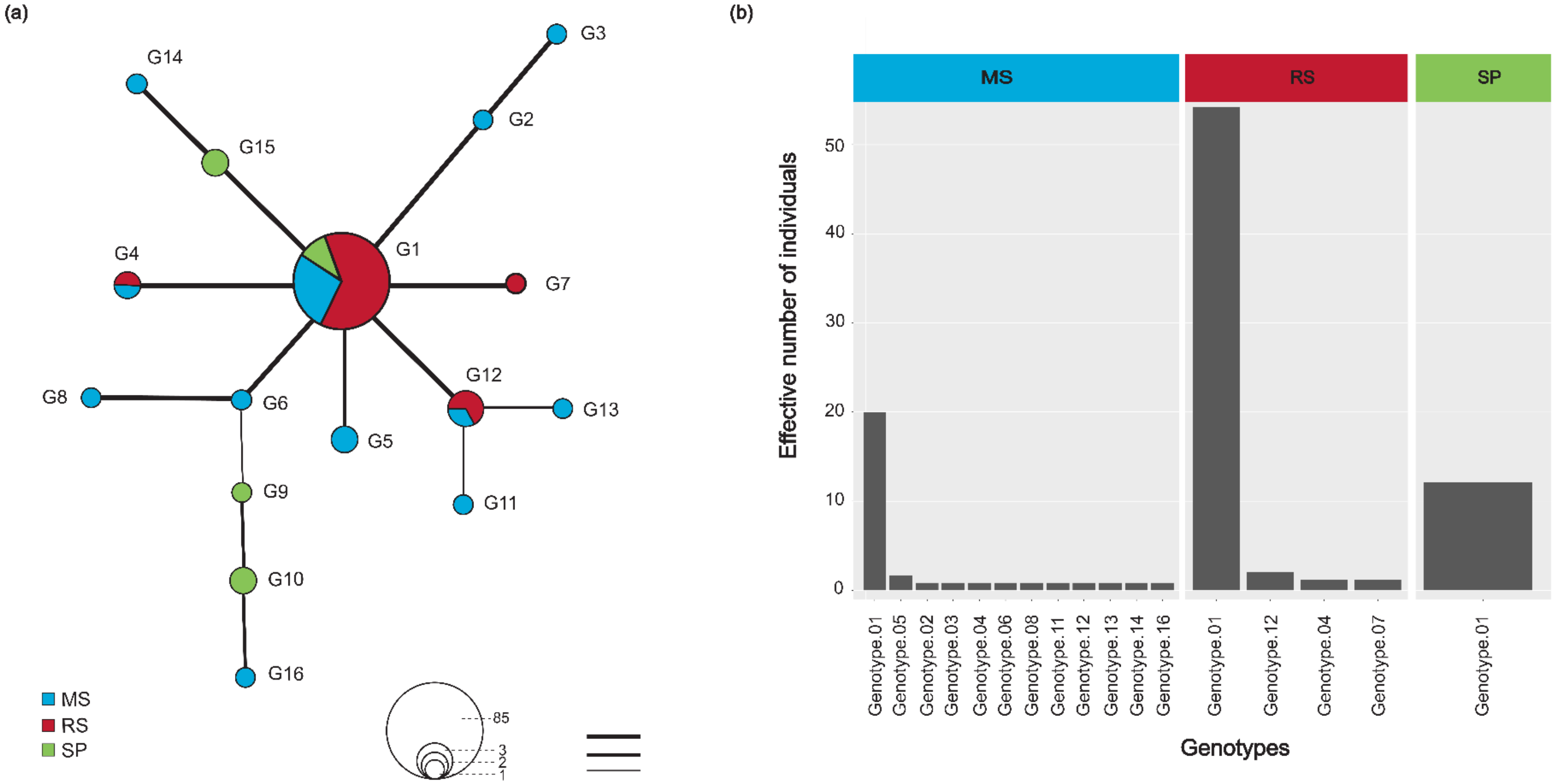

Figure 2. 3. Genotypes of *Erwinia psidii* populations from Brazil. (a) Genotypes network of isolates from *Eucalyptus* spp. and guava collected in Mato Grosso do Sul, Rio Grande do Sul and São Paulo. Each color represents a state. The size of the circle represents the number of isolates in the genotypes. (b) Genotypes present in the *Eucalyptus* spp. populations from Mato Grosso do Sul, Rio Grande do Sul and São Paulo.

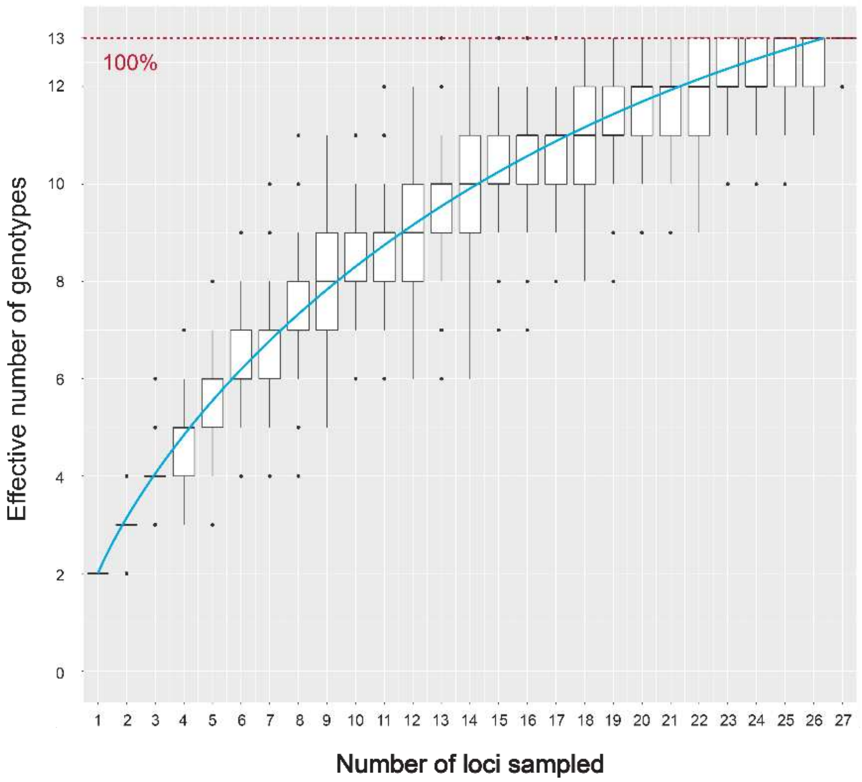

Figure 2. 4. Genotype accumulation curves for *Erwinia psidii* populations from Brazil. Dashed lines indicate 100% of the number of genotypes identified in each population. The number of loci was randomly sampled (1,000 times).

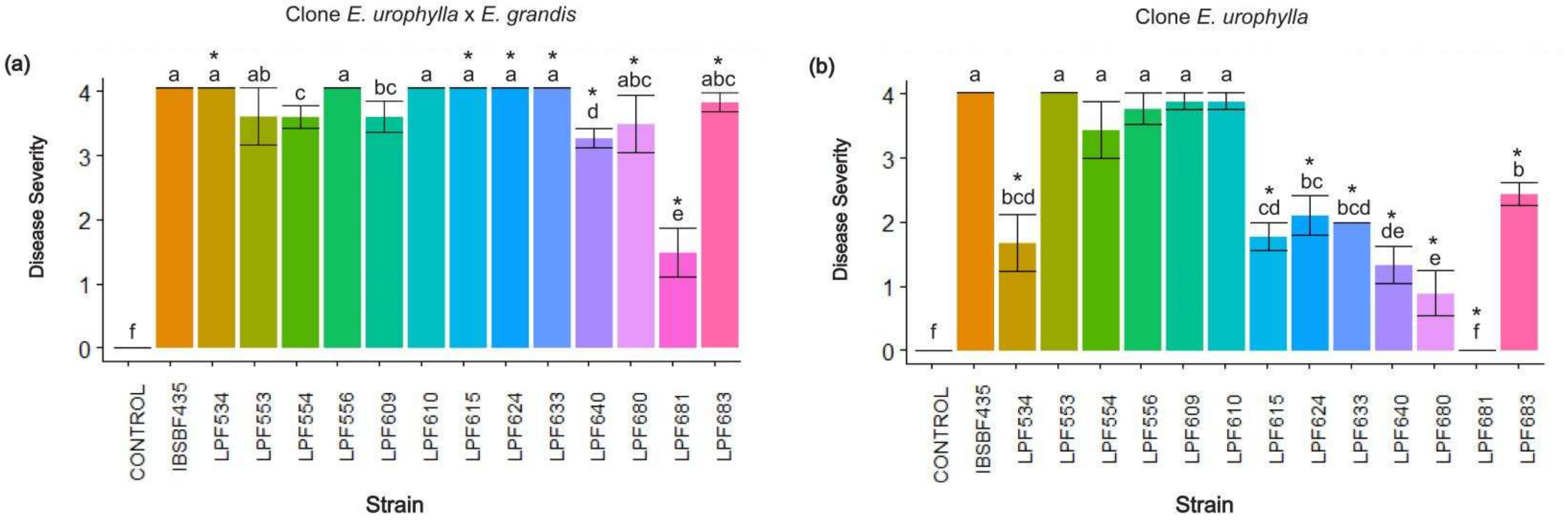

Figure 2. 5. Disease severity on *Eucalyptus urophylla* × *E. grandis* and *E. urophylla* clones inoculated with *Erwinia psidii* isolates. (a) *E. urophylla* × *E. grandis*; (b) *E. urophylla*. Bars indicate mean rating values of nine repetitions with vertical lines indicating standard errors. Means of bars with the same letter are not significantly different within the same clone (Kruskal-Wallis, 95% significance level). Asterisks indicate significant differences between clones for the same isolate (Wilcoxon Test).

SUPPLEMENTARY MATERIAL

Table S2. 1. Bacterial isolates used in this study.

N	Isolate	Species	Host	Collection site	Tissue
1	**LPF528**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
2	**LPF 529**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
3	**LPF530**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
4	**LPF 531**	*Erwinia psidii*	*E. dunnii*	Guaíba- RS	Leaf
5	**LPF 533**	*Erwinia psidii*	*E. dunnii*	Guaíba- RS	Leaf
6	**LPF 545**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Leaf
7	**LPF 546**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
8	**LPF 547**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
9	**LPF 548**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
10	**LPF 549**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
11	**LPF 550**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
12	**LPF 552**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
13	**LPF 553**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
14	**LPF 554**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
15	**LPF 556**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
16	**LPF557**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
17	**LPF 606**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Stem
18	**LPF 607**	*Erwinia psidii*	*E. urophylla*	Três Lagoas- MS	Stem
19	**LPF 608**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Stem
20	**LPF 609**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Leaf
21	**LPF 610**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Branch/Leaf
22	**LPF 611**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Andradina- SP	Branch/Leaf
23	**LPF 612**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
24	**LPF 613**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
25	**LPF 614**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
26	**LPF 615**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
27	**LPF 616**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
28	**LPF 617**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
29	**LPF 618**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
30	**LPF 619**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf

Table S2. 1. Bacterial isolates used in this study (cont).

N	Isolate	Species	Host	Collection site	Tissue
31	**LPF 620**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
32	**LPF 621**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
33	**LPF 622**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
34	**LPF 623**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Root
35	**LPF 624**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Root
36	**LPF 625**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
37	**LPF 626**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Stem
38	**LPF 627**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
39	**LPF 628**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
40	**LPF 629**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
41	**LPF 630**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Stem
42	**LPF 631**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
43	**LPF 632**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
44	**LPF 633**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Stem
45	**LPF 634**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
46	**LPF 635**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Stem
47	**LPF 636**	*Erwinia psidii*	*Eucalyptus* sp.	Brasilândia- MS	Branch/Leaf
48	**LPF 637**	*Erwinia psidii*	*Eucalyptus* sp.	Brasilândia- MS	Stem
49	**LPF 638**	*Erwinia psidii*	*Eucalyptus* sp.	Itapetininga- SP	Branch/Leaf
50	**LPF 639**	*Erwinia psidii*	*Eucalyptus* sp.	Itapetininga- SP	Stem
51	**LPF 640**	*Erwinia psidii*	*Eucalyptus* sp.	Conceição- SP	Branch/Leaf
52	**LPF 641**	*Erwinia psidii*	*Eucalyptus* sp.	Conceição- SP	Stem
53	**LPF 642**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
54	**LPF 643**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
55	**LPF 644**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
56	**LPF 645**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
57	**LPF 646**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
58	**LPF 647**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
59	**LPF 648**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
60	**LPF 649**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem
61	**LPF 650**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
62	**LPF 651**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
63	**LPF 652**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem

Table S2. 1. Bacterial isolates used in this study (cont).

N	Strain	Species	Host	Collection site	Tissue
64	**LPF 653**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
65	**LPF 654**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
66	**LPF 655**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
67	**LPF 656**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
68	**LPF 657**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
69	**LPF 658**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
70	**LPF 659**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
71	**LPF 660**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
72	**LPF 661**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
73	**LPF 662**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
74	**LPF 663**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
75	**LPF 664**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
76	**LPF 665**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem
77	**LPF 666**	*Erwinia psidii*	*Eucalyptus* sp.	Brasilândia- MS	Stem
78	**LPF 667**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
79	**LPF 668**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
80	**LPF 669**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
81	**LPF 670**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
82	**LPF 671**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
83	**LPF 672**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Stem
84	**LPF 673**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
85	**LPF 674**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
86	**LPF 675**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
87	**LPF 676**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
88	**LPF 677**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
89	**LPF 678**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Stem
90	**LPF 679**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
91	**LPF 680**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Andradina - SP	Stem
92	**LPF 681**	*Erwinia psidii*	*Psidium guajava*	Vista alegre do alto - SP	Stem
93	**LPF 682**	*Erwinia psidii*	*Psidium guajava*	Vista alegre do alto - SP	Stem
94	**LPF 683**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé-SP	Stem
95	**LPF 684**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé-SP	Stem
96	**LPF 685**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé-SP	Branch/Leaf

Table S2. 1. Bacterial isolates used in this study (cont).

N	Strain	Species	Host	Collection point	Tissue
97	**LPF 686**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
98	**LPF 687**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
99	**LPF 688**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
100	**LPF 689**	*Erwinia psidii*	*E. saligna*	Campo-Limoeiro- RS	Branch/Leaf
101	**LPF690**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
102	**LPF691**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
103	**LPF534**	*Erwinia psidii* (Type strain)	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
104	**IBSBF435**	*Erwinia psidii* (Type strain)	*Psidium guajava*	Valinhos- SP	-
105	**IBSBF454**	*Erwinia psidii*	*Psidium guajava*	Valinhos- SP	-
106	**IBSBF493**	*Erwinia psidii*	*Psidium guajava*	Itariri- SP	-

Table S2. 2. Percentage of polymorphic Rep-PCR loci of *Erwinia psidii* populations.

Rep Marker	Size (bp)	% polymorphic loci	Total loci
ERIC	300-1000	64,28	14
REP	250- 5000	52,94	17
BOX	400-3000	66,66	15

Table S2. 3. Genotypes of *Erwinia psidii*, populations from Mato Grosso do Sul, Rio Grande do Sul and São Paulo obtained from *Eucalyptus* spp. and *Psidium guajava*.

Genotype	Isolate
G1	LPF534 (RS), LPF606 (MS), LPF607 (MS), LPF608 (MS), LPF611 (SP), LPF612 (RS), LPF613 (RS), LPF614 (RS), LPF616 (RS), LPF617 (RS), LPF618 (RS), LPF619 (RS), LPF620 (RS), LPF622 (RS), LPF623 (MS), LPF624 (MS), LPF625 (MS), LPF626 (MS), LPF627 (MS), LPF628 (MS), LPF629 (MS), LPF631 (MS), LPF634 (MS), LPF637 (MS), LPF638 (SP), LPF639 (SP), LPF640 (SP), LPF641 (SP), LPF642 (RS), LPF643 (RS), LPF644 (RS), LPF645 (RS), LPF646 (RS), LPF647 (RS), LPF648 (RS), LPF649 (RS), LPF650 (RS), LPF652 (RS), LPF653 (RS), LPF654 (RS), LPF655 (RS), LPF656 (RS), LPF657 (RS), LPF658 (RS), LPF659 (RS), LPF660 (RS), LPF661 (RS), LPF662 (RS), LPF663 (RS), LPF664 (RS), LPF665 (RS), LPF666 (MS), LPF667 (RS), LPF668 (RS), LPF669 (RS), LPF671 (RS), LPF672 (RS), LPF673 (RS), LPF674 (RS), LPF675 (RS), LPF676 (RS), LPF677 (RS), LPF678 (RS), LPF679 (RS), LPF680 (SP), LPF683 (SP), LPF684 (SP), LPF685 (SP), LPF686 (SP), LPF687 (SP), LPF688 (SP), LPF689 (RS), LPF548 (MS), LPF546 (MS), LPF545 (MS), LPF533 (RS), LPF531 (RS), LPF529 (RS), LPF528 (RS), LPF550 (MS), LPF552 (MS), LPF557 (MS), LPF690 (RS), LPF691 (RS)
G2	LPF630 (MS)
G3	LPF633 (MS)
G4	LPF621 (RS), LPF636 (MS)
G5	LPF632 (MS), LPF635 (MS)
G6	LPF547 (MS)
G7	LPF670 (RS)
G8	LPF549 (MS)
G9	IBSBF435 (SP)
G10	IBSBF454 (SP), IBSBF493 (SP)
G11	LPF610 (MS)
G12	LPF615 (RS), LPF651 (RS), LPF553 (MS)
G13	LPF554 (MS)
G14	LPF609 (MS)
G15	LPF681 (SP), LPF682 (SP)
G16	LPF556 (MS)

Table S2. 4. Genotypes of *Erwinia psidii*, populations from Mato Grosso do Sul, Rio Grande do Sul and São Paulo obtained from *Eucalyptus* spp.

Genotype	Isolate
G1	LPF534 (RS), LPF606 (MS), LPF607 (MS), LPF608 (MS), LPF611 (SP), LPF612 (RS), LPF613 (RS), LPF614 (RS), LPF616 (RS), LPF617 (RS), LPF618 (RS), LPF619 (RS), LPF620 (RS), LPF622 (RS), LPF623 (MS), LPF624 (MS), LPF625 (MS), LPF626 (MS), LPF627 (MS), LPF628 (MS), LPF629 (MS), LPF631 (MS), LPF634 (MS), LPF637 (MS), LPF638 (SP), LPF639 (SP), LPF640 (SP), LPF641 (SP), LPF642 (RS), LPF643 (RS), LPF644 (RS), LPF645 (RS), LPF646 (RS), LPF647 (RS), LPF648 (RS), LPF649 (RS), LPF650 (RS), LPF652 (RS), LPF653 (RS), LPF654 (RS), LPF655 (RS), LPF656 (RS), LPF657 (RS), LPF658 (RS), LPF659 (RS), LPF660 (RS), LPF661 (RS), LPF662 (RS), LPF663 (RS), LPF664 (RS), LPF665 (RS), LPF666 (MS), LPF667 (RS), LPF668 (RS), LPF669 (RS), LPF671 (RS), LPF672 (RS), LPF673 (RS), LPF674 (RS), LPF675 (RS), LPF676 (RS), LPF677 (RS), LPF678 (RS), LPF679 (RS), LPF680 (SP), LPF683 (SP), LPF684 (SP), LPF685 (SP), LPF686 (SP), LPF687 (SP), LPF688 (SP), LPF689 (RS), LPF548 (MS), LPF546 (MS), LPF545 (MS), LPF533 (RS), LPF531 (RS), LPF529 (RS), LPF528 (RS), LPF550 (MS), LPF552 (MS), LPF557 (MS), LPF690 (RS), LPF691 (RS)
G2	LPF630 (MS)
G3	LPF633 (MS)
G4	LPF621 (RS), LPF636 (MS)
G5	LPF632 (MS), LPF635 (MS)
G6	LPF547 (MS)
G7	LPF670 (RS)
G8	LPF549 (MS)
G11	LPF610 (MS)
G12	LPF615 (RS), LPF651 (RS), LPF553 (MS)
G13	LPF554 (MS)
G14	LPF609 (MS)
G16	LPF556 (MS)

Table S2. 5. Results of ANOVA for the area under the disease progress curve (AUDPC) obtained by inoculation of *Erwinia psidii* isolates on clones of *E. urophylla* × *Eucalyptus grandis* and *E. urophylla*.

Experiment 1					
	Df	Sum sq	Mean Sq	F value	Pr(>F)
isolate	14	104327	7452	26.416	< 2e-16 ***
clone	1	29909	29909	106.024	< 2e-16 ***
isolate:clone	14	21680	1549	5.489	4.62e-08 ***
Residuals	121	34133	282		

Experiment 2					
	Df	Sum sq	Mean Sq	F value	Pr(>F)
isolate	14	114943	8210	51.34	< 2e-16***
clone	1	10584	10584	66.19	2.14e-12***
isolate:clone	14	29906	2136	13.36	< 2e-16***
Residuals	90	14392	160		
Signif. codes: 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1**					

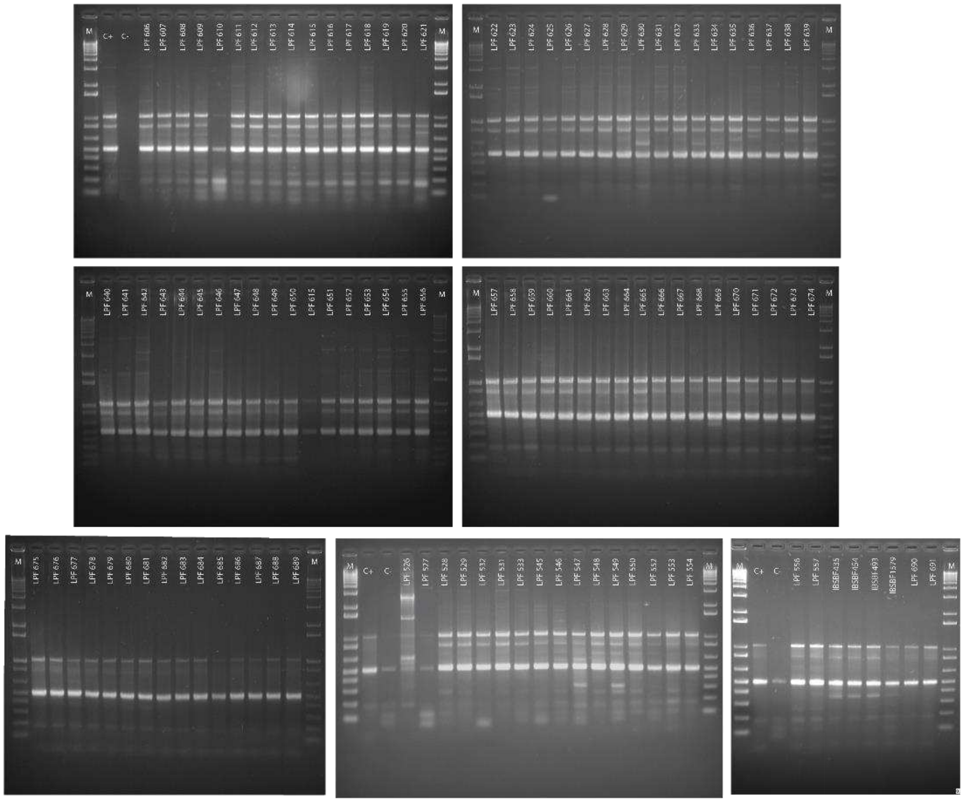

Figure S2. 1. ERIC-PCR profiles of *Erwinia psidii* isolates from Brazil. C+, positive control (DNA from type strain LPF534); C-, negative control (water); M, Molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

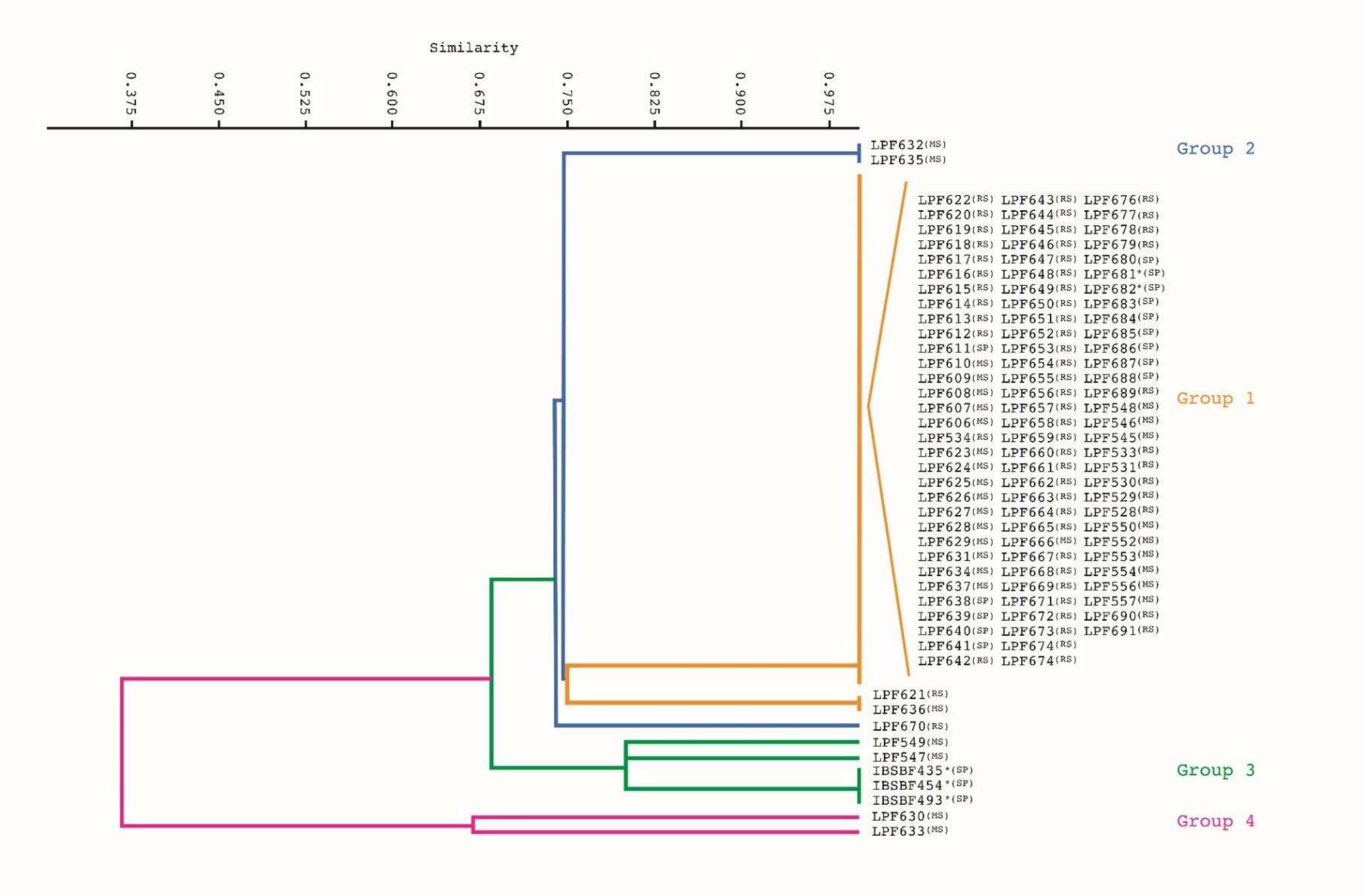

Figure S2. 2. ERIC-PCR dendrogram of *Erwinia psidii* isolates obtained from *Eucalyptus* spp. and *Psidium guajava*. The differences between band profiles are indicated by percentage of similarity. The dendrogram was constructed using the Jaccard similarity coefficient and the UPGMA clustering method. Asterisks indicate isolates obtained from *P. guajava*.

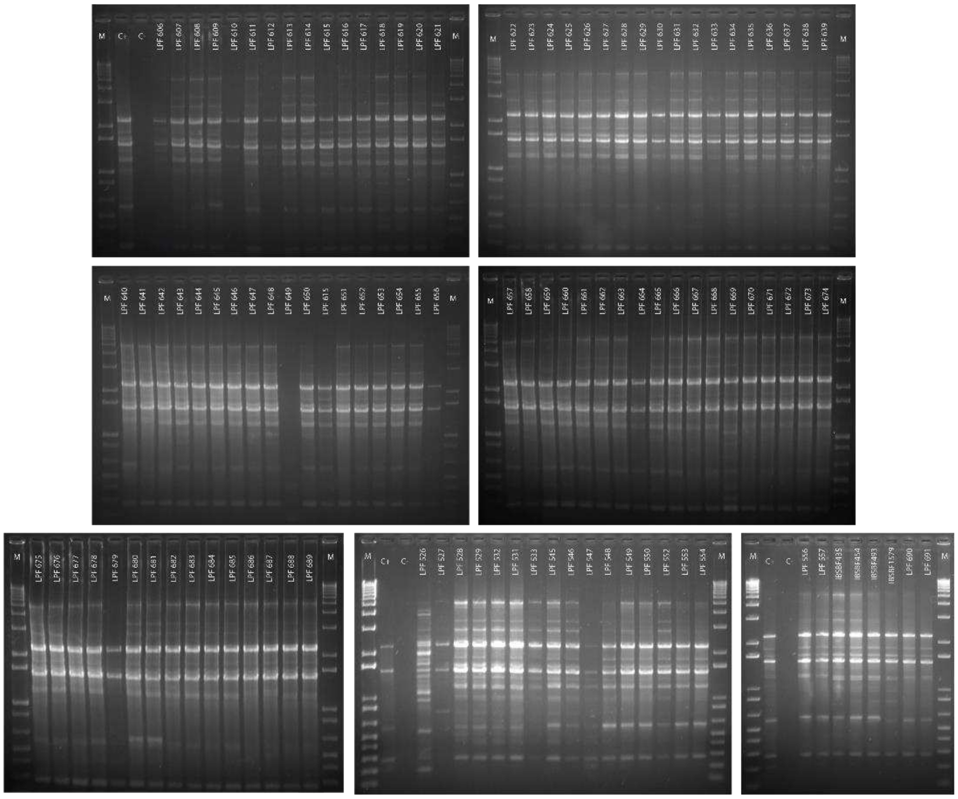

Figure S2. 3. REP-PCR profiles of *Erwinia psidii* isolates from Brazil. C+, positive control (DNA from type strains LPF534); C-, negative control (water); M, Molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

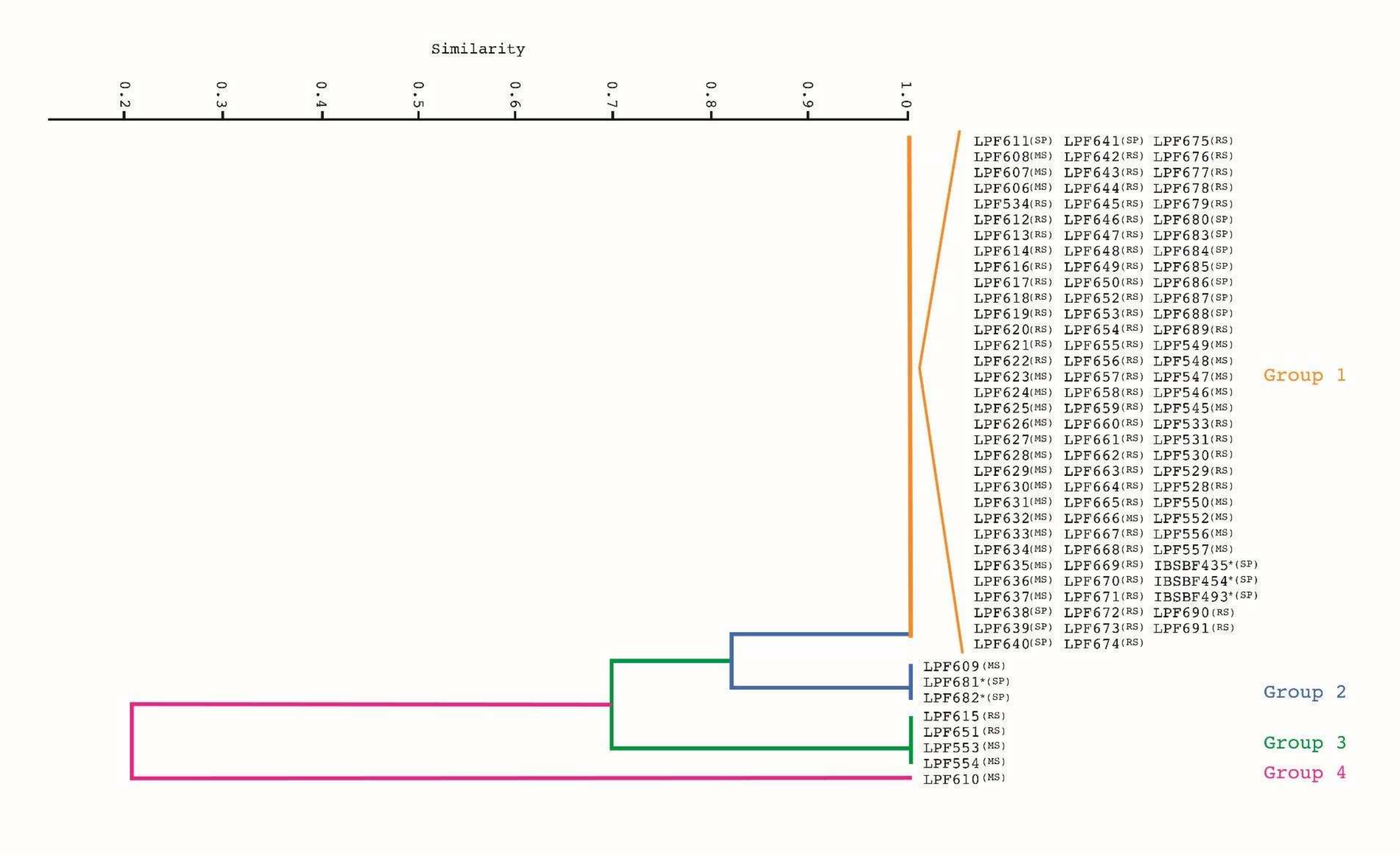

Figure S2. 4. REP-PCR dendrogram of *Erwinia psidii* isolates obtained from *Eucalyptus* spp. and *Psidium guajava*. The differences between band profiles are indicated by percentage of similarity. The dendrogram was constructed using the Jaccard similarity coefficient and the UPGMA clustering method. Asterisks indicate isolates obtained from *P. guajava*.

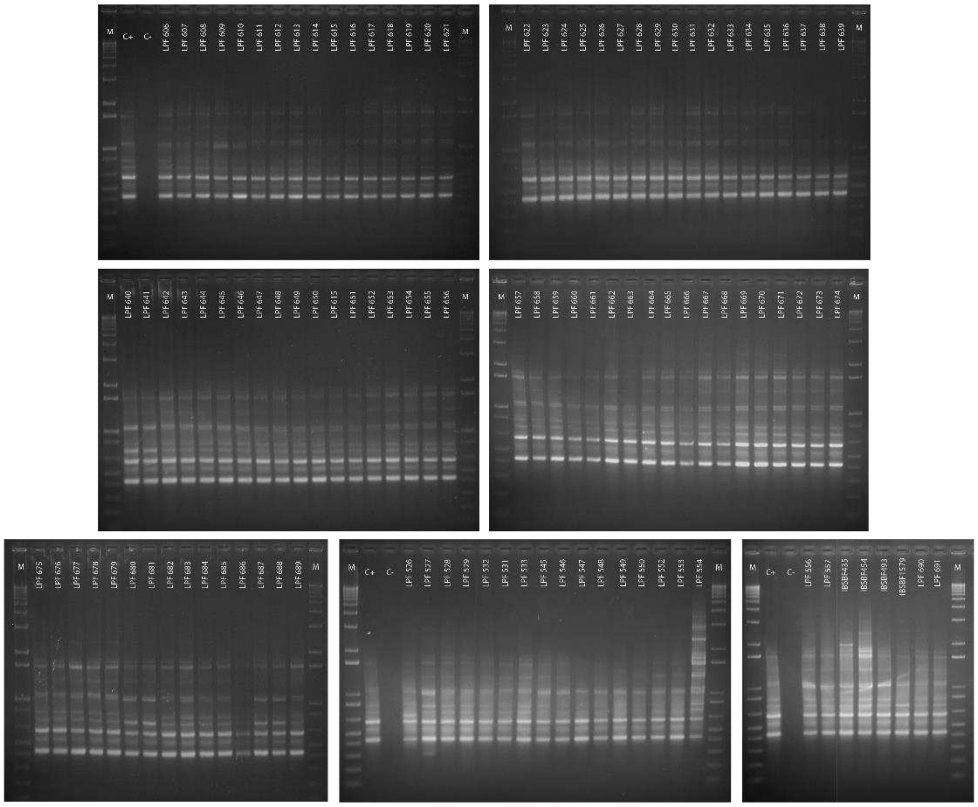

Figure S2. 5. BOX-PCR profiles of *Erwinia psidii* isolates from Brazil. C+, positive control (DNA from type strains LPF534); C-, negative control (water); M, Molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

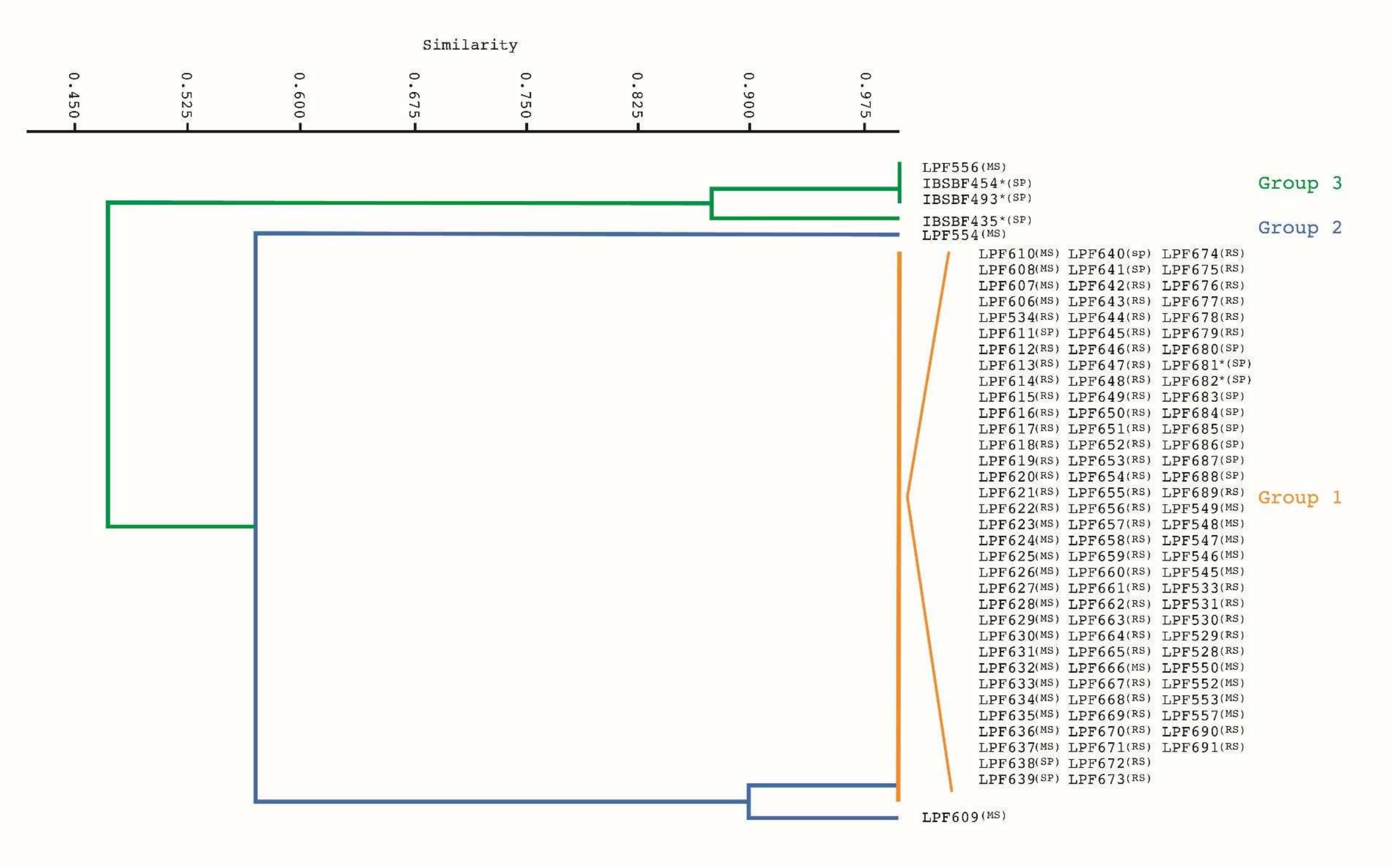

Figure S2. 6. BOX-PCR dendrogram of *Erwinia psidii* isolates obtained from *Eucalyptus* spp. and *Psidium guajava*. The differences between band profiles are indicated by percentage of similarity. The dendrogram was constructed using the Jaccard similarity coefficient and the UPGMA clustering method. Asterisks indicate isolates obtained from *P. guajava*.

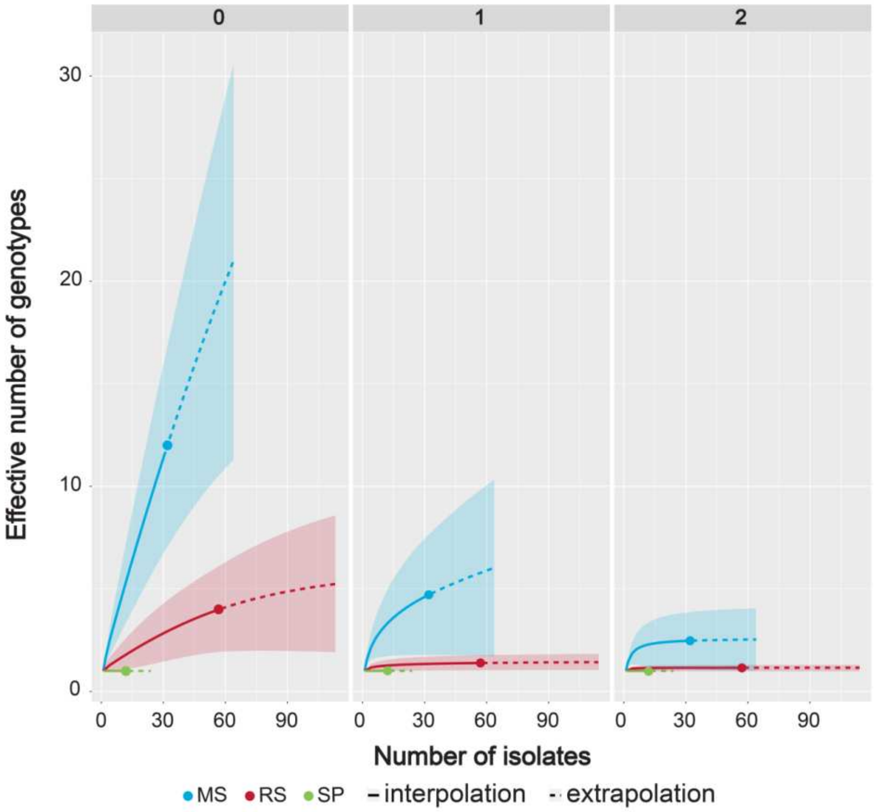

Figure S2. 7. Diversity accumulation curves for different sample sizes of the Hill numbers or effective number of genotypes of orders (0), (1) and (2) estimated for the *Erwinia psidii* populations from Brazil. Dash areas represent 95% confidence intervals for populations from Mato Grosso do Sul, Rio Grande do Sul and São Paulo. The 0, 1 and 2 numbers correspond to genotype richness, Shannon's index, and Simpson's index, respectively. Solid lines correspond to rarefaction (interpolation) and dashed lines to extrapolation curves to the base sample size of 57 individuals. The 95% confidence intervals were obtained by 200 bootstrap replicates.

ARTICLE 3: Agglutination test for *Erwinia psidii* is successful, fast and specific for diagnosing dieback in *Eucalyptus* spp.

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ABSTRACT

Dieback, caused by *Erwinia psidii* is currently one of the most severe emergent diseases in *Eucalyptus* spp. in Brazil. Diagnosis of dieback and wilting currently relies on the isolation of *E. psidii* from plant samples and the use of molecular techniques, which is labor and time-consuming. Considering the need for a reliable, rapid and simple method for detecting the pathogen in asymptomatic plants, in this study we obtained an *E. psidii*-specific polyclonal antibody and developed an agglutination test. The antiserum was produced against the *E. psidii* type strain LPF534 and tested against 101 *E. psidii* isolates from *Eucalyptus* spp., three *E. psidii* isolates from *P. guajava*, seven endophytic bacteria from *Eucalyptus* spp. three non-pathogenic bacteria isolated from *Eucalyptus* spp. phylogenetically related to the genus *Erwinia* and two bacterial isolates pathogenic to *Eucalyptus* spp. Detection limit, specificity and sensitivity threshold were also determined. The antiserum Anti-Ep reacted positively with all strains of *E. psidii*. Cross-reactions were not detected in non-pathogenic isolates obtained from eucalypt and its detection limit was approx. $1x10^5$ colony forming units (CFU)/mL. The relative sensitivity and specificity of the agglutination test was similar to those of plate isolation and polymerase chain reaction (PCR) using specific primers (Ep2L-Ep2R). The relative sensitivity of the agglutination test was 100% and its specificity was 100%. These results show that the agglutination test is rapid, precise, non-expensive and can be used for accurate diagnosis of dieback and wilt in eucalypt caused by *E. psidii*.

Keywords: *Psidium guajava*, polyclonal antibodies, dieback.

INTRODUCTION

Erwinia psidii Rodrigues Neto, Robbs & Yamashiro was considered a restricted guava bacterium. However, in recent years, this pathogen has been identified as the causal agent of dieback and wilting in *Eucalyptus* spp. in Uruguay, Argentina (Coutinho et al., 2011) and Brazil (Arriel et al., 2014). In addition, disease symptoms in *Carica papaya* L. have also been associated with *E. psidii* in Thailand (Chai et al., 2017). In Brazil, in certain locations and periods of the year, the disease can infect almost 100% of the plants leading to loss of apical dominance and possibly growth decrease (Arriel et al., 2014). Due to the disease's highly destructive effects, it is necessary to have highly sensitive and rapid methods for diagnosis, focused on efficient disease control.

Currently, diagnosis of this disease relies on the use of phenotypic methods (i.e., cultural and biochemical tests) and detection of bacterial DNA by polymerase chain reaction (PCR), which is time-consuming and relatively expensive. In the field, the diagnosis is made based on the symptoms that sometimes can be confused with other diseases, such as the wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi. Before reporting *E. psidii* in eucalypt, the etiology of bacterial wilt was attributed to *R. solanacearum* based solely on the bacterial oozing and, in many cases, the disease was incorrectly diagnosed (Ferraz et al., 2016).

The development of fast and efficient diagnostic methods is essential to prevent dissemination of the bacterium through symptomatic or asymptomatic plants both in nurseries and in the field. Agglutination assays have been widely used in biology and medicine due to their simplicity, specificity and quick results, which are available within 10 min. In addition, it does not require special equipment, and is cost effective (Dubey et al., 2015; Picardeau et al., 2014). Agglutination test is widely used to detect pathogens that cause important diseases and that need to be quickly diagnosed or when an elevated quantity of saprophytic microorganisms is present in a sample (Idelevich et al, 2014). In veterinary medicine, it is commonly used for diagnosing diseases of big and small animals (Hafez et al., 2015). Therefore, it is also possible to use it in phytopathology laboratories.

The objective of this study was to develop and validate a quick and simple agglutination test for the detection of *E. psidii*, that allows the early detection of the bacterium in asymptomatic plants. Detection limit, specificity, and sensitivity of the method were determined and compared with those of standard diagnostic methods currently used in laboratories.

MATERIAL AND METHODS

Bacterial strains

One hundred and one isolates of *E. psidii* from symptomatic *Eucalyptus* spp. plants from three Brazilian states, three isolates from *P. guajava*, seven endophytic bacteria from *Eucalyptus* spp. three non-pathogenic bacterial isolates from *Eucalyptus* spp. phylogenetically related to the genus *Erwinia* and two pathogenic bacterial isolates from *Eucalyptus* spp. (*Ralstonia solanacearum* and *Xanthomonas campestris*), were analyzed in this study. These strains were obtained from the Collection of Plant Pathogenic Bacteria of the Forest Pathology Laboratory/Bioagro of the Universidade Federal de Viçosa-CPBFPL-UFV and Collection of Plant Pathogenic Bacteria at Instituto Biológico (IBSBF), Campinas, SP, Brazil (Table S3.1).

Immunization of rabbits for the production of anti-*E. psidii*

A rabbit (New Zealand breed) was immunized with 100 μg of antigen emulsified at a 1:1 ratio with incomplete Freund's adjuvant (Sigma). This procedure was repeated on days 21 and 40 after the first immunization. Subsequent immunizations were performed with 50 mcg of antigen emulsified at a 1:1 ratio with incomplete Freund's adjuvant (Sigma). At the end of the immunization protocol, on the 47th day, with the objective of obtaining hyperimmune serum, the rabbit received an intramuscular injection of a combination of anesthetic and muscle relaxant: 10 mg/kg Propofol and 0.25 mL/kg xylazine 2%. Under anesthetic and muscle relaxant effect, the rabbit’s blood was withdrawn by cardiac puncture (approximately 44 to 70 mL).

Preparation of anti-*E. psidii*

Purified polyclonal antibodies obtained from the rabbit were dialyzed in phosphate buffer (10mM Na_2HPO_4 pH 7.5) for 4 h in a cold chamber at 4 °C and their concentration adjusted to 1 mg/mL and stored at -20 ºC.

Agglutination test

Bacterial cultures stored in 30% glycerol (-80 °C) were streaked on solid 523 medium and the plates incubated for 48 h at 28 °C. Subsequently, a bacterial suspension was prepared with one individual colony in 100 μL of sterile distilled water. To perform the test, 10 μL of the bacterial suspension and 10 μL of the antibody were placed on a glass slide and mixed manually for 5 min until the formation of small or larger clumps.

Validation of specificity and sensitivity

The specificity and sensitivity of the antibodies were tested with one hundred and sixteen isolates of *E. psidii* (Table S3.1). To confirm the results, PCR was conducted using specific primers (Ep2L/2R). (Silva et al., 2015). A bacterial suspension was prepared by mixing one individual colony of each isolate and 100 µL of sterile distilled water. Amplification was performed in 15 μL of reaction mix containing: 5 U/μL GoTaqDNA polymerase (Promega), 0.75 mM $MgCl_2$, 1 mM each dNTP, 1 mM each primer and, 2 µl of bacterial suspension and 2.5 µL of ultrapure water. The PCR was performed with cycling conditions as follows: initial denaturation at 95 °C for 3 min followed by 32 cycles of 94 °C for 1 min, 64 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min (Silva et al., 2015) The reactions were performed on a Veriti^{TM}96 Thermal Cycler (Life Technologies, USA). Amplification bands were separated by electrophoresis on a 1% agarose gel, visualized by staining with ethidium bromide, and the gels photographed with a L.PIX camera (Loccus Biotechnology, Brazil). The sizes of the amplified fragments were estimated by comparison with the molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

Detection limit

The lowest detection limit of the agglutination test was done with the type strain LPF534. One isolated colony was kept on solid LB medium at 28 °C for 24 h. The suspension was then centrifuged at 11,180 *g* for 3 min, then the pellet was suspended again in 1 mL of sterile distilled water and the optical density (OD_{600}) measured. Subsequently, serial dilutions were made and finally 10 µL of each of the dilutions were mixed with 10 µL of the antiserum to evaluate the reaction.

Ethical and legal aspects

The experiments were conducted in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation and were submitted to the Animal Ethics Committee of the Federal University of Viçosa and certified with the process number 47/2016, in accordance with the actual Brazilian legislation law 11,794 of October 8, 2008. Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI. The Forest Pathology Laboratory/Bioagro and Immunochemistry and Glycobiology have the Certificate of Quality in Biosafety CQB No 0024/97.

RESULTS

Antibody specificity and sensitivity

The titer obtained from the dilution of the antibody (1:8), based on the original concentration, was satisfactory for the agglutination test. Subsequently, the exact titer was determined with bacterial isolates in the laboratory using plate isolation, PCR amplification and the agglutination test (Table 3.1). The analyzed samples of *Eucalyptus* spp. and *P. guajava* plants showed symptoms of dieback and tissue darkening. It was possible to isolate on solid culture medium, all the colonies morphologically similar to *E. psidii*, which were confirmed using PCR with specific primers. They also showed positive result in the agglutination test.

The antiserum Anti-Ep reacted positively against all strains of *E. psidii* tested, including strains from *P. guajava*. There was no reaction with strains of phytobacteria tested that belong to other genera, species or patovares (Figure 3.1, Figure 3.2).

Compared to polymerase chain reaction (PCR) using specific primers (Ep2L-Ep2R) (Silva et al., 2015), the specificity and sensitivity of the agglutination test was 100 % efficient. All isolates that showed a positive reaction in the agglutination test amplified a fragment of 200 bp in the PCR (data not shown).

Lower detection limit

According to the growth curve of *E. psidii* an optical density (OD_{600}) of 1.15 correspond to 10^7 CFU/mL (Arriel, 2012). From this concentration, serial dilutions were subsequently made until agglutination was detected. It was possible to show agglutination up to dilution 10^{-2}, equivalent to 10^5 CFU/mL. These results indicate that it is possible to detect *E. psidii* in samples with concentration above 10^5 CFU/mL.

Different agglutination patterns were found according to the concentration of the bacterial suspension, allowing this to generate semi-quantitative results. When the suspension has approximately 10^7 CFU/mL the semi-quantitative value is +++, 10^7 CFU/mL= ++, 10^6 CFU/mL= + and $<10^5$= Negative result (Figure 3.3).

DISCUSSION

Development of rapid diagnostic methods for reliable detection of *E. psidii* is essential to prevent its dissemination and establishment in new areas and to prevent its dissemination through symptomatic or asymptomatic plants, both in nurseries and in the field. Currently, diagnosis of this disease relies on the use of phenotypic methods (i.e., cultural and biochemical tests) and detection of bacterial DNA by polymerase chain reaction (PCR), which is time-consuming and relatively expensive. In the field, the diagnosis is made based on the symptoms that sometimes can be confused with other diseases, such as the wilt caused by *R. solanacearum*. Before reporting *E. psidii* in eucalypt, the etiology of bacterial wilt was attributed to *R. solanacearum* based solely on the bacterial oozing and in many cases, the diagnostic incorrect (Ferraz et al., 2016).

The agglutination test developed in this study represents a rapid and low-cost alternative compared to PCR, combines the advantages of high specificity and sensitivity and easiness to operate. The antiserum Anti-Ep reacted positively against all strains of *E. psidii* tested, including isolates from *P. guajava*. However, for its use in guava, validation with endophytic bacteria is recommended, because cross-reactions with other immunological tests were evidenced (Teixeira et al., 2008).

Some studies have shown that at low bacterial concentration, the agglutination test is not feasible for the target organism (Fronczek, 2013). Our results, showed similar sensitivity when compared with other agglutination tests designed for the detection of bacterial pathogens, but the specificity of our anti-serum was significantly higher than that previously found for *Streptococcus pneumoniae* (Altun et al., 2016). Although our method has a higher sensitivity than other agglutination tests, it still needs improvement by employing microspheres called "latex beads", which have demonstrated to be rather efficient for antibody absorption (Mahat et al., 2014).

In conclusion, this study demonstrated that the agglutination test allows detecting *E. psidii* with high specificity and sensitivity, compared to other tests used for disease diagnosis in humans and animals. Agglutination test is very simple, fast and easily replicable, being on par with gold standard methods employed for identifying *E. psidii*. It is also suitable to limited laboratory facilities. The test has commercial potential as a low-cost alternative method for detection of bacteria in symptomatic or asymptomatic plants both in nurseries and in the field.

ACKNOWLEDGEMENTS

The authors would like to thank the Organization of American States (OAS) and the Coimbra Group of Brazilian Universities (GCUB) for the Doctoral Scholarship provided.

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Table 3. 1. Comparison of agglutination test, plate isolation and PCR for detection of *Erwinia psidii* in naturally infected plant samples.

	Positive samples/Total samples		
Strains	**Plate isolation**	**PCR specific primers for *E. psidii***	**Agglutination Test**
Erwinia psidii from *Eucalyptus* spp.	101/101	101/101	101/101
Erwinia psidii from *Psidium guajava*	3/3	3/3	3/3
Non-pathogenic bacterial isolates in *Eucalyptus* spp.	3/3	0/3	0/3
Endophytic bacteria from *Eucalyptus* spp.	7/7	0/7	0/7
Ralstonia solanacearum	1/1	0/1	0/1
Xanthomonas campestris	1/1	0/1	0/1
Total	116/116	106/116	106/116

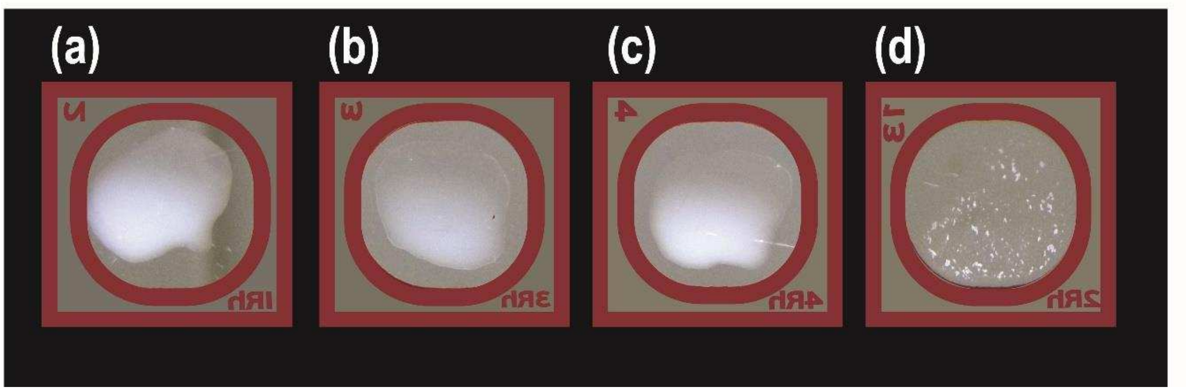

Figure 3. 1. Results of the agglutination test with non-pathogenic bacterial isolates from *Eucalyptus* spp. (a) *Pectobacterium carotovorum* subsp. *carotovororum*; (b) *Pectobacterium atrosepticum*; (d) *Dickeya chrysanthemi* pv. *chrysanthemi*; and (e) *Erwinia psidii* LPF534.

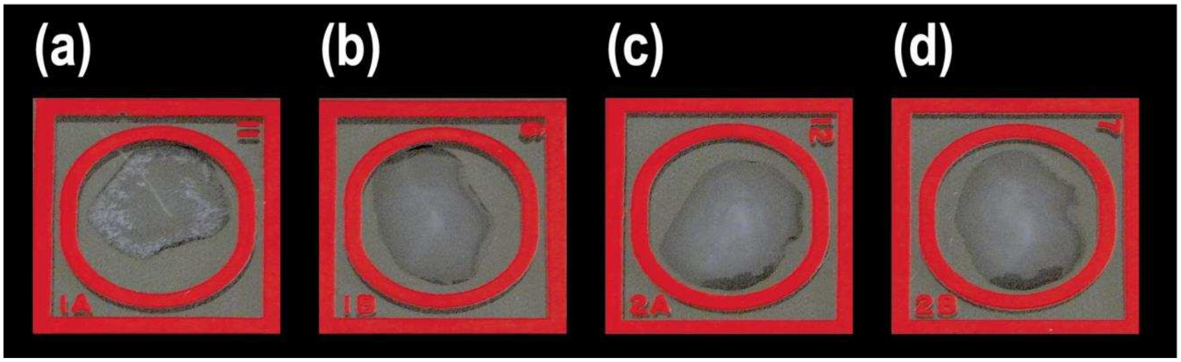

Figure 3. 2. Reaction of the agglutination test with pathogenic bacterial isolates from *Eucalyptus* spp. (a) *Erwinia psidii* LPF534; (b) *Ralstonia solanacearum*; (c) *Pantoea agglomerans*; and (d) *Xanthomonas axonopodis*.

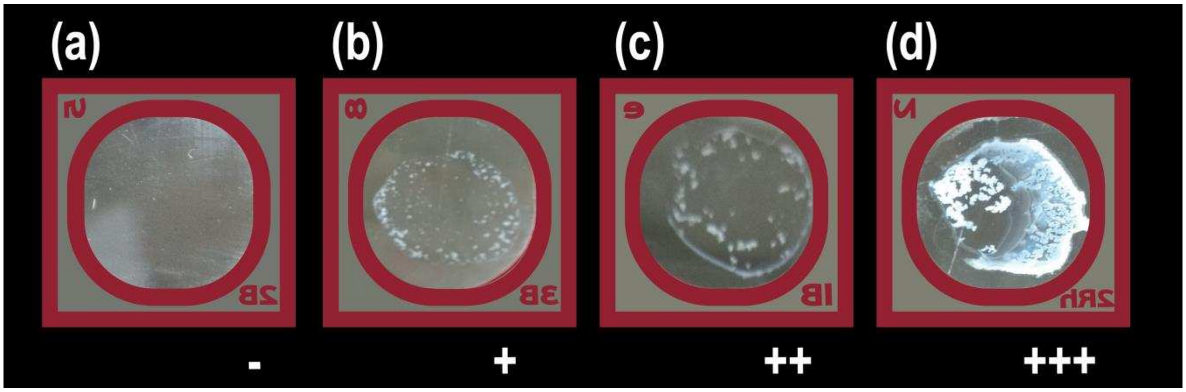

Figure 3. 3. Variations of the agglutination reactions from negative (-) to semi-quantitative (+ to +++). (a) Negative result; (b) Positive + for 10^5 CFU/mL; (c) Positive ++ for 10^6 CFU/mL; and (d) +++ for 10^7 CFU/mL.

SUPPLEMENTARY TABLE

Table S3. 1. Bacterial isolates used in this study.

N	Isolate	Species	Host	Collection site	Tissue
1	**LPF528**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
2	**LPF 529**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
3	**LPF530**	*Erwinia psidii*	*E. dunnii*	Rosário do Sul- RS	Branch
4	**LPF 531**	*Erwinia psidii*	*E. dunnii*	Guaíba- RS	Leaf
5	**LPF 533**	*Erwinia psidii*	*E. dunnii*	Guaíba- RS	Leaf
6	**LPF 534**	*Erwinia psidii* (Type strain)	*E. dunnii*	Guaíba- RS	Leaf
7	**LPF 545**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Leaf
8	**LPF 546**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
9	**LPF 547**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
10	**LPF 548**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
11	**LPF 549**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
12	**LPF 550**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
13	**LPF 552**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Stem
14	**LPF 553**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
15	**LPF 554**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
16	**LPF 556**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	Branch
17	**LPF 557**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas-MS	stem
18	**LPF 606**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	stem
19	**LPF 607**	*Erwinia psidii*	*E. urophylla*	Três Lagoas- MS	stem
20	**LPF 608**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Stem
21	**LPF 609**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Leaf
22	**LPF 610**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Três Lagoas- MS	Branch/Leaf
23	**LPF 611**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Andradina- SP	Branch/Leaf
24	**LPF 612**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
25	**LPF 613**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
26	**LPF 614**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
27	**LPF 615**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
28	**LPF 616**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
29	**LPF 617**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
30	**LPF 618**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
31	**LPF 619**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf

Table S3. 1. Bacterial isolates used in this study (cont).

N	Isolate	Species	Host	Collection site	Tissue
32	**LPF 620**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
33	**LPF 621**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
34	**LPF 622**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
35	**LPF 623**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Root
36	**LPF 624**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Root
37	**LPF 625**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
38	**LPF 626**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Stem
39	**LPF 627**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
40	**LPF 628**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
41	**LPF 629**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Branch/Leaf
42	**LPF 630**	*Erwinia psidii*	*Eucalyptus* sp.	Selviria- MS	Stem
43	**LPF 631**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
44	**LPF 632**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
45	**LPF 633**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Stem
46	**LPF 634**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Branch/Leaf
47	**LPF 635**	*Erwinia psidii*	*Eucalyptus* sp.	Três Lagoas- MS	Stem
48	**LPF 636**	*Erwinia psidii*	*Eucalyptus* sp.	Brasilândia- MS	Branch/Leaf
49	**LPF 637**	*Erwinia psidii*	*Eucalyptus* sp.	Brasilândia- MS	Stem
50	**LPF 638**	*Erwinia psidii*	*Eucalyptus* sp.	Itapetininga- SP	Branch/Leaf
51	**LPF 639**	*Erwinia psidii*	*Eucalyptus* sp.	Itapetininga- SP	Stem
52	**LPF 640**	*Erwinia psidii*	*Eucalyptus* sp.	Conceição- SP	Branch/Leaf
53	**LPF 641**	*Erwinia psidii*	*Eucalyptus* sp.	Conceição- SP	Stem
54	**LPF 642**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
55	**LPF 643**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
56	**LPF 644**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
57	**LPF 645**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
58	**LPF 646**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
58	**LPF 647**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
60	**LPF 648**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
61	**LPF 649**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem
62	**LPF 650**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
63	**LPF 651**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
64	**LPF 652**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem

Table S3. 1. Bacterial isolates used in this study (cont).

N	Strain	Species	Host	Collection site	Tissue
65	**LPF 653**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
66	**LPF 654**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
67	**LPF 655**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Stem
68	**LPF 656**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
69	**LPF 657**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Root
70	**LPF 658**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
71	**LPF 659**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
72	**LPF 660**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Guaíba- RS	Branch/Leaf
73	**LPF 661**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
74	**LPF 662**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
75	**LPF 663**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
76	**LPF 664**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Branch/Leaf
77	**LPF 665**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Stem
78	**LPF 666**	*Erwinia psidii*	*Eucalyptus*	Brasilândia- MS	Stem
79	**LPF 667**	*Erwinia psidii*	*E. saligna*	Guaíba- RS	Root
80	**LPF 668**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
81	**LPF 669**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
82	**LPF 670**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
83	**LPF 671**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
84	**LPF 672**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Stem
85	**LPF 673**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
86	**LPF 674**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
87	**LPF 675**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
88	**LPF 676**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
89	**LPF 677**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
90	**LPF 678**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Stem
91	**LPF 679**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Root
92	**LPF 680**	*Erwinia psidii*	*E. urophylla* × *E. grandis*	Andradina - SP	Stem
93	**LPF 681**	*Erwinia psidii*	*Psidium guajava*	Vista alegre do alto - SP	Stem
94	**LPF 682**	*Erwinia psidii*	*Psidium guajava*	Vista alegre do alto - SP	Stem
95	**LPF 683**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Stem
96	**LPF 684**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Stem
97	**LPF 685**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf

Table S3. 1. Bacterial isolates used in this study (cont).

N	Strain	Species	Host	Collection point	Tissue
98	**LPF 686**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
99	**LPF 687**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
10	**LPF 688**	*Erwinia psidii*	*E. grandis*	Campo-Santana-Itararé- SP	Branch/Leaf
101	**LPF 689**	*Erwinia psidii*	*E. saligna*	Campo-Limoeiro- SP	Branch/Leaf
102	**LPF690**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
103	**LPF691**	*Erwinia psidii*	*Eucalyptus* sp.	Guaíba- RS	Branch/Leaf
104	**IBSBF435**	*Erwinia psidii* (Type strain)	*Psidium guajava*	Valinhos-SP	-
105	**IBSBF424**	*Pectobacterium atrosepticum*	*Solanum tuberosum*	-	-
106	**IBSBF863**	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	-	-
107	**IBSBF231**	*Dickeya chrysanthemi* pv. *chrysanthemi*	*Chrysanthemum*	-	-
108	**UFV32**	*Ralstonia solanacearum*	*Eucalyptus* sp.	Bahia	Wilt
109	**LPF560**	*Xanthomonas axonopodis* pv. *eucalypti*	*E. grandis x E. urophylla*	Aracruz- Espírito Santo	Leaf spot
110	**LPF698**	*Pantoea dispersa*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
111	**LPF699**	*Enterobacter cloaceae*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
112	**LPF700**	*Novosphingobium panipatense*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
113	**LPF701**	*Paenibacillus cookii*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
114	**LPF702**	*Curtobacterium* spp.	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
115	**LPF703**	*Pantoea agglomerans*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic
116	**LPF704**	*Pantoea dispersa*	*E. urophylla* × *E. grandis*	Andradina- SP	endophytic