RODRIGO GALVÃO DE FREITAS

QUANTIFICATION, MOLECULAR CHARACTERIZATION AND AGGRESSIVENESS OF *Ralstonia solanacearum* SPECIES COMPLEX IN *Eucalyptus* SPP.

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*.

Orientador: Acelino Couto Alfenas

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Jorge Luis Badel Pacheco
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APROVADA: 28 de janeiro de 2021.

Assentimento:

Rodrigo Galvão de Freitas
Autor

Acelino Couto Alfenas
Orientador

To my parents, Rui and Maria Luisa,
to my sister Janaína and brother Ricardo,
to my niece Marcelinha and
to Diego, who always belivied in me,
I dedicate.

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RESUMO

FREITAS, Rodrigo Galvão de, D.Sc., Universidade Federal de Viçosa, janeiro de 2021. **Quantificação, caracterização molecular e agressividade do complexo de espécies *Ralstonia solanacearum* em *Eucalyptus* spp.** Orientador: Acelino Couto Alfenas. Coorientadores: Lúcio Mauro da Silva Guimarães, Jorge Luis Badel Pacheco e Poliane Alfenas-Zerbini.

A murcha bacteriana causada por *Ralstonia solanacearum* é uma importante doença do eucalipto em regiões quentes e úmidas em todo o mundo. A bactéria exibe considerável variação quanto à gama de hospedeiros, origem geográfica, patogenicidade e às propriedades fisiológicas. A dispersão da bactéria para o campo ou para outros viveiros de eucalipto ocorre principalmente por material vegetal infectado, porém assintomático. O uso de material de propagação livre do patógeno, bem como o plantio de genótipos resistentes são atualmente as únicas estratégias utilizadas para o controle da doença. Portanto, o conhecimento dos filotipos e da variabilidade genética em populações dessa bactéria é importante para a implementação de medidas de controle eficientes. Neste trabalho, desenvolveu-se um eficiente protocolo de PCR em tempo real baseado em corante intercalante para detectar a bactéria em plantas assintomáticas de eucalipto, bem como investigar sua movimentação nos tecidos de clones com diferentes níveis de resistência. Além disso, identificamos filotipos, sequevares e genótipos de 93 isolados de *Ralstonia,* obtidos de mudas e árvores de eucalipto cultivadas em diferentes regiões do Brasil. Posteriormente, foram investigados os padrões de distribuição de sequevar e sua relação com a agressividade da bactéria em clones de eucalipto. Através da técnica de PCR em tempo real, observou-se que a bactéria translocou acropetal e basipetalmente em plantas inoculadas e assintomáticas do clone resistente, assim como em plantas sintomáticas do clone suscetível. No entanto, uma menor concentração bacteriana foi detectada nos tecidos do clone resistente, onde, por meio de microscopia eletrônica de varredura, não se observou biofilme bacteriano obstruindo os vasos do xilema. Por meio de análises moleculares, constatou-se que, assim como *R. solanacearum* (filotipo II), *R. pseudosolanacearum* (filotipo I) também causa murcha em plantas de eucalipto. Entretanto, *R. pseudosolanacearum* é geneticamente uniforme, o que indica uma provável recente introdução desse filotipo no país. Diferentemente de *R. pseudosolanacearum*, *R. solanacearum* é filogeneticamente diversa, e não há correlação entre sequevar e origem geográfica. A agressividade dos isolados variou entre os clones de eucalipto testados, o

que reforça a importância da caracterização molecular e de estudos de agressividade da população do patógeno, a fim de embasar a seleção de material resistente. O método de qPCR desenvolvido neste estudo pode ser valioso para detecção do patógeno durante o diagnóstico da doença, assim como para sua quantificação nos tecidos da planta. O presente estudo expande o conhecimento da variabilidade genética do complexo de espécies de *Ralstonia solanacearum* em *Eucalyptus* spp.

Palavras-chave: Murcha bacteriana. PCR em Tempo Real. Eucalipto.

ABSTRACT

FREITAS, Rodrigo Galvão de, D.Sc., Universidade Federal de Viçosa, January, 2021. **Quantification, molecular characterization and aggressiveness of *Ralstonia solanacearum* species complex in *Eucalyptus* spp.** Advisor: Acelino Couto Alfenas. Co-advisors: Lúcio Mauro da Silva Guimarães, Jorge Luis Badel Pacheco and Poliane Alfenas-Zerbini.

Bacterial wilt caused by *Ralstonia solanacearum* is a serious disease of eucalypt in humid and high temperature areas worldwide. The bacterium exhibits considerable variation in the host range, geographic origin, pathogenicity and physiological properties. Spreading of the bacterium in the field or to other eucalypt nurseries occurs mainly by infected but asymptomatic plant material. The use of pathogen-free propagating material as well as planting of resistant genotypes are currently the only strategies used for the disease control. Therefore, knowledge of the phylotype composition and genetic variability in populations of this bacterium is useful for implementing effective control measures. In this work, we developed an efficient intercalating dye-based real-time PCR protocol to detect the bacterium in asymptomatic eucalypt plants as well as to investigate its movement in tissues of clones with different levels of resistance. In addition, we identified phylotypes, sequevars and genotypes of 93 *Ralstonia* isolates, obtained from cuttings and eucalypt trees grown in different regions of Brazil. Subsequently, sequevar distribution patterns and their relationship with the aggressiveness of the bacterium in eucalypt clones were investigated. Through the real-time PCR technique, we found that the bacterium translocates acropetally and basipetally in inoculated but asymptomatic plants of the resistant clone as in plants of the symptomatic susceptible one. Nevertheless, a lower bacterial concentration was detected in the tissues of the resistant clone, where, through the scanning electron microscope, no bacterial biofilm was observed obstructing the xylem vessels. With the molecular characterization, we found that, like *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotype I) also causes eucalypt wilt. However, *R. pseudosolanacearum* is genetically uniform, which indicates a probable recent introduction of this phylotype in the country. Unlike *R. pseudosolanacearum*, *R. solanacearum* is phylogenetically diverse, and there is no correlation between sequevar and geographic origin. Isolate aggressiveness varied between the eucalypt clones tested, reinforcing the importance of conducting molecular and aggressiveness characterizations of the pathogen population, in order to support the selection of resistant material. The

qPCR method developed in this study could be valuable for pathogen detection during disease diagnosis as well as pathogen quantification in plant tissue. The present study expands the knowledge of the variability of the *Ralstonia solanacearum* species complex in *Eucalyptus* spp.

Keywords: Bacterial wilt. Real time PCR. Eucalypt.

SUMMARY

GENERAL INTRODUCTION

In Brazil, the eucalypt crop is of great economic, environmental and social importance. It, currently occupies an area of approximately 6.97 million hectares and with an average annual volumetric increase of 35.3 m^3 ha^{-1} $year^{-1}$ (IBÁ, 2020). The increasing productivity of planted genetic materials, the clone adaptability in the new forest frontiers and the control of pests and diseases, are among the big challenges for maintaining and expanding the eucalypt crop in the country (Abad et al., 2013).

Ralstonia solanacearum (Smith) Yabuuchi et al. (1995) is a destructive bacterial plant pathogen that causes bacterial wilt in more than 450 species of plants from different botanical families (Wicker et al., 2007). It is a species complex that exhibits considerable variation in host range, geographic origin, pathogenicity and physiological properties (Denny, 2006). Due to its high variability, it is historically divided into five races (Buddenhagen et al., 1962), five biovars (Hayward, 1994), four phylotypes (Fegan and Prior, 2005), 60 sequevars (Lowe-Power et al., 2020), clades or ecotypes (Wicker et al. 2012). More recently, Safni et al. (2014), supported by Prior et al. (2016), proposed splitting the *R. solanacearum* complex into three species. Based on phylotyping, genomics, and proteomics analyses, they named phylotypes I and III as *R. pseudosolanacearum* sp. nov.; phylotype II remained as *R. solanacearum;* and phylotype IV was classified as *R. syzygii*, including the three subspecies, *Ralstonia syzygii* subsp. *syzygii*, *Ralstonia syzygii* subsp. *celebesensis* and *Ralstonia syzygii* subsp. *indonesiensis*.

The first report of this disease in eucalypt was in Brazil in the 1980s, in the municipality of Prata, Minas Gerais, in plantations of *Eucalyptus grandis* (Sudo et al., 1983). In 2005, the bacterial wilt resulted in high losses in eucalypt nurseries in the states of Bahia, Espírito Santo, Maranhão, Minas Gerais, and Pará, totaling an estimated loss of US$ 27 million (Alfenas et al., 2009), without considering losses resulting from management changes, use of less adapted genotypes and delays in planting schedules. Brazil is a putative center of origin of *Ralstonia solanacearum* (phylotype II) (Wicker et al., 2012). However, *R. pseudosolanacearum* (phylotype I) has also been found associated with solanaceous crops, such as tomato, aubergine, long pepper and bell pepper (Santiago et al., 2017). Up to now, only phylotype II has been reported causing bacterial wilt in eucalypt in Brazil, but phylotype I was also reported causing wilt in this crop in Africa and Asia (Carstensen et al., 2016). Whether the pathogenicity of these two bacterial

species towards *Eucalyptus* is similar is unknown and requires further investigation (Carstensen et al., 2016).

Species of *Ralstonia* are soilborne and infection generally occurs through the roots (Hayward, 1991). After entry into the host tissue they rapidly multiply, invade the root cortex and vascular parenchyma cells intercellularly, before spreading to the xylem vessels (Vasse et al., 1995). In these vessels, they degrade the xylem wall components and spread to the aerial parts of the host eventually blocking the water transport system (Agrios, 2005; Carstensen et al., 2016). In mini-clonal stumps of eucalypt in clonal hedges, external symptoms of bacterial wilt include leaf necrosis, darkening at the stem base, discoloration of internal tissue, wilting and root death. However, cuttings may have a latent infection, in which the xylem has low concentrations of bacterial cells and visualization of wilt symptoms is not possible. When latently infected, asymptomatic cuttings transplanted to the field usually begin to wilt at three to four months after planting (Alfenas et al., 2006).

Thus, in order to certify that rooted cuttings for planting are pathogen-free, it is necessary to use a method capable of detecting the bacterium, even when present in low concentrations. Traditional methods, such as bacterial isolation and morphological characterization, are time consuming and can result in an underestimation of the actual population of the bacterium (Elphinstone et al., 1996; Yun et al., 2010). In this this context, a sensitive and specific molecular method such as real-time PCR could improve the reliability in detection of *R. solanacearum.* However*,* to our knowledge, there is no real-time PCR assay for detection and quantification this bacterium directly in samples of infected eucalypt tissues.

In addition of planting pathogen-free rooted cuttings in inoculum-free areas, the disease can also be controlled by the use of resistant clones. This represents one of the best management strategies applied in large areas and has no environmental impact (Fonseca et al., 2010). Variations in resistance to bacterial wilt was observed for different eucalypt species (Dianese and Dristig, 1993). Nevertheless, for effective and durable resistance in the field, it is necessary to know the genetic and physiological variability of the pathogen. Characterization of *R. solanacearum* isolates through molecular techniques in phylotypes, sequevars and clones, is well accepted in the scientific community (Fegan & Prior, 2005), allows a better understanding of the variability of the pathogen and its consequences for the management of bacterial wilt. *R. solanacearum* from eucalypt in

Brazil has a high genetic diversity, which is a challenge for breeding aiming at resistance to the disease (Fonseca et al. 2014)

The present Thesis consists of three chapters. In the first chapter, I developped a real-time PCR protocol for detection of *R. solanacearum* in *Eucalyptus* sp. and investigated the bacterium movement in tissues of two eucalypt clones differing in resistance. The second chapter aimed to report and characterize isolates of *R. pseudosolanacearum* causing bacterial wilt in *Eucalyptus* in Brazil for the first time. Finally, the third chapter aimed to molecularly characterize a large collection of *Ralstonia* spp. isolates obtained from *Eucalyptus,* to determine their phylotypes, sequevars their geographic distribution as well as to assess the aggressiveness of a select group of isolates representative of the pathogen population.

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Article 1: Validation and use of a qPCR procedure to quantify the spread of *Ralstonia solanacearum* in susceptible and resistant eucalypt plants

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is a serious disease of eucalypt in humid and high temperature areas worldwide. Spreading of the bacterium in the field or to other nurseries occurs mainly by infected but asymptomatic plant material. The use of pathogen-free propagating material as well as planting of resistant genotypes are currently the only strategies used for disease control. Therefore, a reliable and sensitive method for detection of low titers of *R. solanacearum* in infected plant tissue is essential for the success of management programs. In this work, we adapted an efficient intercalating dye-based real-time PCR protocol to detect the bacterium in asymptomatic eucalypt plants as well as to investigate its movement in eucalypt clones CLR172 and CLR371, which exhibit resistance and susceptible phenotypes, respectively. We found that the bacterium translocates acropetally and basipetally in inoculated but asymptomatic cuttings of the resistant clone as in cuttings of the symptomatic susceptible one. Nevertheless, a smaller concentration of bacterial DNA was detected in tissues of the resistant clone. Mature biofilms occluding the xylem vessels were present in the susceptible clone whereas only single cells or small aggregates were observed in the resistant clone. This work contributes to improve our knowledge of the colonization process of *R. solanacearum* in eucalypt clones with different levels of susceptibility and to understand how the defense mechanisms against bacterial wilt

in *Eucalyptus* work. Our findings could aid in the selection of the best eucalypt clones to be used in wilt disease management programs.

Keywords: Bacterial wilt, biofilm, colonization, eucalypt

INTRODUCTION

Ralstonia solanacearum (Smith) Yabuuchi et al. (1995), causal agent of bacterial wilt in more than 200 plant species within 50 different botanical families, may cause substantial losses of economically important agricultural crops in tropical and subtropical regions worldwide (Hayward 1994). In eucalypt, the first report of wilt disease was in Brazil in the 1980s, in the municipality of Prata, Minas Gerais state (Sudo et al. 1983). Subsequently, bacterial wilt has been recorded in most eucalypt-producing regions worldwide (Ferreira et al., 2018), becoming one of the main diseases of this crop.

Leaf necrosis, darkening and bark cracking at the stem base, xylem discoloration, wilt and root death are the main disease symptoms of trees in the field. Asymptomatic shoots collected from infected mother trees in the field and used for making propagating cuttings are the main source of primary inoculum of *R. solanacearum* in nurseries (Alfenas et al., 2006). Rooted cuttings may have latent infections in which the xylem has low titers of bacterial cells, which when transplanted in the field usually begin to show wilt symptoms three to four months after planting (Alfenas et al., 2006).

Considering the possibility that rooted cuttings with latent infections can be source of inoculum during the implantation of mini-clonal hedges and to certify that the cuttings for planting are pathogen-free, it is necessary to have reliable and sensitive methods for *R. solanacearum* detection. Traditional methods, such as bacterial isolation on culture media and morphological characterization, are time consuming and can result in an underestimation of the actual population of the bacterium (Elphinstone et al., 1996; Chen et al., 2010). Furthermore, *R. solanacearum* is relatively slow growing, has an undistinctive colony morphology on non-selective medium, is significantly out-numbered by saprophytic microbes present in field samples (Poussier et al. 2002), and can become viable but not culturable (van Elsas et al., 2005), hindering its isolation. Rapid methods, such as the test of bacterial exudation from stem tissue and the commercially available immunoassay strips for *R. solanacearum* detection are very practical, but require a

relatively high population of bacterial cells in plant tissue to provide positive results (Denny, 2006).

To deal with these limitations, sensitive and specific molecular methods such as conventional polymerase chain reaction (cPCR), quantitative real-time PCR (qPCR) and loop-mediated isothermal amplification (LAMP) have been developed to improve reliability in the detection of *R. solanacearum* (Tran et al. 2016a; Cellier et al. 2017). Although LAMP is sensitive, rapid and inexpensive (Okiro et al., 2019), real-time PCR has the advantages of enabling automation, being suitable for large-scale sample processing and being highly accurate for quantification of the target DNA (Weller et al., 2000). However, to our knowledge, there was no real-time PCR assay available for *R. solanacearum* detection and quantification directly in samples of infected eucalypt tissue, which may have been caused by the inhibition of the DNA polymerase by the high content of polyphenolics and polysaccharides present in the plant tissue (Suzuki et al., 2003).

Besides planting of pathogen-free rooted cuttings, the use of resistant clones also is an important measure for bacterial wilt control in eucalypt (Alfenas et al., 2009). The bacterial colonization may vary depending on the clone, which may be related mainly to the level of resistance and the time of colonization (Mafia et al., 2012). Assessments of resistance levels of eucalypt clones have only been carried out in a qualitative manner (Mafia et al., 2012). In this regard, qPCR may be an important alternative not only for quantitative resistance assessment but also for understanding relevant aspects of the plant-pathogen interaction, such as the mechanisms underlying the resistance response as well as pathogen concentration maintained during latent infections, which could contribute to the selection of clones with less resistance instability (Lebeau et al., 2011).

R. solanacearum belongs to a species complex along with two other species*: R. pseudosolanacearum* and *R. syzygii* (Safni et al., 2014). Both *R. solanacearum* and *R. pseudosolanacearum* have already been reported causing bacterial wilt in *Eucalyptus* (Carstensen et al., 2016; Freitas et al., 2020). In this study, we adapted a real-time PCR protocol for detection of bacteria of the *R. solanacearum* species complex affecting eucalypt and investigated the movement of a *R. solanacearum* strain in host tissue of two clones with different levels of resistance. Subsequently, scanning electron microscopy analysis were performed to determine whether the difference in bacterial concentration between the two clones was related to biofilm formation in host tissues.

MATERIALS AND METHODS

Bacterial strains

Three strains of *R. solanacearum* sensu stricto and nine strains of other bacterial species, isolated from eucalypt, were retrieved from the Culture Collection of the Laboratory of Forest Pathology/Bioagro of the Universidade Federal de Viçosa and used to determine the primers specificity (Table S1). Bacterial DNA was extracted from cultures grown on 523 medium (Kado and Heskett, 1970) for 24 h using the Wizard Genomic DNA Purification Kit (Promega, Madison, U.S.A.) as recommended by the manufacturer.

Primers selection and PCR amplification

The specificity of five primer pairs developed by Opina et al. (1997) and Chen et al. (2010) to detect strains of the *R. solanacearum* species complex were tested in conventional PCR (cPCR) (Table 1) with DNA from ten bacterial species (Table S1). To eliminate the risk of false positives, purified DNA from eucalypt tissue was tested.

Conventional PCR was performed in a final volume of 15 µL containing 2.0 µL of DNA (15 ng μL^{-1}), 1.0 µL of each primer (10 µM), 7.5 µl of GoTaq® Colorless Master Mix (Promega) and ultrapure distilled water. For primers 759/760, the amplification consisted of an initial denaturation at 94 °C for 3 min, annealing at 53 °C for 1 min and extension at 72 °C for 90 s, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. For primers developed by Chen et al. (2010), the program consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95°C for 20s, 61°C for 25 s, 72 °C for 35 s, and final extension at 72 °C for 5 min. The amplification was performed using a Veriti 96 thermocycler (Applied Biosystems) and the PCR products were analyzed on a 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide (1.0 µg mL^{-1}) and visualized in an L-PIX gel documentation system (Loccus Biotechnology).

After confirmation of the primers specificity in the cPCR, they were tested in real-time PCR (qPCR) and the best primer pair chosen based on the following criteria: reaction efficiency closest to 100%, high sensitivity, absence of primer dimer formation and absence non-specific amplification products. Efficiency and sensitivity were evaluated

by generating a standard curve using genomic DNA from the strain LPF710 (phylotype II, sequevar 41), which belongs to the species *R. solanacearum* sensu stricto. For that, the mass (ng) corresponding to one genomic unit of DNA was estimated considering a *R. solanacearum* genome size of 5.8 Mb (Hayes et al., 2017) and a 1.09 x 10^{-12} ng mean mass of each nucleotide, resulting in 6.3 x 10^{-6} ng per genomic unit. Then, a DNA solution was prepared to contain the mass equivalent to 10^7 genomic units μL^{-1} with the help of a NanoDrop 2000c spectrophotometer (Thermo Scientific) and used to make serial 1:10 dilutions ranging from 10^7 to 10^0 genomic units μL^{-1}. The qPCR assays were carried out in a CFX–96 BioRad thermocycler using a 12 µL reaction volume containing 2 µL of DNA solution, 6 µL of GoTaq qPCR Master Mix (Promega) and 0.3 µL (10 µM) of each primer. The amplification conditions included an initial denaturation step at 95 °C for 2 min, followed by 40 cycles, each one consisting of 15 s at 95°C and 60 s at 60 °C. The assays were repeated twice with three replicates for each dilution. To verify the absence of primer dimers or nonspecific amplification products, a melting curve was generated by heating from 60 °C to 95 °C with 1 °C increment, 30 s dwell time and a plate reading at each temperature.

Detection of bacteria of the *R. solanacearum* species complex in asymptomatic eucalypt plants

After selecting the best primer pair for qPCR, an assay was performed to detect bacteria of the *R. solanacearum* species complex directly from tissue of asymptomatic eucalypt plants. For this, 150 rooted cuttings of three clones (50 each) collected in a nursery in São Paulo state were processed in bulks containing ten plants each, totaling five bulks of each clone. For each bulk, five tissue fragments along the stem of each rooted cutting were removed and mixed in a 2.0 mL microcentrifuge tube. These fragments were frozen in liquid nitrogen, macerated in TissueLyser II (Qiagen®) at 25Hz for 2 min and a total of 10 mg used for total DNA extraction.

Total DNA was extracted by the CTAB method (Doyle and Doyle, 1990) with some modifications (CTAB extraction buffer: 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, with 2% polyvinyl pyrrolidone and 2% β-mercaptoethanol added just before use). A volume of 700 µL of the CTAB buffer was added into the tube, containing 10 mg of the macerated fragments and incubated at 65ºC for 30 min, gently mixing by inversion every 10 min. A total of 500 µL of chloroform:isoamylalcohol (24:1)

were added to the tubes and gently mixed. Samples were centrifuged for 10 min at 13,200 rpm and then 450 µL of the supernatant were transferred to a new tube containing 450 µL of cold isopropanol (-20ºC). After 2 h, samples were gently mixed by inversion and centrifuged at 13,200 rpm for 10 min. The pellet was washed with 70% ethanol, air-dried at room temperature and resuspended in ultrapure distilled water. The DNA was quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific) and the concentration was adjusted to 2.5 ng μL^{-1} to conduct the qPCR and cPCR, as described above.

For qPCR, the equation generated by the standard curve was used to quantify the bacterium in plant tissue. Samples were considered positive when the Ct values obtained were within the limits of the standard curve. DNA amplification from each sample (bulk) was performed in triplicate. Since *Ralstonia* spp. are difficult to isolate from infected asymptomatic samples (Poussier et al. 2002,van Elsas et al., 2005), to confirm the results obtained in PCR, the samples were also tested by using immunoassay strips (Agdia, Elkhart, IN). As a positive control, a sample of a eucalypt plant artificially inoculated with *R. solanacearum* showing symptoms of wilt was used. As a negative control, a sample of a eucalypt plant not inoculated with the pathogen was employed.

Translocation and quantification of *R. solanacearum* in eucalypt tissue

To evaluate the movement and concentration of *R. solanacearum* in plant tissue, the strain LPF710 was inoculated into two *Eucalyptus* clones, CLR371 (*E. urophylla*) and CLR172 (*E. urophylla x E. grandis*), previously classified as susceptible and resistant to bacterial wilt, respectively (Lúcio M. S. Guimarães, personal communication).

For inoculum preparation, the strain was grown on triphenyl-tetrazolium-chloride (TTC) medium (Kelman, 1954) at 28 °C. After 48 h, smooth, fluid colonies with white edges and red centers were transferred onto casamino acid-peptone-glucose medium (CPG) and grown at 28 °C for 48 h. Then, 0.85% (w/v) sodium chloride solution was added to the plate and the bacterial cells scraped from the medium surface with a Drigalski spatula. Finally, the absorbance at 540 nm of the bacterial suspension was adjusted to 1.0, which corresponds approximately to 1 x 10^9 CFU/mL (Fonseca et al., 2016).

In a first experiment, thirty-days-old rooted cuttings of the two clones were inoculated by injection of the bacterial cell suspension at mid-height of the stem (Fonseca et al., 2016) and the bacterial population (as the number of genomic units) in the host

tissue was estimated at 0, 2, 4, 6, 8, 10, and 12 days after inoculation (dai). At each evaluation time, stem fragments were collected at successive 2-cm distance increments from above and below the inoculation point, starting at 0 cm at day 0 and ending at 12 cm at 12 dai. Three plants from each clone were inoculated with the bacterial cell suspension for each evaluation time and one plant was treated with 0.85% (w/v) sodium chloride solution to serve as control. All plants were maintained in a growth chamber at 28 ± 2 °C with a 12-h photoperiod and light intensity of 80 μmoL s^{-1} m^{-2} in a completely randomized design.

Ten milligrams of tissue fragments macerated in TissueLyser II (Qiagen) were used to extract total DNA using the CTAB method as aforementioned. The DNA concentration was adjusted to 2.5 ng μL^{-1} and used in qPCR under the same conditions previously described. Amplification from each sample was conducted in triplicates. The standard curve generated for selection of the best primer pair (described above) was also used to quantify the bacterial population in the samples collected.

In a second experiment, plants of the two tested eucalypt clones were inoculated and stem fragments collected at the same incubation periods as described for the first experiment. However, in this experiment the samples were collected only at 6 and 12 cm above and below the inoculation point at all evaluation times. In this case, it was possible to estimate the bacterial concentration in the resistant clone at 24 dai, but it was not possible for the susceptible clone since the plants usually exhibited complete wilt by 12 dai. Total DNA from the samples was extracted and bacterial DNA concentrations in plant tissue quantified by qPCR as in the first experiment.

For both experiments, comparisons of bacterial concentrations in each collection point between the two clones were performed using the Kruskal–Wallis test with post hoc t-test for non-normal distributions at $P \leq 0.05$ with the package Easyanova (Arnhold, 2013) for R v. 3.5.3 (R Core Team Development, 2019).

Biofilm formation in *Eucalyptus* tissue

To determine whether the bacterium forms biofilm in the xylem vessels of CLR172 and CLR371 eucalypt clones, fragments of the main stem (approx. 0.5 cm length) were collected from 6 cm below and above the inoculation point at 12 dai in three plants of each clone. Fragments at the same distances were also collected from uninoculated plants of the two clones to serve as controls. The plant tissue fragments were

transferred to 2.0 mL microcentrifuge tubes containing modified Karnovsky fixative (2.5% vv^{-1} glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) and kept in a refrigerator until use.

Before microscopy observation, the tissue fragments were dehydrated in an alcohol series (30%, 50%, 70%, 90% and 100%; the last wash was repeated three times) and subjected to critical point drying in CO_2 using a Critical Point Dryer device (model CPD030). Then, fragments were sectioned with a sterile stainless-steel razor blade to obtain 2 mm-thick sections that were mounted on aluminum stubs using double-sided carbon tape and covered with gold plating using a sputter coater device (model Q150R S, Quorum). Subsequently, the samples were analyzed using a Scanning Electron Microscope (LEO 1430VP model) to obtain the electron micrographs.

RESULTS

Primer selection and sensitivity

Among the primers tested, the pair Rs199F/Rs199R exhibited specificity for *R. solanacearum* DNA amplification in cPCR as well as best reaction efficiency (101.6%) (Table 1), high sensitivity, absence of primer dimer formation as well as absence of non-specific amplification products in qPCR**.** The detection limit of this primer pair was at a total of 1 x 10^1 genomic units μL^{-1} (Figure S1A). A melting curve showed a single peak at a T_m of 82 °C, indicating lack of nonspecific amplification (Figure S1B). A standard curve was generated covering serial Ct values that spanned from 10^7 to 10^1 genomic units μL^{-1} (Figure S1C). While the detection limit of *R. solanacearum* DNA in qPCR was at 10^1 genomic units μL^{-1}, in the cPCR it was 10^3 genomic units μL^{-1} (Figure 1). Therefore, qPCR was 100 times more sensitive than cPCR in specifically detecting *R. solanacearum* DNA. The equation generated by the standard curve was thereafter used (Figure S1C) to quantify the bacterial DNA in eucalypt tissue. Bacterial concentrations were estimated in genomic units of *R. solanacearum* per nanograms of total DNA (genomic units/ng total DNA).

Detection of bacteria of the *R. solanacearum* species complex in asymptomatic eucalypt plants

Among 15 sample bulks of eucalypt tested, seven were positive for the *R. solanacearum* species complex by using qPCR, a number close to that obtained with detection by immunoassay strips, which yielded six positive sample bulks (Table 2, Figure S2). In contrast, none of the sample bulks tested positive by cPCR (Table 2, Figure S3). The bacterial concentrations as estimated by qPCR in samples from asymptomatic plants ranged from 0.84 x 10^1 to 1.22 x 10^3 genomic units/ng total DNA, while for the samples from plants showing wilt symptoms, which served as a positive control, the bacterial concentration was 2.80 x 10^5 genomic units/ng total DNA (Table 2). Negative results using all three detection methods were obtained for the other eight sample bulks, indicating that bacteria of the *R. solanacearum* species complex were either not present or were present at concentrations below the limit of detection under the conditions used here. Only for bulk 2 of clone 3, the results of real-time PCR did not match with the immunoassay strip test; whereas qPCR gave a positive result the immunoassay strips gave negative (Table 2 and Figure S2).

Translocation and quantification of *R. solanacearum* in eucalypt tissue

Unlike the resistant clone (CLR172), plants of the susceptible clone (CLR371) showed initial wilting symptoms at 8 dai and completely wilted and died at 12 dai (Figure 2). *R. solanacearum* was detected in plant tissue fragments of the two clones at all distances from the inoculation point throughout the evaluation period, in both upward and downward directions (Figure 3). However, in the resistant clone, the bacterial concentration was always smaller than in the susceptible one. The largest bacterial concentration detected in the resistant clone averaged 1.95 x 10^4 genomic units/ng total DNA at 4 dai and 4 cm below the inoculation point, while the largest concentration in the susceptible clone had an average of 2.12 x 10^5 genomic units/ng total DNA at 6 dai and 6 cm above the inoculation point (Table 3).

As the bacterium was detected in both resistant and susceptible clones at all distances from the inoculation point tested, we aimed to determine whether the bacterial cells would take the same time to reach plant tissue at 6 and 12 cm away from the inoculation point in both clones. As in the first experiment, the susceptible clone

completely wilted at 12 dai whereas the resistant one was apparently healthy, although the bacterium was detected in both clones at 6 cm and 12 cm from the inoculating point, respectively at 2 and 4 dai (Figure 4). Nevertheless, the bacterial concentration in the resistant clone was smaller than in the susceptible one. The average of the maximum bacterial concentration detected in the resistant clone was 3.18 x 10^4 genomic units/ng total DNA at 10 dai and 6 cm above the inoculation point, while in the susceptible clone the average of the maximum concentration was 3.29 x 10^5 genomic units/ng total DNA at 4 dai and 6 cm above the inoculation point (Table 4). Since the resistant clone remained symptomless, tissue fragments taken at 6 and 12 cm from the inoculation point were also analyzed at 24 dai. The results showed that the bacterial concentration was practically the same as that at 12 dai (Figure 4).

Biofilm formation in *Eucalyptus* tissue

At 6 cm above and below the inoculation point, mature bacterial biofilm causing total obstruction was observed in the xylem vessels of the susceptible clone CLR371 (Figure 5A-B). In contrast, only small aggregates of bacterial cells were observed around the vessel of the resistant clone CLR172 (Figure 5C-D). In longitudinal sections, it was possible to observe bacterial communities immersed in an exopolysaccharide (EPS) matrix which formed the biofilms in the xylem vessels of the susceptible clone (Figure 6A-B), while in the resistant clone only single and dispersed bacterial cells were observed (Figure 6C). In addition, tyloses were observed in response to bacterial infection only in some xylem vessels of the resistant clone (Figure 6D).

DISCUSSION

In this study, we adapted a specific and sensitive real-time PCR method using an intercalating dye to detect bacteria of the *R. solanacearum* species complex in eucalypt tissue. The primer pair Rs199F/Rs199R (Chen et al. 2010) was chosen for exhibiting better reaction efficiency, absence of primer dimer formation, specificity, and greater sensitivity. This method was 100 times more sensitive than conventional PCR in detecting *R. solanacearum* DNA. Unlike our work, Chen et al. (2010) chose the primer pair RSF/RSR to detect *R. solanacearum* in infected tomato plants and quantified the bacterium in tomato, tobacco, mulberry, and ginger tissues. It is likely that the

performance of the primers in the PCR could have been influenced by the amplification conditions in different laboratories, such as the intercalating dye, the buffer and the thermocycler used.

The high sensitivity of the qPCR method described here was confirmed by detecting bacteria of the *R. solanacearum* species complex in asymptomatic eucalypt rooted cuttings. In a total of 15 sample bulks, seven were qPCR positive. Although the serological method used for comparison was also able to detect bacteria of the complex, in four of the six qPCR positive samples the bands were very faint (Figure S2), which could go unnoticed or generate doubts about them being false positives. Furthermore, for one sample qPCR provided a positive result whereas bacteria of the *R. solanacearum* species complex were not detected by the immunostrip assay. Thus, in the case of asymptomatic plants carrying very small bacterial concentrations, qPCR can be more reliable than the other methods tested here. In addition, qPCR enables quantification of bacterial populations in the host tissue.

The use of TaqMan probes to detect *R. solanacearum* in soil and other plant hosts (Weller et al., 2000; Thammakijjawat et al., 2006; Huang et al., 2009; Stulberg et al., 2016) has been shown highly specific and sensitive and has the potential for multiplexing, which is not possible when using unspecific DNA intercalating dyes in qPCR assays (Cao and Shockey, 2012). However, in our work we used a feasible, simpler and less costly method, which is still more efficient than the most commonly used methods, such as bacterial isolation on semi-selective medium, conventional PCR and serological tests. With respect to the latter, although immunoassay strip assays are faster and easier to execute, sometimes give false positive results, since anti-sera are not totally specific (Poussier et al., 2002), often causing doubts in the interpretation of the results when weak bands appear.

As it was demonstrated histologically in a previous work for *Erwinia psidii* in eucalypt (Caires et al., 2020), our work showed that *R. solanacearum* translocates acropetally and basipetally in both susceptible and resistant eucalypt clones. We detected the bacterium in host tissue at all tested distances from the inoculation point and found that the tissue colonization may occur at a similar speed in both resistant and susceptible clones. On the other hand, the bacterial population was larger in the susceptible clone, although the bacterium reached a relatively large concentration (10^4 genomic units/ng total DNA) at various distances from the inoculation point in the resistant clone without causing disease symptoms. It is likely that the pathogen did not reach a population size

large enough to overcome the immunity barriers of the resistant clone. In addition, it was also possible that the bacterium was not able to form a structured biofilm inside tissue of the resistant host. Consistent with this interpretation, in a scanning electron microscopy analysis of plant tissue at 12 dai, we did not observe mature biofilms in the xylem vessels of the resistant clone, only single bacterial cells or small aggregates. Quorum sensing and biofilm formation are often tightly linked, that is, biofilm formation is dependent on bacterial population density, and their interaction is very often central to the plant pathogenicity of many bacterial species (Nadell et al., 2008).

The growth of *R. solanacearum* inside plant tissues, particularly during disease development, leads to the formation of colonies that develop into mature biofilms (Morris and Monier, 2003). Tran et al. (2016b) found that *R. solanacearum* formed dense biofilm structures inside tomato xylem vessels. Biofilm development contributes to the virulence of phytopathogenic bacteria through various mechanisms and a high level of multiplication leads to wilting symptoms as a result of reduced sap flow caused by the presence of a large number of bacterial cells and exopolysaccharide (EPS) slime in some xylem vessels (Genin and Denny, 2012). The reason for the lack of symptoms in the resistant clone CLR172 despite carrying some bacterial population loads is still unknown. However, given the results obtained in this study, it is tempting to speculate that they might be related to an impairment of biofilm formation due to less bacterial multiplication. In addition, our results indicate that one of the resistance mechanisms of the CLR172 clone was the formation of tyloses, which are formed in response to infection by vascular pathogens and result in the invagination of the protoplasm of parenchyma cells adjacent to the xylem vessels to contain the pathogen advance.

It has already been shown that *R. solanacearum* can live for extended periods in xylem vessels of tolerant plant cultivars at moderately high cell densities without triggering symptoms (Lowe-Power et al., 2018). How plants with these latent infections are able to remain symptomless is still not fully understood. Lebeau et al. (2011), assessing tomato, pepper and eggplant phenotypes on the basis of percentage of wilted plants and colonization index, distinguished two mechanisms of plant defense against bacterial wilt: plant resistance based on (1) limitation of bacterial colonization and (2) capacity to survive despite the presence of bacteria in the vessels (latent infection). Transmission electron microscopy showed that resistant tomato LS-89 developed electron-dense materials in stem xylem vessels and apposition layers in xylem parenchyma cells that restricted stem colonization by *R. solanacearum* (Nakaho and

Allen, 2009). In contrast, in tomato cultivar CRA66, whose resistance derived from a different wild source (Lebeau et al., 2011), the bacterium was found colonizing large meta-xylem vessels and the plant was unable to restrict bacterial spread within the vasculature (Caldwell et al., 2017). From the results found in our study, the resistance of the CLR172 clone does not appear to be based on restriction of tissue colonization by *R. solanacearum*; the plant is able to remain symptomless even with the bacteria in the vessels. However, when compared with equivalent tissue of the susceptible clone, there was a reduction in bacterial concentration, which could have a negative effect on biofilm formation.

This is the first work to quantify and prove the translocation of *R. solanacearum* in tissue of susceptible and resistant eucalypt clones. The results presented here, show differential pathogen populations dynamics in the resistant and susceptible eucalypt clones, as indicated by a 10- to 100-fold lower population size in the resistant clone at the evaluated distances for the inoculation point. Evaluation of eucalypt resistance to *R. solanacearum* in breeding programs would benefit from an examination of not only wilt symptoms but also bacterial colonization in asymptomatic plants, since the resistance can be manifested as either an ability to adapt to a bacterial colonization of the xylem vessels or, conversely, an ability to contain the bacterium in the lower parts of the plant (Grimault et al., 1994, Lebeau et al., 2011). Whether these mechanisms of resistance are related to loss of productivity (tolerance) remains undetermined. Given the importance of the xylem tissue for resistance (Caldwell et al., 2017), deciphering the complex transcriptional events that occur within this tissue is an important next step in our understanding of the defense mechanisms of *Eucalyptus* spp. to *R. solanacearum.*

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Table 1. Primers tested in this study

Primer	Sequence (5'-3')	Amplicon size	Target gene	Efficiency (%)	Reference
Rs185F	CCGAAAGCAGACTACAACC	185 bp	*egl*	78.4	Chen et al. (2010)
Rs185R	ACCTTCCCCTGGACAAAAT				
Rs199F	AGTAACTCGGCTGTTCTTT	199 bp	ITS	101.6	Chen et al. (2010)
Rs199R	TATTCGCTTGACCCTATAA				
Rs224F	TAGTTGTTGGGGATTCATTT	224 bp	16S rRNA	93.6	Chen et al. (2010)
Rs224R	CCTGTGTCCACTTTCTCTTT				
RSF	GTGCCTGCCTCCAAAACGACT	159 bp	Deacetylase	88.0	Chen et al. (2010)
RSR	GACGCCACCCGCATCCCTC				
759	GTCGCCGTCAACTCACTTTCC	283 bp	Deacetylase	77.3	Opina et al. (1987)
760	GTCGCCGTCAGCAATGCGGAATCG				

Table 2. Results of qPCR, cPCR and immunoassay strip (Agdia) detection of *R. solanacearum* **i**n samples of asymptomatic eucalypt rooted cuttings collected in São Paulo state, Brazil

Clone	Sample bulk	Concentration (genomic units / ng DNA total)	qPCR	Immunoassay strip	cPCR
1	1	-[a]	Negative	Negative	Negative
	2	-	Negative	Negative	Negative
	3	-	Negative	Negative	Negative
	4	0.84×10^1	**Positive**	**Positive**	Negative
	5	5.08×10^1	**Positive**	**Positive**	Negative
2	1	3.50×10^1	**Positive**	**Positive**	Negative
	2	-	Negative	Negative	Negative
	3	-	Negative	Negative	Negative
	4	8.20×10^1	**Positive**	**Positive**	Negative
	5	-	Negative	Negative	Negative
3	1	1.22×10^3	**Positive**	**Positive**	Negative
	2	2.84×10^1	**Positive**	Negative	Negative
	3	6.12×10^2	**Positive**	**Positive**	Negative
	4	-	Negative	Negative	Negative
	5	-	Negative	Negative	Negative
Positive control		2.80×10^5	**Positive**	**Positive**	**Positive**
Negative control		-	Negative	Negative	Negative

[a] qPCR signal was negative or the *R. solanacearum* concentration was smaller than the detection limit under the tested conditions.

Table 3. Concentration of *Ralstonia solanacearum* strain LPF710 in tissues of resistant (CLR172) and susceptible (CLR371) eucalypt clones determined by qPCR

Days after inoculation	Distance (cm)[a]	Concentration (clone CLR172)[b]	Concentration (clone CLR371)[b]
12	12	4.21 (± 3.02) x 10^2 b	2.54 (± 1.54) x 10^4 a
10	10	7.07 (± 1.68) x 10^2 b	5.82 (± 2.44) x 10^4 a
8	8	3.41 (± 3.69) x 10^2 b	1.13 (± 0.42) x 10^5 a
6	6	2.03 (± 0.34) x 10^3 b	2.11 (± 0.26) x 10^5 a
4	4	1.29 (± 0.29) x 10^4 b	9.78 (± 5.29) x 10^4 a
2	2	2.80 (± 2.38) x 10^3 b	2.32 (± 1.24) x 10^4 a
0	0	3.76 (± 2.79) x 10^2 a	3.80 (± 2.15) x 10^2 a
2	-2	7.01 (± 2.46) x 10^3 b	2.15 (± 0.35) x 10^4 a
4	-4	1.95 (± 0.24) x 10^4 b	1.05 (± 0.38) x 10^5 a
6	-6	4.90 (± 2.20) x 10^3 b	5.57 (± 1.58) x 10^4 a
8	-8	1.34 (± 0.33) x 10^3 b	5.84 (± 4.50) x 10^4 a
10	-10	1.69 (± 0.21) x 10^3 b	2.08 (± 0.23) x 10^4 a
12	-12	2.20 (± 1.31) x 10^2 b	4.06 (± 0.16) x 10^4 a

[a] Centimeters above (positive values) and below (negative values) the inoculation point.
[b] Average concentration (genomic units/ng total DNA) of three plants. Mean concentration within each row followed by the same lowercase letter are not significantly different according to the t-test for nonparametric distributions ($P \leq 0.05$).

Table 4. Concentration of *Ralstonia solanacearum* strain LPF710 in tissues of resistant (CLR172) and susceptible (CLR371) eucalypt clones determined by qPCR

Distance (cm)[a]	Days after inoculation	Concentration (clone CLR172)[b]	Concentration (clone CLR371)[b]
12	0	**Negative**[c]	**Negative**
	2	**Negative**	**Negative**
	4	3.53 (± 0.83) x 10^3 b	1.22 (± 0.08) x 10^5 a
	6	1.31 (± 0.06) x 10^4 b	1.83 (± 0.49) x 10^5 a
	8	1.97 (± 0.79) x 10^3 b	1.28 (± 0.49) x 10^5 a
	10	1.25 (± 0.71) x 10^4 b	9.38 (± 0.43) x 10^4 a
	12	1.04 (± 0.83) x 10^3 b	1.19 (± 0.78) x 10^5 a
	24	3.60 (± 2.37) x 10^2	Not evaluated
6	0	**Negative**	**Negative**
	2	3.94 (± 2.72) x 10^1 a	1.82 (± 1.65) x 10^2 a
	4	1.63 (± 0.34) x 10^4 b	3.29 (± 0.48) x 10^5 a
	6	1.75 (± 1.10) x 10^4 b	1.45 (± 0.38) x 10^5 a
	8	9.42 (± 2.55) x 10^3 b	1.37 (± 0.34) x 10^5 a
	10	3.18 (± 1.73) x 10^4 b	2.80 (± 1.20) x 10^5 a
	12	2.93 (± 1.49) x 10^4 b	1.69 (± 1.20) x 10^5 a
	24	1.49 (± 0.60) x 10^4	Not evaluated
-6	0	**Negative**	**Negative**
	2	6.40 (± 4.99) x 10^1 a	9.75 (± 9.64) x 10^2 a
	4	4.22 (± 1.34) x 10^3 b	1.31 (± 0.06) x 10^5 a
	6	6.78 (± 4.44) x 10^3 b	1.01 (± 0.22) x 10^5 a
	8	9.07 (± 0.17) x 10^3 b	5.96 (± 0.46) x 10^4 a
	10	1.08 (± 0.32) x 10^4 b	1.81 (± 1.13) x 10^5 a
	12	1.06 (± 0.88) x 10^4 b	2.29 (± 1.37) x 10^5 a
	24	6.23 (± 2.37) x 10^3	Not evaluated
-12	0	**Negative**	**Negative**
	2	**Negative**	**Negative**
	4	2.21 (± 0.74) x 10^3 b	8.50 (± 0.40) x 10^4 a
	6	1.27 (± 0.48) x 10^4 b	9.51 (± 4.09) x 10^4 a
	8	1.40 (± 0.37) x 10^4 b	3.31 (± 0.93) x 10^4 a
	10	1.69 (± 1.10) x 10^4 b	1.32 (± 0.53) x 10^5 a
	12	4.28 (± 2.92) x 10^3 b	5.59 (± 0.12) x 10^4 a
	24	3.42 (± 2.43) x 10^3	Not evaluated

[a] Centimeters above (positive values) and below (negative values) the inoculation point.
[b] Mean concentration (genomic units/ng DNA total) of three plants. Mean concentration within each row followed by the same lowercase letter are not significantly different according to the t-test for nonparametric distributions ($P \leq 0.05$).
[c] qPCR signal was negative or the *R. solanacearum* concentration was smaller than the detection limit under the tested conditions.

PCR Test	Genomic units/µL						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Real-time PCR (Ct)	14.52	17.68	20.94	24.56	28.38	31.51	33.11
Conventional PCR							

Figure 1. Sensitivity of real-time PCR (qPCR) and conventional PCR (cPCR) for detecting purified genomic DNA of *Ralstonia solanacearum* strain LPF710. Ct refers to the qPCR cycle number in which the fluorescence signal generated by the amplification of the target DNA crosses the threshold line in the exponential amplification phase of the reaction.

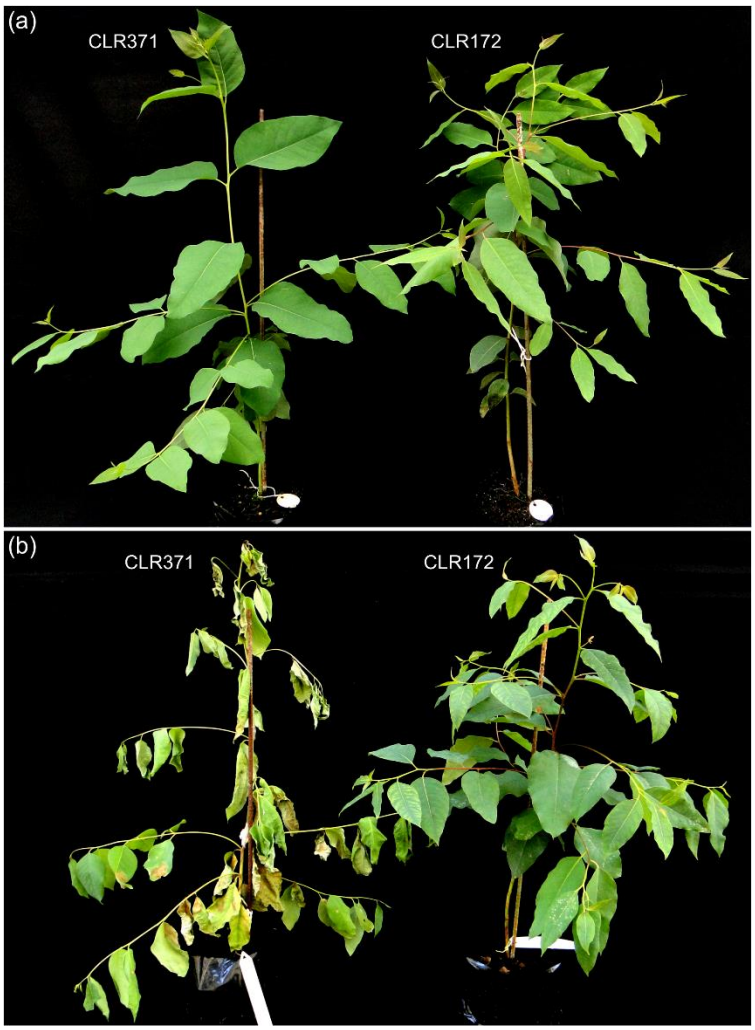


Figure 2. Response of eucalypt clones CLR371 (susceptible) and CLR172 (resistant) to *Ralstonia solanacearum* strain LPF710 inoculation at 0 (a) and 12 (b) days after inoculation (dai).

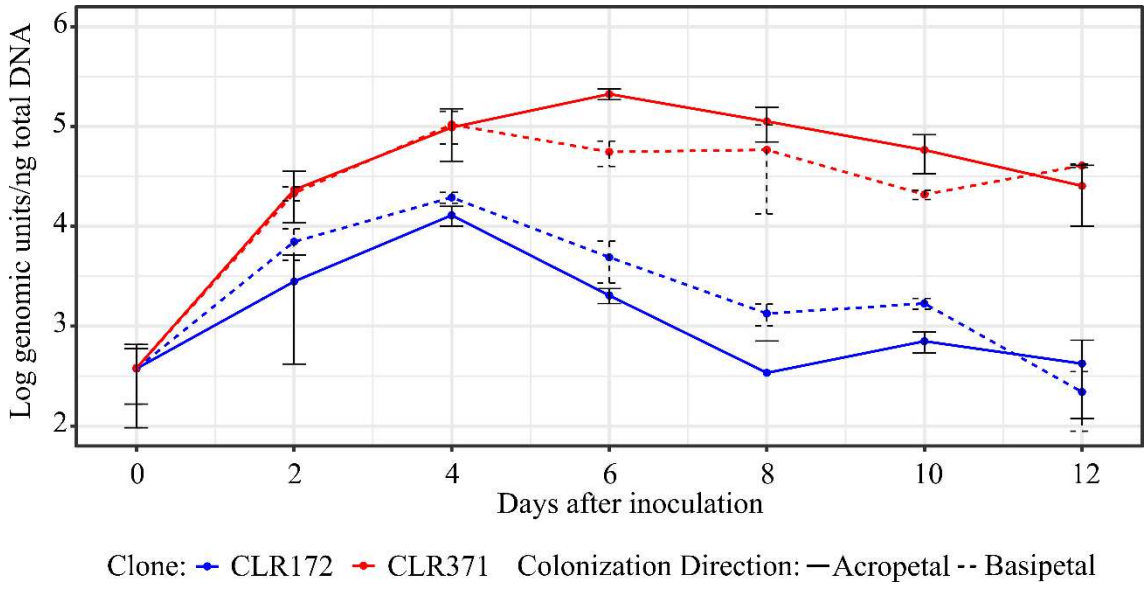


Figure 3. Concentration (genomic units/ng DNA) of *Ralstonia solanacearum* strain LPF710 in the stem tissues of resistant (CLR172) and susceptible (CLR371) eucalypt clones determined by qPCR using primer pair Rs199F/Rs199R from 0 to 12 days after inoculation (dai). For each day, stem fragments were taken 2 cm above (acropetal) and below (basipetal) the collection distance of the preceding evaluation time, starting from the inoculation point at day 0 up to 12 cm above and below the inoculation point at 12 dai.

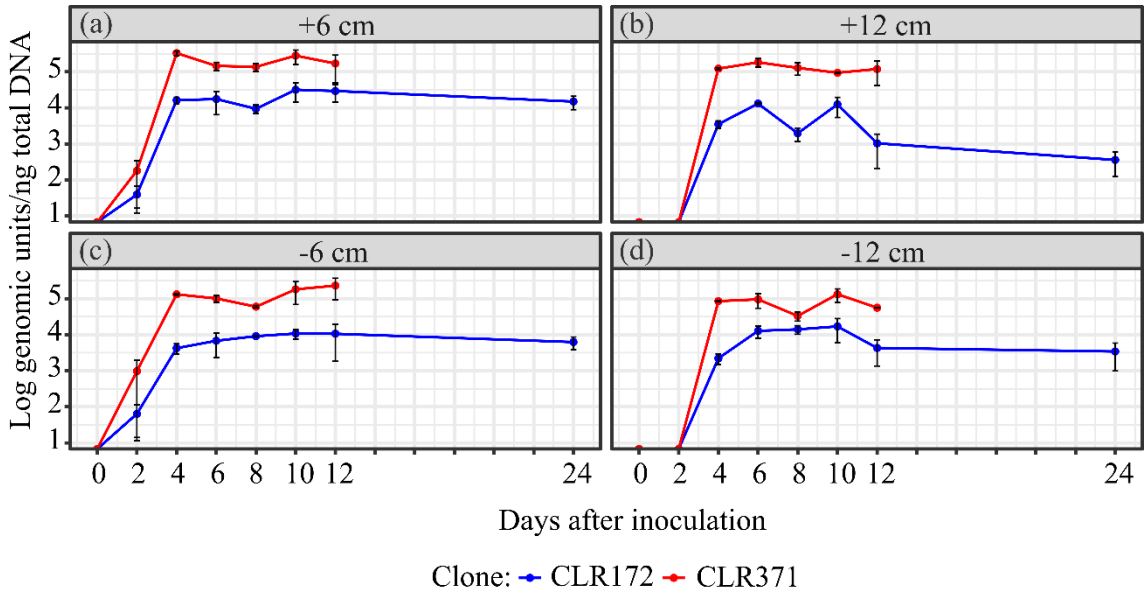


Figure 4. Concentration (genomic units/ng total DNA) of *Ralstonia solanacearum* strain LPF710 in the stem tissues of resistant (CLR172) and susceptible (CLR371) eucalypt clones determined by qPCR using primer pair Rs199F/Rs199R. (a) 6 cm and (b) 12 cm above the inoculation point; (c) 6 cm and (d) 12 cm below the inoculation point. For clone CLR172, an additional evaluation was performed at 24 dai.

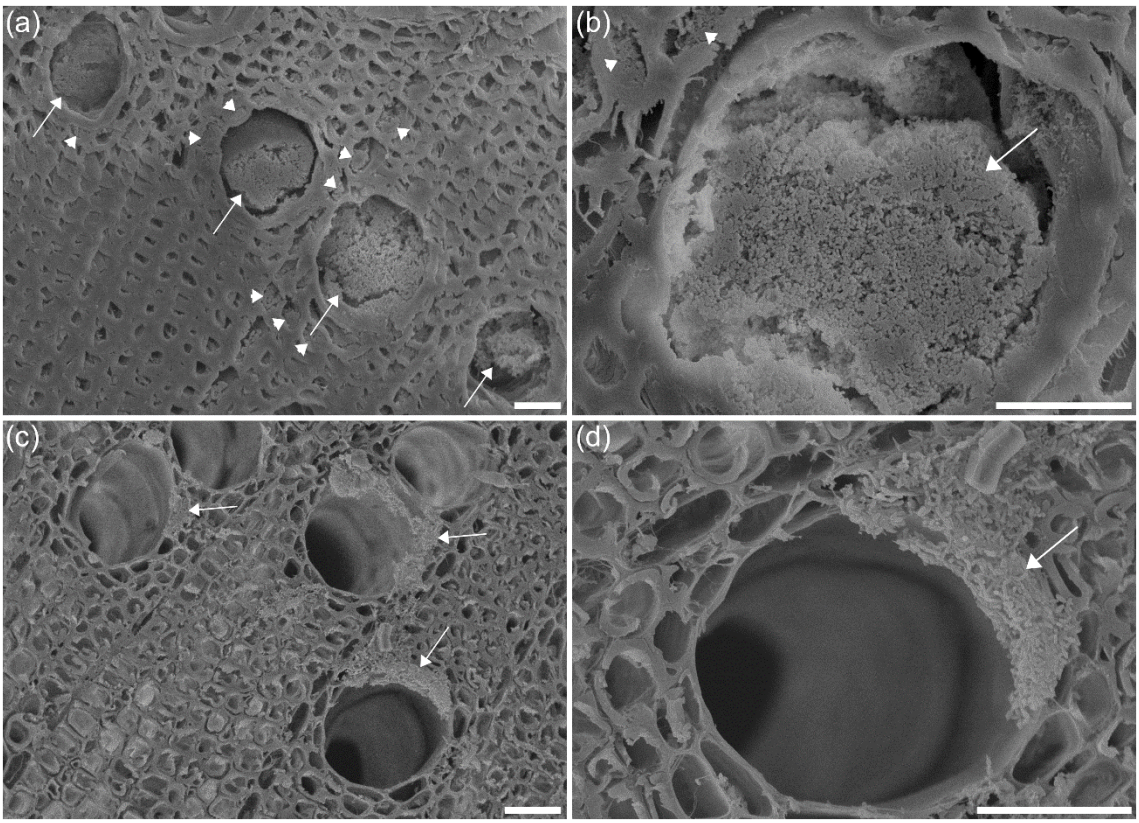


Figure 5. Scanning electron micrographs of transverse sections of the stem tissues of eucalypt clones inoculated with *R. solanacearum* at 12 days after inoculation and 6 cm above the inoculation point. (a-b) Susceptible clone (CLR371) with mature biofilm causing total obstruction of the xylem (arrows), parenchyma and fiber cells (arrowheads). (c-d) Resistant clone (CLR172) with small aggregates of bacterial cells around the xylem vessels (arrow). Scale bars = 20 µm.

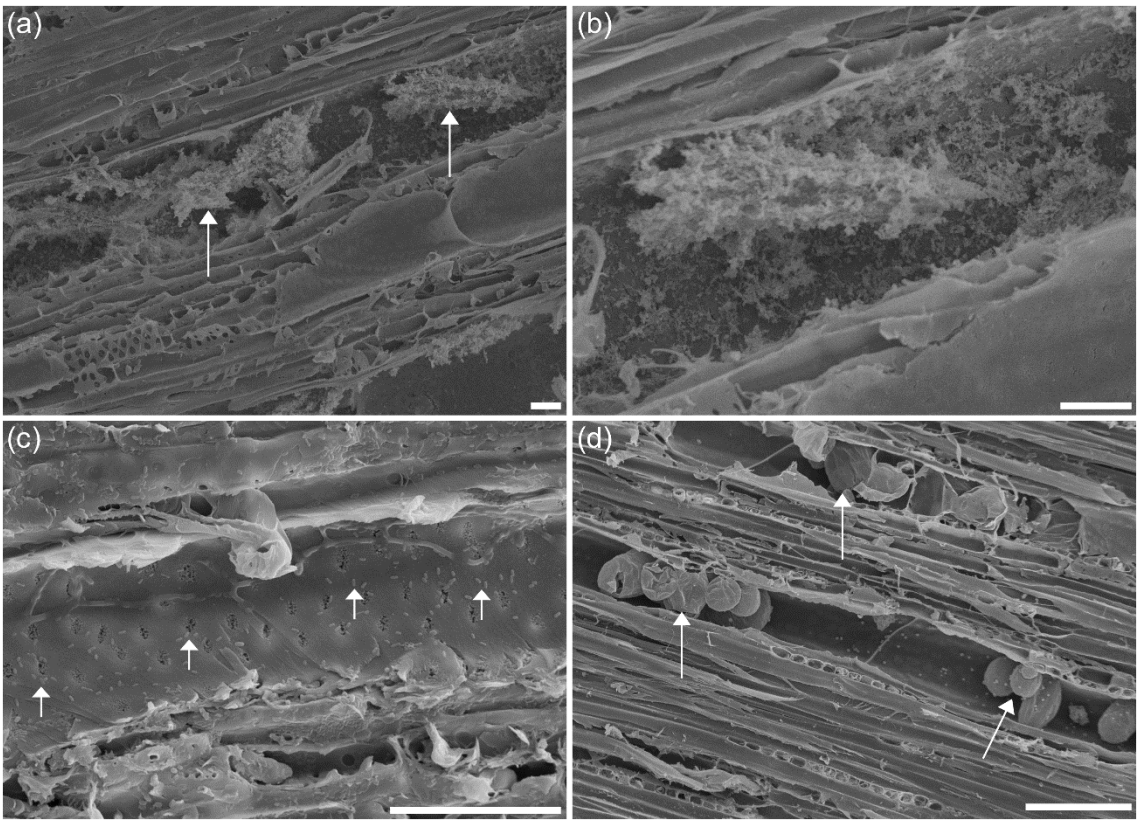


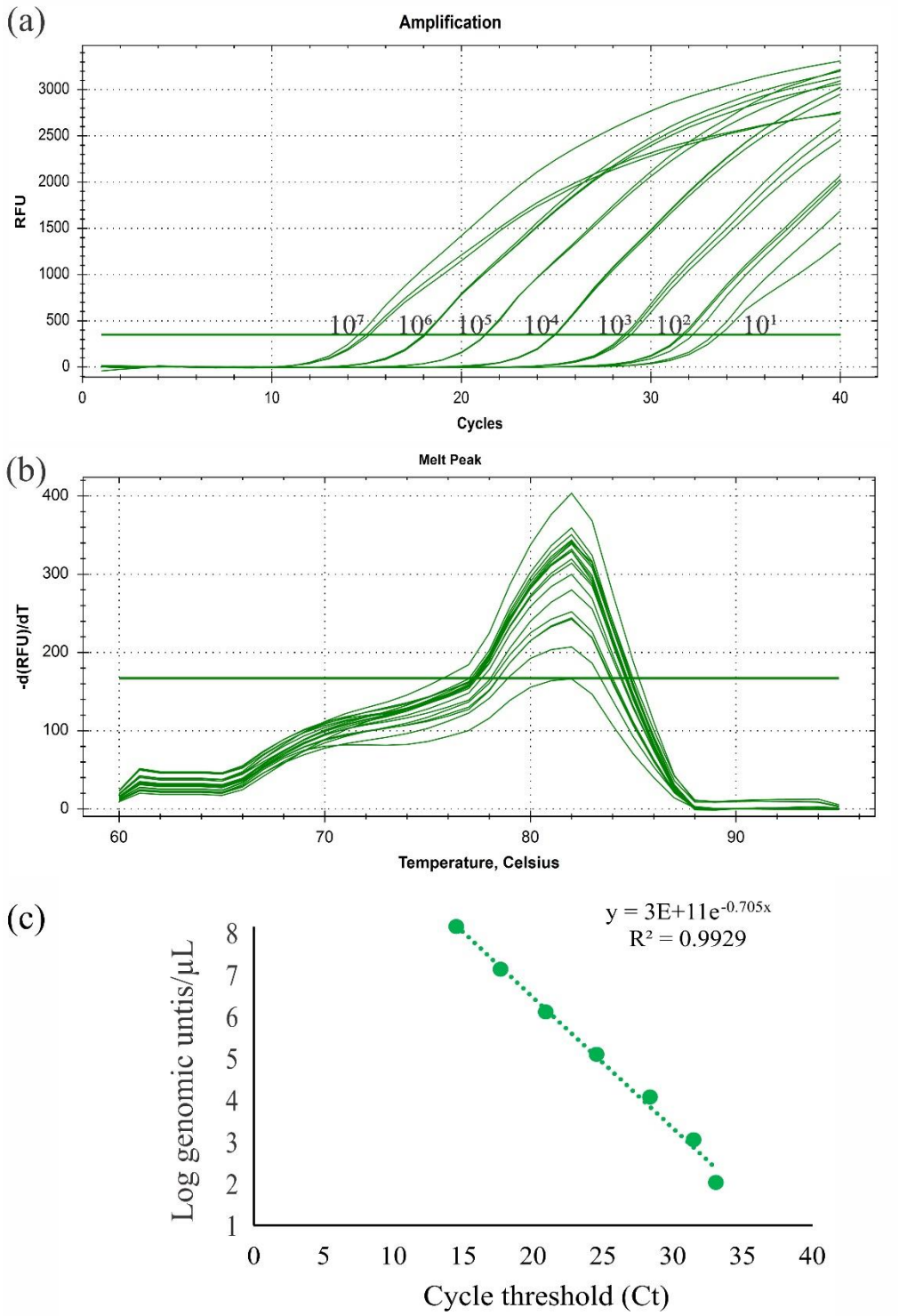
Figure 6. Scanning electron micrographs of longitudinal sections of the stem tissues of eucalypt clones inoculated with *R. solanacearum* at 12 days after inoculation and 6 cm above the inoculation point: (a-b) Susceptible clone (CLR371) with bacterial communities immersed in an exopolysaccharide (EPS) matrix (arrows) forming biofilms inside the xylem vessels. (c and d) Resistant clone (CLR172) with isolated bacterial cells (arrows) and tyloses formation (arrows) inside the xylem cells, respectively. (a-c) Scale bars = 20 μm; (d) Scale bar = 100 μm.

SUPPORTING INFORMATION

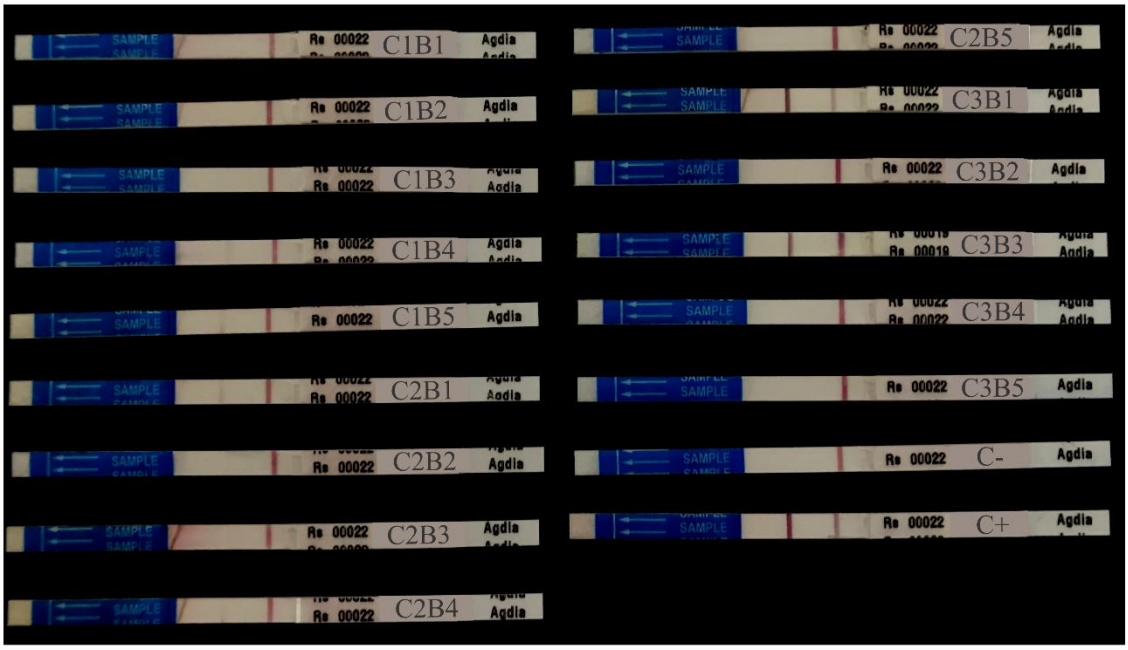
Supplementary Table S1. Strains of *Ralstonia solanacearum* and other bacterial species isolated from eucalypt used in this study

Bacterial strains	**Species**	**Geographic origin***
LPF710	*Ralstonia solanacearum*	Alagoas
LPF711	*Ralstonia solanacearum*	São Paulo
LPF713	*Ralstonia solanacearum*	Maranhão
LPF587	*Xanthomonas axonopodis*	Espírito Santo
LPF597	*Xanthomonas axonopodis*	Rio Grande do Sul
LPF599	*Xanthomonas axonopodis*	Paraná
LPF534	*Erwinia psidii*	Rio Grande do Sul
LPF558	*Erwinia psidii*	São Paulo
LPF610	*Erwinia psidii*	Mato Grosso do Sul
LPF698	*Pantoea dispersa*	São Paulo
LPF699	*Enterobacter cloaceae*	São Paulo
LPF700	*Novosphingobium panipatense*	São Paulo
LPF701	*Paenibacillus cookii*	São Paulo
LPF702	*Curtobacterium* sp.	São Paulo
LPF703	*Pantoea agglomerans*	São Paulo
1904	*Pseudomonas* sp.	-

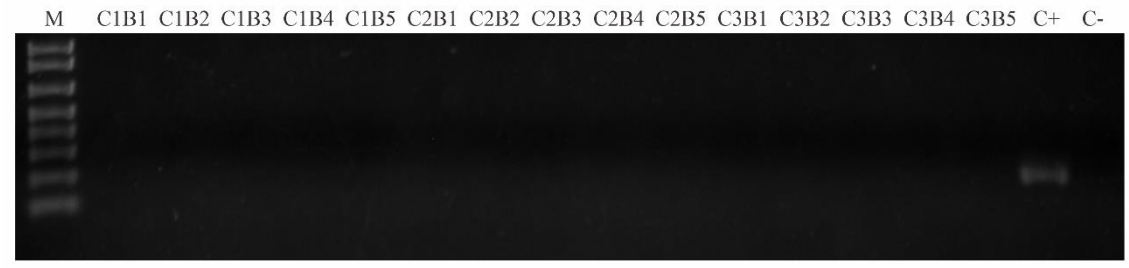
* Brazilian states.



Supplementary Figure S1. Amplification (a), melting curve (b) and standard curve (c) of qPCR with primers Rs199F/Rs199R and purified genomic DNA from *R. solanacearum* strain LPF710. Linear regression equation of Ct (x) on genomic units (y) was: Y = $3E+11e^{-0.705x}$. The y axis indicates number of genomic units/µL. RFU, relative fluorescent units. T, temperature. Ct, qPCR cycle number in which the fluorescence signal generated by the amplification of the target DNA crosses the threshold line in the exponential amplification phase of the reaction.



Supplementary Figure S2. Immunoassay strip tests (Agdia, Elkhart, IN) conducted to detect *Ralstonia solanacearum* **i**n samples of asymptomatic eucalypt rooted cuttings from São Paulo state, Brazil. Positive samples were: clone 1 (C1) bulks 4 and 5 (B4, B5); clone 2 (C2) bulks 1 and 4 (B1, B4); and clone 3 (C3), bulks 1 and 3 (B1, B3). C-: healthy eucalypt plant, C+: eucalypt with wilt symptom.



Supplementary Figure S3. Lack of amplification of *Ralstonia solanacearum* DNA by conventional PCR using primers Rs199F/Rs199R from samples of asymptomatic eucalypt rooted cuttings from São Paulo state, Brazil. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); C1B1 to C3B5: clone 1 bulk 1 to clone 3 bulk 5. C+: eucalypt with wilt symptom, C-: healthy eucalypt plant.

Article 2: Detection and characterization of *Ralstonia pseudosolanacearum* infecting *Eucalyptus* sp. in Brazil

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ABSTRACT

Ralstonia solanacearum sensu lato causes bacterial wilt in many agronomic crops and tree species economically important worldwide. It is a species complex that has been divided into phylotypes and sequevars, commonly related to geographic distribution. Knowledge of the phylotype composition and genetic variability in populations of this phytopathogenic bacterium is useful for implementing effective control measures. In a survey conducted in 2019, six bacterial strains were obtained from wilted *Eucalyptus urophylla* trees in plantations located in the municipality of Dom Eliseu, Pará state, Brazil. Multiplex PCR based on the internal transcribed spacer (ITS) indicated that the bacterial strains belonged to two different species, namely *R. pseudosolanacearum* (phylotype I) and *R. solanacearum* (phylotype II). In a phylogenetic analysis, the nucleotide sequence of the endoglucanase (*egl*) gene from eucalypt strains of phylotype I clustered together with sequevar 18 sequences from GenBank. Separation of the strains into two different species was confirmed by repetitive element palindromic PCR (rep-PCR). Pathogenicity tests demonstrated that the *R. solanacearum* and *R.*

pseudosolanacearum strains recovered from *E. urophylla* cause disease in both tomato and eucalypt plants. Until now, only *R. solanacearum* (Phylotype II) has been reported causing wilt symptoms on *Eucalyptus* spp. in Brazil. Therefore, the presence of *R. pseudosolanacearum* and a need for better understanding of its genetic and aggressiveness variability as well as possible differences between the two species should be considered in breeding programs aimed at the deployment of host resistance.

Keywords: Bacterial wilt, eucalypt, *Ralstonia solanacearum*

INTRODUCTION

Ralstonia solanacearum (Smith) Yabuuchi is one of the most important plant pathogenic bacteria in the world. It causes bacterial wilt (BW) in plants of over 50 botanical families in tropical, subtropical and some temperate areas (Elphinstone, 2005). It is considered a species complex that exhibits considerable variation in host range, geographic origin, pathogenicity and physiological properties (Denny, 2006).

Fegan & Prior (2005) proposed a classification of the *R. solanacearum* species complex based on the amplification of the partial sequences of the rRNA internal transcribed spacer (ITS) region. This molecular classification scheme, aimed at allocating strains into phylotypes, makes use of multiplex PCR. Depending on the size of the resulting amplicons, a strain can be allocated into one of four phylotypes, which are commonly related to their geographic origins. Phylotype I contains strains mainly originated from Asia, phylotype II includes strains mainly from the Americas, phylotype III strains typically originated from Africa and nearby islands, while phylotype IV strains are primarily from Indonesia. In addition, phylotypes can be further subdivided into sequevars based on the polymorphism of the endoglucanase (*egl*) gene, adding an even more refined sequence-based classification of strains of the *R. solanacearum* species complex (Poussier et al., 2000). As a result of its separation into two distinct clusters in *egl*-based phylogenetic analyses, phylotype II has been divided into phylotypes IIA and IIB (Cellier & Prior, 2010; Gutarra et al., 2017).

Using polyphasic analyses, Safni et al. (2014) suggested the division of the *R. solanacearum* species complex into three distinct species, which are to some extent linked to the phylotype groups described by Fegan & Prior (2005). Safni et al. (2014) proposed to retain phylotype II as *R. solanacearum*, which includes the type strain IBSBF292, and

to reclassify phylotypes I and III as *R. pseudosolanacearum*, and phylotype IV as *R. syzygii.* Prior et al. (2016) used genomic comparisons, proteomic analyses and metabolic characterization to support the division of the complex into the three distinct species.

Brazil is considered a putative center of origin of *Ralstonia solanacearum* (phylotype II) (Wicker et al., 2012). However, *R. pseudosolanacearum* (phylotype I) has also been found associated with solanaceous crops, such as tomato, aubergine, long pepper and bell pepper (Santiago, Lopes, Caetano-Anollés & Mizubuti, 2017). Until now, only phylotype II has been reported causing BW in eucalypt in Brazil, but some reports indicate that phylotype I can also cause wilt symptoms in eucalypt in Africa and Asia (Carstensen, Venter, Wingfield & Coutinho, 2016).

The geographic separation of these two *Ralstonia* species indicates that their association with *Eucalyptus* sp. may have occurred independently of one another and raises questions regarding the epidemiology and etiology of bacterial wilt symptoms. A clear understanding of the geographic distribution of these two bacterial species is crucial for establishment of both worldwide and local containment strategies. Whether the pathogenicity of these two bacterial species towards *Eucalyptus* is similar is not known and requires further investigation (Carstensen, Venter, Wingfield & Coutinho, 2016). This work demonstrates that *R. pseudosolanacearum* is present in Brazil causing BW in *Eucalyptus* sp., information highly relevant for disease management.

MATERIAL AND METHODS

Bacterial isolation

Bacterial strains were obtained from one-year-old trees of *Eucalyptus urophylla* showing wilt symptoms and discoloration of internal tissue. During a survey conducted in February 2019 in plantations located in Dom Eliseu municipality, Pará state, Brazil, a high incidence of the disease was observed and ten trunk samples were randomly collected. In addition, some wilted rooted cuttings were found in a eucalypt nursery located in the same region and a strain was isolated from one of the cuttings. Samples were transported for processing at the Laboratory of Forest Pathology/Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil.

Infected eucalypt tissue was washed in running tap water, surface sterilized with 90% alcohol by flaming and internal fragments aseptically removed. The fragments were macerated in 1.0 mL of sterile water in a TissueLyser II (Qiagen®) at 15 Hz for 90 s and

the macerate plated on modified semiselective medium from South Africa (SMSA) (Elphinstone, Hennessy, Wilson & Stead, 1996). Strain purification was conducted by streaking cells from individual colonies with morphology typical of *R. solanacearum* on casamino acid-peptone-glucose medium (CPG) (Kelman 1954). Cell suspensions of purified strains were prepared in 30% (v/v) glycerol and stored at -80 °C in the Culture Collection of the Laboratory of Forest Pathology/Bioagro (CPBFPL- UFV).

Strain identification

Bacterial DNA was extracted from 24 h cultures grown on CPG medium using the Wizard Genomic DNA Purification Kit (Promega) as recommended by the manufacturer**.** To confirm that strains belonged to the *R. solanacearum* species complex, PCR amplification of a *ca*. 280-bp fragment was performed using species complex-specific primers 759 and 760 (Opina et al., 1997). The reaction was performed in a final 15 µL volume containing 2.0 µL DNA (15 ng μL^{-1}), 7.5 µL of GoTaq® Master Mix (Promega), 1.0 µL of each primer (10 µM) and ultrapure distilled water. The amplification was performed using a Veriti 96 thermocycler (Applied Biosystems) with a program that consisted of initial denaturation at 94 °C for 3 min, annealing at 53 °C for 1 min and extension at 72 °C for 90 s, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were analyzed on a 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide (1.0 µg mL^{-1}) and visualized in an L-PIX gel documentation system (Loccus Biotechnology).

Phylotype classification

Classification of bacterial strains into phylotypes was conducted by multiplex PCR using a set of four forward primers (Nmult - 21:1 F, Nmult - 21:2 F, Nmult - 22: InF and Nmult - 23: AF), each specific for a particular phylotype and one reverse primer (Nmult - 22: RR) common to all phylotypes (Fegan & Prior, 2005). The reaction mix contained 2.0 µL DNA (15 ng μL^{-1}), 9.2 µL of GoTaq® Master Mix (Promega), 0.5 µL of each forward primer (10 µM) and 1.0 µL of reverse primer (10 µM) in a final volume of 25 µL. The PCR amplifications were performed using a Veriti 96 thermocycler (Applied Biosystems) with a program consisting of an initial denaturation at 96 °C for 3

min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The PCR products were resolved on a 1% (w/v) agarose gel in TAE buffer, stained with ethidium bromide (1.0 µg mL^{-1}) and analyzed using an L-PIX gel documentation system (Loccus Biotechnology). Strains IBSBF292 and GMI1000 were used as a references for phylotype II and I, respectively (Fonseca et al., 2014).

Sequevar classification

A phylogenetic analysis based on the partial sequence of the *egl* gene was performed to determine to which sequevar the strains belonged to. To this end, the *egl* gene was amplified by PCR using primers ENDO-F and ENDO-R (Ji et al., 2007) in a 25-µL reaction containing 2.0 µL DNA (15 ng μL^{-1}), 12.5 µL of GoTaq® Master Mix (Promega), 1.5 µL of each primer (10 µM) and ultrapure distilled water. Amplification was performed in a Veriti 96 thermocycler (Applied Biosystems) using the program: initial denaturation at 96 °C for 9 min, 30 cycles at 95 °C for 1 min, 70 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed on a 1% (w/v) agarose gel and stained with ethidium bromide (1.0 µg mL^{-1}). After confirmation of the expected size, the amplicons were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and the final product was sequenced in an ABI PRISM 3100 sequencer (Applied Biosystems) using the same primers used for PCR amplification. Contigs were assembled with SeqAssem (SequentiX - Digital DNA Processing) and subjected to multiple alignment with *egl* sequences from strains representing different sequevars, covering all phylotypes described in the literature, and deposited in GenBank (Table 1) using Muscle (Edgar, 2004) and MEGA 7.0 (Kumar, Stecher & Tamura, 2016) followed by manual adjustments.

Bayesian inference was used to construct a phylogenetic tree based on the *egl* sequences with the program MrBayes 3.2 (Ronquist et al., 2012). The substitution model was chosen based on the Akaike information criterion (AIC) in Mega 7.0 (Kumar, Stecher & Tamura, 2016). The *a posteriori* probability of tree distribution was calculated using the MCMC (Metropolis-coupled Markov chain Monte Carlo) algorithm, with two chains from a random tree and one million generations, discarding the first 25% of the trees. The phylogenetic tree was viewed and edited with Figtree 1.4

(http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.48.1 (https://inkscape.org/pt-br/release/inkscape-0.48.1/).

Clustering based on rep-PCR

In order to further verify the classification of the strains obtained from eucalypt into different phylotypes, they were subjected to rep-PCR analysis using the primers REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-ICGITTATCIGGCCTAC-3') for REP-PCR, ERIC1R (5'-ATGTAAGCTCCTGGGGATTCA-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') for ERIC-PCR, and BOXA1R (5'-CTACGGCAAGGCGACGCTGAC-3') for BOX-PCR (Louws, Fulbright, Stephens & Bruijn, 1994). All amplifications were conducted in a reaction that contained 2.0 µL DNA (15 ng μL^{-1}), 7.5 µL of GoTaq® Master Mix (Promega) and 1.5 µL of each primer (10 µM), adjusting the total volume with ultrapure distilled water to 15 µL for REP-PCR and ERIC-PCR and to 20 µL for BOX-PCR. The PCR was performed in a Veriti 96 thermocycler (Applied Biosystems) programmed for an initial denaturation at 95 °C for 7 min, 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min, and a final extension at 65 °C for 15 min for ERIC-PCR and BOX-PCR. For REP-PCR, the same program was used, except that an annealing temperature of 44 °C for 8 min was used. The products were analyzed on a 2% (w/v) agarose gel, stained with ethidium bromide (1.0 µg mL^{-1}) and visualized using a L-PIX gel documentation system (Loccus Biotechnology). The PCR with each primer set was performed three times to confirm the reproducibility of the banding patterns. Fragment profiles obtained with the BOX, ERIC and REP primers were used to construct binary matrices, assigning a value of 1 to the presence of a band and 0 to its absence. Nonreproducible and weak bands were excluded from the analysis. The results obtained with BOX, ERIC and REP (rep-PCR) were combined and analyzed with PAST 3.06 (Hammer, Harper & Ryan, 2001). Tree construction was conducted using the Dice similarity coefficient (Dice, 1945) and the unweighted pair group method with arithmetic means (UPGMA).

Pathogenicity tests

The pathogenicity of strains of phylotypes I and II collected from eucalypt in this study was tested by inoculating plants of clone CLR371 (*Eucalyptus urophylla*). The *R.*

solanacearum strain LPF710 was used as a positive control. Strain LPF710 was previously characterized and is routinely used in the selection of eucalypt clones resistant to bacterial wilt (Lúcio M. S. Guimarães, personal communication). Plants were inoculated by injection of a bacterial cell suspension at the base of the stem following the methodology described by Fonseca et al. (2016). For inoculum preparation, the strains were grown on triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954) at 28 °C. After 48 h, smooth, fluid colonies with white edges and red centers were transferred onto CPG medium and grown at 28 °C for 48 h. Then, bacterial cells were removed from the medium by adding 0.85% (w/v) sodium chloride solution and scraping them from the surface with a Drigalski spatula. Finally, the absorbance at 540 nm of the bacterial suspension was adjusted to 1.0.

Thirty-days-old cuttings clonally propagated from CLR371 plants were inoculated with either *R. solanacearum* or *R. pseudosolanacearum* strains. Each bacterial strain was individually inoculated into five plants. The same number of plants were treated with 0.85% (w/v) sodium chloride solution instead of bacterial suspension to serve as controls. Inoculated plants were kept in a growth chamber at 28 ± 2 °C with a 12-h photoperiod and light intensity of 40 μmoL s^{-1} m^{-2} in a completely randomized design. Wilt symptoms in all plants were evaluated daily for up to 15 days after inoculation (dai). In addition, bacterial exudation from five fragments of stem tissue collected from each wilted or symptomless plant was evaluated under a light microscope at 100× magnification. Bacteria were re-isolated from inoculated plants by plating tissue macerates on SMSA medium (Elphinstone, Hennessy, Wilson & Stead, 1996), colony-purified and their identity as either *R. pseudosolanacearum* or *R. solanacearum* confirmed by ITS-based multiplex PCR.

Pathogenicity of the strains collected from eucalypt was also tested on tomato plants (*Solanum lycopersicum* cv. Santa Clara) since this plant species is considered an indicator host (Carstensen, Venter, Wingfield & Coutinho, 2016) and disease symptoms appear faster. The inoculation was conducted essentially as described above for eucalypt.

RESULTS

Bacterial isolation

A total of five bacterial strains were obtained from eucalypt trunk samples collected in two different farms in Dom Eliseu municipality. Each strain was isolated

from a different infected tree. An additional strain (LPF755) was obtained from a cutting of *E. urophylla* from a nursery located in the same region (Table 2). On SMSA medium the morphology of the colonies was smooth and fluid, with white edges and red centers. As for the *R. solanacearum* type strain IBSBF292, used as a positive control, a 280-bp fragment was amplified by primers 759 and 760 when PCR was conducted with DNA of all six strains obtained from infected eucalypt tissue (Figure S1), indicating that they belonged to the *R. solanacearum* species complex.

Phylotype determination

Of the six strains of the *R. solanacearum* species complex obtained from infected eucalypt tissue, phylotype-specific multiplex PCR amplified a 144-bp fragment, typical of phylotype I, from DNA of four strains (Table 2 and Figure S2). In contrast, a 372-bp fragment, typical of phylotype II, was amplified from DNA of the other two strains. (Figure S2). As expected a 372-bp band was amplified from DNA of the *R. solanacearum* IBSBF292 strain and a 144-bp band was amplified from DNA of the *R. pseudosolanacearum* GMI1000 reference strain. These results indicated that strains LPF750, LPF752, LPF753 and LPF754 infecting *E. urophylla* in Brazil belonged to *R. pseudosolanacearum*.

Sequevar classification

After manual trimming, a 637-bp sequence alignment of the *egl* gene was used for phylogenetic reconstruction, of which 160 positions were variable. The best evolutionary model selected by Mega 7.0 for Bayesian analysis by AIC was GTR + I + G. Phylogenetic analysis produced a well-resolved tree, in which strains obtained from infected eucalypt plants were assigned a phylogenetic position that was entirely consistent with their phylotype determination using multiplex PCR (Figure 1). Strains LPF751 and LPF755 clustered together with strains previously classified as phylotype IIA; strain LPF751 was most closely related to sequevar 50 whereas strain LPF755 was most closely related to sequevar 41. On the other hand, all four strains of *R. pseudosolanacearum* isolated from eucalypt plants in this study clustered together with strains of sequevar 18 (Figure 1).

Clustering based on rep-PCR

The number of well-defined and reproducible bands amplified by BOX, ERIC and REP primers varied from three to eight, seven to thirteen and six to thirteen, respectively, depending on the strain. The sizes of the bands amplified varied between 200 and 3000 bp for ERIC, 300 and 1500 bp for BOX and 250 and 3000 bp for REP. When the three markers were combined (BOX+ERIC+REP), a total of 69 bands were polymorphic and considered for phylogenetic analysis. A dendrogram obtained combining the three markers together (BOX+ERIC+REP) indicated that strains LPF750, LPF752, LPF753 and LPF754, classified by phylotype and sequevar typing as *R. pseudosolanacearum,* were highly clonal and clustered together with phylotype I reference strain GMI1000, with 100% similarity (Figure 2 and Figure S3). They formed a group separate from strains LPF751 and LPF755, which clustered together with phylotype II reference strain IBSBF292, although with only 25% similarity. Furthermore, the similarity between strains LPF751 and LPF755, based on results of rep-PCR, was only 52%.

Pathogenicity test

Considering that, based on results of both sequevar typing and rep-PCR, the four eucalypt strains of phylotype I are highly clonal, the LPF750 and LPF752 strains were selected to confirm their pathogenicity. Strains LPF751 and LPF755 of phylotype IIA were also included for pathogenicity comparisons. All strains tested caused wilt symptoms in tomato plants at five days after inoculation (dai) (Figure 3), whereas plants treated with sodium chloride solution remained healthy over the evaluation period. The disease symptoms resembled those typically caused by strains of the *R. solanacearum* species complex in tomato plants.

All eucalypt plants inoculated with bacterial strains exhibited wilt symptoms within 10 dai (Figure 4) while plants treated with sodium chloride solution did not. Wilt symptoms initiated at the plant apex and progressed towards the base, which was followed by defoliation. At 15 dai, plants were completely wilted (Figure 4). Besides wilt symptoms, stem tissue collected from inoculated plants displayed abundant bacterial exudation when examined under the microscope. Strains with morphological characteristics on SMSA medium identical to those inoculated were re-isolated from

symptomatic eucalypt plants and their identity confirmed by multiplex PCR, in fulfillment of Koch's postulates.

DISCUSSION

In this study, we demonstrated that, like *R. solanacearum*, *R. pseudosolanacearum* also causes eucalypt wilt in Brazil. According to Lopes & Rossato (2018), the latter species is already distributed in 17 Brazilian states and associated with wilt symptoms in solanaceous plants, mainly in Northern and Northeastern regions. Nonetheless, the pathogenicity of this bacterial species to eucalypt in the country had not previously been reported. Here, *R. pseudosolanacearum* was detected in the two farms sampled in the Dom Eliseu municipality, indicating that the bacterium may be spreading and infecting additional host plant species. In Santa Tereza farm, a nearby locality, a *R. solanacearum* strain was also obtained from eucalypt, which indicates the sympatry of the two bacterial species infecting the same host plant.

The two species of the *R. solanacearum* complex were clearly distinguished by multiplex PCR and rep-PCR. Furthermore, insight into the genetic variability of the phylotype I strains obtained from eucalypt plants was obtained by sequevar typing and rep-PCR. In a phylogenetic tree based on the *egl* sequence, the strains clustered together along with strains of sequevar 18 (including the reference strain GMI1000), with no evidence of genetic diversity revealed. In the rep-PCR analysis, the eucalypt strains exhibited the same banding pattern as the phylotype I reference strain GMI1000, which was isolated from tomato plants in French Guiana. In a previous study, Santiago, Lopes, Caetano-Anollés & Mizubuti (2017) identified only this sequevar among 48 strains of *R. pseudosolanacearum* from solanaceous plants, indicating no genetic diversity and a probable recent entry of this phylotype in Brazil.

The opposite occurs with the two phylotype IIA strains collected from eucalypt plants in this study, which exhibited only a 52% similarity with each other based on rep-PCR and were separated into two distinct sequevars based on the *egl* gene sequence. These results are consistent with the high genetic diversity observed for *R. solanacearum sensu stricto* population in Brazil (Santiago, Lopes, Caetano-Anollés & Mizubuti, 2017), some strains of which remain without sequevar designation (Fonseca et al., 2014). This high genetic variability may be related to the fact that Brazil is a likely center of origin of

this bacterial species. In this study, *Ralstonia* strains collected from eucalypt consistently grouped into two distinct groups, indicating that they are phylogenetically different.

Phylotype I strains of *R. solanacearum sensu lato* typically originate from Asia while phylotype II strains primarily originate from the Americas (Fegan & Prior, 2005). This suggests that the *R. pseudosolanacearum* and *R. solanacearum* strains associated with bacterial wilt of *Eucalyptus* spp. in Brazil may have originated independently from Asia and the Americas, respectively. An evolutionary study conducted by Wicker et al. (2012) indicated that phylotype I possesses many features of a relatively recent (as evidenced by its low genetic diversity) and recombinogenic lineage that has spread over long distances very rapidly, most probably as latent infections in exchanged plant material. Its worldwide distribution in Asia, Africa, Central America, South America and Oceania, including relatively recently formed islands (Buddenhagen, 1986; Elphinstone, 2005), also suggests its better fitness to establish in tropical conditions.

The results of this study indicate that the diversity of etiological agents of bacterial wilt of eucalypt in Brazil has been underestimated. So far, only *R. solanacearum* (Fonseca et al., 2014) and *Erwinia psidii* Rodrigues Neto, Robbs & Yamashiro (Arriel et al., 2014) have been documented as agents responsible for bacterial wilt symptoms in the country. Underestimation of the presence of *Eucalyptus* pathogens masked by an inability to discriminate between cryptic species, such as those of the *R. solanacearum* species complex, can negatively impact both disease management and quarantine measurements seeking to prevent their introduction into new geographic areas (Wingfield et al., 2008; Wingfield, Brockeroff, Wingfield & Slippers, 2015). Now, that *R. pseudosolanacearum* has been found in this study, a clear understanding of the genetic diversity and virulence of the pathogens is of prime importance to succeed in controlling the eucalypt wilt diseases. This is extremely important considering the pathogenicity of the eucalypt phylotype I strains towards tomato, a crop widely distributed in different geographic areas worldwide, and their suggested better fitness to adapt to tropical conditions (Buddenhagen, 1986; Elphinstone, 2005).

As for the two *Ralstonia* species associated with bacterial wilt of eucalypt found in this study, further investigation into their pathogenicity mechanisms is necessary to provide researchers with enhanced knowledge that could facilitate the selection of resistant eucalypt clones (Carstensen, Venter, Wingfield & Coutinho, 2016). Possible differences in the pathogenicity strategies utilized by these two bacterial species should

be taken into consideration in breeding programs in order to design novel and effective disease control alternatives appropriate for each pathogen.

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Table 1. Strains of the *Ralstonia solanacearum* species complex used in phylogenetic analyses.

Phylotype	Sequevar	Strain	Geographic origin	Host	GenBank accession No.
I	18	RS07	Brazil	*Piper hispidinervum*	KT629942
I	18	GMI1000	French Guiana	*Solanum lycopersicum*	AF295251
I	18	lbz1	Indonesia	*Eucalyptus* sp.	KT317626
I	18	RS344	Brazil	*Solanum lycopersicum*	KT629980
I	14	RSC3	China	*Eucalyptus* sp.	KT317616
I	31	CK	Dem. Rep. of Congo	*Eucalyptus* sp.	KT317623
I	17	lbz7	Indonesia	*Eucalyptus* sp.	KT317627
I	46	lbz11	Indonesia	*Eucalyptus* sp.	KU180284
IIA	50	RS297	Brazil	*Eucalyptus* sp.	KT629888
IIA	50	T1-UY	Uruguay	*Solanum lycopersicum*	GU295049
IIA	40	UW469	Brazil	*Solanum lycopersicum*	AF295269
IIA	24	B34	Brazil	*Musa* sp.	GQ907154
IIA	41	RS298	Brazil	*Eucalyptus* sp.	KT629855
IIA	41	CRM39	Indonesia	*Solanum lycopersicum*	EF439726
IIA	7	IBFBF292	U.S.A	*Solanum lycopersicum*	HM142850
IIB	1	RS66	Brazil	*Solanum lycopersicum*	KT630658
IIB	26	CIP240	Brazil	*Solanum lycopersicum*	EF647739
IIB	54	RS448	Brazil	*Capsicum* sp.	KT630017
IIB	28	RS60	Brazil	*Solanum lycopersicum*	KT629982
IIB	4	RS214	Brazil	*Musa* sp.	KT629997
III	-	UW386	Nigeria	*Solanum lycopersicum*	DQ657606
III	20	J25	Kenya	*Solanum tuberosum*	AF295279
III	19	JT525	Reunion	*Pelargonium*	AF295279
IV	10	UW443	Indonesia	*Musa* sp.	KF357880
IV	10	Psi7	Indonesia	*Solanum lycopersicum*	EF371804
IV	8	ACH732	Australia	*Solanum lycopersicum*	GQ907150

Table 2. Classification of *Ralstonia* strains obtained from infected *Eucalyptus urophylla* trunk tissue collected in different farms in Dom Eliseu, Pará, Brazil.

Strain	Locality	Latitude	Longitude	Multiplex PCR fragment size (bp)	Phylotype	Species	GenBank accession No.
LPF750	Farm Santa Tereza	-4.0461	-47.711	144	I	*R. pseudosolanacearum*	MN496142
LPF751	Farm Santa Tereza	-4.0461	-47.711	372	II	*R. solanacearum*	MN496147
LPF752	Farm Terra Roxa	-4.0544	-47.693	144	I	*R. pseudosolanacearum*	MN496143
LPF753	Farm Terra Roxa	-4.0544	-47.693	144	I	*R. pseudosolanacearum*	MN496144
LPF754	Farm Terra Roxa	-4.0515	-47.694	144	I	*R. pseudosolanacearum*	MN496145
LPF755*	Eucalypt nursery	-4.2911	-47.609	372	II	*R. solanacearum*	MN496146

* Obtained from rooted cutting

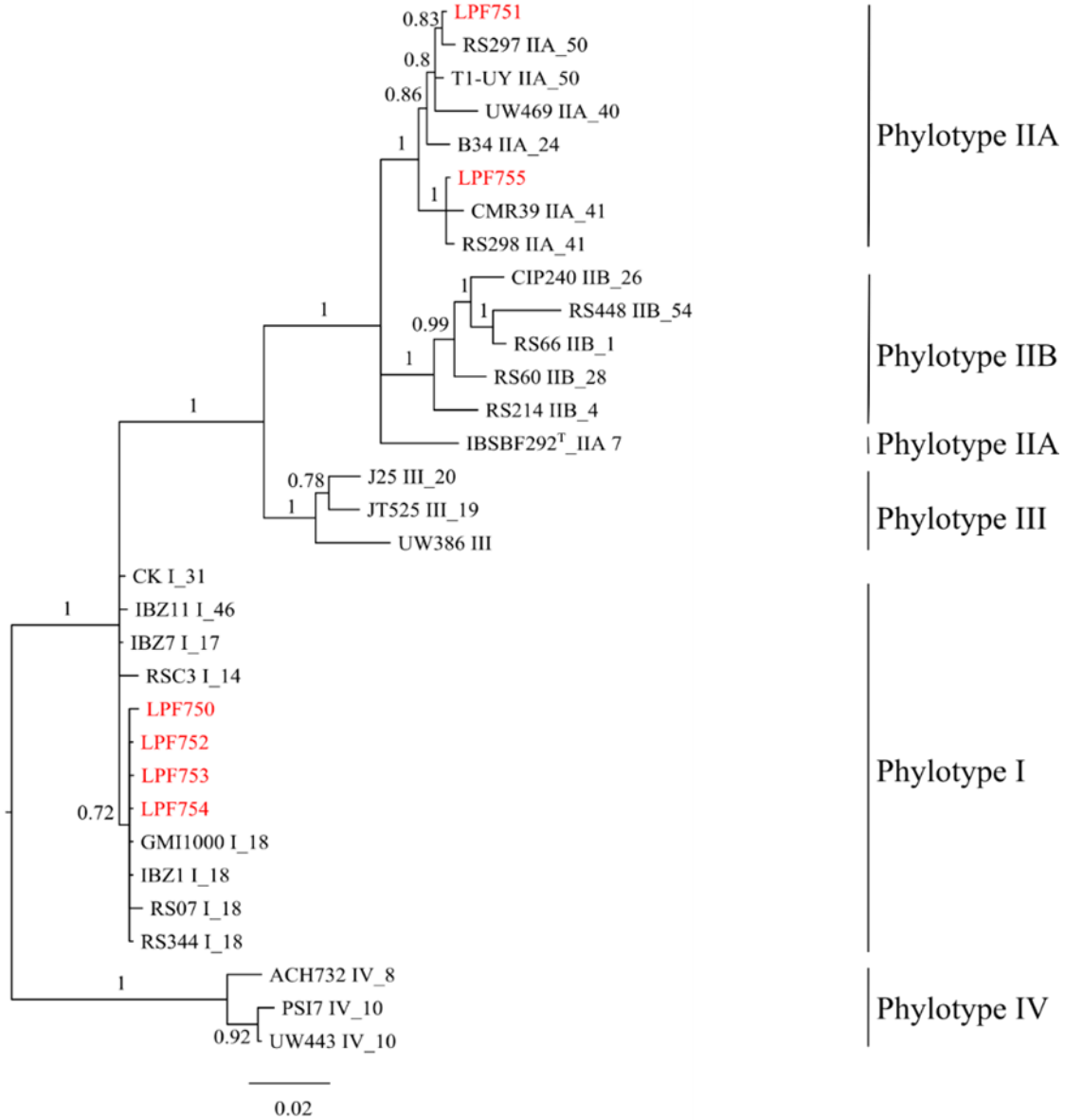


Figure 1. Phylogenetic tree based on *egl* gene sequences from strains of the *Ralstonia solanacearum* species complex constructed using Bayesian inference. The *a posteriori* probability values are indicated on the branches. Phylotypes and sequevars are sequentially indicated after the names of the strains. The bar indicates the fraction of substitutions per site. The strains isolated in this study are highlighted in red and those obtained from GenBank are shown in black.

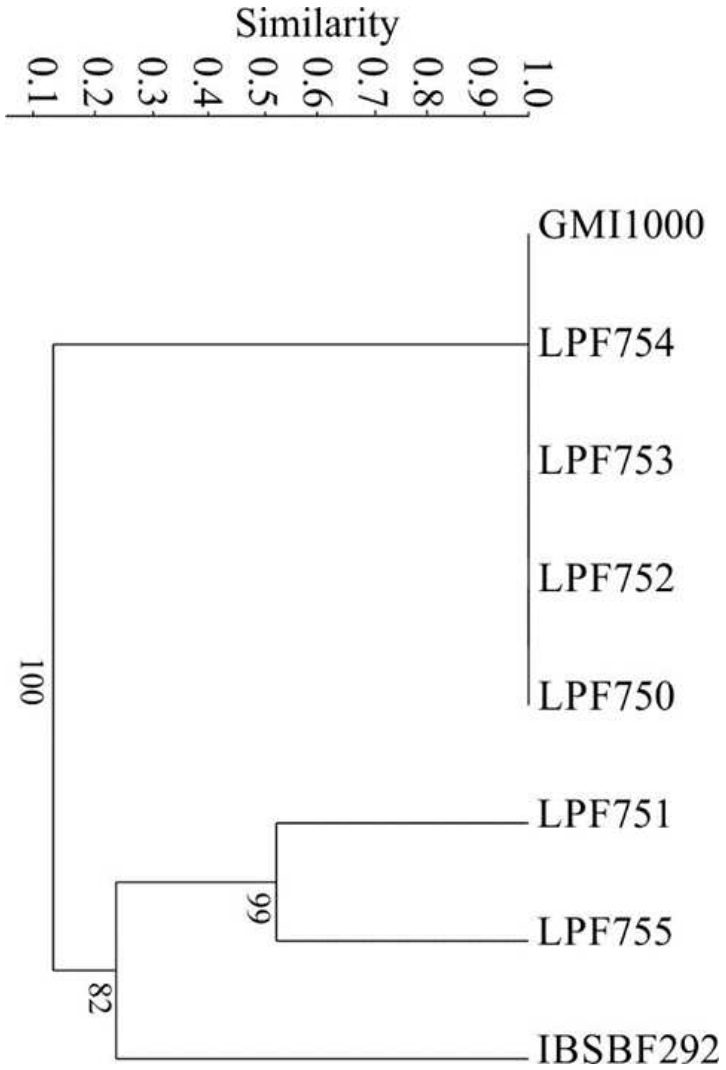


Figure 2. Dendrogram of rep-PCR profiles of four strains of *Ralstonia pseudosolanacearum* and two strains of *R. solanacearum* from *Eucalyptus* fields in Dom Eliseu, Pará, Brazil. The tree was generated with combined ERIC, REP and BOX-PCR band profiles. The differences among profiles are indicated by the percentage of similarity. The dendrogram was constructed using the Dice's similarity coefficient and UPGMA clustering method. GMI1000 is the reference strain for *R. pseudosolanacearum* and IBSBF292 is the reference strain for *R. solanacearum*.

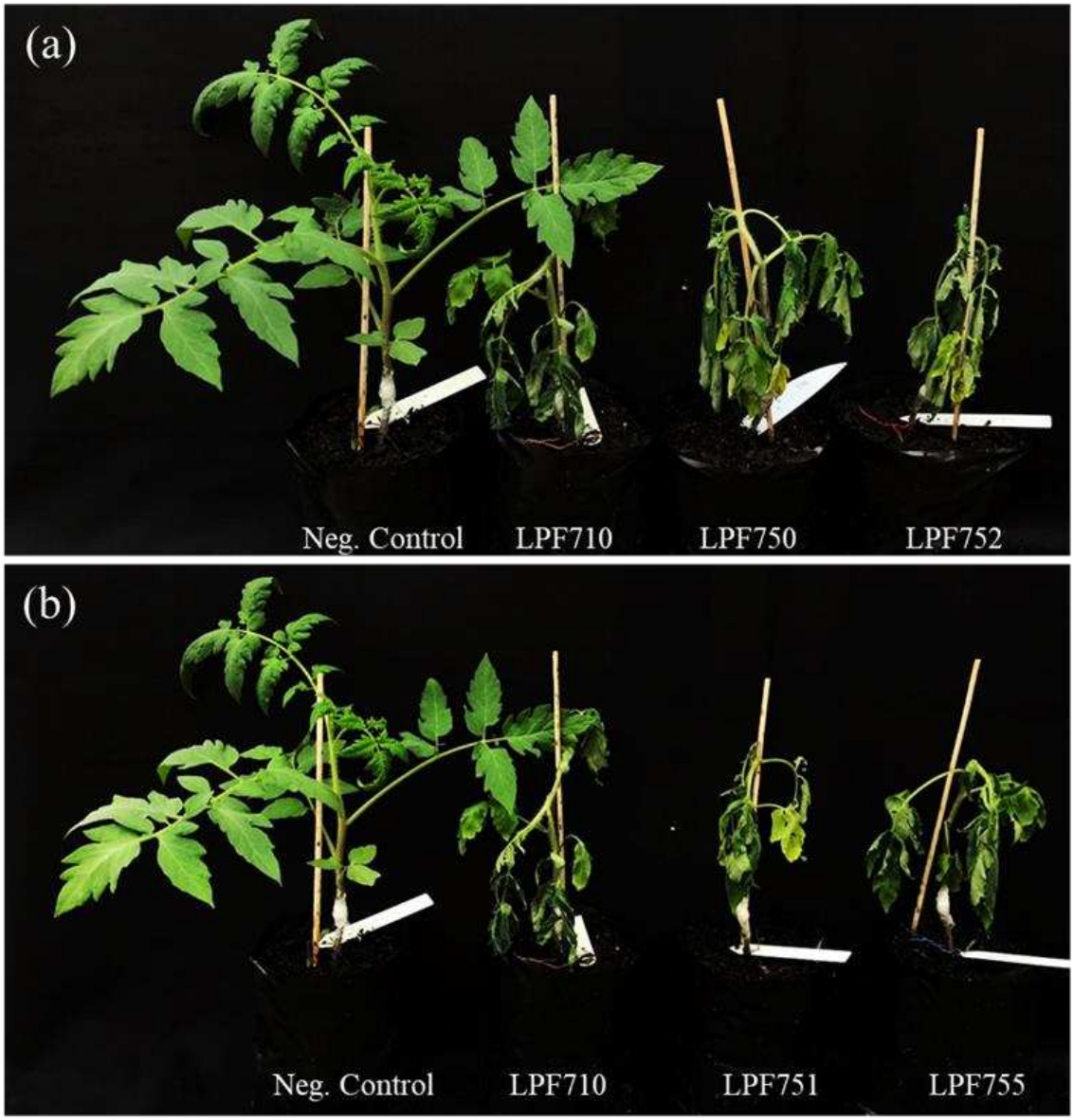


Figure 3. Wilt symptoms in tomato plants (*Solanum lycopersicum* cv Santa Clara) caused by strains of the *Ralstonia solanacearum* species complex collected in Dom Eliseu, Pará, Brazil. Neg. control: treated with 0.85% (w/v) sodium chloride solution; LPF710: positive control strain (*R. solanacearum*). (a) LPF750 and LPF752: phylotype I strains (*R. pseudosolanacearum*). (b) LPF751 and LPF 755: phylotype IIA strains (*R. solanacearum*). Pictures were taken at 5 dai.

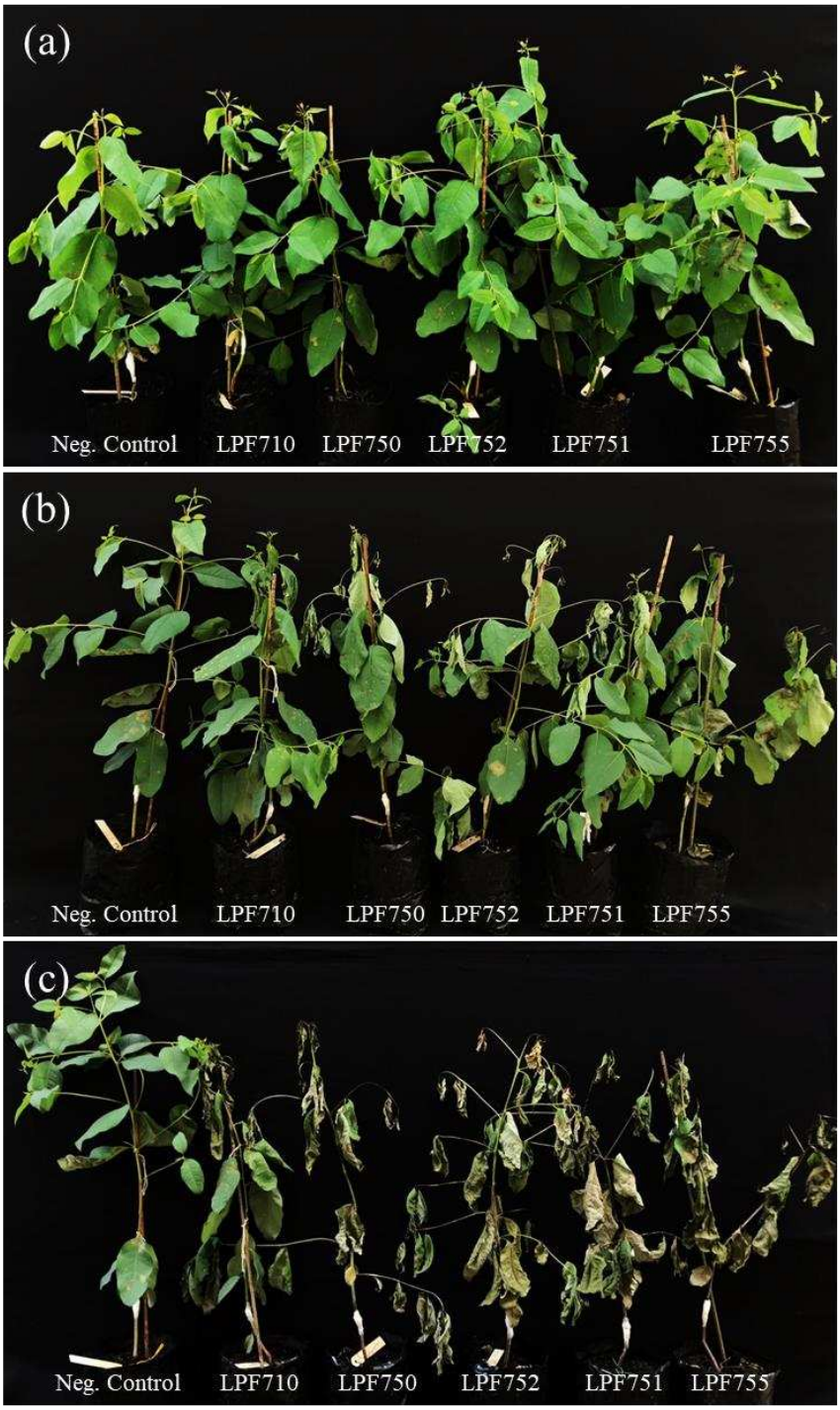
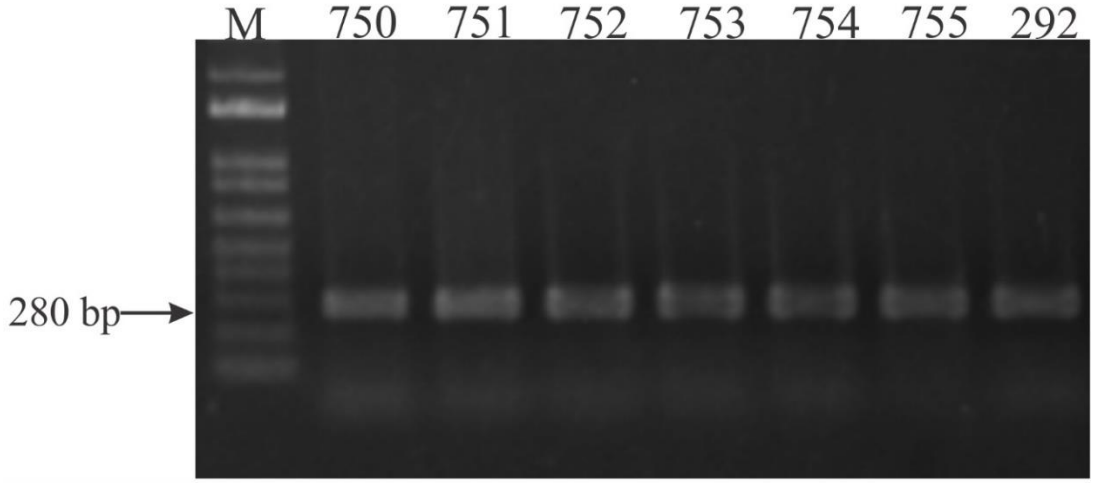
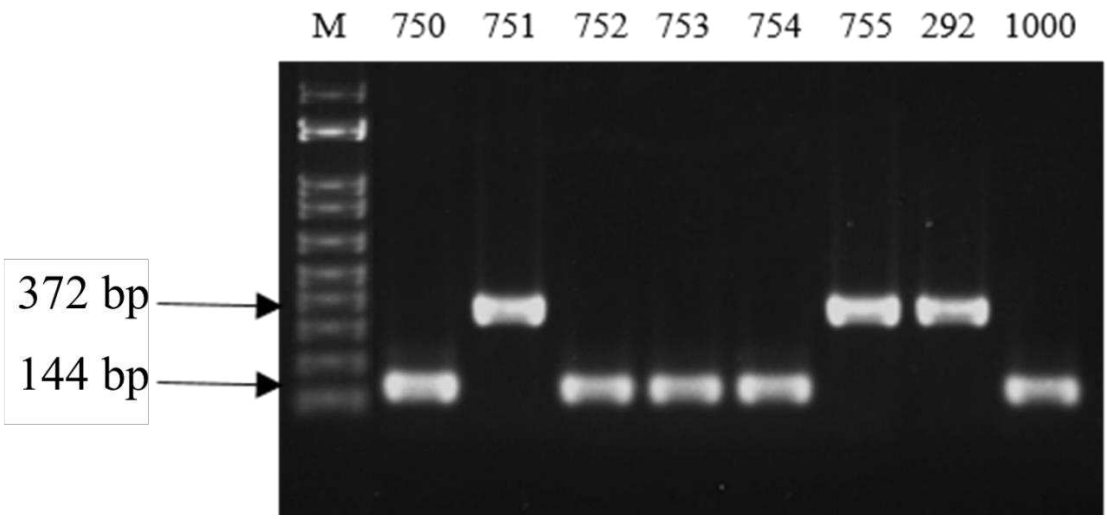


Figure 4. Wilt symptoms in eucalypt plants caused by strains of the *Ralstonia solanacearum* species complex collected in Dom Eliseu, Pará, Brazil. Neg. control: treated with 0.85% (w/v) sodium chloride solution. LPF 710: positive control strain (*R. solanacearum*); LPF750 and LPF752: phylotype I strains (*R. pseudosolanacearum*); LPF751 and LPF755: phylotype II strains (*R. solanacearum*). Pictures were taken at (a) 0 dai; (b) 10 dai and (c) 15 dai.

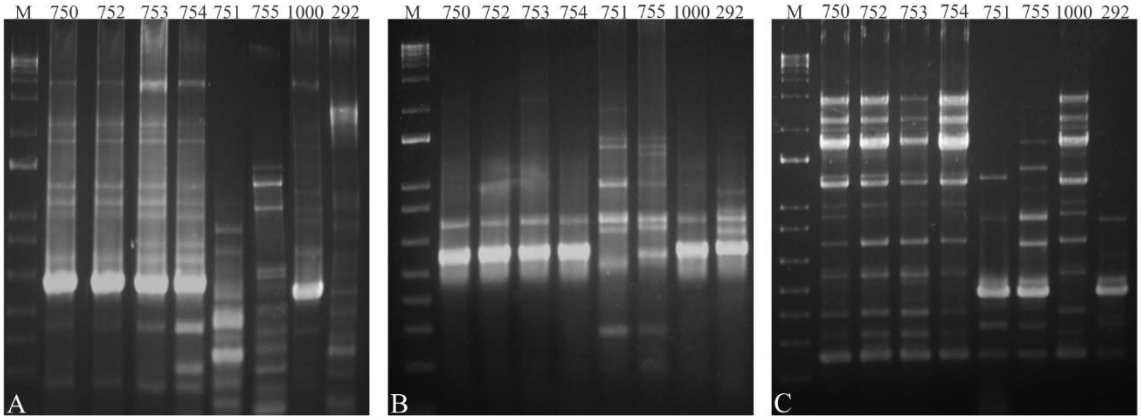
SUPPORTING INFORMATION



Supplementary Figure S1. Products amplified by PCR with primers 759/760 from DNA of strains of the *Ralstonia solanacearum* species complex. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); 750 – 755: LPF750 to LPF755 strains isolated from *Eucalyptus* in Dom Eliseu, Pará, Brazil; 292: *R. solanacearum* strain IBSBF292, used as positive control.



Supplementary Figure S2. Products amplified by multiplex PCR from DNA of strains of the *Ralstonia solanacearum* species complex to determine their phylotype. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); 750, 752, 753 and 754: LPF strains of phylotype I (*R. pseudosolanacearum*); 751 and 755: LPF strains of phylotype IIA (*R. solanacearum*); 292: *R. solanacearum* type strain IBSBF292 (phylotype IIA); 1000: *R. pseudosolanacearum* reference strain GMI1000 (phylotype I).



Supplementary Figure S3. Banding patterns generated by rep-PCR using ERIC (A), BOX (B), and REP (C) primers and DNA from strains of the *Ralstonia solanacearum* species complex. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); 750, 752, 753 and 754: LPF strains of phylotype I (*R. pseudosolanacearum*); 751 and 755; LPF strains of phylotype IIA (*R. solanacearum*). 292: *R. solanacearum* type strain IBSBF292 (phylotype IIA); 1000: *R. pseudosolanacearum* reference strain GMI1000 (phylotype I).

Article 3. Molecular characterization and aggressiveness of the *Ralstonia solanacearum* species complex from *Eucalyptus* spp. in Brazil

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ABSTRACT

Bacterial wilt, one of the world's most destructive diseases in many crops, including eucalypt, is caused by four distinct phylogenetic lineages of the *Ralstonia* species complex, recently classified in three distinct species: *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotype I and III) and *R. syzygii* (phylotype IV). In this study, we characterized 93 *Ralstonia* isolates obtained from eucalypt grown in different Brazilian regions using phylotype and sequevar designations and genomic fingerprinting with BOX-PCR. In addition, we evaluated the aggressiveness of a select group of isolates in two eucalypt clones differing in resistance. We found that 89 isolates belong to *R. solanacearum* (phylotype II) and four to *R. pseudosolanacearum* (phylotype I). Unlike *R. pseudosolanacearum*, *R. solanacearum* was phylogenetically diverse and no correlation was found between sequevar and geographic origin. Most isolates grouped with reference isolates of phylotype IIA sequevar 41, whereas a few others clustered in phylotype IIB, mainly sequevar 4NPB, which is an emergent variant described affecting eucalypt for the first time. Isolates of *R. solanacearum* phylotype IIB were less aggressive to clone CLR371 (susceptible) whereas the *R. pseudosolanacearum* isolate tested was the only one pathogenic to the CLR172 (resistant) clone. Isolate aggressiveness varied between the eucalypt clones tested. The results of this study reinforce the importance of

conducting molecular and aggressiveness characterization of the pathogen population to develop management strategies aimed at the deployment of host resistance in eucalypt breeding programs.

Keywords: Bacterial wilt, endoglucanase, management, rep-PCR, phylotype, resistance.

INTRODUCTION

Bacterial wilt (BW), caused by *Ralstonia solanacearum* species complex (RSSC) is one of the world's most destructive plant diseases, which has also caused great negative impacts on eucalypt in nurseries and plantations in Brazil. In 2005, there were an estimated loss of about US$ 27 million, without considering costs imposed by changes in management practices, replacement of genotypes used for planting and delays in planting schedules (Alfenas et al., 2009). *Ralstonia* infection causes permanent wilt as a result of xylem vessels colonization and host cell wall degradation (Mafia et al., 2012). BW was first reported in *Eucalyptus* in 1980 in Prata, State of Minas Gerais (Sudo et al., 1983). Few years later, another focus of the disease was detected in Monte Dourado, State of Pará, where approximately 30% of plants wilted (Dianese & Takatsu, 1985). In addition to Brazil, in the early 1980s BW in eucalypt was also reported in China (Cao, 1982). Since these initial reports, this disease has been reported from countries in South America, Africa, Southeast Asia and Australia (Coutinho et al., 2000; Fonseca et al., 2016).

Currently, *R. solanacearum sensu lato* (s.l.) is composed of four distinct phylogenetic lineages grouped into three species: *R. solanacearum* (phylotype II – originated from America), *R. pseudosolanacearum* (phylotype I – originated from Asia and phyloype III – originated from Africa) and *R. syzygii* (phylotype IV – originated from Indonesia) (Safni et al., 2014; Fegan & Prior 2005). Until 2020, *R. solanacearum* was the only species reported infecting *Eucalyptus* in Brazil (Fonseca et al., 2014). However, *R. pseudosolanacearum* phylotype I was recently reported causing wilt in eucalypt plantations (Freitas et al., 2020).

In addition to classification in species and phylotypes, further separation into sequevars based on endoglucanase (*egl)* gene sequences added a more detailed classification scheme of the species complex (Poussier et al., 2000). The *egl* gene codes for a protein that partially degrades host cell walls and is located on the megaplasmid (Roberts et al., 1988; Castillo & Greenberg, 2007). A new sequevar is identified when

two or more isolates cluster separately from all known reference strains and exhibit less than 1% variation of the partial *egl* gene sequence (Fegan & Prior, 2005; Wicker et al., 2012). A sequevar may contain isolates with similar patterns of virulence or common geographical origins (Fegan & Prior 2005). Based on *egl* phylogenetic analyses, phylotype II was been divided into phylotypes IIA and IIB (Fegan & Prior 2006). Lineages within particular sequevars can be determined by genomic fingerprinting techniques such as repetitive element palindromic PCR (rep-PCR) (Fegan & Prior, 2005). The RSSC encompasses a great diversity of ecotypes, defined as groups of isolates ecologically similar (Cohan, 2006), sharing similar host ranges, and causing similar disease symptoms under similar climatic conditions (Wicker et al., 2012). Based on the hierarchical classification, genetic variants of the RSSC can be clearly distinguished, providing important insights into the pathogen genetic variability and giving more appropriate tools to identify the causal agent of BW (Li et al., 2016) and a better understanding of the implications for disease management.

Planting of pathogen-free rooted cuttings and use of resistant genotypes are the best management strategies to control BW in *Eucalyptus* (Fonseca et al., 2016). However, a high genetic variability of the RSSC found in Brazil (Fonseca et al., 2014; Santiago et al., 2020) may hamper the effective development of resistant clones in breeding programs. The correct taxonomic classification of the pathogen and a good knowledge of its genetic and aggressiveness variability is a key factor when undertaking epidemiological studies of this species complex that could contribute to disease management. Studies of pathogen aggressiveness provide valuable information on the evolutionary potential of the pathogen and the risk of plant resistance breakdown (Cabrefiga & Montesinos, 2005). In addition, they allow the selection the most aggressive isolates to be used in inoculations for screening genotypes with effective resistance. Because of the high genetic diversity of *R. solanacearum* associated with solanaceous plants (Santiago et al., 2020) and the recent detection of *R. pseudosolanacearum* also causing BW in eucalypt (Freitas et al., 2020), we hypothesized that a study of the genetic variability of the RSSC population associated with eucalypt in Brazil could provide important information to help guide studies aimed at establishing appropriate disease management strategies. As such, this work aimed to molecularly characterize a large collection of RSSC isolates obtained from *Eucalyptus,* to determine to which phylotypes and sequevars they belong, to gain insights into their geographic distribution as well as to assess the aggressiveness variability of a select group.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

A total of 91 RSSC isolates obtained from *Eucalyptus* in Brazil and belonging to the Laboratory of Forest Pathology/Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil (Table S1) were used in this study. Of these, 21 isolates were previously molecularly characterized (Fonseca et al., 2014, Freitas et al., 2020). The remaining 70 isolates were obtained from eucalypt mini-stumps in clonal hedges or from infected trees in the field. Additionally, two isolates (RSB1 and RSB2) studied by Xavier et al. (2018) were included in the analyses. Bacterial DNA was extracted from cultures grown on CPG medium for 24 h using the Wizard Genomic DNA Purification Kit (Promega) as recommended by the manufacturer**.**

Phylotype designation

Multiplex PCR (Fegan & Prior 2005) was used to assign the isolates to phylotypes, and hence, to a validly published bacterial species. Four forward primers were used: Nmult 21:1F, Nmult 21:2F, Nmult 22:Inf and Nmult 23:AF, with one primer in the reverse direction, Nmult 22:RR (Fegan & Prior, 2005). The reaction mix contained 2.0 µl DNA (15 ng/µl), 9.2 µl of GoTaq® Master Mix (Promega), 0.5 µl of each forward primer (10 µM) and 1.0 µl of reverse primer (10 µM) in a final volume of 25 µl. The PCR amplifications were performed using a Veriti 96 thermocycler (Applied Biosystems) with a program consisting of an initial denaturation at 96 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The PCR products were resolved on a 1.5% (w/v) agarose gel in TAE buffer stained with ethidium bromide (1.0 µg/ml) and analysed using an L-PIX gel documentation system (Locus Biotechnology). Identification of phylotypes I, II, III and IV was based on the amplification of expected 144, 372, 91 and 213 bp fragments, respectively.

Sequevar designation

A phylogenetic analysis based on the partial sequence of the *egl* gene was performed to determine the sequevar of the isolates studied. Sequences from the Genbank were used for the isolates previously characterized (Fonseca et al., 2014; Freitas et al.,

2020). For the other isolates, PCR amplification of a 750 bp region of *egl* was performed using the Endo-F and Endo-R primers (Ji et al., 2007). The reaction was carried out in a final volume of 25-µL reaction containing 2.0 µL DNA (15 ng μL^{-1}), 12.5 µL of GoTaq® Master Mix (Promega), 1.5 µL of each primer (10 µM) and ultrapure distilled water. Amplification was performed in a Veriti 96 thermocycler (Applied Biosystems) using the program: initial denaturation at 96 °C for 9 min, 30 cycles at 95 °C for 1 min, 70 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. PCR products were purified using GFX™ PCR DNA and the Gel Band Purification (GE Healthcare) kit, according to the manufacturers' instructions, and sequenced by ABI PRISM 3100 sequencer (Applied Biosystems) using the same Endo-F and Endo-R primers.

Phylogenies were reconstructed using Bayesian inference based on the *egl* sequences with the program MrBayes 3.2 (Ronquist et al., 2012). Akaike information criterion (AIC) in Mega 7.0 (Kumar et al., 2016) was used to choose the substitution model. Markov chain Monte Carlo analysis was conducted with two chains starting from a random tree topology and lasting two millions generations, discarding the first 25% of the trees. The phylogenetic tree was viewed and edited with Figtree 1.4 (http://tree.bio.ed.ac.uk/softw are/figtree/) and Inkscape 0.48.1 (https://inksc ape.org/pt-br/release/inksc ape-0.48.1/). Contigs were assembled with SeqAssem (SequentiX - Digital DNA Processing) and subjected to multiple alignment with *egl* sequences from strains representing different sequevars, collected from different hosts, described in the literature and deposited in GenBank (Table S2). Alignment was conducted using Muscle (Edgar, 2004) and MEGA 7.0 (Kumar et al., 2016) followed by manual adjustments.

Two ecotypes are currently distinguished within sequevar 4, banana Moko disease-causing strains (sequevar 4) and strains designed as Not Pathogenic to Banana, (4NPB), a recently emerged group that does not wilt banana despite its phylogenetic accommodation in sequevar 4 (Prior & Fegan, 2005). Therefore, to distinguish between these two ecotypes in this study, a *Musa*-specific multiplex PCR (Mmx-PCR), which distinguishes sequevars 3, 4, 4NPB and 6, was performed. The multiplex PCR contained a set of four primer pairs (Mus35-F/Mus35-R; Mus20-F/Mus20-R; Mus06-F/Mus06-R; and Si28-F/Si28-R). The expected amplicon sizes for sequevars 3, 4, 4NPB and 6 are 400, 351 + 167 bp (two bands), 351, and 220, respectively. Isolates that do not belong to the *Musa* group (sequevar 3, 4, or 6) do not produce any PCR product when subjected to Mmx-PCR. The reaction mix contained 2.0 µl DNA (15 ng/µl), 9.2 µl of GoTaq® Master

Mix (Promega), 0.5 µl of each primer (10 µM) in a final volume of 25 µl. The PCR amplifications were performed using a Veriti 96 thermocycler (Applied Biosystems) with a program consisting of an initial denaturation at 96 °C for 5 min, followed by 30 cycles of 94 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min (Prior & Fegan, 2005). The PCR products were resolved on a 1.5% (w/v) agarose gel in TAE buffer, stained with ethidium bromide (1.0 µg/ml) and analyzed using an L-PIX gel documentation system (Locus Biotechnology).

Genomic fingerprinting

To classify RSSC isolates into clonal lineages, aliquots of genomic DNA were used as templates to generate repetitive element PCR genomic fingerprints with the BOX A1R primer (5′-CTACGGCAAGGCGACGCTGAC-3′) (Louws et al., 1994). The PCR amplification was conducted in a reaction containing 2.0 µL DNA (15 ng μL^{-1}), 7.5 µL of GoTaq® Master Mix (Promega) and 1.5 µL of primer (10 µM), adjusted to the total volume of 20 µL with ultrapure distilled water. The reaction was performed in a Veriti 96 thermocycler (Applied Biosystems) programmed for an initial denaturation at 95 °C for 7 min, 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min, and a final extension at 65 °C for 15 min. PCR products were analysed on a 2% (w/v) agarose gel at 70V for 4 h 30 min, stained with ethidium bromide (1.0 µg/ml) and photographed using a L-PIX gel documentation system (Loccus Biotechnology) under UV light. Thiourea 200 µM was added to both agarose gel and electrophoresis buffer (TAE) to ensure minimal DNA degradation (Fawley & Wilcox, 2002). To evaluate the reproducibility of banding patterns, the PCR was performed three times. A binary matrix was constructed based on the presence (1) or absence (0) of the amplified fragments. Non-reproducible and weak bands were excluded from the analysis. The results obtained were analysed with PAST 3.06 (Hammer et al., 2001) and tree construction was conducted using the Dice similarity coefficient (Dice, 1945) using the unweighted pair group method with arithmetic means (UPGMA).

Strain aggressiveness

The aggressiveness of a select group of isolates, belonging to different sequevars and species, was evaluated on two eucalypt clones, CLR172 (resistant) and CLR371

(susceptible), both *Eucalyptus urophylla*. Plants were inoculated by injection of a bacterial cell suspension at the base of the stem as previously described (Fonseca et al. 2016). For inoculum preparation, the strains were grown on triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954) at 28 °C. After 48 h, smooth, fluid colonies with white edges and red centers were transferred onto CPG medium and grown at 28 °C for 48 h. Then, bacterial cells were removed from the medium by adding 0.85% (w/v) sodium chloride solution and scraping them from the surface with a Drigalski spatula. Finally, the absorbance at 540 nm of the bacterial suspension was adjusted to 1.0, which corresponds approximately to 1 x 10^9 colony forming units (CFU)/mL (Fonseca et al., 2016).

Forty-day-old clonally propagated cuttings were inoculated with one isolate of *R. pseudosolanacearum* and seven *R. solanacearum* isolates (four of phylotype IIA and three of phylotype IIB) selected based on the molecular characterization. Each bacterial isolate was individually inoculated into five plants of each clone. The same number of plants were treated with 0.85% (w/v) sodium chloride solution instead of bacterial suspension to serve as control. Inoculated plants were kept in a growth chamber at 28 ± 2 °C with a 12-h photoperiod and light intensity of 80 μmoL s^{-1} m^{-2} in a completely randomized design. Wilt symptoms in all plants were evaluated daily for up to 35 days after inoculation (dai). At the end of the experiment, bacterial exudation from five fragments of stem tissue collected every 6 cm from the inoculation point (up to 24 cm above and 12 cm below) from each plant, was evaluated under a light microscope at 100× magnification.

RESULTS

Phylotypes

Of the 93 isolates analysed in this study, a 144 bp fragment, corresponding to phylotype I (*R. pseudosolanacearum*), was amplified only from the four isolates previously characterized by Freitas et al. (2020). A 372 bp fragment, corresponding to phylotype II (*R. solanacearum*), was amplified from the remaining 89 isolates (Table S1).

Sequevars

After manual trimming, a 634-bp sequence alignment of the *egl* gene was used for phylogenetic reconstruction, of which 125 positions were variable. The best evolutionary model selected by Mega 7.0 for Bayesian analysis by AIC was GTR + I + G. The four isolates of *R. pseudosolanacearum* grouped together in a single clade with strain GMI1000 of phylotype I sequevar 18 (Figure 1). All other isolates of the RSSC were distributed among different sequevars of phylotype II, revealing high sequevar diversity. Partial sequencing of the *egl* gene made it possible to confirm the formation of subclades IIA and IIB (Figure 1).

In the subclade IIA, 38 isolates from Pará grouped together with other 13 isolates from different states in sequevar IIA_41, which is the clade with the greatest number of isolates. Eight isolates, also from different states, clustered with the reference strain of phylotype IIA sequevar 38. Isolate UFV32 clustered with phylotype IIA sequevar 37 and isolates UFV34, UFV48, UFV55 and UFV60 did not group in any clade. Isolate AMC22, from Amapá, formed a separate clade along with three isolates from Santa Catarina whereas isolates UFV20 and UFV18 grouped in a separate clade. However, these two clades could not be assigned to any previously known sequevar.

In the subclade IIB, the isolates LPF711 from São Paulo and LPF761 from Minas Gerais, clustered with the reference strain IBSBF1712 (sequevar IIB_27). The isolate LPF727 from São Paulo was not part of any clade and the three isolates from Amapá (LPF805, LPF806 and LPF807) grouped together in a clade with no sequevar designation. The isolate IBSBF624 from Pará clustered with a reference strain of sequevar IIB_26. Fifteen isolates from different states clustered with strains previously classified as sequevar IIB_4. A Mmx-PCR revealed that a specific 351-bp band was amplified from all eucalypt isolates of sequevar IIB_4 analyzed in this study (Figure S1), indicating that they belong to sequevar IIB_4NPB.

Genomic fingerprinting

The number of well-defined and reproducible bands amplified by BOX-PCR varied from 4 – 12 bands, depending on the strain. The sizes of the bands varied between 190 and 2000 (Figure S2). A total of 29 bands were polymorphic and considered for phylogenetic analysis, which allowed identification of 20 haplotypes, excluding the

reference strains GMI1000 and IBSBF292. The isolates were separated into four major groups (Figure 2). The first group was composed by the four *R. pseudosolanacearum* isolates, which grouped with 100% similarity with the reference strain GMI1000. The second group was formed by isolates from different geographic origins which exhibited 100% similarity to the emergent ecotype IIB_4NPB. The third group was composed by phylotype IIA isolates, which shared 70% similarity among them. The isolates of the sequevar IIA_41, with the exception of isolate LPF717, showed 100% similarity among them, even though they originated in several regions of Brazil. The fourth group was formed by the other isolates from phylotype IIB, which had over 60% similarity. The *R. solanacearum* type strain IBSBF292, previously classified in phylotype II, did not cluster with any group.

Strain aggressiveness

The isolate LPF710 (sequevar IIA_41), identified as the most aggressive, induced wilting in all plants of the susceptible clone CLR371 at 15 dai. The other three isolates of phylotype IIA; LPF755 (IIA_41), LPF751 (IIA_38) and AMC22 (IIA_ND), as well as the isolate of *R. pseudosolanacearum* LPF752 (I_18), wilted all plants at 20 dai. The two isolates of phylotype IIB_4NPB, LPF718 and LPF721, were able to wilt only three of the five replicates by the end of the experiment at 35 dai. Finally, the isolate LPF807 (IIB_ND) did not wilt any plant and was considered as non-aggressive (Figure 3). All withered plants presented lesions in the internal tissues and oozed bacteria from stem at all distances from the inoculation point analysed. Although the isolates LPF718 (IIB_4NPB) and LPF721 (IIB_4NPB) did not cause wilt in two plants, they caused internal darkening of the tissues and ooze at all points analysed. The isolate LPF807 (IIB_ND) was the only one that did not cause any symptoms of darkening, however, bacterial ooze was observed at up to 18 cm above and at 12 cm below the inoculation point in all plants.

None of the isolates of *R. solanacearum* phylotype II tested was able to cause wilt or lesion in the inner stem tissues of any of the plants of the resistant clone CLR172 (Figure 4A-G). For most isolates, there was bacterial ooze at the inoculation point but not at several distances from it. Two plants inoculated with the isolates LPF751 (IIA_38) and AMC22 (IIA_ND) showed bacterial ooze at 6 and 12 cm above the inoculation point. However, for the isolate of *R. pseudosolanacearum* LPF752, two plants showed slowly

progressive wilt symptoms at 28 dai, but these plants did not die by 35 dai (Figure 4H). Only these two plants showed internal darkening of the tissues, but four plants showed bacterial ooze in all evaluated points; one exuded bacteria at up to 12 cm below and 18 cm above de inoculation point.

DISCUSSION

The results of this work demonstrate that *R. solanacearum* (phylotype II) is the main species associated with bacterial wilt of *Eucalyptus* in Brazil. It is a phylogenetically diverse species exhibiting no correlation between sequevar designation and geographic origin. Phylogenetic analysis of the partial sequencing of the *egl* gene showed that some clades were formed by isolates from different states, indicating that different sequevars are spread throughout Brazil. Infected planting material, either from eucalypt or other hosts, may contribute to its dissemination. According to Wicker et al. (2012), several lines of evidence indicate that Brazil is the origin center of this phylotype, which, afterwards, differentiated into subclades IIA and IIB. The authors claim that phylotype IIB is nearly clonal, but the phylotype IIA is recombinogenic, highly diverse and expanding its geographic distribution.

Most isolates of the subclade IIA clustered in sequevar 41, including UFV7 and AMC76, previously classified in this sequevar (Fonseca et al. 2014). Santiago et al. (2017) detected isolates of sequevar 41 in different hosts, including eucalypt, as well as from different Brazilian regions. Albuquerque et al. (2014) also detected this sequevar in banana in the Northeastern region, suggesting a possible wide host range. Isolates IBSBF623, IBSBF625, IBSBF2131, UFV12 and UFV13 did not cluster in a defined sequevar in a previous work (Fonseca et al. 2014), however in our study they clustered in sequevar 38 along with three other isolates. For designation of isolates to this sequevar, Fonseca et al. (2014) used a different reference strain, CIP120, which is currently classified as sequevar 50 (Gutarra et al., 2017). The strain IBSBF625 had already been classified as sequevar 38 (Carstensen et al., 2016), which was corroborated by the results of our work. The previously characterized isolate UFV32 (Fonseca et al., 2014) is the only one that still remains classified as sequevar 37.

The other isolates of subclade IIA did not form a clade with any of the reference strains and remained without sequevar designation. Isolates AMC22, IBSBF2777, IBSBF2568, and IBSBF2576 as well as isolates UFV20 and UFV18 grouped in a single

clade. Due to the low support of the branches, no new sequevars were assigned to these clades. Additional studies with a larger number of isolates are necessary to better delimit the formation of these clusters. Sequevar 50, widely distributed in Brazil and associated with eucalypt and solanaceous plants (Santiago et al., 2017), was not detected in this work.

The strain IBSBF624 of subclade IIB, previously characterized by Fonseca et al. (2014), is the only one that still remains classified as sequevar 26. Santiago et al. (2017) detected sequevar 27 only in eucalypt and in the Northern region of the country, which suggested it to have a narrow host range. In this study, we detected two isolates of this sequevar from the Southeastern region, indicating that it may be more widespread. The three isolates from Amapá, which probably belong to a new sequevar, clustered in a clade supported by a branch with 83% a posteriori probability. The wide host range sequevar 56, recently reported by Santiago et al. (2017) in eucalypt, was not found here.

Sequevar IIB_4NPB, a genotype variant that was first described in the French West Indies in anthurium (Wicker et al., 2007), was detected in 15 isolates. This emerging ecotype does not infect banana but is highly aggressive to eggplant, pepper and tomato, and already overcame a resistance present in tomato 'Hawaii 7996' (Wicker et al., 2007). In an agressiveness test conducted by Lebeau et al. (2011), a strain of this sequevar from *Heliconia caribea* in Martinique overcame the resistance expressed in 26 out of 30 accessions tested. In Brazil, the first report of sequevar IIB_4NPB was in 1998 in *Cucumis sativus* (Wicker et al., 2007), but it is currently widespread in Latin America. Santiago et al. (2017) reported for the first time the occurrence of *R. solanacearum* IIB_4NPB in sassafras oil-producing *Piper hispidinervum* (long pepper). These authors claimed that this variant seemed to be restricted to the Northern region, but our results showed its occurrence in the Northeastern, Central and Southeastern regions as well. This is the first report of this variant causing wilt in *Eucalyptus* both in field and nursery plants. This new report increases the range of crops affected by this *R. solanacearum* variant.

Phylotype I, *R. pseudosolanacearum*, was first reported in eucalypt in Brazil in the state of Pará (Freitas et al., 2020). Although Lopes & Rossato (2018) claimed that this species is widely distributed in 17 Brazilian states causing wilt symptoms in solanaceous plants, in our work we identified this species in only four among 93 isolates studied. Santiago et al. (2017) identified only sequevar 18 among 48 *R. pseudosolanacearum* isolates from solanaceous plants in Brazil, indicating no genetic diversity and a probable

recent entry of this phylotype in this country. This observation is supported by results of the present study.

Genomic fingerprint with BOX-PCR formed four major groups composed by *R. pseudosolanacearum* isolates, the emergent ecotype IIB_4NPB, phylotype IIA isolates and phylotype IIB isolates. Despite belonging to phylotype IIB, 4NPB isolates have less than 20% similarity with the other isolates of this phylotype. The *R. pseudosolanacearum*, IIB_4NPB and IIA_41 (except for the strain LPF717) groups have isolates with 100% similarity among them, indicating the presence of a single clonal lineage. Using the same technique with RSSC populations from tomato and potato in Brazil, Santiago et al. (2020) found three major groups. One group was composed by *R. pseudosolanacearum* isolates, a second group was formed by *R. solanacearum* isolates predominantly from the Southern, Southeastern and Central regions, and a third group was formed by isolates of *R. solanacearum* from the Northern and Northeastern regions. In their work, the subpopulation of the Northern region could be clearly differentiated from those of all other geographic regions. However, no relationship between the isolates clustering and geographic origin was observed in our study.

The high genetic diversity of the Brazilian isolates of *R. solanacearum* raises important issues for disease management. As with many pests and diseases, the most desirable approach for efficient control of this disease in *Eucalyptus* is breeding for resistance (Wingfield et al., 2013) and knowledge on the aggressiveness of isolates is important for the selection of resistant genetic material. To investigate whether there is variation of aggressiveness between the two species of *Ralstonia* and among the different sequevars, eight strains were chosen to be inoculated in two eucalypt clones, previously classified as susceptible (CLR371) and resistant (CLR172) to bacterial wilt.

Isolates of phylotype IIA and one of *R. pseudosolanacearum* were more aggressive on clone CLR371 than isolates from phylotype IIB, as also found in previous studies for tobacco (Gutarra et al. 2017). In our study, all selected isolates of phylotype IIA were able to cause wilt in all plants during the evaluation period, showing no significant differences in aggressiveness among them. The isolate of phyloype IIB LPF807, although did not cause any disease symptoms, caused plant to ooze bacteria at distances far from the inoculation point, which may result in a negative effect in later plant development. It is worth noting that the isolates of phylotype IIB were less aggressive in this clone, but their aggressiveness may vary according to the genetic background of the inoculated plant. This is especially important for the 4NPB variant,

which is already known to be very aggressive in other crops (Lebeau et al., 2011), but we still do not know its potential to infect *Eucalyptus*. The isolate of *R. pseudosolanacearum* was the only one capable of causing symptoms in plants of clone CLR172. These results suggest the existence of differences in the ability to cause disease between the two *Ralstonia* species in this particular clone and reinforces the importance of the molecular characterization and correct identification of RSSC strains for selection of genotypes resistant to bacterial wilt. Nonetheless, additional investigation with a larger number of strains from both species is necessary to unravel any aggressiveness difference between the two *Ralstonia* species.

In summary, the present study expands the knowledge of the variability of the RSSC in *Eucalyptus* in Brazil. The emergence of new sequevars might be a result of a continuous and rapid evolution of this pathogen. Santiago et al. (2017) hypothesized that the high mutation and recombination rates contributed to originate new sequevars. In addition, the results of this work show that isolates of the RSSC infecting eucalypt in Brazil exhibit genetic and aggressiveness variation, reinforcing the importance of a clear understanding of the prevalence of the bacterial genetic variants and their aggressiveness in particular geographic areas in order to develop efficient management strategies for bacterial wilt of eucalypt. Studies involving a larger number of genetically diverse strains and a larger number of eucalypt clones and species are pivotal in the search for sources of resistance and correct selection of resistant genetic material.

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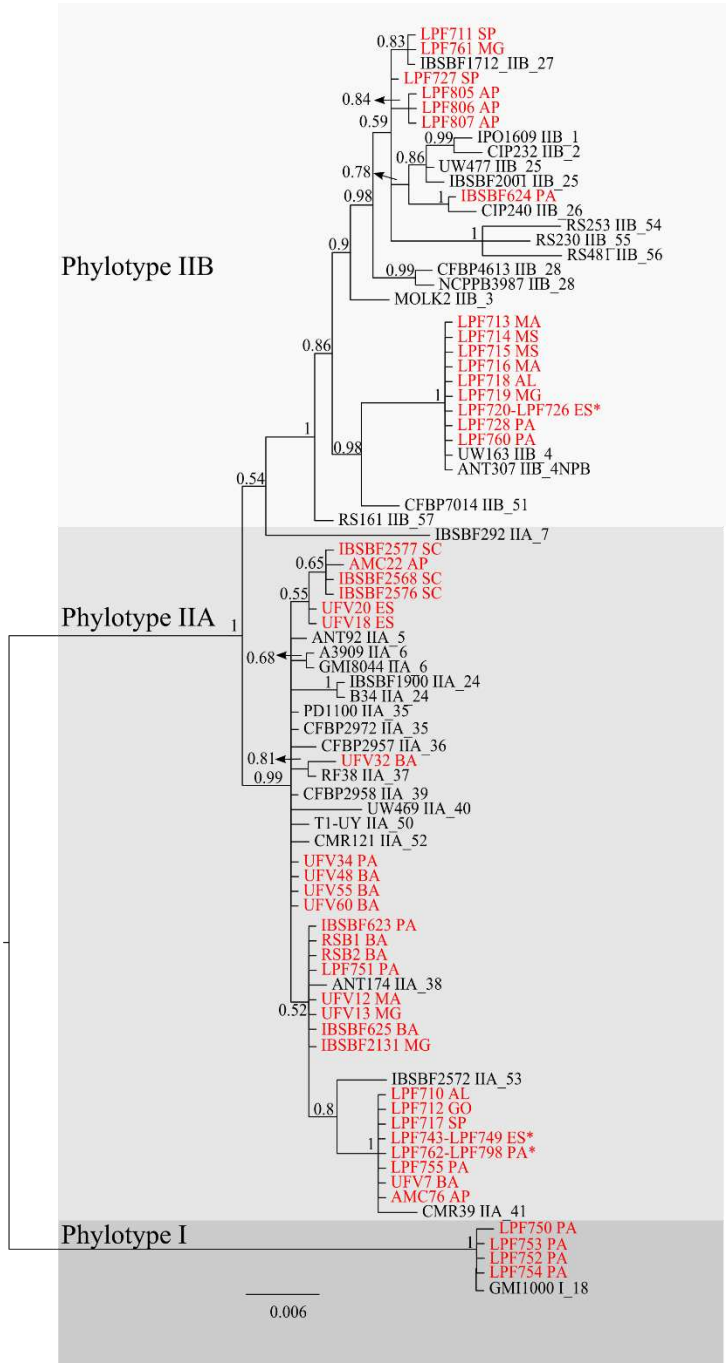


Figure 1. Phylogenetic tree based on partial *egl* gene sequences from strains of the *Ralstonia solanacearum* species complex constructed using Bayesian inference. The *a posteriori* probability values are indicated on the branches. The bar indicates the fraction of substitutions per site. Strains from the Laboratory of Forest Pathology / Universidade Federal de Viçosa are highlighted in red with their codes followed by the origin state. Reference strains are shown in black followed by phylotype_sequevar designations. Asterisks: LPF720-LPF726 represents a group of seven isolates, LPF743-LPF749 represents a group of seven isolates and LPF762-LPF798 represents a group of 37 isolates, which were named using consecutive LPF numbers.

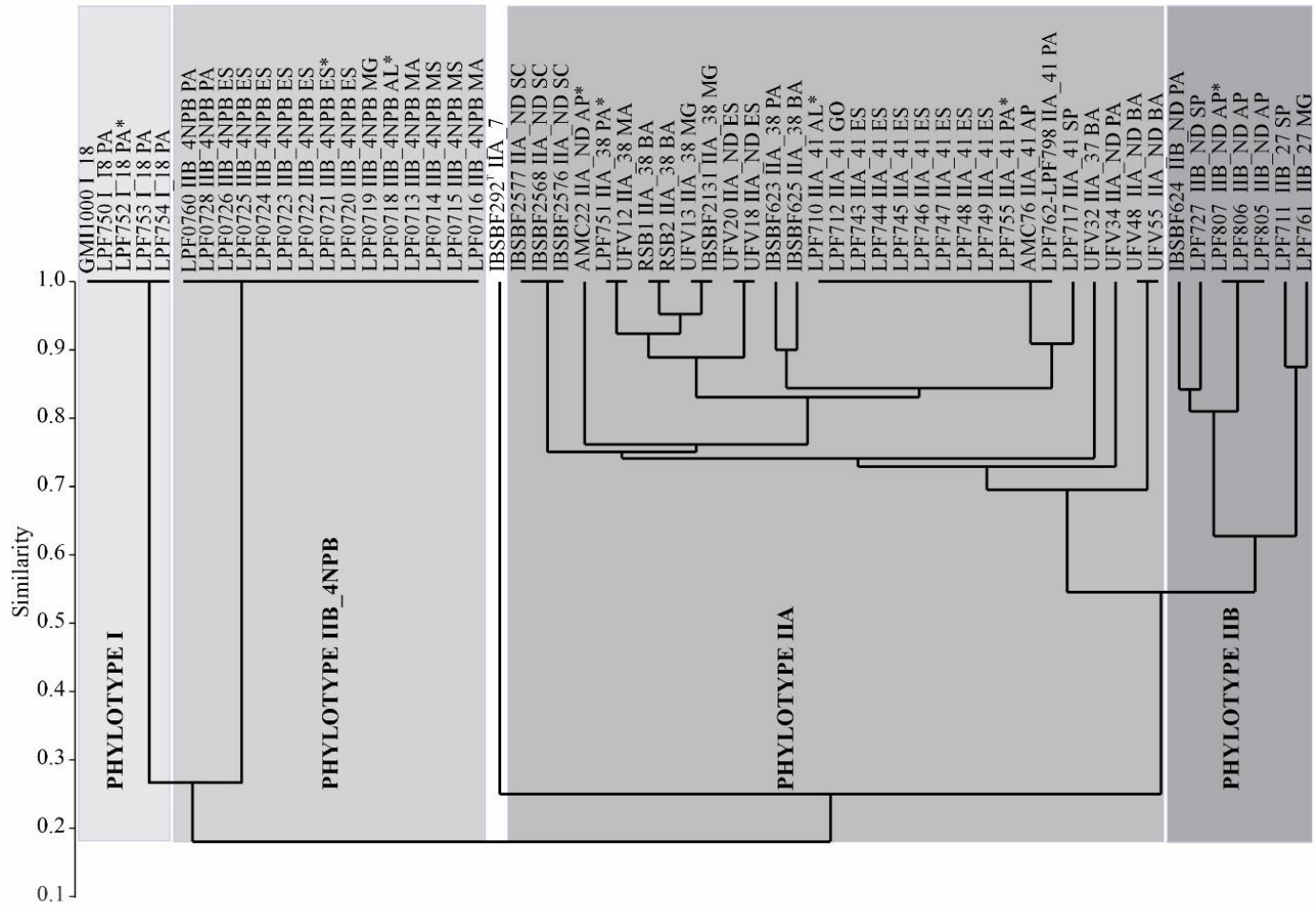


Figure 2. Dendrogram based on BOX-PCR profiles of strains of the *Ralstonia solanacearum* species complex from *Eucalyptus* in Brazil. Differences among profiles are indicated by the percentage similarity. The dendrogram was constructed using the Dice's similarity coefficient and UPGMA clustering method. Strains codes are followed by phylotype_sequevar designations and their origin states. ND: sequevar not determined. GMI1000 is the reference strain for *R. pseudosolanacearum* and IBSBF292 is the reference strain for *R. solanacearum*. Asterisk: Strains used for aggressiveness tests.

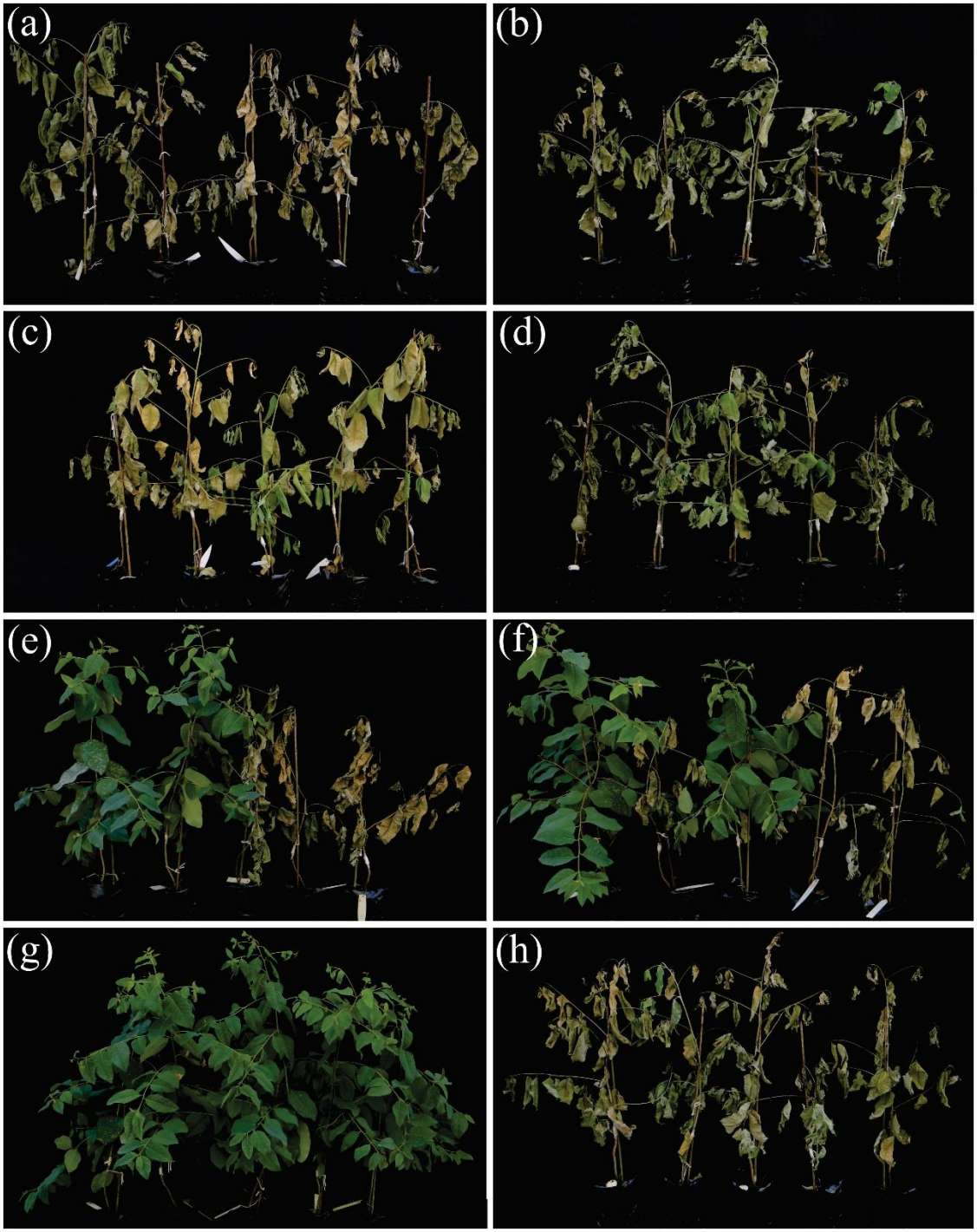


Figure 3. Aggressiveness of strains of the *Ralstonia solanacearum* species complex in *Eucalyptus* clone CLR371 at 35 dai. (a) LPF710 IIA_41; (b) LPF755 IIA_41; (c) LPF751 IIA_38; (d) AMC22 IIA_ND; (e) LPF718 IIB_4NPB; (f) LPF721 IIB_4NPB; (g) LPF807 IIB_ND and (h) LPF752 I_18. Strain codes are followed by phylotype_sequevar designations. ND: sequevar not determined.

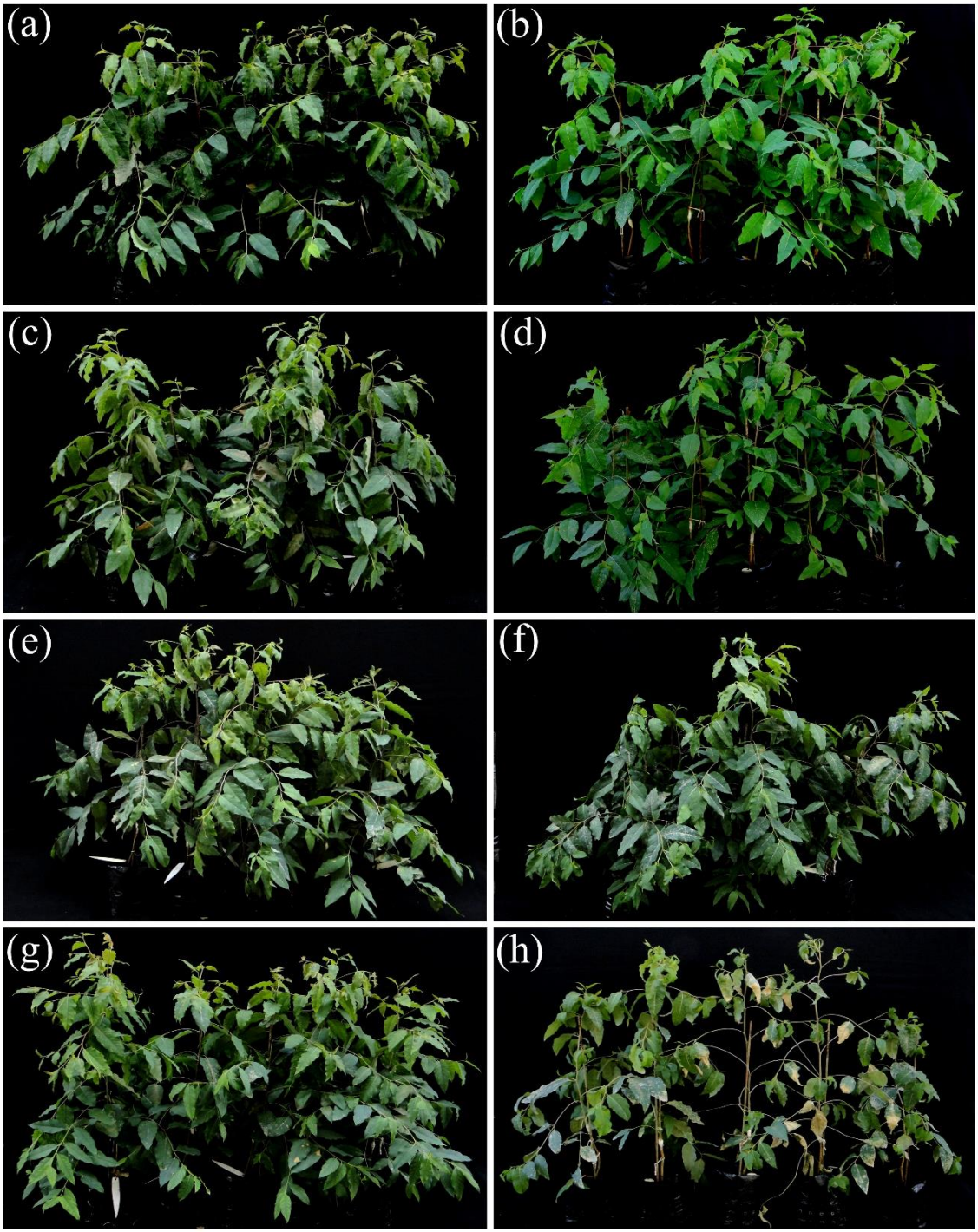


Figure 4. Aggressiveness of strains of the *Ralstonia solanacearum* species complex in *Eucalyptus* clone CLR172 at 35 dai. (a) LPF710 IIA_41; (b) LPF755 IIA_41; (c) LPF751 IIA_38; (d) AMC22 IIA_ND; (e) LPF718 IIB_4NPB; (f) LPF721 IIB_4NPB; (g) LPF807 IIB_ND and (h) LPF752 I_18. Strain codes are followed by phylotype_sequevar designations. ND: sequevar not determined.

SUPPORTING INFORMATION

Supplementary Table S1. Strains of the *Ralstonia solanacearum* species complex isolated from *Eucalyptus* sp. in Brazil and used in this study.

Strain	Origin	Material	Phylotype_ sequevar	*egl* GenBank Acession	Collection year
UFV32[a]	Bahia	Ministump	IIA_37	KF357864	2005
UFV48[a]	Bahia	Ministump	IIA_ND	KF357866	2005
UFV55[a]	Bahia	Ministump	IIA_ND	KF357867	2005
UFV13[a]	Minas Gerais	Ministump	IIA_38	KF357860	2005
UFV12[a]	Maranhão	Ministump	IIA_38	KF357859	2005
UFV18[a]	Espírito Santo	Ministump	IIA_ND	KF357861	2005
UFV20[a]	Espírito Santo	Ministump	IIA_ND	KF357862	2005
UFV34[a]	Pará	Ministump	IIA_ND	KF357865	2005
IBSBF623	Pará	-	IIA_38	MW381114	1987
IBSBF624[b]	Pará	-	IIB_26	KF357871	1987
IBSBF625[b]	Bahia	-	IIA_38	KF357872	1987
IBSBF2131[b]	Minas Gerais	-	IIA_38	KF357874	-
IBSBF2568[b]	Santa Catarina	-	IIA_ND	KF357875	-
IBSBF2576[b]	Santa Catarina	-	IIA_ND	KF357876	-
IBSBF2577	Santa Catarina	-	IIA_ND	MW381115	-
AMC76[a]	Amapá	-	IIA_41	KF357870	-
AMC22[a]	Amapá	-	IIA_ND	KF357869	-
RSB1[c]	Bahia	-	IIA_38	MW381133	-
RSB2[c]	Bahia	-	IIA_38	MW381134	-
LPF710	Alagoas	Trunk	IIA_41	MW381111	2014
LPF711	São Paulo	Trunk	IIB_ND	MW381162	2013
LPF712	Goiás	Ministump	IIA_41	MW381112	2014
LPF713	Maranhão	Trunk	IIB_4NPB	MW381163	2015
LPF714	Mato Grosso do Sul	Trunk	IIB_4NPB	MW381164	2015
LPF715	Mato Grosso do Sul	Trunk	IIB_4NPB	MW381165	2015
LPF716	Maranhão	Ministump	IIB_4NPB	MW381166	2016
LPF717	São Paulo	Trunk	IIA_41	MW381113	2016
LPF718	Alagoas	Trunk	IIB_4NPB	MW381167	2016
LPF719	Minas Gerais	Cutting	IIB_4NPB	MW381168	2017
LPF720	Espírito Santo	Ministump	IIB_4NPB	MW381169	2017
LPF721	Espírito Santo	Ministump	IIB_4NPB	MW381170	2017
LPF722	Espírito Santo	Ministump	IIB_4NPB	MW381171	2017
LPF723	Espírito Santo	Ministump	IIB_4NPB	MW381172	2017
LPF724	Espírito Santo	Ministump	IIB_4NPB	MW381173	2017
LPF725	Espírito Santo	Ministump	IIB_4NPB	MW381174	2017
LPF726	Espírito Santo	Ministump	IIB_4NPB	MW381175	2017

Supplementary Table S1. (continued)

LPF727	São Paulo	Trunk	IIB_ND	MW381176	2018
LPF728	Pará	Ministump	IIB_4NPB	MW381177	2018
LPF743	Espírito Santo	Ministump	IIA_41	MW381116	2018
LPF744	Espírito Santo	Ministump	IIA_41	MW381117	2018
LPF745	Espírito Santo	Ministump	IIA_41	MW381118	2018
LPF746	Espírito Santo	Ministump	IIA_41	MW381119	2018
LPF747	Espírito Santo	Ministump	IIA_41	MW381120	2018
LPF748	Espírito Santo	Ministump	IIA_41	MW381121	2018
LPF749	Espírito Santo	Ministump	IIA_41	MW381122	2018
LPF750[d]	Pará	Trunk	I_18	MN496142	2019
LPF751[d]	Pará	Trunk	IIA_38	MN496147	2019
LPF752[d]	Pará	Trunk	I_18	MN496143	2019
LPF753[d]	Pará	Trunk	I_18	MN496144	2019
LPF754[d]	Pará	Trunk	I_18	MN496145	2019
LPF755[d]	Pará	Rooted cutting	IIA_41	MN496146	2019
LPF760	Pará	Ministump	IIB_4NPB	MW381178	2019
LPF761	Minas Gerais	Ministump	IIB_ND	MW381179	2019
LPF762	Pará	Trunk	IIA_41	MW381123	2019
LPF763	Pará	Trunk	IIA_41	MW381124	2019
LPF764	Pará	Trunk	IIA_41	MW381125	2019
LPF765	Pará	Trunk	IIA_41	MW381126	2019
LPF766	Pará	Trunk	IIA_41	MW381127	2019
LPF767	Pará	Trunk	IIA_41	MW381128	2019
LPF768	Pará	Trunk	IIA_41	MW381129	2019
LPF769	Pará	Trunk	IIA_41	MW381130	2019
LPF770	Pará	Trunk	IIA_41	MW381131	2019
LPF771	Pará	Trunk	IIA_41	MW381132	2019
LPF772	Pará	Trunk	IIA_41	MW381135	2019
LPF773	Pará	Trunk	IIA_41	MW381136	2019
LPF774	Pará	Trunk	IIA_41	MW381137	2019
LPF775	Pará	Trunk	IIA_41	MW381138	2019
LPF776	Pará	Trunk	IIA_41	MW381139	2019
LPF777	Pará	Trunk	IIA_41	MW381140	2019
LPF778	Pará	Trunk	IIA_41	MW381141	2019
LPF779	Pará	Trunk	IIA_41	MW381142	2019
LPF780	Pará	Trunk	IIA_41	MW381143	2019
LPF781	Pará	Trunk	IIA_41	MW381144	2019
LPF782	Pará	Trunk	IIA_41	MW381145	2019
LPF783	Pará	Trunk	IIA_41	MW381146	2019
LPF784	Pará	Trunk	IIA_41	MW381147	2019

Supplementary Table S1. (continued)

LPF785	Pará	Trunk	IIA_41	MW381148	2019
LPF786	Pará	Trunk	IIA_41	MW381149	2019
LPF787	Pará	Trunk	IIA_41	MW381150	2019
LPF788	Pará	Trunk	IIA_41	MW381151	2019
LPF789	Pará	Trunk	IIA_41	MW381152	2019
LPF790	Pará	Trunk	IIA_41	MW381153	2019
LPF791	Pará	Trunk	IIA_41	MW381154	2019
LPF792	Pará	Trunk	IIA_41	MW381155	2019
LPF793	Pará	Trunk	IIA_41	MW381156	2019
LPF794	Pará	Trunk	IIA_41	MW381157	2019
LPF795	Pará	Trunk	IIA_41	MW381158	2019
LPF796	Pará	Trunk	IIA_41	MW381159	2019
LPF797	Pará	Trunk	IIA_41	MW381160	2019
LPF798	Pará	Trunk	IIA_41	MW381161	2019
LPF805	Amapá	Trunk	IIB_ND	MW381180	2019
LPF806	Amapá	Trunk	IIB_ND	MW381181	2019
LPF807	Amapá	Trunk	IIB_ND	MW381182	2019

[a] Strains previously characterized by Fonseca et al. (2014).

[b] Strains from Instituto Biológico, São Paulo, previously characterized by Fonseca et al. (2014).

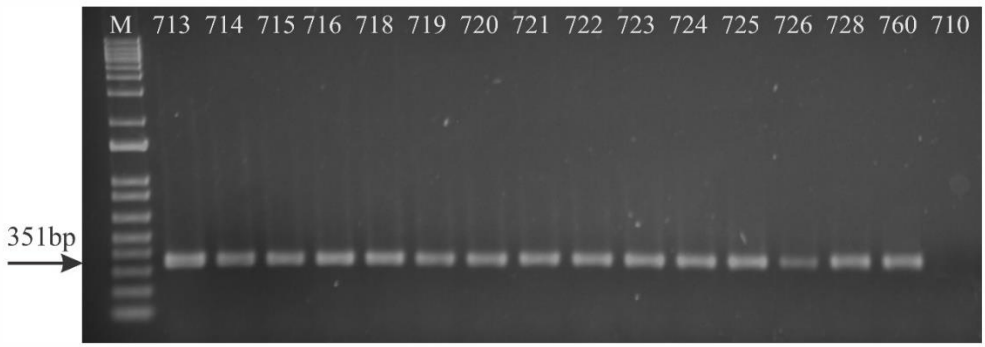
[c] Strains obtained from Xavier et al. (2018).

[d] Strains previously characterized by Freitas et al. (2020).

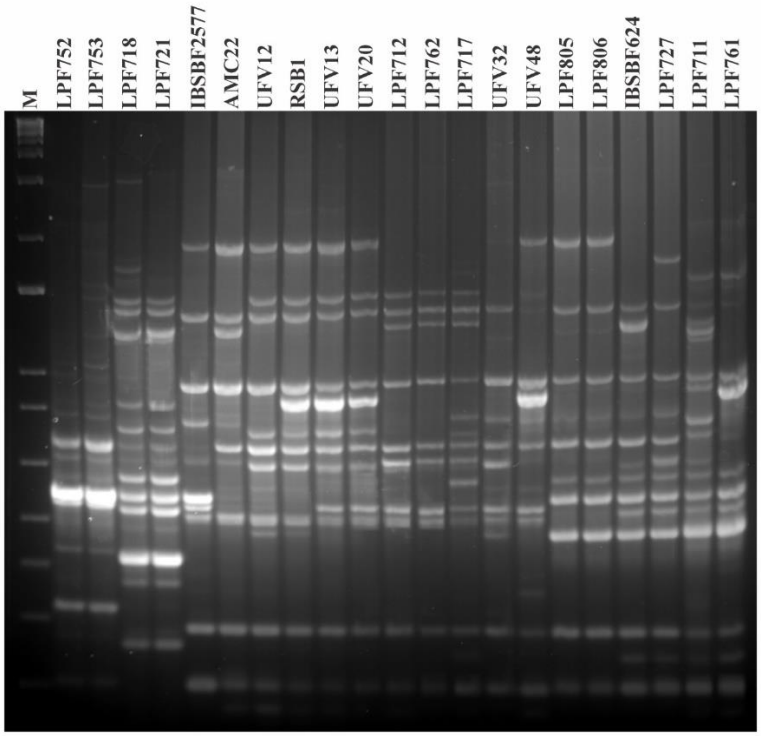
ND: Sequevar not determined.

Supplementary Table S2. Strains of the *Ralstonia solanacearum* species complex used as reference in phylogenetic analyses.

Phylotype	Sequevar	Strain	Origin	Host	GenBank Accession
I	18	GMI1000	French Guiana	*Solanum lycopersicum*	AF295251
IIB	1	IPO1609	Netherlands	*Solanum tuberosum*	EF371814
IIB	2	CIP232	Chile	*Solanum tuberosum*	EF647734
IIB	3	MOLK2	Philippines	*Musa* sp.	EF371841
IIB	4	UW163	Peru	*Musa* sp.	GU295052
IIB	4NPB	ANT307	Martinique	*Anthurium* sp.	EF371813
IIA	5	ANT92	Martinique	-	EF371828
IIA	6	GMI8044	Grenada	*Musa sp.*	GU295013
IIA	6	A3909	Hawaii	*Heliconia*	EF371812
IIA	7	IBSBF292	U.S.A	*Solanum lycopersicum*	HM142850
IIA	24	B34	Brazil	*Musa* sp.	GQ907154
IIA	24	IBSBF1900	Brazil	*Musa* sp.	EF371839
IIB	25	UW477	Peru	*Solanum tuberosum*	AF295260
IIB	25	IBSBF2001	Brazil	*Solanum lycopersicum*	GU295017
IIB	26	CIP240	Brazil	*Solanum lycopersicum*	EF647739
IIB	27	ISBSF1712	Brazil	*Pelargonium hortorum*	EF371833
IIB	28	NCPPB3987	Brazil	*Solanum tuberosum*	AF295261
IIB	28	CFBP4613	Brazil	*Solanum tuberosum*	GU294982
IIA	35	CFBP2972	Martinique	*Solanum tuberosum*	EF371809
IIA	35	PDI100	Egypt	*Solanum tuberosum*	GU295043
IIA	36	CFBP2957	Martinique	*Solanum lycopersicum*	EF371807
IIA	37	RF38	Trinidad	*Solanum lycopersicum*	JF702309
IIA	38	ANT174	Martinique	*Canna indica*	EF371835
IIA	39	CFBP2958	Guadeloupe	*Solanum lycopersicum*	AF295266
IIA	40	UW469	Brazil	*Solanum lycopersicum*	AF295269
IIA	41	CRM39	Indonesia	*Solanum lycopersicum*	EF439726
IIA	50	T1-UY	Uruguay	*Solanum lycopersicum*	GU295049
IIB	51	CFBP7014	Trinidad	*Anthurium* sp.	EU726798
IIA	52	CMR121	Cameroon	*Solanum lycopersicum*	EF439725
IIA	53	IBSBF2572	Brazil	*Musa* sp.	KF875427
IIB	54	RS253	Brazil	*Solanum tuberosum*	KT630047
IIB	55	RS230	Brazil	*Solanum tuberosum*	KT630057
IIB	56	RS481	Brazil	*Eucalyptus* sp.	KT630065
IIB	57	RS161	Brazil	*Solanum tuberosum*	KT630005



Supplementary Figure S1. 351 pb-fragment amplified by multiplex PCR from DNA of isolates of *Ralstonia solanacearum* sequevar 4, indicating that they belong to ecotype 4NPB. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); 713-760: LPF strains that belong to phylotype IIB and sequevar 4NPB (IIB_4NPB); 710: LPF strain that do not belong to the *Musa* group (sequevar 3, 4, or 6) used as negative control.



Supplementary Figure S2. Representative banding patterns of the four major groups formed by BOX-PCR using DNA from strains of the *Ralstonia solanacearum* species complex. M: molecular marker, 1 Kb Plus DNA Ladder (GeneRuler^{TM}); LPF752-LPF753: *R. pseudosolanacearum* strains; LPF718-LPF721: phylotype IIB and sequevar 4NPB (IIB_4NPB) strains; IBSBF2577-UFV48: phylotype IIA strains; and LPF805-LPF761: phylotype IIB strains.

FINAL REMARKS

From the results obtained, it can be concluded that:

1- In the first article of this study we adapted a qPCR protocol useful to detect and quantify *R. solanacearum,* showing that the bacterium translocates acropetally and basipetally in both susceptible and resistant eucalypt clones. However, differential pathogen populations dynamics were observed, with mature biofilms occluding the xylem vessels in the susceptible clone whereas only single cells or small aggregates were observed in the resistant one.

2- The second article demonstrates that, like *R. solanacearum*, *R. pseudosolanacearum* (phylotype I) also causes eucalypt wilt in Brazil, however, no genetic diversity was observed. The two bacterial species should be taken into consideration in genetic breeding programmes and studies involving the aggressiveness of these two species in eucalypt are of great importance for the development of resistant genetic material.

3- The third article expands the knowledge of the variability of *Ralstonia solanacearum* species complex in *Eucalyptus* in Brazil, being *R. solanacearum* (phylotype II) the main species associated with bacterial wilt. There is difference in aggressiveness of the isolates from eucalypt, reinforcing the importance of the molecular characterization to develop management strategies and to screen for effective plant resistance in breeding programs.