Plant Pathol. J. 34(4) : 269-285 (2018)
https://doi.org/10.5423/PPJ.OA.01.2018.0014
pISSN 1598-2254 eISSN 2093-9280

Research Article Open Access

The Plant Pathology Journal

Xanthomonas axonopodis pv. *eucalyptorum* pv. nov. Causing Bacterial Leaf Blight on Eucalypt in Brazil

Hélvio Gledson Maciel Ferraz[1], Jorge Luis Badel[1], Lúcio Mauro da Silva Guimarães[1], Bruna Paolinelli Reis[1], Marcos Rogério Tótola[2], Rivadalve Coelho Gonçalves[3], and Acelino Couto Alfenas[1]*

[1]*Department of Phytopathology, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil*
[2]*Department of Microbiology, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil*
[3]*EMBRAPA Acre, BR 364, Km 14, Zona Rural, CEP 69.908.970, Caixa Postal 321, Rio Branco, AC, Brazil*

(Received on January 30, 2018; Revised on April 2, 2018; Accepted on April 17, 2018)

Bacterial leaf blight is a major disease of eucalypt, especially under nursery conditions. Different bacterial species have been associated with the disease in several countries, and despite its importance worldwide, it is not clear to date whether similar disease symptoms are caused by the same or by different etiological agents. In this study, 43 bacterial strains were isolated from blighted eucalypt leaves collected in different geographic areas of Brazil and inoculated onto a susceptible eucalypt clone. Polyphasic taxonomy, including morphological, physiological, biochemical, molecular, and pathogenicity tests showed that only certain strains of *Xanthomonas axonopodis* caused symptoms of the disease. Strains varied in their aggressiveness, but no correlation with geographic origin was observed. MLSA-based phylogenetic analysis using concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* gene sequences allocated the strains in a well-defined clade, corresponding to Rademarker's group RG 9.6. Inoculation of nineteen plant species belonging to seven botanical families with representative strain LPF 602 showed it to be pathogenic only on *Eucalyptus* spp, and *Corymbia* spp. Based on distinct biochemical and pathogenic characteristics that differentiate the eucalypt strains from other pathovars of the *X. axonopodis* species, here we propose their allocation into the new pathovar *X. axonopodis* pv. *eucalyptorum* pv. nov.

Keywords **:** *Eucalyptus* spp., fatty acid profile, metabolism fingerprinting, multilocus sequence analysis, *Xanthomonas axonopodis*

Handling Associate Editor **:** Sohn, Kee Hoon

Bacterial leaf blight is a major disease of eucalypt, especially under nursery conditions. In the period 2003-2008, the disease caused significant losses of clonal rooted cuttings and mini-stumps in several nurseries of Brazil, estimated at approximately 7.5 million dollars (Alfenas et al., 2009). Symptoms of the disease are characterized by the presence of water-soaked lesions, and interveinal necrosis, frequently accompanied by central perforations. The disease can also affect stems and branches (Neves et al., 2014). Intense defoliation is commonly observed in susceptible genotypes under conditions favorable for the disease.

In Brazil, the first records of bacterial leaf blight of eucalypt date back to the mid-1990s, when *Pseudomonas cichorii* (Pomella et al., 1995) and *Xanthomonas* sp. (Reis et al., 1996) were found to be associated with symptomatic nursery plants in the state of São Paulo. More recently, Gonçalves et al. (2008) reported that *X. axonopodis*, *X. campestris*, *P. syringae*, *P. putida*, *P. cichorii*, *Erwinia* sp. and strains belonging to the Rhizobiaceae family can also be recovered from diseased nursery and field plants in several regions of the country.

Different etiologic agents have been reported to be associated with eucalypt bacterial leaf blight in several other countries. Truman (1974) described similar disease symptoms affecting *Corymbia citriodora* (Hook) Hill and John-

*Corresponding author.
Phone) +55-31-3899-2937, FAX) +55-31-3899-2240
E-mail) aalfenas@ufv.br

son in Australia, and concluded that they were caused by *X. deleyii* subsp. *eucalypti* (Truman) Dye (synonym *X. campestris* pv. *eucalypti*). In South Africa, similar symptoms were observed in different eucalypt species, hybrids, and clones, and the disease was attributed to *Pantoea ananatis* (Serrano) Mergaert et al. (Coutinho et al., 2002) and, more recently, *X. vasicola* was also associated with similar disease symptoms in clones of *Eucalyptus grandis* W. Hill (Coutinho et al., 2015). In Thailand, a similar bacterial blight observed in seedling beds of *Eucalyptus camaldulensis* × *E. deglupta* and *E. grandis* under commercial production was attributed to *X. axonopodis* pv. *eucalypti* (Pothiluk et al., 2013).

Despite the high importance of leaf blight for eucalypt production worldwide, it remains to be conclusively determined whether similar symptoms are caused by the same or by different bacterial pathogens in several countries. In the study reported by Gonçalves et al. (2008), the authors found that isolates of *X. axonopodis, P. cichorii* and others belonging to the Rhizobiaceae family induced typical symptoms of the disease when spray inoculated onto a hybrid clone of *E. urophylla* × *E. maidenii*, which is susceptible to the disease upon natural infection. It is also not clear whether several bacterial pathogens have evolved to infect *Eucalyptus* spp. Recently, Coutinho et al. (2015) suggested that *X. vasicola* underwent a host jump from sugarcane to *E. grandis* in South Africa.

It is currently accepted that the genus *Xanthomonas* consists of at least 28 plant-associated species that infect more than 120 species of monocot and 260 species of dicot plants (Hayward, 1993; Samanta et al., 2013; Young et al., 2010). Successful classification of *Xanthomonas* at the infraspecific level has been accomplished using multilocus sequence analysis (MLSA) (Ah-You et al., 2009; Ferraz et al., 2017; Samanta et al., 2013; Young et al., 2008; 2010). MLSA is a simple and rapid technique that allows accurate allocation of strains in their respective taxonomic groups, in most cases reflecting their classification based on DNA-DNA reassociation techniques (Young et al., 2008).

We conducted an extensive sampling of eucalypt rooted cuttings with symptoms of bacterial leaf blight in different regions of Brazil and a polyphasic approach that included MLSA in order to identify the bacterium responsible for the disease. We found that *Xanthomonas axonopodis* was consistently associated with leaf blight and was the only species, among those recovered in this study, that caused such symptoms on eucalypt. Host range determination and cross-inoculation experiments revealed that the bacterium belongs to a pathovar not previously described.

Materials and Methods

Bacterial strains. Leaf samples were collected between September 2012 and June 2013 from *Eucalyptus* spp. plants showing leaf blight in five geographic regions of Brazil: Southeast, South, Midwest, North and Northeast. For confirmation of the bacterial etiology of the disease, symptomatic leaf tissue was subjected to the exudation test. To this end, fragments of approximately 0.5 × 0.5 cm were taken from the lesion margins and placed on a drop of sterile water on a glass slide and observed under a light microscope (magnification × 200). Leaf tissue showing bacterial exudation was washed with a neutral detergent, rinsed three times with sterile water, and macerated. Loopfuls of the macerate were streaked on solid 523 medium (Kado and Heskett, 1970) for bacterial isolation. Pure cultures were obtained from individual colonies after incubation at 28°C for 48 h. Bacterial strains were preserved in 30% glycerol stocks stored at –80°C for later use.

Pathogenicity tests. Clone CLR368, a *E. urophylla* × *E. globulus* hybrid previously characterized as highly susceptible to bacterial leaf blight (Clonar Resistência a Doenças Florestais, unpublished data), was used to determine the pathogenicity of the strains. For this, 60-day old cuttings were transplanted into 2-dm^3 plastic bags containing the commercial substrate MecPlant (Mecprec Indústria e Comércio Ltda., Telêmaco Borba, PR, Brazil) supplemented with 6 kg m^{-3} of single superphosphate and fertilized with 1.5 kg m^{-3} Osmocote (19 : 6 : 10) (Scotts Australia Pty Ltd., Bella Vista, Australia). Thirty days after planting, cuttings of clone CLR368 were inoculated with suspensions of the bacterial strains prepared from a culture grown on solid 523 medium at 28°C for 24 h. The suspension was adjusted to an OD_{600} = 0.25 (equivalent to 10^8 cfu ml^{-1}) and sprayed onto the eucalypt leaves using a De Vilbiss 1.5 HP air compressor (DeVilbiss Air Power Company, Jackson, TN, USA). Three individual cuttings were inoculated with each strain. The cuttings were maintained in a mist irrigation chamber at 26°C for 24 h before and for 24 h after inoculation. Subsequently, the plants were returned to a greenhouse until the appearance of bacterial blight symptoms. Percent of leaf area affected by the disease was determined using the diagrammatic scale proposed by Gonçalves (2003), choosing the ten leaves with the highest severity and then calculating the mean disease severity for each plant. Such a scale of disease severity assessment was developed by scanning symptomatic leaves at 300 dpi, saving the images in a bmp format and quantifying the af-

fected leaf area with the program Quant v1.0 (Vale et al., 2003). The experiments were conducted in completely randomized designs, the data subjected to ANOVA, and the treatment means compared with the Scott-Knott grouping test ($P \leq 0.05$). Statistical analyses were performed with the SAS software (SAS Institute Inc., Cary, NC, USA).

***16S rDNA* sequencing and analysis.** For DNA extraction, bacterial strains were grown in 5 ml of liquid 523 medium at 28°C for 48 h in the dark. One ml of bacterial culture was transferred to a microfuge tube and centrifuged at 13,000 rpm for 3 min. The DNA was extracted from bacterial cells using the Wizard Genomic DNA Purification kit (Promega Corporation, Mannheim, Germany) according to the manufacturer's instructions. The DNA of each isolate was diluted to a concentration of 10 ng μl^{-1} for conducting the PCR. Primers fd2 (5-AGAGTTTGATCCTGGCTCAG-3) and rP1 (5-ACGGTTACCTTGTTACGACTT-3) were used to PCR amplify the *16S rDNA* region (Weisburg et al., 1991) in a reaction mixture containing 12.5 µl of Dream Taq PCR Master Mix 2X (MBI Fermentas, Hanover, MD), 1.5 µl of 10 µM of each primer, and 2 µl of genomic DNA in a total volume of 25 µl. PCR amplification was performed by an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were confirmed for their expected sizes by running 5 µl on a 1.4% agarose gel at 100 V for 60 min, purified with the PCR kit GFX DNA and Gel Band Purification Kit (GE Healthcare Life Sci., Sao Paulo, SP, Brazil), and their sequences determined in an Applied Biosystems 3500XL Series Genetic Analyser (Applied BioSystems, Foster City, CA) using the same primers used for DNA amplification. The sequences were analyzed using Navigator version 1.0.1 (Applied BioSystems) and compared with sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov) using BLASTN (Altschul et al., 1997).

Multilocus sequence analysis (MLSA). PCR amplifications were carried out with primers specific for the four housekeeping genes *dnaK*, *fyuA*, *gyrB* and *rpoD* (Young et al., 2008) using the same reaction mixture as for amplification of the *16S rDNA* region. PCR amplification was performed with initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 10 min. The sequences of the PCR products were obtained and analyzed as for *16S rDNA* and subsequently aligned using Clustal W v.1.5 (Thompson et al., 1994) followed by manual adjustments in MEGA 5.00 (Tamura et al., 2011), when necessary. Because *dnaK*, *fyuA*, *gyrB* and *rpoD* genes encode proteins, it was verified that the insertion of gaps in the alignment did not alter their protein sequences.

Phylogenetic trees were constructed with sequences of the four housekeeping genes obtained in this work, those reported by Young et al. (2008) for *Xanthomonas* spp., and others extracted from the Genbank (http://www.ncbi.nlm.nih.gov) (Table 1). In the first phylogenetic analysis, sequences from validly published *Xanthomonas* species were used (Bull et al., 2010; 2012). The second phylogenetic analysis was conducted using housekeeping gene sequences of several different *X. axonopodis* pathovars (Table 1). In this study, we adopted the *Xanthomonas* classification and nomenclature proposed by Vauterin et al (1995). When appropriate, the most recently published bacterial names are placed in parentheses.

Bayesian Inference (BI) methods were used to construct phylogenetic trees of concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* gene sequences. BI was performed with MrBayes 3.1.2 (Bayesian Inference of Phylogeny) (Ronquist and Huelsenbeck, 2003). MrModelTest 3.2 was used to choose the substitution model based on the Akaike information criterion (AIC). The probability of an *a posteriori* tree distribution was calculated using an MCMC algorithm (Metropolis-coupled Markov Chain Monte Carlo) of two chains from a random tree with 10 million generations, discarding 25% of the first trees. The Tracer 1.4 program was used to examine the MCMC convergence and effective sample size. FigTree 1.3.1. (http://tree.bio.ac.uk/software) was used to view and edit the phylogenetic trees.

Biochemical and physiological tests. The following tests were performed on the eucalypt strains according to the Laboratory Guide for Identification of Plant Pathogenic Bacteria (Schaad et al., 2001): (i) Ryu's non-staining KOH test for Gram determination; (ii) yellow pigmentation of colonies on YDC (yeast extract-dextrose-calcium carbonate agar) medium; (iii) xanthomonadin production; (iv) fluorescence production on King's B medium; (v) anaerobic growth; (vi) oxidase test; (vii) catalase activity; (viii) utilization of asparagine as a sole carbon and nitrogen source; (ix) urease activity; (x) gelatin liquefaction; (xi) starch hydrolysis; (xii) H_2S production from cysteine; (xiii) esculin hydrolysis; and (xiv) NO_3 reduction.

Metabolic fingerprinting. The Biolog GN microPlate system (Microlog 2, Version 3.5, Biolog Inc.) was used to test the ability of the strains to metabolize different carbon sources. For that, trypticase soy agar (TSA) medium was inoculated with a single colony obtained from a nutrient

Table 1. *Xanthomonas* strains used in multilocus sequence analysis

Bacterial strain	Species name	Synonym	GenBank accession number			
			dnaK	*fyuA*	*gyrB*	*rpoD*
ICMP 196[a]	*Xanthomonas albilineans*		-	-	EU498963	EU499082
ICMP 35[a]	*X. arboricola* pv. *juglandis*		EU498750	EU498852	EU498951	EU499070
ICMP 50[a]	*X. axonopodis* pv. *axonopodis*		EU498751	EU498853	EU498952	EU499071
CFBP 6369	*X. axonopodis* pv. *allii*		CM002866	CM002866	CM002866	CM002866
ICMP 5718[b]	*X. axonopodis* pv. *alfalfae*	*X. alfalfae* subsp. *alfalfae*	EU498792	EU498894	EU499001	EU499120
ICMP 8432	*X. axonopodis* pv. *aurantifolii*	*X. fuscans* subsp. *aurantifolii*	EU498811	EU498913	EU499027	EU499146
ICMP 194[b]	*X. axonopodis* pv. *begoniae*		EU498757	EU498859	EU498962	EU499081
ICMP 444[b]	*X. axonopodis* pv. *cajani*		EU498767	EU498869	EU498973	EU499092
ICMP 24[a]	*X. axonopodis* pv. *citri*	*X. citri* subsp. *citri*	EU498749	EU498851	EU498950	EU499069
ICMP 10009	*X. axonopodis* pv. *citrumelo*	*X. alfalfae* subsp. *citrumelonis*	EU498826	EU498926	EU499042	EU499162
DXD 01	*X. axonopodis* pv. *commiphorae*		JN898928	JN621250	JN621253	JN621257
LMG 695	*X. axonopodis* pv. *dieffenbachiae*		CP014347	CP014347	CP014347	CP014347
ICMP 5732[b]	*X. axonopodis* pv. *glycines*		EU498794	EU498896	EU499003	EU499122
ICMP 217	*X. axonopodis* pv. *malvacearum*	*X. citri* subsp. *malvacearum*	EU498760	EU498862	EU498966	EU499085
LMG 941	*X. axonopodis* pv. *mangiferaeindicae*		CAHO01000008	HQ590941	CAHO01000002	CAHO01000012
ICMP 5741[b]	*X. axonopodis* pv. *manihotis*		EU498796	EU498898	EU499006	EU499125
ICMP 5834[b]	*X. axonopodis* pv. *phaseoli*	*X. phaseoli*	EU498802	EU498904	EU499015	EU499134
ICMP 239[a]	*X. axonopodis* pv. *phaseoli* (biovar. *fuscans*)	*X. fuscans* subsp. *fuscans*	EU498761	EU498863	EU498967	EU499086
NCPPB 466	*X. axonopodis* pv. *punicae*		JN790906	JN790914	JN790930	JN790938
ICMP 3031	*X. axonopodis* pv. *ricini*		EU498782	EU498884	EU498991	EU499110
ICMP 5757[b]	*X. axonopodis* pv. *vasculorum*		EU498798	EU498900	EU499011	EU499130
ICMP 333[b]	*X. axonopodis* pv.*vignicola*		EU498764	EU498866	EU498970	EU499089
ICMP 109[a]	*X. axonopodis* pv. *vesicatoria*	*X. euvesicatoria*	EU498754	EU498856	EU498955	EU499074
ICMP 16690[a]	*X. axonopodis* pv. *vesicatoria*	*X. perforans*	EU498844	EU498944	EU499059	EU499179
ICMP 12545[a]	*X. bromi*		EU498837	EU498937	EU499052	EU499172
ICMP 6541	*X. campestris* pv. *campestris*		EU498747	EU498849	EU498948	EU499067
ICMP 204[a]	*X. cassavae*		EU498759	EU498861	EU498965	EU499084
ICMP 9513[a]	*X. codiaei*		EU498822	EU498922	EU499038	EU499158
ICMP 2299[a]	*X. cucurbitae*		EU498780	EU498882	EU498989	EU499108
ICMP 16775[a]	*X. cynarae*		EU498846	EU498946	EU499061	EU499181
ICMP 2415[a]	*X. dyei* pv. *dysoxyli*		EU498781	EU498883	EU498990	EU499109
ICMP 5715[a]	*X. fragariae*		EU498791	EU498893	EU499000	EU499119
ICMP 16689[a]	*X. gardneri*		EU498843	EU498943	EU499058	EU499178
ICMP 453[a]	*X. hortorum*		EU498769	EU498871	EU498975	EU499094
ICMP 189[a]	*X. hyacinthi*		-	-	EU498960	EU499079
ICMP 8683	*X. melonis*		EU498816	EU498916	EU499032	EU499152
ICMP 3125[a]	*X. oryzae* pv. *oryzae*		EU498784	EU498886	EU498993	EU499112
ICMP 570[a]	*X. pisi*		EU498770	EU498872	EU498976	EU499095
ICMP 5816[a]	*X. populi*		EU498801	EU498903	EU499014	EU499133
ICMP 16916[a]	*X. sacchari*		-	-	EU499063	EU499183

Table 1. Continued

Bacterial strain	Species name	Synonym	GenBank accession number			
			dnaK	*fyuA*	*gyrB*	*rpoD*
ICMP 6774[a]	*X. theicola*		-	-	EU499020	EU499139
ICMP 5752[a]	*X. translucens* pv. *translucens*		-	-	EU499009	EU499128
ICMP 3103[a]	*X. vasicola*		EU498783	EU498885	EU498992	EU499111
ICMP 63[a]	*X. vesicatoria*		EU498753	EU498855	EU498954	EU499073
ICMP 17033[a]	*Stenotrophomonas maltophilia*		EU498848	-	EU499066	EU499186

CFBP- Collection Française de Bactéries Phytopathogènes
ICMP- International Collection of Microorganisms from Plants
LMG- BCCM/LMG Bacteria Collection, Laboratory for Microbiology
NCPPB- National Collection of Plant Pathogenic Bacteria
[a]Type strain of species
[b]Pathotype strain

agar (NA) culture and incubated at 28°C for 24 h. Then, a bacterial suspension was prepared in saline solution, the absorbance adjusted to match the turbidity standards of the Biolog GN MicroPlate system, and 150 µl pipetted into each well. The microplates were incubated at 28°C and the reactions rated as positive or negative at 48 h of incubation.

Fatty acid analysis. The Microbial Identification System (MIS; MIDI Inc., Newark, DE, USA) was used to determine the whole cell fatty acid methyl ester (FAME) profiles of the eucalypt strains. Fatty acids were extracted from cultures on trypticase soy agar (BD BBL, São Paulo, Brazil) grown at 28°C for one day according to the procedures described in the MIS Handbook. Extracts were analyzed with a Hewlett-Packard 7890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA), which provided automatic identification and quantification of fatty acids by comparison with internal FAME standards. Fatty acid profiles of eucalypt strains were compared to those of reference strains deposited in the MIS database. To this end, similarity indices were generated by the Sherlock MIS software comparing the fatty acid profiles of eucalypt strains with those of known *Xanthomonas* species and pathovars deposited in the MIS database.

Host range and cross-inoculation. Bacterial strains isolated from eucalypt leaves clustered together in Rademaker's group RG 9.6 (Rademaker et al. 2005). Therefore, LPF 602 was selected to represent the strains isolated from *Eucalyptus* spp. Strain LPF 602 was used to inoculate plants belonging to different botanical families. Myrtaceae: *Eucalyptus grandis* W. Hill, *E. urophylla* S. T. Blake, *E. robusta* Sm., *E. saligna* Smith, *E. globulus* Labill., *E. cloeziana* F. Muell, *E. camaldulensis* Dehnh, *Corymbia maculata* (Hook.) K. D. Hill & L. A. S. Johnson, *Psidium guajava* L., *Myrciaria jaboticaba* (Vell.) O. Berg. and *Eugenia jambolana* Lam.; Rosaceae: *Prunus persica* (L.) Batsch; Caricaceae: *Carica papaya* L.; Fabaceae: *Phaseolus vulgaris* L. and *Pisum sativum* L.; Solanaceae: *Solanum lycopersicum* Mill.; Rutaceae: *Citrus limon* (L.) Burm. f.; Euphorbiaceae: *Mabea fistulifera* Mart. and Anacardiaceae: *Schinus terebinthifolius* Raddi. Twenty plants of each species were inoculated with bacterial suspensions of strain LPF 602.

Also, cross inoculation experiments were conducted with LPF 602 and strains of the *X. axonopodis* pathovars phylogenetically most closely related to the eucalypt strains and that clustered in Rademaker's group RG 9.6. Based on the results of MLSA, it was decided to inoculate strain LPF 602 onto plants of common bean (*Phaseolus vulgaris* L.) and acid lime (*Citrus* × *aurantiifolia* (Christm.) Swingle). Representative strains of *X. axonopodis* pv. *vignicola* (IBSBF 1739 = NCPPB 1838 = ICMP 333 = ATCC 11648), *X. axonopodis* pv. *aurantifolii* (*X. fuscans* subsp. *aurantifolii*) (IBSBF 380 = NCPPB 3654 = CFBP 2905), and *X. axonopodis* pv. *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*) (UNB 772) were inoculated onto rooted cuttings of clone CLR368 (*E. urophylla* × *E. globulus*) as well as on susceptible plants of their corresponding host species. Plants of common bean were inoculated separately with *X. axonopodis* pv. *vignicola* and *X. axonopodis* pv. *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*), because it is susceptible to both pathovars. Plant maintenance, inoculum preparation and inoculation were conducted as described for pathogenicity tests. Bacterial strains inoculated onto their respective host species served as positive controls for disease.

Table 2. *Xanthomonas axonopodis* strains collected in Brazil

Strain	Host species	Collection city/State*	Brazilian region	Growth conditions	GenBank accession number				
					16S rDNA	*dnaK*	*fyuA*	*gyrB*	*rpoD*
LPF564	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Nursery	KY288830	KY287807	KY287848	KY287889	KY460467
LPF565	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Nursery	KY288831	KY287808	KY287879	KY287890	KY460503
LPF566	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Nursery	KY288832	KY287809	KY287849	KY287891	KY460504
LPF567	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Nursery	KY288833	KY287810	KY287880	KY287892	KY460468
LPF568	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Field	KY288834	KY287811	KY287850	KY287893	KY460469
LPF569	*Eucalyptus urophylla*	Curvelo/ MG	Southeast	Field	KY288835	KY287812	KY287851	KY287894	KY460470
LPF571	*Eucalyptus urophylla*	Niquelândia/ GO	Midwest	Nursery	KY288837	KY287814	KY287852	KY287896	KY460472
LPF572	*Eucalyptus urophylla*	Niquelândia/ GO	Midwest	Nursery	KY288838	KY287815	KY287853	KY287897	KY460473
LPF573	*E. grandis x E. urophylla*	Niquelândia/ GO	Midwest	Field	KY288839	KY287816	KY287854	KY287898	KY460501
LPF574	*E. grandis x E. urophylla*	Niquelândia/ GO	Midwest	Field	KY288840	KY287817	KY287855	KY287899	KY460474
LPF575	*E. urophylla x E. maidenii*	Guaíba/ RS	South	Field	KY288841	KY287818	KY287856	KY287900	KY460475
LPF577	*Eucalyptus grandis*	Paracatu/ MG	Southeast	Nursery	KY288842	KY287819	KY287857	KY287901	KY460476
LPF578	*Eucalyptus grandis*	Paracatu/ MG	Southeast	Nursery	KY288843	KY287820	KY287858	KY287902	KY460477
LPF582	*E. grandis x E. urophylla*	Limeira/ SP	Southeast	Nursery	KY288847	KY287824	KY287860	KY287906	KY460481
LPF588	*E. grandis x E. urophylla*	Aracruz/ ES	Southeast	Field	KY288853	KY287829	KY287864	KY287911	KY460485
LPF589	*E. grandis x E. urophylla*	Aracruz/ ES	Southeast	Nursery	KY288854	KY287830	KY287865	KY287912	KY460486
LPF590	*E. grandis x E. urophylla*	Santana/ AP	North	Nursery	KY288855	KY287831	KY287866	KY287913	KY460487
LPF591	*E. grandis x E. urophylla*	Santana/ AP	North	Nursery	KY288856	KY287832	KY287867	KY287914	KY460488
LPF592	*Eucalyptus grandis*	Santana/ AP	North	Field	KY288857	KY287833	KY287868	KY287915	KY460489
LPF593	*E. urophylla x E. globulus*	São José do Triunfo/ PR	South	Nursery	KY288858	KY287844	KY287869	KY287927	KY460490
LPF594	*E. urophylla x E. globulus*	São José do Triunfo/ PR	South	Nursery	KY288859	KY287845	KY287870	KY287916	KY460491
LPF595	*E. urophylla x E. globulus*	São José do Triunfo/ PR	South	Nursery	KY288860	KY287834	KY287871	KY287917	KY460492
LPF599	*Eucalyptus saligna*	São José do Triunfo/ PR	South	Nursery	KY288864	KY287838	KY287874	KY287921	KY460495
LPF600	*E. grandis x E. urophylla*	São José do Triunfo/ PR	South	Nursery	KY288865	KY287839	KY287875	KY287922	KY460496
LPF601	*E. grandis x E. urophylla*	Tocantins	North	Nursery	KY288866	KY287840	KY287876	KY287923	KY460497
LPF602	*E. grandis x E. urophylla*	Teixeira de Freitas/ BA	Northeast	Field	KY288867	KY287841	KY287877	KY287925	KY460500

*AP- Amapá, BA- Bahia, ES- Espírito Santo, GO- Goiás, MG- Minas Gerais, PR- Paraná, RS- Rio Grande do Sul and SP- São Paulo.

Results

Bacterial isolation. Forty-three bacterial strains were obtained from eucalypt leaves with symptoms of bacterial blight. Yellow colonies were repeatedly isolated from symptomatic leaves after 24-48 h of incubation on solid 523 medium. In some cases, white cream colonies were also observed within the first 24 h of incubation. However, these bacterial colonies were not pathogenic to clone CLR368. Only pathogenic strains were selected for further characterization in this study.

Pathogenicity on eucalypt. Twenty-six of the 43 bacterial

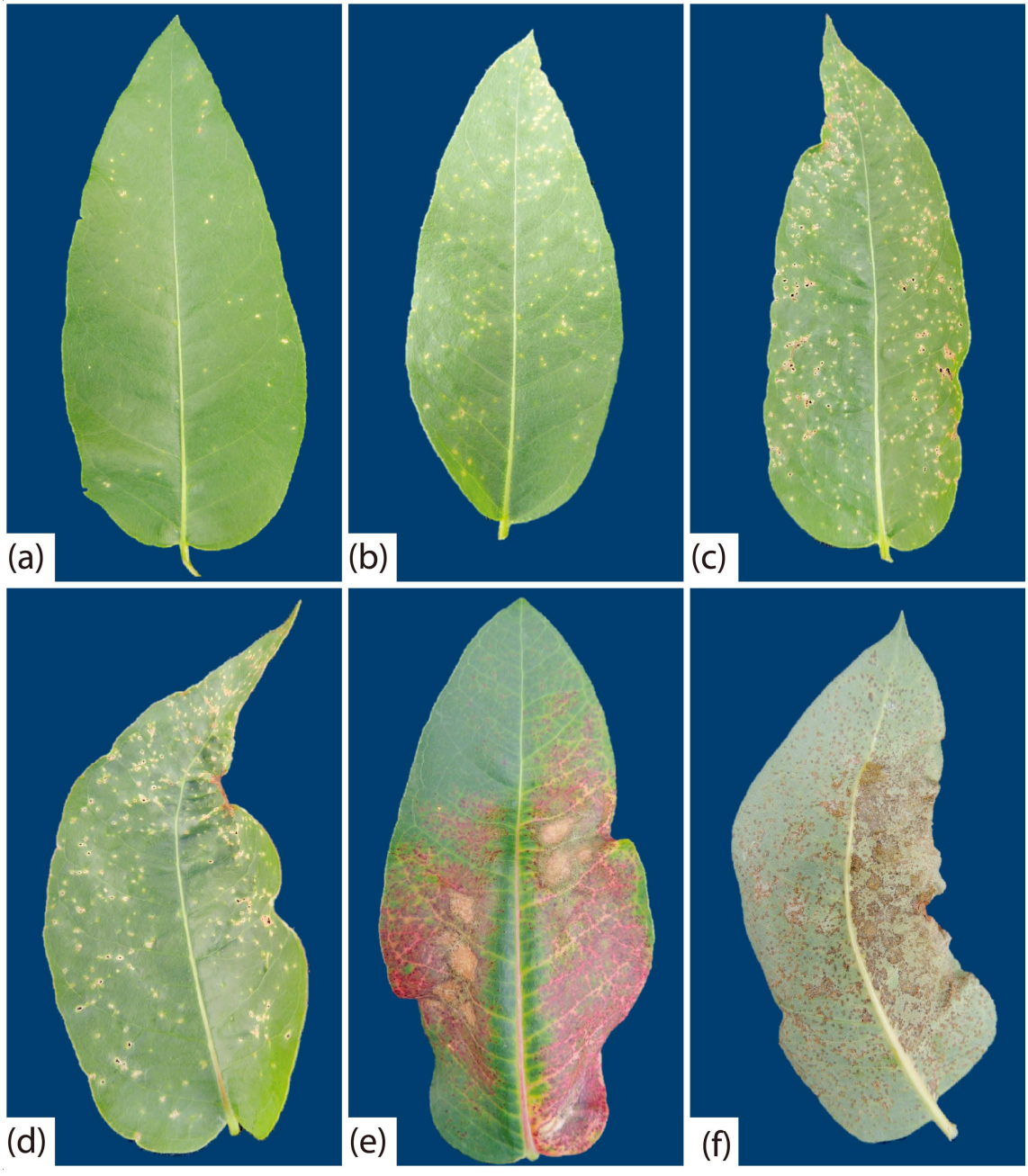

Fig. 1. Symptoms caused by *Xanthomonas axonopodis* in clone CLR368 of *Eucalyptus urophylla* × *E. globulus*. (a) Small chlorotic lesions caused by LPF 588; (b) coalescing lesions caused by LPF601; (c) perforation in the centers of lesions caused by LPF 573; (d) tanned injuries caused by LPF 591; (e) and (f) necrotic and rough lesions on the adaxial and abaxial sides of the leaf caused by LPF 594. Pictures were taken at 23 days after inoculation.

strains obtained from symptomatic leaves were pathogenic when inoculated by spraying onto clone CLR368. The pathogenic strains were assigned accession numbers and deposited in the collection of phytopathogens of the Forest Pathology Laboratory at the Universidade Federal de Viçosa (Table 2). All strains caused the same symptoms, but they varied in aggressiveness (evolution rate of disease symptoms). The first symptoms of the disease were observed at seven days after inoculation (dai). Initially, lesions were small, chlorotic, and scattered on the leaf blade (Fig. 1A), and with the progress of the disease they became necrotic and coalescent (Fig. 1B). In some leaves, it was observed detachment of tissue from the center of the lesions, distortion of the leaf and tanning of the limb (Fig. 1C-E). On the abaxial side, necrotic and rough lesions were observed (Fig. 1F). No bacterial leaf blight symptoms were observed on control plants. Morphological characteristics and *gyrB* gene sequences of bacterial strains re-isolated in pure culture from inoculated plants were the same as those obtained from naturally infected tissue, in fulfilment of Koch's postulate. The strains varied in aggressiveness, the most aggressive being LPF 594, and LPF 595, and the least aggressive being LPF 599, LPF 565, LPF 582 and LPF 600 (Fig. 2).

Analysis of partial *16S rDNA* sequences. Partial *16S rDNA* sequences of approximately 1200 bp were obtained for each strain. Blast searches in the NCBI data base (http://www.ncbi.nim.nih.gov) revealed that the *16S rDNA* sequences of strains isolated from eucalypt show similarity with those of several *Xanthomonas*. For example, the LPF 565 sequence showed 100% identity with those of *X. axonopodis* pv. *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*) (CP023294), *X. axonopodis* pv. *vignicola* (CP022270), and *X. axonopodis* (KY982974). The LPF 582 sequence showed 100% identity with those of *X. axonopodis* pv. *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*) (CP023294), *X. axonopodis* pv. *vesicatoria* (*X. euvesicatoria*) (KX512836), and *X. campestris* (KU163445). And, the LPF 602 sequence showed higher than 99% identity with those of *X. axonopodis* pv. *dieffenbachiae* (KM576803), *X. axonopodis* pv. *citrumelo* (*X. alfalfae* subsp. *citrumelonis*) (CP002914), and *X. campestris* pv. *viticola* (JQ513818). Therefore, all strains were identified as belonging to the genus *Xanthomonas*.

Multilocus sequence analysis (MLSA). Partial sequences of 834, 657, 693, and 702 bp for *dnaK*, *fyuA*, *gyrB* and *rpoD*, respectively, were obtained for each strain and used in phylogenetic analyses. The total length of the alignment for the concatenated sequences of the four genes was 2886 bp. For the first phylogenetic analysis using sequences from validly published *Xanthomonas* species, the number of conserved sites was 648 for *dnaK*, 442 for *fyuA*, 321 for *gyrB*, and 402 for *rpoD*. The number of variable sites was 186 for *dnaK*, 215 for *fyuA*, 372 for *gyrB*, and 300 for *rpoD*, and the number of parsimony informative sites were 74, 144, 226, and 196 for *dnaK*, *fyuA*, *gyrB*, and *rpoD*, respectively. The best evolutionary model selected by MrModeltest 2.3 for Bayesian analysis by AIC was the General Time Reversible plus Invariant site plus Gamma (GTR + I + G) for all four genes. In this analysis, all pathogenic strains recovered from eucalypt grouped together with the type strain of the *X. axonopodis* species (Fig. 3).

We then performed a phylogenetic analysis to position the eucalypt strains within the *X. axonopodis* species using gene sequences of pathovars previously described and available in the GenBank. In this case, the number of con-

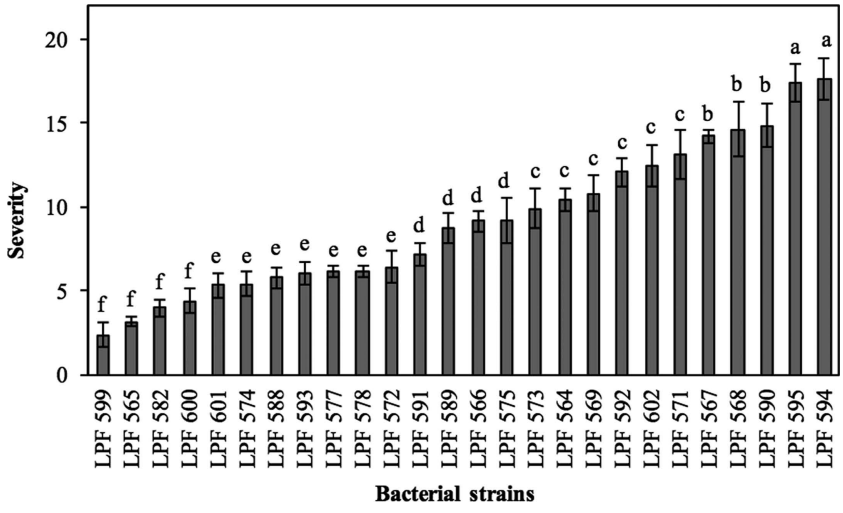

Fig. 2. Severity of bacterial leaf blight in rooted cuttings of clone CLR368 (*Eucalyptus urophylla* × *E. globulus*) inoculated with strains of *Xanthomonas axonopodis*. Bars represent the mean and vertical lines the standard error of the mean. Severity was determined as percent of leaf area affected by the disease as in Gonçalves (2003). Means followed by the same letter are not statistically different by the Scott-Knott test ($P \leq 0.05$).

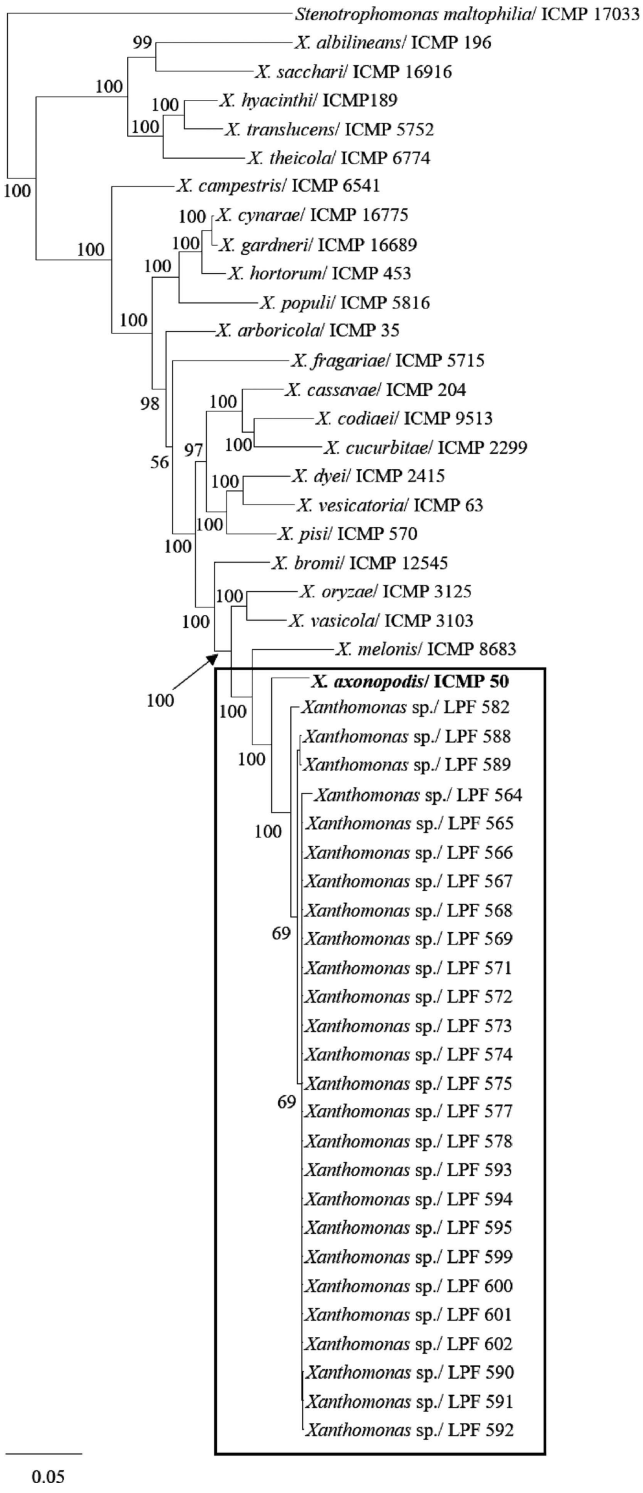

Fig. 3. Phylogenetic tree of concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* nucleotide sequences of diverse *Xanthomonas* species constructed by Bayesian inference. Strains isolated from eucalypt in this study are shown within the rectangle. Posterior probability values are indicated on the branches. Bars indicate the fraction of substitutions per site. *Stenotrophomonas maltophilia* (ICMP 17033) was used as an outgroup.

served sites was 749 for *dnaK*, 535 for *fyuA*, 534 for *gyrB*, and 599 for *rpoD*. The number of variable sites was 85 for *dnaK*, 122 for *fyuA*, 159 for *gyrB*, and 103 for *rpoD*, and the number of parsimony informative sites were 49, 57, 66, and 49 for *dnaK*, *fyuA*, *gyrB*, and *rpoD*, respectively. The best evolutionary model selected by MrModeltest 2.3 for Bayesian analysis by AIC were the GTR + I for *dnaK* and *fyuA*, and GTR + G for *gyrB* and *rpoD*. Our results indicate that all strains pathogenic to eucalypt cluster in RG 9.6 group (Fig. 4) corresponding to the allocation scheme of *X. axonopodis* pathovars proposed by Rademaker et al (2005).

Biochemical and physiological tests. Strains pathogenic to eucalypt were Gram-negative and on YDC agar medium colonies were yellow and mucilaginous with abundant slime formation. Bacterial strains were positive for catalase, starch utilization, H_2S production, esculin hydrolysis, xanthomonadin production, and gelatin liquefaction. They were negative for growth under anaerobic conditions, production of fluorescent pigments on King's B medium, oxidase, urease, nitrate reductase, and utilization of asparagine as a sole source of carbon and nitrogen.

Metabolic fingerprinting. We selected LPF 564, LPF 582, LPF 590, and LPF 602 pathogenic strains recovered from eucalypt to determine their profiles of carbon source utilization using the Biolog system. The profile of most strains was the same as the profile of *X. axonopodis* reported by Vauterin et al. (1995), the exceptions being LPF 582 that was not able to utilize methyl pyruvate, and LPF 582 and LPF 602 that were able to utilize glucuronamide (Table 3). Strains recovered from eucalypt were able to utilize 36 carbon sources and unable to utilize 47 carbon sources, whereas variable results were obtained for 12 carbon sources (Table 3). A reaction was considered positive when all strains metabolized the substrate and negative when no strain metabolized the substrate. A reaction was considered variable when at least one (but not all) strain utilized the substrate.

Fatty acid analysis. Fatty acid profiles of the strains pathogenic to eucalypt were typical of *X. axonopodis* species. Average and standard deviation values of fatty acids identified for the twenty-six strains tested in this study are shown in Table 4. These values were compared with those obtained by Vauterin et al. (1996) for several *Xanthomonas* species. Fatty acid values were considered to be different from those of other *Xanthomonas* spp. when their averages differed by a magnitude greater than the sum of their standard deviations. The value of the fatty acid 17:0 iso

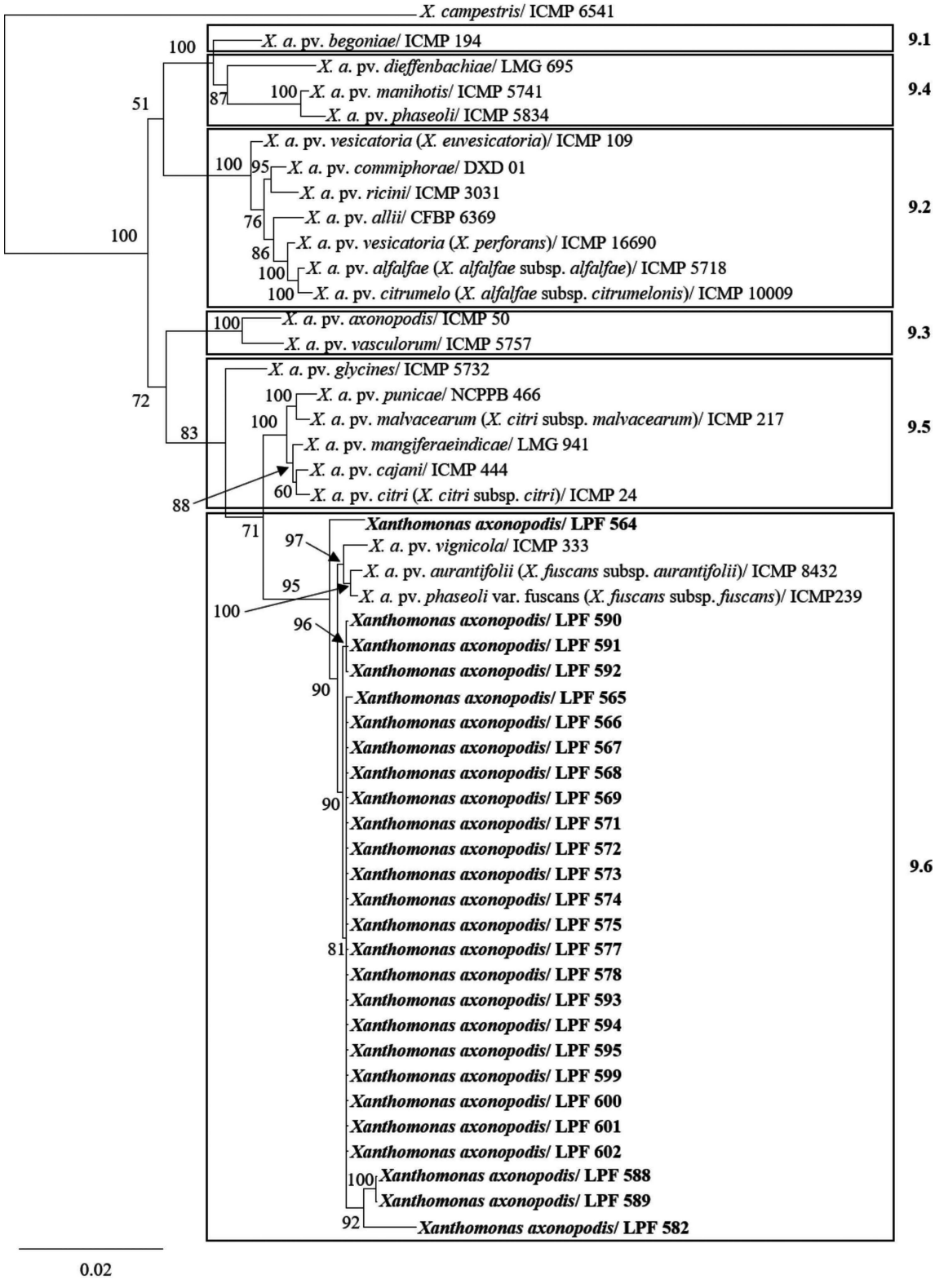

Fig. 4. Phylogenetic tree of concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* nucleotide sequences of *Xanthomonas axonopodis* pathovars constructed by Bayesian inference. Strains isolated from eucalypt in this study are shown in bold. Posterior probability values are indicated on the branches. *X. axonopodis* is divided in groups according to Rademaker et al. (2005) as RG 9.1 to RG 9.6. Bar indicates number of substitutions per site. *X. campestris* pv. *campestris* (ICMP 6541) was used as an outgroup.

Table 3. Characterization of *X. axonopodis* strains based on the Biolog test

Carbon source	LPF 564	LPF 582	LPF 590	LPF 602	Vauterin et al. (1995)*
α-cyclodextrin	-	-	-	-	-
Dextrin	+	+	+	+	+
Glycogen	+	+	+	+	V
Tween 40	+	+	+	+	V
Tween 80	+	+	+	+	V
N-Acetyl-D-galactosamine	-	-	-	-	V
N-acetyl-D-glucosamine	+	+	+	+	V
Adonitol	-	-	-	-	-
L-arabinose	-	-	-	-	V
D-arabitol	-	-	-	-	-
D-cellobiose	+	+	+	+	+
i-erythritol	-	-	-	-	-
D-fructose	+	+	+	+	+
L-fucose	+	+	+	+	V
D-galactose	+	+	+	+	V
Gentiobiose	+	+	+	+	+
α-D-glucose	+	+	+	+	+
m-inositol	-	-	-	-	-
α-D-lactose	-	+	-	-	V
Lactulose	+	+	+	+	V
Maltose	+	+	+	+	+
D-mannitol	-	-	-	-	V
D-mannose	+	+	+	+	+
D-melibiose	-	+	-	-	V
β-methyl-D-glucoside	-	-	-	-	-
D-psicose	+	+	+	+	+
D-raffinose	-	-	-	-	V
L-rhamnose	-	-	-	-	-
D-sorbitol	-	-	-	-	V
Sucrose	+	+	+	+	V
D-trehalose	+	+	+	+	+
Turanose	-	+	-	-	V
Xylitol	-	-	-	-	-
Methyl pyruvate	+	-	+	+	+
Mono-methyl-succinate	+	+	+	+	+
Acetic acid	-	-	-	-	V
Cis-aconitic acid	+	+	+	+	V
Citric acid	-	-	-	-	V
Formic acid	-	-	-	-	-
D-galactonic acid lactone	-	-	-	-	-
D-galacturonic acid	-	-	-	-	-
D-gluconic acid	-	-	-	-	V
D-glucosaminic acid	-	-	-	-	-
D-glucuronic acid	-	-	-	-	-
α-hydroxy butyric acid	-	-	-	-	V
β-hydroxy butyric acid	-	-	-	-	V
γ-hydroxy butyric acid	-	-	-	-	-
p-hydroxy phenylacetic acid	-	-	-	-	-

Table 3. Continued

Carbon source	LPF 564	LPF 582	LPF 590	LPF 602	Vauterin et al. (1995)*
Itaconic acid	-	-	-	-	-
α-keto butyric acid	+	+	-	+	V
α-keto glutaric acid	+	+	+	+	+
α-keto valeric acid	-	-	-	-	-
D,L-lactic acid	-	-	-	-	V
Malonic acid	+	+	+	+	V
Propionic acid	-	-	-	-	V
Quinic acid	-	-	-	-	-
D-saccharic acid	+	+	+	+	V
Sebacic acid	-	-	-	-	-
Succinic acid	+	+	+	+	+
Bromo succinic acid	+	+	+	+	+
Succinamic acid	+	+	+	+	+
Glucuronamide	-	+	-	+	-
L-alaninamide	+	+	+	+	V
D-alanine	+	+	+	+	+
L-alanine	+	+	+	+	+
L-alanylglycine	+	+	+	+	+
L-asparagine	+	-	-	-	V
L-Aspartic acid	+	+	-	+	V
L-Glutamic acid	+	+	+	+	+
Glycyl-L-aspartic acid	+	+	+	+	V
Glycyl-L-glutamic acid	+	+	+	+	+
L-histidine	-	-	-	+	V
Hydroxy-L-proline	+	+	+	+	V
L-leucine	-	-	-	-	V
L-ornithine	-	-	-	-	V
L-phenylalanine	-	-	-	-	-
L-proline	+	+	+	+	V
L-pyroglutamic acid	-	-	-	-	-
D-serine	-	-	-	-	-
L-serine	+	+	+	+	V
L-threonine	+	+	-	-	V
D,L-carnitine	-	-	-	-	-
γ-amino butyric acid	-	-	-	-	-
Urocanic acid	-	-	-	-	V
Inosine	-	-	-	-	V
Uridine	-	-	-	-	V
Thymidine	-	-	-	-	-
Phenyethylamine	-	-	-	-	-
Putrescine	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-
Glycerol	+	+	+	+	V
D,L-α-glycerol phosphate	+	+	-	-	V
Glucose-1-phosphate	+	+	-	-	V
Glucose-6-phosphate	-	-	-	-	V

*Strains of *X. axonopodis* used by Vauterin et al. (1995): (+) indicates that 90% or more of strains are positive, (-) indicates that 90% or more of strains are negative and (V) indicates that 11 to 89% of strains were able to metabolize the carbon source

3OH differentiated strains pathogenic to eucalypt from all reported by Vauterin et al. (1996), except for *X. codiaei*, *X. fragariae*, and *X. albilineans*. The fatty acid 13:0 iso 2OH identified in all strains pathogenic to eucalypt is not in the list proposed by Vauterin et al (1996). The fatty acid profiles of the eucalypt strains were more similar to those of *X. axonopodis* pv. *manihotis*, *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *glycines* with average similarity indices of 0.660, 0.650 and 0.645, respectively.

Host range and cross-inoculation. To gain insights into the host range of *X. axonopodis* strains isolated from eucalypt, we inoculated LPF 602 on different plant species. LPF 602 only caused disease on eucalypt plants belonging to the genera *Eucalyptus* and *Corymbia*. However, it was noted that there is intraspecific host variability in susceptibility to the disease. All plants of *E. cloeziana* and hybrid clones of *E. grandis* × *E. urophylla* and *E. urophylla* × *E. maidenii* were susceptible. At least 50% of the seedlings of *C. maculata* and *E. globulus* and 5% of *E. grandis*, *E. urophylla*, *E. saligna*, *E. camaldulensis* and *E. robusta* exhibited symptoms of bacterial blight. Plants of *Psidium guajava*, *Myrciaria jaboticaba*, *Eugenia jambolana* and other botanical families showed no disease symptoms. Bacteria were isolated from inoculated plants exhibiting disease symptoms and their colony morphologies were identical to those used for inoculation.

The results of cross-inoculation experiments show that *X. axonopodis* pv. *vignicola* (IBSBF 1739), *X. axonopodis* pv. *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*) (UNB 772) and *X. axonopodis* pv. *aurantifolii* (*X. fuscans* subsp. *aurantifolii*) (IBSBF 380) do not cause disease symptoms when inoculated onto eucalypt rotted cuttings. Only strain LPF 602 caused disease in clone CLR368. Overall, the results of this study indicate that the strains pathogenic to *Eucalyptus* spp. belong to *Xanthomonas axonopodis* pv. *eucalyptorum* pv. nov. that have not been previously reported.

Table 4. Fatty acid profile of *Xanthomonas axonopodis* strains isolated from eucalypt

Compound	Fatty acids (%)	Strains (%)
Saturated fatty acids		
10:0	0.897 ± 0.207	100
14:0	2.004 ± 0.333	100
16:0	4.899 ± 0.872	100
17:0	0.078 ± 0.005	15.4
18:0	0.681 ± 0.633	34.6
Unsaturated fatty acids		
15:1 w6c	0.52 ± 0.1559	100
16:1 w5c	0.123 ± 0.023	61.5
16:1 w7c/16:1 w6c	20.91 ± 1.315	100
16:0 10-methyl/17:1 iso w9c	4.76 ± 0.697	100
17:1 w8c	0.878 ± 0.101	100
17:1 w6c	0.192 ± 0.074	61.5
18:1 w9c	0.413 ± 0.089	96.2
18:1 w7c/18:1 w6c	0.504 ± 0.096	100
Hydroxy fatty acids		
10:0 3OH	0.39 ± 0.064	84.6
10:0 2OH	0.173 ± 0.023	23.1
11:0 3OH	0.271 ± 0.049	76.9
12:0 3OH	3.434 ± 0.414	100
16:0 3OH	0.156 ± 0.040	53.8
Branched-chain fatty acids		
11:0 iso	4.765 ± 0.907	100
13:0 iso	0.367 ± 0.058	84.6
14:0 iso	0.395 ± 0.040	100
15:0 iso	30.474 ± 1.533	100
15:0 anteiso	8.504 ± 0.965	100
16:0 iso	1.21 ± 0.244	100
17:0 iso	4.922 ± 0.664	100
17:0 anteiso	0.34 ± 0.058	88.5
Branched-chain unsaturated fatty acids		
15:1 iso F	0.462 ± 0.080	100
Branched-chain hydroxy fatty acids		
11:0 iso 3OH	2.062 ± 0.445	100
12:0 iso 3OH	0.234 ± 0.034	53.8
13:0 iso 3OH	4.998 ± 0.414	100
13:0 iso 2OH	0.327 ± 0.054	77.0
17:0 iso 3OH	0.326 ± 0.038	88.5

Discussion

From samples of eucalypt leaves with symptoms of bacterial blight collected in different geographic regions of Brazil, 26 strains were obtained, which had their pathogenicity confirmed by inoculation onto healthy plants. Symptoms developed on inoculated plants were similar to those observed under field and nursery conditions, and to those described in previous studies (Gonçalves et al., 2008; Neves et al., 2014), ranging from small necrotic lesions to angular leaf spots. However, the most frequently observed symptoms in the inoculated plants were small spots distributed in the leaf blade, whereas under natural field and nursery conditions, the lesions are generally larger and delimited by the leaf ribs. We observed that the strains isolated from eucalypt varied in aggressiveness when inoculated onto clone CLR368 (*E. urophylla* × *E. globulus*). This information is

important for designing strategies to control the disease. For instance, selection of host genotypes resistant to bacterial leaf blight should preferably be conducted by challenging clones of different genetic backgrounds with the most aggressive strains.

Analysis of the *16S rDNA* sequence indicated that all strains that caused disease on eucalypt belonged to the genus *Xanthomonas*, but sequence similarity was observed with strains of the species *X. campestris* and *X. axonopodis*. Thus, it was not sufficient to allocate strains in a particular bacterial species. The analysis of the *16S rDNA* sequence is a simple method that allows rapid identification of strains at the genus level. According to Hauben et al. (1997) because *Xanthomonas 16S rDNA* sequences show limited variability, they do not provide sufficient resolution to differentiate among all species of the genus. It provides even less resolution to differentiate among pathovars of the same species.

The identity of the strains causing eucalypt bacterial blight as *X. axonopodis* was confirmed by results of metabolic and FAME profiling. As expected, we observed metabolic variations among strains, as indicated by the fact that LPF 582 was unable to metabolize methyl pyruvate. We also found that LPF 582 and LPF 602 were able to metabolize glucuronamide. According to Vauterin et al. (1995) methyl pyruvate is used as a carbon source by 99% of *X. axonopodis* strains, whereas glucuronamide is utilized as a sole carbon and nitrogen source by only 8% of *X. axonopodis* strains. The same authors also reported that the fatty acid 13:0 isso 2OH is not commonly found in *X. axonopodis* strains. However, 76.9% of strains recovered in the present study had such a fatty acid. Here, we found that the percentage of 17:0 isso 3OH of the strains pathogenic to eucalypt was different from those of the *X. axonopodis* strains studied by Vauterin et al (1996). These results indicate that strains that cause disease on eucalypt plants have distinct phenotypic characteristics that distinguish them from other *X. axonopodis* pathovars.

Moreover, MLSA also indicated that the eucalypt strains belong to the species *X. axonopodis*. Other bacterial species that have been reported to be associated with similar disease symptoms in Brazil (Gonçalves et al., 2008; Pomella et al., 1995) and in other countries (Coutinho et al., 2002, 2015) were not found in this study. Despite finding other bacterial species associated with the disease symptoms, Gonçalves et al. (2008) pointed *X. axonopodis* as the predominant species causing eucalypt bacterial leaf blight in Brazil and our results confirm such an interpretation. MLSA is considered a more robust alternative to *16S rDNA* sequencing and more flexible than DNA-DNA reassociation methods for classification of bacterial species (Ah-You et al., 2007; Ferraz et al., 2017; Young et al., 2008, 2010). Young et al. (2008) showed that MLSA based on the *dnaK*, *fyuA*, *gyrB* and *rpoD* genes sequences allow discrimination of *Xanthomonas* species with results similar to those obtained by Vauterin et al. (1995) using a DNA-DNA reassociation method. Furthermore, MLSA using these four genes allows separation of *X. axonopodis* pathovars into the six subgroups obtained by Rademaker et al. (2005) using rep-PCR.

When comparing the concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* gene sequences, we observed some genetic variability among *X. axonopodis* strains associated with bacterial leaf blight of eucalypt (Supplementary Fig. 1). However, MLSA revealed that these strains clustered together in only one distinct clade in the phylogenetic tree, corresponding to Rademaker's group RG 9.6 (Rademaker et al. 2005). There are several proposals to reclassify some pathovars of this *X. axonopodis* RG group in other species. For instance, *X. axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *phaseoli* var. fuscans were proposed to be reclassified as *X. fuscans* subsp. *aurantifolii* and *X. fuscans* subsp. *fuscans*, respectively (Schaad et al., 2005, 2006). Pathovars from other RG groups have also been proposed to be reclassified. For example, *X. axonopodis* pv. *vesicatoria*, *X. axonopodis* pv. *alfalfae*, and *X. axonopodis* pv. *citrumelo*, belonging to RG 9.2, were proposed to be reclassified as *X. euvesicatoria*, *X. alfalfae* subsp. *alfalfae* and *X. alfalfae* subsp. *citrumelo*, respectively (Jones et al., 2004; Schaad et al., 2005). In contrast, no reclassifications have been proposed for pathovars of RG 9.4, in which *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *manihotis* are included.

Some of the species recently proposed by Jones et al. (2004) and Schaad et al. (2005, 2006), such as *X. alfalfae*, *X. citri*, *X. euvesicatoria*, *X. fuscans,* and *X. perforans*, are not clearly differentiated from *X. axonopodis* (Young et al., 2008). The separation of these newly proposed species from *X. axonopodis* was mainly based on results of DNA-DNA reassociation studies. Young et al. (2008) hypothesized a possible disagreement between MLSA and the DNA-DNA reassociation method used by Jones et al. (2004) and Schaad et al. (2005) with regard to the circumscription of species. According to Young et al. (2008), the stringency of the DNA-DNA reassociation method, and perhaps its high experimental error, may be responsible for the discrepancy. There is also the possibility that MLSA and DNA-DNA reassociation methods do not give equivalent results when studying some groups of strains (Young

et al., 2008), and this could be the case for *X. axonopodis*. Therefore, the taxonomic classification and nomenclature widely accepted and used by Vauterin et al. (1995) and Rademaker et al. (2005) were adopted to allocate the strains pathogenic to *Eucalyptus* spp. isolated in this study to the species *Xanthomonas axonopodis*.

MLSA analysis showed that strains pathogenic to *Eucalyptus* spp. are more closely related to the *X. axonopodis* pathovars *vignicola*, *aurantifolii* (*X. fuscans* subsp. *aurantifolii*), and *phaseoli* var. fuscans (*X. fuscans* subsp. *fuscans*). LPF 602, a representative of the pathogenic strains isolated in this study, caused disease only in plants belonging to the genera *Eucalyptus* and *Corymbia*, which suggests specialization of the pathogen to the *Myrtaceae* family. Cross-inoculation experiments revealed that the host range of strain LPF 602 is different from any other pathovar that has been previously reported, and most importantly, is distinctively pathogenic to the plant species from which it was isolated.

The first description of a bacterium causing leaf blight of eucalypt was made by Truman (1974). The bacterium was classified as *Xanthomonas eucalypti* Truman, then as *X. campestris* pv. *eucalypti* (Truman, 1974) Dye 1978 and finally transferred to *X. dyei* pv. *eucalypti* (Truman, 1974) Young et al., 2010. Our phylogenetic analyses indicate that the strains of *X. axonopodis* pathogenic to *Eucalyptus* and *Corymbia* reported in this study are distantly related to *X. dyei*. Hence, we propose to accommodate strains isolated from eucalypt and belonging to Rademaker's group RG 9.6 (Rademaker et al., 2005) as *X. axonopodis* pv. *eucalyptorum* pv. nov.

Description of *Xanthomonas axonopodis* pv. eucalyptorum pv. nov. *Xanthomonas axonopodis* pv. *eucalyptorum* (eu'ca'lip'to'rum. N.L. gen. n. *eucalyptorum* of *Eucalyptus*, referring to the isolation source of the type strain).

The phenotypic description of the pathovar is based on pathotype strain LPF 602 (Table 2). Bacterial strains are Gram negative by KOH test. Colonies are yellow and mucilaginous with abundant slime formation on YDC agar. Strains are positive for H_2S, and xanthomonadin production, catalase, gelatin liquefaction, starch utilization, and esculin hydrolysis. They are negative for growth under anaerobic conditions, production of fluorescent pigments on King's B medium, utilization of asparagine as a sole source of carbon and nitrogen, oxidase, urease, and NO_3 reductase.

Strain gives positive auxanographic reactions for dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-glucosamine, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, lactulose, maltose, D-mannose, D-psicose, sucrose, D-trehalose, methyl pyruvate, mono-methyl-succinate, cis-aconitic acid, α-keto butyric acid, α-keto glutaric acid, malonic acid, D-saccharic acid, succinic acid, bromo succinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanylglycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxyl-L-proline, L-proline, L-serine and glycerol. Negative reactions are given by α-cyclodextrin, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, m-inositol, α-D-lactose, D-mannitol, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, acetic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy phenylacetic acid, itaconic acid, α-keto valeric acid, D,L-lactic acid, propionic acid, quinic acid, sebacic acid, L-asparagine, L-leucine, L-ornitine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-threonine, D,L-carnitine, γ-amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L,-α glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate.

Strains are distinguished from other *Xanthomonas* species by MLSA based on concatenated *dnaK*, *fyuA*, *gyrB* and *rpoD* gene sequences. They can be differentiated from other *X. axonopodis* strains because of their proportion of 17:0 iso 3OH and 13:0 iso 2OH fatty acids. Pathotype strain is LPF 602, which was isolated from infected eucalypt tissue in the municipality of Teixeira de Freitas in the state of Bahia, Brazil, and causes bacterial leaf blight on *Eucalyptus* spp. and *Corymbia* spp. GeneBank accession numbers of the *16S rDNA*, *rpoD*, *dnaK*, *gyrB*, and *fyuA* sequences of the pathotype strain are KY288867, KY287841, KY287877, KY287925, and KY460500, respectively.

Acknowledgments

The authors are grateful to the biotechnological company Clonar Resistência a Doenças Florestais for providing the plant material and the inoculation facilities, to Minas Gerais State Foundation for Research Development (FAPEMIG) and National Council for Scientific and Technological Development (CNPq) for financial support. The authors would like to thank Dra. Marisa Alvares Velloso Ferreira for kindly providing strain UNB 772 for cross inoculation

experiments.

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