Etiology of bacterial leaf blight of eucalyptus in Brazil

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ABSTRACT

Bacterial leaf blight of eucalyptus is initially characterized by water soaked, angular, amphigenous and interveinal lesions, concentrated along the main vein, at the edges or scattered on the leaf blade. As the disease progresses, the lesions become brown to pale, and when young leaves are infected leaf cut areas at the edges or perforations at the center of the lesions may appear due to abortion of the necrotic area. Eventually, necrosis may be found on petiole and twigs. Leaf fall commonly occurs on highly susceptible genotypes due to the early senescence of diseased leaves. Precise diagnosis is accomplished by bacterial exudation from leaf sections placed in a water drop under light microscope (200 x). Twenty-five bacterial isolates from Amapá (2), Bahia (4), Minas Gerais (2), São Paulo (9), Pará (3), Mato Grosso do Sul (1), and Rio Grande do Sul (4) States, which induced hypersensitive reaction (HR) in non-host plants and were pathogenic to eucalyptus, when inoculated by inoculum injection, were identified by biochemical assays, using carbon sources (MicroLog™ BIOLOG) and sequence analysis (16S rDNA). Ten isolates were identified as *Xanthomonas axonopodis*, four as *X. campestris*, four as *Pseudomonas syringae*, two as *P. putida*, two as *P. cichorii*, one as *Erwinia* sp., and two were similar to bacterial genera of Rhizobiaceae. When spray inoculated on intact plants of eucalyptus, only *X. axonopodis, P. cichorii* and isolates of the Rhizobiaceae family induced typical symptoms of the disease and were considered pathogenic. In Brazil, *X. axonopodis* seems to be the most widespread species causing the bacterial leaf blight of *Eucalyptus* spp.
Keywords: *Eucalyptus*, *Xanthomonas*, *Pseudomonas*, *Erwinia*, *Rhizobiaceae*.

RESUMO

Etiologia da mancha foliar bacteriana em eucalipto no Brasil

A mancha foliar bacteriana do eucalipto caracteriza-se inicialmente por lesões encharcadas do tipo anasarca, internervurais, angulares e anfígenas, concentradas ao longo da nervura principal, nas margens da folha ou distribuídas aleatoriamente sobre o limbo. Com o progresso da doença, as lesões adquirem aspecto ressecado e coloração marrom a palha, podendo conter orifícios no centro da lesão ou áreas recortadas do limbo em conseqüência do aborto da área necrosada, principalmente em folhas mais jovens. Eventualmente pode haver necrose em pecíolo e ramos. A doença culmina com a desfolha devido à senescência precoce das folhas infectadas. O diagnóstico inequívoco é realizado por meio de exsudação de pus bacteriano a partir de fragmento de folha infectada, sob microscópio óptico de luz (200 x). Vinte e cinco isolados oriundos dos estados do Amapá (2), Bahia (4), Minas Gerais (2), São Paulo (9), Pará (3), Mato Grosso do Sul (1) e Rio Grande do Sul (4) indutores de reação de hipersensibilidade em plantas não-hospedeiras e, patogênicos ao eucalipto em testes de injeção de suspensão bacteriana no mesófilo foliar, foram identificados por meio de testes bioquímicos, utilização de fontes de carbono e seqüenciamento do rDNA16S. Dez foram identificados como *Xanthomonas axonopodis*, quatro como *X. campestris*, quatro como *Pseudomonas syringae*, dois como *P. cichorii*, dois como *P. putida*, um como *Erwinia* sp. e dois foram similares a gêneros da família Rhizobiaceae. Nos testes de inoculação por atomização de suspensão bacteriana, apenas isolados de *P. cichorii*, *X. axonopodis* e os similares a membros da família Rhizobiaceae foram patogênicos a eucalipto. *X. axonopodis* é provavelmente o agente etiológico predominante da mancha foliar bacteriana de *Eucalyptus* spp., no Brasil.
Palavras-chave: *Eucalyptus*, *Xanthomonas*, *Pseudomonas*, *Erwinia*, *Rhizobiaceae*.

INTRODUCTION

Bacterial blight was first recorded in eucalyptus in the 1970s, when Truman (1974) described dieback in *Corymbia citriodora* (Hook) Hill & Johnson, caused by *Xanthomonas campestris* pv. *eucalypti* (Truman) Dye in Sydney, Australia. About 20 years later, Pomella *et al.* (1995) reported leaf blight for the first time in Brazil, caused by *Pseudomonas cichorii* (Swingle) Stapp in seedlings of *Eucalyptus grandis* W. Hill in a nursery in São Paulo state. Subsequently, the disease was also recorded in nursery and field conditions, associated with various bacterial species in Brazil (Alfenas *et al*, 2001; Gonçalves *et al*., 2001; Reis *et al*., 1996), in Argentina (Ferreira *et al*., 2001; Gonçalves

et al., 2001), Paraguay (Ferreira *et al*., 2001) and Uruguay (personal communication AC Alfenas 2001 – UFV, Viçosa, MG). In South Africa, a disease with similar symptoms to that occurring in Brazil was attributed to *Pantoea ananatis* (Serrano) Mergaert *et al*. (Coutinho *et al*., 2002). Although blight occurs in most eucalyptus-growing regions of Brazil, reports are limited to abstracts at congresses, and these are generally inconclusive regarding the identification of the disease's etiological agent. The present study, therefore, aimed to identify the causal agent of bacterial leaf blight of eucalyptus by pathogenicity, biochemical tests, and molecular analyses of cultures obtained from the main Brazilian eucalyptus-growing regions.

MATERIAL AND METHODS

Symptomology and isolation of the bacteria

The description of symptoms and signs of the disease was based on observation of samples of naturally infected eucalyptus leaves and twigs obtained from 18 samples/plant collected in eucalyptus nurseries or plantations in the states of Amapá, Bahia, Mato Grosso do Sul, Minas Gerais, Pará, Rio Grande do Sul, and São Paulo. After stereo-microscopic examination, lesions with no fungal sporulation were submitted to the pus exudation test in a water-drop (Mafia *et al.,* 2007) and observed under light microscope (200 x). Leaves that were positive for the exudation test were washed in running water and neutral detergent; fragments of tissue taken from the edges of the lesion and disinfested in a sodium hypochlorite solution at 0.5% of Cl_2 were macerated in a porcelain plate containing sterile water. The resulting suspension was spread on Kado & Heskett medium 523 (Kado & Heskett, 1970) in Petri dishes. After 48h of incubation, in the dark at 28ºC, morphologically distinct colonies were transferred to solid medium 523 in test tubes (15 x 1.5cm), which were incubated as above. After 24h, pure cultures were emulsified in glycerin and frozen at –80ºC (Moore *et al.*, 1988). The isolates were liophylized in 10% p/v of trealose:peptona (3:1) v/v and stored in laboratory environment (Dhingra & Sinclair, 1995).

Hypersensitive reaction (HR), pathogenicity, and colony morphology of the cultures

Of 500 isolated bacterial cultures, 90 (five from each of the 18 samples) were inoculated by injection of the inoculum in tomato plants (*Solanum lycopersicum*), tobacco (*Nicotiana tabaco*), sweet-pepper (*Capsicum annuum*), coffee (*Coffea arabica*) and eucalyptus (*Eucalyptus* spp.). For inoculation in eucalyptus, when there were no plants of the host species or a clone of the one from which the culture was isolated, hybrid clones of *E. grandis* x *E. urophylla* and *E. urophylla* x *E. maidenii* were used. These clones were chosen because of their high susceptibility to the bacteriosis, under natural infection. Plants with 4-6 pairs of leaves, cultivated in polyethylene pots with 2 L of soil: cattle manure mixture (3:1),

containing 4g/L of NPK (4:14:8), were inoculated by injecting the suspension of bacterial cells. Each isolate was inoculated in three leaves from each of the species, with inoculum suspension in saline solution at 0.1 of $D.O._{600\,nm}$ (approximately 10^8 ufc/mL), obtained from cultures in solid medium 523 (Kado & Heskett, 1970) with 24 h of incubation at 28ºC, in the dark. Leaves from control plants were inoculated with sterile saline solution. After inoculation, plants were kept at 25ºC, under a 12 h photoperiod at 4 $\mu mol\ of\ photons\ s^{-1}.m^{-2}$. Rapid necrosis and drying in the infiltrated leaf area, 24h after inoculation, was considered as a hypersensitivity reaction (HR) (Klement *et al.*, 1964). Evolution of the symptoms in the form of necrosis in the infiltrated area, 12 days after inoculation, with bacterial pus exudation was considered evidence of the isolate's pathogenicity; the isolate was re-isolated for a pure culture so as to complete the Koch's postulates.

Nine isolates (BSC475a, BSV04, ECLFCAF03, AMPV01, AMP03, RVV11, BSV16, R203BN10, EGS15 and R57BN4), considered pathogenic from the previously described tests, were spray inoculated on seedlings of the original host or, if this was not available, on plants of a hybrid clone of *E. urophylla* x *E. maidenii*, susceptible to the bacteriosis, under natural infection. Ninety-day-old plants previously kept in intermittent mist chamber (1 min of mist every 10 min, at 25ºC for 24 h under photoperiod of 12 h at 4 $\mu mol\ of\ photons.s^{-1}.m^{-2}$), were sprayed with bacterial suspension at 10^8 ufc.mL^{-1}. Control plants were sprayed with sterile saline solution. The inoculated plants were maintained in a mist chamber for 48 h and then in a growth chamber at 28ºC, under a photoperiod of 12 h at 40 $\mu mol\ of\ photons.s^{-1}.m^{-2}$, until symptoms appeared.

Identification of plant pathogenic bacteria

Morphology of the colonies was described after cultivation in medium 523 (Kado & Heskett, 1970) for 72h at 28ºC in the dark. Twenty-five morphologically distinct cultures, which induced a hypersensitive reaction in non-host plants and were pathogenic to eucalyptus by inoculum injection, were identified by biochemical tests, consumption of carbon sources (MicroLog™ BIOLOG), and 16S rDNA sequencing.

Identification at the genus level was based on Gram and KOH tests, fluorescence in King B medium, growth at 33ºC in YDC medium (Yeast Dextrose Calcium – yeast extract - dextrose – calcium carbonate), consumption of asparagin, production of xantomonadin (Schaad, 2001), and use of 95 carbon sources (Jones *et al*., 1993). For the carbon source tests, the isolates were cultivated in BUG™ Agar medium (BIOLOG Universal Growth Agar) at 28ºC and, after 24 h of incubation, the samples were analyzed in a plate reader with a 540 nm filter (Titerk Multiskan® PLUS). The results were compared to the BIOLOG system database and identification was carried out using BIOLOG's Microlog version 4.0. Similarity rates above 0.5 indicated positive results for the identification of each isolate (BIOLOG, MicroLog™ System 4.0, User Guide).

For similarity rates below 0.5, the test was repeated at least once to confirm results.

Identification of the bacteria by phylogenetic analysis was based on partial or complete sequences of the gene *rrs*, corresponding to 16S ribosomal RNA (Hauben *et al*., 1997). Twenty-five bacterial isolates were cultivated in 10 mL of liquid medium 523 (Kado & Heskett, 1970) at 28ºC, in the dark. After 48 h of incubation, the bacterial growth was centrifuged (Eppendorf™ microcentrifuge, mod. 5415C) at 6,000 rpm for 2 min, at room temperature. The genomic DNA of the sedimented bacterial cells was extracted (Ausubel *et al.* 1992) and quantified in agarose gel at 1% in Tris Acetate EDTA (TAE), stained with ethyl bromide (0.5µg/ml) and photo-documented in Eagle Eye II (Stratagene™).

For the PCR reactions, 100 ng of genomic DNA was mixed with 1 unit of Taq polymerase (Boehringer Mannheim, Germany), 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM of $MgCl_2$ 0.1 mM of each dNTPs and 10 pmoles of each oligonucleotide (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3') (Weinsburg *et al.,* 1991). The PCR product was quantified in agarose gel - Tris Acetate EDTA at 1%. Products amplified to the expected size were cut from the gel and the DNA was purified (Concert™ Gel Extraction System kit), following manufacturer's instructions (GIBCO BRL®- Life Technologies Inc.). The DNA was quantified and cloned in plasmid vector pGEM® -T or easy vector pGEM® -T (Promega Co.). Recombinant plasmids were transferred to competent cells of *Escherichia coli* DH 5α (Hanahan, 1983) for thermal shock. Two clones from each isolate were stored in glycerol (25%) at –80ºC. The plasmidial DNA was extracted and the 16S rDNA was sequenced in a MegaBace™ 1000 capillary sequencer (Amersham Biosciences). To obtain the complete sequences, internal oligonucleotides were drawn from partial sequences.

The complete sequence for each gene was obtained on the DNA Man 4.0 program (Lynnon BioSoft). Nucleotide sequences for the studied isolates were stored in GenBanK (http://www.ncbi.nlm.nih.gov/Genbank) and compared to sequences for other organisms (Benson *et al.,*1999) using the BLAST program (National Center for Biotechnology Information, U.S. National Institutes of Health, Bethesda, MD). Species with greatest similarity were considered for phylogenetic analysis, and specific names were updated according to Garrity *et al.* (2002). Sequences were aligned in the CLUSTALW program and phylogenetic analysis carried out in the MEGA 2.1 program. A genetic distance matrix was obtained with Kimura's two-parameter index (Kimura, 1980) and the dendrogram was drawn by the neighbor-joining method (Saitou & Nei, 1987). Analyses were carried out separately for each isolate. Each dendrogram was submitted to bootstrap analysis with 2,000 repetitions (Felsenstein, 1985). Sequences greater than 1,400 pb were considered in species determination and partial sequences over 500 pb were considered for definition of genus. The species was defined by considering the greatest rate of identity within the grouping (Garrity *et al.*, 2002).

RESULTS

Symptomology and isolation of the bacteria

The disease symptoms may vary depending on leaf age, development stage of the lesion and species of *Eucalyptus*, but in general they are characterized by water soaked, angular, interveinal, and anphighenous lesions (Figure 1 A-B), which evolve into necrotic brown to straw-colored lesions, sometimes with chlorotic or reddish edges, distributed over the limb or just on one half of the leaf blade, but especially along the main vein and on the edges of the leaf (Figure 1 C-E). Deformities in the leaf limb and abortion of the lesioned area are usually observed, resulting in cut or perforated limbs (Figure 1 F). Unequivocal diagnosis is accomplished by bacterial cell exudation from leaf sections placed in a water drop under light microscope (200 x) (Figure 1 G). Defoliation commonly occurs on highly susceptible genotypes due to the early senescence of infected leaves (Figure 1 H–I). The pathogen may also infect the terminal twigs of the plant, causing die-back (Alfenas *et al*., 2004). Among 500 pure cultures obtained from infected leaves, 90 were selected for further studies.

### Hypersensitive reaction (HR), pathogenicity, and colony morphology of the cultures

Among the 90 isolates that were initially evaluated, 78 elicited HR in at least one of the tested species or were pathogenic in eucalyptus when inoculated by infiltration of bacterial suspension. Among the HR-positive, only five did not cause symptoms on the eucalyptus plants. Based on the positive results of HR and pathogenicity tests by inoculum injection, twenty five isolates were selected for identification (Table 1). Of the ten isolates selected for spray inoculations, only six were pathogenic inducing typical symptoms of the bacterial blight (Table 1). All the pathogenic bacteria were re-isolated from the lesioned tissues, to complete Koch's postulates.

The isolates selected for identification showed a wide variability in colony morphology and were grouped in four classes: 1) circular, smooth, high, convex, shiny yellow and mucous colonies; 2) circular, smooth, high, convex, shiny beige and mucous colonies; 3) circular, smooth, low, convex colonies, translucent, and fluorescent in King B medium; and 4) circular, smooth, low, convex, light beige colonies, translucent and shiny. Of the 25 isolates studied, 14 displayed a typical biochemical pattern of the genus *Xanthomonas*, eight of *Pseudomonas*, one of *Erwinia* and two isolates (RVV11 and BSV16) were distinct from any genus commonly known as a leaf pathogen (Table 2). The isolate R203BN10 was similar to the genus *Pseudomonas*, but did not fluoresce in King´s B medium.

Identification of plant pathogenic bacteria

With the exception of R57BN4, RVV11, and BSV16, the other isolates were identified by use of carbon sources. Of the 14 isolates of the genus *Xanthomonas*, ten were identified as *Xanthomonas axonopodis* Starr & Garces emend. Vauterin *et al.*, and four as *X. campestris* (Pammel) Dowson emend.
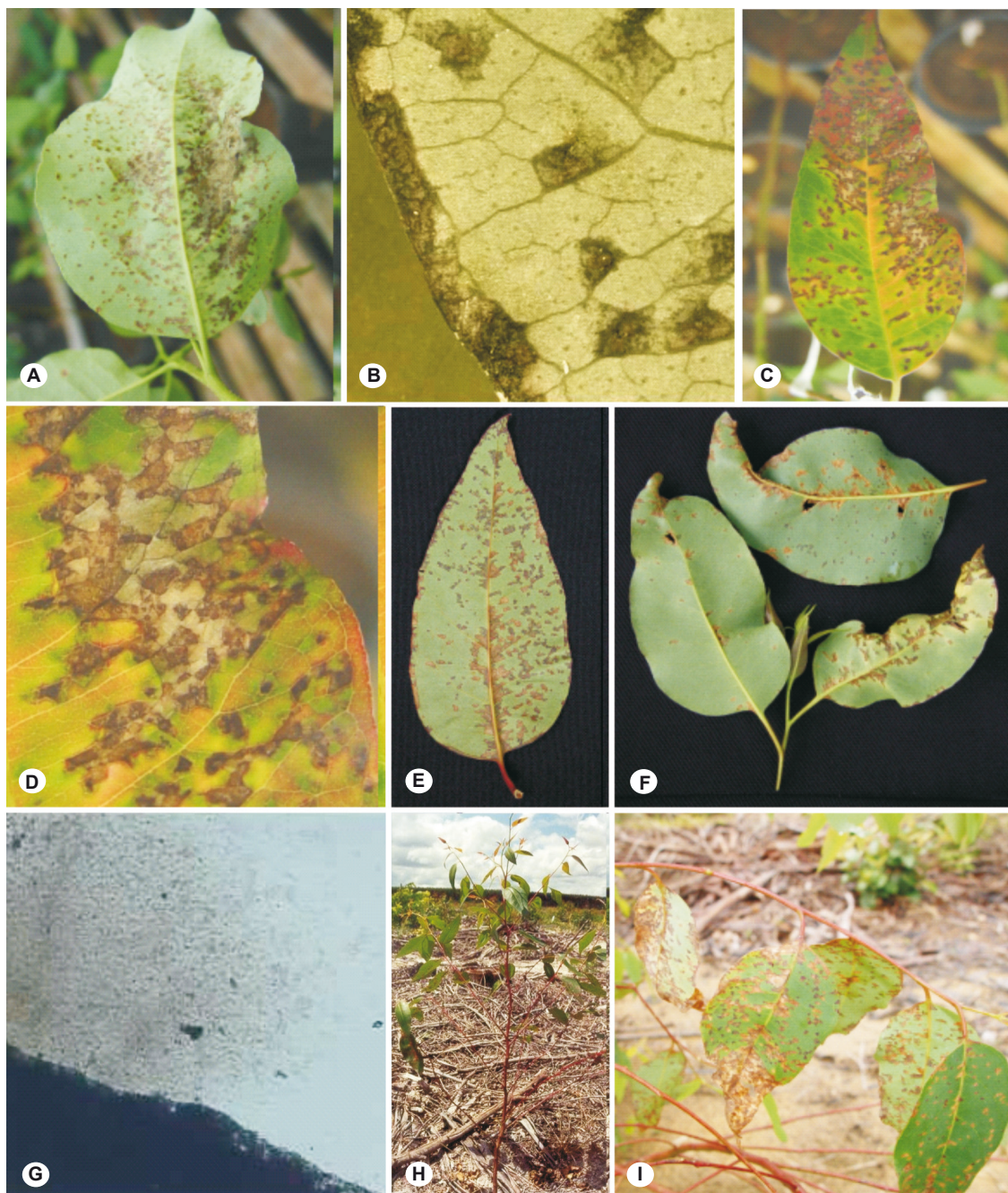
FIG. 1 - Symptoms of bacterial leaf blight in *Eucalyptus* spp.: **A.** Lesions at initial stage.; **B.** Detail of water soak; **C.** Interveinal angular lesions; **D.** Detail of angular necrotic lesions with chlorotic or reddened edges; **E.** Lesions concentrated along the main nerve and at the limb margins; **F.** Perforation, deformity and cutting of leaf limb; **G.** Microscopic exudation of bacterial pus; **H.** Defoliation; **I.** Detail of diseased branch.

Vauterin *et al*. Of the eight isolates of *Pseudomonas,* four were identified as *Pseudomonas syringae* van Hall, two as *P. cichorii* (Swingle) Stapp and two as *P. putida* (Trevisan) Migula (Table 2).

With the exception of the CAF05 isolate, it was possible to amplify a fragment of approximately 1500 pb.

The complete sequences varied between 1,496 and 1,558 pb. For some clones, only partial sequences were obtained between 400 and 1,000 pb. Comparisons of the 16S rDNA sequences (Figure 2) confirmed the identification of the bacterial isolates based on biochemical and consumption of carbon source tests (Table 2).

TABLE 1 - Bacterial cultures obtained from plants of *Eucalyptus* spp., inoculated in tobacco plants (*Nicotiana tabacum*), coffee (*Coffea arabica*), sweet pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), and eucalyptus (*Eucalyptus* spp.)

Isolate	Host	Region	Age (months)	Place	Inoculated plants				
					Tobacco	Coffee	Sweet pepper	Tomate	Eucalyptus
AMP03	*E. grandis*	Tartarugalzinho (Porto Grande-AP)	06	Field	HR	-	HR	HR	++
AMPV01	*E. grandis* x *E. urophylla*	Tartarugalzinho (Porto Grande-AP)	02	Nursery	-	-	HR	HR	+
BSV04	*E. grandis* x *E. urophylla*	Teixeira de Freitas – BA	02	Nursery	-	-	-	HR	++
BSV16	*E. grandis* x *E. urophylla*	Teixeira de Freitas – BA	02	Nursery	HR	HR	HR	HR	++
BSC475a	*E. grandis* x *E. urophylla*	Teixeira de Freitas - BA	06	Field	-	-	-	HR	++
BSC23	*E. grandis* x *E. urophylla*	Teixeira de Freitas - BA	06	Field	HR	HR	-	HR	+
CAF05	*E. grandis*	Bom Despacho MG	02	Nursery	HR			HR	+
ECLCAF03	*E. cloeziana*	Dionísio – MG	02	Nursery	-	-	-	HR	++
ESS01	*E. saligna*	Itapetininga – SP	12	Field	HR	HR	-	HR	+
ESS08	*E. saligna*	Itapetininga – SP	12	Field	HR	-	HR	HR	+
EGS09	*E. grandis*	Itapetininga – SP	12	Field	HR	HR	-	HR	+
EGS15	*E. grandis*	Itapetininga – SP	12	Field	HR	-	HR	HR	+
EUS11	*E. urophylla*	Itapetininga SP	12	Field	HR		HR	HR	+
EUS14	*E. urophylla*	Itapetininga SP	12	Field	HR	HR		HR	+
IP1-05	*E.grandis* x *E.urophylla*	Mogi Guaçu – SP	02	Nursery	HR	HR	HR	HR	+
IP1-36	*E.grandis* x *E.urophylla*	Mogi Guaçu – SP	02	Nursery	HR	-	-	HR	+
IP2-23	*E.grandis* x *E.urophylla*	Mogi Guaçu – SP	02	Nursery	-	-	-	HR	+
MSF	*E. grandis*	Três Lagoas – MS	06	Field	-	-	-	HR	+
MST07	*E. grandis*	Monte Dourado - PA	06	Field	HR	-	HR	HR	+
MST09	*E. grandis*	Monte Dourado – PA	06	Field	-	-	-	HR	+
MRP03	*E. grandis*	Tartarugalzinho (Porto Grande-AP)	06	Field	HR	-	HR	HR	+
R57BN4	*E. grandis x E. urophylla x E. robusta*	Barra do Ribeiro - RS	18	Field	HR	-	HR	HR	+
R203BN10	*E. robusta*	Barra do Ribeiro - RS	18	Field	HR	-	HR	HR	+
R57 5	*E. robusta*	Barra do Ribeiro RS	18	Field	HR	-	HR	HR	+
RVV11	*E urophylla* x *E maidenii*	Barra do Ribeiro - RS	02	Nursery	HR	HR	HR	-	++

HR = hypersensitivity reaction; - = HR absent or non-pathogenic to eucalyptus when inoculated by injection; + = pathogenic to eucalyptus when inoculated by injection. ++ pathogenic to eucalyptus when inoculated by injection and spraying of inoculum.

Identification of isolates RVV11 and BSV16 is inconclusive. Isolate RVV11 had 94-97% of similarity with *Rhizobium* sp. (Frank 1879) Frank 1889, *Agrobacterium tumefaciens* (Smith & Towsend 1907) Conn 1942 and *Alpha proteobacterium* Zengler *et al*. Compared to other plant pathogenic bacteria, the similarity indexes were relatively low, at 41% with *E. psidii*, 74% with *X. axonopodis*, 75% with *Pantoea ananatis* (Serrano 1928), and 76% with *Pseudomonas syringae*. Isolate BSV16 showed 95% similarity with *Rhizobium* spp., *Allorhizobium undicola* de Lajudie *et al*., *Agrobacterium tumefaciens*, *A. radiobacter* (Beijerinck & van Delden) Conn emend. Sawada *et al*. and *A. proteobacterium*.

DISCUSSION

Bacterial leaf blight is currently one of the most important leaf diseases in the eucalyptus culture. It occurs mainly in nursery, but also in the field. When in nursery, the leaf lesions and defoliation make the plants unsuitable for transplanting to the field. Between 2003 and 2008, about 105,500 mini-stumps used for cutting production and 16.5 million infected rooted cuttings were discarded in the states of Espírito Santo, Bahia, Goiás, Minas Gerais, and Rio Grande do Sul, with losses of about US $ 10,000,000.00 (personal information, A.C. Alfenas, 2008. UFV, Viçosa, MG).

Bacterial leaf blight differs from leaf blight caused by fungi or abiotic agents, described and illustrated for *Eucalyptus* spp. (Ferreira, 1989; Ferreira & Milani, 2001; Alfenas *et al.,* 2004) by the microscopic exudation of bacterial pus in a water drop (Alfenas *et al*., 2004). Among the fungal diseases, the leaf spot caused by *Kirramyces epicoccoides* (Cooke & Massee) J. Walker, B. Sutton & Pascoe, which occurs in completely expanded leaves, can be confused with bacterial blight, because of the angular and interveinal lesions in both diseases. However, they

TABLE 2 - Biochemical and molecular identification at the genus and species level of bacteria associated with leaf blight of *Eucalyptus* spp.

Isolate	Biochemical tests						M.C.C.**	Genus identified	Microlog™ Biolog		rDNA16S		
	GRAM	Asp.*	Xant.*	Anf.*	M.G.*	Fluor.*			P (%)/ SIM	Species identified	% identity*** fragment size (bp)	Nº of accession****	Species identified
AMP03	-	+	-	-	-	+	3	*Pseudomonas*	100 / 0.7	*P. cichorii*	98 (1542)	EF101305	*P. cichorii*
AMPV01	-	-	+	-	+	-	1	*Xanthomonas*	91 / 0.8	*X. campestris*	96 (1000)	EF101306	*Xanthomonas* sp.
BSV04	-	-	+	-	+	-	1	*Xanthomonas*	91 / 0.8	*X. axonopodis*	98 (1549)	EF101307	*X. axonopodis*
BSV16	-	+	-	-	-	-	2	Undetermined	-	Undetermined	95 (1485)	EF101308	Rhizobiaceae
BSC475a	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.8	*X. axonopodis*	99 (1549)	EF101967	*X. axonopodis*
BSC23	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.5	*X. axonopodis*	97 (500)	EF101968	*Xanthomonas* sp.
CAF05	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.6	*X. axonopodis*	-	-	-
ECLCAF03	-	-	+	-	+	-	1	*Xanthomonas*	96 / 0.6	*X. axonopodis*	95-97^1 (1000)	EF101969	*Xanthomonas* sp.
ESS01	-	-	+	-	+	-	1	*Xanthomonas*	99 / 0.6	*X. axonopodis*	89-98^1 (500)	EF101970	*Xanthomonas* sp
ESS08	-	+	-	-	-	+	3	*Pseudomonas*	74 / 0.6	*P. syringae*	98(1000)	EF101971	*P. syringae*
EGS09	-	-	+	-	+	-	1	*Xanthomonas*	97 / 0.6	*X. campestris*	98 (1565)	EF101972	*X. campestris*
EGS15	-	+	-	-	-	+	3	*Pseudomonas*	92 / 0.8	*P. syringae*	98 (1550)	EF101973	*P. syringae*
EUS11	-	+	-	-	-	+	3	*Pseudomonas*	72 / 0.6	*P. syringae*	90-98^1 (500)	EF101974	*Pseudomonas* sp.
EUS14	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.6	*X. campestris*	98 (1548)	EF101975	*X. campestris*
IP1-05	-	+	-	-	-	+	3	*Pseudomonas*	100 / 0.7	*P. cichorii*	98 (1526)	EF101976	*P. cichorii*
IP1-36	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.8	*X. axonopodis*	99 (1546)	EF101977	*X. axonopodis*
IP2-23	-	-	+	-	+	-	1	*Xanthomonas*	100 / 0.8	*X. axonopodis*	92-96^1 (500)	EF101978	*Xanthomonas* sp.
MSF	-	-	+	-	+	-	1	*Xanthomonas*	91 / 0.8	*X. axonopodis*	98 (1544)	EF101979	*X. axonopodis*
MST07	-	-	+	-	+	-	1	*Xanthomonas*	88 / 0.6	*X. axonopodis*	- (500)	EF101980	*Xanthomonas* sp.
MST09	-	+	-	-	-	+	3	*Pseudomonas*	98 / 0.6	*P. putida*	- (1000)	EF101981	*Pseudomonas* sp.
MRP03	-	+	-	-	-	+	3	*Pseudomonas*	100 / 0.6	*P. putida*	99 (1537)	EF101982	*P. putida*
R57BN4	-	+	-	+	-	-	4	*Erwinia*	-	Undetermined	93-97^1 (1000)	EF101983	*Erwinia* sp.
R203BN10	-	+	-	-	-	-	3	*Pseudomonas*	86 / 0.5	*P. syringae*	98 (1538)	EF101984	*P. syringae*
R57.5	-	-	+	-	+	+	1	*Xanthomonas*	100 / 0.8	*X. campestris*	95-98^1 (500)	EF101985	*Xanthomonas* sp.
RVV11	-	+	-	-	-	-	2	Undetermined	-	Undetermined	94-97^1 (900)	EF101986	Rhizobiaceae

* Asp. = Asparagin; Xant. = Xanthomonadin; Anf. = Facultative anaerobiosis; M.G. = Mucous growth in YDC/33°C medium; Flor. = Fluorescence in King´s B medium.
**M. C. C. = Morphological class of the colony: 1) circular, smooth, high, convex, shiny yellow and mucous colonies; 2) circular, smooth, high, convex, shiny beige and mucous colonies; 3) circular, smooth, low, convex colonies, translucent and fluorescent in King B medium; and 4) circular, smooth, low, convex, light beige colonies, translucent and shiny:
*** Greatest value of identity with sequences of grouped species. P (%) / SIM = Probability / Similarity. (-) data not obtained.
**** Gen Bank.
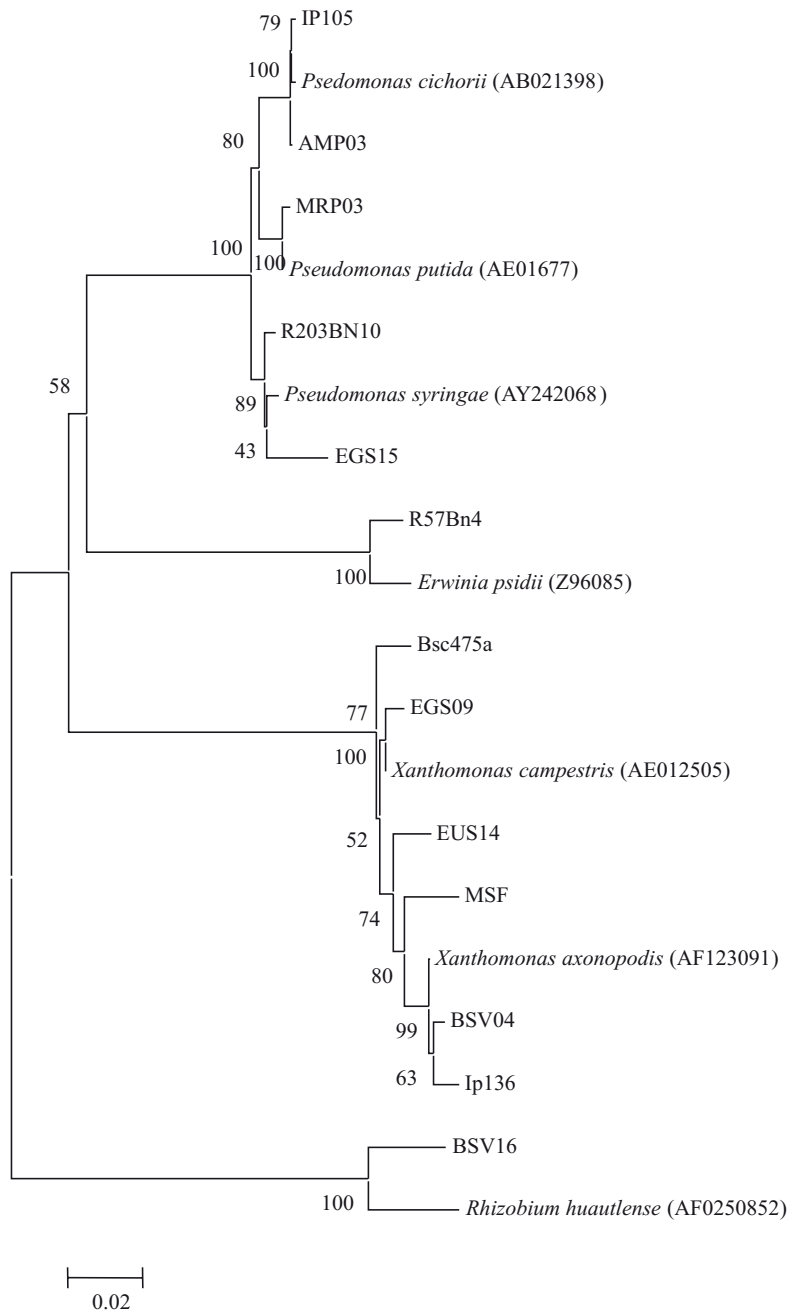
FIG. 2 - Phylogenetic dendrogram of complete 16S rDNA sequences of phytopathogenic bacteria isolates from leaf blight in eucalyptus. Topology constructed in the Mega 2.1 program by the neighbor-joining method and bootstrap analysis on 2,000 trees.

are distinguished by the fungus' dark sporulation, absence of water soaked necrosis and of microscopic bacterial pus exudation from the lesions (Alfenas *et al.*, 2004).

   With the infiltration of bacterial cells in the host's leaf mesophyll (HR-test), there is a rapid development of

the disease and plant pathogenic isolates can be selected (Schaad *et al.*, 2001). However, not all isolates considered pathogenic to eucalyptus, when inoculated by injection, caused symptoms when inoculated by spraying the inoculum suspension. Of the ten isolates tested, only six were

pathogenic by spray inoculation. This is expected because the injection of bacterial cells in the leaf tissue eliminates the pre-penetration barriers to the infectious process. Bacteria that cause leaf blight respond to chemical stimuli at their entry points – stomata, hydathodes, and lenticells – to penetrate and multiply in the intercellular spaces (Swings & Civerolo, 1993, Melotto *et al.*, 2006). Others need wounds on the host to stimulate their mobility and penetration (Josenhans & Suerbaum, 2002).

Of the 25 isolates selected for identification by biochemical and molecular analyses, 14 were included in genus *Xanthomonas*, eight in *Pseudomonas*, one in *Erwinia,* and two in the family Rhizobiaceae. Based on the use of carbon sources, ten of the isolates of *Xanthomonas* were identified as *X. axonopodis* and four as *X. campestris*. Identification of four isolates identified as *X. axonopodis* (BSV04, BSC475a, IP1-36 and MSF) was confirmed by the 16S sequences of the 16S rDNA region (98 to 99% of identity with sequences of *X. axonopodis*). The identity of isolate BSC475a was also confirmed by analysis of the fatty acid profile, while that of the two (EUS14 and EGS09) of the four isolates of *X. campestris* was confirmed by complete sequences of 16S rDNA (98% of identity with sequences of *X. campestris*). Based on phylogenetic analyses, these two isolates formed a group with six species of *Xanthomonas*, including *X. campestris*. Based on partial rDNA sequence the other two isolates were identified, only at the genus level.

Described by Truman (1974), *Xanthomonas eucalypti*, named *X. campestris* pv. *euclypti* by Dye in 1978, was considered the causal agent of die-back in *Eucalyptus* in Australia. The profile of the use of carbon sources by the strain type of this bacterium is distinct from those of *Xanthomonas* identified in the present work, indicating that they belong to different taxa.

Among the eight isolates of *Pseudomonas* identified, four are *P. syringae*, two *P. cichorii*, and two *P. putida*. Isolates MRP03 and MST09, which induced a hypersensitivity reaction in *N. tabacum* and *S. lycopersicum* and necrosis when infiltrated in leaves of *E. urophylla* x *E. maidenii*, were identified as *P. putida*, but they were not pathogenic by spray inoculation. The identification of *P. cichorii* in this study confirms the preliminary report of this species as one of the causal agent of leaf blight of eucalyptus in Brazil (Pomella *et al.*, 1995).

Isolates BSV16 and RVV11 seem to belong to a new taxon, although analysis of the 16S rDNA sequence shows that they are similar to species of the Rhizobiaceae family (*Rhizobium* sp., *A. tumefaciens*, *A. radiobacter, Alpha proteobacterium* and *Allorhizobium undicola*). These isolates used all 95 carbon sources tested, and attempts to identify isolate RVV11 by fatty acid profile analysis (Sherlock MIS system) were also inconclusive (data not shown), considering the absence of referential fatty acids in their database. The Rhizobiaceae family includes symbiotic nitrogen-fixing species, non-symbiotic species

and rhizogenic, and tumorogenic species (Young *et al.,* 2001). Conclusive classification of bacterial isolates RVV11 and BSV16, which are pathogenic to eucalyptus, requires more detailed biochemical and genetic characterization. Along with biochemical tests, total DNA re-association should be carried out with taxonomically close species and representative specimens from each genus of plant pathogenic bacteria.

In South Africa, a disease with similar symptoms to that found in Brazil was attributed to *P. ananatis* (Coutinho *et al.*, 2002). Among the bacteria that were isolated and identified in the present work, only isolate R57BN4, identified as *Erwinia* sp., was similar to this group of bacteria. Based on 16S rDNA sequences of this isolate, about 97% of identity was found with the four isolates studied in South Africa (AF364844 -LMG 20106; AF364845 - LMG 20105; AF364846 - LMG 20104; AF364847 - LMG 20103). Despite responding positively to the pathogenicity test when inoculated by injection in this study, isolate R57BN4 was not pathogenic when inoculated by inoculum spraying on intact eucalyptus leaves, raising doubts about the role of this bacterium as a primary pathogen on eucalyptus.

Although there are several species of bacterium associated with leaf blight of eucalyptus in Brazil, only *X. axonopodis*, *P. cichorii* and members of the Rhizobiaceae family were pathogenic when spray inoculated in intact eucalyptus leaves. *P. cichorii* was found in Mogi Guaçu (São Paulo) and in the Tartarugalzinho region (Amapá), and members of the *Rhizobiaceae* family were only detected in the Teixeira de Freitas region (Bahia) and in Guaíba (Rio Grande do Sul). *X. axonopodis* was reported in all regions except in Amapá, and seems to be the predominant species in nurseries and in the field in all the main Brazilian eucalyptus-producing regions.

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