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Bacteria from savanna-like Cerrado soils tolerant of pH and salinity and antagonistic to *Ralstonia solanacearum*

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ABSTRACT Microorganisms that inhabit the soil play several ecological functions, such as organic matter decomposition, particle aggregation and biological control. The objective of this study was to verify the presence of bacteria tolerant of temperature, pH and salinity in savanna-like Cerrado soils, besides evaluating their antagonistic potential to *Ralstonia solanacearum*, the causal agent of bacterial wilt of various plant species. Sixty-one bacterial isolates were obtained, and 30 of them (none at high temperatures) showed any in vitro antagonism against the plant pathogen in question. Among these, 10 isolates were selected for characterization and identification by sequencing of 16S rRNA. The cultural and morphological characteristics and tests performed were corroborated by the sequencing, which led to the identification of two bacterial genera: *Enterobacter* and *Bacillus*.*Enterobacter* sp. were obtained from pH 5 and 7. On the other hand, *Bacillus* sp. were obtained from pH 3, 5, 9, 10 and 10 and 15% of NaCl. Both bacterial genera observed in savanna-like Cerrado soils are already recognized for their physiological versatility and occurrence in different habitats, as well as their importance in the biological control of pests and pathogens or in the promotion of plant growth, which indicates that the soils of this region are also important sources of microorganisms for the most diverse purposes. These bacterial isolates are stored in the Collection of Bacteria from the Department of Plant Pathology of the University of Brasilia and are available for future studies.

KEYWORDS : acidophilic, alkalophilic, halophilic, bacterial wilt, biocontrol.

1. Introduction:

Microorganisms that inhabit the soil play several ecological functions, such as organic matter decomposition, particle aggregation and biological control (Siqueira, 1993; Moreira & Siqueira, 2006). Among these organisms, bacteria constitute the largest group, and may vary according to the type of soil, management, isolation methods employed (Brandão, 1992) or agricultural practices (Pereira et al., 1999, Carneiro et al., 2004). The savanna-like Cerrado soils are characterized by low fertility and pH due to high concentrations of aluminum, iron, manganese and low levels of phosphorus, calcium and magnesium (Oliveira et al., 2005).

The ability to adapt to environmental changes is one of the most intriguing features of life on Earth (Santos et al., 2001). Extremophilic microorganisms are adapted to surviving in particular ecological niches, such as high temperatures, pH extremes, high concentrations of salt, pressure, high gamma or ultraviolet radiation (Niehaus et al., 1999). When speaking of extremophilia, the first association would be with prokaryotes; however, extremophilic organisms appear in the three domains of living beings, not constituting a phylogenetic characteristic, although all hyperthermophiles are members of Archaea and Bacteria. Among the eukaryotes are common psychrophiles and acidophiles (Rothschild & Mancinelli, 2001). On the other hand, non-thermophilic members belonging to Archaea also inhabit other terrestrial niches (Nicol & Schleper, 2006; Simon et al., 2000), such as the roots of plants. In a pioneering study of microbial diversity in Amazon soils without the need of cultivation, Berneman & Triplett (1997) observed some members of Archaea, Phylum Crenarchaeota.

Aiming to describe the bacterial diversity of soils of the Brazilian Atlantic Forest through independent molecular methods of cultivation, Faoro (2006) observed a predominance of the Acidobacteria phylum, created to encompass acidophilic bacteria in the Bacteria Domain. The phylum with the second greatest number of representatives was Proteobacteria. In the semi-arid region of Cariri (Paraíba), Gorlach-Lira & Coutinho (2007) isolated mesophilic and thermophilic bacteria from soil and rhizosphere associated with *Aristida adscensionis* L., and some isolates were observed growing at up to 70 ºC.

Bacterial diseases constitute a challenge to the growing of *Eucalyptus* spp., and may limit the use of susceptible clones (Cunha et al., 2006). Among these diseases, bacterial wilt caused by *Ralstonia solancearum* may become a problem in clonal nurseries (Alfenas et al., 2006) or even in the field (Marques et al., 2012), under favorable conditions. It is postulated that wilt is not a primary disease but results from the reduction of plant defense by factors such as stress (Coutinho and Wingfield, 2017). However, some studies have reported the management of the disease with the use of soil or rhizosphere bacteria (Moura & Romeiro, 1999; Ran et al., 2005; Cunha et al., 2006).

In view of the above, it was the objective of this study to verify the presence of bacteria tolerant of temperature, pH and salinity in savanna-like Cerrado soils, besides their antagonistic potential to *Ralstonia solanacearum*, the causal agent of bacterial wilt in several plants.

2. Material and Methods:

2.1 Obtaining of soil samples

Five different types of soils were collected at a depth of 0-20 cm, previously georeferenced (Lacerda et al., 2007), on the Água Limpa farm of the University of Brasilia (near Vargem Bonita, DF): Yellow Red Latosol (YRL, cultivated with citrus), Red Latosol (RL, uncultivated), Cambisol (CAMB, uncultivated), Mellean Gleysol (GLEI, cultivated with pasture) and Organosol (ORGA, cultivated with pasture).

2.2 Bacterial isolation

One gram of each soil sample was inoculated into 50 mL of bacteriological medium 523 (Kado & Heskett, 1970) liquid and titrated to pH 3.0; pH 5.0; pH 7.0 and pH 9.0 or supplemented with 5, 10 and 15% of NaCl. Then, the material was incubated on a rotary shaker at 150 rpm and 28 ºC or maintained at 50 ºC and 60 ºC, for isolation of bacteria at high temperatures. After this time, 1 mL was plated in solid 523 medium and incubated for 48 h. The different bacteria were isolated individually by the streaking method, according to their cultural characteristics, and preserved in sterile water and glycerol at -20 ° C.

2.3 Biochemical, morphological and cultural characterization

The selected isolates were submitted to the following tests: Gram, glucose oxidation / fermentation, catalase, fluorescent pigment production (King's B medium) and asparagine as a source of carbon and nitrogen. All tests were performed according to the methodologies described by Mariano et al. (2005). Morphological and cultural characteristics were also observed, such as pigmentation, staining and shape visualization under optical microscope.

2.4 In vitro tests - Prospection of double-layer diffusion antibiosis

Initially the isolates were cultured for 48 h at 28 ° C in 523 medium. Four isolates were then transferred at equidistant points to another Petri dish containing solid 523 medium. The plates were incubated under the above conditions until they showed evident growth. In a laminar flow chamber the plates were inverted and each lid was lined with 90 mm filter paper disks. With the aid of a micropipette, 1 ml of chloroform was added to the paper disks. After 20 min the paper discs were removed and the plates opened for 30 min to remove chloroform residues (Figure 1). Simultaneously, a suspension of *R. solanacearum* (strain UnB 1359, 2T biovar, from eucalyptus) at a concentration of

approximately 10 x 10^8 CFU / mL (equivalent to McFarland's Scale 7) was prepared, from which 25 µL were withdrawn and added to 5 ml of semi-solid (0.8%) flux medium (48 ° C). After returning to the normal position, the plates were covered with the semi-solid flux medium previously inoculated with *R. solanacearum* (with care to form a homogeneous layer over the entire surface.) Two days after inoculation, the formation or not of an inhibition halo was observed (Romeiro, 2007, with modifications). Trials were performed in triplicate.

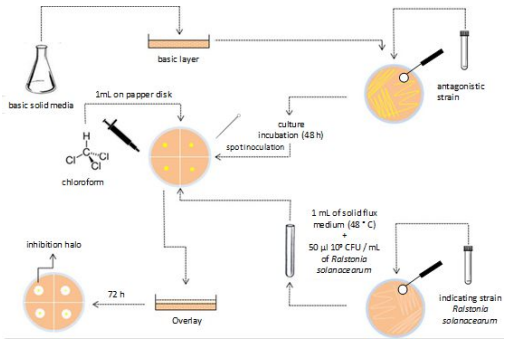

Figure 1. Stages of the in vitro double layer diffusion antibiosis test for selection of bacteria antagonistic to *Ralstonia solanacearum*, according to Romeiro 2007, with modifications.

2.5 Identification on partial 16S rRNA gene-based sequencing

The isolates selected from the in vitro tests were sent to In Vitro Palm Consultoria, Estudo e Desenvolvimento Biológico - LTDA, located in Piracicaba (São Paulo), for partial sequencing of the 16S rRNA gene and identification at the genus level.

3. Results:

3.1 Obtained isolates

From the collected soil samples, a total of 61 bacterial isolates were obtained under the previously described conditions (Table 1).

Table 1. Bacterial isolates obtained from the five different types of soils of savanna-like Cerrado, under the respective conditions imposed.

Type of soil / Condition	ORGA*	GLEI	CAMB	YRL	RL	Total
	Number of isolates obtained					
pH 3	3	2	1	3	1	10
pH 5	1	1	1	1	1	5
pH 7	2	3	1	1	1	8
pH 9	-	-	1	1	1	3
pH 10	1	1	1	1	-	5
50 ºC	1	1	1	1	1	5
60 ºC	1	1	2	1	1	5
5% NaCl	1	1	1	1	1	6
10% NaCl	1	1	2	1	1	10
15% NaCl	1	1	1	1	-	4
Total						61

***ORGA: organosol; GLEI: gleisol; CAMB: cambisol; YRL: yellow red latosol; RL: Red latosol.**

3.2 In vitro inhibition test

Of the 61 bacterial isolates obtained, 30 exhibited some inhibitory activity of *Ralstonia solanacearum* (Figure 2). Of these, 10 were randomly selected to continue their characterization and identification studies.

3.3 Biochemical, morphological and cultural characterization

The biochemical tests revealed that six bacterial isolates were Gram positive and four Gram negative, none produced fluorescent pigment, four were oxidative and six fermentative, all were catalase positive, and the cells were rods. The color of the colonies (Figure 2) varied from cream to white and they were of mucoid to dry consistency (Table 2).

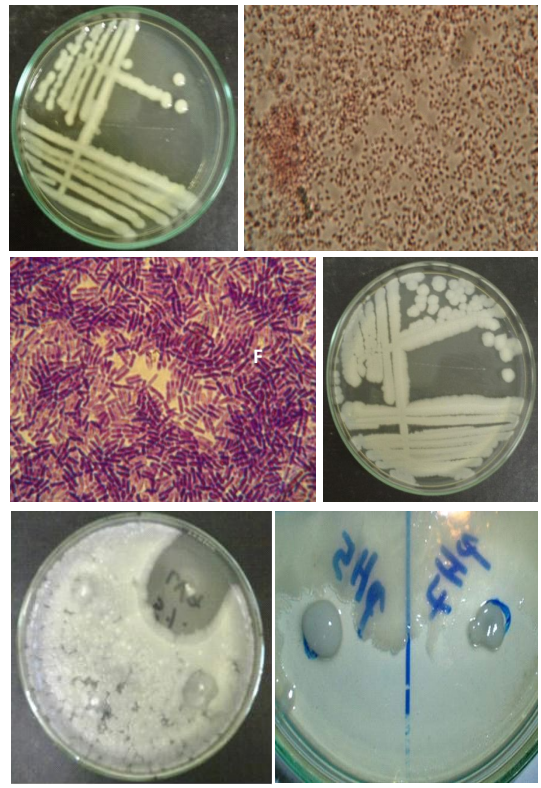

Figure 2. Morphological and cultural characterization and antagonism of bacteria isolated from savanna-like Cerrado soils.

A. Mucoid colonies with a bright cream coloration of the isolate pH 7 ORGA (2),
B. Small rod Gram-positive cells from isolate 15% GLEI (2),
C. dry, white and opaque colonies of the 15% GLEI isolate (2) and
D. Gram-negative rods of the 15% GLEI isolate (2). In vitro test, by double layer diffusion antibiosis against *Ralstonia solanacearum*, showing inhibition halos formed by
E. Isolate 15% GLEI (2) and
F. isolate right pH 7 CAMB and left pH 5 ORGA.

Table 2. Characteristics of bacterial isolates obtained from savanna-like Cerrado soils, under different conditions of pH and salinity, capable of inhibiting the in vitro growth of *Ralstonia solanacearum*.

Isolate	Tests					Morphological and cultural characteristics		
	Gram	King's B	O/F[1]	Catalase	Asparagine	Cells format	Color / Brightness	Consistency
pH 3 GLEI (2)	+[3]	-	O	+	-	R[1]	white / opaque	dry
pH 5 ORGA	-	-	F	+	-	R	cream / opaque	mucoid
pH 5 LV	+	-	O	+	-	R	white / opaque	mucoid
pH 7 ORGA (2)	-	-	F	+	+	R	cream / bright	mucoid
pH 7 CAMB	-	-	F	+	+	R	cream / bright	mucoid
pH 7 LV	-	-	F	+	+	R	cream / bright	mucoid
pH 9 LVA	+	-	O	+	-	R	white / opaque	dry
pH 10 GLEI	+	-	F	+	-	R	white / opaque	dry
10% LVA	+	-	F	+	+	R	white / opaque	mucoid
15% GLEI (2)	+	-	O	+	-	R	white / opaque	dry

[1]O-Oxidative; F- Fermentative
[2]Rods
[3] (+) positive results, (-) negative results in the respective tests*3.4 Identification on partial 16S rRNA gene-based sequencing*

The 16S rRNA partial sequence of the bacterial isolates showed 99%

similarity with the *Bacillus* and *Enterobacter* genera deposited in GenBank (Table 3).

Table 3. Identification of bacterial isolates obtained from savanna-like Cerrado soils by sequencing part of the 16S rRNA gene.

Isolates	Name of Collection	Genus
pH 3 GLEI (2)	UnB 1366	*Bacillus* sp.
pH 5 ORGA	UnB 1367	*Enterobacter* sp.
pH 5 RL	UnB 1368	*Bacillus* sp.
pH 7 ORGA (2)	UnB 1369	*Enterobacter* sp.
pH 7 CAMB	UnB 1370	*Enterobacter* sp.
pH 7 RL	UnB 1371	*Enterobacter* sp.
pH 9 YRL	UnB 1372	*Bacillus* sp.
pH 10 GLEI	UnB 1373	*Bacillus* sp.
10% YRL	UnB 1374	*Bacillus* sp.
15% GLEI (2)	UnB 1375	*Bacillus* sp.

4. Discussion:

Among the 61 bacterial isolates that were able to grow in different pH and salinity conditions, half of them had some inhibitory activity against *R. solanacearum*, strain UnB 1359 from eucalyptus. In the same line, some studies have reported the use of soil or rhizosphere bacteria in the control of eucalyptus diseases. Moura & Romeiro (1999) evaluated the in vitro activity of actinomycetes isolated from different soil types as antagonists to *R. solanacearum*, including a eucalyptus isolate, and most showed intermediate activity of inhibition of this plant pathogen. Isolates of *R. solanacearum* from eucalyptus also had their growth inhibited by *Pseudomonas putida* and *Pseudomonas fluorescens* obtained from rhizospheric soil (Ran et al., 2005). Similarly, Cunha et al. (2006) were successful in the in vitro control of *Pseudomonas chichorii*, the causal agent of eucalyptus leaf spot, by *B. subtilis* rhizobacteria.

The molecular identification of soil isolates indicated that they belong to two Phyla of the Bacterial Domain: Firmicutes and γ-Proterobacteria. The first comprises the genus *Bacillus*, from the family Bacillaceae, which are Gram and catalase positive organisms, with rod-shaped cells. Their colonies vary greatly, mainly according to the composition of the culture medium used, and generally they are apigmented, rhizoid, smooth or rough, tending to grow fast in culture dishes. They exhibit a great diversity and physiological ability, which varies between species. They are psychrophilic to thermophilic, aerobic or anaerobic facultative, growing in a range of 5 to 65 ^{o}C, are acidophilic to alkalophilic, varying between pH 5.7 and 6.8, moderate halophiles to halophiles supporting 2-10% NaCl (Garrity et al., 2005). The observations in biochemical tests, morphology and cultural characteristics confirm the diversity reported in this genus, since the strains grew at pH 3.0, pH 5.0 and pH 9.0, at 10 and 15% of NaCl. In this study, two of the *Bacillus* strains were fermentative, UnB 1373 and UnB 1374. Ibrahim et al. (2007) obtained isolates of *B. halodurans*, from soil and water samples in a sodium lake in Egypt, growing in medium supplemented with 15% of NaCl and pH ranging from 8.0-11.0. Bacteria belonging to *Bacillus* species were observed by Venugopalan et al. (2008) in compost with optimal growth at 60 ° C. Here bacteria were also isolated from higher temperatures, growing at 50 and 60 ° C, but they were not identified because they did not inhibit the growth of the phytopathogen in question. The main habitat of these organisms is soil, but they are widely distributed in nature, including as pathogens of humans (Rezende-Lago et al., 2004), of animals (Santos et al., 2008), of insects (Polanczyk et al., 2003) and of plants (Garrity et al., 2009).

The members of the second Phylum belong to the genus *Enterobacter*, family Enterobacteriaceae, are Gram negative, also have cells in the form of rods, are catalase positive, anaerobic facultative, with colonies smooth, mucoid or dry as observed. The optimal growth is at 30 ° C, but some species are apt to grow at 37 and 45 ° C. They are also found in natural environments including water, waste, vegetables and soil. Pathogens of importance for humans in respiratory infections (*Enterobacter* spp.), some are symbionts in maize, nitrogen fixers and plant pathogen suppressants (*E. cloacae*), endophytes in innumerable plants (*E. asburiae*), and there are also plant pathogenic species such as *E. nimipressuralis* (rotting wood in elm tree), *E. cancerogenus* (causing poppy canker) (Garrity et al., 2005). Other species act on phosphate solubilization (Stamford et al., 2005) and plant growth-promoting (Oliveira et al., 2003; Assumpção et al., 2009). Interestingly, the four isolates obtained from this genus all originate from pH 5 and 7. The enterobacterium *Escherichia*, for example, which is the genus type of Enterobacteriaceae, grows at pH ranging from 5.0 to 9.0 (Garrity et al. al., 2005).

5. Conclusions:

In savanna-like Cerrado soils several bacterial isolates were observed that were capable of growing under acidophilic, alkalophilic, thermophilic and halophilic conditions. However, not all were able to inhibit the growth of plant pathogenic bacterium *Ralstonia solanacearum*.

Among the 10 identified antagonistic bacteria, four belong to the genus *Enterobacter* and six to *Bacillus*. Only representatives of the latter genus were able to grow under halophilic conditions.

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