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ORIGINAL PAPER

Efficient nitrogen-fixing *Rhizobium* strains isolated from amazonian soils are highly tolerant to acidity and aluminium

Paulo Ademar Avelar Ferreira · Cleide Aparecida Bomfeti · Bruno Lima Soares · Fatima Maria de Souza Moreira

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Abstract One of the most cultivated and consumed vegetables in Brazil is the common bean, *Phaseolus vulgaris* L. The symbiosis of this plant species with nitrogen-fixing bacteria that are adapted to the stresses commonly found in tropical soils can increase production. The aim of this study was to evaluate the symbiotic effectiveness of bacterial strains from soils under different land uses in the Amazon region. Further, rhizobia tolerance to acidity and aluminium and the involvement of some possible physiological mechanisms of such tolerance were also investigated. In assessing the efficiency of biological nitrogen fixation, inoculation with strains UFLA04-195, UFLA04-173 and UFLA04-202, belonging to the genus *Rhizobium*, resulted in greater plant growth, higher shoot nitrogen content and good nodulation compared to the inoculation with the strain CIAT 899 (*R. tropici*), and to the mineral nitrogen control or *Burkholderia fungorum* strains that nodulated or not bean plants. These efficient strains grew better at pH 5.0 than at pH 6.0 or pH 6.9; they also tolerated up to 1 mmol l^{-1} of Al^{3+} and showed an increased production of exopolysaccharides where the growing rates were less (pH 6.0 and pH 6.9). With respect to aluminium, the highest production of EPS produced greater tolerance to this element. Taken together, these results indicate that the strains evaluated in this study were tolerant to acidity and aluminium; they appeared to have developed resistance mechanisms such as EPS production and a resistant cell outer membrane (indicated by resistance to polymyxin and methyl violet). As these strains also gave increased yields of the host species, further studies on whether to recommend these strains as inoculants are already underway.

Keywords Exopolysaccharides · Resistance to polymyxin B · Resistance to methyl violet · *Phaseolus vulgaris*

P. A. Avelar Ferreira · C. A. Bomfeti · B. Lima Soares · F. M. de Souza Moreira (✉)
Departamento de Ciência do Solo, Setor de Microbiologia e Bioquímica do Solo, Universidade Federal de Lavras, Caixa Postal 3037, Lavras, Minas Gerais CEP 37200-000, Brazil
e-mail: fmoreira@dcs.ufla.br

C. A. Bomfeti
Instituto de Ciência e Tecnologia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, Minas Gerais CEP 39803-371, Brazil

Introduction

Bean (*Phaseolus vulgaris* L.) is an important crop in Brazilian agriculture, and it represents the main source of protein for the low-income population of the country. Brazil is the largest world producer and consumer of the common bean; a total of 3,265,000 tons were produced with an average yield of 942 kg ha^{-1} in the harvest 2010 (CONAB 2010).

This bean plant can associate with nitrogen-fixing bacteria, commonly called rhizobia. This association enables the plant to secure part of its nutritional nitrogen requirements through biological nitrogen fixation (BNF), which leads to a decrease in both production costs and nitrogen fertilizer pollution. Therefore, it is economically and ecologically advantageous.

The efficiency of the interaction between rhizobia and bean plants depends not only on the combination of bacteria and plant variety, but also on the environmental conditions where the symbiosis occurs. In the case of

tropical soils, conditions such as high temperatures, low pH, low nutrient levels and high levels of aluminium can reduce the rhizobia soil populations, impairing the BNF process (Wood 1995; Igual et al. 1997; Lima et al. 2009). This is especially true in *Phaseolus vulgaris*, a species known to be sensitive to these stressors (Vieira 2006).

The Amazon region contains a large number of nodulating plant species in areas where human intervention has not occurred (Moreira et al. 1992) and the soils of these areas display characteristics representative of tropical soils. Therefore, these areas can be considered a potential source of inoculant strains for vegetable plants, including beans. Strains derived from these soils have been selected and recommended for the cultivation of cowpea bean, and they have been used successfully in other areas (Lacerda et al. 2004; Soares et al. 2006a, b; Almeida et al. 2010).

The underlying mechanisms that result in differences in acid tolerance in rhizobia strains have not been well defined (Correa and Barneix 1997; Graham et al. 1994). However, several authors have suggested that this tolerance is due to the ability of acid tolerant rhizobia strains to maintain a consistent cytoplasmic pH (Chen et al. 1993a, b; Goss et al. 1990; O'Hara et al. 1989). Differences in lipopolysaccharide membrane composition and exclusion and extrusion of protons (Chen et al. 1993a, b); accumulation of cellular polyamines (Fujihara and Yoneyama 1993); protein synthesis (Hickey and Hirshfield 1990); membrane lipids modification (Rojas-Jiménez et al. 2005) and accumulation of molecules that offer protection to oxidative stress, such as glutathione (Riccillo et al. 2000; Muglia et al. 2007), are also associated with cell growth in acidic conditions. Acid-adapted cells were also found to be more tolerant to certain surface-active agents like crystal violet and polymyxin, whose uptake depends on the permeability of the outer membrane (Leyer and Johnson 1993).

According to Cunningham and Munns (1984), *Rhizobium* strains that produce greater amounts of exopolysaccharides (EPS) are more tolerant to acidic conditions when compared to strains that produce a lesser amount. Barberi et al. (2004) and Miguel and Moreira (2001) also found similar results for strains of the genus *Bradyrhizobium*; they correlated the higher EPS production by these bacteria with their better growth under acidic culture conditions.

The aim of this study was to evaluate the symbiotic effectiveness of inoculating *P. vulgaris* L. with strains from soils under different land uses that are representative of the Amazon region. In addition, rhizobia tolerance to acidity and aluminium were examined, as were possible some physiological mechanisms of such tolerance, i.e., EPS production and those related to the ability of cells to tolerate compounds that interact with the bacterial cell surface, specifically, resistance to the hydrophobic dye methyl violet and the antibiotic polymyxin B, were examined.

Materials and methods

Measurement of symbiotic efficiency

Fourteen fast growing rhizobial strains isolated from nodules of the promiscuous trap plant *Macroptilium atropurpureum* (siratro) from the previous work of Lima et al. (2009) were evaluated regarding their symbiotic efficiency with common beans. Origin and cultural characteristics of these strains are shown in Table 1.

Experiments were conducted in a greenhouse during February and March of 2008 using Leonard's jars. The top of the jar contained a 1:2 mixture of sand (150 ml) and vermiculite (300 ml), the bottom contained a mineral solution previously described in Hoagland and Arnon (1950). This solution had a low concentration of mineral nitrogen (5.25 mg l^{-1}) obtained from NH_4NO_3 and KNO_3.

The statistical design was completely randomised, with seventeen treatments and five replications. The treatments consisted of the 14 strains to be tested, the strain approved by the Ministry of Agriculture, Livestock and Supply as bean inoculant, CIAT899^T (*Rhizobium tropici*), which is well known for its high efficiency (symbiotic nitrogen fixation) and acid tolerance (Graham et al. 1994), and two controls that were not inoculated. One of these controls had the low N concentration of the solution and the other received mineral nitrogen to a concentration up to 210 mg of nitrogen per jar in the form of NH_4NO_3.

The bean cultivar used was the BRS-MG Talismã, carioca grain type, launched in 2002; it is resistant to anthracnose and common mosaic virus, and is also moderately resistant to angular leaf spot (Ramalho et al. 2002). Before planting, seeds were superficially disinfested with 70% ethanol for 5 min and 1% sodium hypochlorite for 3 min and then they were washed six times in sterile distilled water. Once germinated, four seeds were transferred to each Leonard's jar and each seed was inoculated with 1×10^8 rhizobia cells from bacterial cultures that were in exponential phase (1×10^8 cells ml^{-1}). After 5 days of germination, thinning was performed leaving only two plants per jar.

Plants were harvested during the flowering period at 38 days to assess the following variables: number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM) and nitrogen accumulation in shoots (NAS). The nitrogen accumulated in shoots (NAS) was calculated by multiplying the weight of dry shoots by the nitrogen content, which was measured by the semi micro-kjedahl method as described by Sarruge and Haag 1979.

Statistical analysis

All data were subjected to analysis of variance, using the statistical analysis software Sisvar version 4.0 (Ferreira 2000).

Table 1 Origin, cultural characteristics, and identification of the strains isolated from nodules of siratro (*Macroptilium atropurpureum*)*

Strains	Origin (LUS, coordinates GPS and soil characteristics of the site)					Cultural characteristics		Identification (based on the most similar sequence found in GenBank)				Accession number in GenBank
	LUS[a]	Longitude	Latitude	pH	$Al^{3+}cmol_c\ Kg^{-1}$	G.R[b]	pH[c]	Species	Similarity (%)	bp[d]	Accession number	
UFLA04-195[e]	FA	386933.89	9514270.62	4.0	9.4	F	Acid	*R. miluonense*	100	678	EF061096	JF412048
UFLA04-122[e]	PF	433449.00	9518075.51	4.5	7.5	F	Acid	*B. fungorum*	100	655	NR025058	JF412046
UFLA04-202[e]	P	387128.16	9514279.79	5.2	2.3	F	Neutral	*R. miluonense*	100	689	EF061096	JF412049
UFLA04-173[e]	AGRI	9514279.79	9512607.25	5.1	2.5	I	Neutral	*R. miluonense*	100	606	EF061096	JF412047
UFLA04-227[e]	AGRI	399088.64	9512729.06	5.6	0.2	F	Neutral	*B. fungorum*	100	594	NR025058	JF412051
UFLA04-226[e]	AGRO	433281.71	9518598.90	5.3	0.4	F	Acid	*B. fungorum*	100	709	NR025058	JF412050
UFLA04-155[e]	FI	433388.76	9518509.63	4.6	3.4	F	Acid	*B. fungorum*	100	631	NR025058	GU144370
UFLA04-228[e]	FI	398948.32	9512557.48	4.4	6.7	F	Acid	*B. fungorum*	100	654	NR025058	JF412052
UFLA04-229[e]	P	387134.63	9514079.79	5.3	2.2	F	Neutral	*B. fungorum*	100	718	NR025058	JF412053
UFLA04-231[e]	P	387122.42	9514384.36	5.3	3.5	F	Acid	*B. fungorum*	100	692	NR025058	JF412054
UFLA04-233[e]	AGRI	399264.14	9512583.50	4.9	2.5	F	Acid	*B. fungorum*	100	626	NR025058	JF412056
UFLA04-232[e]	P	387023.57	9514375.50	5.2	2.3	F	Neutral	*B. fungorum*	100	631	NR025058	JF412055
UFLA04-234[e]	AGRI	399088.64	9512729.06	5.6	0.2	F	Neutral	*B. fungorum*	100	614	NR025058	JF412057
UFLA04-21[e]	AGRI	399025.44	9512442.01	4.9	5.4	F	Acid	*Burkholderia* sp.	99	–	FJ534643	FJ534643
CIAT899	Colombia	–	–	–	–	F	Acid	*R. tropici*	–	–	–	–

* This specie was used as a "trap" and it was inoculated with soils under different land uses from Alto Solimões, AM, Brazil

[a] LUS: Secondary forest in advanced stage of regeneration (FA), agriculture (AGRI), primary forest (PF), pasture (P), agroforest (AGRO), secondary forest in the early stages of regeneration (FI)

[b] Growth rate (as time of appearance of isolated colonies) faster, F (2–3 days), intermediate, I (4–5 days)

[c] pH of the culture medium

[d] Base pair

[e] Lima et al. (2009)

Treatment means were grouped by the Scott-Knott test at 5% probability (Scott and Knott 1974). The values of variables, number of nodules (NN) and nodules dry matter (NDM) were previously transformed by the formula $(X + 0.5)^{0.5}$.

Genetic identification of strains

The strains were grown in 79 liquid medium for 3 days at 28°C. Then the DNA of each strain was extracted using the ZR Fungal/Bacterial DNA Kit (Zymo Research Corp.). The 16S rDNA of the strains was amplified by PCR using the primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). A 2-µl aliquot of extracted DNA was used in polymerase chain reaction (PCR) with a final volume of 50 µl per reaction. The final concentrations of the reagents, per reaction, were 0.2 µM of each primer 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane 1991; Pepi et al. 2007; Osman et al. 2010; Ahmad et al. 2009), 2.5 mM of magnesium chloride, PCR buffer at 1×, 0.2 µM of each dNTP, and 0.02 U Taq DNA polymerase (Taq DNA polymerase, Invitrogen). The amplification reaction was carried out in an Eppendorf Mastercycle® thermocycler under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation (94°C for 40 s), annealing (55°C for 40 s), extension (72°C for 1.5 min), and one final extension at 72°C for 7 min. An aliquot of each PCR reaction (20 µl) was analyzed using a 1% (w/v) agarose gel with TAE buffer and ethidium bromide staining (5 µg ml^{-1}). PCR products purification and DNA sequencing (with the 27F primer) was performed by Macrogen with a 37 30 × 1 sequencer.

To perform sequence alignments using the ClustalW program, we obtained similar sequences from GenBank using Basic Local Alignment program Search Tool (NCBI). The aligned sequences were used for phylogenetic analysis. For this purpose, we used the Neighbour-Joining method with the Kimura 2-parameter (Kimura 1980) in the MEGA 4.1 program (Tamura et al. 2007); a bootstrap with a minimum of 2,000 replications was applied.

Testing acidity and aluminium tolerance in vitro

All strains were tested previously for their tolerance to acidity and aluminium in tests using solid "79" culture medium (Fred and Waksman 1928) by simply streaking them on the surface of this medium with pH 5, 6 or 6.9 or aluminium concentrations 0, 0.5, 1 or 2 mmol l^{-1}, added as $AlCl_3.6H_2O$ before autoclaving.

The strains UFLA04-195, UFLA04-173 and UFLA04-202, that showed the highest symbiotic efficiency in the initial greenhouse experiments, were evaluated for their tolerance to acidity and aluminium (Al^{3+}) in tests using liquid culture medium. Strain CIAT899 was also included as a control. Isolated colonies of each strain were inoculated in "79" liquid medium (Fred and Waksman 1928) without the bromothymol blue dye and grown until they reached an optical density (OD) of 0.5 at 560 nm. One millilitre of inoculum was then transferred to 100 ml of culture medium "79" supplemented with HEPES (1.3 mg l^{-1} N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) and MES [1.1 mg l^{-1} of 2-(N-morpholino) ethane sulfonic acid] (Cole and Elkan 1973); the pH of the media had previously been adjusted with 2 mol l^{-1} HCl to pH 5.0, 6.0 or 6.9. The influence of Al^{3+} on growth was evaluated in liquid media containing 0.0, 0.5, 1.0 and 2.0 mmol l^{-1} of Al^{3+}, added as $AlCl_3{\cdot}6H_2O$ before autoclaving; the final pH was adjusted to 4.5. Strains were grown under agitation at 110 rpm at 28°C. Assessments were made using the successive 10-fold dilutions method as described by Miles and Misra (1938). The numbers of colony forming units (CFU) were counted at intervals of 6, 12, 24, 48, 72, 96 and 120 h of incubation. The number of CFU was calculated by the direct enumeration of colonies present on the plates for each dilution. Regression equations were obtained by the Tablecurve v.5.01 program (Jandel Scientific Corporation), obtaining the equations of best fit and assessing for F test significance. Data were analyzed by ANOVA and means (Scott-Knott) by using the statistical program SISVAR (Ferreira 2000).

Resistance to polymyxin B and methyl violet

To examine resistance to polymyxin B and methyl violet, we used the methodology described by Leyer and Johnson (1993). Cells from the four strains (UFLA04-195, UFLA04-173 and UFLA04-202 and CIAT 899) that were most efficient in promoting plant growth were cultured in "79" medium (pH 6.9) and incubated at 28°C in a shaker set at 110 rpm. When they reached the logarithmic phase (OD 560 = 0.5), cultures were treated with methyl violet (25 mg l^{-1}) or polymyxin B (10 mg l^{-1}). Cell viability was determined by the method of successive decimal dilutions for counting the number of colony forming units (CFU) described by Miles and Misra (1938) after 0, 20, 40 and 60 min of incubation. The acid sensitive strain UFLA04-234 (tested in solid medium) was used as negative control.

Measurement of exopolysaccharide production

EPS extraction was performed for the four strains (UFLA04-195, UFLA04-173 and UFLA04-202 and CIAT 899) grown in liquid medium "79" with the same values of pH and concentrations of Al^{3+} tested previously in the

experiments of tolerance. First, strains were cultured for 120 h at 28°C with agitation. EPS was extracted from the culture medium in the stationary phase of bacterial growth by centrifugation at 13,000 g at 4°C for 30 min to remove bacterial cells. Next, three times its volume of cold ethanol was added to the supernatants; mixtures were then kept refrigerated for 24 h to precipitate the EPS. After filtering and EPS collection, preparations were kept in an oven for 48 h at 70°C. EPS production was assessed by total dry weight of product divided by the dry weight of cells separated by centrifugation.

Results

Measurement of rhizobia symbiotic efficiency

In the symbiotic efficiency experiments, plants inoculated with strains UFLA04-195, UFLA04-173, and UFLA04-202 caused the highest yield of shoot dry matter (SDM). These results were similar to the reference strain (CIAT899) and higher than other treatments, except for the control treatment that received 210 mg of nitrogen (NH_4NO_3 and KNO_3) per jar. With respect to nitrogen accumulation in shoots (NAS), UFLA04-195, UFLA04-173, and UFLA04-202 strains and strain CIAT899 behaved similarly to the control treatment with 210 mg N (Fig. 1).

The highest values for nodule number (NN) and nodules dry matter (NDM) were obtained with strains UFLA04-195, UFLA04-173, and UFLA04-202, whose behaviours were similar to strain CIAT899. Strains UFLA04-155, UFLA04-231, UFLA04-234, UFLA04-226, UFLA04-233, UFLA04-232, and UFLA04-228 did not form nodules on the root systems of inoculated plants. Strains UFLA04-21, UFLA04-229, UFLA04-122, and UFLA04-227 formed nodules on root systems of plants but were symbiotically inefficient. Although strain UFLA04-155 did not form root nodules, it was able to induce some plant growth compared to the control plants with low amount of nitrogen. This was most likely caused by a mechanism other than the symbiotic nitrogen fixation; additionally, this effect may have been limited by the low nitrogen content in the substrate. Moreover, the controls did not show nodules on their roots, indicating that there was no contamination of the experiment (Fig. 2).

Genetic identification of strains

Comparison of the 16S rDNA sequences of the 15 strains tested using the data available in Genbank revealed that they are phylogenetically similar to sequences of nitrogen-fixing bacteria and known members of the genera *Rhizobium* and *Burkholderia* (Fig. 3).

The similarities of the sequences obtained from the studied species with the Genbank accessions varied from 99 to 100% (Table 1). Strains UFLA04-202, UFLA04-195, and UFLA04-173, belonging to the genus *Rhizobium*, were able to efficiently nodulate the species *Phaseolus vulgaris*. However, strains UFLA04-227, UFLA04122, and UFLA04-229, which were classified together with *Burkholderia fungorum*, were able to nodulate bean plants despite not having high symbiotic efficiency. This was true for strain UFLA04-21, which was also classified as *Burkholderia* species. The new sequences were submitted to GenBank and received the accession numbers listed in Table 1.

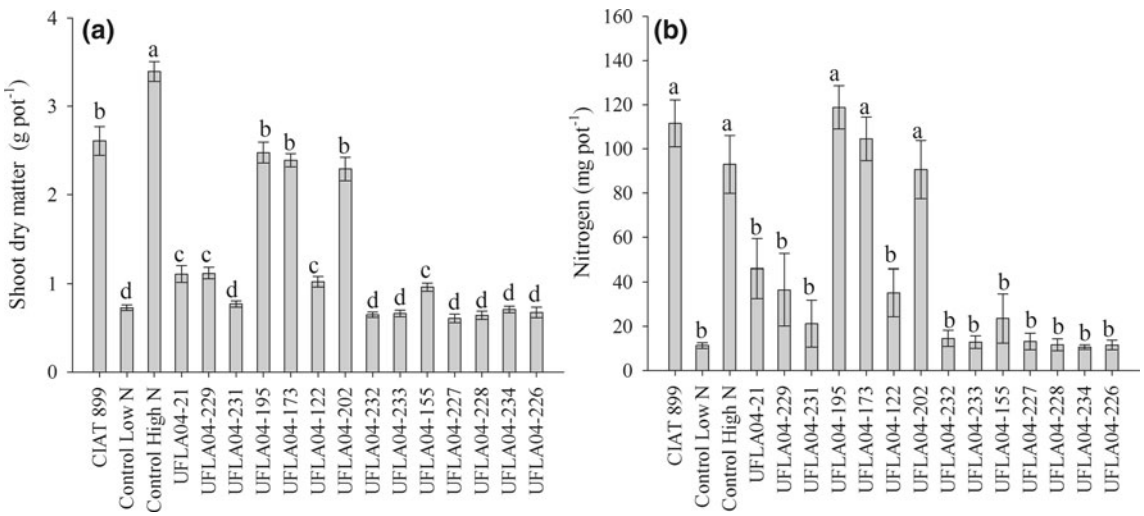

Fig. 1 Shoot dry matter (**a**) and nitrogen accumulation in shoots (**b**) of bean cv. Talismã inoculated with different rhizobia strains. *Same letters* indicate mean of the same group by Scott-Knott test at 5% probability

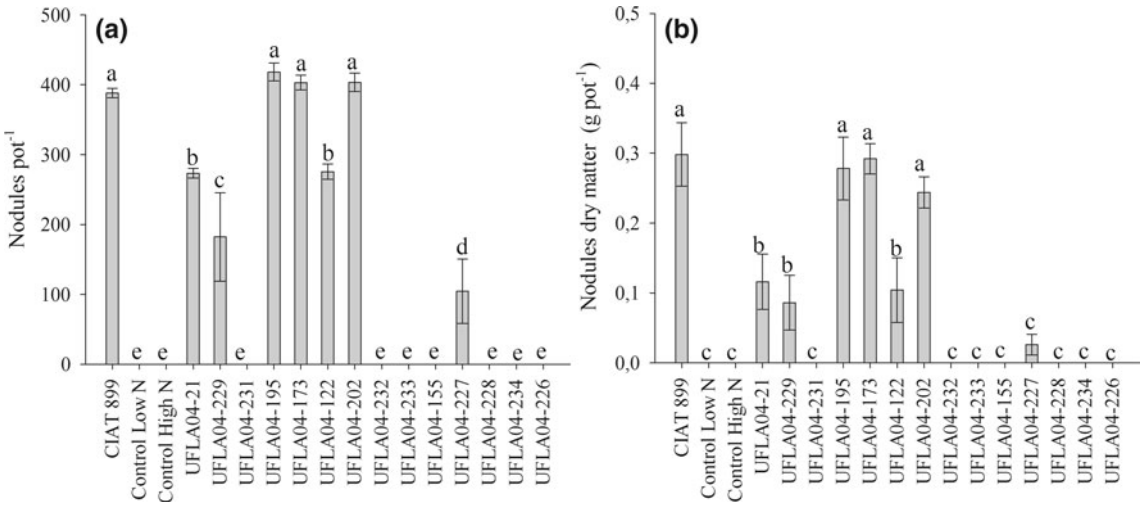

Fig. 2 Number of nodules (**a**) and dry matter of nodules (**b**) of bean cv. Talismã inoculated with different rhizobia strains. *Same letters* indicate mean for the same group by Scott-Knott test at 5% probability

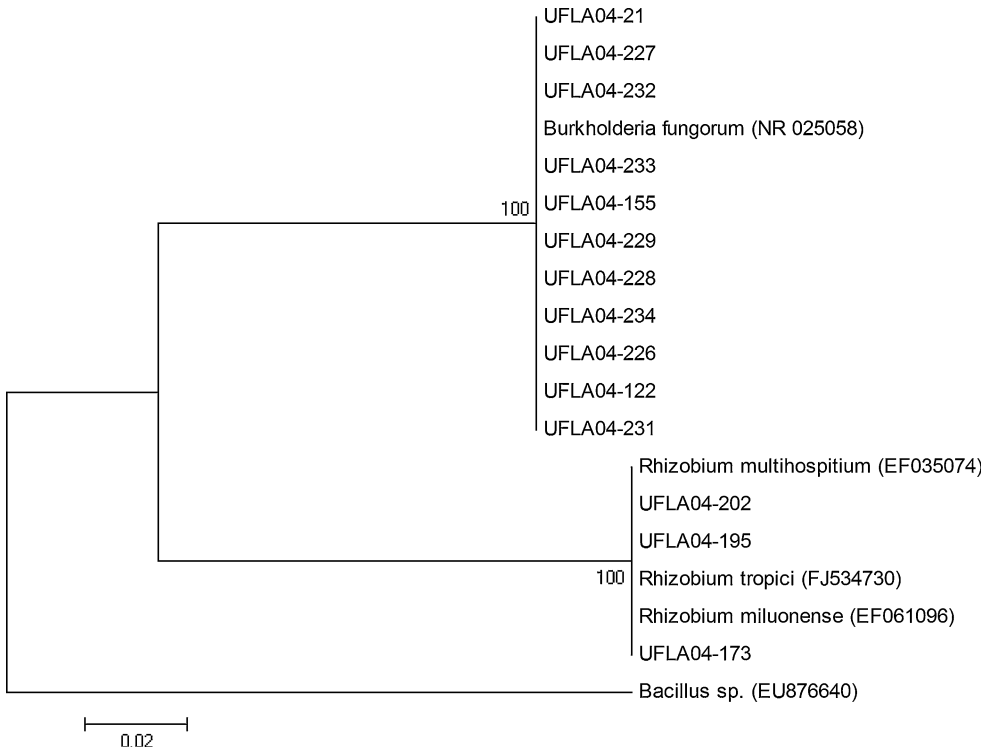

Fig. 3 Neighbour-Joining phylogeny for 16S rDNA of different rhizobia strains. The root for the analysis was *Bacillus* sp. The size of the alignment sequence was 418 bp. The bootstrap percentage support was 2,000 replications

Tolerance to acidity and aluminium in vitro

Strains UFLA04-234 and UFLA04-227 grew only in pH 6.9, and at none of the aluminum concentrations in solid media. The other strains (UFLA04-195, UFLA04-122, UFLA04-202, UFLA04-173, UFLA04-155, UFLA04-226, UFLA04-228, UFLA04-229, UFLA04-231, UFLA04-233, UFLA04-232, UFLA04-21and CIAT899) grew at all values of pH and until the concentration of 0.5 mmol l^{-1} of Al^{3+} in solid media.

Strains UFLA04-195, UFLA04-202, UFLA04-173 (*Rhizobium* sp.), and CIAT899 (*R. tropici*) exhibited better growth at pH 5.0 in liquid media according to Scott-Knott Test at 5% of significance (Fig. 4). Interestingly, strain UFLA04-173 (*Rhizobium* sp.) had a higher growth rate at pH 6.0 during the first 24 h, but after 48 h, its growth at pH 5.0 and 6.9 resembled that observed at pH 6.0. At pH 5.0, strain UFLA04-195 reached stationary phase at 24 h. However, for cultures grown at pH 6.0 and 6.9, the occurrence of a stationary phase was not well defined; after

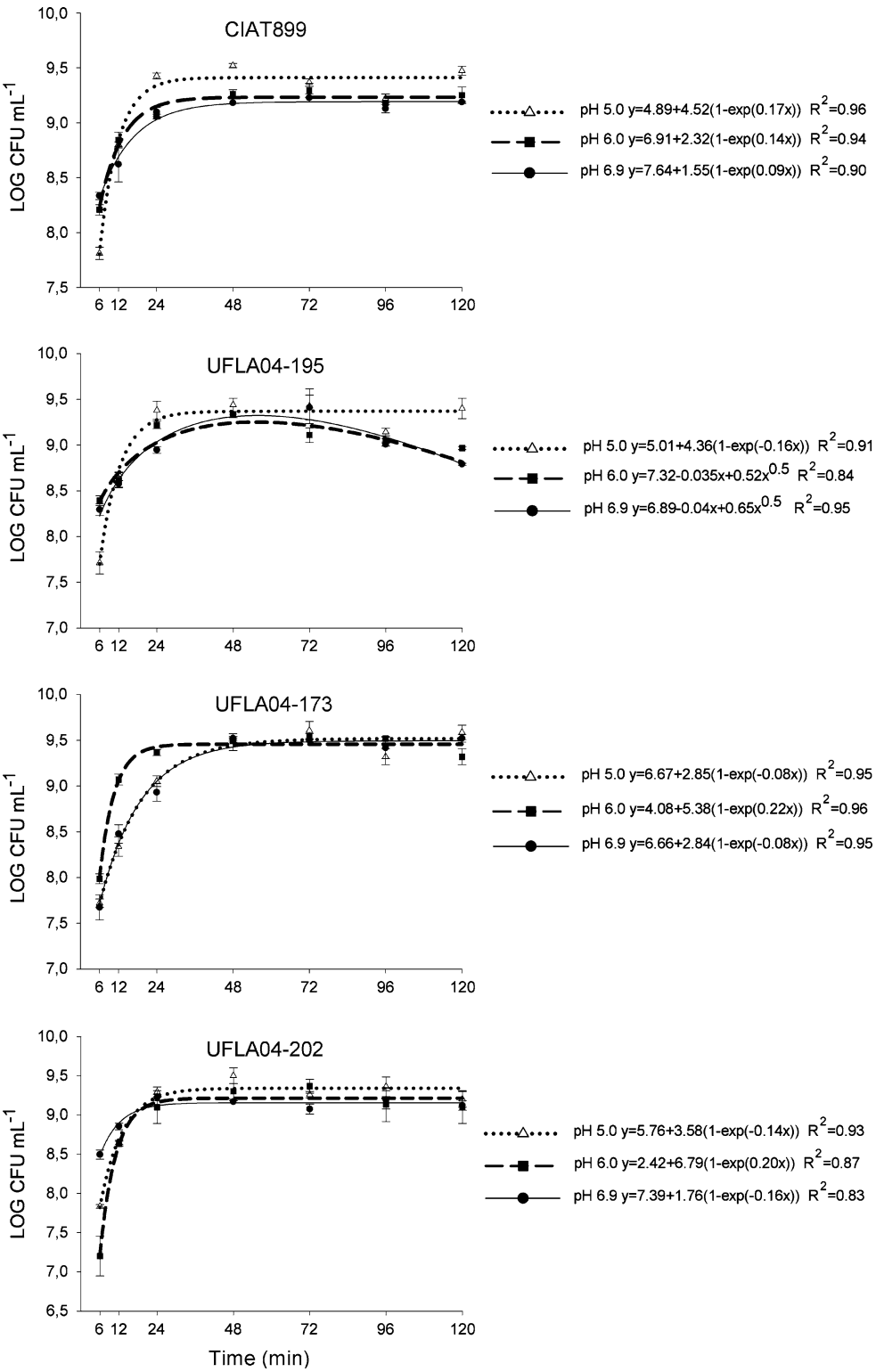

Fig. 4 Growth of strains CIAT899, UFLA04-173, UFLA04-195, and UFLA04-202 in liquid medium "79" as function of time and pH of cultivation

48 h of culture, there was a marked decrease in bacterial population. From the graph, the bacterial growth curves of the strains UFLA04-202 and CIAT899 seems to be essentially similar in the different pH (Fig. 4).

Regarding the effect of aluminium (Fig. 5), strains CIAT899, UFLA04-202, and UFLA04-195 had similar growth at concentrations 0.0 and 0.5 mmol Al^{3+} l^{-1}. At 0.5 mmol Al^{3+} l^{-1}, strain UFLA04-173 showed a decrease in growth rate at 48 h.

At a concentration of 1.0 mmol Al^{3+} l^{-1}, strain CIAT899 grew exponentially until 72 h, with a subsequent reduction. With strain UFLA04-195, there was a decrease

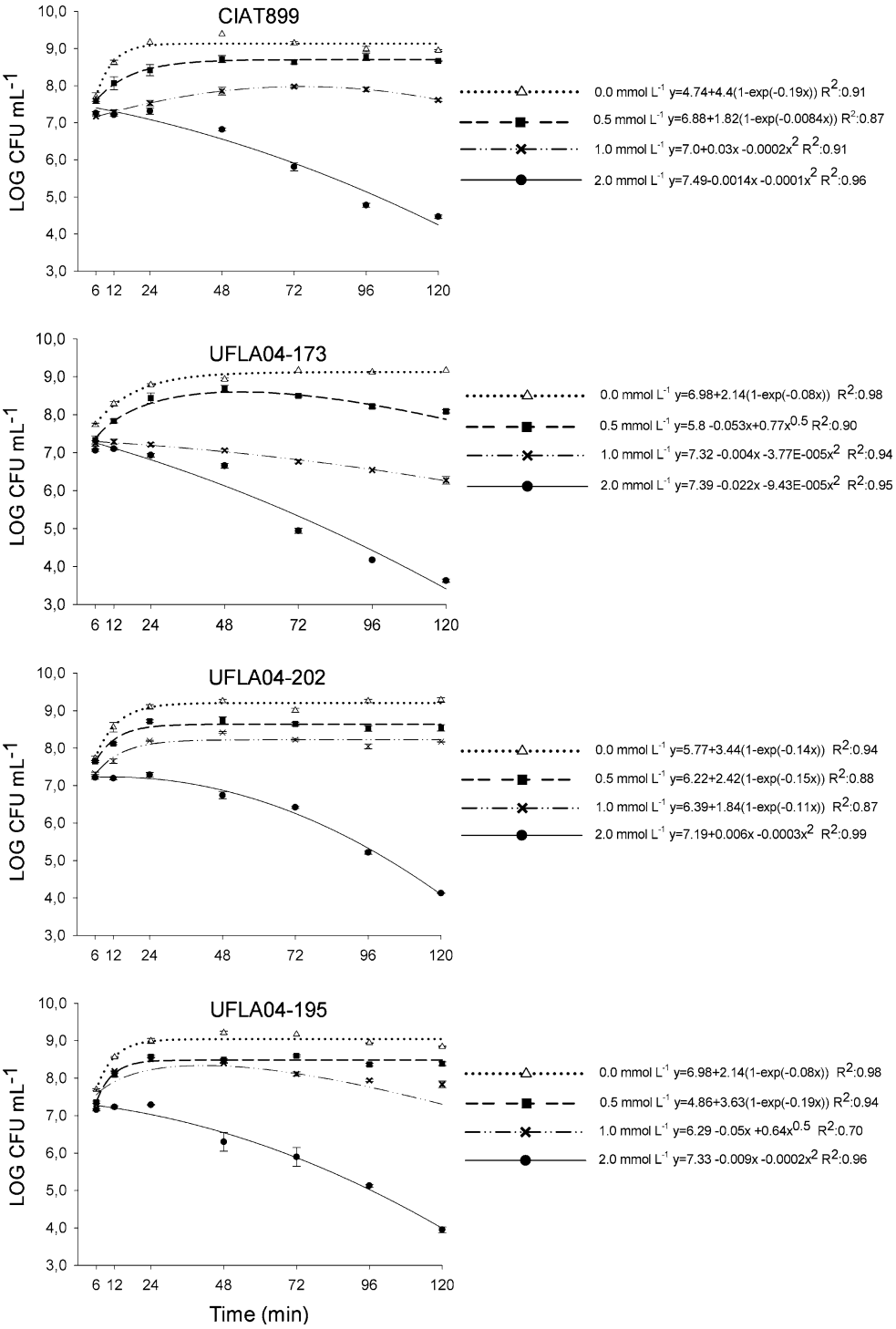

Fig. 5 Growth of strains CIAT899, UFLA04-173, UFLA04-195, and UFLA04-202 in liquid media as a function of different concentrations of Al^{3+} in the culture medium

in growth rate observed as early as after 48 h. Cell multiplication was not observed in strain UFLA04-173 at 1.0 mmol Al^{3+} l^{-1}; there was a sharp reduction in the number of CFU ml^{-1} throughout the study period. Regarding UFLA04-202 strain, the steady growth starting at 24 h in 1.0 mmol Al^{3+} l^{-1} indicates a greater tolerance of this strain compared to the others. There was no growth of the strains CIAT899, UFLA04-202, UFLA04-173, and UFLA04-195 at a concentration of 2.0 mmol Al^{3+} l^{-1} (Fig. 5). These result demonstrate a clear connection between the chemical characteristics of the soils (high acidity, very high aluminium concentration), from which strains were isolated, and their tolerance to these stresses (Table 1).

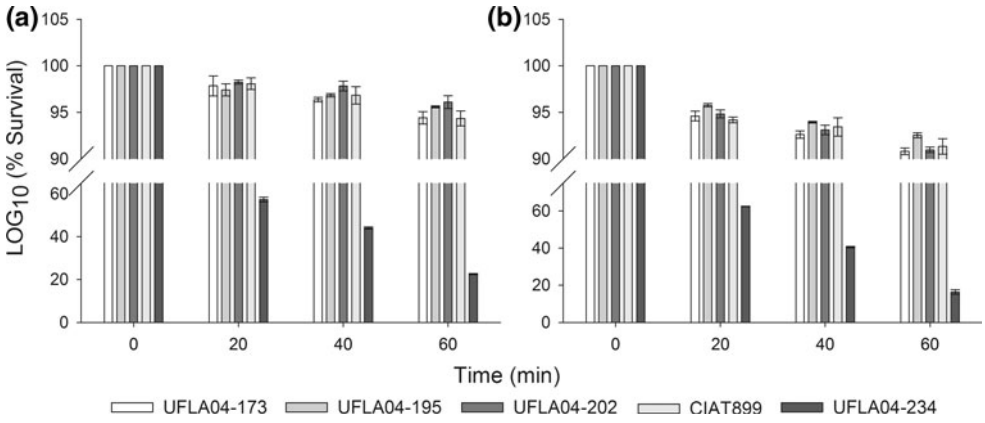

Fig. 6 Survival of rhizobia strains adapted to acidity during exposure to 25 mg of methyl violet (**a**) or 10 mg of polymyxin B (**b**) per litre

Resistance to polymyxin B and methyl violet

The ability of cells to tolerate compounds that interact with the bacterial cell surface was evaluated; specifically, resistance to the hydrophobic dye methyl violet and the antibiotic polymyxin B were examined. When exposed to 25 mg of crystal violet per litre, the four strains adapted to acidic conditions were very tolerant to the action of this dye (Fig. 6a). Approximately 94, 96, 96 and 94% of the log of the original population of cells of strains UFLA04-195, UFLA04-173, UFLA04-202, and CIAT899, respectively, remained viable after 60 min of exposure to methyl violet.

The addition of 10 mg per litre of the antibiotic polymyxin B affected the survival of strains UFLA04-195, UFLA04-173, UFLA04-202 and CIAT 899 in only 9.1, 7.5, 9.0 and 8.7%, respectively, even after 60 min of exposure to this antimicrobial agent (Fig. 6b). In contrast, the acid non adapted rhizobial strains UFLA04-234 after exposed to methyl violet (Fig. 6a) and polymyxin B (Fig. 6b), decreased about 80 and 84% after 60 min respectively.

EPS production

The amount of EPS per gram of bacterial cell strains produced by UFLA04-195, UFLA04-173, UFLA04-202, and CIAT899 was higher at pH 6.9 when compared with other pH values; the lowest production was observed at pH 5.0 (Table 2). In the case of strain UFLA04-202, EPS production at pH 6.9 was almost twice that observed for pH 5.0.

Table 2 Amount of exopolysaccharide (g EPS g cell^{-1}) produced by strains UFLA04-195, UFLA04-173, UFLA04-202, and CIAT899 at pH 5.0, 6.0 and 6.9

Strain	pH 5.0 g EPS g cell^{-1}	pH 6.0	pH 6.9
UFLA04-195	2.43 ± 0.19	3.30 ± 0.33	3.37 ± 0.18
UFLA04-173	3.86 ± 0.23	4.00 ± 0.29	4.32 ± 0.14
UFLA04-202	4.00 ± 0.5	5.52 ± 0.2	7.40 ± 0.3
CIAT899	2.73 ± 0.1	4.78 ± 0.1	5.26 ± 0.1

The lowest EPS production was correlated with better growth. Strains UFLA04-195, UFLA04-202, and CIAT899 showed better growth when cultured at pH 5.0. Better growth indicates a greater adaptation to this condition, and these strains consequently produced smaller amounts of EPS. In the case of UFLA04-173, there were no major differences in growth rate at different pH; consistent with this, EPS production did not change substantially over the different pH values.

The strains and UFLA04-173, and UFLA04-202 produced a greater amount of EPS in relation to strain UFLA04-195 at tested pH values. This lower EPS production by strain UFLA04-195 at pH 5.0 may be related to the origin of this strain; it was isolated from soil with pH 4.0, making it more likely to be adapted to acidic conditions. The other strains were isolated from soil samples with a pH of 5.1.

Assessment of EPS production in liquid media with different concentrations of Al^{3+} (Table 3) showed that this was high in medium without addition of Al^{3+}. At the concentration of 2.0 mmol Al^{3+} l^{-1}, no EPS production could be detected once there was a marked reduction in bacterial growth.

At concentrations of 0.5 and 1.0 mmol Al^{3+} l^{-1}, strain UFLA04-202 produced a larger amount of EPS compared to the other strains tested; this strain also showed better growth under these conditions. Increased concentrations of aluminium in the culture medium did not result in an increased production of EPS by strains UFLA04-202, UFLA04-173, UFLA04-195, and CIAT899. These results indicate a positive correlation between EPS production and tolerance to Al^{3+} for these strains, which showed better growth when EPS production could be detected. Indeed, strain UFLA04-173 showed very low or no production of EPS in the presence of 0.5, 1.0 and 2.0 mmol Al^{3+} l^{-1}, which reflected the less growth of this strain.

Table 3 Amount of exopolysaccharide (g EPS g cell^{-1}) produced by strains UFLA04-195, UFLA04-173, UFLA04-202, and CIAT899 cultured in different concentrations of aluminium

Strain	0.0 mmol g EPS g cell^{-1}	0.5 mmol	1.0 mmol	2.0 mmol
UFLA04-195	1.93 ± 0.1	1.73 ± 0.1	0.73 ± 0.09	0.00
UFLA04-173	5.57 ± 0.17	0.52 ± 0.1	0.00	0.00
UFLA04-202	3.95 ± 0.12	3.72 ± 0.07	2.66 ± 0.1	0.00
CIAT899	4.11 ± 0.14	1.26 ± 0.11	0.76 ± 0.1	0.00

Discussion

Rhizobium strains UFLA04-195, UFLA04-173, and UFLA04-202 as well as the strain CIAT899, an approved inoculant in Brazil, were inoculated onto bean plants and demonstrated high symbiotic efficiency. These strains were isolated from acidic soils with high concentrations of aluminium in different land uses in the Amazon region. The dry matter of nodules positively correlated with the accumulation of nitrogen in the shoot ($r = 0.82$; $P < 0.01$). Significant positive correlations between nodule mass and the amount of biologically fixed nitrogen have also been reported by Döbereiner et al. (1966).

The nitrogen in the shoots of bean plants inoculated with these strains were within the sufficiency range indicated by Malavolta et al. (1997); it was above the critical level of 30 g kg^{-1}. Similar results were found for beans inoculated with *Rhizobium* strains isolated from different regions of Brazil and Venezuela and the Amazon region, which showed levels of nitrogen similar to the strain CIAT899, which has been recommended as an inoculant (González et al. 2008; Soares et al. 2006b).

In this study, the strains examined belonged to two different groups, which included species of the genera *Rhizobium* and *Burkholderia*. The strains belonging to the genus *Burkholderia* were previously isolated from soil by using siratro as trap and were latter authenticated in its trap host (Lima et al. 2009). *Burkholderia* genus may consist of endophytic bacteria from nodules as described by Li et al. (2008). These authors reported that some non-symbiotic endophytic bacterial strains isolated from soybean root nodules, when reinoculated were not able to form nodules on the host plant. Moreover, the inoculation of nodule endophytes had no significant effects on the growth and nodulation of soybean. In our study, however, from 11 strains tested, four strains of the genus *Burkholderia* were able to nodulate beans. Recently, four bacterial strains isolated from root nodules of *Phaseolus vulgaris* grown in soil from Morocco were identified as *Burkholderia phymatum* (Talbi et al. 2010). This was the first report of a *Burkholderia* species nodulating the common bean. Consistent with this, our data show that bacteria of the genus *Burlholderia* isolated from acidic soils in the Amazon region are able to form nodules when inoculated onto *Phaseolus vulgaris*.

The three most efficient strains were grouped into the cluster comprised of *R. tropici*, *R. miluonense* and *R. multihospitium* by 16S rDNA sequencing. These strains showed a better ability to grow in culture medium with pH 5.0, similar to the pH of soil from which they were isolated. In studies done with unidentified strains isolated from cowpea nodules in acidic soils from Iranduba and Presidente Figueiredo, in the Amazon region, it was shown that 47 and 25%, respectively, of the isolates were tolerant to acidic conditions in YMA ("79") medium (Hara and Oliveira 2004, 2005). A positive correlation between soil pH and acid pH tolerance in culture medium was also reported for *Mesorhizobium* strains isolated from nodules of chickpea plants grown in acidic soils in the southern region of Portugal (Rodrigues et al. 2006). There are several published reports showing the different ranges of acidity tolerance among *Rhizobium* species. However, no literature exists concerning pH and aluminium tolerance for *R. multihospitium* and *R. miluonense*.

After 120 h of cultivation, the pH of the cultures was reduced compared to the initial pH for all strains, which is typical of the genus *Rhizobium*. Similar results were found by Correa and Barneix (1997) for the strain *Mesorhizobium loti*, suggesting that tolerance to low pH is not related to the release of compounds that alkalize the medium but is instead related to the presence of factors that protect the cells from high concentrations of extracellular protons. Additionally, several authors have reported that the production of proteins such as glutathione provides protection against this oxidative stress in *R. tropici*. They also claim that such proteins are essential for these species to grow in extreme environmental conditions (Riccillo et al. 2000; Muglia et al. 2007).

Although the three highly efficient strains isolated from Amazonian soil were tolerant to aluminium in the culture medium, a marked decrease in bacterial growth was observed at 2 mmol Al^{3+} l^{-1} (2,000 µmol l^{-1}). However, other isolates from acidic soils of the Amazon were able to grow on YMA with up to 2 $cmol_c$ Al^{3+} l^{-1} (6,600 µmol l^{-1}) (Hara and Oliveira 2004; 2005). Probably this difference in the Al

tolerance is due to the availability of aluminium in the medium. We used liquid medium where the tolerance is usually much lower than in solid medium. In another studies from a total of 155 rhizobial strains isolated from bean growing areas in the state of São Paulo, Brazil, 77 strains were able to grow on solid medium supplemented with a maximum concentration of 100 μmol Al^{3+} l^{-1} (Vargas and Denardim 1992) which is very low. In the South Brazil, 50 μmol Al^{3+} l^{-1} was not able to affect the growth of 10 isolates from *Acacia* nodules in liquid medium (Vargas et al. 2007). The only study found about the effect of increased concentrations of aluminium in liquid medium showed that fast growing strain *Sinorhizobium meliloti* RMP5 could tolerate up to 100 μmol Al^{3+} l^{-1}, which was more tolerant than the *Bradyrhizobium* strain PMP1 that grew in only 25 μmol l^{-1}. This result occurred because Al^{3+} adversely affected the production and activity of nitrogenase, hydrogenase, nitrate reductase and nitrite reductase in both strains (Arora et al. 2010). Thus, the strains tested in our study are able to tolerate Al concentration much higher than others already reported.

Aluminium is a metal that can be toxic to nitrogen-fixing bacteria at high concentrations in the soil or in culture medium; it can cause changes in cellular metabolism that affect bacterial growth and survival. Although the nature of the process and location of Al^{3+} activity still remains uncertain, it is known that Al^{3+} ion binding to DNA can interfere with cell division (Johnson and Wood 1990; Wood 1995). Considering that sensitive and tolerant strains have the same potential to bind Al^{3+}, a DNA repair mechanism may exist in tolerant strains. DNA synthesis in tolerant strains of *M. loti* was not affected by Al^{3+} (Johnson and Wood 1990), but 7.5 μmol Al^{3+} l^{-1} $^{+}$ reduced nod*A* gene expression in *R. leguminosarum* bv. *trifolii* strains, which then affected BNF (Richardson et al. 1988).

There is evidence that the acidic tolerance found in several *Rhizobium* strains, is related to the structure of the outer membrane (Correa and Barneix 1997). The resistance to crystal violet, whose penetration depends on cell membrane permeability, was exhibited by strains UFLA04-195, UFLA04-173, and UFLA04-202. Cells of *R. tropici* and *M. loti*, which are acid tolerant, demonstrate a significantly greater tolerance (85 and 60%), to crystal violet when compared to susceptible strains (20 and 10%) (Graham et al. 1994; Correa and Barneix 1997). The strains studied in this work showed a higher survival rate (Fig. 6) when grown in the presence of crystal violet compared to strains previously examined.

The strains that were adapted to acidic conditions were also tolerant to the antibiotic polymyxin B, which interacts with the lipid phosphatidylethanolamine, causing a breach in the cell membrane. *Salmonella typhimurium* bacteria adapted to acidity were also very tolerant to this compound (Leyer and Johnson 1993). These results additionally indicate that the adaptation to acidity may be partly linked to structural changes in the bacterial cell membrane.

Differences in EPS production, resulting from pH variation, were observed for the three efficient strains, as well as for the control strain (CIAT 899). Generally, when pH conditions negatively affected the bacterial growth, higher EPS production was observed. In other words, strains CIAT899, UFLA04-202, and UFLA04-195, which showed slower growth at pH 6.9, produced a greater quantity of EPS at this pH. In the case of strain UFLA04-173, which showed no differences in bacterial growth at pH 5.0, 6.0 and 6.9, there were no significant differences in EPS production.

In several rhizobia, a positive and significant correlation between the amount of EPS and tolerance to acidity has been reported (Cunningham and Munns 1984; Miguel and Moreira 2001; Barberi et al. 2004; Bomfeti et al. 2011). The data in this study indicated that UFLA04-173, UFLA04-202, and UFLA04-195 strains, because they came from an environment with high acidity, developed efficiently at pH 5.0 and that the highest EPS production at pH 6.9 should be associated with minor adjustment to this condition. These results demonstrate that increased EPS production may be related to an adaptive response to stressful environmental conditions for microbial growth of these strains, which in the case of strains adapted to acidity, the conditions were higher values of pH.

EPS production at different concentrations of aluminium showed a positive correlation with strain growth; there was no EPS production by strains when growth was drastically affected by high concentrations of Al^{3+}. Strain UFLA04-202 tolerated up to 1 mmol l^{-1} (1,000 μmol l^{-1}) of Al^{3+} in the medium and showed higher EPS production compared to the other strains at this concentration and at 0.5 mmol l^{-1} (500 μmol l^{-1}) Al^{3+}. However, Kingsley and Bohlool (1992), who evaluated EPS production in a strain of *R. leguminosarum* bv *phaseoli* found that in media containing 0 to 300 μM of Al^{3+}, the aluminium tolerant strain showed no changes in EPS production. The same authors also analysed the behaviour of a mutant strain (*exo*$^{-}$) unable to produce EPS. The mutant strain was also tolerant to Al^{3+} compared to wild type, indicating that the Al^{3+} tolerance of this strain was not related to EPS production. These findings conflict with our results suggesting that EPS production is related to tolerance to Al^{3+} in the acid-tolerant strains studied. However, there are probably other mechanisms acting to create tolerance to aluminium.

Other mechanisms of aluminium tolerance found in rhizobia populations appear to be related to the decreased amount of negative charge on the cell surface reducing the amount of Al^{3+} bound to the cell (Bushby 1990), reduced accumulation of inorganic phosphate inside the cell, which neutralises the effects of aluminium by forming insoluble

complexes that are not biologically toxic (Mukherjee and Asanuma 1998), and increased levels of potassium and phosphorus associated with the maintenance of internal pH (Mukherjee and Asanuma 1998; Watkin et al. 2003).

Although the strains evaluated in this study were tolerant to acidity and aluminium, they still need to be tested in symbiosis with the plant in the presence of these limiting factors in the soil field condition. The possibility of obtaining strains tolerant to soil acidity, and especially Al^{3+}, could provide increased yields of the host plant, resulting in a more efficient nitrogen fixation. However, it is important that the plant is well adapted to these soil conditions; therefore, studies evaluating symbiotic effectiveness and tolerance to adverse environmental factors must continue.

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References

Ahmad N, Johri S, Abdin MZ, Qazi GN (2009) Molecular characterization of bacterial population in the forest soil of Kashmir, India. World J Microbiol Biotechnol 25:107–113

Almeida ALG, Alcântara RMCM, Nóbrega RSAE, Abrahão RS, Leite LFC, Silva JAL, Nobrega JCA (2010) Cowpea cv. BR17 Gurguéia yield inoculated with symbiotic diazotrophic bacteria in Piauí. Agrária 5:364–369

Arora NK, Khare E, Singh S, Maheshwari DK (2010) Effect of Al and heavy metals on enzymes of nitrogen metabolism of fast and slow growing rhizobia under explanta conditions. World J Microb Biot 26:811–816

Barberi A, Moreira FMS, Florentino LA, Rodrigues MID (2004) Growth of *Bradyrhizobium elkanii* strain Br 29 in culture media with different pH values. Ciênc Agrotec 28:397–405

Bomfeti CA, Florentino LA, Guimarães AP, Cardoso PG, Guerreiro MC, Moreira FMS (2011) Exopolysaccharides produced by the symbiotic nitrogen-fixing bacteria of *Leguminosae*. Rev Bras Ciênc Solo 35:657–671

Bushby HVA (1990) The role of bacterial surface charge in the ecology of root nodule bacteria: a hypothesis. Soil Biol Biochem 22:1–9

Chen HC, Richardson AE, Rolfe BG (1993a) Studies on the physiological and genetic basis of acid tolerance in *Rhizobium leguminosarum* biovar *trifolii*. Appl Environ Microbiol 59:1798–1804

Chen HC, Gartner E, Rolfe BG (1993b) Involvement of genes on a megaplasmid in the acid-tolerant phenotype of *Rhizobium leguminosarum* biovar *trifolii*. Appl Environ Microbiol 59:1058–1064

Cole MA, Elkan GH (1973) Transmissible resistance to penicillim G, Neomycin, and cloramphenicol in *Rhizobium japonicum*. Antimicrob Agents Chemother 4:248–253

CONAB (Companhia Nacional de Abastecimento) (2010) Safra Grãos 2009/2010. Disponível: http://www.conab.gov.br/conabweb/download/safra/FeijaoTotalSerieHist.xls>

Correa OS, Barneix AJ (1997) Cellular mechanisms of pH tolerance in *Rhizobium loti*. World J Microb Biot 13:153–157

Cunningham SD, Munns DN (1984) The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. Soil Sci Soc Am J 48:1273–1276

Döbereiner J, Arruda NB, Penteado AF (1966) Evaluation of nitrogen fixation in legumes by regression of N_2 on total plant weight of nodules. Pesq Agropec Bras 1:233–237

Ferreira DF (2000) Análises estatísticas por meio do SISVAR para windows versão 4.0. In: Reunião anual da Região Brasileira da Sociedade Internacional de Biometria. São Carlos. programas e Resumos… UFSCar, 45:235

Fred EB, Waksman SA (1928) Laboratory manual of general microbiology. McGraw-Hill Book, New York, p 143

Fujihara S, Yoneyama DT (1993) Effects of pH and osmotic stress on cellular polyamine contents in the soybean rhizobia *Rhizobium fredii* p220 and *Bradyrhizobium japonicum* A 1017. Appl Environ Microbiol 59:1104–1109

González TO, Campanharo JC, Lemos EGM (2008) Genetic characterization and nitrogen fixation capacity of *Rhizobium* strains on common bean. Pesq Agropec Bras 43:1177–1184

Goss TJ, O'hara GW, Dilworth MJ, Glenn AR (1990) Cloning, characterization, and complementation of lesions causing acid sensitivity in Tn*5*-induced mutants of *Rhizobium meliloti* WSM419. J Bacteriol 172:5173–5179

Graham PH, Draeger K, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, Naarons SR, Quinto C (1994) Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can J Microbiol 40:198–207

Hara FAZ, Oliveira LA (2004) Características fisiológicas e ecológicas de isolados de rizóbios oriundos de solos ácidos e álicos de Presidente Figueiredo, Amazonas. Acta Amazon 34:343–357

Hara FAZ, Oliveira LA (2005) Características fisiológicas e ecológicas de rizóbios oriundos de solos ácidos de Iranduba, Amazonas. Pesq Agropec Bras 40:667–672

Hickey EW, Hirshfield IN (1990) Low-pH-induced effects on patterns of protein synthesis and on internal pH in *Escherichia coli* and *Salmonella typhimurium*. Appl Environ Microbiol 56:1038–1045

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. California Agricultural Experiment Station, Berkeley, p 32

Igual JM, Rodriguez-Barrueco C, Cervantes E (1997) The effects of aluminum on nodulation and symbiotic nitrogen fixation in *Casuarina cunningamiana Miq*. Plant Soil 190:41–46

Johnson AC, Wood M (1990) DNA, a possible site of action of aluminum in *Rhizobium* spp. Appl Environ Microbiol 56:3629–3633

Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kingsley MT, Bohlool BB (1992) Extracelular polysaccharide is not responsible for aluminum tolerance of *Rhizobium leguminosarum* bv. *phaseoli* CIAT899. Appl Environ Microbiol 58:1095–1101

Lacerda AM, Moreira FMS, Andrade MJB, Soares ALL (2004) Yield and nodulation of cowpea inoculated with selected rhizobia strains. Rev Ceres 51:67–82

Lane DJ (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics, Wiley, New York, pp 115–148

Leyer GJ, Johnson EA (1993) Acid adaptation induces crossprotection against environmental stresses in *Salmonella typhimurium*. Appl Environ Microbiol 59:1842–1847

Li JH, Wang ET, Chen WF, Chen WX (2008) Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. Soil Biol Biochem 40:238–246

Lima AS, Nóbrega RSA, Barberi A, Silva K, Ferreira DF, Moreira FMS (2009) Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon region as indicated by nodulation of siratro (*Macroptilium atropurpureum*). Plant Soil 320:1–19

Malavolta E, Vitti GC, Oliveira AS (1997) Avaliação do estado nutricional de plantas: princípios e aplicações, 2 edn. Potafos

Miguel DL, Moreira FMS (2001) Influence of médium and peat pH on the behaviour of *Bradyrhizobium* strains. R Bras Ci Solo 25:873–883

Miles AA, Misra SS (1938) The estimations of the bacteriocidal power of the blood. J Hyg 38:732–749

Moreira FMS, Silva MF, Faria SM (1992) Occurrence of nodulation in legume species in the Amazon region of Brazil. New Phytol 121:563–570

Muglia CI, Grasso DH, Aguilar OM (2007) *Rhizobium tropici* response to acidity involves activation of glutathione synthesis. Microbiol 153:1286–1296

Mukherjee SK, Asanuma S (1998) Possible role of cellular phosphate pool and subsequent accumulation of inorganic phosphate on the aluminum tolerance in *Bradyrhizobium japonicum*. Soil Biol Biochem 30:1511–1516

O'hara GW, Goss TJ, Dilworth MJ, Glenn AR (1989) Maintenance of intracellular pH and acid tolerance in *Rhizobium meliloti*. Appl Environ Microbiol 55:1870–1876

Osman O, Tanguichi H, Ikeda K, Park P, Tanabe-Hosoi S, Nagata S (2010) Copper-resistant halophilic bacterium isolated from the polluted Maruit Lake. Egypt. J App Microbiol 108:1459–1470

Pepi M, Volterrani M, Renzi M, Marvasi M, Gasperini S, Franchi E, Focardi SE (2007) Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization. J Appl Microbiol 103:2299–2308

Ramalho MAP, Abreu AFB, Carneiro JES, Goncalves FMA, Santos JB, Peloso MJD, Faria LC, Carneiro GES (2002) O "Talismã" de sua Lavoura de Feijoeiro. EMBRAPA/CNPAF 4

Riccillo PM, Muglia CI, Bruijn FJ, Roe AJ, Booth IR, Aguilar OM (2000) Glutathione is involved in environmental stress responses in *Rhizobium tropici*, including acid tolerance. J Bacteriol 182:1748–1753

Richardson AE, Simpson RJ, Djordjevic MA, Rolfe BJ (1988) Expression of nodulation genes in *Rhizobium leguminosarum* bv. *trifolii* is affected by low pH and by Ca21 and Al ions. Appl Environ Microbiol 54:2541–2548

Rodrigues CS, Laranjo M, Oliveira S (2006) Effect of Heat and pH Stress in the Growth of Chickpea Mesorhizobia. Curr Microbiol 53:1–7

Rojas-Jiménez K, Sohlenkamp C, Geiger O, Martínez-Romero E, Werner D, Vinuesa P (2005) A ClC chloride channel homolog and ornithine-containing membrane lipids of *rhizobium tropici* ciat899 are involved in symbiotic efficiency and acid tolerance. Mol Plant Microbe Interact 18:1175–1185

Sarruge JR, Haag HP (1979) Análises químicas em plantas. ESALQ/USP, 27

Scott AJ, Knott MA (1974) Cluster analysis method for grouping means in the analysis of variance. Biometrics 30:507–512

Soares ALL, Pereira JPAR, Ferreira PAA, Vale HMM, Lima AS, Andrade MJB, Moreira FMS (2006a) Agronomic efficiency of selected rhizobia strains and diversity of native nodulating populations in Perdões (MG—Brazil). I—cowpea. R Bras Ci Solo 30:795–802

Soares ALL, Ferreira PAA, Pereira JPAR, Vale HMM, Lima AS, Andrade MJB, Moreira FMS (2006b) Agronomic efficiency of selected rhizobia strains and diversity of native nodulating populations in Perdões (MG—Brazil). II—Beans. R Bras Ci Solo 30:803–811

Talbi C, Delgado MJ, Girard L, Ramírez-Trujillo A, Caballero-Mellado J, Bedmar EJ (2010) *Burkholderia phymatum* Strains Capable of Nodulating *Phaseolus vulgaris*Are Present in Moroccan Soils. Appl Environ Microbiol 76:4587–4591

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Vargas AAT, Denardin ND (1992) Tolerance to acidity and soil aluminum by rhizobia strains of beans isolated in São Paulo state, Brazil. R Bras Ci Solo 16:337–342

Vargas LK, Lisboa BB, Scholles D, Silveira JRP, Jung GC, Granada CE, Nevesa G, Braga MM, Negreiros T (2007) Genetic diversity and symbiotic efficiency of black wattle nodulating rhizobia in soils of Rio Grande do Sul State, Brazil. R Bras Ci Solo 31:647–654

Vieira C (2006) Adubação mineral e calagem. In: Vieira C, Paula Jr TJ, Borém A (eds.) Feijão, 2 edn. Atual.—Viçosa: Ed. UFV, pp 115–142

Watkin ELJ, O'Hara GW, Glenn AR (2003) Physiological responses to acid stress of an acid-soil tolerant and acid-soil sensitive strain of *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 35:621–624

Wood M (1995) A mechanism of aluminum toxicity to soil bacteria and possible ecological implications. Plant Soil 171:63–69