

## Cowpea symbiotic efficiency, pH and aluminum tolerance in nitrogen-fixing bacteria

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### Introduction

Cowpea (*Vigna unguiculata*) is an important crop in northern and northeastern Brazil, because it is an excellent source of nutrients and carbohydrates for the poor and underprivileged that produces and consumes it. Seventy percent of all beans produced in northern and northeastern Brazil is cowpea (Santos et al., 2000), but this crop is continually expanding into other areas in Brazil. The production of cowpeas is based on very low inputs and use of marginal soils that are phosphorus- and nitrogen (N)-deficient. Therefore, current yields in Brazil are very low: between 400 and 500 kg ha<sup>-1</sup>. To increase productivity, cowpea cultivation may be associated with nitrogen-fixing bacteria that provide its partial or total nitrogen fertilization. This would reduce production costs and prevent environmental pollution caused by the manufacture and misuse of nitrogen-based fertilizers. Several researchers have demonstrated the ability of these bacteria to provide N to crops with yields similar to 70 kg ha<sup>-1</sup> of N-urea (Soares et al., 2006; Lacerda et al., 2004) or 50 kg N-urea ha<sup>-1</sup> (Almeida et al., 2010).

Although cowpea is considered a promiscuous species for its ability to nodulate with several bacterial species and genera (Moreira, 2008), the maximum yield is obtained with strains belonging to *Bradyrhizobium* genus (Almeida et al., 2010; Chagas Junior et al., 2010; Costa et al., 2011; Lacerda et al., 2004; Moreira, 2006; Soares et al., 2006; Zilli et al., 2006). Soil characteristics, such as

ABSTRACT: Cowpea (*Vigna unguiculata*) cultivation in northern and northeastern Brazil provides an excellent source of nutrients and carbohydrates for the poor and underprivileged. Production surplus leads to its consumption in other regions of Brazil and also as an export commodity. Its capacity to establish relationships with atmospheric nitrogen-fixing bacteria is crucial to the reduction of production costs and the environmental impact of nitrogen fertilizers. This study assessed the symbiotic efficiency of new strains of symbiotic nitrogen-fixing bacteria with cowpea and their tolerance to pH and aluminum. Twenty-seven strains of bacteria from different soils were evaluated under axenic conditions. These strains were compared to the following inoculant strains: INPA03-11B, UFLA03-84 and BR3267 and two controls that were not inoculated (with and without mineral nitrogen). Six strains and the three strains approved as inoculants were selected to increase the dry weight production of the aerial part (DWAP) and were tested in pots with soil that had a high-density of nitrogen-fixing native rhizobia. In this experiment, three strains (UFLA03-164, UFLA03-153, and UFLA03-154) yielded higher DWAP values. These strains grow at pH levels of 5.0, 6.0, 6.8 and at high aluminum concentration levels, reaching 10<sup>9</sup> CFU mL<sup>-1</sup>. In particular UFLA03-84, UFLA03-153, and UFLA03-164 tolerate up to 20 mmol<sub>c</sub> dm<sup>-3</sup> of Al<sup>3+</sup>. Inoculation with rhizobial strains, that had been carefully selected according to their ability to nodulate and fix N<sub>2</sub>, combined with their ability to compete in soils that are acidic and contain high levels of Al, is a cheaper and more sustainable alternative that can be made available to farmers than mineral fertilizers.

Keywords: *Vigna unguiculata*, *Bradyrhizobium*, *Burkholderia*, Native rhizobia

pH and Al<sup>3+</sup>, may compromise symbiotic efficiency and plant development. Ph values below 5.0 are reported to be deleterious for nodulation and nitrogen fixation (Apunu and Dhar, 2006; Mukherjee and Asanuma, 1998). On the other hand, four strains of *Bradyrhizobium*, that are approved as inoculants to the following: *Glycine max* (BR29, SEMIA587), *Vigna unguiculata* (INPA3-11B) and *Enterolobium contortisiliquum* (BR4406), all grow satisfactorily in pH values of 5.0; 6.0 and 6.8 (Miguel and Moreira, 2001; Barberi et al., 2004). Therefore, selection of bacteria with greater tolerance to different soil acidities and Al<sup>3+</sup> concentrations is essential to maximizing symbiotic efficiency.

The diversity of soils and climates ensures a variety of native bacteria adapted to these diverse conditions. Moreover, in this edaphoclimatic diversity, the N<sub>2</sub>-fixing capacity of bacteria might significantly differ between strains, and thus require more appropriate selection under certain conditions. This study assessed the symbiotic efficiency of 27 bacterial strains isolated from different locations, as well as their tolerance to pH and Al<sup>3+</sup> as compared to the three strains approved as inoculants.

### Materials and Methods

The evaluation of strains efficiency comprised the first two steps in the methodology usually applied for this purpose: sterilized Leonard jars and non-sterilized pots containing soil.

### Strain efficiency under axenic conditions

The first experiment was performed in Leonard jars in a greenhouse (Vincent, 1970), between 25 June 2007 and 16 Aug 2007. The experimental design was completely randomized, consisting of three replicates of 32 treatments. These treatments were inoculation with each one of the 27 strains (Table 1) and the three strains approved as inoculants for cowpea cultivation by the Brazilian Ministry of Agriculture Livestock and Supply (MAPA) (INPA 03-11B, UFLA 03-84 and BR 3267), and two non-inoculated controls, one without (C) and one with mineral N (C+N). N was supplied three times at 10-day intervals beginning 15 days after emergence, for a total treatment of 210 mg N ( $\text{NH}_4\text{NO}_3$ ) per jar. A 1:1 mixture of sand (250 mL) and vermiculite (250 mL) was placed in the upper part of the Leonard jar; 4-fold diluted Jensen's nutrient solution was placed at the bottom ( $0.2 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$ ;  $0.2 \text{ g L}^{-1} \text{ MgSO}_4 \cdot \text{H}_2\text{O}$ ,

$0.2 \text{ g L}^{-1} \text{ NaCl}$ ,  $1 \text{ g L}^{-1} \text{ CaHPO}_4$ ,  $0.1 \text{ g L}^{-1} \text{ FeCl}_3 \cdot 6\text{H}_2\text{O}$ ;  $2.86 \text{ mg L}^{-1} \text{ H}_3\text{BO}_3$ ;  $2.03 \text{ mg L}^{-1} \text{ MnSO}_4 \cdot 4\text{H}_2\text{O}$ ;  $0.22 \text{ mg L}^{-1} \text{ ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ;  $0.08 \text{ mg L}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; and  $0.09 \text{ mg L}^{-1} \text{ Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ ). The nutrient solutions and jars were autoclaved for 1 h at  $1.5 \text{ kg cm}^{-2}$ ,  $127 \text{ }^\circ\text{C}$ .

Cowpea seeds were superficially disinfected with 98 % alcohol for 30 s, followed by 2 % sodium hypochlorite for 2 min. The seeds were washed six times with sterile distilled water to remove the residues of previous treatments. Then the seeds were immersed in sterile distilled water for two hours. Germination occurred on petri dishes where the seeds were placed with paper film and sterile moistened cotton and maintained in a growth chamber at  $28 \text{ }^\circ\text{C}$  for 24 h.

The cowpea variety used for the experiment was BR-17 Gurgueia. After sterilization and disinfection, four pre-germinated seeds were placed in each jar. These were then inoculated with 1 mL bacteria dur-

Table 1 – Origin and identification of the strains UFLA 03-84, INPA 03-11B and BR 3267, which are currently approved as inoculants for the cowpea bean (*Vigna unguiculata* (L.) Walp) and of the 27 other strains.

Strain	Origin Location/ SUT <sup>(1)</sup>	Growth characteristics in 79 medium <sup>(2)</sup>		Characteristics of the soil <sup>(3)</sup>			Identification <sup>(4)</sup>
		G.R	pH	pH	Al <sup>3+</sup>	m%	
UFLA 03-84	Theobroma,RO/P	S	alkaline	6.1	0	0	<i>Bradyrhizobium</i> sp.
INPA 03-11B	Manaus AM/Terra Firme	S	alkaline	–	–	–	<i>Bradyrhizobium elkanii</i>
BR 3267	—	S	alkaline	–	–	–	<i>Bradyrhizobium. Japonicum</i>
UFLA 03-05	Benjamim Constant AM/FS	S	alkaline	4.3	52	25	Not identified
UFLA 03-14	Benjamim Constant AM/FS	S	neutral	4.9	28	51	<i>Enterobacter</i> sp.
UFLA 03-15	Benjamim Constant AM/FS	S	acidic	4.3	52	25	<i>Burkholderia fungorum</i>
UFLA 03-17	Benjamim Constant AM/FS	I	acidic	4.3	52	25	<i>Burkholderia fungorum</i>
UFLA 03-18	Benjamim Constant AM/FS	S	acidic	4.3	52	25	<i>Pseudomonas</i> sp.
UFLA 03-19	Benjamim Constant AM/FS	F	neutral	4.9	28	51	<i>Burkholderia fungorum</i>
UFLA 03-20	Benjamim Constant AM/FS	S	neutral	4.3	52	25	<i>Burkholderia fungorum</i>
UFLA 03-21	Benjamim Constant AM/P	S	neutral	4.9	28	51	Not identified
UFLA 03-22	Benjamim Constant AM/P	S	acidic	4.9	28	51	<i>Pseudomonas koreensis</i>
UFLA 03-23	Benjamim Constant AM/P	S	neutral	4.9	28	51	<i>Pseudomonas koreensis</i>
UFLA 03-26	Benjamim Constant AM/P	F	acidic	4.9	28	51	<i>Pseudomonas</i> sp.
UFLA 03-27	Benjamim Constant AM/P	S	acidic	4.9	28	51	<i>Enterobacter</i> sp.
UFLA3-153	Poços de Caldas MG/MR	S	alkaline	6.7	0	0	<i>Bradyrhizobium</i> sp.
UFLA3-154	Poços de Caldas MG/MR	S	alkaline	7.5	0	0	<i>Burkholderia fungorum</i>
UFLA3-163	Poços de Caldas MG/MR	S	alkaline	5.1	0	0	<i>Bradyrhizobium elkanii</i>
UFLA3-164	Poços de Caldas MG/MR	S	alkaline	5.1	0	0	<i>Bradyrhizobium elkanii</i>
UFLA3-165	Poços de Caldas MG/MR	S	alkaline	5.1	0	0	<i>Acinetobacter genomosp</i>
UFLA3-170	Poços de Caldas MG/MR	S	alkaline	6.6	0	0	<i>Bradyrhizobium elkanii</i>
UFLA3-172	Poços de Caldas MG/MR	S	neutral	5.7	00.7	1.76	<i>Bradyrhizobium elkanii</i>
UFLA04-0110	Theobroma- RO/AGRI	S	alkaline	6.6	0	0	<i>Burkholderia fungorum</i>
UFLA04-1309	Pedro Peixoto, AC/F	S	alkaline	6.1	0	0	<i>Burkholderia fungorum</i>
UFLA04-1020	RECA, AC/AGRO	S	alkaline	5	1	21	<i>Burkholderia fungorum</i>
UFLA04-0314	Theobroma, RO/F	S	alkaline	4.7	9	35	<i>Burkholderia fungorum</i>
UFLA04-321	Theobrama, RO/F	S	alkaline	4.7	9	35	<i>Bradyrhizobium japonicum</i>
UFLA04-546	Theobroma RO/P	S	alkaline	5.3	4	16	<i>Burkholderia fungorum</i>
UFLA04-559	Theobroma RO/P	S	alkaline	5.3	4	16	<i>Burkholderia fungorum</i>
UFLA04-885	Ji-Paraná, RO/P	S	alkaline	6.1	0	0	<i>Pseudomonas koreensis</i>

<sup>(1)</sup>AM: Amazonas state, MG: Minas Gerais state, AC: Acre state; RO-Rondonia State - SF: Secondary forest, P: Pasture, AGRO: Agroforestry, AGRI: Agriculture; F: Fallow, MR: Bauxite mining areas after recovery of vegetation, BC: beans crop; <sup>(2)</sup>Growth characteristics in 79 medium: G.R. Growth rate – F: fast (2 to 3 days) - I: intermediate (4 to 5 days), S Slow (6 to 10 days); pH of culture medium after growth; <sup>(3)</sup>Characteristics of the soil: pH, Al<sup>3+</sup> concentration (mmol<sub>c</sub> dm<sup>3</sup>) and Al<sup>3+</sup> saturation (m%) in the soil from where the strain was isolated; <sup>(4)</sup>Oliveira-Longatti et al. (2014).

ing the log phase of growth ( $10^9$  cells  $\text{mL}^{-1}$ ) cultured in medium 79 (Fred and Waksman, 1928). In the non-inoculated control and in the absence of mineral N, only 1 mL sterile medium 79 was added. Ten days after germination, the seedlings were thinned, leaving two plants per jar. After seeding, a 2.0 cm layer of a sterile mixture of sand, benzene, and paraffin (5:1:0.015) was placed in the jar to avoid contamination.

Solution levels were periodically replenished with sterile Jensen nutrient solution. After 52 days, at maximum flowering, plants were harvested to assess dry weight of the aerial part (DWAP), number of nodules (NN), dry weight of nodules (DWN), and N accumulation in the aerial part (NAAP). Data were analyzed using the analysis of variance method as per Sisvar 5.3. Values for NN and DWN were previously transformed into  $(X + 0.5)^{0.5}$ .

### Strain efficiency in pots with soil

This experiment was performed in a greenhouse, from 11 Feb 2008 to 11 May 2008 in plastic pots (5.0  $\text{dm}^3$ ). The soil was a Haplic Cambisol, collected in the municipality of Itutinga-MG ( $21^\circ 23' 29''$  S;  $44^\circ 39' 13''$  W), with *Brachiaria* (*Urochloa decumbens*) cover and no leguminous plants. The soil was obtained from the arable layer (0 to 20 cm), air-dried, conditioned, homogenized and passed through a 4-mm sieve before use as a substrate. The chemical characteristics of the soil prior to liming and fertilization were: pH in  $\text{H}_2\text{O}$  (1:2.5) 5.8; P (Mehlich 1) 2.3  $\text{mg dm}^{-3}$ ;  $\text{K}^+$  (Mehlich 1) 58  $\text{mg dm}^{-3}$ ;  $\text{Ca}^{2+}$  15  $\text{mmol}_c \text{dm}^{-3}$ ;  $\text{Mg}^{2+}$  8  $\text{mmol}_c \text{dm}^{-3}$ ;  $\text{Al}^{3+}$  0  $\text{mmol}_c \text{dm}^{-3}$ ; H + Al 29  $\text{mmol}_c \text{dm}^{-3}$  (extracted by 1 M KCl); Sum of Bases 25  $\text{mmol}_c \text{dm}^{-3}$ ; effective Cation Exchange Capacity (CEC) 25  $\text{mmol}_c \text{dm}^{-3}$ ; potential CEC pH 7 53  $\text{mmol}_c \text{dm}^{-3}$ ; aluminum saturation 0 %; base saturation 45 %; organic matter (2 M sulfuric acid + 5 M sodium dichromate) 30  $\text{g kg}^{-1}$ .

The base saturation method was used to calculate the amount of lime in order to increase the base saturation to 60 %. Dolomitic lime was applied 30 days before sowing, while maintaining soil humidity in the field capacity to allow the reaction to occur. In all plots, fertilization was carried out with 300, 300, 40, 0.8, 1.5, 3.6, 5.0, and 0.15  $\text{mg dm}^{-3}$  K, P, S, B, Cu, Mn, Zn, and Mo, respectively (Malavolta et al., 1989). The sources were Super Simple phosphate [ $3\text{Ca}(\text{H}_2\text{PO}_4)_2$ ], Potassium Sulfate ( $\text{K}_2\text{SO}_4$ ), Boric Acid ( $\text{H}_3\text{BO}_3$ ), Copper Sulfate ( $\text{CuSO}_4$ ), Manganese Sulfate ( $\text{MnSO}_4$ ), Zinc Sulfate ( $\text{ZnSO}_4$ ), and Sodium Molybdate ( $\text{Na}_2\text{MoO}_4$ ).

The experimental design was completely randomized and consisted of three replicates and 11 treatments with the strains that demonstrated the best capacity to induce dry weight of the aerial part (DWAP) in experiment 1, in addition to three strains approved as inoculants for cowpea cultivation (UFLA 03-84, INPA 03-11B, and BR 3267) and two non-inoculated controls, without (C) and with mineral N [C+N; 500  $\text{mg}$ / pot of  $\text{NH}_4\text{NO}_3$ . Mineral N was divided into two applications

(at sowing and 20 days after emergence)]. The cowpea used in the experiment was BR-17 Gurguéia.

After sterilization and disinfection as previously described, pre-germinated seeds were distributed four per pot and inoculated with 1 mL bacteria ( $10^9$  cells  $\text{mL}^{-1}$ ) grown in medium 79 (Fred and Waksman, 1928) and added during log-phase growth. In the non-inoculated control and without addition of mineral N, only 1 mL sterile medium 79 was added. Ten days after germination, the plants were thinned, leaving two per pot. Plants were harvested at maximum flowering to assess dry weight of the aerial part (DWAP), number of nodules (NN), dry weight of nodule (DWN), and N accumulation in the aerial part (NAAP). Data were analyzed by the analysis of variance method as per Sisvar 5.3.

### Rhizobia native populations density of soil

The experiment was conducted in Leonard jars (Vincent, 1970), from 15 Feb 2008 to 15 Mar 2008 to assess the most probable number of native soil rhizobia in experiment 2. The experimental design was completely randomized, and consisted of three replicates of eight treatments. The treatments were 1-mL inoculations of  $10^{-1}$  to  $10^{-6}$  serial dilutions of soil suspension and two non-inoculated controls, one without (C) and one with mineral N (C+N). At the control C + N, N was supplied three times at 10-day intervals, 15 days after emergence, for a total treatment of 210  $\text{mg N}$  ( $\text{NH}_4\text{NO}_3$ ) per jar. The preparation of Leonard jars and seeds was performed as described in experiment 1.

To estimate the most probable number (MPN) of rhizobia cells in the three collected samples the MPNES (most probable number estimate) program was used after recording the presence (positive) and absence (negative) of rhizobia in each dilution (Woomer et al. 1988). Data including dry weight in the aerial part (DWAP), dry weight of nodules (DWN), and number of nodules (NN) were compared by the analysis of variance method in Sisvar 5.3.

### Strain tolerance to pH and Al

The three strains with better symbiotic efficiency in soil pots, together with UFLA 03-84, INPA 03-11B, and BR 3267, were evaluated for their tolerance to acidity and aluminum ( $\text{Al}^{3+}$ ) in liquid culture medium. Isolated colonies from each strain were inoculated in medium 79 (Fred and Waksman, 1928) without the bromothymol blue dye and grown until they reached an optical density and incubated to optical density ( $\text{OD}_{560}$ ) of 0.5 at 560 nm.

A 1 mL inoculum was transferred into 100 mL culture medium 79 supplemented with HEPES (1.3  $\text{mg L}^{-1}$  N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) and MES [1.1  $\text{mg L}^{-1}$  2-(N-morpholino) ethane sulfonic acid] (Cole and Elkan, 1973), with pH adjusted with HCl 2  $\text{mol L}^{-1}$  to 5.0, 6.0, and 6.9. The influence of  $\text{Al}^{3+}$  was evaluated by adding 0, 5, 10, and 20  $\text{mmol}_c \text{L}^{-1}$   $\text{Al}^{3+}$  as  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  to the medium before autoclaving,

followed by adjustment to pH 4.5. Strains were grown subjected to shaking at 120 rpm at 28 °C. Colony forming units (CFU) were counted by using serial dilutions as described by Miles et al. (1938). Aliquots of 20 µL were collected at intervals of 24, 48, 72, 96, 120, 144, 168, and 192 h incubation. The number of CFUs was assessed by directly counting the number of colonies on the plates at each dilution.

## Results

### Strain efficiency under axenic conditions

In the experiment conducted in Leonard jars, four groups based on the means were identified for the production of dry weight in the aerial part (DWAP) of cowpea plants (Table 2). In the first group, only the C+N control yielded higher values. In the second group, two strains, UFLA 03-164 and UFLA 03-153, showed DWAP values lower than 28 % and 40 %, respectively, in comparison to the nitrogen control. The third group was formed by strains recommended as inoculants for cowpea cultivation (UFLA 03-84, INPA03-11B, and BR 3267) and four strains (UFLA 03-154, UFLA 03-21, UFLA 03-165, and UFLA 04-885), all of which yielded DWAP higher than the control without mineral N, although these values were lower than the yields provided by strains in the second group.

Eighteen strains yielded similar numbers of nodules ( $p < 0.05$ ) as the strains approved as inoculants for cowpea (UFLA 03-84, INPA 03-11B, and BR 3267). The non-inoculated controls (with and without mineral nitrogen) produced no nodules, indicating the absence of contamination (Table 2). The six strains with higher DWAP values included strains UFLA 03-164 and UFLA 04-0885 that had DWN lower than the four other strains (Table 2). UFLA 03-164 and UFLA 03-153 have nitrogen accumulation in the aerial part similar to the nitrogen fertilizer control. In the three other treatments, UFLA 03-154, UFLA 04-885, and UFLA 03-165 were included in the group of UFLA 03-84 (Table 2).

### Strain efficiency in plots with soil and native population density of soil rhizobia

In the experiment carried out in pots with soil in the greenhouse to evaluate the efficient of each strain, UFLA 03-164, UFLA 3-154, and UFLA 03-153 excelled in promoting higher DWAP, with values similar to those of nitrogen-fertilized controls and those inoculated with a MAPA-approved strain, INPA 03-11B (Table 3). Only UFLA 03-153 showed higher NN values; and was included in the BR 3267 group. UFLA 03-154 was in the lower group, although it did not differ from INPA03-11B (Table 3).

No difference in DWN was noted among treatments ( $p < 0.05$ ), except for the nitrogen-fertilizer control, which developed a few small nodules only.

Nitrogen accumulation in the aerial part was higher in the nitrogen-fertilizer control, thus differing from

the other treatments. The control without mineral nitrogen and inoculation showed DMAP, DMN, and NAAP values similar to those provided by BR 3267 and UFLA 03-84 (Table 3). In the evaluation of the native population (Table 4) nodulation occurred up to dilution  $10^{-6}$ , and the DWAP in the dilution  $10^{-1}$  up to  $10^{-3}$  was no different ( $p < 0.05$ ) from the N-fertilizer control indicating a highly efficient native population and a high density of soil rhizobia.

### Tolerance to pH and Aluminum "in vitro"

The strains grew efficiently at different pH values (Figure 1). At pH 5.0, all strains exceeded  $10^9$  CFU mL<sup>-1</sup>, indicating a good capacity to tolerate acidic conditions.

Table 2 – Number of nodules per jar (NN), dry weight of the aerial part (DWAP), dry weight of nodules (DWN), and nitrogen accumulation in the aerial part (ANAP) of cv. BR17-Gurgueia cowpea inoculated with different strains in Leonard jars under axenic conditions.

Treatment	NN	DWAP	DWN	ANAP
		g jar <sup>-1</sup>	mg jar <sup>-1</sup>	
UFLA 03-14	3 b	0.4 d	0 c	7.6 c
UFLA 04-0110	21 b	0.4 d	6.6 c	7.6 c
UFLA 04-0546	34 b	0.4 d	26.6 c	10.1 c
UFLA 03-26	44 b	0.4 d	3.3 c	9.7 c
UFLA 03-19	17 b	0.4 d	10 c	10.2 c
UFLA 03-23	60 a	0.4 d	3.3 c	5.7 c
UFLA 03-05	0 b	0.4 d	0 c	7 c
C*	0 b	0.4 d	0 c	4.5 c
UFLA 03-17	8 b	0.5 d	10 c	3 c
UFLA 03-18	33 b	0.5 d	66 c	3.5 c
UFLA 03-22	38 b	0.6 d	20 c	5.6 c
UFLA 04-0321	56 a	0.7 d	90 b	13.5 c
UFLA 04-1309	86 a	0.7 d	83.3 b	28 c
UFLA 03-172	57 a	0.7 d	96.6 b	34.4 c
UFLA 03-170	56 a	0.8 d	63.3 b	30.7 c
UFLA 03-15	53 a	0.8 d	53.3 b	32.9 c
UFLA 03-163	57 a	0.8 d	83.3 b	39.3 c
UFLA 03-20	93 a	0.8 d	86.6 b	21.6 c
UFLA 05-0559	56 a	0.9 d	150 a	21.3 c
UFLA 03-27	76 a	0.9 d	146.6 a	39 c
UFLA 04-1020	36 b	0.9 d	70 b	40.9 c
UFLA 04-0314	69 a	0.9 d	76.6 b	20.3 c
UFLA 04-885	130 a	1.1 c	103.3 b	43.8 c
INPA03-11B	142 a	1.2 c	196.6 a	41.4 c
BR 3267	106 a	1.2 c	146.6 a	44.3 c
UFLA 03-165	95 a	1.4 c	163.3 a	64.7 b
UFLA 03-21	77 a	1.5 c	233.3 a	59 b
UFLA03-84	171 a	1.8 c	256.6 a	72.8 b
UFLA 03-154	72 a	1.9 c	233.3 a	80.8 b
UFLA 03-153	98 a	2.5 b	273.3 a	117.4 a
UFLA 03-164	85 a	3 b	96.6 b	121.8 a
C+N*	0 b	4.2 a	0 c	126.9 a
CV%	42	48	68	60

In each column, means followed by the same letter belong to the same group (Scott-Knott test,  $p < 0.05$ ). \*Controls without inoculation in the absence (C) and presence of mineral nitrogen (C+N) on the bottom of the jar. N was added at a rate of 210 mg N-NH<sub>4</sub>NO<sub>3</sub> jar<sup>-1</sup>.

Table 3 – Number of nodules per jar (NN), dry weight of the aerial part (DWAP), dry weight of nodules (DWN), and nitrogen accumulation in the aerial part (ANAP) of cv. BR17-Gurgueia cowpea, inoculated with different strains. Experiments performed in pots with non-sterilized soil.

Treatment	NN	DWAP	DWN	ANAP
		g jar <sup>-1</sup>	mg jar <sup>-1</sup>	
BR 3267	145 a	13 b	456.6 a	345 b
UFLA 03-84	94 b	13.2 b	560 a	375 b
UFLA 3-165	97 b	13.3 b	453.3 a	306b
C*	38 c	14.1 b	416.6 a	330 b
UFLA04-885	72 b	14.3 b	553.3 a	293 b
UFLA 03-21	119 b	14.4 b	463 a	195 b
UFLA 3-153	210 a	15.4 a	533 a	186 b
UFLA 3-154	49 c	15.8 a	336.6 a	316 b
UFLA 3-164	120 b	16.4 a	536.6 a	313.4 b
INPA03-11B	102 b	16.5 a	533 a	469 b
C+N*	6 d	17.4 a	0 b	719.4 a
CV%	34	7	33	23

In each column, means followed by the same letter belong to the same group (Scott-Knott test,  $p < 0.05$ ). \*Controls without inoculation in the absence (C) and presence of mineral nitrogen (C+N) on the bottom of the jar. N was added at a rate of 500 mg N-NH<sub>4</sub>NO<sub>3</sub> per pot.

Table 4 – Number of nodules per jar (NN), dry weight of the aerial part (DWAP) and dry weight of nodules (DWN), of cv. BR17-Gurgueia cowpea, inoculated with suspensions of the soil used in the previous experiment. Experiments performed in Leonard jars with three replicates.

Treatment	NN	DWAP	DWN
		g jar <sup>-1</sup>	mg jar <sup>-1</sup>
C*	0 c	163 b	0 c
Dilution 10 <sup>-6</sup>	31 b	290 b	20 c
Dilution 10 <sup>-5</sup>	32 b	443 b	26 c
Dilution 10 <sup>-4</sup>	44 b	570 b	73.3 b
Dilution 10 <sup>-3</sup>	47 b	966 a	83.3 b
Dilution 10 <sup>-2</sup>	92 a	1396 a	73.3 b
Dilution 10 <sup>-1</sup>	98 a	1720 a	140 a
C+N*	0 c	1393 a	0 c
CV (%)	49	39	56

In each column, means followed by the same letter belong to the same group (Scott-Knott test,  $p < 0.05$ ). \*Controls without inoculation in the absence (C) and presence of mineral nitrogen (C+N) on the bottom of the jar. N was added at a rate of 210 mg N-NH<sub>4</sub>NO<sub>3</sub> jar<sup>-1</sup>.

Table 5 – Final pH after growing six strains in medium 79 for 192 h at different initial pH and Al<sup>3+</sup> concentrations

pH	UFLA 03-84	INPA 03-11B	BR 3267	UFLA 03-154	UFLA 03-164	UFLA 03-153
5	5.6	5.8	5.7	5.8	5.9	5.8
6	6.4	6.3	6.3	6.2	6.4	6.5
6.8	7.11	7.04	6.7	7	7.2	7.1
[Al <sup>3+</sup> ] mmol <sub>c</sub> mL <sup>-1</sup>	UFLA 03-84	INPA 03-11B	BR 3267	UFLA 03-154	UFLA 03-164	UFLA 03-153
0	5.3	5.2	5.4	5.1	5.6	5.5
5	5	4.7	3.1	5.4	5.7	5.5
10	5.6	4.8	4.9	4.1	5.6	5.4
20	5	4.6	4.1	3.9	4.5	5.9

However, these values were not reached at the same time. UFLA 03-164 had values higher than 10<sup>9</sup> CFU mL<sup>-1</sup>, at 72 h, whereas INPA 03-11, UFLA 03-153, and UFLA 03-154 reached these values only at 96 h. UFLA 03-84 presented similar values only at 144 h. At pH 6.0, growth above 10<sup>9</sup> CFU mL<sup>-1</sup> was faster with UFLA 03-153, UFLA 03-164, and BR 3267 at 96 h; for UFLA 03-84 and INPA 03-11B, these values were reached at 120 h. At pH 6.9, the maximum number of cells was more rapidly obtained (96 h) with UFLA 03-153, UFLA 03-164, and BR 3267, followed by INPA 03-11B at 120 h and UFLA 03-84 and UFLA 03-164 at 144 h. UFLA 03-164 generated a greater increase in pH, from pH 5.0 to pH 5.95, in the culture medium. However, all the strains increased the pH (Table 5). This increase was more significant when the strains were grown at pH 5.0 and 6.0.

In the aluminum tolerance test (Figure 2) at pH 4.5 in the absence of Al<sup>3+</sup>, three strains (UFLA 03-84, INPA 03-11B, and BR 3267) grew satisfactorily, reaching more than 10<sup>9</sup> CFU mL<sup>-1</sup> at 96 h; UFLA 03-153 reached this value at 120 h. UFLA 03-154 and UFLA 03-164 reached 10<sup>8.90</sup> CFU mL<sup>-1</sup> and 10<sup>8.86</sup> CFU mL<sup>-1</sup> at 144 h and showed a higher tolerance to pH 4.5 in the absence of Al<sup>3+</sup>. Addition of 5 mmol<sub>c</sub> dm<sup>-3</sup> of Al<sup>3+</sup> prevented all strains from reaching 10<sup>9</sup> CFU mL<sup>-1</sup>, although some strains reached 10<sup>8</sup> CFU mL<sup>-1</sup> at 120 h. This indicates high adaptability to acidic pH and to low concentrations of Al<sup>3+</sup>. The only exception was UFLA 03-154, which showed limited growth at 5 mmol<sub>c</sub> dm<sup>-3</sup>. For concentrations of 10 mmol<sub>c</sub> dm<sup>-3</sup> of Al<sup>3+</sup>, all strains, except UFLA 3-154, appeared to be tolerant, with values reaching around 10<sup>8.0</sup> UFC mL<sup>-1</sup>. For concentrations of 20 mmol<sub>c</sub> Al<sup>3+</sup> only strains UFLA3-84, UFLA 3-153 and UFLA 3-164 were able to grow, although less than at lower Al<sup>3+</sup> concentrations. There was no relationship between the origin of the strains, i.e., the soil characteristics from where they were isolated (Table 1) and their tolerance to acidity and aluminum.

## Discussion

The DWAP of the six strains studied were lower than the C+N ( $p < 0.05$ ), but were similar to the three bacterial strains approved as inoculants for cultivation. Lima et al. (2005) used Leonard jars for experiments performed in the spring/summer and found that UFLA

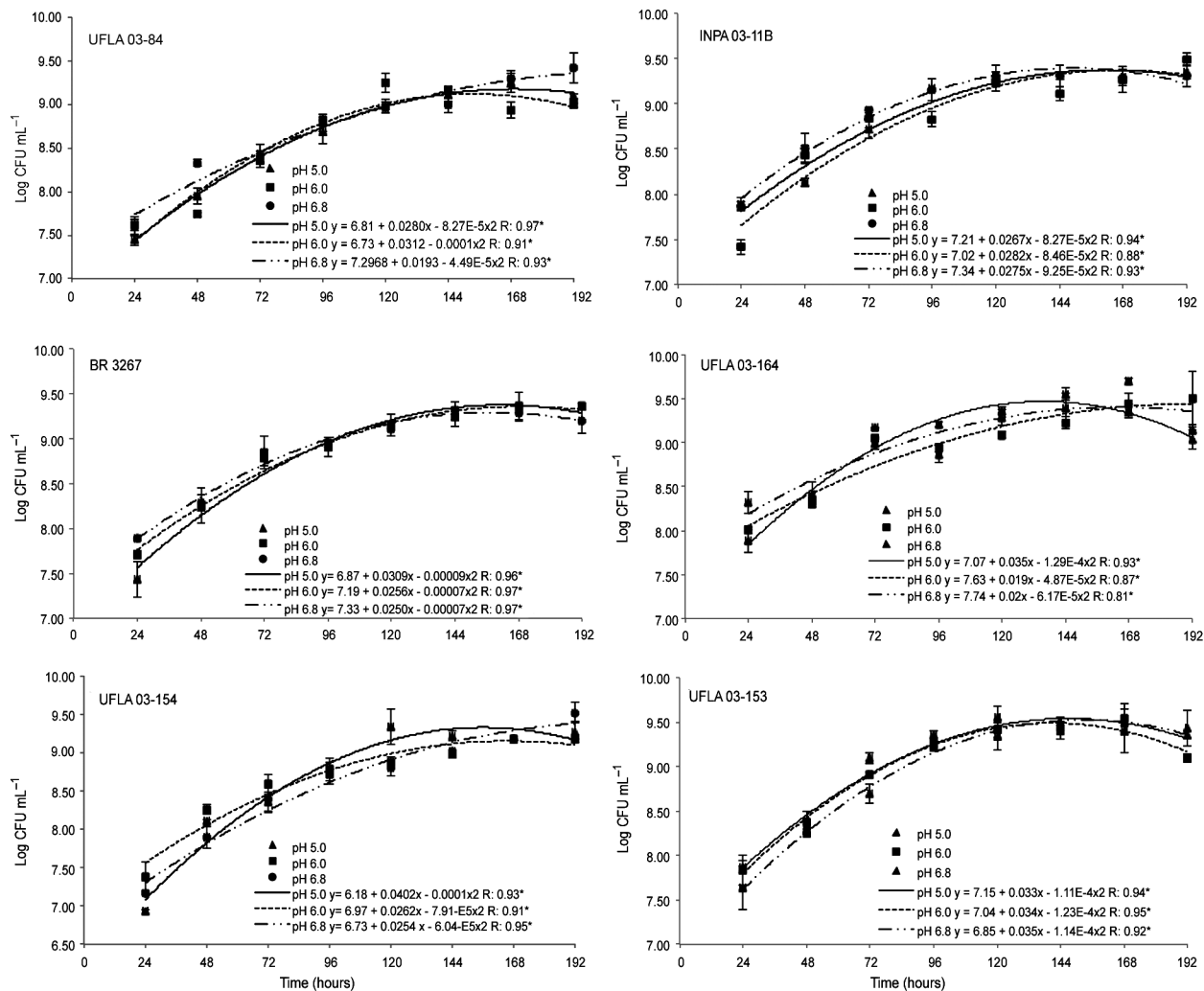


Figure 1 – Growth curve of diverse strains at different pH values. \* Significant at 5 % probability (F-test).

04-885 and UFLA 03-84 belong to the same symbiotic efficiency group in comparison to controls treated with mineral nitrogen. This suggested that even under axenic conditions, climate effects might impact strain efficiency, in contrast to experiments carried out in the autumn/winter. DWAP positively correlated with DWN ( $r: 0.42; p < 0.01$ ), as observed by Fernandes et al. (2003), and with N accumulation ( $r: 0.93; p < 0.01$ ). Positive correlation was also observed between DWN and N accumulation ( $r: 0.56; p < 0.01$ ), thus confirming the data obtained by Döbereiner et al. (1966), Fernandes and Fernandes (2000), and Lima et al. (2005).

The soil used in the pots had a high density of native rhizobia, including strains that can fix nitrogen when associated with cowpea. In this experiment, UFLA 3-153, UFLA 3-154, UFLA 0-164, and INPA 03-11B were similar to C+N. The high efficiency of INPA 03-11B has also been observed by other authors in field experiments. Costa et al. (2011), Lacerda et al. (2004) and Soares et al.

(2006) showed INPA 03-11B has been included in the same control group that received nitrogen fertilization. Costa et al. (2011), also showed that UFLA 03-153 has been included in this group.

C+ N treatment was higher than inoculated strains in Leonard jars condition (Table 2). However, in pots with soil C+ N treatment was similar to inoculant strains (Table 3). This happens because plant response to mineral N is faster than to inoculation. However, when more time is given for plants to develop, and this includes the conditions for this development, responses become similar. Nitrogen treatment similar to strain inoculation also occurs in the field when yields are evaluated (Soares et al., 2006).

The native population of rhizobia efficiently fixed nitrogen under controlled condition, with DWAP values similar to C+N. Density of these native populations can be considered high: from  $2 \times 10^5$  to  $4 \times 10^6$  CFU per gram of soil. These are usual values for soils, since the

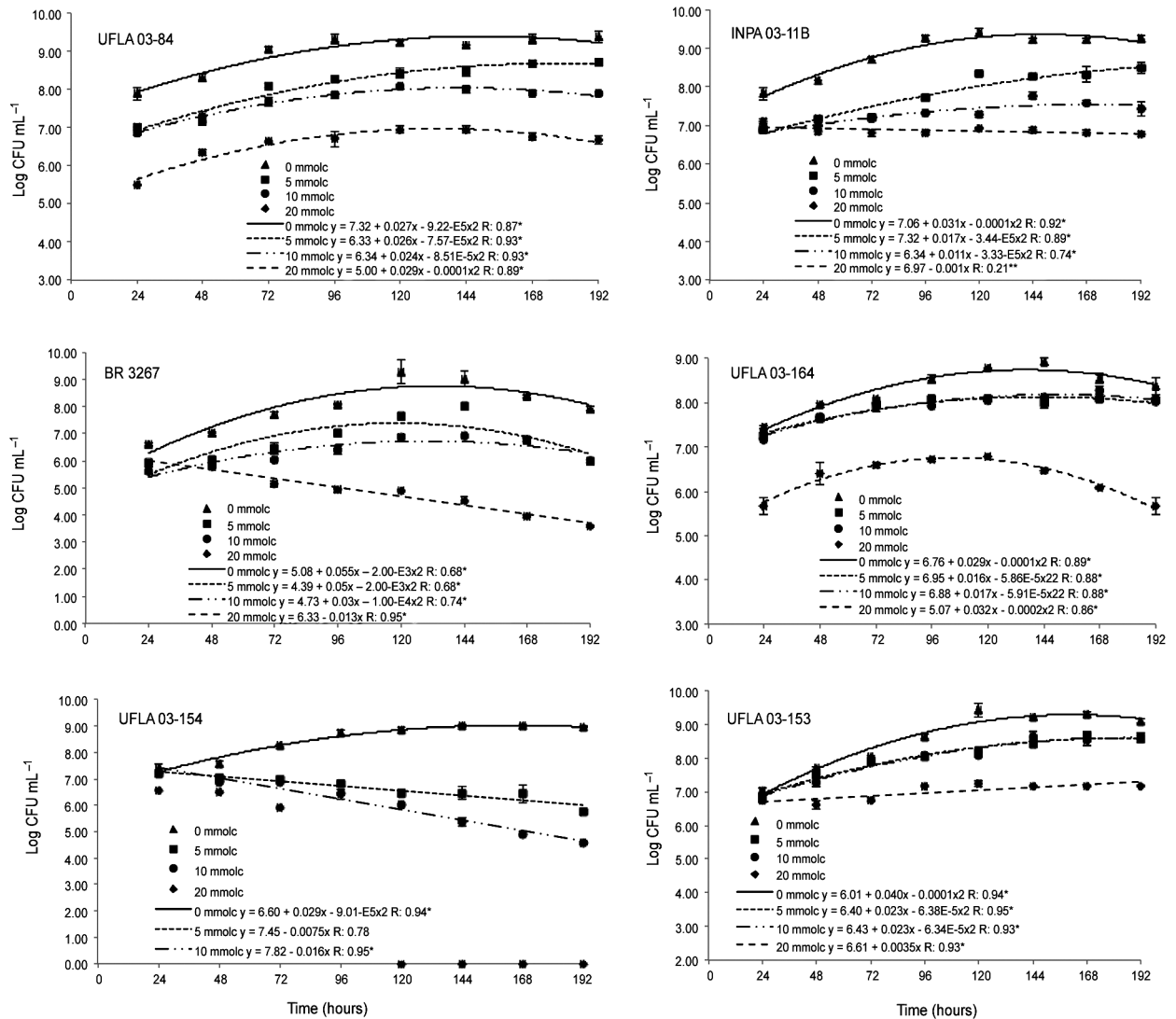


Figure 2 – Growth curve of diverse strains at different aluminum concentrations. Significant (F-test,  $p < 0.05$ ), \*\* not significant (F-test,  $p < 0.05$ ).

native population of rhizobia can reach up to  $10^7$  CFU per gram (Hirsch, 1996). Martins et al. (2003) observed  $10^4$  CFU per gram of soil after the second year of rhizobial inoculation in cowpea cultivations in the northeast of Brazil and Soares et al. (2006) showed native populations to be about 23 and 38 CFU per gram of soil in southeast Brazil. Considering the high numbers of native populations, we have to prove that the inoculated strains in soil were the ones that induced higher production of DWAP. Thus, the patterns of root infection by INPA 03-11B, UFLA 03-153, C+N, and C (Figure 3) treatments were compared.

Roots in the C+N had no nodules, due to inhibition by mineral nitrogen (Figure 3A). Figure 3B shows the pattern of nodulation of control by native soil bacterial that were distributed throughout all roots, thus differing from the INPA 03-11B and UFLA 03-153 inocu-

lation treatments, which yielded a pattern of infection at the base of the shoot, typical of a cell concentration derived from inoculation (Figures 3C and 3D).

UFLA 3-153 and UFLA 0-164 belongs to *Bradyrhizobium* genus, as well as INPA 3-11B, UFLA3-84 and Br3267, the strains currently approved by MAPA as cowpea inoculants (Table 1). UFLA 03-154 belongs to the genus *Burkholderia*. Nodulation of cowpea by *Burkholderia* strain UFLA 03-216 was reported previously (Guimarães et al., 2012). However, this strain was inefficient in terms of symbiotic nitrogen fixation with cowpea. Thus, this is the first report of the soil symbiotic efficiency of this genus in cowpea. Nodulation by unusual genera such as *Enterobacter*, *Pseudomonas*, *Acinetobacter* corroborates findings previously reported (Guimarães et al., 2012; Marra et al., 2012). This nodulation was confirmed by Koch's postulates, i.e. pure cultures were re inoculated

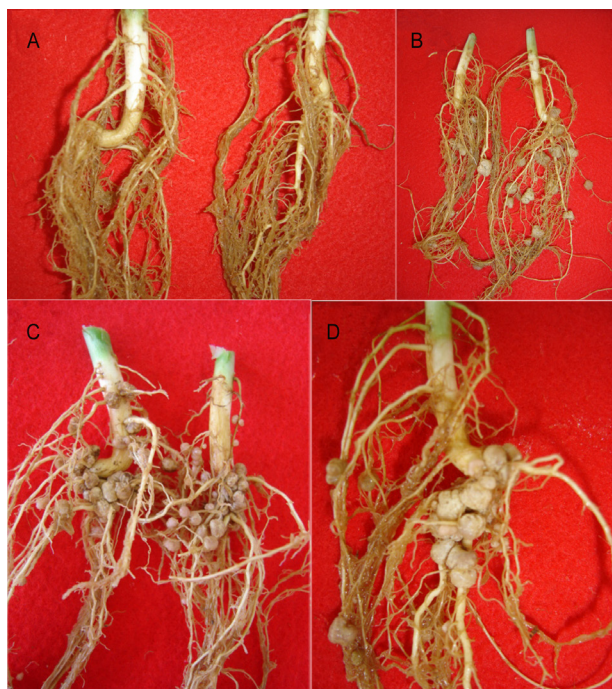


Figure 3 – Nodulation in the roots of the four treatments used in the soil pot experiment; (A) treatments that were not inoculated and received mineral nitrogen fertilization; (B) treatments that were not inoculated and did not receive mineral nitrogen fertilization in the soil; (C) and (D) treatments that were inoculated and did not receive nitrogen fertilization in the soil (C-INPA 03-11B and D-UFLA 03-154).

under axenic conditions and reisolated from nodules. Besides, contamination could be detected by low quality of sequences, which was not the case. Bacterial endophytes of nodules may become symbiotic by horizontal gene transfer from symbiotic bacteria (Li et al., 2008; Shiraishi et al., 2010).

Shiraishi et al. (2010) detected occurrence of nodulation in *Robinia pseudoacacia* by *Pseudomonas* sp. and the presence of *nodA*, *nifH* and *nifHD* in this genus. Shiraishi et al. (2010) also demonstrated that *nodA* genes from *Pseudomonas*, *Agrobacterium*, *Burkholderia* and *Mesorhizobium* isolates, from the same soil, had high genetic relationship. Further studies looking for the presence of nodulation and nitrogen fixation genes must confirm their taxonomic position of our strains.

All the strains studied were tolerant to acidity, although they grew at different rates (Figure 1). Graham et al. (1994) showed that *Bradyrhizobium* strains grow on solid media at pH 4.25 although with a late growth. The variation in time to maximum bacterial growth might be related to membrane permeability (Correa and Barneix, 1997). Chen et al. (1993 a,b) suggest that tolerance to acidity is related to the ability to maintain the cytoplasmic pH unchanged.

The strains isolated from bauxite mining areas, where the pH of the soil is between 5.13 and 7.5, seem to have acquired adaptation mechanisms for growth at pH 5.0; the strains recommended as inoculants were isolated from Amazonian acid soils (INPA 03-11B and UFLA 03-84). Ferreira et al. 2012 showed *Rhizobium* strains from the same origin were highly tolerant to acidity and up to 1 mmol Al<sup>3+</sup> L<sup>-1</sup>. At varying Al<sup>3+</sup> concentrations bacterial growth yielded different changes in medium pH (Table 5). However, all alkalized in the media, a characteristic typical of *Bradyrhizobium* genus. Tolerance to pH and acidity may differ among strains, as Cunningham and Munns (1984) reported that production of exopolysaccharides might coincide with increased acid tolerance. *R. miluonense* isolated from the acidic soil of the Amazon showed high tolerance to acidity and aluminum, an effect that might be related to plasma membrane permeability (Ferreira et al., 2012).

The adaptation of tropical *Bradyrhizobium* strains to acidity was also verified previously. Miguel and Moreira (2001) observed that four *Bradyrhizobium* strains grew better at pH 6.0. Barberi et al. (2004) showed that *B. elkanii* BR 29 grew better at pH 6.8 or 5.5 supplemented with calcium. Our results show that UFLA 03-153, UFLA 03-154 and UFLA 03-164 are also adapted to acidity and highly efficient in symbiotic N<sub>2</sub> fixation with cowpea. Besides, UFLA 03-153 and UFLA 03-164 are also tolerant to high Al<sup>3+</sup> concentrations. Thus, they should be assessed regarding agronomic efficiency in the field, in both acidic and neutral soil. The good performance of inoculant strain UFLA3-84 in various soils must be due to its tolerance to various antibiotics produced by other microorganisms (Florentino et al., 2012) as well as its adaptation to acidity and high aluminum contents in soil.

## Conclusions

*Bradyrhizobium* strains UFLA3-164, UFLA3-153, and *Burkholderia* strain UFLA3-154 isolated from bauxite mining areas, have high nitrogen-fixing capacity in symbiosis with cowpea and are good competitors with native soil rhizobia, and are thus recommended for field experiments.

All strains grow well at pH 5.0, 6.0, and 6.8. In particular, UFLA 03-84, UFLA 03-153, and UFLA 03-164 grow in media supplemented with up to 20 mmol Al<sup>3+</sup> dm<sup>-3</sup>.

Inoculation with rhizobial strains that have been carefully selected according to their ability to nodulate and fix N<sub>2</sub>, combined with their ability to compete in soils that are acidic and contain high levels of Al, is a cheaper and more sustainable alternative that can be made available to farmers than mineral N fertilizers.

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