Universidade Federal Rural do Semi-Árido
Pró-Reitoria de Pesquisa e Pós-Graduação
http://periodicos.ufersa.edu.br/index.php/sistema

ISSN 0100-316X (impresso)
ISSN 1983-2125 (online)

SYMBIOTIC EFFICIENCY OF RHIZOBIA STRAINS WITH COWPEA IN SOUTHERN MARANHÃO[1]

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ABSTRACT - The objective of this study was to evaluate the symbiotic efficiency of three novel nitrogen-fixing bacterial strains with cowpea cv. BRS Guariba as forage. Two experiments were carried out in the field in Balsas and São Raimundo das Mangabeiras, MA, in a randomized block design with seven treatments and four replications: three novel strains efficient in symbiotic nitrogen fixation with cowpea (UFLA 3-153, UFLA 3-154 and UFLA 3-164), two strains already approved as inoculants by MAPA (UFLA 3-84/SEMIA 6461 and INPA 3-11B/SEMIA 6462), and two controls without inoculation, with and without mineral N. Symbiotic efficiency of strains was determined by the number of nodules, nodules dry matter, shoot dry matter, relative efficiency, crude protein content and shoot N content. Strains UFLA 3-154 and UFLA 3-164 were the most efficient in Balsas, whereas UFLA 3-153 and UFLA 3-164 were the most efficient in São Raimundo das Mangabeiras. Strains UFLA 3-153, UFLA 3-154 and UFLA 3-164 were more efficient in symbiotic nitrogen fixation with cowpea than the strains already approved as inoculants UFLA 3-84 and INPA 3-11B in the climatic and edaphic conditions of southern Maranhão. UFLA 3-164 is the most efficient strain in this region.

Keywords: *Vigna unguiculata*. Biological nitrogen fixation. Diazotrofic bacteria. Forage.

EFICIÊNCIA SIMBIÓTICA DE ESTIRPES DE RIZÓBIO EM FEIJÃO-CAUPI NO SUL DO MARANHÃO

RESUMO - O objetivo deste trabalho foi avaliar a eficiência simbiótica de três novas estirpes de bactérias fixadoras de N2 em feijão-caupi cv. BRS Guariba como forrageira. Foram conduzidos dois experimentos em campo, em Balsas e São Raimundo das Mangabeiras, MA, em delineamento em blocos casualizados com sete tratamentos e quatro repetições, sendo: três novas estirpes eficientes em feijão-caupi (UFLA 3-153, UFLA 3-154 e UFLA 3-164), duas estirpes já aprovadas como inoculantes pelo MAPA (UFLA 3-84/SEMIA 6461 e INPA 3-11B/SEMIA 6462) e dois controles sem inoculação, com e sem N mineral. A eficiência simbiótica das estirpes foi determinada pelo número e massa seca de nódulos, produção de matéria seca da parte aérea, eficiência relativa, teor e acúmulo de proteína bruta e nitrogênio na parte aérea. As estirpes UFLA 03-154 e UFLA 3-164 destacaram-se na eficiência simbiótica de N_2 em Balsas e a UFLA 3-153 e UFLA 3-164 em São Raimundo das Mangabeiras. As estirpes UFLA 3-153, UFLA 3-154 e UFLA 3-164 são mais eficientes em fixar nitrogênio do que as estirpes aprovadas como inoculantes UFLA 3-84 e INPA 3-11B nas condições edafoclimáticas do sul do Maranhão. A UFLA 3-164 é a estirpe mais eficiente em fixar nitrogênio na região estudada.

Palavras-chave: *Vigna unguiculata*. Fixação biológica de nitrogênio. Bactérias diazotróficas. Forragem.

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[1]Received for publication in 03/11/2015; accepted in 03/28/2016.
Extracted from the first author's Master's dissertation.
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INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is a Dicotyledonea plant, which belongs to the Fabaceae family, and is an important protein source in food and feed (PADULOSI; NG, 1997). Its grains present high protein content (18 to 35%), and is rich in lysine, carbohydrates (50 to 65%), vitamins A, C, E and B complex (0.08 to 37 mg 100-1g), fibers (11-16%), ash (3.6 to 4.2%), lipids (0.61 2.2%), gross energy (320.8 to 357 KCAL), depending on the variety (CAMPBELL; EUSTON; AHMED, 2016).

Cowpea is widely cultivated in tropical and subtropical regions, where it plays important role in human and animal nutrition, being an essential component of the production systems in semi-arid regions, mainly in Africa and Brazil (SINGH et al., 2002). With 30 to 50 g kg^{-1} of nitrogen in the dry matter, legumes species, such as cowpea, are used in rotation with other crops to protect the soil, reduce erosion, or as green manure to improve soil fertility and forage production rich in protein (TIMKO et al., 2007; KAMBASHI et al., 2014a).

Cowpea plant is used as animal feed, supplementing the protein content in silage, which are mostly made up of grasses, improving the intake and digestion of low quality forages (OSAFO et al., 2013). Due to its dual purpose, after harvesting the grain for human consumption, cowpea plant is available for animal feed, becoming a low-cost forage as food supplement (KAMBASHI et al., 2014b).

BRS Guariba, with approximately sixty days cycle, is an excellent alternative for the cultivation of grains, forage and/or green manure in rotation, or intercropped with other crops, since it benefits from the biological nitrogen fixation (BNF) in the symbiosis with diazotrophic bacteria, and most part of this nitrogen fixation occurs until the 41st day, corresponding to the flowering stage.

This combination contributes to the low production cost of cowpea, since nitrogen stocks in soil under tropical climate are low, making it the most limiting nutrient in crop productions in these regions, which increases production costs, especially the crops which are more dependent on mineral fertilizers (RANGEL; SILVA, 2007). In these environments, biological nitrogen fixation (BNF) has significant contribution in the economic and environmental viability for production systems, since it reduces the need for costly application of nitrogenous fertilizers.

BNF has contributed to the increase in cowpea yield, which along with other technological strategies, has led to the expansion of the culture to the agricultural frontier regions of cerrado, competing as off-season culture with traditional commodities, such as corn (FREIRE FILHO et al., 2011).

The establishment, development and performance of the mutualistic symbiosis between cowpea and rhizobia are affected by various biological, chemical and physical factors, which may act on the macro and microsymbiont, varying according to their genotypic characteristics, which produces different answers in relation to the diversity of bacteria that nodulate cowpea, as well as the variation in relation to the symbiotic efficiency within each species (MOREIRA; SIQUEIRA, 2006). Thus, soil and climatic factors, management practices, such as no-tillage, the use of pesticides and native rhizobia populations interfere with symbiotic efficiency of efficient strains selected for the culture. Therefore, in order to increase BNF efficiency in cowpea, and to contribute to greater N_2 incorporation in the system, with increase in grain yield, forage quality and/or nitrogen availability as green manure, it is necessary further selection studies on strains with high symbiotic efficiency and which are adaptable to regional conditions.

Therefore, the objective of this study was to evaluate the symbiotic efficiency of three novel nitrogen-fixing bacterial strains in cowpea cv. BRS Guariba as forage.

MATERIAL AND METHODS

Two experiments were carried out in the cerrado region of southern Maranhão, in soil classified as Dystrophic Red-Yellow Latosol (Oxisol), under no-tillage, at Fazenda Cajueiro, in Balsas (MA) (lat. 7°13'S, long. 45°59'W, at 342 m asl), and in soil classified as Dystrophic Yellow Latosol under conventional no-tillage, at the Federal Institute of Education, Science and Technology of Maranhão (IFMA), Campus São Raimundo das Mangabeiras (MA) (lat. 7°02'S, long. 45°30'W, at 258m asl), between January and March, 2013. According to the Thornthwaite classification, the climate is dry sub-humid (C1SA'a'), with average temperature of 27°C, average relative air humidity of 75%, and annual rainfall of 1,200 mm. The average monthly rainfall observed at the weather station of Balsas (Inmet), which covers both municipalities, was 243.4 mm during the experiments (Figure 1). Table 1 shows the chemical properties of the soil of the experimental areas, in the layer 0 to 20 cm.

It was used as experimental design of randomized blocks with seven treatments and four replications (5 inoculant strains + 2 controls without inoculation, with 80 kg ha^{-1} N-urea (+N), and without mineral N (-N)), totaling 28 experimental plots. It was evaluated three novel nitrogen-fixing bacterial strains, previously selected in experiments in axenic conditions and with pots filled with soil (SOARES et al., 2014). Plots consisted of six 6m rows, sown with cowpea cv. BRS Guariba. The first and the sixth rows were considered as border rows.

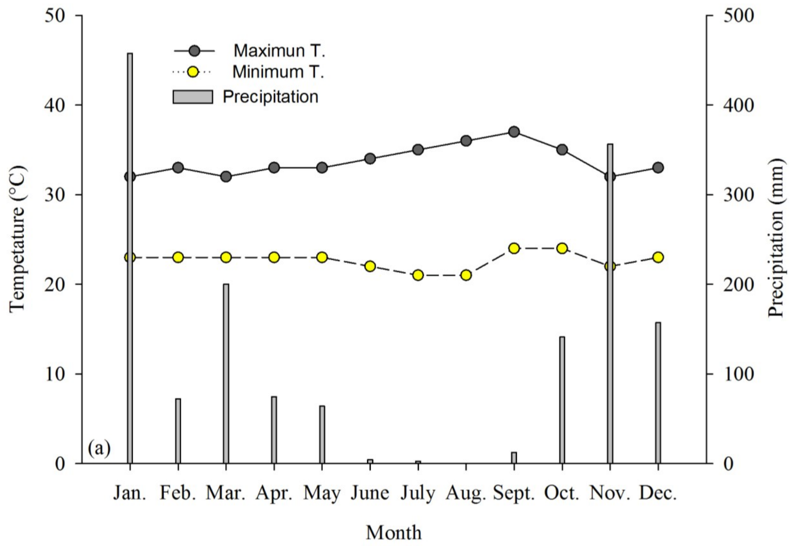

Figure 1. Minimum and maximum temperatures and average monthly rainfall in 2013, Balsas - MA and São Raimundo das Mangabeiras - MA; Source: National Institute of Meteorology – INMET.

Table 1. Chemical analysis of the soil in the layer 0-20 cm before the implementation of the experiments. in Balsas and in São Raimundo das Mangabeiras - MA. (PH in water).

Location	pH	K^+	P	P-Rem	Ca^{2+}	Mg^{2+}	H+Al	Al^{3+}	S	MO
		-mg/dm^3-		mg/L	----------cmol$_c$/dm^3----------				mg/dm^3	dag/kg
Balsas	LVA – Dystrophic Red-Yellow Latosol									
	5.7	64.0	41.6	31.1	2.7	0.6	4.5	0.2	7.0	3.3
São R. das Mangabeiras	LA - Dystrophic Yellow Latosol									
	5.8	38.0	2.3	42.8	0.8	0.5	2.9	0.3	2.9	1.9

The evaluated novel strains were UFLA 03-153, UFLA 03-154 and UFLA 03-164. They were isolated from bauxite mining areas located in the Serra de Poços de Caldas, Minas Gerais, and were classified as *Bradyrhizobium* sp., *Burkholderia fungorum* and *Bradyrhizobium* sp., respectively, and kept in the collection of the Biology, Microbiology and Soil Biological Processes Laboratory (UFLA) (OLIVEIRA-LONGATTI et al., 2013; SOARES et al., 2014). In addition, two other strains approved by MAPA were tested as cowpea inoculant references: UFLA 03-84 – SEMIA 6461 (*Bradyrhizobium* sp.) and INPA 03-11B – SEMIA 6462 (*B. elkanni*).

Through sequence analysis of 77 types of reference multilocus from *Burkholderia* strains, using atpD, gltB, lepA and recA genes, in combination with the 16S 16S rRNA gene, Estrada-de Los Santos et al. (2013) demonstrated that *Burkholderia fungorum* does not group with other species of the *B. cepacia* complex, which are animal pathogens. Strains were grown in glass jars containing 30 ml of liquid culture medium 79 (FRED; WAKSMAN, 1928), also known as YM (Yeast Mannitol) (VINCENT, 1970), at 6.8 pH, under orbital agitation at 100 rpm, at 28 ° C..

Before planting, soil samples were obtained to determine the most probable number (MPN) of rhizobia, with the use of the "Most Probable Number Estimate" (MPNES) (WOOMER at al., 1990). The experiment was carried out in a greenhouse (DCS/ UFLA), and it was used longneck glass bottles with nutrient solution of Hoagland and Arnon (1950), in which it was cultivated cowpea cv. BRS Guariba as trap plant, inoculated with aliquots of soil suspensions (10^{-1} a 10^{-6}) for twenty-five days.

In Balsas, soil analysis (Table 1) showed no deficiency in mineral nutrients. Therefore, fertilization with P_2O_5, K_2O, S and liming with Ca^{2+}, Mg^{2+} were not necessary. In São Raimundo das Mangabeiras, analysis indicated deficiency of some nutrients, and thus it was applied 90 kg ha^{-1} P_2O_5 in the planting furrow before sowing, in the form of superphosphate (18% P_2O_5 and 12% S), and 40 kg ha^{-1} K_2O in the form of potassium chloride (60% K_2O) (ALVAREZ et al., 1999).

For seeds inoculation, peat inoculants were used at a dose of 250g 10^{-1} kg of seeds, with 10% sugar solution to adhere the inoculum to the seeds. The inoculant, which contained strains at the log stage, was standardized to 10^{9} UFC mL^{-1}, and peat in the ratio of 7:4 (w/v). In Balsas, seeds were sown in furrows spaced 0.45 m apart, with 10 seeds per meter, and 7.2 m^2 of useful area in each plot. In São Raimundo das Mangabeiras, spacing was 0.7 m between rows, with 8 seeds per meter, and 11.2 m^2 useful area in each plot.

At the flowering stage (35 days after emergence - DAE), 10 plants were collected from the second and third rows of the useful area of each plot for the evaluation of the following variables: number of nodules (NN), nodules dry matter (NDM), shoot dry matter (SDM), relative efficiency (RE), shoot total content (STC), shoot N accumulation (SNA), total content of crude protein (CP), and crude protein accumulation (CPA). Shoot dry matter and nodules dry matter were determined after drying in a forced-air oven at 65-70°C until constant weight. Shoot dry matter was estimated in kg ha^{-1} from the plants collected in each experimental area. Relative efficiency was calculated by the ratio of shoot dry matter of the inoculated treatment with the shoot dry matter of the nitrogen control [RE = (SDMi/SDM +N) x100].

Total shoot N content was determined by the semi-micro Kjeldahl method. N accumulation in kg ha^{-1} was calculated by multiplying the shoot N content by the shoot dry matter. In the determination of the crude protein, it was multiplied the value of total nitrogen found by the semi-micro Kjeldahl method by a factor (6.25), which converts the nitrogen in protein (crude protein = N_{Total} x $Factor_N$). The calculation of the crude protein accumulation in kg ha^{-1} is similar to the calculation of shoot N accumulation.

Data were subjected to analysis of variance by the F test at 5% probability. Means were compared by the Scott-Knott test, also at 5% probability, using the statistical software Sisvar, version 4.0 (FERREIRA, 2011).

RESULTS AND DISCUSSION

In the experiment carried out in Balsas, all the three new strains evaluated in this study (UFLA 3-153, UFLA 3-154 and UFLA 3-164) were able to nodulate cowpea, and UFLA 3-154 and UFLA 3-164 presented the highest values for number of nodules, and were superior to those of the strains approved as inoculant in cowpea (UFLA 3-84 and INPA 3-11B) (Table 2). In São Raimundo das Mangabeiras, the novel strains were also able to nodulate cowpea, and UFLA 3-153 and UFLA 3-164 promoted greater number of nodules, which was superior to that of the other strains, but did not differ (p> 0 05) from the control (without inoculation and without mineral N) (Table 2).

In cowpea grown in both areas, it was observed low presence of nodules in the treatment without the inoculation with 80 kg ha^{-1} N-urea (+N), which indicates inhibition of rhizobium-cowpea symbiosis by the presence of nitrogen added to the soil. However, there was nodulation in all treatments, including the control without inoculation, indicating the presence of native populations in Balsas and São Raimundo das Mangabeiras. The most probable number of these populations estimated by the method of successive dilutions of soil samples inoculated in the trap plant presented results of 680 and 200 cells g^{-1} soil, respectively, for Balsas and São Raimundo das Mangabeiras. This difference in the number of viable cells between both sampled areas may be related to higher OM content in Balsas, since the area was under no-tillage. UFLA 3-164, which presented the highest number of nodules and nodules dry matter in both experiments, demonstrated ability to adapt to both evaluated soil conditions.

In the nodules dry matter, UFLA 3-154 and UFLA 3-164 strains presented the highest values in Balsas, while in São Raimundo das Mangabeiras, UFLA 3-153 and UFLA 3-164 presented the highest values, together with the treatment without inoculation and without mineral-N (-N) (Table 2). Groups formed in each site were higher than the reference strains (SEMIA 6461 and SEMIA 6462). In São Raimundo das Mangabeiras, results of the number and dry matter of nodules described above demonstrate the ability of the native population to establish symbiosis with cowpea, which is a promiscuous legume species (MOREIRA; SIQUEIRA, 2006; GUIMARÃES et al., 2012; JARAMILLO et al., 2013). The highest values of number of nodules and nodules dry matter of treatments with UFLA 3-153, UFLA 3-154 and UFLA 3-164 strains achieved in both experiments corroborate with Soares et al. (2014), who evaluated the symbiotic efficiency of the same strains in cowpea in axenic conditions and in pots filled with soil. Other authors also found similar results in field experiments (COSTA et al., 2011; FERREIRA et al., 2013). Oliveira-Longatti et al. (2013) showed the resistance of strains UFLA 3-153 and UFLA 3-164 to eleven in vitro antibiotics, which may explain the good adaptation of both strains.

In relation to shoot dry matter and to relative efficiency, UFLA 3-154 and UFLA 3-164 strains were the most effective in promoting plant growth in the experiment carried out in Balsas, surpassing the other treatments (Table 2). In São Raimundo das

Mangabeiras, the highest values of both shoot dry matter and efficiency in promoting growth of cowpea plants were observed for UFLA 3-153 and UFLA 3-164 strains, which were similar to the treatment without inoculation with 80 kg ha^{-1} N (Table 2).

The efficient symbiosis of UFLA 3-153, UFLA 3-154 and UFLA 3-164 strains with cowpea in southern Maranhão, observed in both trials, is relevant for the increase of grain yield, forage quality and/or nitrogen availability as green manure, since strains demonstrated ability to adapt to the conditions of the region with high symbiotic efficiency. These results corroborate those obtained by Soares et al. (2014) in pots filled with soil, who reported increase in nodulation and shoot dry matter in cowpea inoculated with the strains UFLA 3-153, UFLA 3-154 and UFLA3-164. However, Ferreira et al. (2013), working with UFLA 3-154, UFLA 3-164, UFLA 3-84 and INPA 3-11B inoculated with the cowpea cultivar BR 17 Gurgueia, in a field experiment in Itaueira, Piauí, in medium texture Oxisol, with monthly precipitation and average temperature of 102 mm and 28 °C, respectively, found higher relative efficiency for the strain INPA 3 -11B. Differences in the results of efficiency of the studied strains may be due to the different cultivars, to the management practices, such as the use of pesticides, humidity, and competition with native population of soil rhizobia, since the studied strains were under similar soil and climate conditions, with the exception of average rainfall during the experiments.

Table 2. Number of nodules per plant (NN), nodules dry matter (NDM), shoot dry matter (SDM), and relative efficiency (RE), cultivar BRS Guariba of the experiments carried out in Balsas and São Raimundo das Mangabeiras - MA(1).

Treatment	NN	NDM	SDM	RE
	Unit.	g plant^{-1}	kg ha^{-1}	%
		Balsas		
UFLA 3-153	22.5c	0.12b	1.472.7c	63.7d
UFLA 3-154	77.2a	0.29a	2.024.0b	87.6b
UFLA 3-164	65.4a	0.28a	2.038.7b	88.4b
UFLA 3-84	30.9c	0.15b	1.485.6c	64.3d
INPA 3-11B	40.6b	0.20b	1.712.8c	73.8c
Control –N	42.1b	0.20b	1.636.2c	71.1c
Control +N	2.5d	0.00c	2.319.3a	100.0a
CV (%)	8,5	5,9	8,4	1,9
		São Raimundo das Mangabeiras		
UFLA 3-153	42.7a	0.23a	872.6a	86.9a
UFLA 3-154	25.0b	0.12b	662.1b	66.4b
UFLA 3-164	40.7a	0.22a	892.0a	89.7a
UFLA 3-84	22.7b	0.11b	658.8b	65.2b
INPA 3-11B	17.5b	0.09b	497.5b	49.5c
Control –N	38.3a	0.20a	557.8b	54.3c
Control +N	4.5c	0.13c	1010.1a	100.0a
CV (%)	21.5	28.4	8.4	4.3

(1)Means followed by the same letters belong to the same group, according to the Scott-Knott test at 5% probability.

In relation to the total N content and shoot nitrogen accumulation in Balsas, UFLA 3-154 and UFLA 3-164 strains were similar to the treatment without inoculation with 80 kg ha^{-1} N-urea (N +) (Table 3) . In São Raimundo das Mangabeiras, treatment without inoculation with 80 kg ha^{-1} N-urea (+N) was the one which provided the highest total N content and shoot N accumulation, followed by strains UFLA 3-153, UFLA 3-164 and INPA 3-11B for total N content, and UFLA 3-153 and UFLA 3-164 for shoot nitrogen accumulation (Table 3).

For the nitrogen content in the shoots, similar results were found by Costa et al. (2011), in a field experiment in Bom Jesus, southwest Piauí. The authors evaluated the nodulation and yield of cowpea cv. BR 17 Gurgueia inoculated with strains UFLA 3-155, UFLA 3-164, UFLA 3-84 (BR 3302), INPA 3-11B and BR 3267 in Medium Texture Oxisol, with an average temperature of 25 °C. N contents are within the values indicated by Costa et al. (2011), and are higher than those found by Anele et al. (2011).

For the total crude protein content and crude protein accumulation, UFLA 3-154 and UFLA 3-164 strains were similar to the treatment without inoculation with 80 kg ha^{-1} N-urea (+N) in Balsas, while in São Raimundo das Mangabeiras, UFLA 3-153 and UFLA 3-164 strains showed values similar to the treatment without inoculation with 80 kg ha^{-1} N-urea (+N), providing the highest values of total crude protein content and crude protein accumulation in the shoot, followed by the other treatments (Table 3).

In São Raimundo das Mangabeiras, crude protein content in all treatments was higher than those observed in Balsas, which may be related to a dilution effect in Balsas due to higher shoot dry matter. Treatments inoculated with UFLA 3-153 and UFLA 3-164 strains showed the highest crude protein content and crude protein accumulation between inoculated treatments; however, they presented similar values to those of the control (without inoculation with 80 kg ha^{-1} N) in Raimundo das Mangabeiras (Table 3).

Results in both locations are close to those found by Kochhar et al. (1988), when they studied the effect of variety of cowpea in protein content. Cultivar BRS Guariba presented crude protein content between 19.6 and 32.0, and thus it was superior to other forage legumes, such as peanut forage (14.3 to 18%), stylosanthes (9.8 to 16.2%), perennial soybean (12.3%), leucaena (16.3%), alfalfa (17.0%), desmodium (14%) and pigeonpea (20.3%) reported by other authors (Barcellos et al., 2008; Silva et al., 2009), indicating its potential as forage for animal feed in the state of Maranhão.

Table 3. Mean values of shoot nitrogen content (SNC), shoot nitrogen accumulation (SNA), crude protein (CP) and crude protein accumulation (CPA), cultivar BRS Guariba of the experiments carried out in Balsas and São Raimundo das Mangabeiras - MA(2).

Treatment	SNC	SNA	CP	CPA
	%	kg ha^{-1}	%	kg ha^{-1}
		Balsas		
UFLA 3-153	3.1a	46.5b	19.7a	290.8b
UFLA 3-154	3.1a	63.6a	19.6a	397.4a
UFLA 3-164	3.3a	68.2a	20.7a	426.4a
UFLA 3-84	3.3a	48.5b	20.5a	303.0b
INPA 3-11B	3.2a	55.2b	20.2a	344.8b
Control –N	3.2a	53.0b	20.4a	331.6b
Control +N	3.2a	74.4a	20.0a	465.3a
CV (%)	11,4	4,4	4,4	18,6

(2) Means followed by the same letters belong to the same group, according to the Scott-Knott test at 5% probability.

Table 3. Continuation.

Treatment	SNC	SNA	CP	CPA
	%	kg ha^{-1}	%	kg ha^{-1}
São Raimundo das Mangabeiras				
UFLA 3-153	4.9a	43.3b	30.4a	264.4a
UFLA 3-154	4.3b	28.8c	27.2b	180.2b
UFLA 3-164	4.8a	42.8b	30.0a	267.7a
UFLA 3-84	4.0b	26.2c	24.9b	163.9b
INPA 3-11B	4.5a	22.4c	28.3a	140.1b
Control –N	4.0b	21.5c	25.1b	134.6b
Control +N	5.1a	51.7a	32.0a	323.1a
CV (%)	5.0	14.5	2.3	14.5

(2) Means followed by the same letters belong to the same group, according to the Scott-Knott test at 5% probability.

CONCLUSIONS

Strains UFLA 3-153, UFLA 3-154 and UFLA 3-164 are more efficient in nitrogen fixation than the strains approved as inoculants UFLA 3-84 and INPA 3-11B in cowpea in southern Maranhão.

UFLA 3-164 is the most efficient strain for nitrogen fixation in the soil and climatic conditions of southern Maranhão.

ACKNOWLEDGMENTS

The authors thank the Federal Institute of Education, Science and Technology of Maranhão (IFMA), the Federal University of Lavras (UFLA), CNPq and CAPES for the financial support to carry out this work, and for the scholarships awarded to students and researchers.

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