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Combined inoculation of rhizobia on the cowpea development in the soil of Cerrado[1]

Inoculação combinada de rizóbios sobre o desenvolvimento do feijão-caupi em solo de Cerrado

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ABSTRACT - Biological nitrogen fixation (BNF) plays an important role in the cowpea cultivation. This study aimed to assess the foliar levels of chlorophyll and the yield components of cowpea subjected to combined inoculation of rhizobia in Cerrado soil. The experiment was carried out in field conditions, from December of 2014 to the march of 2015, in Rondonópolis, Mato Grosso, using randomized block design with ten treatments and three replicates. Were tested the single strains MT8 and MT15 (both of *R. tropici*), MT16 (*R. leguminosarum*), BR3267 (*Bradyrhizobium japonicum*), the strains combinations MT8+MT15, MT8+MT16, MT15+MT16, and MT8+MT15+MT16, nitrogen fertilization (70 kg ha^{-1} of N-urea), and absolute control (without inoculation of rhizobia and without nitrogen fertilization). Data were subjected to analysis of variance and the averages were compared by orthogonal contrast and F test ($p \leq 0.05$). Were assessed the Falker chlorophyll index (at the 40 and 60 days after sowing), number and dry matter of nodules (at the 40 days after sowing), number of pods per plant, grains yield, the concentration and accumulation of nitrogen in grains, and crude protein. The number of pods per plant was increased 33.6% in the combination MT8+MT15. The same effect was observed for the yield grain, which presented an increase of 8.7%, 13.8%, and 16.7% in the combinations MT8+MT16, MT15+MT16, and MT8+MT15, respectively. The nitrogen accumulation in the grains increased 42.7% with the inoculation of MT15 strain. The cowpea responds positively to the usage of combinations of rhizobia strains.

Key words: Symbiotic N_2 fixation. Inoculant. *Rhizobium tropici*. *Vigna unguiculata*.

RESUMO - A fixação biológica de nitrogênio (FBN) desempenha um importante papel no cultivo do feijão-caupi. Este estudo objetivou avaliar os teores foliares de clorofila e os componentes de produção do feijão-caupi submetido à inoculação combinada de rizóbios em solo de Cerrado. O experimento foi realizado em condições de campo, de dezembro de 2014 a março de 2015, em Rondonópolis, Mato Grosso, utilizando delineamento em blocos casualizados, com dez tratamentos e três repetições. Foram testadas as estirpes MT8 e MT15 (ambas de *Rhizobium tropici*), MT16 (*R. leguminosarum*), BR3267 (*Bradyrhizobium japonicum*), as combinações das estirpes MT8+MT15, MT8+MT16, MT15+MT16 e MT8+MT15+MT16, adubação nitrogenada (70 kg ha^{-1} de N-ureia) e controle absoluto (sem inoculação de rizóbios e sem adubação nitrogenada). Os dados foram submetidos à análise de variância e as médias foram comparadas por contraste ortogonal e teste F ($p \leq 0.05$). Foram avaliados o índice de clorofila Falker (aos 40 e 60 dias após a semeadura), número e massa seca de nódulos (aos 40 dias após a semeadura), número de vagens por planta, rendimento de grãos, concentração e o acúmulo de nitrogênio nos grãos e a proteína bruta. O número de vagens por planta foi incrementado em 33,6% na combinação MT8+MT15. O mesmo efeito foi observado para o rendimento de grãos, que apresentou aumento de 8,7%, 13,8% e de 16,7% nas combinações MT8+MT16, MT15+MT16 e MT8+MT15, respectivamente. O acúmulo de nitrogênio nos grãos foi incrementado em 42,7% com a inoculação da estirpe MT15. O feijão-caupi responde positivamente à inoculação combinada de estirpes de rizóbio.

Palavras-chave: Fixação simbiótica de N_2. Inoculante. *Rhizobium tropici*. *Vigna unguiculata*.

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INTRODUCTION

In Brazil, cowpea is cultivated in an area of 1.3 million ha in North, Northeast, and Central-West regions, with a total production of 482,000 tons yr^{-1} (RUFINI *et al.*, 2014). This crop plays an important role in the feed of the most of the population at those regions, supplying the daily demand of vitamins, minerals, and proteins (FRIGO *et al.*, 2014).

Particularly, the biological nitrogen fixation (BNF) can contribute to the nutrition of cowpea, once this process replaces up to 80 kg ha^{-1} of N, increasing remarkably the cowpea yield in the Brazilian agroecosystems (MELO; ZILLI, 2009; RUFINI *et al.*, 2014).

For cowpea inoculation, Brazilian farmers can use four strains recommended by Ministry of Agriculture, Livestock and Supply belonging to the genus *Bradyrhizobium*. Therefore, the recommended strains are BR 3262 (SEMIA 6464), BR 3267 (SEMIA 6462), INPA3-11B (SEMIA 6463), and UFLA3-84 (SEMIA 6461) (ZILLI *et al.*, 2009). However, cowpea is able to form, promiscuously, symbiosis with diverse rhizobia strains and, thus, limiting the commercial use of inoculants (ZILLI *et al.*, 2006).

Alternatively, a strategy for increasing the efficiency of inoculants recommended to cowpea is the use of combinations of rhizobia strains. Owing to little information existent about this technique for cowpea crop, studies have been necessary for understanding the contribution of combined rhizobia strains in the same formulation. Traditionally, are well-known studies involving the combination of rhizobia strains with mycorrhizal fungi (LIMA *et al.*, 2011; OMIROUA; FASOULAB; IOANNIDES, 2016), or rhizobia strains with plant growth promoting bacteria (PGPB) (ARAÚJO *et al.*, 2010; RODRIGUES *et al.*, 2013a).

According to Trabelsi and Mhamdi (2013), the use of combined rhizobia inoculation (two or more strains) can be beneficial to the establishment of bacteria present in the inoculant at the soil in relation to native rhizobia population, and it also increases the efficiency of BNF, avoiding the technical problems of the single strains inoculation. In addition, nitrogen participates of chlorophyll structure and correlates positively with nitrogen content in the leaves of cowpea (MORAIS; FONTES; GONÇALVES, 2013).

In this context, this study aimed to assess the effect of combined inoculation of rhizobia strains on the foliar chlorophyll levels and the yield components of cowpea grown in Cerrado soil.

MATERIAL AND METHODS

Experimental location and soil characteristics

The experiment was carried out from December 2014 to March 2015, at the experimental site of Federal University of Mato Grosso, Rondonopolis *Campus*, located at latitude 16º27’44” S and longitude 54º34’48” W, 290 m. According to Köppen classification (ALVARES *et al.*, 2013), the local climate is type Aw (humid tropical, with a temperature average of 25.6 °C, rainy summer with average rainfall amongst 1,400 to 1,500 mm yr^{-1}, and dry winter). The climate data during the experimental period are shown in Figure 1.

The chemical and physical properties of soil were determined through sampling at the depth of 0-0.20 m, according to the methodology proposed by Embrapa (2011). The soil was classified as Red Oxisol according to Brazilian system of soil classification (EMBRAPA, 2013). The physicochemical properties of the soil are shown in Table 1.

Figure 1 - Minimum and maximum averages of temperature and average rainfall in Rondonopolis (Mato Grosso State) during the experimental period. Source of data: Nacional Institute of Meteorology (Brazil) - INMET

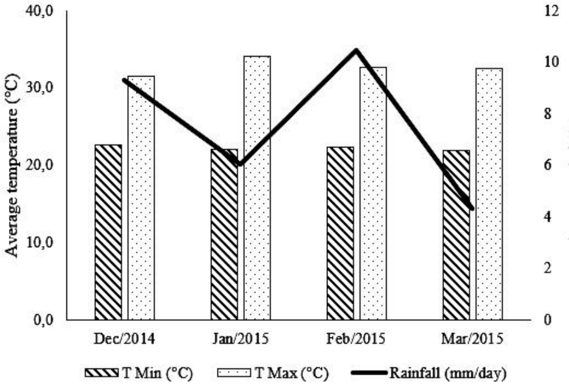

Table 1 - Physicochemical properties from the soil at the depth of 0.0-0.20 m

	Chemical characteristics										Particle size		
Depth	pH	Organic Matter	P	K	Ca	Mg	Al	H+Al	CTC	V	Sand	Silt	Clay
	($CaCl_2$)	----- g kg^{-1} -----	-- mg dm^{-3} --		--------------- cmol$_c$ dm^{-3} -------------					%	--------- g/kg --------		
0-0.20 m	4.5	28.0	3.5	65	1.2	0.9	0.5	4.9	7.2	31.7	350	150	500

Experimental design and treatments

The experimental design used was in randomized block, with ten treatments and three replicates. The treatments consisted of inoculations with three strains isolated from cowpea (MT8 and MT15 of *Rhizobium tropici*, and MT16 of *R. leguminosarum*), a recommended strain for cowpea (BR3267, of *Bradyrhizobium japonicum*), four combinations (MT8+MT15, MT8+MT16, MT15+MT16, and MT8+MT15+MT16), nitrogen fertilization (70 kg N ha^{-1}, using urea as source), and absolute control (without rhizobia inoculation and without N-urea application).

Inoculant preparation

The strains were cultivated using the YMA solid medium (in 1000 mL of distilled water: 10 g of mannitol, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.5 g of yeast extract, 5 mL of 0.5% blue bromothymol in 0.2N KOH, 16 g of agar) with pH 6.8 (FRED; WAKSMAN, 1928). Initially, the strains were multiplied in Petri plates, in a bacteriological incubator (72 h, 28 °C). After this, strains were transferred to Erlenmeyer flasks containing 150 mL of YMA liquid media for obtaining of bacterial broth, which was maintained in benchtop shaker (28 °C and 80 rpm) for 24 h. The peat-based inoculant was prepared in the proportion of 35 g of peat to 10 mL of bacterial broth when strains were in log phase of growth, aiming a minimal concentration of 10^9 viable cells g^{-1} inoculant, which was applied to seed little before sowing (FRIGO *et al*., 2014).

Used cultivar

The BRS Tumucumaque cultivar was used, which was obtained of two lineages crossing (TE96-282-22G and IT87D-611-3). According to Embrapa (2014), this cultivar presents a cycle of 65-75 days, semi-straight branches, purple colored pod, and average grain yield of 1,100 kg ha^{-1}, with white and reniform grains containing about 23.5% of protein, 60.5% of iron, and 51.6% of zinc. Furthermore, it is adapted to cultivation in diverse Brazilian states, such as Alagoas, Amazonas, Maranhão, Mato Grosso, Pará, Pernambuco, Piauí, Rio Grande do Norte, Rondônia, Roraima, and Sergipe.

Soil preparation and fertilization

The site of study was prepared with a light harrow, and the soil acidity was corrected by dolomitic limestone application (80.3% of effective neutralizing power), aiming to elevate the soil bases saturation to 50%. All treatments received phosphorus fertilization at doses of 120 kg P_2O_5 ha^{-1} (simple superphosphate), potassium fertilization at doses of 40 kg K_2O ha^{-1} (potassium chloride), and micronutrients fertilization at doses of 20 kg ha^{-1} of FTE (Fritted Trace Elements), containing (Zn - 15%; Mn - 30%; Cu - 20%; B - 9%; Fe - 3.5%; Mo - 0.1%) according recommendations for crop (FRIGO *et al*., 2014).

The urea was applied in plots of the treatment with nitrogen fertilization in a single dose at 15 days after sowing. The BRS Tumucumaque cultivar was sowed in the plots after inoculation with the density of ten seeds per meter. The experimental plots they consisted of 5 m x 3 m (15 m^2), being spaced 0.5 m inter-row with 1.0 m far from each other, wherein were used 9 m^2 as the useful area to assess the studied parameters.

Studied parameters and determinations

Were evaluated the Falker chlorophyll index at the 40 and 60 DAS (days after sowing), number and dry matter of nodules per plant, the number of pods per plant, grain yield, nitrogen concentration and accumulation in grains, and crude protein.

For measure the Falker chlorophyll index, a portable chlorophyll meter was used, model ClorofiLOG® CFL 1030, in which the readings were performed in five leaves per plant. Two readings were performed, the first one being in flowering period (40 DAS), and the second one being in grain filling period (60 DAS), wherein five plants were sampled for each reading.

The nodule number was estimated through removal and counting of nodules present in roots of ten plants at the 40 DAS. The dry matter of nodules was estimated through the weighing of nodular material in semi-analytical weighing scale, which was previously oven-dried (72 h, 65 °C).

The number of pods per plant was estimated by counting the pods from ten chosen randomly plants. All cowpea plants were harvested to estimate the grain yield, being the grain moisture initially standardized at 13% by oven method at 105 °C, according to Brazilian rules for seed analysis (BRASIL, 2009).

The concentration of nitrogen and crude protein in grains was estimated by Kjeldahl method, according to the methodology described by Frigo *et al.* (2014). The nitrogen accumulation in the grains was calculated by multiplying weight of the sample by N percentage obtained in titration, divided by 1000.

Statistical analysis

In order to investigate the effects of combined inoculation of rhizobia strains on the foliar chlorophyll levels and the yield components of cowpea, the data were subjected to analysis of variance (ANOVA) using the statistical software SISVAR Version 5.3 (FERREIRA, 2011). The average of the treatment was compared by orthogonal contrasts and F test ($p<0.05$). The use of orthogonal contrasts makes possible a detailed analysis of experimental data, being useful to estimate the main and nested effects, as well as interaction effects in comparisons between average groups (NOGUEIRA, 2004).

The contrasts were established as the following: (C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15.

RESULTS AND DISCUSSION

The results of the Falker chlorophyll index at flowering period (40 DAS) of the cowpea plants did not present significant differences in contrasts accomplished among the treatments (Table 2).

Based on the equation ($Y = 17.695 + 0.3362x - 0.0015x^2$, with $R^2 = 0.89$), proposed by Morais, Fontes and Gonçalves (2013), the observed values of nitrogen levels in the leaves of cowpea plants varied from 32.84 g kg^{-1} in the MT16 treatment to 33.50 g kg^{-1} in the BR3267 treatment.

According to Crusciol *et al.* (2007), values between 30-50 g kg^{-1} indicates a satisfactory nutritional status of the plants, and shows the cowpea plants presented an adequate amount of nitrogen at the leaves. This indicates the effectiveness of the rhizobia strains, and reinforces the positive correlation between chlorophyll index (Falker) and the nitrogen content in the plant tissues as an indicator of the nutritional state of plants (MORAIS; FONTES; GONÇALVES, 2013).

In a similar study, Frigo *et al.* (2014) evaluated the productive characteristics of cowpea inoculated with rhizobia lineages in Red Oxisol during the flowering period, and they observed significant difference between nitrogen fertilization treatment (63.21) and BR3267 strain (57.85). According to these authors, the plant response was associated with the availability of nitrogen in the urea form, which allowed the major uptake of this nutrient.

In the present study, the high soil moisture probably contributed to more nitrogen leaching in depth, reducing the availability of this nutrient and its uptake by plants, according to climate data observed in February 2015, once was the period with most rainfall (Figure 1), corroborating the conclusions of Rufini *et al.* 2014, who they observed that nitrogen fertilization can be substituted by BNF, decreasing the nitrogen losses in agroecosystems.

In the second reading (60 DAS), the absolute control presented the highest value of Falker chlorophyll index,

Table 2 - Average values of chlorophyll index assessed at flowering period (40 days after sowing)

Treatments	Average	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	64.48	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	64.68	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	67.14	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	64.06	-1	-1	-1	3	0	0	0	0	0
MT15	67.42	-1	-1	1	0	0	0	-1	-1	-1
MT8	67.19	-1	-1	1	0	0	0	-1	-1	1
MT16	64.53	-1	-1	1	0	0	0	-1	2	0
BR3267	68.00	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	64.51	-1	8	0	0	0	0	0	0	0
Absolute control	66.99	9	0	0	0	0	0	0	0	0
Estimate		1.21	-1.42	1.69	-1.37	2.56	0.19	1.62	-2.77	-0.23
F values		0.38^{ns}	0.31^{ns}	0.07^{ns}	0.36^{ns}	0.12^{ns}	0.91^{ns}	0.29^{ns}	0.09^{ns}	0.89^{ns}
CV (%)					3.39					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15.; ^{ns} - Non-significant contrast by F-test when compared to others treatments (C1, $p \leq 0.05$). In this same reading, the MT15 strain also presented significant difference in relation to MT8 strain (C9, $p \leq 0.05$). In other contrasts, have not were observed significant difference amidst treatments (Table 3).

According to the equation proposed by Morais, Fontes and Gonçalves (2013), the plants of absolute control presented 33.70 g kg^{-1} of nitrogen in their leaves and an increase of 7.1% in the chlorophyll index, whereas the plants of MT15 treatment presented 33.49 g kg^{-1} of nitrogen in their leaves and an increase of 4.65%, both in relation to nitrogen fertilization. This result shows that the measurement with chlorophyll meter is capable of informing the nitrogen levels in the leaves of cowpea plants.

The high chlorophyll index of absolute control plants can be related to the small size of this plants in relation to plants of other treatments, once these plants were not inoculated, neither received nitrogen fertilizer. At the 60 DAS (grain filling period), decreases in the chlorophyll index are expected during grain filling period, once nitrogen is remobilized from leaves to the grains (FRIGO *et al.*, 2014), which, in this study, led to a low nitrogen accumulation in the grains (11.86 kg ha^{-1}) in these plants.

Cowpea is able to form, promiscuously, a symbiosis with soil native rhizobia, besides the population of soil native rhizobia may vary in composition and effectiveness (ZILLI *et al.*, 2006). In a study with cowpea inoculation, Rufini *et al.* (2014) did not observe significant differences in the nitrogen accumulation in the grains, probably due to inefficient BNF by rhizobia strain of soil.

The highest nitrogen accumulation in the grains (65.30 kg ha^{-1}) in MT15 treatment suggest the effectiveness of this strain in the fixation of nitrogen, and and corroborates the observation of Melo and Zilli (2009), where the BNF contributes for an adequate cowpea nutrition in Brazilian agroecosystems, and may replaces the nitrogen fertilization in the cowpea cultivation.

The number of nodules did not present significant differences in contrasts accomplished amongst treatments (Table 4).

Regarding the number of nodules, the results testified that nodulation of cowpea can be affected by the association of this crop with strains of soil native rhizobia. The presence of nodules in the absolute control plants (uninoculated plants) proves this hypothesis, suggesting native rhizobia strains established in soil where this experiment was conducted, being supported by the results found by Zilli *et al.* (2006), who proved the existence of a large genetic diversity of rhizobia capable of nodulate the cowpea in the soil of Brazilian Cerrado.

Table 3 - Average values of chlorophyll index assessed at grain filling period (60 days after sowing)

Treatments	Average	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	64.86	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	65.78	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	65.65	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	65.54	-1	-1	-1	3	0	0	0	0	0
MT15	67.04	-1	-1	1	0	0	0	-1	-1	-1
MT8	62.23	-1	-1	1	0	0	0	-1	-1	1
MT16	62.49	-1	-1	1	0	0	0	-1	2	0
BR3267	67.16	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	64.06	-1	8	0	0	0	0	0	0	0
Absolute control	68.64	9	0	0	0	0	0	0	0	0
Estimate		3.66	-1.03	-0.72	0.10	0.33	0.91	3.24	-2.14	-4.81
F values		0.02*	0.49^{ns}	0.47^{ns}	0.94^{ns}	0.84^{ns}	0.64^{ns}	0.05^{ns}	0.22^{ns}	0.02*
CV (%)		3.7								

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15; ^{ns} and * – Non-significant contrast, and significant at 5% of probability by F-test, respectively

Table 4 - Average number of nodules in cowpea plants subjected to combined inoculation of rhizobia strains in Cerrado soil

Treatments	Average	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	28.53	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	33.52	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	31.43	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	23.43	-1	-1	-1	3	0	0	0	0	0
MT15	20.70	-1	-1	1	0	0	0	-1	-1	-1
MT8	27.73	-1	-1	1	0	0	0	-1	-1	1
MT16	20.76	-1	-1	1	0	0	0	-1	2	0
BR3267	28.23	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	24.63	-1	8	0	0	0	0	0	0	0
Absolute control	30.43	9	0	0	0	0	0	0	0	0
Estimate		38.77	-21.62	-48.75	-77.33	4.00	50.00	51.66	-34.50	70.33
F values		0.44^{ns}	0.67^{ns}	0.16^{ns}	0.17^{ns}	0.94^{ns}	0.46^{ns}	0.35^{ns}	0.55^{ns}	0.30^{ns}
C.V. (%)		30.44								

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15; ^{ns} - Non-significant contrast by F-test

These results corroborates the ones reported Rufini *et al.* (2014), who tested the agronomic efficiency of rhizobia strains approved as inoculants in symbiosis with cowpea in Lavras (Minas Gerais), in a Eutroferric Oxisol, and observed similar nodulation amongst uninoculated plants in absolute control (259 nodules per plant) and plants inoculated with strains UFLA 03-84 (210 nodules per plant), INPA 03-11B (268 nodules per plant) and BR3267 (282 nodules per plant). Similar to nodulation, these authors observed similar dry matter of nodules amongst the inoculated and uninoculated treatments.

Similar result was observed for the dry matter of nodules, in which do not presented significant differences in contrasts accomplished amongst treatments (Table 5).

The absence of significant difference may be related to the diverse size of nodules within the same treatment. The values observed in MT8+MT16 (1.16 g plant^{-1}), MT8 (1.03 g plant^{-1}) and absolute control (1.00 g plant^{-1}) treatments, show that these treatments showed higher nodules in relation to those found in other treatments, as in the absolute control, confirming the existence of a miscellany of rhizobia strain able to form symbiosis with this legume in the soils of Cerrado (ZILLI *et al.*, 2006).

These results also corroborate those found by Rebeschini *et al.* (2014), who assess the effect of inoculation of rhizobia and nitrogen application on cowpea in two traditionally growing area of bean (Cafeara and Florestopolis, both of Parana state, Brazil), in a ultisol, and they did not observe significant difference between inoculated and uninoculated treatments, neither between the areas wherein the experiment was carried out.

The number of pods per plant presented significant difference in the contrast comparing the MT8+MT15 combination in relation to MT8+MT15+MT16, MT8+MT16 and MT15+MT16 combinations (C4, $p \leq 0.001$). In comparison involving single inoculations, MT15 strain presented significant difference in relation to MT8 strain, (C9, $p \leq 0.05$) (Table 6).

The values observed in the present study for the MT8+MT15 combination (33.7% greater than treatment with 70 kg ha^{-1} N-urea) and in the MT15 strain (15% greater than treatment with 70 kg ha^{-1} N-urea) was greatest those reported by Farias *et al.* (2016), who evaluate the agronomic efficiency of two strains approved as inoculants for cowpea, in Maranhão State, and observed significant differences for the number of pods per plant in the control (with 80 kg ha^{-1} mineral N-urea) the value of 4.83 pods per plant, following for strain UFLA03-153 with 4.09 pods per plant.

These observations can be explained based on the interaction legume-rhizobia-environment, which makes possible to obtain further representative responses in the field, and due to the nitrogen supplying during the pre-flowering and grain filling periods, once that are the periods when to occur major assimilation rates of CO_2 by plants, increasing the pod yield (MOREIRA; SIQUEIRA, 2006; OLIVEIRA *et al.*, 2012).

Table 5 - Average values of dry matter of nodules in cowpea plants subjected to rhizobia inoculation in Cerrado soil

Treatments	Average (g plant^{-1})	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	0.93	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	1.16	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	0.96	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	0.66	-1	-1	-1	3	0	0	0	0	0
MT15	0.53	-1	-1	1	0	0	0	-1	-1	-1
MT8	1.03	-1	-1	1	0	0	0	-1	-1	1
MT16	0.66	-1	-1	1	0	0	0	-1	2	0
BR3267	0.76	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	0.63	-1	8	0	0	0	0	0	0	0
Absolute control	1.00	9	0	0	0	0	0	0	0	0
Estimate		0.18	-0.20	-0.18	-0.35	-0.08	0.23	0.02	-0.11	0.50
F values		0.42^{ns}	0.36^{ns}	0.23^{ns}	0.16^{ns}	0.75^{ns}	0.44^{ns}	0.92^{ns}	0.20^{ns}	0.11^{ns}
C.V. (%)					43.82					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) – MT8 vs MT15; ^{ns} - Non-significant contrast by F-test

Table 6 - Average values of number of pods per plant of cowpea plants subjected to rhizobia inoculation in Cerrado soil

Treatments	Average	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	4.13	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	4.93	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	4.23	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	6.23	-1	-1	-1	3	0	0	0	0	0
MT15	5.36	-1	-1	1	0	0	0	-1	-1	-1
MT8	3.80	-1	-1	1	0	0	0	-1	-1	1
MT16	4.53	-1	-1	1	0	0	0	-1	2	0
BR3267	4.53	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	4.66	-1	8	0	0	0	0	0	0	0
Absolute control	4.66	9	0	0	0	0	0	0	0	0
Estimate		-0.04	-0.05	-0.32	1.80	-0.30	0.80	-0.03	-0.05	-1.56
F values		0.92^{ns}	0.92^{ns}	0.37^{ns}	0.00**	0.63^{ns}	0.27^{ns}	0.95^{ns}	0.93^{ns}	0.04*
CV (%)					18.46					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15. ^{ns}, **, and *- Non-significant contrast, significant at 0.1%, and significant at 5% of probability by F-test, respectively

Analysis of the grain yield data showed that all nitrogen sources influenced this parameter when compared to absolute control (C1, $p \leq 0.001$). Of all combinations, the MT8+MT15 presented highest value of grain yield, followed by the combination MT15+MT16 and MT8+MT16, which were highest in relation to MT8+MT15+MT16 combination (C5, $p \leq 0.05$). In other contrasts did not observed significant difference (Table 7).

Table 7 - Average values of grain yield in cowpea plants subjected to rhizobia inoculation in Cerrado soil

Treatments	Average (kg ha^{-1})	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	1354	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	1293	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	663	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	1388	-1	-1	-1	3	0	0	0	0	0
MT15	1295	-1	-1	1	0	0	0	-1	-1	-1
MT8	1005	-1	-1	1	0	0	0	-1	-1	1
MT16	1190	-1	-1	1	0	0	0	-1	2	0
BR3267	1110	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	1189	-1	8	0	0	0	0	0	0	0
Absolute control	284	9	0	0	0	0	0	0	0	0
Estimate		-881.2	26.98	-24.66	284.1	-660.3	-60.79	-53.69	39.98	-290.4
F value		0.00***	0.86^{ns}	0.81^{ns}	0.12^{ns}	0.00***	0.77^{ns}	0.75^{ns}	0.82^{ns}	0.18^{ns}
CV (%)					18.79					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) – MT8 vs MT15.; ^{ns}, **, and *- Non-significant contrast, significant at 0.1%, and significant at 5% of probability by F-test, respectively

The MT8+MT15 combination presented an increase of 16.7% in relation to nitrogen fertilization treatment, whereas the combinations MT15+MT16 and MT8+MT16 stand out regarding combination MT8+MT15+MT16, with increases of 13.8% and 8.7% in relation to nitrogen fertilization treatment, respectively. These results corroborate the positive effect of the use of combined rhizobia strains allows the effective establishment in soil compared to native rhizobia strains (TRABELSI; MHAMDI, 2013).

This results are similar to those found by Farias *et al.* (2016), who evaluated the agronomic efficiency of strains approved as inoculants for cowpea and observed highest grain yields in the inoculated treatment UFLA03-153 (1.153,41 kg ha^{-1}), differing to the control (uninoculated and with 80 kg ha^{-1} mineral N-urea), which presented the highest grain yield of all treatments.

Regarding nitrogen concentration and crude protein in grain, significant difference was observed in C9 contrast ($p \leq 0.05$), in which the MT15 strain was found 50.40 g kg^{-1} of nitrogen concentration in grain and 315 g kg^{-1} of crude protein in grain. For others, contrasts did not observe significant differences between treatments (Table 8).

The increase observed in MT15 strain for both parameters was 30.9% in relation to treatment with nitrogen fertilization, and confirmed that the BNF contributes to obtaining high nitrogen concentration and crude protein in grain.

The values found in crude protein in grain were higher than expected for BRS Tumucumaque (235.3 g kg^{-1}) (EMBRAPA, 2014). The results of this study differ from those found by Ddamulira *et al.* (2015) in other Brazilian cultivars of cowpea, who observed values of 252 g kg^{-1} (BRS Carijó), 258 g kg^{-1} (BRS Tapaihum), and 280 g kg^{-1} (BRS Pujante).

The data of nitrogen accumulation in the grains showed that all nitrogen sources influenced this parameter (C1, $p \leq 0.001$). The MT8+MT15 combination presented significant differences (C4, $p \leq 0.05$) when compared to all others combinations. The MT8+MT16 and MT15+MT16 combinations were significantly higher regarding to triple combination MT8+MT15+MT16 (C5, $p \leq 0.01$). The inoculated plants with MT15 strain presented significant difference in relation to MT8 strain (C9, $p \leq 0.05$). In other contrasts did not was observe significant differences (Table 9).

The MT8+MT15 combination presented an increase of 27.8% in relation to nitrogen fertilization, whereas the MT8+MT16 and MT15+MT16 combinations presented an increase of 23.3% and 14.5% in relation to nitrogen fertilization treatment, respectively. The MT15 strain presented an increase of 42.7% in relation to treatment with nitrogen fertilization. These results show that the BNF process is responsible for the largest accumulation of this nutrient in cowpea plants (RODRIGUES *et al.*, 2013b).

Different results were found by Rufini *et al.* (2014), who evaluate the agronomic efficiency of rhizobia strains approved as inoculant for cowpea in a Rhodic Eutrudox in Lavras (Minas Gerais) and did not observe significant differences among the inoculated and noninoculated treatments. This response shows that inoculation of single rhizobia strains, in some cases, it not influences positively the nitrogen accumulation in the grains of cowpea (FRIGO *et al.*, 2014).

Table 8 - Average values of nitrogen concentration and crude protein in grains of cowpea plants subjected to rhizobia inoculation in Cerrado soil

Nitrogen concentration in grains

Treatments	Average (g kg^{-1})	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	41.30	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	43.40	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	42.70	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	42.00	-1	-1	-1	3	0	0	0	0	0
MT15	50.40	-1	-1	1	0	0	0	-1	-1	-1
MT8	37.80	-1	-1	1	0	0	0	-1	-1	1
MT16	49.00	-1	-1	1	0	0	0	-1	2	0
BR3267	40.60	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	38.50	-1	8	0	0	0	0	0	0	0
Absolute control	43.40	9	0	0	0	0	0	0	0	0
Estimate		0.54	-4.9	2.1	-0.46	0.35	2.1	-5.13	4.9	-12.6
F values		0.88^{ns}	0.22^{ns}	0.42^{ns}	0.91^{ns}	0.93^{ns}	0.68^{ns}	0.24^{ns}	0.29^{ns}	0.03*
CV (%)					11.73					

Crude protein in grais

Treatments	Average (g kg^{-1})	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	258.12	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	271.25	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	266.86	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	271.25	-1	-1	-1	3	0	0	0	0	0
MT15	315.00	-1	-1	1	0	0	0	-1	-1	-1
MT8	231.87	-1	-1	1	0	0	0	-1	-1	1
MT16	306.25	-1	-1	1	0	0	0	-1	2	0
BR3267	253.75	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	240.62	-1	8	0	0	0	0	0	0	0
Absolute control	271.25	9	0	0	0	0	0	0	0	0
Estimate		0.29	-3.11	0.98	0.58	0.21	1.31	-3.06	3.28	-8.31
F values		0.89^{ns}	0.19^{ns}	0.52^{ns}	0.81^{ns}	0.93^{ns}	0.66^{ns}	0.23^{ns}	0.23^{ns}	0.02*
CV (%)					10.98					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15; ^{ns} e *- Non-significant contrast, and significant at 5% of probability by F-test, respectively

Table 9 - Average values of nitrogen accumulation in the grains of cowpea subjected to rhizobia inoculation in Cerrado soil

Treatments	Average (kg ha^{-1})	Contrast and coefficients								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
MT15+MT16	55.82	-1	-1	-1	-1	-1	-1	0	0	0
MT8+MT16	56.42	-1	-1	-1	-1	-1	1	0	0	0
MT8+MT15+MT16	28.45	-1	-1	-1	-1	2	0	0	0	0
MT8+MT15	58.47	-1	-1	-1	3	0	0	0	0	0
MT15	65.30	-1	-1	1	0	0	0	-1	-1	-1
MT8	39.07	-1	-1	1	0	0	0	-1	-1	1
MT16	56.36	-1	-1	1	0	0	0	-1	2	0
BR3267	43.12	-1	-1	1	0	0	0	3	0	0
70 kg N ha^{-1}	45.73	-1	8	0	0	0	0	0	0	0
Absolute control	11.86	9	0	0	0	0	0	0	0	0
Estimate		-37.99	-4.64	1.16	11.57	-27.66	0.59	-10.45	4.18	-26.23
F values		0.000***	0.35^{ns}	0.71^{ns}	0.05*	0.001*	0.92^{ns}	0.07^{ns}	0.46^{ns}	0.002*
CV (%)					13.61					

(C1) - Absolute control vs other treatments; (C2) - Nitrogen fertilization vs strains; (C3) - single strains vs combined strains; (C4) - MT8+MT15 vs MT8+MT15+MT16, MT8+MT16, and MT15+MT16; (C5) - MT8+MT15+MT16 vs MT8+MT16 and MT15+MT16; (C6) - MT8+MT16 vs MT15+MT16; (C7) - BR3267 vs MT8, MT15 and MT16; (C8) - MT8 vs MT15 and MT16; (C9) - MT8 vs MT15; - Non-significant contrast, significant at 0,01%, and significant at 5% of probability by F-test, respectively

CONCLUSION

1. The combined inoculation of rhizobia strains can be used to improve the cowpea cultivation, and present potential for increase the grain yield of this crop;
2. The combinations MT8+MT15, MT8+MT16, and MT15+MT16 were capable to provide for cowpea plants a nitrogen amount equivalent to nitrogen fertilization with 70 kg N ha^{-1} urea.

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