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Coinoculation of diazotrophic bacteria in upland rice, cultivar BRS Esmeralda

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ABSTRACT
The objective of this study was to evaluate the efficiency of inoculation of associative bacteria and the coinoculation of rhizobia in upland rice. The experiment was conducted under greenhouse conditions. The experiment was conducted in a completely randomized design with seven treatments: commercial inoculant (CI), co-inoculation with four rhizobia strains: MT16, MT23, MT08, MT15 and two controls (nitrogen - 50 mg dm-3 nitrogen in the form of urea, and absolute without nitrogen and without inoculation), with 5 replicates, totaling 35 experimental units. The cultivar of rice used was BRS Esmeralda. The variables analyzed were: Falker chlorophyll index, number of tillers, plant height, dry shoot mass, grain mass, root volume, root dry mass and total dry mass. The SISVAR statistical program was used for data analysis. Statistical differences were observed between treatments. The results showed that the co-inoculated rice plants presented beneficial results similar to the nitrogen control for the variables plant height, Falker chlorophyll index, root dry mass, root volume and number of tillers. In addition, all treatments presented superior performance when compared to the absolute control. Therefore, the inoculation of associative diazotrophic bacteria coinoculated with rhizobia showed a beneficial effect on the development of rice plants.

Keywords: Rhizobium, Nitrogen, *Oryza sativa* L.

1 INTRODUCTION

Rice (*Oryza* sativa L.) is a staple food for about billions of people and is of paramount economic importance to many developing countries. Consumed and cultivated in all continents, it stands out for its production and cultivation area, playing an important role in social and economic levels (EMBRAPA, 2023). Considering the perspective that by 2050 there will be 9 billion people on the planet, and that rice is an important food for the human population, it is essential to increase production, minimizing costs and improving procedures of less ecological damage (GODFRAY et al., 2012).

In the rice crop, nitrogen is the nutrient absorbed in the greatest amount in its cycle. Because of this, high doses of nitrogen are usually used to increase production. However, one of the consequences of this high application in rice crops is the risk of losses due to volatilization raising the cost of crop production (KNOBLAUCH, 2011).

To partially meet the nitrogen demand in the cultivation of irrigated rice and reduce the volume of mineral fertilizer applied, an efficient method may be biological nitrogen fixation (BNF), which consists of inoculation with diazotrophic bacteria that have as a characteristic the ability to fix atmospheric nitrogen in the soil, making it available to plants (BIANCHET et al., 2013; HUNGARY, 2011).

The motivation for the search for microorganisms capable of fixing nitrogen directly from the atmosphere has been expanding due to the fact that the high cost of production linked to the great loss of nitrogen to the environment and the risks in its application, thus reducing the pollution of water, soils and the cost of production (LADHA et al., 2003).

Diazotrophic bacteria can contribute to plant development through the synthesis of phytohormones, such as auxins, ethylene, gibberellins and cytokinins (ALI et al., 2009; MENHAZ; LAZAROVITS, 2006). Studies conducted by Guimarães et al. (2003) showed the possibility of using endophytic diazotrophic bacteria as a biological input, improving the availability of nutrients, promoting growth and improving the efficiency of nitrogen application in rice crops.

According to Ladha et al. (2003) several studies have shown that certain flooded rice genotypes can obtain a certain part of the nitrogen necessary for their development through the BNF process.

Therefore, due to the great economic importance of this crop, the growing interest in the study of the quantification of BNF has been increasing, seeking to partially replace the industrialized nitrogen fertilizer, allowing a reduction in the cost of production and encouraging the development of cultivars in the state of Mato Grosso.

Thus, the objective of this study was to evaluate the efficiency of associative bacteria inoculation and rhizobia coinoculation in upland rice.

2 MATERIAL AND METHODS

The experiment was conducted in a greenhouse at the Federal University of Rondonópolis – MT, at the geographical coordinates: 54°34'45" West longitude, 16°27'48" South latitude and altitude of 284 m. The climate of the region is classified as Aw (tropical climate with dry season in winter) according to the Köppen and Geiger model.

Initially, soil was collected, making a superficial cleaning of the area where the soil was collected at a depth of 0-20 cm. The soil type used was the dystrophic Red Latosol. It was sieved in a sieve with 2 mm mesh and allocated in plastic bags, after collection a soil sampling was made to be taken in the laboratory to be done the analysis of the same.

After physicochemical analyses, soil liming was performed to adjust the pH, and then the soil was submitted to phosphate, potassium and micronutrient fertilization. Subsequently, it was allocated in 35 pots with a capacity of 4 dm^3 of soil. Soil chemical analysis as shown in Table 1.

Table 1- Chemical analysis of the soil at the depth of 0.00-0.20 m.

ph	P	K+	Ca2+	Mg2+	H	To the	In	MO	CTC	SB (S)
CaCl2	... mg dm^{-3}...		.... cmolc $.dm^{-3}$............				%	g dm^{-3}	.... cmolc dm^{-3}....	
4,0	1,4	23	0,4	0,2	5,4	0,8	9,7	27,1	6,8	0,7

P = available phosphorus; Exchangeable K+, Ca2+ and Mg2+; H and Al = potential acidity; CTC = cation exchange capacity at pH 7.0; V = base saturation; OM = organic matter; SB = sum of bases.

The experiment was conducted keeping the soil at 80% of the maximum water retention capacity. The moisture (or vessel) retention capacity was determined according to the methodology described by Bonfim-Silva et al. (2011). In the laboratory, vessels with perforations at their base and of identical volumes to the experiment were used, with three replications. They were filled with air-dried fine earth (TFSA), weighed and later placed in a basin containing two-thirds of water, allowing capillary saturation. After twenty-four hours the vessels were removed, drained and weighed again, by means of the difference in weights the vessel capacity in moisture retention was determined.

Phosphate and potassium fertilization was performed before sowing in all treatments, except for the absolute control, at doses of 200 mg dm-3 of P2O5, 80 mg dm-3 of K2O. For the nitrogen control, urea was used as a nitrogen source in the recommendation of 50 mg dm-3.

Micronutrient fertilization (FTE) was performed at a dose of 30 mg dm^3 in each experimental unit except for the absolute control.

The experimental design was completely randomized, with seven treatments and five replications, totaling 35 experimental units. The treatments were composed of commercial inoculant - IC (*Azospirillum brasilense*, containing the strains AbV5 and AbV6), four strains of rhizobia: MT16, MT 23, (*Rhizobium leguminosarum), MT08, MT 15 (*R. tropici*), and combinations (IC+MT16, IC+MT23, IC+MT08, IC+MT15*). The other treatments were absolute control and nitrogen control (50 mg dm-3 in the form of urea).

10 rice seeds were sown per pot of the cultivar BRS Esmeralda, and then thinning was carried out, leaving four plants per experimental unit.

The choice of the cultivar BRS Esmeralda was due to the high quality and productive potential of grains, which are long and fine, with good appearance. It is moderately resistant to the main diseases, has greater tolerance to water stress and low risk of lodging (EMBRAPA, 2018).

The bacteria used in the experiment were multiplied in YMA culture medium (Hungary, 1994), and an aliquot of 5 mL containing 109 CFU $^{mL-1}$ was applied in the experimental units, close to the root system of the plants. This process refers to rhizobia strains (MT08, MT15, MT16 and MT23). The inoculation with A. brasilense because it is a commercial product, followed the manufacturer's recommendation.

Data collection was performed in the vegetative stages, flowering and grain filling, and in these periods the variables analyzed were plant height, number of tillers and Falker chlorophyll index. After 119 days of sowing, shoot dry mass, root dry mass and total dry mass, root volume and grain mass were evaluated.

The data were submitted to analysis of variance and later the Tukey test was used at 5% probability to compare the means, with the aid of the statistical software SISVAR (FERREIRA, 2011).

3 RESULTS AND DISCUSSION

For the variable plant height, a statistical difference was observed between the treatments. Coinoculation showed similar results to nitrogen control and commercial inoculant in all evaluations performed. The treatments that contained the combination of the commercial inoculant with TM 08 and MT 23 presented means up to 20% higher than the absolute control (Table 02).

Table 2- Height of rice plants, cultivar BRS Esmeralda, coinoculated with diazotrophic bacteria.

Treatments	30 DAS	40 DAS	55 DAS	70 DAS
MT08+Commercial inoculant	20,17 a	21,97 a	22,67 a	37,42 a
MT16+Commercial inoculant	20,40 a	22,40 a	22,97 a	33,68 a
MT15+Commercial inoculant	19,75 a	21,77 a	23,00 a	30,55 a
MT23+Commercial inoculant	20,40 a	21,70 a	22,75 a	35,00 a
Commercial inoculant	20,27 a	22,12 a	23,55 a	36,55 a
Nitrogen control	22,25 a	23,07 a	23,08 a	29,12 a
Absolute witness	4,70 b	6,35 b	7,72 b	10,75 b
CV (%)	7,16	5,63	8,35	16,38

Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05.

For this study, the coinoculated treatments remained similar to the treatments corresponding to the commercial inoculant alone and the nitrogen control, differing from the results presented by Silva et al. (2006), where the coinoculated treatments in cowpea presented lower averages in relation to the treatments without coinoculation, suggesting a higher energy expenditure for biological nitrogen fixation and nodule production, and consequently, reduction in plant growth.

These data corroborate with the study by Viana (2012), in which the inoculation with *Herbaspirillum seropedicae* in rice plants, cultivar BRS Tropical, showed an increase of 3.5% in the height of these plants.

Mazzuchelli et al. (2014) worked with the coinoculation of *Bacillus subtilis* and *A. brasilense* in the corn crop, noting greater development in relation to control, thus corroborating the importance of the role of diazotrophic bacteria in promoting the growth of plants, especially those that produce grains.

Regarding the Falker chlorophyll index, there was a significant difference between treatments. In the first evaluation, carried out 30 days after sowing (DAS), for the coinoculated plants and corresponding to the MT16+Commercial Inoculant treatment, a mean similar to the nitrogen control was observed, being higher than the absolute control. In the second reading performed at 40 DAS, the nitrogen treatment stood out from the others, however the difference between the absolute control and the others was smaller when compared to the previous reading. The nitrogen control in the third reading, 55 days after sowing, continued to present the highest average. In the last reading, there was no statistical difference between the treatments (Table 03).

Table 3- Falker chlorophyll index of rice plants inoculated with associative bacteria and coinoculated with rhizobium.

Treatments	**30 DAS**	**40 DAS**	**55 DAS**	**70 DAS**
T 01	43,04 b	30,09 from	28,47 from	25,96 a
T 02	45,95 from	31,78 from	27,97 from	26,42 a
T 03	44,62 b	31,00 from	28,06 from	27,39 a
T 04	42,48 b	32,34 from	27,00 from	26,96 a
IC	44,67 b	28,70 from	25,76 b	23,27 a
TN	49,85 a	46,22 a	33,37 a	29,50 a
TA	19,36 c	23,05 b	27,57 from	29,65 a
CV (%)	6,01	28,51	12,02	13,1

Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05. T 01 *(MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum* + Commercial Inoculant), T03 (MT15 *Rhizobium tropici* + Commercial Inoculant), T 04 (MT23 *R. legumisorarum* + *Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).*

Regarding the absolute control, all coinoculated treatments presented higher means in the first two readings. In the subsequent ones, the circumstance that there is no statistical difference between them may be related to the time factor. At the last reading the control treatment was not in the same vegetative stage as the others due to its later initial development. According to Stroschein et al. (2011), evaluating the characterization and influence of rhizobia isolated from alfalfa on the germination and initial development of rice seedlings, they found that all rice seeds inoculated with rhizobia showed an initial increase in the percentage of twinning compared to the control treatment, in addition to increasing the twinning speed index of the seeds.

The treatment containing the MT16 strain (T 02) presented in the first reading an average higher than 55% compared to the absolute control. According to Baldani (1996) studies of inoculation in rice, showed promising results for the inoculation of plants with strains of *Burkholderia* sp. (11 to 20%), and *H. seropedicae* (increases of 17-19% of nitrogen derived from BNF), in a vessel experiment.

Basi (2013) in a study on the association of nitrogen and A. brasilense in cover in the *corn crop did not find integeration of the nitrogen fertilization of cover with the inoculation, but it was found, by the average of the results, increases of 0.96 and 1.05% with the presence of* A. brasilense in the seeds and in the sowing furrow and in the total chlorophyll content. As well as in this study where some averages of the treatments coinoculated in the first readings showed an increase of up to 60% in the chlorophyll index compared to the control treatment.

Regarding the dry mass of the shoot, there was a significant difference between the treatments. The nitrogen control was the treatment that promoted the highest average. The other treatments did not present statistical difference among themselves, and all coinoculated treatments were superior in relation to the absolute control. Thus, it can be noted that the bacteria positively affected the development of the aerial part of the plants (Table 04).

The variable dry mass of roots also showed significant difference between treatments compared to the control. The other coinoculated treatments remained at the same level of the nitrogen control, showing a beneficial action of the bacteria in the root development of the plants (Table 04).

Table 4 – Dry mass of the shoot and dry mass of roots of rice plants inoculated with associative bacteria and coinoculated with rhizobium.

Treatment	**Shoot dry mass**	**Dry mass of roots**
T 01	23,92 b	19,76 a
T 02	24,28 b	18,56 a
T 03	24,36 b	18,26 a
T 04	23,84 b	18,84 a
TN	33,66 a	23,32 a
IC	21,68 b	16,82 a
TA	05,00 c	03,12 b
CV (%)	12,35	29,52

Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05. T 01 *(MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum* + Commercial Inoculant), T03 (MT15 *Rhizobium tropici* + Commercial Inoculant), T 04 (MT23 *R. legumisorarum* + *Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).*

According to Macedo et al. (2015), coinoculation with *A. brasilense* and *R. tropici* had a significant effect on the production of shoot and root dry matter in bean plants.

The data obtained are in agreement with those found by Bulegon et al. (2014), where evaluating the height of soybean plants for the cultivar TURBO coinoculated with *Bradyrhizobium and*

Azospirillum, *showed that the hormonal activity of these organisms is positive for the greater development of the aerial and root part, providing a larger diameter and greater dry mass of the shoot.*

The results of the results found in this study differ from the studies developed by Bárbaro et al. (2009), which verified that the coinoculation of *A. brasilense and* Bradyrhizobium *did not influence the development of shoot dry mass, root and nodulation in soybean plants.*

When determining the total dry mass, it was observed that there was a significant difference between the treatments. The nitrogen control presented the highest mean. The other coinoculated treatments. As well as the treatment with the commercial inoculant, they had similar averages, but all stood out in relation to the absolute control, which obtained means below the other treatments (Table 05).

Table 5 - Total dry mass of rice plants inoculated with associative bacteria and coinoculated with rhizobium.

Treatment	**Total dry mass (g)**
T 01	43,68 b
T 02	42,84 b
T 03	42,62 b
T 04	42,78 b
TN	56,98 a
IC	38,50 b
TA	8,12 c
CV (%)	16,37

Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05. T 01 *(MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum* + Commercial Inoculant), T03 (MT15 *Rhizobium tropici* + Commercial Inoculant), T 04 (MT23 *R. legumisorarum* + *Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).*

The coinoculated treatments presented averages up to 5 times higher than the control treatment, however the nitrogen control presented the highest mean among the treatments, corroborating the study done by Bianchet et al. (2013), where they evaluated the effect of simple and mixed inoculation with diazotrophic bacteria in irrigated rice cultivar under different nitrogen levels. They concluded that plants that received treatments with isolates of diazotrophic bacteria produced 18% less dry mass of the area and 26% of the root phytomass of rice plants that received nitrogen fertilization.

Guimarães et al. (2013) evaluating the production of rice inoculated with diazotrophic bacteria labeled with induced resistance to the antibiotic streptomycin, showed that in the rice cultivar IR42, inoculated with the strain ZAE94 of *Hesbaspirillum seropediacae* There was a 50% increase in the dry mass of plants.

For the variable root volume, a statistically significant difference was observed between the treatments. The treatments containing the commercial inoculant coinoculated with the bacteria MT 08 (T 01), MT 16 (T 02) and MT 23 (T 04) presented a volume up to 4 times higher when compared to the absolute control, thus indicating as in the variable of dry mass of the root, efficiency of the treatments in the root development of the plants (Table 06).

Table 5- Root volume of rice plants inoculated with associative bacteria and coinoculated with rhizobium.

Treatment	**Root volume (cm3)**
T 01	116,40 a
T 02	115,60 a
T 03	96,20 a
T 04	121,20 a
TN	133,40 a
IC	115,00 a
TA	25,60 b
CV (%)	21,66

Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05. T 01 *(MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum* + Commercial Inoculant), T03 (MT15 *Rhizobium tropici* + Commercial Inoculant), T 04 (MT23 *R. legumisorarum* + *Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).*

These data corroborate those presented by Bianchet et al. (2015) evaluating the vegetative development of irrigated rice affected by inoculation with *Azospirillum* and application of mineral nitrogen, showed that the area and root volume were affected by the interaction between cultivar and inoculation. The plots of the rice cultivar SCS 115 CL when they were inoculated with the isolates UDESC AI 27 and UDESC AI 32 of the genus *Azospirllum*, presented higher root volume than the non-inoculated treatment.

These results differ from those reported by Bianchet et al. (2013) showed that with the inoculation of diazotrophic bacteria in irrigated rice cultivation, where the inoculated treatments presented volume and the root area smaller in relation to the non-inoculated treatment.

In the evaluations made in relation to the number of tillers, there was a significant difference between the treatments. In the first evaluation, performed at 30 days after sowing, the treatment containing the bacterium MT 08 (T 01) presented the highest mean. In the second evaluation made at the end of the reproductive stage, the nitrogen control provided a higher mean than the others, and in both the absolute control showed lower performance. The increase in the number of tillers was up to 80% in relation to the treatment without inoculation (Table 07).

Table 6 - Number of tillers of rice plants inoculated with associative bacteria and coinoculated with rhizobia

Treatments	1st Review	2nd Review
T 01	4,60 a	5.6 from
T 02	4,00 from	5,75 from
T 03	3,95 from	5,2 b
T 04	3,9 b	4,95 b
TN	4,45 from	6,4 a
IC	3,95 from	5,4 b
TA	1,00 c	1,00 c
CV (%)	9,12	9,41

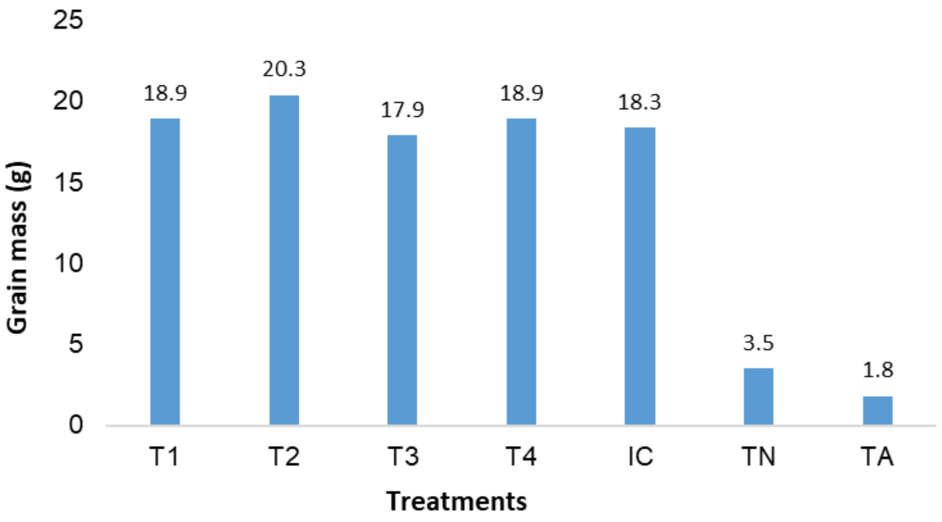
Values followed by the same letter in the column indicate that the treatments do not differ statistically from each other by the Tukey test at the significance level of 0.05. T 01 *(MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum* + Commercial Inoculant), T03 (MT15 *Rhizobium tropici* + Commercial Inoculant), T 04 (MT23 *R. legumisorarum* + *Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).*

The results differ from those found by Mendes et al. (2011), working with wheat cultivar found that there were no significant differences between the treatments with reduction of nitrogen fertilization and inoculation of *A. brasilense* for the number of tillers, results that do not corroborate with this study.

Sala et al. (2008) obtained similar results to this study evaluating in wheat the interaction of nitrogen fertilization and diazotrophic bacteria, where they observed a greater contribution of inoculation in the vegetative period to plant tillering.

Regarding seed weight, the coinoculated treatments presented the highest averages, standing out in relation to the absolute control and nitrogen. (Figure 05).

Figure 1 – Mass of grains of rice plants inoculated with associative bacteria and coinoculated with rhizobium.



T 01 (MT08 Rhizobium tropici + Commercial Inoculant), T 02 (MT16 R. leguminosarum + *Commercial Inoculant), T03 (MT15* Rhizobium tropici + *Commercial Inoculant), T 04 (MT23* R. legumisorarum + Commercial Inoculant), IC (Commercial Inoculant), TN (Nitrogen Control), TA (Absolute Control).

The results presented corroborate studies conducted by Guimarães et al. (2010) evaluating diazotrophic bacteria and nitrogen fertilization in rice cultivars. The authors found that for the cultivar IR42, there were increases in grain production in relation to the absolute control in all plants inoculated with *H. seropedicae* and *Burkholderia* sp. followed by nitrogen fertilization corresponding to 50 kg N ha^{-1}.

The coinoculated treatments showed a grain mass superior to the nitrogen control and the control, verifying the results of Garcia (2016), evaluating predecessor cultures and inoculation of *A. brasiliense* in upland rice and winter beans in succession inoculated with *R. tropici,* It was observed that there was a significant effect of inoculation, where the inoculated treatment obtained an increase of 19% in the yield of rice grains in relation to the treatment without inoculation of *A. brasilense*.

Lima et al. (2011) observed that inoculation of corn seeds with *Bacillus subtilis* improved plant development and increased plant grain yield.

Mazzuchelli et al. (2014), concluded that corn yield was higher in the treatment with inoculation of *A. brasilense* in the seeds, presenting a productivity of about 12 bags per hectare higher than the control treatment.

Chaves et al. (2016), when working with the Abv 5 and Abv6 strains of *A. brasiliense* to evaluate the efficiency of inoculation in rice crop, reported a greater increase in rice grain yield due to inoculation.

4 CONCLUSION

The inoculation of associative diazotrophic bacteria coinoculated with rhizobia strains showed a beneficial effect, promoting rice growth and development in all variables analyzed.

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