Co-inoculation with *Azospirillum brasilense* promotes growth in forage legumes[1]

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ABSTRACT

Studies have shown the synergism of co-inoculation with symbiotic and non-symbiotic N2-fixing bacteria, efficiently contribute to plant development. However, for crops such as forage legumes, data on the use of co-inoculation are still incipient, requiring studies to identify the contribution of this technique for application in pasture areas, thus ensuring greater sustainability. The objective of this study was to evaluate the effects of co-inoculation of *Azospirillum brasilense* (Ab-V5) and native rhizobia isolated from two forage legumes, *Crotalaria spectabilis* and *Lupinus albus*. Two experiments were installed in pots with 8dm³. Both were conducted in a randomized block design (DBC) with four replications and an 8 x 2 factorial scheme with six bacterial strains and two control treatments without inoculation (with presence or absence of mineral nitrogen), co-inoculated or not with the Ab-V5 strain. Plants were cultivated until the time of flowering when shoot (SDM) and root dry matter (RDM), number (NN) and nodule dry matter (NDM), and relative symbiotic efficiency (RSE) were evaluated. Isolates 05-21 and 06-03, for *C. spectabilis* and *L. albus*, respectively, indicated higher potential to promote plant growth when co-inoculated with Ab-V5, showing potential to act as substitutes for nitrogenous chemical fertilizers, reducing costs and increasing the sustainability of production.

Keywords: agriculture; degradation; sustainability.

INTRODUCTION

The increase in the world population, associated with a higher demand for food, has raised concerns about the sustainability of the production chains. In Brazil, pastures are characterized by extensive grazing, and it is commonly responsible for soil degradation and productivity losses over the years. The use of chemical fertilizers is a direct technique to recover these areas (Carvalho *et al.*, 2017). However, it is considered unsustainable from an economic and environmental point of view.

With increasingly demanding consumers, there is the necessity of new alternatives to reduce and optimize the use of nitrogen fertilizers (Aguirre *et al.*, 2020). These alternatives can promote improvements in soil characteristics and increases production. In this context, the use of N2-fixing bacteria stands out, which can significantly contribute with nitrogen (N) to the plant metabolic demand and the enrichment of this element to the soil (Moreira & Siqueira, 2006). Recently, studies have shown the feasibility of using co-inoculation of N_2-fixing bacterial strains (Hungria *et al.*, 2015; Galindo *et al.,* 2018; Wang *et al.,* 2019).

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According to Hungria *et al.* (2015), co-inoculation can improve crop performance, respecting sustainability demands in economic, social, and environmental terms. Inoculation with *Azospirillum* may represent a key component for degraded pasture recovery programs, providing a higher accumulation of nitrogen in the biomass, in addition to helping with carbon sequestration (Hungria *et al.*, 2016).

Aguirre *et al.* (2020) highlight the use of the associative bacterium *Azospirillum brasilense*, which, in addition to its ability to fix nitrogen, also produces phytohormones responsible for root growth, increasing the density, length, and volume of roots (Moreira *et al.*, 2010). Considering that, in Brazil, there are about 175 million hectares of pasture (Lapig, 2017), the impact of any increase in the efficiency of N is considered significant, both in terms of production and recovery of these areas (Aguirre *et al.*, 2020).

Co-inoculation with Azospirillum, by promoting root growth, consequently, increases the infection sites for rhizobia strains (Cassán *et al.*, 2009), providing better productivity in legumes such as soybean (Glycine max) and common beans (Phaseolus vulgaris L.) (Hungria *et al.*, 2013). Galindo *et al.* (2018) also found a positive effect of co-inoculation with A. brasilense in two soybean cultivars, positively influencing not only yield but also crop profitability. Most studies concerning co-inoculation are related to soybean crops, especially because they are of economic interested to agribusiness.

However, the plant growth promoting bacteria (PGPR) which increases the efficiency in one legume may noy necessarily show the same response with different species (Korir *et al.,* 2017). Therefore, these unstable responses to co-inoculation emphasize the need of identifying appropriate combinations of rhizobia strain and PGPR.

This research aimed to evaluate the effects of co-inoculation of *Azospirillum brasilense* (Ab-V5) and native rhizobia isolated from two forage legumes, *Crotalaria spectabilis* and *Lupinus albus*.

MATERIAL AND METHODS

The rhizobia strains used in the experiment were isolated by Terra (2018) from the legumes *Crotalaria spectabilis* and *Lupinus albus*, classified as efficient, intermediate, and inefficient in the previous authentication and cross inoculation experiments. Table 1 shows the origin of the strains, classification in terms of efficiency, and morphological characteristics in medium 79 (Fred & Waksman, 1928).

The rhizobia strains were cultivated in liquid medium 79 for three days until the log phase of growth, with approximately 10^8 cells mL^{-1}. The *A. brasilense* strain, used in the co-inoculation, was cultivated in FAM liquid medium (Magalhães & Döbereiner, 1984) for three days, enough time to reach the log growth phase.

Two different experiments were installed in a greenhouse, the first for *C. spectabilis* isolates and the second for *L. albus* (Table 1). The experiments were in a randomized block design (DBC), with four replications, in a factorial scheme (8x2), with six treatments with the rhizobia mentioned in table 1, and two control treatments, one with mineral nitrogen (70 mg. L^{-1} N-NH_4 NO_3) and another without nitrogen and inoculation, co-inoculated or not with the Ab-V5 strain.

The experiments were installed in pots containing 8 dm^3 of sterilized soil, whose chemical characterization (Embrapa, 2017) presented the following results: pH (H_2O) = 5.2; P = 10 mg dm^{-3}; K^+ = 119 mg dm^{-3}; Ca^{2+} = 1.5 cmol dm^{-3}; Mg^{2+} = 0.9 cmol dm^{-3}; Al^{3+} = 0.2 cmol dm^{-3}; sum of bases (SB) = 2.6 cmolc dm^{-3}; Cation Exchange Capacity (CEC) = 6.0 cmolc dm-3; base saturation (V%) = 40.0; aluminum saturation (m%) = 10 and organic matter (M.O) = 22 g kg^{-1}.

Liming was done with dolomitic limestone according to the method of raising the base saturation to 60%. The soil was moistened and incubated in plastic bags to increase the limestone reaction speed. After 30 days, macronutrients (phosphorus and potassium) were added following the recommendation by Novais *et al.* (1991).

Ten days after fertilization, four seeds were sown per pot, and after germination, only two plants were left per pot. The inoculated treatments applied one mL of bacterial suspension per seed, and the co-inoculated one, there was also one mL of *A. brasilense* bacterial suspension. The treatment with mineral nitrogen had two applications, 10 and 25 days after plant emergence, of 35 mg.L^{-1} N-NH_4NO_3, totaling 70 mg.L^{-1} N-NH_4NO_3 during cultivation.

After the flowering period, the following parameters were evaluated: shoot dry matter (SDM) and root dry matter (RDM), number (NN) and nodule dry matter (NDM), and relative symbiotic efficiency (RSE).

An adaptation of the formula by Bergersen *et al.* (1971) was used to calculate the relative symbiotic efficiency (RSE%). In this research, the SDM value of the treatment containing mineral N and inoculated with *A. brasilense* (Ab-V5) was adopted, divided by the others, and multiplied by 100, as indicated below.

$$RSE\% = \frac{SDM\ N\ mineral\ with\ Ab-V5}{SDM\ treataments} * 100 \quad (1)$$

Table 1: Identification, origin, efficiency data, and morphological characteristics in medium 79 of *Crotalaria spectabilis* and *Lupinus albus* isolates used in the co-inoculation experiment with *Azospirillum brasilense*

Identification of strains	Origin	Efficiency	Morphological characterization		
			pH[1]	Colony color[2]	EPS[3]
Isolated strains of *C. spectabilis*					
05-01	Alfenas	Efficient	Ac	y	++++
05-03	Alfenas	Inefficient	Al	Cr	+++
05-16	Alfenas	Intermediary	Al	y	+
05-21	Alfenas	Efficient	N	Cr	++
05-23	Alfenas	Intermediary	Ac	y	+++
05-25	Alfenas	Inefficient	Ac	Cr	++++
Isolated strains of *L. albus*					
06-01	Três Pontas	Inefficient	Ac	y	+++
06-03	Alfenas	Efficient	Ac	y	++++
06-09	Três Pontas	Intermediary	N	Cr	+++
06-17	Alfenas	Inefficient	N	Cr	++
06-21	Três Pontas	Intermediary	N	y	+++
06-27	Alfenas	Efficient	N	y	++

[1]Reaction of pH in culture medium after the growth of the strain, evaluated by the color change of the indicator (N – Neutral; Ac – Acid; Al – Alkali).

Data on SDM, RDM, NN, NDM, and RSE were submitted to analysis of variance using the statistical analysis program Sisvar, version 5.3. Treatment means were compared by the Scott-Knott test at 5% probability.

RESULTS AND DISCUSSION

For both legume species the co-inoculation had positive effect (Tables 2 and 3). For *C. spectabilis*, the 05-21 strain (Table 2) stands out, which when co-inoculated, presented superior results in all parameters, including when compared to the control treatment with mineral N. Regarding SDM and RDM there was an increase of 16% and 36%, respectively, when the strain 05-21 was co-inoculated with *A. brasilense* in comparison with the N control treatment.

This strain, associated with *A. brasilense*, showed a symbiotic efficiency (RSE) of 116,41%. Without co-inoculation the same strain had a RSE of 75,71%, indicating a positive response to co-inoculation and showing potential for replacement of mineral N, attending the nutritional needs of the plant, and promoting its growth.

L. albus also had a positive interaction, with higher values in the presence of co-inoculation (Table 3). The strain 06-03 had better results, being inferior only to the treatment with mineral N, in the parameters of RDM and SDM. Regarding SDM and RDM the strain when co-inoculated with *A. brasilense* had results that represent 91,24% and 78,41% of the values found in N mineral treatment.

The strain still obtained an RSE of 92,39% when compared to the control treatment with mineral N, highlighting its potential to supply nitrogen to the plants as well as their development.

Cassán *et al.* (2009) verified that *A. brasilense* produces root growth regulator compounds, such as indoleacetic acid (IAA), justifying better responses in co-inoculated treatments, both for *L. albus* and *C. spectabilis*. The results of SDM and RDM with co-inoculation also show that the beneficial effect of the association of isolated rhizobia with *Azospirillum brasilense* is mainly due to the bacteria's capacity to produce growth hormone (Bárbaro *et al.*, 2008).

According to Ferlini (2006), the co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* in soybean increases production. The same was observed in this study for *L. albus* and *C. spectabilis*. There were some treatments where co-inoculation resulted in higher values of MSPA and MSR, highlighting their potential not only in fixing nitrogen but also in stimulating plant growth and production.

Table 2: Shoot dry matter (SDM) and root dry matter (RDM), number (NN) and nodule dry matter (NDM), and relative efficiency (RSE) values of *Crotalaria spectabilis* cultivated with different treatments co-inoculated or not with *Azospirillum brasilense*, and in the presence or absence of N mineral

TREAT	SDM		RDM		NN		NDM		RSE	
	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc
Presence	2,17Ab	2,44 Ba	0,92 Bb	1,31 Ba	0,00 Da	0,00 Da	0,00 Da	0,00 Fa	88,92 Ab	100 Ba
Absence	1,08 Ca	1,24 Ea	0,67 Cb	0,95 Ca	26,00 Ab	41,00 Aa	0,03 Ba	0,03 Da	44,48 Ca	50,93 Ea
05-01	2,05 Aa	2,22 Ca	0,44 Db	1,32 Ba	28,00 Aa	26,00 Ba	0,03 Bb	0,08 Aa	84,29 Aa	91,18 Ca
05-03	0,31 Da	0,43 Fa	0,19 Ea	0,27 Ea	6,00 Ca	8,00 Ca	0,02 Ca	0,02 Ea	12,62 Da	17,68 Fa
05-16	2,07 Aa	1,76 Db	0,52 Db	0,76 Da	19,00 Bb	32,00 Ba	0,03 Bb	0,05 Ca	85,22 Aa	72,65 Db
05-21	1,84 Bb	2,84 Aa	1,32 Ab	1,79 Aa	35,00 Ab	55,00 Aa	0,05 Ab	0,06 Ba	75,71 Bb	116,41 Aa
05-23	1,01 Ca	1,19 Ea	0,62 Cb	0,82 Da	18,00 Ba	24,00 Ba	0,03 Ba	0,02 Ea	45,12 Ca	49,05 Ea
05-25	1,24 Ca	1,20 Ea	0,22 Ea	0.24 Ea	10,00 Cb	18,00 Ba	0,03 Bb	0,04 Da	50,75 Ca	49,29 Ea

TREAT: treataments. Means followed by the same letter, uppercase in the column and lowercase in the row, do not differ statistically by the Scott-Knott test (5%).

Corroborating these results, Molla *et al.* (2001), in a laboratory experiment to evaluate the potential to improve root growth and nodulation in soybean co-inoculated with *Azospirillum* and *Bradyrhizobium*, observed that *Azospirillum* has the potential to stimulate root growth significantly.

These results are relevant, because root growth is an important parameter as it contributes to water and nutrient absorption by plants and enhances soil structure reducing compaction problems commonly found in degraded pasture areas. Also, the higher production of shoot dry matter contributes to biomass production and consequently improves nutrients availability in these regions. For NN and NDM (Table 2 and 3), in both legumes the co-inoculated treatments were superior to those in which the rhizobia acted in isolation. Cassán *et al.* (2009) support these results, reporting that the number of nodules and the percentage of nodulated plants was higher in soybean plants co-inoculated with *B. japonicum* and *A. brasilense*, attributing these results to the excretion of metabolic products by *A. brasilense*.

This was also observed by Molla *et al.* (2001), where *Azospirillum* improved nodule initiation and development in soybean plants by co-inoculation with *Bradyrhizobium*. Thus, there is an increase in nodulation and a higher root growth in response to the positive interaction between bacteria (Ferlini, 2006).

Table 3: Table 3: Shoot dry matter (SDM) and root dry matter (RDM), number (NN) and nodule dry matter (NDM), and relative efficiency (RSE) values of *Lupinus albus* cultivated with different treatments co-inoculated or not with *Azospirillum brasilense*, and in the presence or absence of N mineral

TREAT	SDM		RDM		NN		NDM		RSE	
	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc	Absence Coinoc	Presence Coinoc
Presence	2,13Ab	2,74 Aa	1,30 Aa	1,39 Aa	0,00 Da	0,00 Ga	0,00 Ea	0,00 Da	78,31 Ab	100 Aa
Absence	0,26 Ea	0,39 Fa	0,74 Ca	0,85 Ca	29,00 Ca	37,00 Fa	0,16 Da	0,23 Ca	9,56 Ea	14,27 Ea
06-01	0,87 Da	0,88 Ea	0,83 Ca	0,85 Ca	25,00 Cb	36,00 Fa	0,25 Ca	0,33 Ba	32,14 Da	32,68 Da
06-21	0,73 Da	0,86 Ea	0,42 Ea	0,48 Ea	63,00 Ba	52,00 Ea	0,39 Ba	0,38 Ba	26,78 Da	31,64 Da
06-09	1,27 Ca	1,36 Da	0,56 Da	0,67 Da	64,00 Ba	71,00 Da	0,37 Ba	0,39 Ba	47,30 Ca	50,03 Ca
06-03	2,31 Aa	2,50 Ba	1,07 Ba	1,09 Ba	81,00 Aa	88,00 Ca	0,56 Aa	0,62 Aa	85,29 Aa	92,39 Aa
06-17	1,33Cb	1,66 Ca	0,71 Ca	0,83 Ca	96,00 Aa	107,00 Ba	0,44 Ba	0,45 Ba	48,88 Cb	61,69 Ba
06-27	1,57 Bb	1,89 Ca	0,67 Cb	0,81 Ca	96,00 Ab	128,00 Aa	0,51 Ab	0,64 Aa	57,79 Bb	69,71 Ba

TREAT: treataments. Means followed by the same letter, uppercase in the column and lowercase in the row, do not differ statistically by the Scott-Knott test (5%).

In general, the results found in this study corroborate several other studies that have been carried out in Brazil, especially with soybeans. The practice of co-inoculation can be considered a key component of degraded pasture recovery programs, representing a new technique to improve crop productivity, and contributing to sustainable practices in agricultural systems.

CONCLUSION

For the co-inoculation analysis, treatments 06-03 and 05-21, for *L. albus* and *C. spectabilis*, respectively, presented higher values of SDM, RDM, and RSE. Thus, indicating a better potential for nitrogen supply to the plant, as well as promoting plant growth when co-inoculated with *Azospirillum brasilense*.

Plant and root growth, in association with improvements in the physicochemical and biological characteristics of the soil, as a consequence of co-inoculation with plant growth-promoting bacteria, contribute to the recovery of degraded areas and constitute an alternative to more sustainable agricultural practices.

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REFERENCES

Aguirre PF, Giacomini SJ, Olivo CJ, Bratz VF, Quatrin MP & Schaefer FL (2020) Biological nitrogen fixation and urea-N recovery in ‘Coastcross-1’ pasture treated with *Azospirillum brasilense*. Pesquisa Agropecuária Brasileira, 55:01-10.

Bárbaro IM, Brancalião SR, Ticelli M, Miguel FB & Silva JAA (2008) Técnica alternativa: coinoculação com *Azospirillum* e *Bradyrhizobium* visando incremento de produtividade da cultura da soja no Norte do Estado de São Paulo. Available at: <http://www.infobibos.com/Artigos/2008_4/coinoculacao/index.htm>. Accessed on: July 20th, 2021.

Bergersen FJ, Brockwell J, Gibson AH & Schwinghamer EA (1971) Studies of natural populations and mutants of *Rhizobium* in the improvement of legume inoculants. Plant and Soil, 35:03-16.

Carvalho WTV, Minghin DC, Gonçalves LC, Villanova DFQ, Maurício RM & Pereira RVG (2017) Pastagens degradadas e técnicas de recuperação: revisão. Pubvet, 11:947-1073.

Cassán F, Perring D, Sgroy V, Masciarelli O, Penna C & Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). European Journal of Soil Biology, 45:28-35.

Ferlini HA (2006) Co-Inoculación en Soja (*Glicyne max*) con *Bradyrhizobium* japonicum y *Azospirillum brasilense*. Available at: <https://www.engormix.com/agricultura/articulos/co-inoculacion-en-soja-t26446.htm>. Accessed on: January 11th, 2021.

Embrapa - Empresa Brasileira de Pesquisa Agropecuária (2017) Manual de **método**s de análises de solos. 3ª ed. Brasília, Embrapa. 577p.

Fred EB & Waksman SA (1928) Laboratory Manual of General Microbiology. New York, McGraw-Hill. 145p.

Galindo SF, Teixeira Filho MCM, Buzetti S, Ludkiewicz MGZ, Rosa PAL & Tritapepe CA (2018) Technical and economic viability of co-inoculation with *Azospirillum brasilense* in soybean cultivar in the Cerrado. Revista Brasileira de Engenharia Agrícola e Ambiental, 22:51-56.

Hungria M, Nogueira MA & Araújo RS (2016) Inoculation of *Brachiaria* spp. with the plant growth-promoting bacterium *Azospirillum brasilense*: An environment-friendly component in the reclamation of degraded pastures in the tropics. Agriculture, Ecosystems & Environment, 221:125-131.

Hungria M, Nogueira MA & Araújo RS (2015) Soybean Seed Co-Inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: A New Biotechnological Tool to Improve Yield and Sustainability. American Journal of Plant Sciences, 6:811-817.

Hungria M, Nogueira MA & Araújo RS (2013) Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. Biology and Fertility of Soils, 49:791-801.

Korir H, Mungai NW, Thuita M, Hamba Y & Masso C (2017) Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. Frontiers in Plant Science, 8:01-10.

Lapig - Laboratório de Processamento de Imagens e Geoprocessamento (2017) Programa pastagem. Available at: <http://maps.lapig.iesa.ufg.br/lapig.html>. Accessed on: May 10th, 2021.

Magalhães FMM & Döbereiner J (1984) Occurrence of Azospirillum amazonense in some Amazonian (Brazil) ecosystems. Revista de Microbiologia, 15:246-252.

Molla AH, Shamsuddin ZH, Halimi MS, Morziah M & Puteh AB (2001) Potential for enhancement of root growth and nodulation of soybean co-inoculated with *Azospirillum* and I in laboratory systems. Soil Biology & Biochemistry, 33:457-463.

Moreira FMS, Silva K, Abrahão RS & Carvalho F (2010) Bactérias diazotróficas associativas: diversidade, ecologia e potencial de aplicações. Comunicata Scientiae, 1:74-79.

Moreira FMS & Siqueira JO (2006) Microbiologia e bioquímica do solo. 2ª ed. Lavras, Editora UFLA. 744p.

Novais RF, Neves JCL & Barros NF (1991) Ensaio em ambiente controlado. In: Oliveira AJEA (Ed.) Métodos de Pesquisa em Fertilidade do Solo. Brasília, Embrapa-SEA. p.189-253.

Terra ABC (2018) Diversidade de rizóbios em áreas de pastagens de *Brachiaria decumbes:* eficácia e sinergismo da coinoculação com *Azospirillum brasilense* em leguminosas. Master Dissertation. Universidade José do Rosário Vellano, Alfenas. 51p.

Wang Z, Chen Z, Xu Z & Fu X (2019) Effects of Phosphate-Solubilizing Bacteria and N2-fixing Bacteria on Nutrient Uptake, Plant Growth, and Bioactive Compound Accumulation in Cyclocarya paliurus (Batal.) Iljinskaja. Forests, 10:772-786.