EVALUATION OF WATER STRESS AND CO-INOCULATION OF *AZOSPIRILLUM BRASILENSE* AND *RHIZOBIUM TROPICI* IN BEANS (*PHASEOLUS VULGARIS L.*)

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ABSTRACT: Climate change has caused major changes in abiotic factors, with water stress as the greatest threat to agricultural production. The measures aimed at alleviating the problems caused by this limiting production factor have occurred through the adoption of sustainable strategies, especially microbial biotechnology, which uses the interactions between the microorganism and the plant, ensuring productive quality and inducing plant resistance to stresses biotic and abiotic. The objective of the present work was to evaluate the biological nitrogen fixation and the development of bean seedlings, with co-inoculation of two types of inoculants, which were subjected to water stress by different pot capacities. The experiment was conducted in a greenhouse, at Universidade Paranaense - UNIPAR, from April to June 2019. The experimental design was completely randomized (DIC), with 5 replications, 16 treatments and 80 experimental units. The cultivar used was SCS Riqueza. The parameters evaluated were pot capacity (25%, 50%, 75% and 90%); small, large and total nodules, shoot and root length, dry and fresh weight, total carbon and nitrogen. The evaluation of the morphological parameters of the bean seedlings indicated that the co-inoculation technique promoted beneficial effects for the dry mass parameters of shoot, nodule and root. The analysis of the percentage of carbon and nitrogen in the tissues of the seedlings provided an increase in the concentration of these elements in treatments that involved co-inoculation (Azospirillum brasilensis and Rhizobium tropici) with pot capacities of 25 and 75% (CV), demonstrating that the association of microorganisms is beneficial in the limiting water situation.

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KEYWORDS: Pot capacity; Biological nitrogen fixation; Co-inoculation, Water stress.

AVALIAÇÃO DO ESTRATÉGIO DE ÁGUA E CO-INOCULAÇÃO DE *AZOSPIRILLUM BRASILENSE* E *RHIZOBIUM TROPICI* IN BEANS (*PHASEOLUS VULGARIS L.)*

RESUMO: A mudança climática tem causado grandes mudanças nos fatores abióticos, sendo o estresse hídrico a maior ameaça à produção agrícola. As medidas destinadas a aliviar os problemas causados por este fator limitante de produção ocorreram através da adoção de estratégias sustentáveis, especialmente a biotecnologia microbiana, que utiliza as interações entre o microorganismo e a planta, garantindo a qualidade produtiva e induzindo a resistência da planta ao estresse biótico e abiótico. O objetivo do presente trabalho foi avaliar a fixação biológica de nitrogênio e o desenvolvimento de mudas de feijão, com co-inoculação de dois tipos de inoculantes, que foram submetidos ao estresse hídrico por diferentes capacidades de vaso. A experiência foi realizada em uma estufa, na Universidade Paranaense - UNIPAR, de abril a junho de 2019. O projeto experimental foi completamente randomizado (DIC), com 5 réplicas, 16 tratamentos e 80 unidades experimentais. A cultivar utilizada foi a SCS Riqueza. Os parâmetros avaliados foram a capacidade do vaso (25%, 50%, 75% e 90%); nódulos pequenos, grandes e totais, comprimento do rebento e da raiz, peso seco e fresco, carbono total e nitrogênio. A avaliação dos parâmetros morfológicos das mudas de feijão indicou que a técnica de co-inoculação promoveu efeitos benéficos para os parâmetros de massa seca do turião, nódulo e raiz. A análise da porcentagem de carbono e nitrogênio nos tecidos das mudas proporcionou um aumento na concentração destes elementos nos tratamentos que envolveram a co-inoculação (Azospirillum brasilensis e Rhizobium tropici) com capacidades de vaso de 25 e 75% (CV), demonstrando que a associação de microorganismos é benéfica na situação limite da água.
PALAVRAS-CHAVE: Capacidade da panela; Fixação biológica de nitrogênio; Co-inoculação; Estresse hídrico.

EVALUACIÓN DEL ESTRÉS HÍDRICO Y LA COINOCULACIÓN DE *AZOSPIRILLUM BRASILENSE* y *RHIZOBIUM TROPICI* EN FRIJOLES (*PHASEOLUS VULGARIS L.)*

RESUMEN: El cambio climático ha provocado importantes cambios en los factores abióticos, siendo el estrés hídrico la mayor amenaza para la producción agrícola. Las medidas encaminadas a paliar los problemas causados por este factor limitante de la producción se han producido mediante la adopción de estrategias sostenibles, especialmente la biotecnología microbiana, que utiliza las interacciones entre el microorganismo y la planta, asegurando la calidad productiva e induciendo la resistencia de la planta a los estreses bióticos y abióticos. El objetivo del presente trabajo fue evaluar la fijación biológica de nitrógeno y el desarrollo de plántulas de frijol, con la co-inoculación de dos tipos de inoculantes, que fueron sometidos a estrés hídrico por diferentes capacidades de maceta. El experimento se realizó en un invernadero, en la Universidade Paranaense - UNIPAR, de abril a junio de 2019. El diseño experimental fue completamente al azar (DIC), con 5 repeticiones, 16 tratamientos y 80 unidades experimentales. El cultivar utilizado fue SCS Riqueza. Los parámetros evaluados fueron capacidad de maceta (25%, 50%, 75% y 90%); nódulos pequeños, grandes y totales, longitud de brotes y raíces, peso seco y fresco, carbono y nitrógeno total. La evaluación de los parámetros morfológicos de las plántulas de frijol indicó que la técnica de coinoculación promovió efectos beneficiosos para los parámetros de masa seca de brotes, nódulos y raíces. El análisis del porcentaje de carbono y nitrógeno en los tejidos de las plántulas proporcionó un aumento en la concentración de estos elementos en los tratamientos que involucraron la coinoculación (Azospirillum brasilensis y Rhizobium tropici) con capacidades de maceta de 25 y 75% (CV), demostrando que la asociación de microorganismos es beneficiosa en la situación de agua limitante.
PALABRAS CLAVE: Capacidad de la maceta; Fijación biológica de nitrógeno; Coinoculación; Estrés hídrico.

1. INTRODUCTION

Beans (*Phaseolus vulgaris* L.) contribute to Brazilian food security, as they are part of the population's basic diet. Therefore, it has great economic importance. Furthermore, it is a food that has a high protein value and is rich in vitamins, minerals, iron and carbohydrates (Foster-Powell, 2002). The United States Department of Agriculture (USDA) recognizes the importance and quality of beans as a staple food and in 2010 included this legume as an alternative source to meat in the North American diet. (USDA Dietary Guidelines, 2010).

In Brazil, the cultivation of this legume is done by small, medium and large farmers and the annual production was 3.1 million tons in the 2020/2021 harvest (CONAB 2020), being the third largest producer in the world (FAOSTAT, 2017). For Brazil to maintain the surplus of this legume, the increase in productivity is a fundamental point and for that, the nutritional balance is essential. One of the most important nutrients for this crop is nitrogen and the main sources of this nutrient are nitrogen fertilization and inoculation from diazotrophic bacteria. The use of nitrogen fertilization, besides being expensive, can generate an unnecessary environmental problem (Cantarella, 2007). The use of inoculation by diazotrophic bacteria has been a practice that gains prominence every year, as it is efficient, ecologically correct and a cheap practice (Ferreira et al. 2020). However, only the use of nitrogen fertilization is not an exclusive factor for increasing productivity in common bean plants. it can be affected by biotic and abiotic stressors, which often tend to influence, hinder and reduce plant growth and development. According to the report by the Intergovernmental Panel on Climate Change (IPCC), the global climate scenario tends to undergo intense change, with strong evidence of climate change through increases in temperatures and variations in rainfall regimes (IPCC, 2007).

The demand for water in beans varies throughout the production cycle (Lima et al., 2006), with minimal amounts at germination to maximum amounts in the flowering and pod formation phase, which may decrease from the beginning of maturation (Bastos et al., 2008). In beans, water deficit reduces stomatal conductance and increases diffuse resistance to water vapor, by closing the stomata, reducing transpiration and, consequently, the supply of CO2 for photosynthesis (Oliveira et al., 2005), leading to significant changes in root and shoot growth and thickness (Gray and Brady 2016), in addition to altering enzymatic activities related to nitrogen (N) and carbon (C) metabolism (Mantonvani et al. 2015). All these factors can minimize the bean yield, causing great harm to the producer (Custódio et al., 2009).

Sustainable strategies have been studied and applied as tools that help the production system to minimize both biotic and abiotic stressful effects, promoting increased productivity (CARD et al., 2016; TANG, 2017; BILAL et al., 2018). Among the most used technologies to minimize the effects of water stress, we can mention microbial biotechnology. Microbial biotechnology is based on the natural relationships that exist between microorganisms and plants. The techniques used have been widely studied, as it is a natural, sustainable and economic strategy in the development of production technologies for the industrial and agribusiness sector (BIANCHI, GERMINO e SILVA, 2016; FUKAMI et al., 2016; ULLAH et al., 2019).

Plant growth-promoting rhizobacteria (PGPR) are beneficial because they associate with plants from root exudates, however, in most cases, without invading plant tissues. Many of these species are known to improve nutritional intake (including nitrogen intake) in crops such as wheat, maize, ferns, sugarcane and rice (Mus et al. 2016). In addition, these microorganisms can be considered anti-stress agents, as they aid in the production of important hormones such as indole acetic acid (IAA).

Rhizobia comprise several genera of nitrogen-fixing bacteria (NFB), which induce the formation of nodules in the roots of leguminous plants. When the symbiotic association is established, the rhizobia begin to reduce N_2 to NH_3, supplying nitrogen to the plant and, in exchange, receiving photosynthate. NFB is a renewable source of N and can complement and even replace chemical fertilizers (Garg and Geetanjali, 2007). The symbiosis between legumes and rhizobia accounts for about 20-25% of the N rate into the ecosystem cycle (GRUBER e GALLOWAY, 2008), that is, the application of this technique in the production system tends to reduce the effects caused by the inappropriate use of chemical fertilizers in the global nitrogen cycle, global warming and surface water contamination.

The symbiotic association between bean culture with rhizobia and biological nitrogen fixation is widely known (Soares et al., 2006; Almeida et al., 2010; Costa et al., 2011). However, nodulation and NFB are influenced by several edaphoclimatic factors, among which the availability of water and nutrients stands out (Gualter et al., 2008; Bonilla; Bolanõs, 2009; Silva et al., 2010). NFB is a complex process that is affected by abiotic stress, especially when there is water deficit and high temperatures (Hungria & Vargas, 2000). Among the rhizobia species, the most recommended for use in the bean crop is the species Rhizobium tropici (Peralta et al. 2016). This species is considered stable and more stress tolerant than most rhizobia species available to farmers. It has the ability to withstand high temperatures and more acidic environments, being more adapted to Brazilian soils, both tropical and subtropical (Graham, 1992). Inoculation techniques are consolidated in the agricultural production sector, however other strategies have been developed, such as co-inoculation or mixed inoculation, which has the ability to enhance the effects of the adopted microorganisms on productivity (Htwe et al. 2019). In bean crops, co-inoculation of Rhizobium and Azospirillum can increase the amount of fixed N and grain yield (Peres et al. 2018).

Thus, the aim of this study was to evaluate the growth and development of bean seedlings, in addition to the carbon and nitrogen content, subjected to inoculation and co-inoculation, applying the microorganisms *Azospirillum brasilensis* and *Rhizobium tropici* under the effect of different pot capacities.

2. MATERIAL AND METHODS

The experiment was conducted in a greenhouse, at Universidade Paranaense - UNIPAR, city of Umuarama , Brazil, from April to June 2019.

The soil used was collected in the city of Tapejara-PR, with 50% soil and 50% medium texture sand in the composition. The soil was mixed and autoclaved in a vertical autoclave for 2 hours at 121ºC. Subsequently, the soil was packed in plastic pots with a capacity of 3 kg.

The experimental design was completely randomized (DIC), with 5 replications, 16 treatments and 80 experimental units. The composition of the treatments adopted in the experiment was in a factorial scheme considering the first factor the pot capacity (P.C) (25, 50, 75 and 90%); and the second factor Inoculation (absent, individual inoculation and co-cultivation of *Azospirillum brasilensis* and *Rhizobium tropici*) as shown in table 1.

Table 1- Treatments performed in the experiment, reporting field capacity and type of inoculant used

Treatment	Pot Capacity	Inoculant
1° Control	25%	None
2° Control	50%	None
3° Control	75%	None
4° Control	90%	None
5° Inoculated	25%	*Rhizobium tropici*
6° Inoculated	50%	*Rhizobium tropici*
7° Inoculated	75%	*Rhizobium tropici*
8° Inoculated	90%	*Rhizobium tropici*
9° Inoculated	25%	*Azospirillum brasilensis*
10° Inoculated	50%	*Azospirillum brasilensis*
11° Inoculated	75%	*Azospirillum brasilensis*
12° Co-cultivation	90%	*Azospirillum brasilensis*
13° Co-cultivation	25%	*Rhizobium + Azospirillum*
14° Co-cultivation	50%	*Rhizobium + Azospirillum*
15° Co-cultivation	75%	*Rhizobium + Azospirillum*
16° Co-cultivation	90%	*Rhizobium + Azospirillum*

The strains applied as inoculants were *Azospirillum brasilense* (strains AbV5 and AbV6) and *Rhizobium tropici* (SEMIA 4077 and SEMIA 4078), and were applied to seeds just before planting, using peat inoculant. The cultivar used was SCS Riqueza. Five seeds were planted per pot, and after germination, manual thinning was done, leaving only one plant per pot.

Water availability occurred daily from planting to stage V2. After this period, which was approximately 10 days after germination, water stress started with different pot capacities, as shown in table 1, that is, measuring the field capacity of a pot weighing 3 kg, we obtained a weight 300 g of water, totaling 3,300 kg with 100% capacity. Thus, it was possible to obtain the other capacities with their respective percentages: The vessels with their P.C respectively, were irrigated daily, up to the corresponding weight: 90% P.C: 3,270 kg; 75% P.C: 3.225 kg; 50% P.C: 3,150 kg; 25% P.C: 3.075 kg.

During the stress period, a nutrient solution by Hoagland and Arnon (1950) was used, with no nitrogen. When the plants reached stage R5, that is, the pre-flowering period, the plant material was collected.

The collected material was divided into roots, stems and leaves, obtaining fresh mass of both, with the aid of an analytical balance, (Root Fresh Weight (RFW), Fresh weight of aerial part (FWS)); number and size of nodules in the roots (total nodule (TN), large nodule (LN), small nodule (SN)); and Length of root and aerial part, in cm, (Aerial part length (APL) and Root Length (RL)). After obtaining dry mass, the material was ground in a knife mill to obtain carbon and nitrogen content.

2.1 Carbon and nitrogen analysis

Analysis of carbon and nitrogen was obtained through digestion. Approximately 200 mg of ground dry matter was used in a test tube, to which 700 mg of the digester mixture and 5 ml of sulfuric acid were added. After this procedure, the test tube with the material was placed in the digester block until it reached 450 °C for a period of approximately 4 hours until the sample reached a light color. After digestion and after the samples cooled to room temperature, 10 ml of distilled water was added.

The samples were then taken to the nitrogen distiller. The tubes were placed in the rack, the metering cup valve was opened. Then 15N Sodium Hydroxide was added in the measuring cup, until the sample turned dark. After this process, the measuring cup was closed and the beaker containing 10 ml of indicator was placed in the collection support and the distiller was turned on, leaving the material until it reached a volume of 40 to 50 ml inside the Becker. After distillation, the sample with 3 drops of the indicator was titrated with 0.05 N HCL.

Data were subjected to analysis of variance using the ASSISTAT program (2017) and means were compared using the Scott Knott test, with 5% probability.

3. RESULTS

The evaluation of morphological parameters were analyzed separately, as well as the interaction of factors that make up each treatment. The results obtained for the factors in isolation against water stress and inoculation are shown in Table 2.

Table 2- Statistical results of treatments performed under different pot capacities and different inoculations with nitrogen-fixing bacteria

	RFW	APL	RL	LN	SN	FWAP	TN
Control 25	0.6605 a	31.9781 a	21.2344 a	1.0513 a	1.8255 a	1.2509 a	1.9646 a
Control 50	0.6318 a	26.1347 b	21.4677 a	1.1169 a	1.7168 a	1.1997 a	1.8685 a
Control 75	0.7849 a	28.7712 ab	25.1074 a	1.1081 a	2.0157 a	1.2282 a	2.0883 a
Control 90	0.6774 a	31.3390 ab	24.8609 a	1.1275 a	2.1551 a	1.3113 a	2.2882 a
				Inoculation			
Control	0.7150 a	26.6132 b	22.6107 a	1.0268 b	1.8053 a	1.1209 b	1.8372 a
Azospirillum	0.6811 a	27.7422 b	22.9077 a	1.0930 ab	1.7837 a	1.2281 ab	1.9569 a

Rizobium	0.6412 a	30.3467 ab	22.2673 a	1.1146 ab	1.9959 a	1.2540 ab	2.1291 a
Co-inoculation	0.7174 a	33.5209 a	24.8847 a	1.1694 a	2.1283 a	1.3870 a	2.2864 a
CV%	34,72%	22,52%	25,53%	12%	27,72%	20,47%	26,46%

RFW – Root Fresh Weight; APL - Aerial part length; RL - Root Length; LN - large nodule; SN - small nodule; FWS - Fresh weight of Aerial part; TN - total nodule; CV– Coefficient of variation.

When evaluating the pot capacity, it was noted that all treatments had a positive increase in shoot growth, except for the treatment with 50% P.C. This treatment showed greater susceptibility to attack by diseases such as whitefly and tripes. We attribute this to the high intensity of these insects due to the presence of other cultures in the greenhouse where the experiment was carried out.

In terms of bean inoculation, the variables that stood out were: Aerial part length, large nodules and root fresh weight. Although there is no statistical difference for these variables when comparing co-inoculation or individual inoculation, it is noted that co-inoculation increased Aerial part length by approximately 10%, when compared to inoculation with *Rhizobium tropici* (recommended species for the bean crop), which, being converted into cultivated area, tends to increase productivity.

Nodule size was influenced by inoculation and co-inoculation with variation between 6.44% (*Azopirillum brasilense*); 8.55 % (*Rhizobium tropici*) and 13.88 % with co-inoculation. If these nodules are effective, there is a possibility of promoting biological nitrogen fixation.

Root fresh weight was also influenced by inoculation and co-inoculation. It was influenced so much that there was an expressive variation of 9.56% (*Azopirillum brasilense*); 11.87% (*Rhizobium tropici*) and 23.73% with co-inoculation, when compared to the control.

3.1 Carbon analysis

Statistical analyzes revealed that for carbon percentage, (Table 3), within each factor, there was no statistical difference. However, when the interaction of the factors was evaluated, it can be observed that there was a statistical difference between the water stress factors and types of inoculation techniques. The means of treatments with Azospirilum at 25 and 50% of pot capacity had a statistical lower percentage of carbon than all other variables evaluated. For NFB co-inoculation, the 90% pot capacity treatment had a higher percentage of carbon when compared to the other pot capacity treatments. For the control and *Rizobium*, only the 90% pot capacity had a lower value compared to the other treatments.

Table 3- Result of Average Analysis of Carbon concentration (%) in the interaction between P.C and microbial biotechnology techniques evaluated in stage R5.

Treatment	Carbon concentration (%)			
	25%	50%	75%	90%
Control	11.3619 aA*	11.1552 abA	11.6341 aA	11.5925 aA
Azospirillum	11.9512 aA	10.3138 bC	10.6681 bBC	11.3795 aAB
Rhizobium	11.0323 aA	11.3743 aA	11.5785 abA	11.3889 aA
Co-inoculation	11.3940 aA	11.6379 aA	11.6174 aA	11.5981 aA
CV (%)	3.68			

*p < 0,01

3.2 Nitrogen analysis

The nitrogen analysis showed that in most treatments where there was inoculation of microorganisms, either *A. brasilense, R.tropici* or both in co-inoculation, there was an increase in the percentage of nitrogen, regardless of the pot capacities. The most insignificant results were those obtained without inoculation, that is, the control treatment (Table 4).

Table 4 – Result of Average analysis of Nitrogen concentration (%) in the interaction between P.C and microbial biotechnology techniques evaluated in stage R5.

Treatment	Nitrogen Concentration (%)			
	25%	50%	75%	90%
Control	0.3480 cA	0.3563 bA	0.3607 cA	0.3582 bA
Azospirillum	0.3688 bAB	0.3604 abB	0.3818 abA	0.3699 abAB
Rhizobium	0.3814 abA	0.3705 abA	0.3672 bcA	0.3778 aA
Co-inoculação	0.4005 aA	0.3792 aB	0.3996 aA	0.3757 abB
CV (%)	2.35			

*p < 0,01

4. DISCUSSION

The use of bacteria of the genus *Rhizobium* and *Azospirillum*, with the co-inoculation technique, considerably increase nodulation, root growth, fresh weight of roots and length of aerial part, which provided greater absorption of water and nutrients, which stimulates effective gain in carbon and nitrogen, which would consequently stimulate greater grain yield. Bacteria can promote plant growth and are considered anti-stress agents (BIANCHI, GERMINO e SILVA, 2016. Therefore, there is substantial interest in the use of this microbial biotechnology, which uses the interactions between microorganism and plant, aiming at productivity and resistance to biotic and abiotic stresses (CARD et al., 2016; TANG, 2017; BILAL et al., 2018).

The effect of water stress can be minimized with the application of microbial biotechnology techniques, especially through co-inoculation between species of *Bradyrhizobium* and *Azospirillum*. These were applied to Soybean (*Glycine max*) in the vegetative phase, by spray, and even with the low rainfall in the flowering and fructification stage, it allowed for greater grain productivity (TONIATO et al., 2020). This increased production is a consequence of synergisms between free-living bacteria and nitrogen-fixing bacteria that have the ability to carry out the NFB process. NFB in natural cycle tends to aggregate in the nitrogen cycle about 20 - 25% in the system (MONTEIRO et al., 2019).

A. brasilense has a positive secondary effect on this synergism due to the production of AIA, maintaining the plant's metabolic activity, allowing the growth of secondary roots, and reducing the negative effect of the production of the stress indicator hormone in plants: Abscisic acid (ABA) (FERLINI, 2006). Understanding the signaling processes of plants in response to water deficit is very complex, as there is no single route considered universal. Thus, knowledge about the mechanisms of reaction of plants to water stress is important to create strategies to aid drought resistance, which can be efficient in maintaining plant development (MARTINS et al., 2018).

Studies show that co-inoculation can produce a multiple effect, with superior productivity results when compared to inoculation techniques performed alone (Hungria et al. 2013, Carvalho et al. 2020; Pastor-Bueis et al. 2021). In bean crop, it has been shown that co-inoculation of Rhizobium and Azospirillum can increase the amount of aerial part dry mass, nodule dry mass, root dry mass, fixed N and grain yield (CARDOSO e FERREIRA, 2021).

In the present study, it can be seen that co-inoculated plants with a pot capacity of 25% obtained, in most variables, the same evaluations as plants with a pot capacity of 75%, showing that there was a positive effect despite the water stress. The interaction between pot capacity factors and microbial biotechnology techniques demonstrate the potential application of this tool as an important step for plant development, especially in the accumulation of N and C for the benefit of the morphological parameters of plants, which can be converted in productivity. Plants, and especially legumes, have a high demand for N. This nutrient is the basic element for photosynthesis and plant respiration, being a constituent of amino acids, a chlorophyll molecule and numerous enzymes that are associated with vital processes for growth and plant development (MARSCHNER, 1995; MALAVOLTA, 2006). This study shows the importance of microbial inoculation technology to aid in the supply of nitrogen and consequently increase the productivity of legumes, even in situations of abiotic stress.

5. CONCLUSION

The evaluation of the morphological parameters of growth and development of bean seedlings indicated that the microbial biotechnology technique of co-inoculation promoted beneficial effects for the parameters: aerial part dry mass, nodule dry mass and root dry mass.

The analysis of the percentage of carbon and nitrogen in the tissues of the seedlings showed an increase in the concentration of these elements, with emphasis on treatments involving the co-inoculation of the microorganisms *Azospirillum brasilensis* and *Rhizobium tropici* with a pot capacity of 25 and 75%, demonstrating that the association of these microorganisms is beneficial in the limiting water situation.

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