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Co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* in cowpea under salt stress[1]

Coinoculação com *Bradyrhizobium* ssp. e *Azospirillum brasilense* em feijão caupi sob estresse salino

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HIGHLIGHTS:

Diazotrophic bacteria increase cowpea grain yield.
SEMIA 6462/6463 and SEMIA AbV5/AbV6 strains do not attenuate the deleterious effects of salinity on cowpea nodulation.
Bacterial co-inoculation may become a more sustainable alternative to obtain atmospheric nitrogen.

ABSTRACT: Plants grown under salinity are subject to many morphological and physiological disorders. In this context, practices that can enable the use of saline water are essential. The deleterious effects of salinity can be mitigated by using beneficial microorganisms, especially diazotrophic bacteria. In this context, this study aimed to evaluate the effect of bacterial inoculation and co-inoculation using *Bradyrhizobium* spp. and *Azospirillum brasilense* on the growth, nodulation and production of cowpea under salt stress. The experiment was conducted using a randomized block design, in a 4 × 5 factorial arrangement, corresponding to four nitrogen sources (N1 - without nitrogen and without inoculant; N2 - fertilization with mineral nitrogen and without inoculant; N3 - inoculation of *Bradyrhizobium* spp.; and N4 - co-inoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense*) and five values of electrical conductivity of irrigation water - ECw (0.4, 1.9, 3.4, 4.9 and 6.4 dS m^{-1}), with five replicates. Co-inoculation with *Azospirillum brasilense* and *Bradyrhizobium* spp. favors the growth, production and nodulation of cowpea plants up to ECw of 0.4 dS m^{-1}. Increase in the electrical conductivity of irrigation water negatively affects the growth, production and nodulation of cowpea plants in Corujinha variety, regardless of the nitrogen source used.

Key words: *Vigna unguiculata* L. Walp., salinity, biological nitrogen fixation

RESUMO: Plantas cultivadas sob salinidade estão sujeitas a muitos distúrbios morfológicos e fisiológicos. Neste sentido, práticas que possam viabilizar o uso de águas salinas são essenciais. Os efeitos deletérios da salinidade podem ser mitigados ao utilizar microrganismos benéficos, especialmente bactérias diazotróficas. Nesse contexto, objetivou-se avaliar o efeito da inoculação e co-inoculação bacteriana utilizando *Bradyrhizobium* spp. e *Azospirillum brasilense* no crescimento, nodulação e produção de feijão-caupi sob estresse salino. O experimento foi conduzido em blocos casualizados, em arranjo fatorial 4 × 5, sendo quatro fontes de nitrogênio (F1 - sem nitrogênio e sem inoculante; F2 - adubação com nitrogênio mineral e sem inoculante; F3 - inoculação de *Bradyrhizobium* ssp. e F4 - coinoculação de *Bradyrhizobium* ssp. e *Azospirillum brasilense*) e cinco valores de condutividade elétrica da água de irrigação - CEa (0,4; 1,9; 3,4; 4,9 e 6,4 dS m^{-1}), com cinco repetições. A coinoculação com *Azospirillum brasilense* e *Bradyrhizobium* favoreceu o crescimento, a produção e a nodulação de plantas de feijão caupi quando irrigadas até CEa de 0,4 dS m^{-1}. O aumento da condutividade elétrica da água de irrigação afetou negativamente o crescimento, a produção e a nodulação de plantas de feijão Caupi da variedade Corujinha independentemente da fonte de nitrogênio utilizada.

Palavras-chave: *Vigna unguiculata* L. Walp, salinidade, fixação biológica de nitrogênio

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Introduction

Brazil is the third largest producer of cowpea in the world, with production of 2.9 million tons in the 2021/2022 harvest, with the Northeast region corresponding to 21.8% of its production (CONAB, 2021).

The cowpea crop is considered adapted to climatic conditions of the Northeastern semi-arid region (Costa et al., 2017). However, this region has low rainfall and, when coupled with its high temperatures, it favors salinization of the soil, consequently making groundwater have saline conditions and, in periods of water scarcity, becoming one of the only options for replacement (Cruz et al., 2016; Santos et al., 2018).

The use of saline water in agriculture reduces the osmotic and water potential of soil, decreasing water availability and nutrient absorption by plants (Silva et al., 2022). Cowpea is classified as moderately tolerant to salinity, with a salinity threshold of 2.3 dS m^{-1} (Barbosa et al., 2021a). When evaluating cowpea, some growers found that growth, phytomass (Sá et al., 2018) and nodular (Lima et al., 2007) functions are affected with increasing electrical conductivity (ECw) of the irrigation water.

Legumes have the ability to acquire N from the atmosphere through biological nitrogen fixation (BNF), a process that occurs in specialized structures called nodules (Galindo et al., 2021). Root nodules can supply approximately 200 kg ha^{-1} of nitrogen to cowpea plants, causing a positive soil balance of 92 kg ha^{-1}, which can replace the use of chemical fertilizers (Costa et al., 2017; Kyei-Boahen et al., 2017). In this context, co-inoculation of Bradyrhizobium spp. with plant growth-promoting bacteria (PGPB), can increase the mobility of nitrogen (N), bringing positive effects to plants (Arora et al., 2017).

In this context, this study aimed to evaluate the effect of bacterial inoculation and co-inoculation using *Bradyrhizobium* spp. and *Azospirillum brasilense* on the growth, nodulation and production of cowpea under salt stress.

Material and Methods

The experiment was conducted from July to September 2020, in a protected environment (greenhouse), belonging to the Academic Unit of Agricultural Engineering (UAEA) of the Federal University of Campina Grande (UFCG), located in the municipality of Campina Grande, Paraíba - PB, Brazil, whose geographic coordinates are 7° 13' 11" South latitude, 35° 53' 31" West longitude and altitude of 550 m. The greenhouse used was of the arch type, 30 m long and 21 m wide, with a ceiling height of 3.0 m, with a low-density polyethylene cover (150 microns). The data of air temperature (maximum and minimum) and mean relative humidity of air during the experimental period are presented in Figure 1.

The treatments consisted of the combination of two factors, being four nitrogen sources (N1 - without nitrogen and without inoculant; N2 - fertilization with mineral nitrogen and without inoculant; N3 - inoculation of *Bradyrhizobium* spp.; and N4 - co-inoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense*) and five values of electrical conductivity of irrigation water - ECw (0.4, 1.9, 3.4, 4.9 and 6.4 dS m^{-1}) distributed in a randomized block design in a 4 × 5 factorial arrangement, with five replicates and one plant per plot, totaling 100 experimental units. Electrical conductivity values were based on the salinity threshold value of the crop (Barbosa et al., 2021a).

The experiment was conducted using plastic pots adapted as drainage lysimeters, with capacity of 20 L, filled with a 0.5-kg layer of crushed stone, followed by 23 kg of soil classified as Entisol, collected at 0-30 cm depth, from the municipality of Lagoa Seca - PB, whose physical-chemical characteristics were determined according to Teixeira et al. (2017): exchangeable Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Al^{3+} + H^{+} = 2.60, 3.66, 0.16, 0.22 and 1.93 $cmol_c$ kg^{-1}, respectively; pH (water:soil, 1:2.5) = 5.9; ECse = 1.0 dS m^{-1}; organic matter = 1.36 dag kg^{-1}; P = 6.80 mg kg^{-1}; sand, silt and clay = 732.9, 142.1, and 125.0 g kg^{-1}, respectively; bulk density = 1.39 kg dm^{-3}; moisture content at 33.42 and 1519.5 kPa = 11.98 and 4.32 dag kg^{-1}, respectively.

Basal fertilization with NPK was performed according to the recommendation of fertilization for pot experiments, contained in Novais et al. (1991), applying 100 mg N, 300 mg P_2O_5 and 150 mg K_2O kg^{-1} using urea (45% N), single superphosphate (20% P_2O_5, 20% Ca and 12% S) and KCl (60% K_2O), respectively. Nitrogen fertilization was only applied in N2 - fertilization with mineral nitrogen (urea) and without inoculant, whereas the other treatments only received

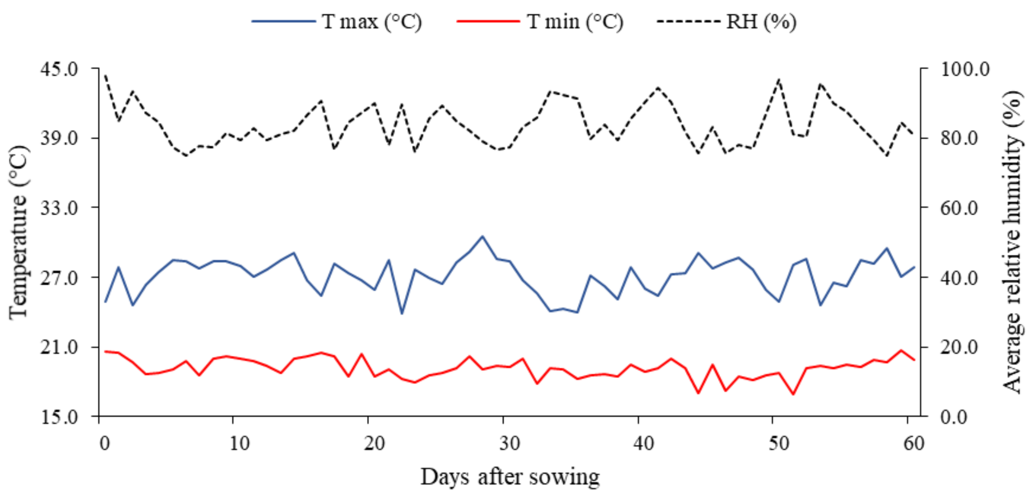

Figure 1. Maximum (T max) and minimum (T min) air temperatures and average relative air humidity (RH) in the internal area of the greenhouse

phosphorus (300 mg P_2O_5) and potassium (150 mg K_2O kg^{-1}). Only half of the recommendation was applied in basal fertilization, and the remainder was applied as top-dressing, at 10 days after sowing (DAS).

The seeds were inoculated with *Bradyrhizobium* spp. strains (SEMIA 6462/6463) and *Azospirillum brasilense* (AbV5/AbV6), provided by the company Total Biotecnologia, based in Curitiba - PR, Brazil. For treatments inoculated with *Bradyrhizobium* spp., the quantity and methodology recommended by Total Biotecnologia (10 mL of liquid inoculant per kg of seeds), described on the product's label, was used. The seeds were mixed with the inoculant until they were fully and uniformly coated. Co-inoculation was performed using both types of microorganisms in the same proportions of inoculation, as recommended by the company Total Biotecnologia. Soon after inoculations and co-inoculations, the seeds were sown in polyethylene pots containing the previously described substrate.

The experiment was conducted with heirloom seeds of cowpea (*Vigna unguiculata* L. Walp.), 'Corujinha', commonly used in the Northeast region. Five seeds were sown in each pot, and thinning was performed ten days after sowing (DAS), leaving only one plant per pot.

The irrigation waters with five values of electrical conductivity (0.4, 1.9, 3.4, 4.9 and 6.4 dS m^{-1}) were prepared by dissolving the NaCl, $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ salts, in the equivalent proportion of 7:2:1, respectively, in local-supply water (ECw = 0.4 dS m^{-1}). This proportion is commonly found in sources of water used for irrigation in small properties in the Northeast region (Medeiros et al., 2003). Irrigation waters were prepared considering the relationship between ECw and the concentration of salts (Richards, 1954), according to Eq. 1:

$$Q = 10 \times \text{ECw} \tag{1}$$

where:
Q - quantity of salts to be added (mmol$_c$ L^{-1}); and,
ECw - electrical conductivity of water (dS m^{-1}).

After sowing, irrigation was performed daily, at 5 p.m., aiming to maintain the soil moisture content close to field capacity, applying in each container the water according to treatment, and the volume to be applied was determined based on the water need of the plants, estimated by water balance, given by Eq. 2:

$$\text{VI} = \frac{(\text{Va} - \text{Vd})}{(1 - \text{Lf})} \tag{2}$$

where:
Vl - water volume to be applied in irrigation (mL);
Va - volume applied in the previous irrigation (mL);
Vd - volume drained (mL); and,
Lf - leaching fraction of 0.20, applied every 15 days to prevent the accumulation of salts in the soil.

Plant height (PH), stem diameter (SD), number of leaves (NL) and leaf area (LA) were evaluated at 60 DAS. PH was obtained with a tape measure, by measuring from the soil level to the apical meristem of the plant. SD was measured two centimeters above the collar with a caliper. NL was counted considering leaves with length greater than 3 cm. LA was estimated by measuring the midrib length of each leaf, according to the methodology described by Grimes & Carter (1969), through Eq. 3:

$$\text{Y} = \sum 0.4322 \times \text{X} \times 2.3002 \tag{3}$$

where:
Y - leaf area per plant; and,
X - leaf midrib length.

At 60 DAS, nodulation and production of cowpea were also evaluated based on: number of pods per plant (NPP), number of grains per pod (NGP), total production (TP), nodule fresh matter (NFM) and nodule dry matter (NDM). NPP was estimated by counting the total number of pods per plant; NGP is an average number obtained by dividing the total number of grains counted by the total number of pods; TP was estimated considering the total mass of the harvested grains (g); NFM was determined at the end of the experiment at 60 DAS, when the nodules were manually removed and weighed (g); and NDM was determined by drying the nodules in an oven at 65 °C for 72 hours and then weighing them (g).

The multivariate structure of the results was evaluated by means of principal component analysis (PCA), synthesizing the amount of relevant information contained in the original data set in a smaller number of dimensions, resulting from linear combinations of the original variables generated from the eigenvalues ($\lambda \geq 1.0$) in the correlation matrix, explaining a percentage greater than 10% of the total variance (Govaerts et al., 2007).

After PCA, the original data of the variables of each component were subjected to multivariate analysis of variance by the Hotelling test (Hotelling, 1947) at $p \leq 0.05$. Only variables with correlation coefficient greater than or equal to 0.6 were maintained in each PCA. Statistica software v. 7.0 (Statsoft, 2004) was used for the statistical analyses.

Results and Discussion

According to the multivariate analysis of variance (Table 1), there was a significant effect of the interaction between nitrogen sources (NS) and the electrical conductivity of irrigation water (ECw) for the two principal components (PC1 and PC2).

The eigenvalues and percentage of variation explained for each component (Table 1) represented together 90.5% of the total variation. PC1 explained 79.2% of the total variance, formed by most of the variables analyzed, except for plant height (PH). PC2 represented 11.3% of the remaining variance, formed by the PH variable.

The two-dimensional projections of the effects of treatments and variables in the first and second principal components (PC1 and PC2) are presented in Figures 2A and B. In the first principal component (PC1), a process possibly characterized

Table 1. Eigenvalues and percentage of total variance explained in the multivariate analysis of variance (MANOVA) and correlations (r) between original variables and the principal components

	Principal components PC1	PC2
Eigenvalues (λ)	7.13	1.02
Percentage of total variance (S^2%)	79.2	11.3
Hotelling test (T^2) for nitrogen sources (NS)	0.01	0.01
Hotelling test (T^2) for electrical conductivity of irrigation water (ECw)	0.01	0.01
Hotelling test (T^2) for interaction (NS × ECw)	0.01	0.01

PCs	Correlation coefficient PH	SD	NL	LA	NPP	NGP	TP	NFM	NDM
PC1	-0.45	-0.93	-0.92	-0.94	-0.98	-0.94	-0.98	-0.86	-0.89
PC2	-0.82	-0.25	-0.19	-0.08	0.08	0.22	0.05	0.32	0.27

Treatments	Mean values PH	SD	NL	LA	NPP	NGP	TP	NFM	NDM
N1S1	79.40	8.62	57.80	4125.55	3.40	68.20	18.80	9.28	1.11
N1S2	67.00	7.72	45.40	3524.65	4.20	67.20	11.62	8.35	1.06
N1S3	34.20	7.56	23.80	1561.69	1.80	19.40	5.31	6.43	0.70
N1S4	27.40	6.50	13.00	1078.72	1.20	8.20	2.08	0.98	0.16
N1S5	24.00	5.88	10.80	647.13	1.00	3.60	1.24	0.65	0.14
N2S1	33.60	10.22	81.40	7437.23	6.40	443.80	22.84	10.39	1.39
N2S2	88.40	11.12	44.40	4323.31	6.20	311.20	13.79	9.55	1.42
N2S3	48.60	8.22	29.00	3051.46	2.00	35.80	5.65	7.23	1.06
N2S4	33.20	7.56	20.80	1622.69	1.80	28.20	4.74	1.18	0.32
N2S5	26.40	5.84	8.80	725.82	2.00	15.20	1.73	0.99	0.27
N3S1	24.40	10.88	77.60	8571.61	10.60	1187.00	41.55	29.46	4.45
N3S2	29.00	9.60	44.60	5149.77	7.20	343.40	23.67	21.29	2.93
N3S3	34.60	8.06	17.80	1741.76	3.20	75.60	12.10	15.11	2.18
N3S4	28.40	6.50	14.40	1087.63	1.60	12.00	2.69	7.19	1.13
N3S5	23.60	5.90	15.40	965.56	1.80	12.40	2.20	5.84	1.05
N4S1	77.40	11.76	79.00	7244.02	12.20	1052.00	48.89	33.83	7.46
N4S2	40.60	9.16	47.80	4084.39	7.60	422.00	17.41	30.02	4.07
N4S3	38.80	7.66	26.60	2326.12	4.20	128.60	8.80	20.90	2.71
N4S4	29.40	6.34	19.60	1075.43	1.60	22.60	4.49	10.94	1.53
N4S5	27.40	5.76	12.00	731.94	1.60	12.00	1.53	8.69	0.96

S1 - 0.4 dS m^{-1}; S2 - 1.9 dS m^{-1}; S3 - 3.4 dS m^{-1}; S4 - 4.9 dS m^{-1}; S5 - 6.4 dS m^{-1}; N1 - Without nitrogen and without inoculant; N2 - Fertilization with mineral nitrogen and without inoculant; N3 - Inoculation of *Bradyrhizobium* spp.; N4 - Co-inoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense*; PH - Plant height - cm; SD - Stem diameter - mm; NL - Number of leaves; LA - Leaf area - cm^2; NPP - Number of pods per plant; NGP - Number of grains per pod; TP - Production - g per plant; NFM - Nodule fresh matter - g; NDM - Nodule dry matter - g

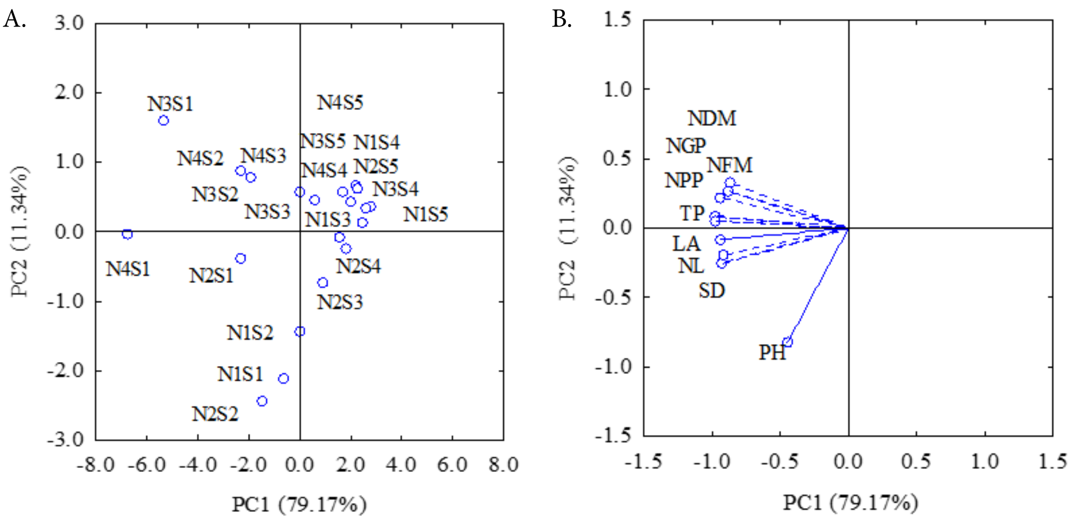

Figure 2. Two-dimensional projection of the scores of the principal components for the factors nitrogen sources (N) and electrical conductivity of irrigation water (S) and the variables analyzed (B) in the two principal components (PC1 and PC2)

by the effect of the interaction between nitrogen sources and the levels of electrical conductivity of irrigation water was identified. The correlation coefficients between SD, NL, LA, NPP, NGP, TP, NFM and NDM were higher than 0.85 (Table 1).

In the principal component 1, it can be noted that cowpea plants grown with co-inoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense* and irrigated using water with ECw of 0.4 dS m^{-1} (N4S1) stood out from those of the other treatments, as they had the highest values (Table 1) of SD (11.76 mm), NL (79), LA (7244.02 cm^2), NPP (12.2), NGP (1052.0), TP (48.89 g per plant), NFM (33.83 g) and NDM (7.46 g).

When comparing the results obtained in N4S1 treatment plants to those of N2S1 treatment plants, increments of 15.1, 90.1, 137.0, 114.0, 225.6 and 437.0% were observed for SD, NPP, NGP, TP, NFM and NDM, respectively, thus pointing to the beneficial effect of bacterial co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*. Leaf area (LA) had the highest average for inoculation with Bradyrhizobium spp., showing increments of 15.25% when compared to the N2S1 treatment.

Similar results were reported by Barbosa et al. (2021b), analyzing the benefits of co-inoculation of soybean with *Azospirillum brasilense* and *Bradyrhizobium* spp., through a meta-analysis, found an increase of 10.6% in the mass of nodules, of 3.2% in grain yield, when compared plants inoculated only with *Bradyrhizobium* spp. In agreement, Pereira et al. (2019), analyzing the improvement in growth and yield of fava beans (*Vicia faba*) by inoculation with *Rhizobium laguerreae*, reported increases in the number of leaves, leaf area, number of pods per plant and number of seeds of 66.1, 114.1, 144.4, and 152.1%, respectively, in inoculated plants, when compared to the control treatment.

Increases in vegetative growth are associated with increased nitrogen supply through biological nitrogen fixation (Ayalew et al., 2022). These effects may be related to the occurrence of heterogeneous nodulation through the increase of the root area, thus increasing the sites of infection (Fukami et al., 2018; Galindo et al., 2018). For Ayalew et al. (2021), the increase in yield variables associated with the use of nitrogen-fixing bacteria is associated with greater growth linked to the accumulation of assimilates, possibly due to increased N nutrition through BNF.

In addition to the ability to fix atmospheric N, bacteria of the genus *Azospirillum* are characterized as plant growth-promoting bacteria (PGPB), enabling beneficial effects for growth through the synthesis of phytohormones, possibly by increasing the capacity of plants to explore the soil more efficiently (Fukami et al., 2018). Studies with common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* L. Walp) indicate that inoculation with Azospirillum brasilense stimulates root exudation of flavonoids, inducing root hair growth and resulting in a significant increase in nodulation and, when subjected to co-inoculation with rhizobia, they increase their production potential, growth and BNF (Dardanelli et al., 2008; Galindo et al., 2020).

According to the principal component 2 (PC2) (Figure 2), cowpea plants cultivated with ECw of 1.9 dS m^{-1}, subjected to mineral fertilization with N, obtained the highest value for PH (88.4 cm). However, the increase in ECw above 0.4 dS m^{-1} negatively affected the growth, nodulation and production of cowpea plants. Similar results were observed by Sá et al. (2018), in a study with cowpea under salt stress (ECw: 0.5; 1.5; 2.5; 3.5 and 4.5 dS m^{-1}), where the variables plant height, stem diameter and number of leaves showed decreases of 38.5% (6.92 cm), 15% (0.95 mm) and 41.64% (3.65), when comparing the lowest (0.5 dS m^{-1}) and the highest (4.5 dS m^{-1}) levels of salinity.

When analyzing cowpea subjected to different values of salinity in irrigation water (0.5; 2.13; 2.94; 3.5 and 5.0 dS m^{-1}), Lima et al. (2007) observed that, for the highest level of ECw (5.0 dS m^{-1}), there were decreases in leaf area, shoot dry matter and in the number of nodules of 65.9% (312.318 cm^2), 66.94% (2.03 g) and 98.71% (35.9), respectively, when compared to the lowest salinity level (0.5 dS m^{-1}).

This result is possibly linked to the negative impact of salinity, due to the reduction in the water flow from the roots to the leaves and a reduction in cell division, affecting the growth of plants subjected to salt stress, and also due to the toxic effects or high osmotic pressures caused by the salt, inhibiting cell division and elongation (Hafez et al., 2020).

When evaluating the antioxidant responses of cowpea inoculated with *Bradyrhizobium* or co-inoculated with *Bradyrhizobium* and PGPB, subjected to salt stress (0.99 and 5.60 mS cm^{-1}), Santos et al. (2018) observed that, even co-inoculated plants, when grown under saline conditions, show reductions in the metabolic characteristics of their nodules. The symbiosis and nodule functions are directly affected by salinity, which can reduce nodulation, decrease nitrogenase activity and biological nitrogen fixation (Silveira et al., 2011).

Conclusions

1. Co-inoculation with *Azospirillum brasilense* and *Bradyrhizobium* spp. favors the growth, production and nodulation of cowpea plants up to ECw of 0.4 dS m^{-1} in irrigation water.

2. Increase in the electrical conductivity of irrigation water negatively affects the growth, production and nodulation of cowpea plants in Corujinha variety, regardless of the nitrogen source used.

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