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Inoculation of nitrogen-fixing bacteria to mitigate the negative effect of brackish water on peanuts

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Abstract: The use of nitrogen-fixing bacteria reduces the use of synthetic nitrogen fertilizers. However, salt stress can negatively affect the establishment of biological nitrogen fixation (BNF). In this context, the aim was to evaluate the use of brackish water at different phenological stages on the gas exchange and mineral composition of inoculated and non-inoculated peanut crops. The experimental design used was completely randomized, following a 5x2 factorial arrangement, with four replications, using five irrigation strategies with brackish water with an electrical conductivity of 5.0 dS m^{-1} applied at different phenological stages of the crop: E1 - at 14 days after sowing - DAS (vegetative stage); E2 - at 25 DAS (flowering stage); E3 - at 36 DAS (gynophore emergence stage); E4 - at 46 DAS (pod formation stage); and E5 - control, and inoculated and non-inoculated plants. The earlier the salt stress is applied, the lower the gas exchange in the peanut crop, with more significant effects up to 36 DAS, the phase of gynophore emergence and elongation, so plants inoculated with rhizobia show greater efficiency in the use of brackish water. Similarly, from 36 DAS onwards, leaf mineral content decreases, with the exception of sodium, which increases. It is recommended to use brackish water associated with the use of *Bradyrhizobium* spp. in the pod formation stage of the peanut crop.

Keywords: *Arachis hypogaea L.*; *Bradyrhizobium*; nodulation; salinity; semi-arid region.

Introduction

Peanut (*Arachis hypogaea L.*) belongs to the Fabaceae family and can be cultivated in almost all types of soil. It can be used for a variety of purposes, from human and animal food to the production of biofuel (Arya et al., 2016), and is one of the main oilseeds produced on a large scale worldwide, and Brazil has an average national yield of 3,604 kg ha^{-1} (Conab, 2022).

In the Northeast of Brazil, where much of the irrigated area is predominantly semi-arid, with irregular rainfall distribution and high evapotranspiration rates, the use of lower quality water such as brackish water is the only alternative to meet the water needs of crops and guarantee agricultural production (Cruz et al., 2021; Sousa et al., 2022).

Excess salts in irrigation water are one of the main abiotic stresses that negatively affect agricultural yields and sustainable development worldwide (Ferreira et al., 2020). The use of brackish water inhibits plant growth by reducing the osmotic potential of the soil solution, restricting the availability of water, which can lead to ionic toxicity and nutritional imbalance (Sousa et al., 2022; Sousa et al., 2023).

Some strategies have been adopted to mitigate the harmful effect of salts in irrigation water on plants. Recently, researchers have been using bacteria as a way to mitigate this problem. The use of bacteria from the genera *Rhizobium spp*. and *Bradyrhizobium spp*. is a way of cultivating peanuts irrigated with brackish water. In addition to fixing the N necessary for better crop yield, the association of the plant with such bacteria can mitigate the effect of salts present in irrigation water by producing organic compounds that act as antioxidants, thus reducing the stress caused (Freitas et al., 2022; Rodrigues et al., 2021).

Study carried out by Lima et al. (2021) in peanut crop inoculated with a mixture of rhizobia SEMIA 630, lot 0810 and SEMIA 6144, lot 0312, of *Bradyrhizobium spp*., isolated, they found that the inoculant attenuated the effect of water salts on gas exchange and water use efficiency. Nascimento et al. (2023) reported that co-inoculation with *Azospirillum brasilense* and *Bradyrhizobium* favored the growth, production and nodulation of cowpea plants when irrigated with water of 0.4 dS m^{-1}, and Egamberdieva et al. (2017) showed that co-inoculation with *Pseudomonas putida* TSAU1 improved plant growth, root architecture, and nitrogen and phosphorus contents of soybeans under salt stress.

Table 1. Meteorological data during the experiment.

Months	RH		T	
	Min	Max	Min	Max
August	45	58	23	30
September	46	60	24	31
October	48	62	25	32

RH- relative humidity; T- temperature; Min- minimum; Max- maximum.

Table 2. Physical-chemical attributes of the soil used in the experiment.

Chemical Attributes												
OM	N	P	K^+	Ca^{2+}	Mg^{2+}	Na^+	$H^+ + Al^{3+}$	Al	SB	CEC	ECse	pH
$g\ kg^{-1}$		-------------------------------- $cmol_c\ kg^{-1}$ ----------------------------									$dS\ m^{-1}$	
4.03	0.24	0.15	0.10	2.50	0.30	0.57	0.33	0.00	3.47	3.78	0.37	7.6
Physical Attributes												
Sand			Silt			Clay		Bulk Density				
--------------------------------- % ---------------------------------								$g\ cm^{-3}$				
80			13			7		1.53				

OM - Organic matter; SB - Sum of bases ($Ca^2 + Mg^{2+} + Na^+ + K^+$); CEC - Cation exchange capacity - [$Ca^2 + Mg^{2+} + Na^+ + K^+ + (H^+ + Al^{3+})$]; pH was measured in aqueous extract (1:2.5).

In this context, the aim was to evaluate the effect of the use of brackish water at different phenological stages on the gas exchange and mineral composition of inoculated and non-inoculated peanut crops.

Results and Discussion

Leaf gas exchange

Based on the means comparison test for photosynthesis (Figure 1A), only at E3 was there a statistical difference, with the inoculated plants being superior. This phase corresponds to the appearance and elongation of the gynophore, when plants require a greater accumulation of phosphorus, a nutrient that promotes greater absorption of nitrogen in the phloem and consequently a higher photosynthetic rate (Prado, 2020); however, the salts present in the irrigation water such as Na^+ cause an antagonistic effect. This result may be associated with lower stomatal opening due to the action of salts, causing reductions in transpiration rates and CO_2 assimilation, that is, salt stress limits gs and the entry of CO_2 into the leaf mesophyll, reducing photosynthesis due to the decrease in the partial pressure of this gas in the intercellular spaces (Dias et al., 2020).

Lima et al. (2021) also observed a reduction in net photosynthesis in peanut genotypes inoculated with rhizobium when subjected to salt stress. A similar trend regarding the reduction in photosynthetic rate in non-inoculated peanut plants under salt stress was also found by Sousa et al. (2021).

According to Figure 1B, salt stress also affected the transpiration of the peanut crop at 36 days after the start of irrigation with brackish water, with the inoculated plants being statistically superior. As a plant defense mechanism, water imbalance causes the stomata to close (estimated by the reduction in gs) in order to prevent water loss through transpiration (E), a factor which explains the reduction in transpiration rates of plants subjected to salt stress for longer periods. The positive effect of inoculation corroborates the study conducted by Lima et al. (2021). These same authors found a reduction in transpiration in peanut plants inoculated with rhizobia SEMIA 630 and SEMIA 6144, from *Bradyrhizobium sp*.

The onset of salt stress at 14 and 25 affected Ci but with less impact in the presence of the inoculant, showing a statistically superior condition (Figure 1C). The partial closure of stomata causes a decrease in internal CO_2 concentration, especially when photosynthesis is maintained, even at low levels. This effect may also be related to limitations that occur in the total water potential caused by excess salts that interfere with gas exchange characteristics, thus reducing stomatal conductance and the rate of CO_2 assimilation (Zahra et al., 2022). A similar trend was observed by Lessa et al. (2021) in peanut plants under salt stress and not inoculated. Lima et al. (2021), when evaluating the effect of salt stress on peanut plants inoculated with a mix of *Bradyrhizobium sp*. rhizobia, also found that inoculated plants obtained a lower internal CO_2 concentration.

For gs (Figure 1D), the control treatment was statistically superior to the others. Salt stress applied throughout the crop cycle caused the stomata to close, i.e., the plants reduced water loss, but limited the uptake and diffusion of CO_2 to the carboxylation sites (Sousa et al., 2021). Similar results were observed by Lessa et al. (2021), who worked with the peanut crop and found a reduction in stomatal conductance with the increase in salts in the irrigation water.

Inoculated plants that received saline water at 14 and 25 DAS showed a reduction in WUE (Figure 1E) when compared to non-inoculated plants. Inoculation with rhizobia was associated with a reduction in WUE, except in the inoculated plants that received saline water at 46 DAS. The reduction in US is related to the drop in transpiration rate (E) caused by the stress applied earlier to the plants. Dourado et al. (2022) describe that the water use efficiency rate expresses the amount of carbon fixed during photosynthesis for each molecule of water that is lost during the process. By closing the stomata to prevent excessive water loss through transpiration, plants are able to utilize the CO_2 accumulated in the substomatal chambers, reducing water loss during water stress (Yan et al., 2016).

As for the SPAD index (Figure 1F), which expresses the chlorophyll content in the leaves, it can be seen that there was a reduction in chlorophyll in non-inoculated plants that

Table 3. Chemical characterization and classification of the irrigation water used in the experiment.

ECw	Ca^{2+}	Mg^{2+}	K^+	Na^+	Cl^-	HCO_3^-	pH	SAR	Classification
dS m^{-1}	---------$mmol_c$ L^{-1}--------				---mmol L^{-1}---		in H_2O	(mmol $L^{-1})^{0.5}$	
5.0	10.55	12.77	3.33	26	25	1.8	7.5	7.32	C_4S_2

SAR—Sodium adsorption ratio.

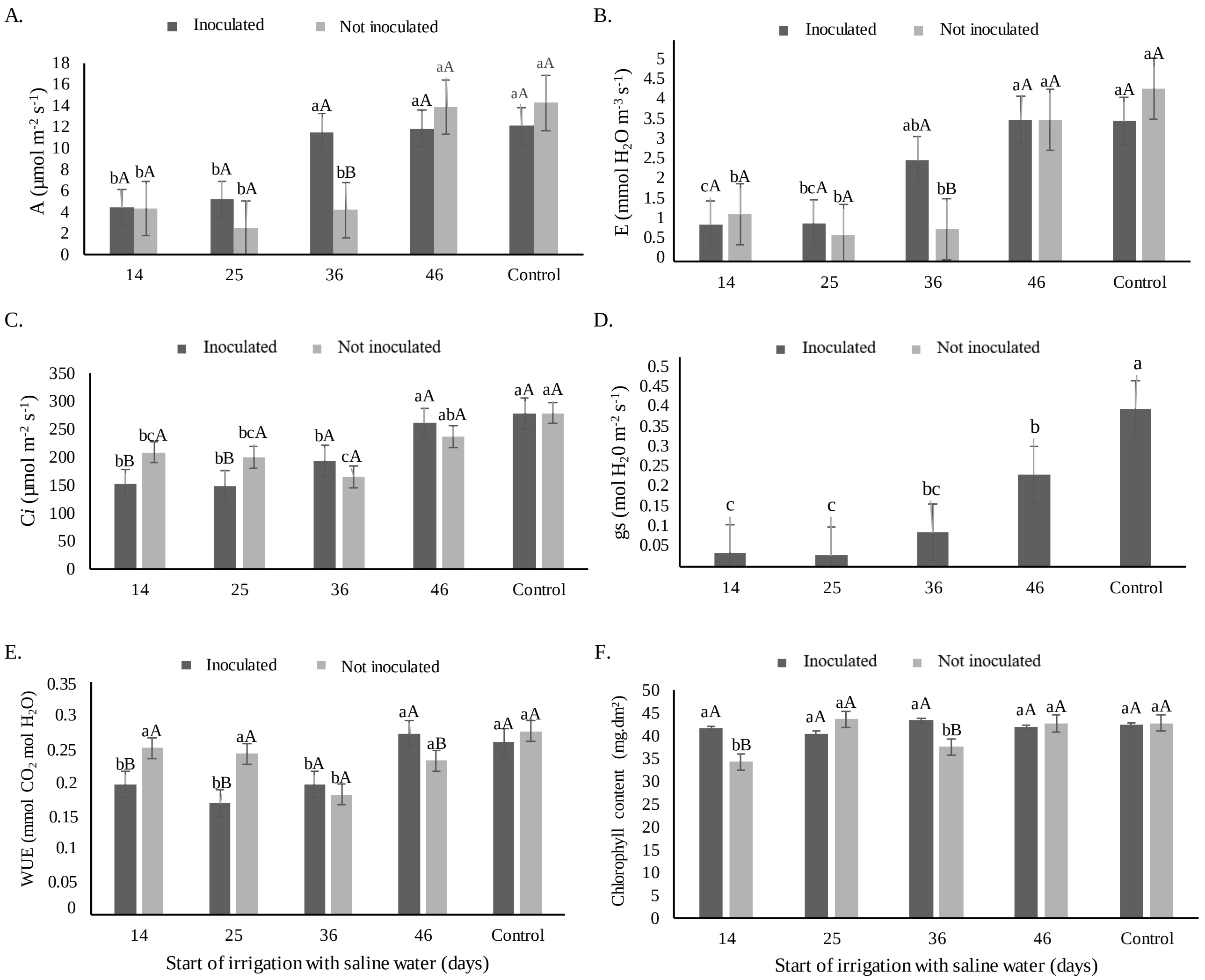

Figure 1. Net photosynthesis (A), transpiration (B), internal CO_2 concentration (C), stomatal conductance (D), water use efficiency (E) and chlorophyll content (F) in the peanut crop as a function of salinity management strategies and inoculation with rhizobia.
In each management strategy, averages with the same lowercase letters do not differ significantly (Tukey; $p < 0.05$); between inoculation treatments, same uppercase letters indicate that there are no differences (Tukey; $p < 0.05$) between inoculated and non-inoculated plants, respectively.

received saline water at 14 and 36 DAS. The results indicate that the application of saline water was more damaging to non-inoculated plants and that this sensitivity was greater when saline water was introduced at the stage of first branch emergence (14 DAS) and flowering (25 DAS).

The relative chlorophyll content is related to the total leaf content of N, which is part of enzymes associated with chloroplasts; however, under these study conditions, the non-inoculated plants, even under conditions of salt stress and although there was not a high abundance, as happened with the strains used in the inoculation, were efficient in performing BNF to provide nitrogen for chlorophyll synthesis (Alvarenga et al., 2019). Lima et al. (2021) also showed better performance in terms of chlorophyll content in peanut plants inoculated and irrigated with brackish water.

Contrary to the data in this study, Sousa et al. (2021) irrigated non-inoculated peanut crop with brackish water and fertilized it with NPK and found that the chlorophyll content was not reduced by salt stress.

Leaf contents

Considering the macronutrient levels present in the aerial part described by Silva et al. (2009) as adequate for peanut cultivation, for the nutrients N, K and Mg (nutrients that showed a statistically significant difference), it can be seen that N was below the estimated average (30 g kg^{-1}) and that K and Mg showed levels considered normal, within the ranges of 17-30 $g.kg^{-1}$ and 3-8 $g.kg^{-1}$, respectively.

For the N content (Figure 2A) there was only a significant difference at 46 DAS, where the non-inoculated plants were

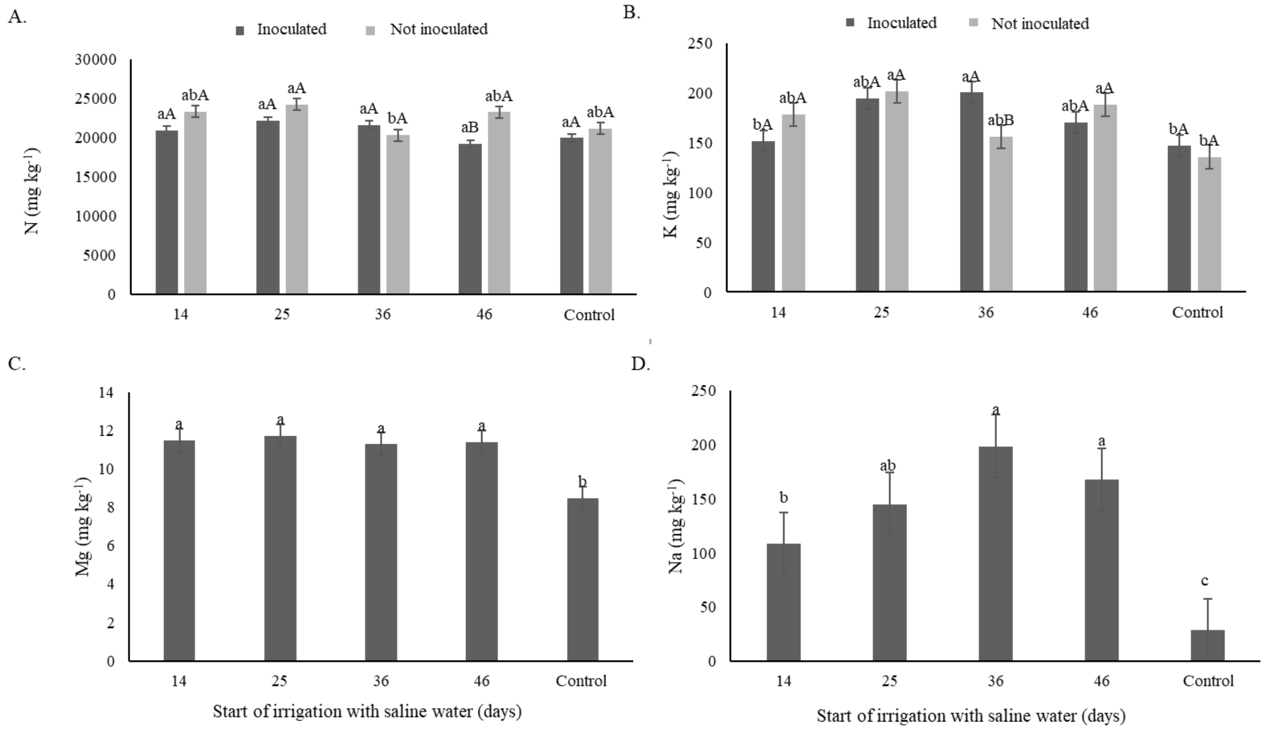

Figure 2. Average values of the leaf contents of nitrogen (A), potassium (B), magnesium (C) and sodium (D) in the leaf dry mass of the peanut crop as a function of salinity management strategies and inoculation with rhizobia.

In each management strategy, averages with the same lowercase letters do not differ significantly (Tukey; $p < 0.05$); between inoculation treatments, same uppercase letters indicate no differences (Tukey; $p < 0.05$) between inoculated and non-inoculated plants, respectively.

statistically superior to the inoculated ones. This is related to the potential of the native microbiota to carry out BNF and/or mineralize soil organic matter. A similar trend was observed by Silva et al. (2022) in non-inoculated peanut plants irrigated with brackish water of 5.0 dS m^{-1} and fertilized with NPK.

For potassium (Figure 2B), inoculation favored a greater accumulation of this nutrient when the salt stress was initiated at 36 DAS, so, for the same period, plants that were not inoculated had lower values when compared to plants that were inoculated. This low content in non-inoculated plants during this period may be related to the phenological state of the crop, which may have translocated the nutrient to other parts of the plant due to its mobility (Prado, 2020). Similarly, Souza et al. (2019) also found a reduction in K levels in noni plants irrigated with water with ECw of 4.0 dS m^{-1} and fertilized with organic compost.

As for magnesium, there was no interaction between the factors evaluated, but there were significant effects for the phenological stage in which saline water was introduced and for inoculation (Figure 2C). Plants in the control treatment (without saline water irrigation) had lower levels of the nutrient. It is worth noting that Mg is essential for photosynthesis, as it is the central atom in the chlorophyll molecule (Stadnik et al., 2023). These results are in line with those reported by Silva et al. (2022), who showed that increasing the salinity of irrigation water increased leaf Mg levels in non-inoculated peanut plants.

The increase in ECw raised Na levels (Figure 2D) in plants that received brackish water at 36 and 46 DAS, which were statistically superior to the others. This may be associated with a greater accumulation of these elements in the substrate and irrigation water. Excess Na in the soil and water can induce nutritional imbalance as a result of the high ionic concentration and inhibition of the absorption of other cations, as reported by Rodrigues et al. (2021).

Materials and methods

Location and climate

The experiment was conducted in a 50% open shade screen on the sides, from August to November 2019, at the University of the International Integration of Afro-Brazilian Lusophony, Redenção, Ceará, Brazil. The local climate is classified as Aw, a tropical climate with dry winters, according to the classification proposed by Köppen (1931). The average annual temperature in the municipality ranges from 26 to 28 °C and the average annual rainfall is 1,062 mm, with rainfall predominating from January to April.

Experimental design and treatments

The experimental design used was completely randomized, following a 5x2 factorial arrangement, with four replications, using five irrigation strategies with brackish water with an electrical conductivity of 5.0 dS m^{-1} applied at different phenological stages of the crop: E1 - 14 days after sowing - DAS (vegetative stage); E2 - 25 DAS (flowering stage); E3 - 36 DAS (gynophore emergence stage); E4 - 46 DAS (pod formation stage); and E5 - control, plants that did not suffer salt stress and plants inoculated and not inoculated with rhizobia mix SEMIA 630, lot 0810 and SEMIA 6144, lot 0312, from *Bradyrhizobium sp*., isolated.

Crop growing

The crop was grown in 8-liter pots containing 12 kg of soil, *Argissolo Eutrófico* (Ultisol) (0-20 cm) with a sandy loam texture, whose chemical characteristics are shown in Table 2.

The seeds used in the experiment came from the peanut cultivar BR-1. After the seedlings emerged and established, they were thinned out, leaving two plants per pot.

The seeds were inoculated using a mix of rhizobial strains recommended for peanuts (SEMIA 630, lot 0810 and SEMIA 6144, lot 0312). The strains were multiplied in a culture medium based on yeast extract, mannitol and agar (YMA). Inoculation was carried out on the seeds using the rhizobia mix in a peat medium and a 20% aqueous solution of gum arabic as an adhesive, with a booster inoculation with liquid culture medium containing the rhizobia mix, in the proportion of 2 mL of broth in the collar of each seedling after thinning.

The full mineral fertilization (for N, P and K) recommended for peanuts by Fernandes (1993) was adopted, corresponding to 15 kg ha^{-1} of N, 62.5 kg ha^{-1} of P_2O_5 and 50 kg ha^{-1} of K_2O. With a stand of 10,000 plants ha^{-1}, the maximum dose per plant in the estimated cycle was: 1.5 g of N; 6.25 g of P_2O_5 and 5.0 g of K_2O.

Irrigation management

Irrigation was carried out manually every day using a container graduated in mm, with a 15% leaching fraction. To prepare the brackish solution of 5.0 dS m^{-1}, the salts NaCl, $CaCl_2$ $2H_2O$ and $MgCl_2$ $6H_2O$ were used in a ratio of 7:2:1, following the relationship between ECw and salt concentration (mmol$_c$ L^{-1} = EC × 10) (Richards, 1954).

The daily estimate of reference evapotranspiration (ETo) was calculated according to the principle of the drainage lysimeter (Bernardo et al., 2019), using equation 1.

$$\mathrm{VI} = \frac{(\mathrm{Vp} - \mathrm{Vd})}{(1 - \mathrm{LF})} \qquad (1)$$

where:

VI - Volume of water to be applied in the irrigation event (mL);

Vp - Volume of water applied in the previous irrigation event (mL);

Vd - Volume of water drained (mL); and,

LF - Leaching fraction of 0.15.

The chemical characteristics of the waters are presented in Table 3.

Leaf gas exchange

Gas exchange measurements were taken at 55 DAS on fully expanded leaves located in the middle third of the plants in the morning, between 09:00 and 11:00 hours, using a portable leaf gas exchange analyzer based on the IRGA system (Infra Red Gas Analyser, LI-6400, LI-COR, USA) to measure the rates of net photosynthesis (A, μmol CO_2 m^{-2}.s^{-1}), stomatal conductance (gs, mol H_2O m^{-2}.s^{-1}), transpiration (E, mmol H_2O m^{-2}.s^{-1}) and water use efficiency (E/A, μmol CO_2/mol H_2O). The SPAD index was also determined using a chlorophyll meter.

Leaf contents

Plant material from the aerial part (collected at 55 DAS) was ground in a mill to determine the macronutrients contained in the aerial part of the plants. Nitrogen (Ntotal) was obtained using the wet digestion procedure, followed by steam distillation and titration to quantify NH_4 using the Semi-micro Kjeldahl method (Miyazawa et al., 2009). The other macronutrients (P, K, Mg, Ca) and Na were determined by dry digestion in a muffle furnace using a 1% HNO_3 solution as an extractant. A 500 mg sample of plant tissue was incinerated in an electric muffle furnace at temperature between 500 and 550 ºC. The resulting ash was dissolved in nitric acid solution. The resulting extract was used to determine P, K, Mg, Ca and Na (Moller et al.,1997). Readings were taken using flame photometry for K and Na, molybdenum blue spectrophotometry for P and atomic absorption spectrophotometry for Mg and Ca (Silva et al., 2009).

Statistical analysis

The variables assessed during the study were analyzed using the Kolmogorov-Smirnov test ($p \leq 0.05$) to assess normality. The data was then subjected to analysis of variance (ANOVA) using the F test ($p \leq 0.05$) and the Assistant 7.7 Beta program (Silva and Azevedo, 2016).

Conclusions

The earlier the salt stress is applied, the lower the gas exchange in the peanut crop, with more significant effects up to 36 DAS, the phase of gynophore emergence and elongation, so plants inoculated with rhizobia show greater efficiency in water use. Similarly, from 36 DAS onwards, leaf mineral content decreases, with the exception of sodium, which increases.

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