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Morphophysiology and gas exchange of pomegranate under salt stress and foliar application of nitrogen[1]

Morfofisiologia e trocas gasosas da romãzeira sob estresse salino e aplicação foliar de nitrogênio

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HIGHLIGHTS:

Salinity reduces growth and gas exchange of pomegranate.
Foliar-applied N dose of up to 1.31 g L^{-1} reduces the harmful effect of salinity on pomegranate growth.
Foliar nitrogen fertilization up to 1.59 g L^{-1} increases net photosynthesis.

ABSTRACT: Salinity is one of the abiotic stresses that affect gas exchange and growth of pomegranate. In this context, the application of nitrogen fertilizer through the leaves can minimize these effects. Thus, the objective of present study was to evaluate the effect of foliar nitrogen fertilization on pomegranate seedlings irrigated with brackish water. The design used was randomized blocks, in an incomplete factorial scheme (Central Composite Design) with five electrical conductivities of irrigation water - ECw (0.50, 1.15, 2.75, 4.35, and 5.00 dS m^{-1}) and five doses of foliar nitrogen fertilization - FNF (0, 0.33, 1.15, 1.97, and 2.30 g L^{-1}), with four replicates and two plants per experimental plot. Plant height, number of leaves, stem diameter, and gas exchange (stomatal conductance, net photosynthesis, intercellular CO_2 concentration, transpiration rate, instantaneous carboxylation efficiency, instantaneous water use efficiency, and intrinsic water use efficiency) were evaluated at 60 days after the beginning of irrigation with saline water. Salinity of irrigation water negatively affects the gas exchange of pomegranate seedlings. Foliar nitrogen fertilization up to 1.31 g L^{-1} improves plant height and number of leaves in pomegranate seedlings under salt stress. Foliar nitrogen fertilization up to 1.59 g L^{-1} increases the net photosynthesis of pomegranate seedlings.

Key words: *Punica granatum* L., photosynthesis, nitrogen, salinity

RESUMO: A salinidade é um dos estresses abióticos que afeta as trocas gasosas e o crescimento de romãzeira. Neste sentido, à aplicação de adubo nitrogenado via foliar poderá minimizar estes efeitos. Com isso, o objetivo do presente estudo foi avaliar o efeito da aplicação foliar com nitrogênio em mudas de romã irrigadas com águas salobra. O delineamento utilizado foi de blocos casualizados, em esquema fatorial incompleto (Composto Central de Box), sendo cinco condutividade elétrica da água - CEa (0,50; 1,15; 2,75; 4,35 e 5,00 dS m^{-1}) e cinco doses de adubação nitrogenada via foliar – ANF (0; 0,33; 1,15; 1,97 e 2,30 g L^{-1}), com quatro repetições. A salinidade da água de irrigação afeta negativamente as trocas gasosas de mudas de romãzeira. A adubação nitrogenada foliar até 1,31 g L^{-1} melhora o crescimento em altura e no número de folhas em mudas de romãzeira sob estresse salino. Adubação nitrogenada foliar até a dose de 1,59 g L^{-1} aumenta a fotossíntese das mudas de romãzeira.

Palavras-chave: *Punica granatum* L., fotossíntese, nitrogênio, salinidade

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Introduction

Pomegranate (*Punica granatum* L.) is a fruit species native to Iran, used as a functional source of food and nutraceuticals, and its cultivation is increasingly expanding in semiarid regions, where soil salinity is a limiting factor (Catola et al., 2016). Short- and long-term salinity adversely affects the growth and physiological processes of this crop (Soares et al., 2021), highlighting the need for techniques to minimize these effects.

Salinity is a serious problem in semiarid regions and irrigated areas, negatively affecting agricultural production. Irrigation with brackish water favors the increase of toxic ions such as sodium (Na^+) and chloride (Cl^-), resulting in decreased osmotic potential, interruption of morphological and physiological processes, ionic toxicity and nutritional imbalance of plants (Soares et al., 2021; Nóbrega et al., 2023).

Excessive absorption of these salts alters the hormonal balance and increases the production of reactive oxygen species, influencing growth and decreasing seedling quality (Bezerra et al., 2019). Salt stress negatively affects gas exchange, causing a reduction in CO_2 diffusion in the chloroplast due to stomatal restrictions, as well as photochemical changes and carbon metabolism, and these effects may vary according to the intensity and duration of stress (Lima et al., 2020).

N is the main essential element constituting amino acids and proteins that protect the plant against abiotic stresses (Coulombier et al., 2020). The role of N against salt stress is associated with the production of solutes, such as proline, which maintains osmotic balance and protects cells against reactive oxygen species (Cerqueira et al., 2019). The beneficial effect of N has already been reported by some authors for *Malpighia emarginata* applied to the soil (Lima et al., 2020), and for *Passiflora edulis* (Pereira et al., 2022) and *Anonna squamosa* L. (Fátima et al., 2023) by foliar application.

Thus, the objective of this study was to evaluate the effect of foliar nitrogen fertilization on pomegranate seedlings irrigated with saline water.

Material and Methods

The experiment was conducted from April to August 2019 in a greenhouse covered with semi-transparent plastic and with screened walls on the sides, with the seedlings arranged on masonry benches at 1.0 m from the floor, at the Agricultural Sciences Center of the Universidade Federal da Paraíba, Areia, Paraíba, Brazil. The municipality is located at the geographical coordinates 6º 58' 00" S and 35º 41' 00" W with an altitude of 575 m. The climate of the region, according to Köppen's classification, is of the As' type, with dry and hot summer and rain in the winter (Alvares et al., 2013). The data of temperature (maximum and minimum), relative air humidity, and precipitation during the experimental period were collected daily and are presented in Figure 1.

The experimental design was in randomized blocks, in an incomplete factorial scheme (Central Composite Design), with five electrical conductivities (0.50, 1.15, 2.75, 4.35, and 5.00 dS m^{-1}) and five doses of foliar nitrogen fertilization (0, 0.33, 1.15, 1.97, and 2.30 g L^{-1}), with four replicates and two plants per replicate, totaling nine combinations (Table 1). The treatments were determined from the Central Composite Design generated from the formula: MNT = 2k + 2*k + 1, where, MNT = minimum number of treatments, k = number of factors.

Table 1. Representative scheme of the combinations and factors (ECw - electrical conductivity of irrigation water; D_{FN} - foliar nitrogen doses) used in the experiment

Treatments	Levels		Doses	
	ECw	D_{FN}	ECw (dS m^{-1})	D_{FN} (g L^{-1})
1	-1	-1	1.15	0.33
2	-1	1	1.15	1.97
3	1	-1	4.35	0.33
4	1	1	4.35	1.97
5	-1.41(α)	0	0.50	1.15
6	1.41(α)	0	5.00	1.15
7	0	-1.41(α)	2.75	2.30
8	0	1.41(α)	2.75	0
9	0	0	2.75	1.15

ECw - Electrical conductivity of irrigation water; ND - Nitrogen doses; α - Distance between each axial point and the center in a central composite design

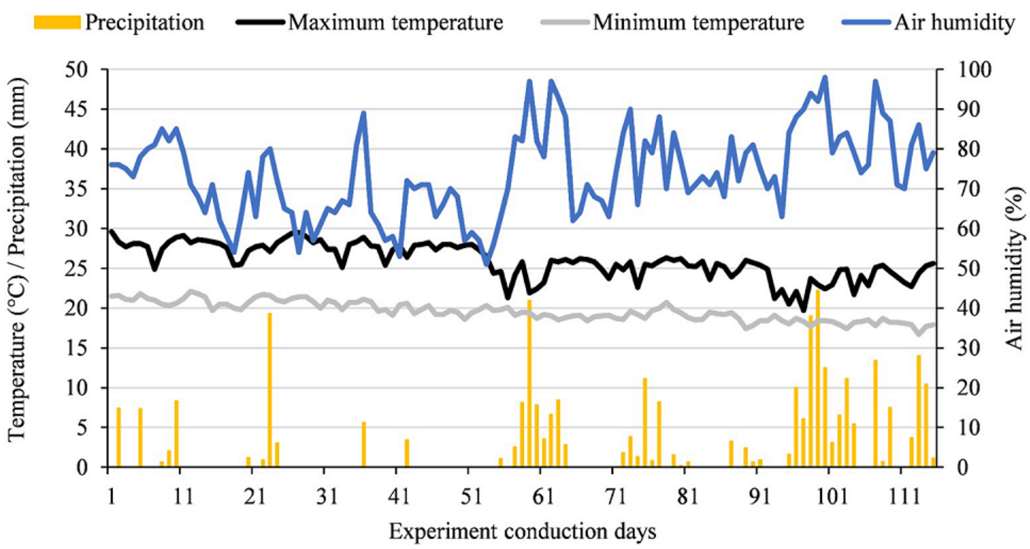


Figure 1. Data of maximum and minimum air temperature, precipitation, and average relative air humidity during the experimental period in greenhouse

Pomegranate seeds cv. Mollar from fruits harvested in the orchard of the Universidade Federal Rural do Semi-Árido (UFERSA) were used. After acquiring the fruits, the seeds were extracted and processed manually with the aid of a #¼ mesh sieve to remove the sarcotesta, to overcome the possible dormancy of the seeds, aiming at the standardization of germination and the establishment of seedlings. Three seeds were used per polyethylene bag, which was kept close to the field capacity from sowing to germination. Seed germination began four days after sowing, extending to 25 days, when seedling emergence was established. At 25 days after sowing, thinning was performed, leaving only one plant per bag, which was considered the most vigorous.

The electrical conductivities of irrigation water (ECw) were obtained by dissolving sodium chloride (NaCl) in water from the supply system (0.5 dS m^{-1}), until the required conductivities were obtained, the values being measured with a microprocessor-based portable conductivity meter (model CD-860, Instrutherm®). The application of brackish water started 25 days after emergence (DAE), with daily manual irrigation. The crop water demand was determined by the drainage lysimetry (Bernardo et al., 2019) method (Eq. 1), with the volume of water lost through evapotranspiration the previous day being replaced daily, thus maintaining the soil at field capacity.

$$VI = \frac{(Va - Vd)}{(1 - LF)} \quad (1)$$

where:

VI - volume of water to be used in the next irrigation (mL);
Va - volume of water applied in the previous irrigation event (mL);
Vd - volume drained (mL); and,
LF - leaching fraction (0.15).

Every 15 days a 15% leaching fraction was applied based on the volume applied in this period, in order to reduce the accumulation of substrate salts.

Nitrogen doses were based on the need for 300 mg per plant, proposed by Novais et al. (1991) for a 1 dm^3 pot, the highest evaluated dose being 400 mg per plant. The commercial product Nitrotecnia-20 (Carbotecnia®), based on urea, with 99 g L^{-1} of N, was used.

Foliar nitrogen fertilization started at 25 DAE, with previous dissolution of the fertilizer in distilled water, followed by application with an atomizer. In total, seven applications were performed, at interval of 10 days. The total volume applied was 175 mL per plant, which provided 0, 58.16, 200, 341.84, and 400 mg of N per plant according to the increase of the evaluated doses, based on study of Fátima et al. (2023).

The polyethylene bags had a capacity of 1.15 dm^3, filled with substrate formed by 85% soil, 10% fine sand and 5% aged manure (Sakazaki et al., 2019). The physical and chemical characteristics of the substrate were evaluated according to methodologies proposed by EMBRAPA (2017) and Richards (1954) (Table 2).

The evaluations were carried out at 90 days after the beginning of the treatment application. Gas exchange was measured on the fourth leaf from the apex to the base, between 9 and 10 a.m. with an infrared gas analyzer – IRGA (LI-6400XT, LI-COR®, Nebraska, USA) with an air flow of 300 μmol s^{-1} and humidity between 50-60%, 400 μmol mol^{-1} of CO_2 and 1200 μmol m^{-2} s^{-1} coupled light source. Stomatal conductance (gs – mol H_2O m^{-2} s^{-1}), net photosynthesis (A – μmol CO_2 m^{-2} s^{-1}), intercellular CO_2 concentration (Ci – mmol CO_2 mol^{-1}), transpiration rate (E – mmol H_2O m^{-2} s^{-1}), instantaneous water use efficiency (WUE – [(μmol CO_2 m^{-2} s^{-1}) (mmol H_2O m^{-2} $s^{-1})^{-1}$]), intrinsic water use efficiency (iWUE - [(μmol CO2 m^{-2} s^{-1}) (mmol H_2O m^{-2} $s^{-1})^{-1}$]), and instantaneous carboxylation efficiency (iCE – [(μmol CO_2 m^{-2} s^{-1}) (mmol CO_2 $mol^{-1})^{-1}$]) were evaluated.

To evaluate the growth of pomegranate seedlings, plant height was analyzed, considering the distance from the collar to the apex of the plant, evaluated with a ruler graduated in cm; stem diameter was measured with a digital caliper and expressed in mm; and the number of leaves was determined by counting fully formed leaves.

Data were subjected to normality (Shapiro-Wilk) and homogeneity of variances (Bartlett) tests. Subsequently, an analysis of variance ($p \leq 0.05$) was performed, and a regression analysis was carried out in cases of significance. The statistical program R (R Core Team, 2021) was used.

Results and Discussion

As observed in the summary of the analysis of variance (Table 3), a significant effect was found for the interaction

Table 2. Physical and chemical composition of the components of substrate used in the experiment

Physical	Value	Fertility	Value	Salinity	Value
Sand (g kg^{-1})	874	pH in water (1: 2.5)	8.10	pH	7.40
Silt (g kg^{-1})	91	P (mg dm^{-3})	65.16	ECse (dS m^{-1})	2.00
Clay (g kg^{-1})	35	K^+ (mg dm^{-3})	1.09	SO_4^{-2} ($mmol_c$ L^{-1})	2.17
Textural class	Sandy	Na^+ ($cmol_c$ dm^{-3})	0.24	Ca^{+2} ($mmol_c$ L^{-1})	6.50
		Al^{+3} ($cmol_c$ dm^{-3})	0.00	Mg^{+2} ($mmol_c$ L^{-1})	17.50
		$H^+ + Al^{+3}$ ($cmol_c$ dm^{-3})	0.99	K^+ ($mmol_c$ L^{-1})	7.67
		Ca^{+2} ($cmol_c$ dm^{-3})	2.88	CO_3^{-2} ($mmol_c$ L^{-1})	0.00
		Mg^{+2} ($cmol_c$ dm^{-3})	0.96	HCO_3^{-2} ($mmol_c$ L^{-1})	17.5
		SB ($cmol_c$ dm^{-3})	5.17	Cl^- ($mmol_c$ L^{-1})	10.00
		CEC ($cmol_c$ dm^{-3})	6.16	SARse ($mmol_c$ $L^{-1})^{0.5}$	1.06
		OM (g kg^{-1})	15.00	ESP (%)	0.30
				Classification	Non saline and non-sodic

OM - Organic matter; SB - Sum of bases ($Na^+ + K^+ + Ca^{2+} + Mg^{2+}$); CEC - Cation exchange capacity = SB + ($H^+ + Al^{3+}$); ECse - Electrical conductivity of the saturation extract; SARse - Sodium adsorption ratio of the saturation extract = $Na^+ \times [(Ca^{2+} + Mg^{2+})/2]^{1/2}$; ESP - Exchangeable sodium percentage ($100 \times Na^+/CEC$)

Table 3. Summary of the analysis of variance for stomatal conductance (gs), net photosynthesis (A), intercellular CO_2 concentration (Ci), instantaneous carboxylation efficiency (iCE), intrinsic water use efficiency (iWUE), instantaneous water use efficiency (WUE), plant height (PH), number of leaves (NL), and stem diameter (SD) of *Punica granatum* L. under foliar nitrogen fertilization (D_{FN}) and salinity of brackish irrigation water (ECw)

Source of variation	GL	Mean square									
		gs	A	Ci	E	iCE	WUE	iWUE	PH	NL	SD
Blocks	3	2.1^{-5ns}	0.34^{ns}	125.7^{ns}	0.09^{ns}	$7.77e^{-6ns}$	0.06^{ns}	31.6^{ns}	2.75^{ns}	45.1^{ns}	0.044^{ns}
Treatment	8	1.2^{-3**}	14.57^{**}	2779.4^{**}	1.37^{ns}	$2.06e^{-4**}$	1.06^{**}	1964.5^{**}	165.74^{**}	1692.7^{**}	2.24^{**}
D_{FN} (L)	1	1.00^{**}	2.10^{**}	33.45^{**}	0.003^{ns}	0.006^{**}	1.08^{**}	32.42^{**}	9.60^{**}	45.79^{**}	1.55^{**}
D_{FN} (Q)	1	0.56^{ns}	2.21^{**}	14.53^{ns}	0.621^{ns}	0.010^{**}	0.21^{ns}	40.96^{**}	5.26^{**}	11.33^{**}	0.28^{*}
ECw (L)	1	2.11^{**}	1.48^{**}	1.41^{ns}	0.434^{ns}	0.005^{**}	0.05^{ns}	25.06^{**}	9.57^{**}	19.99^{**}	0.84^{**}
ECw (Q)	1	0.45^{ns}	1.14^{**}	31.14^{**}	0.326^{ns}	$5.15e^{-4ns}$	0.10^{ns}	22.15^{**}	10.66^{**}	7.28^{*}	0.83^{**}
D_{FN} × ECw	1	0.05^{ns}	0.08^{ns}	0.27^{ns}	0.169^{ns}	$2.45e^{-4ns}$	0.19^{**}	0.22^{ns}	0.58^{**}	2.18^{*}	0.05^{ns}
CV		5.60	6.40	4.80	15.80	9.10	15.40	8.60	4.40	8.50	4.70

* - Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; ^{ns} - Not significant, by F test

between brackish water and foliar nitrogen rates for water use efficiency - WUE, plant height - PH and number of leaves - NL. No significant effect was observed for the transpiration variable - E. Individual effect was observed for the other analyzed variables (Table 3).

For stomatal conductance (gs) and net photosynthesis (A), there was a linear reduction as a function of the salinity of brackish irrigation water, with the highest values (0.076 mol m^{-2} s^{-1} and 7.142 μmol CO_2 m^{-2} s^{-1}, respectively) at the ECw of 0.5 dS m^{-1}, with decreases of 25.3 and 41.5%, at the highest salinity of 5.00 dS m^{-1} (Figures 2A and 2B). The occurrence of these reductions is common in plants subjected to salt stress, closing their stomata as a defense mechanism of the plant. Consequently, CO_2 absorption decreases, reducing the rate of net photosynthesis. This fact was observed by Soares et al. (2021) in pomegranate seedlings and by Silva et al. (2019) in yellow passion fruit, under saline conditions.

The intercellular CO_2 concentration (Ci) was higher (277.70 μmol CO_2 m^{-2} s^{-1}) in seedlings subjected to ECw of 0.5 dS m^{-1}, followed by decreases up to ECw of 4.1 dS m^{-1} (232 μmol CO_2 m^{-2} s^{-1}), reaching decreases equivalent to 16.1% when compared to the values obtained in seedlings subjected to ECw of 0.5 dS m^{-1} (Figure 2C). The reduction of Ci up to the salinity of 4.1 dS m^{-1} shows that the decrease in photosynthesis may be due not only to stomatal factors, but also to factors of non-stomatal nature, such as biochemical alterations in the reduction of energy supply, which supposedly can affect the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), as reported by Sá et al. (2019).

The instantaneous carboxylation efficiency (iCE) was reduced with increasing salinity, with the highest value (0.026) at the ECw of 0.5 dS m^{-1}, followed by decreases that reached 36.1% at the salinity of 5.00 dS m^{-1} (Figure 2D). This marked reduction in iCE with the increase in salinity levels is due to the deleterious effects on the absorption and assimilation of CO_2 by pomegranate seedlings. This directly reflects the reduction of photosynthesis, promoting metabolic changes in the Calvin cycle, preventing carbon from being fixed (Sousa et al., 2016).

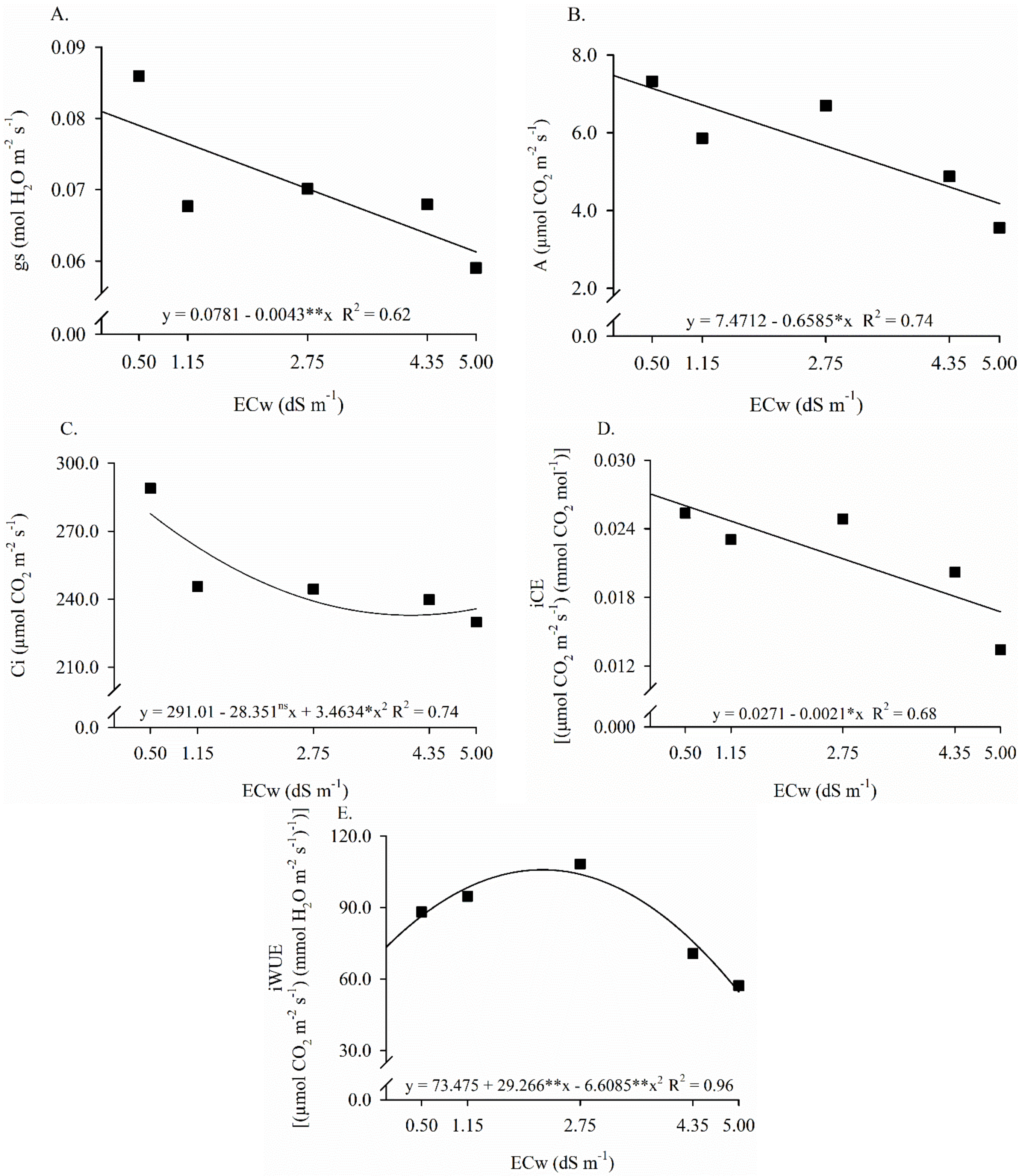
The instantaneous water use efficiency (WUE) ($Z = 3.46 - 0.13^{**}x - 0.08^{**}x^2 - 0.18^{**}y - 0.26^{**}y^2 + 0.29^{**}xy$; $R^2 = 0.54$) was higher at the dose of 0.03 g L^{-1} of N and ECw of 0.52 dS m^{-1}. The occurrence of this effect indicates that N promotes improvements in WUE when pomegranate plants are subjected to high salinity, so when the ECw increases this efficiency is reduced. This behavior is a response to the deleterious effects caused by salt stress, which induces this low water consumption, limiting the absorption of toxic ions, as observed by Nóbrega et al. (2022) in *Mesosphaerum suaveolens*.

The intrinsic water use efficiency (iWUE) increased up to the ECw of 2.20 dS m^{-1}, reaching a value of 105.87 (Figure 2E). The greater iWUE observed in this study is associated with a plant mechanism of adaptation to stress conditions, leading to the maintenance of water in the tissues, as reported by Huang et al. (2015). The negative effects of salinity on gas exchange have been observed in plants of *Solanum lycopersicum* (Talebnejad & Sepaskhah, 2016), *Passiflora edulis* (Silva et al., 2019), *Psidium guajava* L. (Bezerra et al., 2019), and *Punica granatum* L. (Soares et al., 2021), results similar to those found in this study.

The foliar nitrogen fertilization promoted increases in gs, with the highest value obtained being 0.0747 mol m^{-2} s^{-1} at the dose of 1.6 g L^{-1} of N, representing gains of 45.6% when compared to plants of the control treatment (dose 0), indicating the beneficial effect of foliar application of N on the gs of pomegranate plants (Figure 3A). This result may be related to the positive effect of nitrogen on plants, which is an important component in the synthesis of photosynthetic pigments and enzymes, as it improves stomatal regulation and the light saturation point, which explains the increases found for gs in this study (Wang et al., 2016).

Foliar nitrogen fertilization stimulated A and Ci, with the highest values of 6.3457 and 270.26 μmol CO_2 m^{-2} s^{-1} being observed at doses of 1.6 and 1.2 g L^{-1}, representing gains equivalent to 46.9 and 18.8%, respectively (Figures 3B and 3C). This positive effect indicates that the foliar application of nitrogen promoted improvements in the assimilation and internal concentration of CO_2 in pomegranate seedlings. This can be explained by the fact that N is a nutrient that acts in several physiological processes, being involved in the synthesis of chlorophyll, influencing the photosynthetic capacity of the plant (Cerqueira et al., 2019).

The intrinsic water use efficiency (iWUE) was reduced up to the dose of 1.47 g L^{-1} of N, increasing at the highest concentrations (Figure 3D). The occurrence of this effect may be associated with the positive effect of N on stomatal opening, as observed in gs, favoring the diffusion of CO_2 in the cells of the leaf mesophyll, consequently facilitating the loss of water through transpiration, reducing water use efficiency (Talebnejad & Sepakhah, 2016).

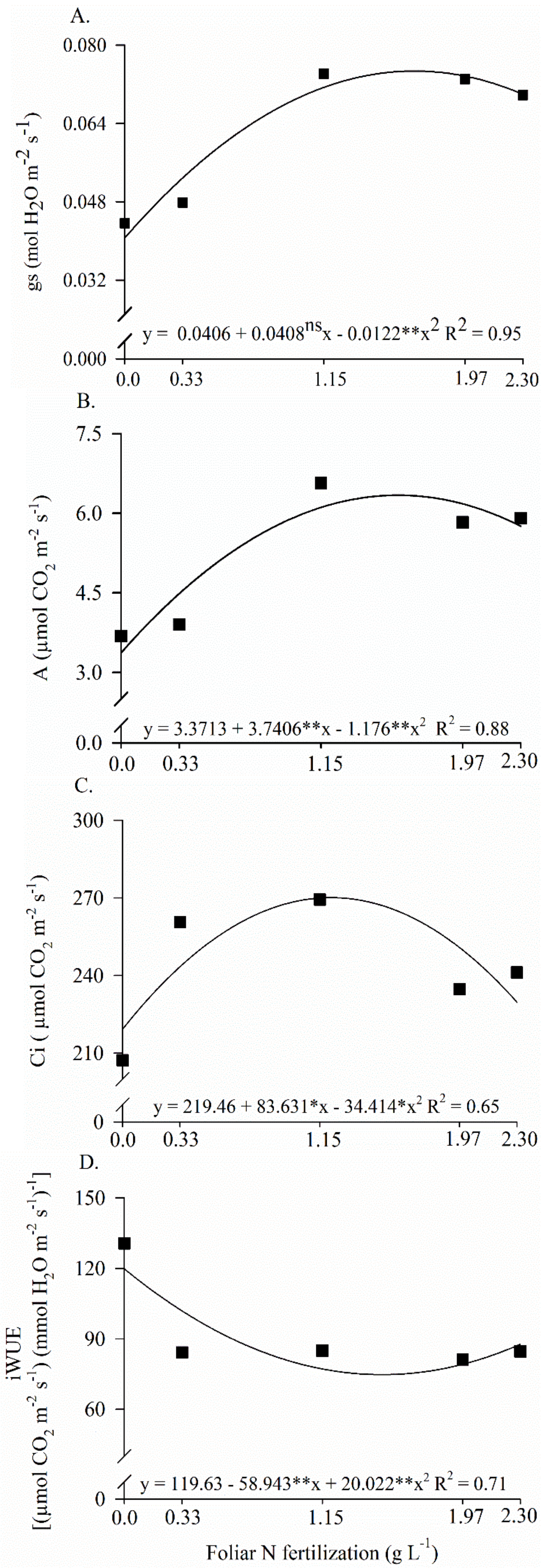


*, ** - Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; ns - Not significant, by F test

Figure 2. Stomatal conductance – gs (A), net photosynthesis – A (B), intercellular CO_2 concentration – Ci (C), instantaneous carboxylation efficiency – iCE (D), and instantaneous water use efficiency – iWUE (E) of *Punica granatum* L. under foliar nitrogen fertilization and salinity of brackish irrigation water (ECw)

For the interaction between factors, foliar nitrogen fertilization and brackish water for plant height and number of leaves, foliar nitrogen fertilization at doses of 1.28 and 1.31 g L^{-1} reduced the deleterious effects of salinity up to the ECw of 0.52 and 1.73 dS m^{-1}, respectively (Figures 4A and 4B).

The attenuating effect of nitrogen on growth in height and number of leaves is an indication that foliar application of nitrogen improves the performance of pomegranate seedlings. This is due to the fact that this nutrient is part of several organic compounds important for plant metabolism (amino acids, proteins, proline), favoring osmotic adjustment (Cerqueira et al., 2019). Foliar nitrogen fertilization attenuates salt stress in yellow passion fruit seedlings – *Passiflora edulis* (Pereira et al., 2022) and sugar apple – *Annona squamosa* L. (Fátima et al., 2023).



*, ** - Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; ns - Not significant, by F test

Figure 3. Stomatal conductance - gs (A), net photosynthesis - A (B), intercellular CO_2 concentration - Ci (C), and intrinsic water use efficiency - iWUE (D) of *Punica granatum* L. under foliar nitrogen fertilization

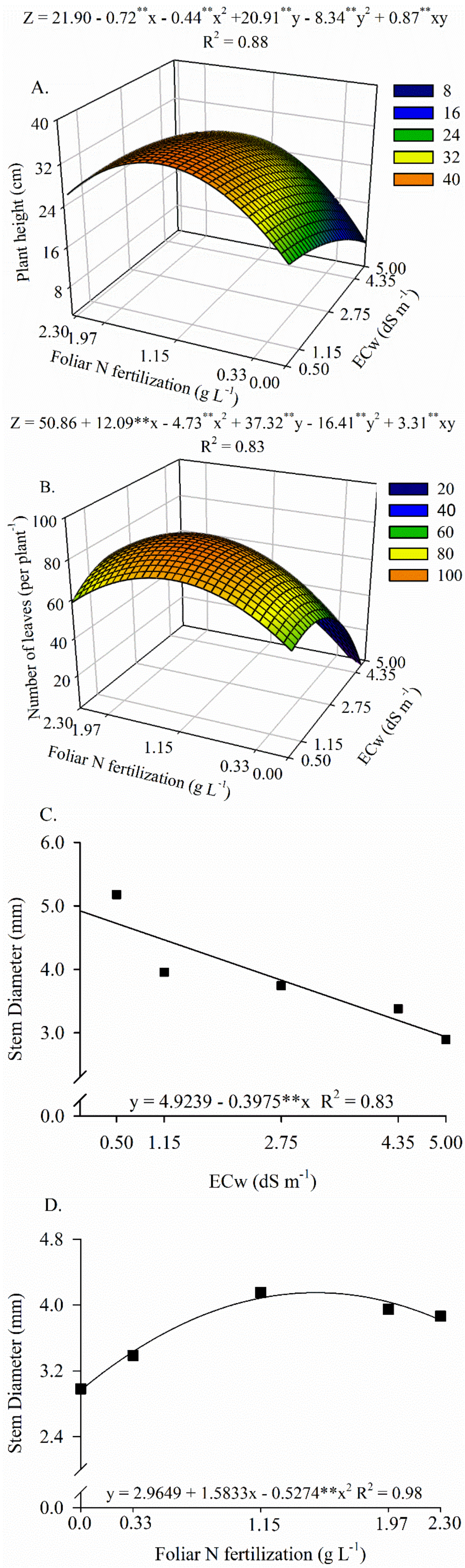


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Figure 4. Plant height (A), number of leaves (B) as a function of interaction between foliar nitrogen fertilization and salinity of irrigation water and stem diameter (C and D) of *Punica granatum* L. under foliar nitrogen fertilization and salinity of irrigation water

Stem diameter was negatively influenced by salinity, with a 37.9% decrease at the highest salinity (5.00 dS m^{-1}) compared to the control (Figure 4C). The reduction in water potential limits water absorption by plants, resulting in less metabolic activity for cell division, which explains the decrease in stem diameter with increased salinity (Veloso et al., 2018).

Foliar nitrogen application promoted an increase in stem diameter up to the dose of 1.50 g L^{-1} of N, with an increase of 23% or equivalent to 1.20 mm being observed when compared to plants subjected to dose 0 (Figure 4D). This increase in stem diameter may be related to the action of nitrogen in favoring the assimilation of CO_2 and, consequently, promoting greater growth and development of plants (Basra et al., 2014).

Conclusions

1. Foliar nitrogen fertilization at doses of 1.28 and 1.31 g L^{-1} of N (Nitrotecnia-20) attenuates the effect of salinity on growth in height and number of leaves in pomegranate seedlings;

2. Irrigation water salinity above 1.7 dS m^{-1} drastically reduces gas exchange in pomegranate seedlings.

3. Net photosynthesis of pomegranate seedlings is stimulated by foliar application of 1.58 g L^{-1} of N.

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