



Salinity stress mitigation by radicular and foliar humic substances application in lettuce plants

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Abstract

Salinity stress constitutes one of the main abiotic stresses that considerably reduces crop yield. An approach to enhance plant growth under salt stress involves the addition of humic substances (HS) to roots or leaves. Here, we evaluated the potential use of BLACKJAK®, an HS-based product, to enhance salt tolerance in lettuce (*Lactuca sativa* L.). For this aim, plants were exposed to salinity (100 mM NaCl), salinity + HS: radicular (R)-HS (0.40 mL/L and 0.60 mL/L) and foliar (F)-HS (7.50 mL/L and 10.00 mL/L), along with a control (without NaCl). Parameters related to plant growth, Na⁺ and K⁺ accumulation, photosynthetic activity, oxidative stress, enzymatic and non-enzymatic antioxidants, as well as proline levels were evaluated. Results showed that R and F-HS considerably enhanced salinity tolerance. In addition, F-HS offered a greater improvement of plant growth in relation to shoot fresh weight, shoot relative growth rate, and foliar area, being 10.00 mL/L the best dose. This tolerance could be associated with reduced Na⁺ translocation to the shoot and enhanced shoot K⁺ accumulation, decreasing Na⁺/K⁺ ratio. Furthermore, HS improved the net photosynthetic rate, Rubisco carboxylation efficiency, and photosystem II performance, and reduced ROS levels and lipid peroxidation. Hence, our data show the potential use of BLACKJAK® to improve lettuce tolerance to salinity, with foliar application slightly better than radicular to achieve this aim, especially at 10.00 mL/L dose.

Keywords Antioxidants · Biostimulant · Humic substances · Lettuce · Photosynthesis · Salt stress

Introduction

One of the major abiotic stresses that considerably reduce plant growth and quality is soil salinity. This environmental problem results from higher salt concentrations, mainly sodium chloride (NaCl), in soil or irrigation water (Mubarak et al. 2021; Shaaban et al. 2023a). It is estimated that salinity affects more than 20% of irrigated land, mainly in arid and semi-arid areas such as the Mediterranean region (Singh et al. 2022a). In the future, a significant portion of these saline soils will undergo cultivation in response to the expanding world population, projected to reach 10 billion people by 2050, and the escalating demand for food (Shah et al. 2021). Several crops are continuously exposed to salinity conditions, which significantly reduce plant growth and crop productivity (Saady et al. 2023b). In this way, annual economic losses due to salinity are estimated at 12 billion USD (Zuluaga et al. 2023). For this reason, different agronomic strategies, including the breeding and evaluation of new varieties (Abd El-Mageed et al. 2022) and the

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application of natural products (Salem et al. 2021), are being studied to increase plant tolerance to salinity.

Osmotic, ionic, and oxidative stress impair plant physiology during exposure to salinity. Besides, the accumulation of soluble salts, and the subsequent reduction in soil water potential, reduces roots' water uptake (Valenzuela et al. 2022). Consequently, one of the responses to osmotic stress is stomatal closure to decrease water loss by transpiration (Makhlouf et al. 2022; Ali et al. 2024b), although it limits CO₂ assimilation and photosynthesis performance (Marriboina and Attipalli 2020). At the cellular level, osmotic adjustment occurs through the synthesis and accumulation of compatible solutes and osmolytes to maintain cellular turgor (El-Metwally et al. 2022a; Ramadan et al. 2023a; Doklega et al. 2024). Proline, betaine, and glycine are examples of solutes synthesized by plants as an osmotic response to different adverse conditions (Hussain et al. 2021; Saady et al. 2023a). In addition, salt stress results in an ionic imbalance associated with high cytosolic accumulation of Na⁺ and Cl⁻, resulting in leaf senescence (Shahzad et al. 2021; Liu et al. 2022). Because of the ionic similarity, Na⁺ tends to replace K⁺, and consequently many enzymatic activities involving K⁺ are disrupted. Thus, some vital processes, such as photosynthesis, are affected by Na⁺ toxicity (Navarro-León et al. 2021).

Reactive oxygen species (ROS), including superoxide radical (O₂⁻) and hydrogen peroxide (H₂O₂), play an important role as stress response signal molecules, at low concentrations (Hadid et al. 2023; Shaaban et al. 2023b). Under high salinity, ROS overproduction is observed in plant cells, which triggers oxidative stress. Nucleic acids, enzyme activities, membrane lipids, photosynthetic apparatus, etc., are damaged by ROS, resulting in cell and plant death (Liu et al. 2022; Mangal et al. 2023). Lipid peroxidation results in malondialdehyde (MDA) generation, therefore, low MDA in plant cells is linked with enhanced salinity stress tolerance (Mousavi et al. 2022). To cope with oxidative stress, different enzymatic and non-enzymatic antioxidants are activated. Superoxide dismutase (SOD) reduces O₂⁻ to H₂O₂ which is detoxified by ascorbate peroxidase (APX), whereas ascorbate (AsA), glutathione (GSH), and phenolic compounds are non-enzymatic antioxidants that contribute to ROS scavenging (Liu et al. 2022; Navarro-León et al. 2023). Therefore, the plant's ability to detoxify ROS and maintain osmotic and ionic homeostasis is essential to overcome salt stress (Chen et al. 2022a, b).

One way to improve salinity stress tolerance is the application of biostimulants or biofertilizers to soil or plant. These natural products are classified in two main groups: microbial (beneficial bacteria and fungi) and non-microbial (protein hydrolysates, plant extracts, seaweed extracts, beneficial elements, biopolymers, chitosan, and humic substances) (Pizzeghello et al. 2020; Ali et al. 2024a; El-Metwally et al.

2022b; Abou El-Enin et al. 2023). Humic substances (HS), such as humic acids (HA), fulvic acids (FA), and humin, represent up to 70% of soil organic matter and have been employed as potential plant biostimulants (Saady et al. 2020; Ore et al. 2023). HS are derived from the chemical and physical degradation of plant and animal waste present in soil, through the "humification" process. In terms of chemical structure, HS are defined as supramolecular colloids formed by the interaction between hundreds of small organic molecules through hydrophobic interactions and hydrogen bonds (Tiwari et al. 2023). HS positively affect primary and secondary metabolism, enhancing plant resistance to different adverse conditions such as salt stress. Thus, HS may enhance root growth and water uptake, nutrient bioavailability and absorption, photosynthesis performance, compatible solutes accumulation, and enzymatic and non-enzymatic antioxidant activity (Ramadan et al. 2023a; Lasheen et al. 2024). All of this translates into ROS scavenging, photosynthetic activity maintenance, preventing cytoplasmic ionic imbalances, and ensuring adequate cellular water status (García et al. 2016; Khaleda et al. 2017; Man-hong et al. 2020; Souza et al. 2021; Abu-Ria et al. 2023; Ramadan et al. 2023b). In this way, several researchers and companies extract HS from various sources such as leonardite, coal, and peat to develop HS-based products and improve plant growth and abiotic stress tolerance in different crops such as lettuce (*Lactuca sativa* L.).

Lettuce is a high-nutrient and low-calorie leafy vegetable with a high economic value (Abd-Elrahman et al. 2022). However, under salinity stress conditions, lettuce production may be reduced because of its sensitivity to salt, prompting researchers to explore strategies to mitigate salt stress damage (Liu et al. 2024). Hence, the objective of the present study was to assess the effectiveness of a HS-based product as a biostimulant to increase salinity stress tolerance in lettuce plants, identifying the possible mechanisms of action of this salt stress alleviation, as well as the differences between both modes of HS application (radicular and foliar).

Materials and methods

Plant material and cultivation conditions

Seeds of lettuce (*L. sativa* L. cv. *Capitata*) were previously germinated and grown in tray cells (3 cm × 3 cm × 3 cm). After 45 days, lettuce seedlings were transferred to pots with vermiculite:perlite (3:1) as substrate, and distributed randomly in a growth chamber under controlled environmental conditions: temperature 25/15 °C (day/night), relative humidity 70%, photoperiod 16/8 h, and 350 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR). Lettuce plants were watered during the experiment

with a complete nutritive solution composed of 1 mM KH_2PO_4 , 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 4 mM KNO_3 , 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 5 μM Fe-chelate (Sequestrene; 138FeG100), 0.25 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 μM HBO_3 , 1 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 0.1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The pH of this solution was adjusted to 5.5–6.

Treatments and experimental design

Treatments started 7 days after transplantation and were maintained for 30 days. A total of six treatments were conducted: (1) Control (nutritive solution only); (2) salinity (nutritive solution and 100 mM NaCl); (3) salinity + radicular-HS (R-HS) 0.40 mL/L (100 mM NaCl and 0.40 mL/L HS added to nutritive solution); (4) salinity + R-HS 0.60 mL/L (100 mM NaCl and 0.60 mL/L HS added to nutritive solution); (5) salinity + foliar-HS (F-HS) 7.50 mL/L (100 mM NaCl added to nutritive solution and spraying leaves with HS 7.50 mL/L); (6) salinity + F-HS 10.00 mL/L (100 mM NaCl added to nutritive solution and spraying leaves with HS 10.00 mL/L). Salinity stress conditions (100 mM NaCl) were established according to previous studies by our research group on lettuce plants grown under the same environmental conditions (Leyva et al. 2011). HS application was conducted using a leonardite-suspension concentrate (SC)-based product called BLACKJAK® provided by Sofbey S.A. (Mendrisio, Switzerland), with acidic pH (4–5) and 30% organic matter. In this way, radicular applications lowered the pH of nutritive solution to 4.5. A total of three HS applications with a periodicity of 10 days were performed. Radicular and foliar-HS doses were selected according to previous results obtained in lettuce plants, in terms of increases in biomass production, nutritional, and antioxidant quality under control conditions (Atero-Calvo et al. 2023). The experimental design comprised a randomized complete block featuring six treatments, each with three replications, and eight plants per replicate.

Plant sampling

Leaves and roots from plants of each treatment were sampled, weighed to measure fresh weight (FW), and subsequently rinsed with distilled water before being dried on filter paper. From each treatment, half of the leaves were preserved at -45°C for future biochemical analysis. The remaining half of the leaves and roots were dried by a forced-air oven at 70°C to obtain dry weight (DW). To determine the relative growth rate (RGR), leaves from each treatment were sampled before the treatments started (initial time, $T_i=0$ days), dried, and weighed (initial DW, DW_i). At the end of the trial (final time, $T_f=30$ days), leaves DW (final DW, DW_f) from each treatment was employed to

estimate RGR as $(\ln DW_f - \ln DW_i)/(T_f - T_i)$ (Navarro-León et al. 2020c). Furthermore, salt tolerance index (STI) was also estimated as $(\text{Total FW of salinity plants} / \text{Total FW of control}) \times 100$ (Navarro-León et al. 2020b). In addition, leaf area was determined using a LI-COR (LI-3000A) optical reader (LI-COR Inc. Nebraska, USA).

Na^+ and K^+ concentrations

Samples of dried leaves and roots (0.15 g) were used to estimate Na^+ and K^+ concentrations through a mineralization process by wet digestion (Wolf 2008). Dry leaves and roots were mineralized using a nitric acid (HNO_3)/perchloric acid (HClO_4) mixture (v/v) with H_2O_2 (30%). After added a total of 20 mL of mili-Q H_2O at the end of the mineralization, the concentration of Na^+ and K^+ was measured by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA).

In addition, the distribution coefficient (DC) of Na^+ and K^+ was calculated as $\text{DC} = [\text{Ion}] \text{ in leaves} / [\text{Ion}] \text{ in roots}$.

Pigment concentrations

Leaves (0.1 g) were macerated in methanol (1 mL). After centrifugation ($5000 \times g$, 5 min), the supernatant was used to measure absorbance at 666, 653, and 470 nm. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids concentrations were calculated following the equations proposed in Wellburn (1994).

Leaf gas exchange parameters

Parameters related to leaf gas exchange were estimated using a LICOR 6800 (IRGA: LICOR Inc. Nebraska, USA), an infra-red gas analyzer. System warmup tests were run before measurements following the manufacturer's recommendations. The environmental conditions of leaf chamber were then adapted to the physiological requirements of lettuce culture, as described previously (Hidalgo-Santiago et al. 2021). Thus, all parameters were measured with CO_2 concentration at $400 \mu\text{mol mol}^{-1}$, relative humidity at 70%, chamber fan mixing speed at 10,000 rpm, 25°C leaf temperature, PAR at $350 \mu\text{mol m}^{-2} \text{s}^{-1}$. Gas exchange measurements were taken between 10.00 a.m. and 02.00 p.m., in fully expanded leaves at the midstem position in six plants per treatment. For each plant, a total of nine measurements were recorded and the mean was used for: net photosynthetic rate (*A*), transpiration rate (*E*), stomatal conductance (*g_s*), and intercellular CO_2 (*C_i*). The equation *A/E* was employed to determine the water use efficiency (WUE).

In addition, after each measurement of these parameters, the Rubisco maximum carboxylation rate (V_{cmax}) was estimated using a rapid *A-C_i* response curve (RACiR). For

this purpose, increasing concentrations of CO₂ (from 10 to 510 μmol mol⁻¹) were applied to lettuce leaf. As CO₂ concentration increased, *A* and *Ci* were recorded every 2 s for 7 min. Furthermore, before the first measurement, a RACiR curve was obtained with the chamber closed and without the leaf to correct data. *V_{cmax}* was estimated through the ‘Plantecophys’ package in R (Duursma 2015) by fitting previously the data following the Farquhar model (Farquhar et al. 1980).

Chl *a* fluorescence measurement

Six leaves from each treatment located in the middle section of the stem were exposed to darkness using a clip for 30 min. Subsequently, Chl *a* fluorescence parameters were estimated through a Chlorophyll Fluorimeter (Handy PEA, Hansatech Ltd., King’s Lynn, Norfolk, UK), by fluorescence induction at 650 nm of red light. These parameters were: Area (the region above the transient curve), Fv/Fm (maximum quantum yield of primary photochemistry), PI_{ABS} (performance index), Φ_{E0} (maximum quantum yield of electron transport), Sm (energy needed to reduce reaction centres, N (the number of times that quinone A (Q_A) is reduced from time 0 to the time of maximum fluorescence), and ETo/RC (the electron flux from Q_A) (Strasser et al. 2004).

Lipid peroxidation and ROS concentrations

The MDA concentration was measured at 532 nm and 600 nm as described by Fu and Huang (2001). In addition, the electrolyte loss test was assayed to determine the electrolyte leakage (EL). Fresh leaves (0.3 g) were placed in tubes with 30 mL of distilled water. After the tubes were vortexed, a conductivity meter (Cond 8; XS Instruments, Italy), was employed to determine the initial conductivity (EC1). Afterwards, an incubation at 100 °C for 20 min was carried out, and the final conductivity (EC2) was measured. Using the formula $EL = EC1/EC2 \times 100$, the EC percentage was determined (Soloklui et al. 2012).

The method described in Kubiś (2008) and based on the nitroblue tetrazolium (NBT) reduction at 580 nm was followed to estimate O₂⁻ concentration, while H₂O₂ was assayed colorimetrically at 350 nm (Junglee et al. 2014).

Antioxidant enzyme activities

The SOD activity determination was performed according to Yu et al. (1998), which is based on the inhibition of NBT. The APX was determined following the method of Rao et al. (1996), measuring H₂O₂ reduction for 5 min at 290 nm.

AsA, GSH, total phenols, and flavonoids

Total and reduced AsA were determined by measuring at 525 nm the Fe³⁺ reduction by AsA. Dehydroascorbate (DHA) was estimated as total AsA–reduced AsA (Law et al. 1983). Total GSH and oxidized GSH (GSSG) were assayed following the methodology of Noctor and Foyer (1998), based on GSSG reduction to GSH through glutathione reductase, and the subsequent DTNB oxidation, measuring the absorbance at 412 nm. Reduced GSH was calculated as total GSH–GSSG.

Total phenols and flavonoids were measured according to (Rivero et al. 2001). Folin–Ciocâlțeu reagent was employed to measure total phenols concentration at 725 nm, using a curve of caffeic acid, while flavonoids were estimated at 415 nm against a curve of rutin.

Proline concentration

0.1 g of leaves were macerated with 1.2 mL of 83% ethanol. After centrifugation (2800×g, 10 min), 1 mL of supernatant was mixture with 4 mL of distilled water, 2.5 mL of ninhydrine, and 2.5 mL of 95% acetic acid were added. After incubation at 100 °C for 45 min, 3 mL of benzene were added, and the absorbance was determined at 515 nm against a curve of proline (Pro) (Irigoyen et al. 1992).

Statistical analysis

The statistical procedure was performed using Statgraphics Centurion 16.1.03 software. A simple analysis of variance (ANOVA) at 95% confidence was conducted to evaluate the data obtained. The means were compared by the Fisher’s least significant difference test (LSD) and the significance levels were indicated as: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS not significant.

Results

Plant biomass, RGR, and STI

Salinity stress conditions without biostimulant application caused a significant reduction in shoot FW (– 75%), shoot DW (– 62%), foliar area (– 64%), shoot RGR (– 40%), root FW (– 38%), and root DW (– 67%) with respect to control treatment. Nevertheless, these reductions were significantly lower in plants subjected to HS: shoot FW (– 61%, – 65%, – 59%, – 55%), shoot DW (– 31%, – 34%, – 37%, – 31%), foliar area (– 53%, – 54%, – 48%, – 46%), shoot RGR (– 14%, – 14%, – 18%, – 14%), root FW (– 2%, – 19%, – 22%, – 18%), and root DW (17%, 6%, 0%, 0%), for R-HS 0.40 mL/L, 0.60 mL/L, and F-HS 7.50 mL/L and

10.00 mL/L, respectively. In addition, although no differences were observed between modes of application in terms of shoot DW, F-HS caused a higher increase in shoot FW, foliar area, and shoot RGR, compared to R-HS, showing a slight increase in these parameters at 10.00 mL/L dose. Concerning STI, it was enhanced in lettuce plants treated with HS (55%, 44%, 78%, 84%, for R-HS 0.40 mL/L, R-HS 0.60 mL/L, F-HS 7.50 mL/L, and F-HS 10.00 mL/L, respectively, compared to lettuce non-treated with HS), (Table 1; Fig. 1).

Na⁺ and K⁺ concentrations, DC, and Na⁺/K⁺ ratio

Under salinity stress without biostimulant application, shoot and root Na⁺ concentrations, as well as Na⁺ DC increased (908%, 188%, and 250%, respectively), compared with control treatment (Fig. 2A–C). However, shoot Na⁺ increased was lower after HS application at R-HS 0.40 mL/L (813%), 0.60 mL/L (801%), and F-HS 10.00 mL/L (801%) (Fig. 2A). In addition, the increase in

root Na⁺ concentration was significantly higher (Fig. 2B) and, subsequently, the increase in Na⁺ DC was lower in HS-treated plants (199%, 193%, 208%, and 184%, at R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and 10.00 mL/L, respectively), with F-HS 10.00 mL/L being the lowest value (Fig. 2C). Furthermore, salt stress reduced shoot K⁺ – 19%), being this reduction lower under biostimulant application (– 7%, – 7%, – 8%, and – 7% at R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and 10.00 mL/L, respectively) (Fig. 2D). Besides, F-HS 7.50 mL/L increased root K⁺ concentration (30%) (Fig. 2E). In this way, K⁺ DC was reduced by 100 mM NaCl application (– 25%), compared with control plants, although this reduction was lower with R-HS 0.40 (– 7%), R-HS 0.60 mL/L (– 12%), and F-HS 10.00 mL/L (– 16%) (Fig. 2F). Thus, salinity stress without biostimulant increased Na⁺/K⁺ ratio in leaves (1140%), whereas this increase was significantly lower in HS-treated plants (869%, 877%, 979%, 886%, for R-HS 0.40 mL/L, R-HS 0.60 mL/L, F-HS 7.50 mL/L, and F-HS 10.00 mL/L, respectively) (Fig. 2G).

Table 1 Effect of salinity stress and HS application on shoot and root biomass, foliar area, shoot RGR, and STI

	Shoot FW	Shoot DW	Foliar area	Shoot RGR	Root FW	Root DW	STI
Control	73.03 ± 2.44a	3.16 ± 0.13a	550.83 ± 28.40a	0.078 ± 0.002a	6.03 ± 0.30a	0.35 ± 0.01b	
100 mM NaCl	18.61 ± 0.96d	1.20 ± 0.05c	199.64 ± 6.91d	0.047 ± 0.001c	3.72 ± 0.08c	0.27 ± 0.01c	26.79 ± 0.77c
100 mM NaCl + R-HS 0.40 mL/L	28.49 ± 0.94bc	2.18 ± 0.13b	260.81 ± 5.83c	0.067 ± 0.002b	5.90 ± 0.26a	0.41 ± 0.02a	41.45 ± 0.86b
100 mM NaCl + R-HS 0.60 mL/L	25.65 ± 0.56c	2.08 ± 0.12b	251.23 ± 9.79c	0.067 ± 0.001b	4.89 ± 0.35b	0.37 ± 0.03ab	38.63 ± 0.96b
100 mM NaCl + F-HS 7.50 mL/L	30.22 ± 1.97b	2.00 ± 0.17b	283.79 ± 5.31b	0.064 ± 0.003b	4.73 ± 0.20b	0.35 ± 0.03b	47.61 ± 1.38a
100 mM NaCl + F-HS 10.00 mL/L	32.72 ± 1.82b	2.17 ± 0.19b	295.64 ± 7.18b	0.067 ± 0.003b	4.84 ± 0.08b	0.35 ± 0.02b	49.20 ± 1.63a
p value	***	***	***	***	***	**	***
LSD _{0.05}	4.55	0.42	40.31	0.006	0.72	0.06	3.35

Shoot FW (Fresh weight), DW (Dry weight), root FW, and DW are expressed as g plant⁻¹; Foliar area is expressed as cm²; Shoot Relative Growth Rate (RGR) is expressed as g g⁻¹ day⁻¹; Salt Tolerance Index (STI) is expressed as %. R-HS Radicular-Humic substances, F-HS Foliar-Humic substances. Values are means ± standard error (n=6). The levels of significance were represented as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Values with different letters indicate significant differences

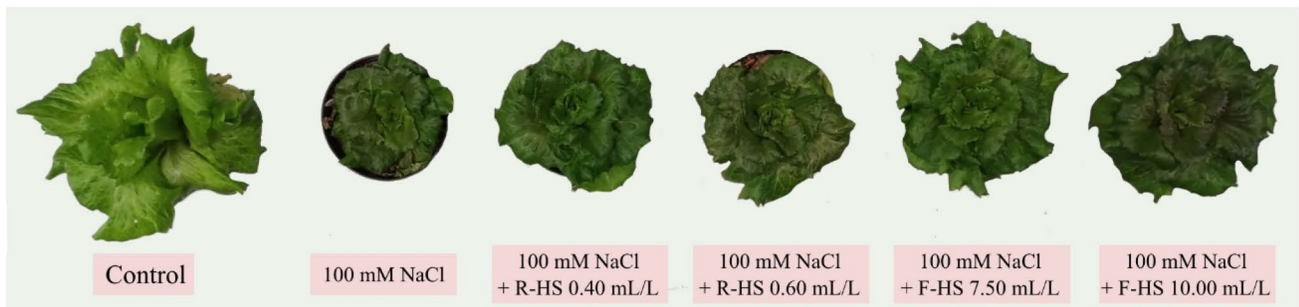


Fig. 1 Effect of salinity stress and HS application on lettuce plants growth. R-HS Radicular-Humic substances, F-HS Foliar-Humic substances

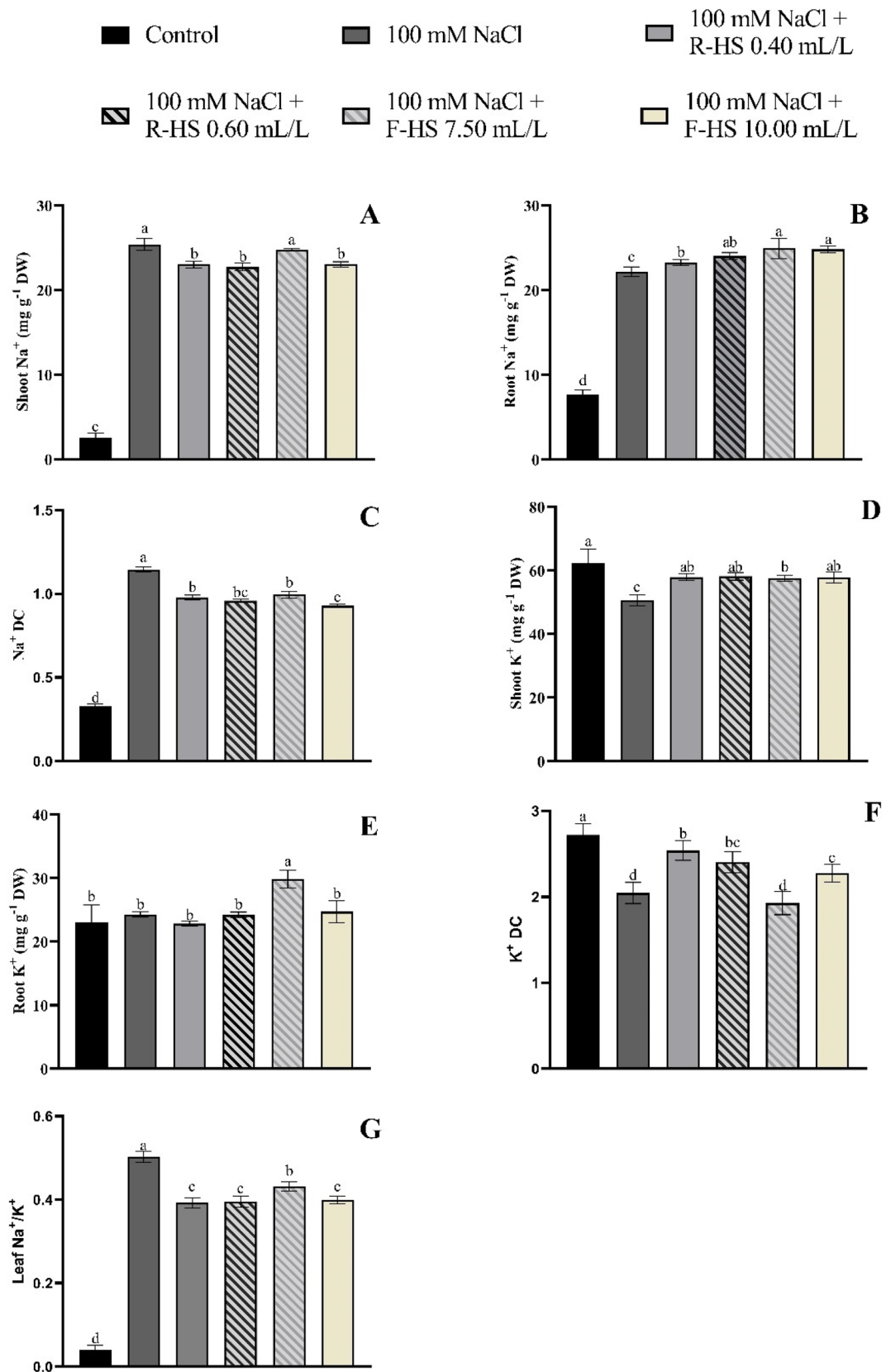


Fig. 2 Effect of salinity stress and HS application on shoot Na^+ (A), root Na^+ (B), Na^+ DC Distribution coefficient (C), shoot K^+ (D), root K^+ (E), K^+ DC (F), and leaf Na^+/K^+ ratio (G). R-HS Radicular-Humic substances, F-HS Foliar-Humic substances. Values are expressed as means \pm standard error ($n=6$). Columns marked with the same letters were not significantly different based on the LSD test ($p < 0.05$)

Photosynthetic pigments concentration

The application of salinity stress without biostimulant did not affect Chl *a*, Chl *b*, and carotenoids concentrations compared with control treatment. Furthermore, R-HS 0.40 and 0.60 mL/L as well as F-HS 10.00 mL/L significantly enhanced Chl *a* (9%, 19%, and 9%, respectively) and *b* (8%, 16%, and 7%, respectively) concentrations, while R-HS 0.60 mL/L also increased carotenoids (7%), with respect to salt stress only. However, F-HS 7.50 mL/L significantly reduced photosynthetic pigments concentration (− 13%, − 11%, and − 19% for Chl *a*, *b*, and carotenoids, respectively), with respect to salt stress without biostimulant (Table 2).

Leaf gas exchange

In general, salinity conditions without biostimulant reduced *A* (− 59%), *E* (− 67%), *Ci* (− 20%), and *gs* (− 82%), compared with plants not supplied with 100 mM NaCl. Nevertheless, the reduction in *A* was lower under HS treatments (− 33%, − 32%, − 34%, and − 19%, at R-HS 0.40 and 0.60 mL/L and F-HS 7.50 and 10.00 mL/L, respectively). Furthermore, plants subjected to F-HS 10.00 mL/L did not show significant differences in *A*, *E*, and *Ci*, compared with control conditions, and increased *gs* with respect to stressed plants without HS. Besides, salinity stress without HS reduced *Vcmax* (− 46%), whereas HS application significantly enhanced it (12%, 14%, 19%, and 28% for R-HS 0.40 and 0.60 mL/L and F-HS 7.50 and 10.00 mL/L, respectively), compared with control conditions. Regarding WUE, a significant increase was found under salinity compared to conditions, presenting HS higher values (49%, 79%, 51%, and 67% at R-HS 0.40 and 0.60 mL/L and F-HS 7.50 and 10.00 mL/L, respectively) (Table 3).

Chl *a* fluorescence

Plants treated with 100 mM NaCl without HS application showed a significant decrease in area (− 42%), Fv/Fm (− 3%), PI_{ABS} (− 11%), Φ_{Eo} (− 5%), Sm (− 21%), N (− 37%), and ETo/RC (− 24%), compared with control conditions. This reduction was significantly lower in plants treated with the leonardite-SC-based product. In addition, all HS doses increased PI_{ABS} compared to control treatment (20%, 10%, 6%, 28% for R-HS 0.40 and 0.60 mL/L and F-HS 7.50

and 10.00 mL/L, respectively), and did not affect Φ_{Eo} , with respect to control treatment, although the higher values of Φ_{Eo} were found after F-HS 10.00 mL/L application (Fig. 3, Table S1).

Oxidative stress indicators

Salinity conditions without biostimulant application increased EL (171%) percentage and MDA (338%) concentration, although this increase was significantly lower in lettuce supplied with HS (128%, 127%, 116%, and 102% for EL, 241%, 257%, 133%, and 165% for MDA, under R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 mL/L and 10.00 mL/L, respectively) (Fig. 4A, B). Consequently, $\text{O}_2^{\cdot-}$ generation was higher after 100 mM NaCl addition without HS (225%), whereas under HS lower $\text{O}_2^{\cdot-}$ was found (205%, 203%, 169%, and 160% for R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 mL/L and 10.00 mL/L, respectively) (Fig. 4C). Similarly, H_2O_2 was increased under salt stress conditions (37%), with lower values in HS-treated plants (9% and 8% for F-HS 7.50 and 10.00 mL/L, respectively), whereas R-HS did not show differences compared with the control (Fig. 4D).

Antioxidant enzyme activities

SOD activity was significantly increased under 100 mM NaCl without HS (14%), compared with control treatment. However, plants treated with all HS doses showed a decrease in SOD activity (− 11%, − 22%, − 30%, and − 30% at R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and 10.00 mL/L, respectively) (Fig. 5A). Concerning APX, and comparing with control plants, salt stress conditions without HS as well as R-HS at 0.40 and 0.60 mL/L enhanced APX activity (27%, 20%, and 20%, respectively), whereas both F-HS did not affect it (Fig. 5B).

Non-enzymatic antioxidants

Total phenols and flavonoids were increased under salinity stress without biostimulant (184% and 88%, respectively) compared with the control treatment. Nevertheless, this increase was lower under HS application for both total phenols (141%, 100%, 73%, 108%) and flavonoids (54%, 45%, 28%, 39%) at R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and 10.00 mL/L, respectively. In addition, total AsA was also enhanced under salt stress (60%, 63%, 39%, 53%, and 58%, for non-treated plants with biostimulant, R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and 10.00 mL/L, respectively), compared with control conditions. Salinity treatment without HS also increased AsA concentration (23%). However, R-HS 0.40 mL/L and F-HS 7.50 mL/L did not affect AsA concentration, whereas R-HS 0.60 mL/L and F-HS 10.00 mL/L decreased it (− 28 and − 30%, respectively),

Table 2 Effect of salinity stress and HS application on Chl a, Chl b, and carotenoids concentrations

	Chl a	Chl b	Carotenoids
Control	0.270 ± 0.002bc	0.144 ± 0.002bc	29.02 ± 0.47c
100 mM NaCl	0.265 ± 0.003c	0.139 ± 0.002c	30.64 ± 0.70bc
100 mM NaCl + R-HS 0.40 mL/L	0.289 ± 0.013b	0.150 ± 0.005b	31.80 ± 0.70ab
100 mM NaCl + R-HS 0.60 mL/L	0.315 ± 0.008a	0.161 ± 0.004a	32.87 ± 0.55a
100 mM NaCl + F-HS 7.50 mL/L	0.230 ± 0.005d	0.128 ± 0.001d	23.41 ± 0.60d
100 mM NaCl + F-HS 10.00 mL/L	0.289 ± 0.004b	0.149 ± 0.001b	31.15 ± 0.73ab
p value	***	***	***
LSD _{0.05}	0.019	0.008	1.79

Chl (Chlorophyll) a and Chl b are expressed as mg g⁻¹ DW; carotenoids are expressed as µg g⁻¹ DW. *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances. Values are means ± standard error (n=9). The levels of significance were represented as ***p < 0.001. Values with different letters indicate significant differences

compared with the control. Moreover, DHA was enhanced under salinity (with or without HS application), being this increment higher in HS-treated plants, and showing F-HS 10.00 mL/L the highest value (180%). Regarding total GSH, salinity without BLAKCJAK® application increased its concentration (92%) compared with the control, whereas this increment was lower in HS-treated plants (47%, 33%, 31%, and 32%, at R-HS 0.40 and 0.60 mL/L, and F-HS 7.50 and

10.00 mL/L, respectively). Besides, salinity stress without biostimulant and salinity stress with R-HS at both doses increased GSH (119%, 40%, and 46%, respectively), compared to control. In addition, salinity enhanced GSSG, showing salt stress without HS and R-HS 0.40 mL/L the largest increase (56% and 57%, respectively) (Table 4).

Pro concentration

Plants subjected to salinity stress conditions and not treated with leonardite-SC showed a significant increase in Pro accumulation (311%) compared with the control treatment. Similarly, plants treated with R-HS 0.60 mL/L also presented an increase in Pro with respect to control (148%), although this increase was lower. However, the remaining HS doses did not significantly affect Pro concentration with respect to control plants, and lower Pro levels were found in F-HS (Fig. 6).

Discussion

Biomass production, RGR, and STI

The main effect of salt stress is plant growth reduction, as observed in different vegetables, including lettuce plants (Leyva et al. 2011; Navarro-León et al. 2020a; Behera et al. 2022; Sardar et al. 2023). In the present study, salt stress reduced plant growth parameters, whereas the application of radicular and foliar-HS caused a lower reduction in lettuce growth, which resulted in higher STI. Moreover, better plant tolerance was observed in plants subjected to F-HS, particularly at 10.00 mL/L, in terms of FW (Table 1,

Table 3 Effect of salinity stress and HS application on leaf gas exchange parameters

	A	E	Ci	gs	Vcmax	WUE
Control	9.35 ± 0.92a	3.58 ± 1.07a	359.55 ± 10.42a	0.148 ± 0.022a	39.91 ± 1.14c	2.29 ± 0.15d
100 mM NaCl	3.84 ± 0.91c	1.18 ± 0.19b	289.39 ± 22.07b	0.027 ± 0.002c	21.39 ± 0.04d	2.92 ± 0.10c
100 mM NaCl + R-HS 0.40 mL/L	6.28 ± 0.62b	1.88 ± 0.11b	295.08 ± 22.61ab	0.056 ± 0.005bc	44.58 ± 1.31b	3.41 ± 0.11b
100 mM NaCl + R-HS 0.60 mL/L	6.34 ± 0.34b	1.52 ± 0.08b	233.31 ± 25.62b	0.042 ± 0.002bc	45.68 ± 0.10b	4.11 ± 0.09a
100 mM NaCl + F-HS 7.50 mL/L	6.21 ± 0.85b	1.69 ± 0.36b	257.01 ± 26.32b	0.047 ± 0.011bc	47.50 ± 0.84ab	3.45 ± 0.04b
100 mM NaCl + F-HS 10.00 mL/L	7.59 ± 0.77ab	2.16 ± 0.02ab	301.09 ± 21.74ab	0.071 ± 0.010b	50.99 ± 2.85a	3.83 ± 0.02a
p value	**	*	*	***	***	***
LSD _{0.05}	2.35	1.51	68.10	0.035	4.33	0.22

A (Net photosynthetic rate) is expressed as µmol m⁻² s⁻¹; E (Transpiration rate) is expressed as mmol m⁻² s⁻¹; Ci (Intercellular CO₂) is expressed as µmol mol⁻¹; gs (Stomatal conductance) is expressed as mol m⁻² s⁻¹; and Vcmax (Rubisco maximum carboxylation rate) is expressed as µmol m⁻² s⁻¹. *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances. Values are means ± standard error (n=6). The levels of significance were represented as *p < 0.05, **p < 0.01, and ***p < 0.001. Values with different letters indicate significant differences

Fig. 3 Effect of salinity stress and HS application on Chl *a* fluorescence parameters. Fv/Fm (Maximum quantum yield for primary photochemistry), PI_{ABS} (Performance index), ΦE_o (Maximum quantum yield for electron transport), Sm (Energy needed to reduce reaction centres), *N* Number of times that quinone A is reduced from time 0 to time the maximum fluorescence is reached, *ETo/RC* The electron flux from quinone A. *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances. All parameters are represented on the same scale, for which data were normalized. Values are means from six data (n=6)

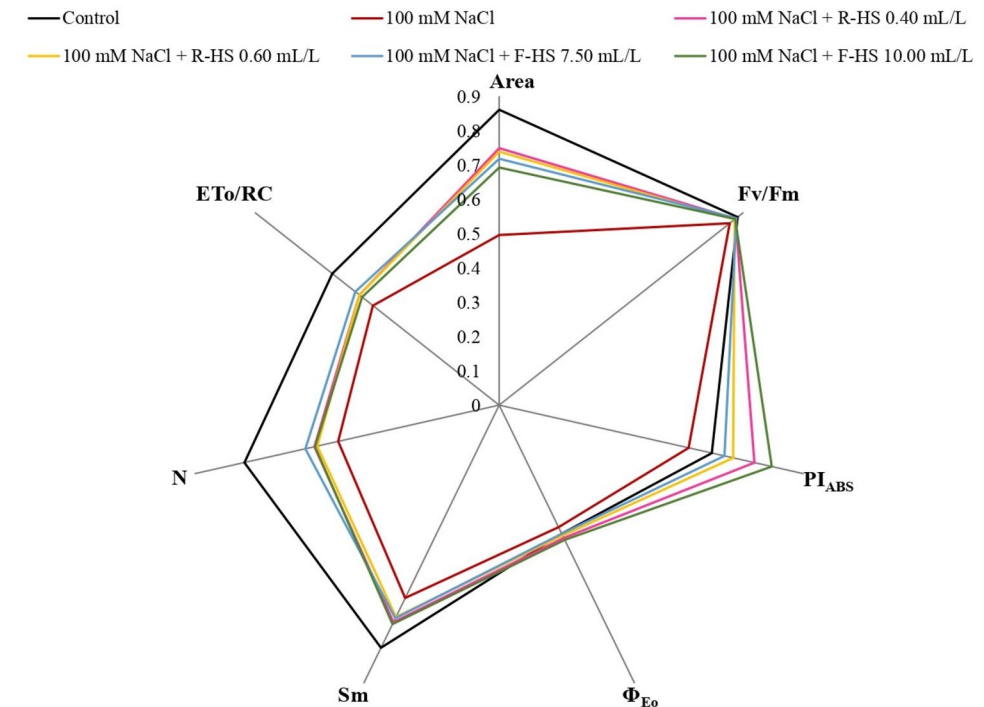


Fig. 1). This fact is of great importance, considering that lettuce is a leafy vegetable mainly consumed fresh (Tan et al. 2020). Our results align with different studies that have shown the potential use of radicular and foliar HS as plant biostimulants to enhance salt tolerance. In this way, Moubarak et al. (2022) found that radicular HA application minimized the negative effect of salinity on lettuce growth. Similar results were shown in strawberry plants by adding HA to nutritive solution (Saidimoradi et al. 2019), in wheat subjected to foliar HA and FA (Sary and Hamed 2021), and in citrus seedlings treated with radicular FA (Zhang et al. 2023). Hence, our findings suggest that the leonardite-SC-based product used in the present study could be a good candidate for increasing lettuce tolerance to salinity stress, being foliar method slightly better than radicular, especially at 10.00 mL/L. These results could be attributed to different physiological mechanisms, including an enhancement in photosynthesis activity, decreasing ROS generation and ionic imbalances, and maintaining cell membrane stability (Acosta-Motos et al. 2017). In addition, among the physiological effects of HS, the induction of root plasma membrane (PM)-H⁺-ATPase, stands out as a mechanism of action to increase root growth, nutrient uptake, and shoot growth (Olaetxea et al. 2019), which could favour lettuce growth under salt stress. Thus, Souza et al. (2021) found an induction of root PM-H⁺-ATPase activity in tomato, thereby preventing salt damage. Thus, BLAKCJAK® application could increase PM-H⁺-ATPase activity, enhancing lettuce growth in saline conditions, although further research is needed.

Na⁺ and K⁺ concentrations and ratio

The toxic effects of salinity stress are primarily attributed to Na⁺, whose increase is correlated with a decrease in essential nutrients such as K⁺. Thus, ionic imbalances caused by salinity negatively impact cell metabolism and essential enzyme activities (Javed et al. 2022). Therefore, reducing Na⁺ uptake and translocation to leaves, while maintaining a low Na⁺/K⁺ ratio leads to increased tolerance to salinity (Navarro-León et al. 2020b). In our experiment, the application of root and foliar-HS caused a reduction in Na⁺ translocation from roots to leaves, especially at F-HS 10.00 mL/L (Fig. 2C). Furthermore, an increase in K⁺ translocation to the shoot was observed, in general, after BLACKJAK® application, resulting in higher K⁺ accumulation (Fig. 2E, F). Consequently, HS caused a lower Na⁺/K⁺ ratio (Fig. 2G), which might aid in salt tolerance.

As previously observed, the application of HS-based products under salinity conditions protects plants from ionic imbalances, thus improving growth under salinity. Thus, commercial HA added to nutrient solution reduced Na⁺/K⁺ ratio in pepper plants subjected to salt stress (Bacilio et al. 2016). Besides, radicular-HA reduced Na⁺ accumulation in shoots, while enhancing K⁺ in two cultivars of strawberry (Saidimoradi et al. 2019). Similar results were subsequently obtained in citrus subjected to FA (Zhang et al. 2023) and in quinoa plants treated with HA (Rekaby et al. 2023), under salt stress conditions. Khaleda et al. (2017) suggested the induction of a sodium influx transporter HIGH-AFFINITY K⁺ TRANSPORTER (HKT1) as a possible mechanism

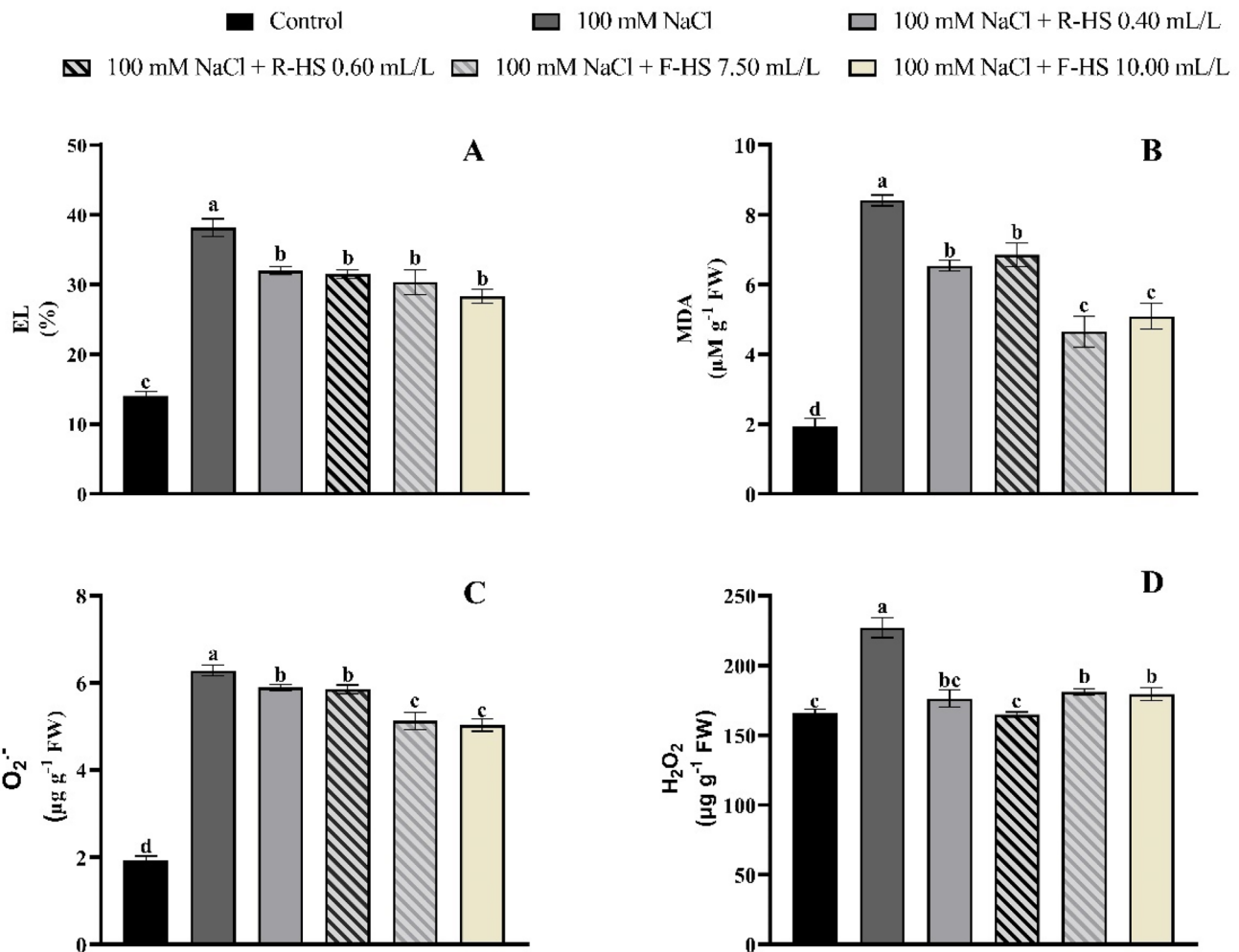


Fig. 4 Effect of salinity stress and HS application on oxidative stress indicators: *EL* Electrolyte leakage (A), *MDA* (Malondialdehyde) (B), *O₂^{·-}* (Superoxide radical) (C), and *H₂O₂* (Hydrogen peroxide) (D). *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances.

Values are expressed as means \pm standard error ($n=9$). Columns marked with the same letters were not significantly different based on the LSD test ($p < 0.05$)

underlying the reduction in Na^+ translocation to shoots after HS application. In this way, HA induced HKT1 in *Arabidopsis thaliana* under saline conditions, reducing the Na^+ flux to shoots, and improving salt tolerance. In addition, Na^+ compartmentalization in vacuoles or apoplast through the induction of membranes transporters, has also been proposed as HS-mechanism of action to mitigate salt stress. Thus, Souza et al (2021) found that HA induced SOS3 (Salt Overlay Sensitive-3) expression in maize plants, suggesting that it could prepare a molecular response to salt stress, reducing cytosol Na^+ content. Although BLACKJAK® reduced Na^+ translocation (Fig. 2C), increased leaves K^+ accumulation (Fig. 2D), and decreased Na^+/K^+ ratio (Fig. 2G), the differences in relation to the negative saline control were not excessively high. For this reason, BLACKJAK® could promote Na^+ compartmentalization in vacuoles or apoplast. Nevertheless, to test this hypothesis, future studies focusing

on the HS induction of membranes Na^+ and K^+ transporters, such as HKT or SOS, are needed to elucidate the mechanism of action of HS to alleviate salt stress.

Photosynthesis performance

Photosynthetic activity reduction is common in plants exposed to salinity due to the inhibition of photosynthetic pigments synthesis, damages to photosynthetic machinery, decreasing electron (e^-) transport flux between photosystem II (PSII) and I (PSI), affecting Rubisco activity and CO_2 assimilation efficiency (Acosta-Motos et al. 2017; Yang et al. 2020; Zahra et al. 2022). In this context, photosynthetic pigments (Chls *a* and *b*, and carotenoids) concentrations could be reduced under salinity (Hamani et al. 2020; Shen et al. 2022; Singh et al. 2022b). However, in the present study, the application of 100 mM NaCl did

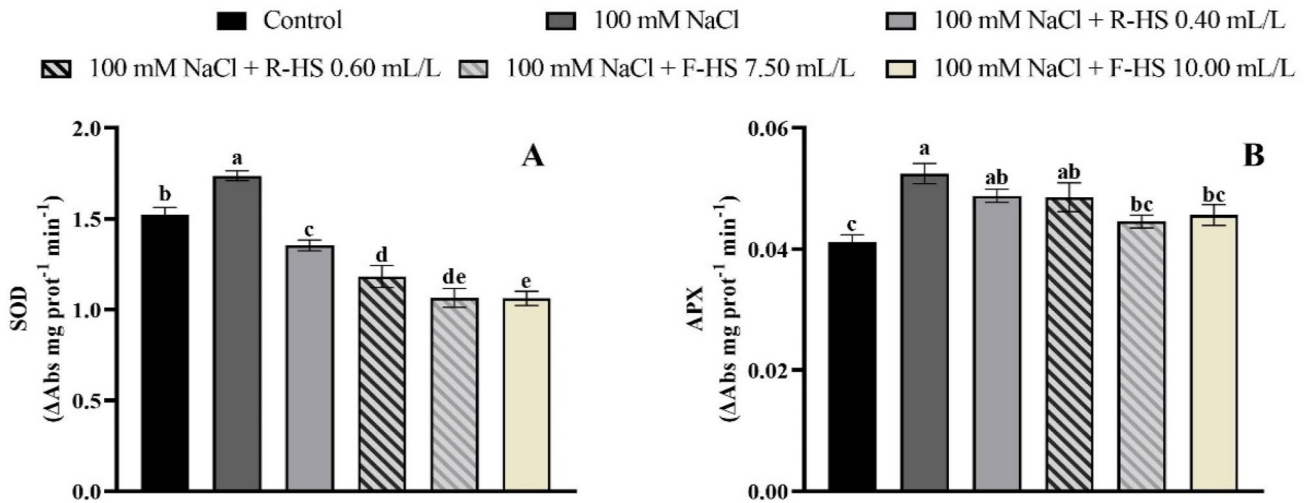


Fig. 5 Effect of salinity stress and HS application on *SOD* Superoxide dismutase (A) and *APX* Ascorbate peroxidase (B) activities. *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances. Values

are expressed as means \pm standard error ($n=9$). Columns marked with the same letters were not significantly different based on the LSD test ($p < 0.05$)

Table 4 Effect of salinity stress and HS application on non-enzymatic antioxidants concentration

	Total Phenols	Flavonoids	Total AsA	AsA	DHA	Total GSH	GSH	GSSG
Control	1.71 \pm 0.08e	1.55 \pm 0.03e	93.31 \pm 4.43d	54.29 \pm 1.61bc	39.03 \pm 2.82e	79.01 \pm 3.16c	45.32 \pm 2.90c	33.69 \pm 0.31d
100 mM NaCl	4.86 \pm 0.12a	2.91 \pm 0.06a	148.94 \pm 1.31ab	66.81 \pm 3.53a	82.13 \pm 2.39d	151.74 \pm 3.64a	99.08 \pm 3.21a	52.66 \pm 0.47a
100 mM NaCl + R-HS 0.40 mL/L	4.12 \pm 0.11b	2.38 \pm 0.06b	151.79 \pm 2.57a	50.67 \pm 0.79c	101.11 \pm 1.82b	116.51 \pm 0.87b	63.48 \pm 3.06b	53.03 \pm 0.20a
100 mM NaCl + R-HS 0.60 mL/L	3.43 \pm 0.05c	2.25 \pm 0.06bc	129.87 \pm 1.65c	39.27 \pm 1.60d	89.50 \pm 0.99c	105.39 \pm 0.79b	65.97 \pm 1.07b	39.42 \pm 1.00c
100 mM NaCl + F-HS 7.50 mL/L	2.96 \pm 0.12d	1.99 \pm 0.06d	142.79 \pm 2.18b	57.55 \pm 2.89b	86.97 \pm 1.90 cd	103.27 \pm 13.25b	56.13 \pm 12.70bc	47.14 \pm 0.66b
100 mM NaCl + F-HS 10.00 mL/L	3.55 \pm 0.08c	2.15 \pm 0.07 cd	147.18 \pm 1.49ab	37.82 \pm 0.43d	109.36 \pm 1.45a	104.19 \pm 1.61b	58.06 \pm 1.18bc	46.13 \pm 1.02b
p value	***	***	***	***	***	*	***	***
LSD _{0.05}	0.28	0.17	7.13	6.01	5.65	16.76	15.93	1.98

Total phenols and flavonoids are expressed as mg g⁻¹ FW, AsA (Ascorbic acid), DHA (Dehydroascorbic acid), and GSH (Glutathione) forms are expressed as μ g g⁻¹ FW. R-HS (Radicular-Humic substances), F-HS (Foliar-Humic substances). Values are means \pm standard error ($n=9$). Values with different letters indicate significant differences

not affect pigments concentration with respect to the control conditions. Besides, HS applied at R-HS at 0.60 mL/L enhanced Chls and carotenoids compared with control plants (Table 2). As has been previously observed, depending on plant species or NaCl concentration, pigments may not be affected by salinity (Cantabella et al. 2017). Thus, in some mint ecotypes, salinity did not affect pigments concentration (Hosseini et al. 2021). Akladios and Mohamed (2018) and Hassanein et al. (2022) also found that HA addition enhanced pigments concentration in salt-stressed pepper and coriander, respectively, with respect to control conditions, which agrees with our results for R-HS 0.60 mL/L.

A correct CO₂ supply is crucial for optimal Rubisco activity, the main enzyme of the photosynthetic process. Under stress conditions, Rubisco activity may be negatively affected. Thus, CO₂ fixation and assimilation and, ultimately, the net photosynthetic rate could be reduced, thereby affecting biomass production and plant development (Yan et al. 2022; Zahra et al. 2022). For this reason, measuring leaf gas exchange parameters through IRGARs analyzers offers us an approximation of the photosynthetic status of plants, which is of great importance, especially in abiotic stress studies (Navarro-León et al. 2023). In the present experiment, plants subjected to root or foliar-HS showed a lower

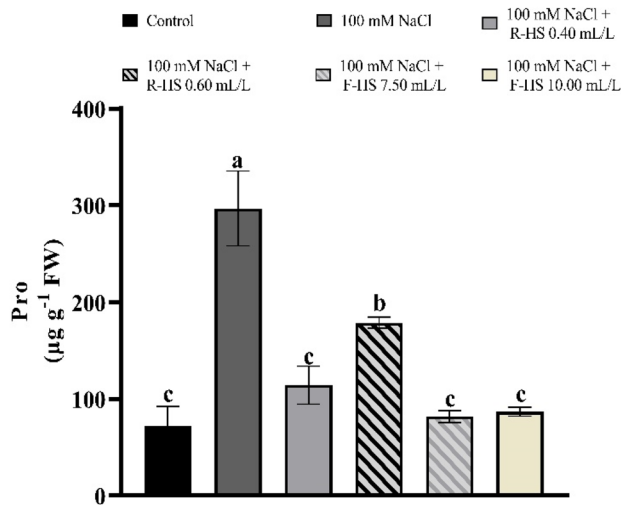


Fig. 6 Effect of salinity stress and HS application on *Pro* Proline concentration. *R-HS* Radicular-Humic substances, *F-HS* Foliar-Humic substances. Values are means \pm standard errors ($n=9$). Columns marked with the same letters were not significantly different based on the LSD test ($p < 0.05$)

reduction of *A* (Table 3). Similarly, it has been reported that HA enhance the photosynthetic rate in tomato plants under 200 mM NaCl (Souza et al. 2021), as well as in perennial ryegrass subjected to 250 mM NaCl (Meng et al. 2023), resulting in higher plant tolerance to salt stress. Additionally, salt-tolerant species display higher *A* under salt conditions with respect to salt sensitive (El-Hendawy et al. 2017; Mahlooji et al. 2018; Yang et al. 2020). A possible explanation for this improved photosynthetic activity is the reduction of toxic Na^+ translocation to leaves through apoplastic barriers (Yang et al. 2020). Hence, the decrease in Na^+ DC observed after HS application (Fig. 2C) could contribute to maintain a higher *A* (Table 3). Particularly, F-HS 10.00 mL/L was the dose that most reduced Na^+ DC and, consequently, did not present differences in *A* with respect to control, showing these plants higher salt tolerance.

In addition, the increased photosynthetic rate in salinity-tolerant plants is associated with enhanced CO_2 fixation efficiency through Rubisco activity (Acosta-Motos et al. 2017; Smith et al. 2023). Thus, in the present experiment, although no differences were observed in stomatal fluxes (*E* or *g_s*) among all the plants subjected to salinity treatments, the increased *V_{max}* found after HS application, especially with F-HS (Table 3) could contribute to maintaining *A* under these conditions. The physiological mechanisms through which HS stimulate plant growth have also been related to the enhancement of Rubisco activity, enabling higher photosynthetic rates and increased crop productivity (Ertani et al. 2019), which could be critical for plant growth under saline conditions (Kaushal 2020). Furthermore, enhanced *A* may explain the increased WUE in plants subjected to the

leonardite-SC-based product (Table 3), contributing to better water status, which is crucial under salinity stress (Sheteiwy et al. 2022). Our results are in line with other studies where HS helped maintain an efficient photosynthetic rate and WUE under abiotic stress, improving Rubisco activity, and conferring plant tolerance. In this way, Rady et al. (2016) reported a significant improvement in WUE and photosynthesis efficiency in cotton plants treated with HA under salt stress. Similarly, Chen et al. (2022a, b) found an increase in Rubisco activity and, consequently, an enhancement in *A* and WUE in two maize genotypes exposed to water deficit.

Photochemical reactions may also be affected by high NaCl concentrations (Zahra et al. 2022). An approximation to PSII activity could be achieved by Chl *a* fluorescence parameters analysis (Garab et al. 2023). Under stress conditions, Chl *a* fluorescence emission is increased and some parameters such as Area, Fv/Fm, and PI_{ABS} are decreased. In this way, Lucini et al. (2015) observed a reduction in the Fv/Fm ratio after the application of 25 mM NaCl to lettuce plants. Kalhor et al. (2018) also found that PI_{ABS} was reduced in lettuce subjected to 80 mM NaCl, whereas 40 and 80 mM NaCl also decreased Fv/Fm. Besides, in salt-tolerant plants, this reduction is lower than in salt-sensitive plants. For this reason, these parameters are usually employed as indicators of salt tolerance (Song et al. 2023). In addition, some Chl *a* fluorescence parameters indicate the efficiency of e^- transport through PSII, as Φ_{E_0} , Sm, N, or ETo/RC, which are decreased under abiotic stress (Kalhor et al. 2018). In the present study, the application of radicular and foliar-HS conferred a maintenance of PSII performance, enhancing e^- transport between PSII and PSI, and increasing Q_A reduction rate (Fig. 3, Table S1), thereby contributing to improved growth under salt stress. The role of HS in PSII status protection under salinity conditions was shown in *Plantago ovata* L. (Gholami et al. 2013), cotton (Rady et al. 2016), and maize plants (Kaya et al. 2018), where HS enhanced maximum quantum yield and performance index, which is in line with our results. In addition, it has also been reported that HA could protect PSII components, such as D1 protein, by decreasing excitation energy (Ozfidan-Konakci et al. 2018). Hence, our data suggest the protection of photosynthesis performance as a possible action mechanism to mitigate salt stress in lettuce by HS, although future research is required to uncover the fundamental molecular mechanisms behind this improvement.

Oxidative stress indicators and antioxidants

Salinity causes oxidative stress through an increase in ROS (i.e., $\text{O}_2^{\cdot-}$ and H_2O_2) production, which trigger damage to cell membranes, resulting in MDA generation and EL (Mousavi et al. 2022; Bai et al. 2023). As previously found, lettuce exposed to salt stress presented an increase in EL

(Hniličková et al. 2019) and MDA (Bai et al. 2023), which reduced plant growth. HS application resulted in reduced ROS generation and lipid peroxidation, showing F-HS doses the lowest O_2^- and MDA levels (Fig. 4A, D). The maintenance of higher K^+ concentrations is usually linked to lower membrane lipid oxidation (Cantabella et al. 2017; Navarro-León et al. 2020b). Therefore, the largest values of shoot K^+ and K^+ DC (Fig. 2D, F), found in lettuce treated with HS compared with stressed plants without HS, could counteract the cell membrane damage caused by salinity. Besides, our data align with other studies that demonstrate a reduction in ROS generation and lipid peroxidation under salinity induced by HS applications (Saidimoradi et al. 2019; Shukry et al. 2023; Zhang et al. 2023).

The generation of ROS under abiotic stress conditions, such as salinity, usually leads to an enhancement in enzymatic antioxidant activity and the biosynthesis of non-enzymatic antioxidants (Acosta-Motos et al. 2017). SOD and APX contribute to detoxify O_2^- and H_2O_2 , respectively, whereas phenolic compounds, AsA, and GSH may also detoxify ROS and reduce lipid peroxidation (Hasanuzzaman et al. 2021; Chourasia et al. 2022). Overall, the application of root and foliar-HS did not enhance the antioxidant capacity of lettuce subjected to salinity stress (Fig. 5A, B, Table 4). As previously reported, salt-tolerant plants present different physiological strategies to cope with salinity: avoid ionic imbalances, improve antioxidant capacity, enhance photosynthetic activity, or biosynthesis of compatible solutes (Acosta-Motos et al. 2017; Feng et al. 2019; Yang et al. 2020). In our experiment, the enhanced tolerance of lettuce treated with HS to salinity caused a reduction in antioxidants activity and biosynthesis, which indicates that salinity effects in these plants could be counteracted by other mechanisms such as improvement in K^+ homeostasis and photosynthetic activity. Similar results were observed by other studies, which demonstrated lower antioxidant enzymes activity (Saidimoradi et al. 2019) and antioxidant biosynthesis (Shukry et al. 2023) in HS-treated plants under saline conditions, showing these plants higher growth and lower oxidative damage.

Pro concentration

Pro concentration is usually increased after salinity stress exposed (De la Torre-González et al. 2018). Pro is an amino acid with multifunctionality under abiotic stress: it is an osmoprotectant, stabilizes protein structure, contributes to maintain cell turgor, and acts as a ROS scavenger (Alvarez et al. 2022). For this reason, Pro concentration is widely used as a salt stress indicator (Cantabella et al. 2017). The highest Pro accumulation was found in lettuce not treated with HS, whereas F-HS did not increase Pro levels (Fig. 6). Higher Pro concentration could indicate plant damage and negatively impact plant

growth (Shukry et al. 2023). Likewise, different studies agreed with our results. Thus, Saidimoradi et al. (2019) showed an increase in plant growth and a reduction in Pro accumulation in strawberry subjected to salinity + HA, suggesting that reduced Pro may indicate salinity stress alleviation. In addition, Shukry et al. (2023) found reduced Pro in maize plants subjected to salt stress and treated with HA, with respect to plants without biostimulant. These authors reported that plants subjected to HA presented higher biomass, despite reduced Pro, and concluded that these plants invested more energy in growth than in Pro biosynthesis. This fact could explain the results obtained in our experiment for both Pro accumulation and antioxidant capacity.

Conclusions

Our results indicate that root and foliar-HS applications at the doses employed enhance salt stress tolerance in lettuce, as evidenced by higher biomass production, shoot RGR, leaf area, and STI. This tolerance could be induced by HS's contribution to ionic homeostasis, reducing Na^+ translocation to the shoot, and increasing shoot K^+ concentration, as well as the improvement in photosynthesis performance. In addition, both root and foliar-HS applications reduced oxidative damage through lower ROS generation and lipid peroxidation. Comparing both modes of application, F-HS caused a better improvement in the analyzed parameters than R-HS, especially at 10.00 mL/L dose. Hence, our data suggest that BLAKCJAK® enhances lettuce growth under salinity stress with both radicular and foliar applications, being foliar mode slightly better. Future research, especially focusing on molecular aspects, would be crucial to understand the physiological mechanisms through which BLACKJAK® induces salt stress tolerance.

Author contributions FM, GM, BB, and JMR conceived and designed research. SAC and ENL conducted experiments. FM and GM provided biostimulant. SAC and ENL analyzed data. SAC wrote the manuscript. FM, GM, BB, and JMR did a critical revision of the article. All authors read and approved the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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