https://doi.org/10.17221/127/2024-PPS

Mitigation of salinity stress effects on *Vicia faba* L. growth and productivity using proline and salicylic acid foliar application

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Citation: El-Beltagi H.S., El-Nady M.F., Ismail A.M., Metwaly M.M.S. (2025): Mitigation of salinity stress effects on *Vicia faba* L. growth and productivity using proline and salicylic acid foliar application. Plant Protect. Sci., 61: 222–241.

Abstract: High soil salinity causes a negative impact on plant growth and lowers crop yields. Thus, pot experiments were conducted to investigate the impact of foliar application of salicylic acid (SA) and proline (Pro), separately and combined, on enhancing salinity tolerance in broad beans. Salinity stress (4.69 ds/m and 6.25 ds/m) significantly reduced plant growth (plant height, leaf area, number of leaf/plant, plant dry weight), chlorophyll pigment content (chlorophyll *a*, *b* or total), relative water content, K/Na ratio, seed yield per plant, and N, P, K, and crude protein content in broad bean seeds. Foliar application of Pro and SA, either individually or in combination, enhanced plant growth parameters, chlorophyll pigment content, endogenous proline levels, phenol content, and the activities of antioxidant enzymes [antioxidant enzymes including catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)]. Additionally, these treatments enhanced plant seed yield, N, P, K, and crude protein levels in the seeds. The combined foliar application of Pro and SA was more effective in mitigating salinity stress's harmful effects than using either substance alone. These findings indicate that foliar application of SA and Pro, either individually or in combination, alleviated the adverse effects of salinity on broad beans, with the combined application proving to be the most effective.

Keywords: chlorophyll pigments; endogenous proline content; antioxidant enzymes activity; relative water content; phenols content; membrane stability

Supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (KFU241386).

https://doi.org/10.17221/127/2024-PPS

Broad bean (*Vicia faba* L.) is the fifth most significant crop in the Fabaceae family, following pea, common bean, chickpea, and lentil (Singh et al. 2013). FAOSTAT (2019) reported that it has grown to 2.6 mil. ha worldwide, yielding an annual production of 5.4 mil. t. This leguminous crop is highly valued for its high protein content (27–40%), carbohydrates (50–60%), minerals, and vitamins (Kumar et al. 2015). Additionally, it enhances agricultural sustainability through its nitrogen-fixing capability, which boosts crop productivity (Barton et al. 2014). In Egypt, the broad bean is one of the main legumes grown. Data from official sources over the past three decades, from 1990 to 2020, reveal that the cultivated area for this crop decreased from around 117 200 ha during 1990–2000 to about 96 800 ha during 2000–2010, indicating a decline of approximately 18%. The cultivated area further decreased to about 40 400 ha from 2010 to 2020, representing a decline of about 65% compared with the first period and about 58% compared to the second period. As for the production quantity, it was noted that it decreased from about 328 000 t on average for the first period to about 314 000 t on average for the second period, reflecting a decline of about 25%. It then decreased to about 136 000 t in the third period, representing a decline of 67% compared with the first period and about 32% compared to the second period (Arafa 2022). At the same time that the cultivated area and production decreased, the population has been increasing, leading to a higher consumer appetite for food products in general or for broad beans in particular. Salt stress (SS) significantly reduces the cultivated areas for broad beans in arid or semiarid regions, like Egypt, resulting in decreased productivity (Abdelraouf et al. 2016).

Globally, soil salinity is a major environmental stress that significantly impacts the growth and development of crop plants throughout their entire life cycle, from seed germination to harvest (El-Mogy & Garchery 2018). This stress significantly reduces the world's food production, causing a 30% loss (Machado & Serralheiro 2017). Approximately one billion hectares of land are affected by soil salinization, and the affected area is growing by 2 mil. ha annually (Lhissoui et al. 2014). Salinity impacts over 7% of the world's land area and around 20% of arable land, particularly in arid or semiarid regions like Egypt (Scudiero et al. 2016). Additionally, it is forecasted that by 2050, more than 50% of the world's arable land will be affected by salinization (Alzahrani et al. 2021).

Salinity negatively affects plant growth through osmotic stress or ionic toxicity (Almeida et al. 2017). Toxic Na^+ and Cl^- ions levels cause nutritional imbalances in plants and create a physiological drought-like condition. This occurs because the high concentrations of these ions lower the osmotic potential of the soil solution (Khan et al. 2019). Consequently, plants struggle with water uptake, resulting in water stress and stunted growth, even when soil moisture is sufficient. In response to the physiological drought induced by high soil salinity, plants trigger the accumulation of compatible solutes, i.e. soluble sugars and proline (Ahmad et al. 2019). Accumulation of these compatible solutes assists plants in preserving water absorption and maintaining balance, helping them adjust to saline environments. Furthermore, the elevated levels of Na^+ in saline conditions lead to excessive generation of reactive oxygen species (ROS) within the cells. This ROS accumulation results in various detrimental effects, including membrane injury, lipid peroxidation, nutrient imbalances, disruption of growth regulator levels, and impairment of enzymatic or metabolic functions (Julkowska & Testerink 2015). These widespread physio-biochemical disruptions ultimately impair photosynthesis and can eventually cause plant death.

In reaction to salt stress from high soil salinity, plants accumulate compatible solutes, i.e. the amino acid proline (Pro), within their vacuoles as a protective response (Annunziata et al. 2017). This accumulation of proline and other compatible solutes helps the plants maintain osmotic balance and avoid cellular dehydration under saline conditions. Osmolytes like Pro generally show elevated levels in salt-tolerant species (Saxena et al. 2019). Proline accumulation helps plants maintain cellular homeostasis by regulating osmotic balance (Reddy et al. 2017). This osmotic regulation is a key adaptive mechanism that allows plants to survive and cope with the saline environment.

Both salicylic acid (SA) and Proline foliar applications have been shown to mitigate the harmful effects of salinity on plants by enhancing various physiological and biochemical processes. Proline plays a crucial role in osmotic regulation and protects cellular structures from the adverse effects of high salt concentrations (Ismail & Helmy 2018). It is attributed to regulating various physiological processes linked to salt stress tolerance (SST). Specifically, proline influences ion transport mechanisms by regulating ion

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channels and transporters, helping plants maintain ion balance under salinity stress conditions (SSC). It also acts as a scavenger of ROS, reducing oxidative damage and supporting cellular redox homeostasis (Okuma et al. 2008). Furthermore, proline affects the functionality of enzymes associated with antioxidant defence, stress signalling pathways, hormone biosynthesis and signalling (Ismail & Helmy 2018; Naliwajski & Sklodowska 2021). Through these diverse mechanisms, proline intensification is a crucial adaptive response that allows plants to better tolerate and cope with high soil salinity.

Salicylic acid functions as a phenolic endogenous phytohormone that controls growth or serves as a defence mechanism against various environmental stresses, particularly SS (Kaya et al. 2002). Under SSC, the accumulation of SA in plants helps activate various stress-responsive pathways or defence mechanisms. This encompasses regulating growth and developmental processes, i.e., improving the plant's capacity to endure the harmful effects of high soil salinity. It enhances growth traits by increasing antioxidants and osmo-protectants, which help alleviate SS's negative effects (Yadu et al. 2017). Besides enhancing antioxidants and osmo-protectants, SA also directly protects plants from oxidative damage, reduces growth limitations, and maintains photosynthesis efficiency under stress conditions (Jogawat 2019). Specifically, SA treatment impacts several physiological processes, i.e. photosynthesis, chlorophyll content, stomatal function, and even seed yield (Khodary 2004; Yildirim et al. 2008). By regulating these key aspects of growth and metabolism, SA enables plants to better adapt to and thrive under high SSC.

Although the effects of salinity on broad bean growth and productivity have been extensively studied, limited research has focused on improving their tolerance to salt stress. This study hypothesized that foliar application of salicylic acid and proline, either individually or in combination, could enhance salt stress tolerance in broad beans (*Vicia faba* L. cv. Sakha 1) by improving photosynthesis, antioxidant activity, ion homeostasis, enzyme activation, and osmo-protection.

MATERIAL AND METHODS

Plant material and growth conditions. Two pot experiments were conducted at the Agricultural Botany Department of Kafrelsheikh University in Egypt under open-air conditions. These experiments took place during the 2021/2022 and 2022/2023 growing seasons, with the following environmental parameters: an average daylight duration of 11–11.5 h, daytime temperatures ranging from 18 °C to 22 °C, nighttime temperatures between 10 °C and 14 °C, and relative humidity levels of 45–55%. These experiments aimed to evaluate the impacts of applying proline L-Proline, 99%, 147-85-3, LANXESS (A Germany Company, India) at 10 mM and salicylic acid 2-hydroxybenzoic acid 98%, C7H6O3, (Merck KGaA, Germany) at 10 mM, either individually or in combination, as foliar applications. These treatments were tested under varying diluted seawater levels (4.69 ds/m and 6.25 ds/m) on various vegetative and reproductive growth traits, water relation aspects, and broad bean's specific chemical and physiological aspects. The soil (clay loam soil) was utilized for this study and analyzed using the methods described by Chapman and Pratt (1978). Comprehensive details about the physicochemical properties of clay loam soil utilized in our experiments are available in our previous article (El-Beltagi et al. 2024).

The experiment consisted of twelve treatments, each replicated four times, and was organized using a split-plot design. Pots (polyethylene), 30 cm in diameter and 40 cm deep, were utilized, with three drainage holes at the bottom plugged with sponges to control drainage. Each pot contained 8 kg of soil. Broad bean seeds (cv. Sakha 1), acquired from the Legume Research Department, Field Crops Institute, Agriculture Research Center, Giza, Egypt, were sown on November 3 and November 6 during the 2021/2022 and 2022/2023 seasons, respectively. Seedlings were thinned to two uniform plants per pot. Additionally, plants received two foliar applications of Pro and SA. The initial application occurred 30 days after sowing, with a second application 15 days later, each concentration applied at a rate of 100 mL per plot (for detail information see Table 1).

Irrigation and fertilization. Each pot was watered with 250 mL of solution every five days, while the control treatments received 250 mL of tap water. Phosphorus and potassium fertilizers were added to the soil before sowing. Calcium superphosphate (15.5% P_2O_5) and potassium sulfate (48% K_2O) were applied at 1.8 g per pot each. Nitrogen was supplied as ammonium sulfate (20.5% N) at a rate of 1.8 g per pot, administered in three separate doses.

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Table 1. The experimental treatments

Factor A	Factor B	Factor A × B
Control (C)	Pro at 10 mM SA at 10 mM Pro + SA combination	C + Pro at 10 mM C + SA at 10 mM C + Pro + SA combination
The first salinity level (S1)	Pro at 10 mM SA at 10 mM Pro + SA combination	S1 + Pro at 10 mM S1 + SA at 10 mM S1 + Pro + SA combination
The second salinity level (S2)	Pro at 10 mM SA at 10 mM Pro + SA combination	S2 + Pro at 10 mM S2 + SA at 10 mM S2 + Pro + SA combination

Factor A (salinity levels), factor B [salicylic acid (SA) and proline (Pro) and their combination], and factor A × B (salinity levels and Pro and SA combination)

Sampling, measurements, and determination top of form. In both seasons of the experimental phase, a single sample was randomly collected for each treatment, with five samples taken per replicate 60 days post-sowing. The following traits were then analyzed.

Growth characters. The following traits were measured: plant height (cm), dry weight of plants (obtained by drying plants in an electric oven at 70 °C for 72 h, recorded in grams per plant), number of leaf/plant, and leaf area (measured utilizing portable laser leaf area meter, CI-02; CID Bio-Science, USA). Relative water content was expressed as a percentage and calculated using the formula described by Kalapos (1994).

Chlorophyll pigments. Sixty days post-sowing, the fourth leaf from the plant tip was collected and submerged in 5 mL of dimethylformamide for extraction. This procedure was utilized to estimate concentrations of chlorophyll pigments (chl. *a*, chl. *b*, or total), measured in μg/g fresh weight, following the methodology described by Moran (1982).

Antioxidant enzymes. To evaluate the activities of antioxidant enzymes, lipid peroxides, tocopherol, or protein levels, 500 mg of fresh leaves were homogenized at –4 °C in 5 cm^3 of 100 mM sodium phosphate buffer (pH 7.5). This buffer contained 1 mM ascorbic acid, 1 mM EDTA, 0.5 M NaCl, and 1% Triton X-100. Homogenate was centrifuged at 20 000 g for 20 min at –4 °C. The supernatant obtained was used to assess enzyme activity, lipid peroxides, or protein content, while crude extracts were used to determine tocopherol levels.

Sixty days after sowing, the fourth leaf from the plant tip was harvested and finely ground using liquid nitrogen for assays measuring antioxidant enzyme activity. The ground tissue was extracted with ice-cold 0.1 M Tris-HCl buffer (pH 7.5), supplemented with 5% sucrose and 0.1% 2-mercaptoethanol, using a buffer volume three times the tissue's fresh weight. Homogenate was centrifuged at 10 000 g for 20 min at 4 °C, and the resulting supernatant was utilized to determine enzyme activity or protein content. All enzyme extraction steps and assays were performed at 4 °C to maintain enzyme stability and prevent denaturation or degradation.

Catalase (CAT, EC 1.11.1.6) activity was measured by the monitoring decomposition of hydrogen peroxide (H_2O_2) at 240 nm, following Aebi's method (1984). Catalase activity was quantified in units (U) per mg of protein, indicating the amount of millimoles (mM) of H_2O_2 decomposed per minute per mg of protein. Peroxidase (POD, EC 1.11.1.7) activity was assessed according to Polle et al. (1994). Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the method of Giannopolitis and Ries (1977), which assesses the SOD-induced inhibition of photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD activity was defined as the content of enzyme required to inhibit 50% of NBT reduction, measured at 560 nm. It was expressed as units (U) per mg of protein, corresponding to micromoles (μmol) of ascorbate consumed per minute per mg.

Proline amount, total phenols, and membrane stability. At 60 days post-sowing, fresh leaves were used to measure the proline amount, expressed in μmol per gram of fresh weight. This measurement was conducted using a Shimadzu UV Vis spectrophotometer (UV-1601, Japan) at a wavelength of 520 nm, following the procedure outlined by Bates et al. (1973) for proline determination. Additionally, the total phenolic compounds in the leaves were evaluated using the methodology outlined by Bessada et al. (2016). Fresh leaves were utilized to estimate membrane stability based on the protocol outlined by Lutts et al. (1996).

Nutrient elements, K/Na ratio, and crude protein. Dried broad bean plants underwent wet digestion with a sulfuric and perchloric acid mixture to estimate N, P, and K levels. Nitrogen levels were estimated using the Kjeldahl method, phosphorus

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levels were measured using a spectrophotometric method, and potassium levels were determined using the flame photometer method, following procedures outlined by Walinga et al. (2013). The K/Na ratio in leaves or crude protein content in dried faba bean seeds was estimated using A.O.A.C. (2000) protocols.

Yield and yield components analysis. At the whole green maturity stage (120 days from sowing), the yield and its components were determined, expressed as pod number/plant and yield (g/plant). Furthermore, the crude protein amount in dried seeds was measured utilizing methods specified by A.O.A.C. (2000).

Statistical analysis. Data analysis was conducted utilizing CoStat version 6.400 (1998–2008) software, following statistical techniques outlined in the methodology based on Gomez and Gomez (1984). The significance level for the least significant difference (LSD) test was set at $\alpha \leq 0.05$. According to Duncan (1955), multiple-range tests were employed to compare treatment means. Additionally, a cluster dendrogram heatmap was created to summarize the findings on the agronomic and biochemical aspects utilizing the online tool ClustVis (https://biit.cs.ut.ee/clustvis/).

RESULTS

Growth characters. The data in Figures 1, 2, and 3 reveal that broad bean plant growth traits, such as plant height, leaf area, number of leaves, or plant dry weight, declined with rising SS levels in both seasons. The reduction percentage in plant height, leaf area or plant dry weight at the first salinity level was 30%, 35% and 29% in the first season and 27%, 25% and 23% in the second season, respectively. The reductions at the second SS level were 42%, 54% and 45% in the first season and 35%, 43% and 31% in the second season, respectively.

However, the Pro and SA applications, either separately or in combination, enhanced these growth parameters under salinity stress and normal condi-

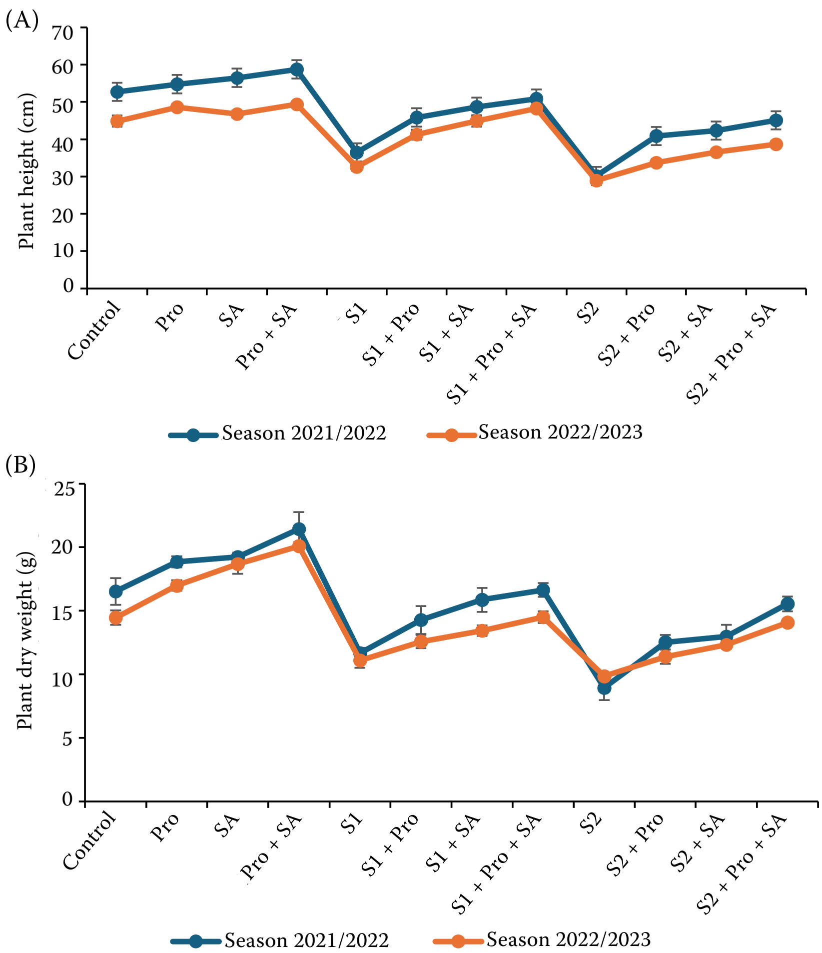

Figure 1. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on growth parameters: (A) plant height and (B) plant dry weight of broad bean

These parameters include plant height and dry weight under different salinity levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons; values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; different letters on each bar indicate significant contrast among treatments ($P < 0.05$)

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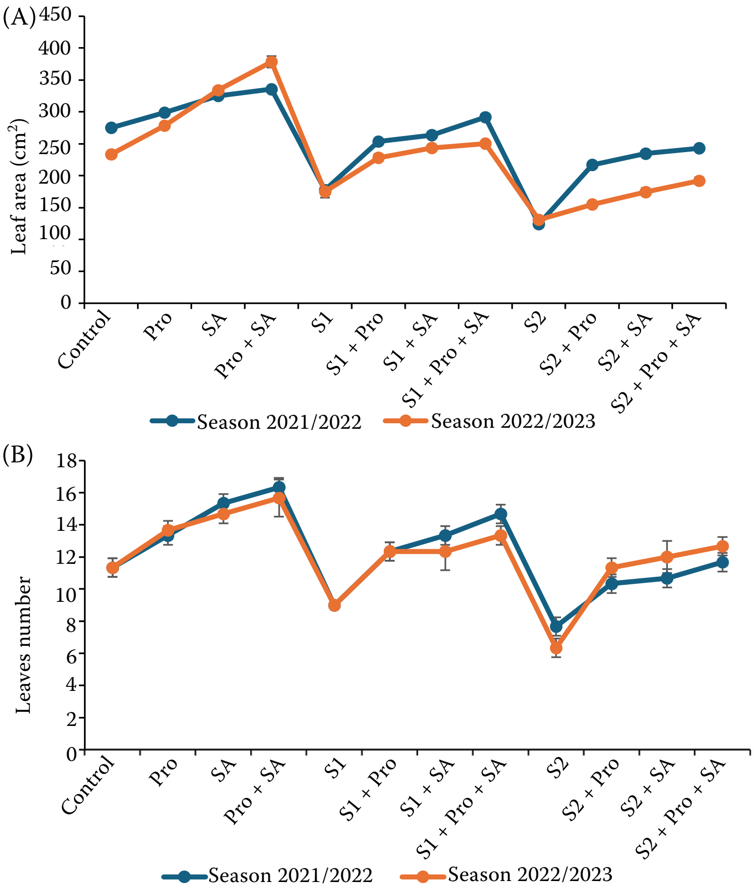

Figure 2. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on growth parameters: (A) leaf area and (B) leaves number of broad bean

These parameters include leaf area and number of leaves per plant under different salinity levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons; values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; different letters on each bar indicate significant contrast among treatments ($P < 0.05$)

tions. While both Pro and SA treatments had beneficial effects, SA treatment was the most effective in both seasons. Furthermore, the combined application of Pro and SA resulted in the highest growth parameter values under both normal or salinity stress conditions (SSC) in both seasons.

The percentage increases in plant height, leaf area, or plant dry weight under optimal conditions were 11%, 21%, and 29% in the first season and 10%, 62%, and 38% in the second season, respectively. Under the first SS level, these increases were 39%, 64%, and 42% in the first season and 48%, 43%, and 30% in the second. Under the second level of salinity stress, the increases were 49%, 95%, and 73% in the first season and 33%, 46%, and 42% in the second season, compared to the untreated of the same SS level.

Chlorophyll pigments content. Data in Figure 3 demonstrated that applying Pro and SA, individually or together, increased chlorophyll content (chl. *a*, *b*, total) in non-saline conditions in both seasons. Under SSC, chlorophyll pigment content decreased as salinity levels increased compared to unstressed plants (control). The decrease in chlorophyll (*a*, *b*, total) at the first salinity level was 33%, 9%, and 32% in the first season and 30%, 23%, and 27% in the second season, respectively. At the second level of SS, the reductions were 42%, 42%, and 42% in the first season and 38%, 36%, and 37% in the second season, respectively. However, the Pro and/or SA application alleviated salinity adverse impacts on chlorophyll pigment content, resulting in higher chlorophyll levels than salinity-stressed controls in both seasons. When comparing Pro and SA treatments, SA alone produced the highest chlorophyll pigment in both SS and non-saline conditions. This enhanced percentage in chlorophyll (*a*, *b*, total) under normal conditions was 35%, 30% and 34% in the first season and 32%, 29% and 31% in the second season, respectively. Under the severe SS levels, the levels increase. *a*, *b*, and total were 46%, 19%, and 38% in the first season and 44%, 36%, and 41% in the second season, respectively, compared with the same level of SS without using SA treatment. However, the combined application of the Pro and SA resulted in the highest overall chlorophyll pigment content during both seasons. These increases in percentage in chlorophyll (*a*, *b* and total) under the first level of salinity were 41%, 43% and 42% in the first season and 41%, 36% and 42%

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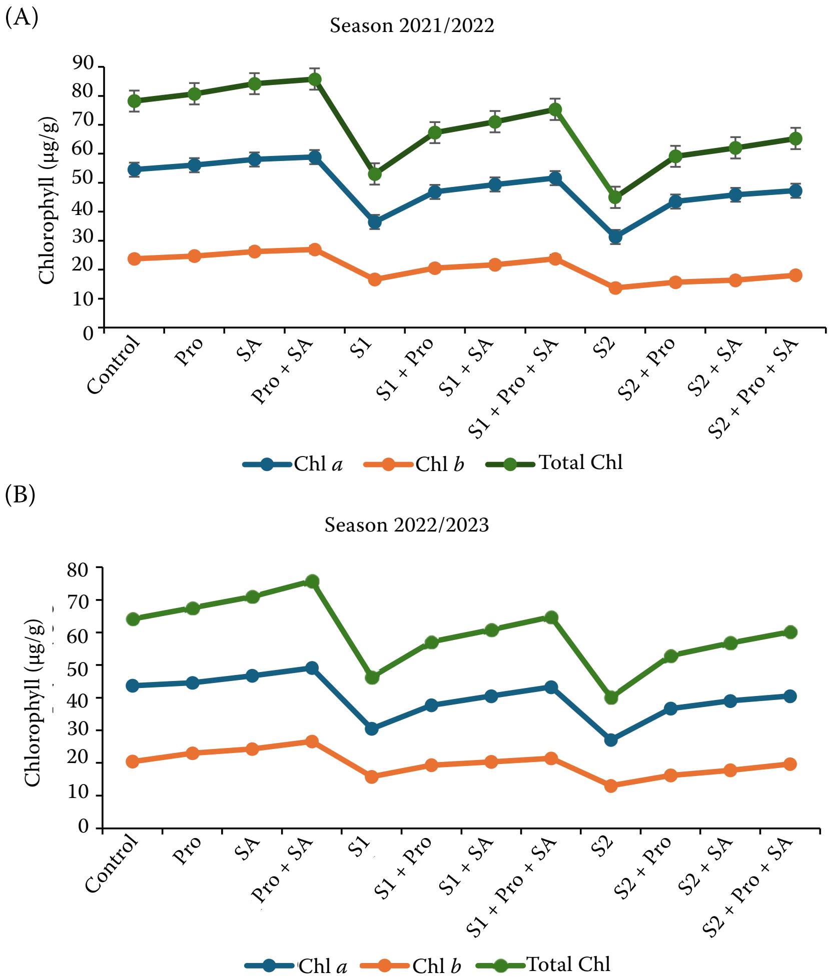

Figure 3. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro+SA), on chlorophyll (*a*, *b*, total) content under various SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the (A) 2021/2022 and (B) 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

in the second season, respectively. The increases at the second salinity level were 51%, 32% and 45% in the first season and 49%, 50% and 50% in the second season, respectively, compared with the same salinity level without treatment.

Endogenous proline content. Under normal conditions, application of Pro and/or SA increased endogenous proline (Endo-Pro) content compared to untreated plants (control) during both seasons (Figure 4). Under SSC, the content of Endo-Pro increased with rising SS levels. Specifically, there was a 55% increase in the first and 67% in the second seasons. The application of Pro, SA, and their combination further increased Endo-Pro content compared to stressed untreated plants (controls). The Pro treatment resulted in higher Endo-Pro content than the SA treatment in the second season. While the Pro + SA application resulted in the highest Endo-Pro content compared to other treatments, with percentage increases of 24% and 32% under normal conditions in the first and second seasons, respectively. Under the first level of SS, the increases were 30% and 15%, and at the second level of SS, the increases were 23% and 16% in the first or second season, respectively.

Membrane stability. Figure 5 shows membrane stability values increased with higher salinity levels in both seasons. The percentage increases were 15% and 20% at the first SS level and 25 and 35% at the second SS level in the first and second seasons, respectively. However, Pro and/or SA application decreased membrane stability under SSC. Among the individual applications, SA led to a greater reduction in membrane stability compared to Pro under each SSC or normal control condition. The combined foliar application of Pro and SA resulted in the greatest reduction in electrolyte leakage. Specifically, the reduction by the combined treatment was 14% and 8% at the first SS level and 16% and 15% at the second SS level in the first or second season, respectively.

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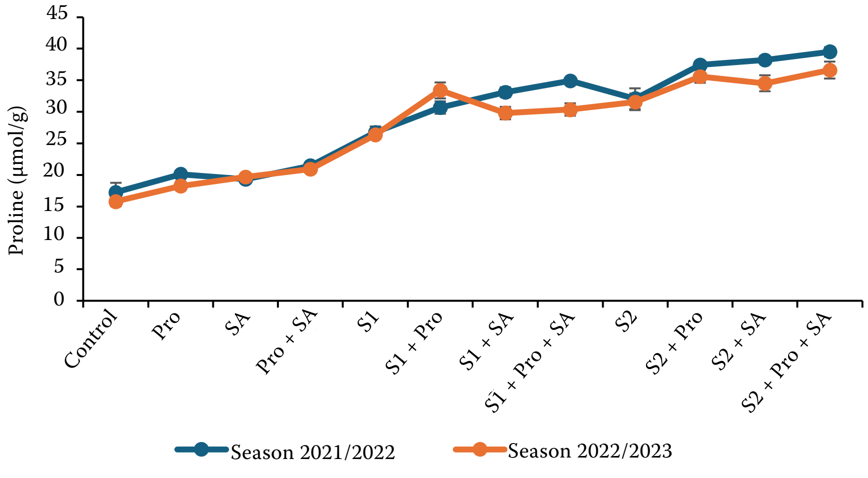

Figure 4. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro+SA), on endogenous proline content under different SS levels [4.69 ds/m (S1) and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on each bar indicate significant contrast among the treatments ($P < 0.05$)

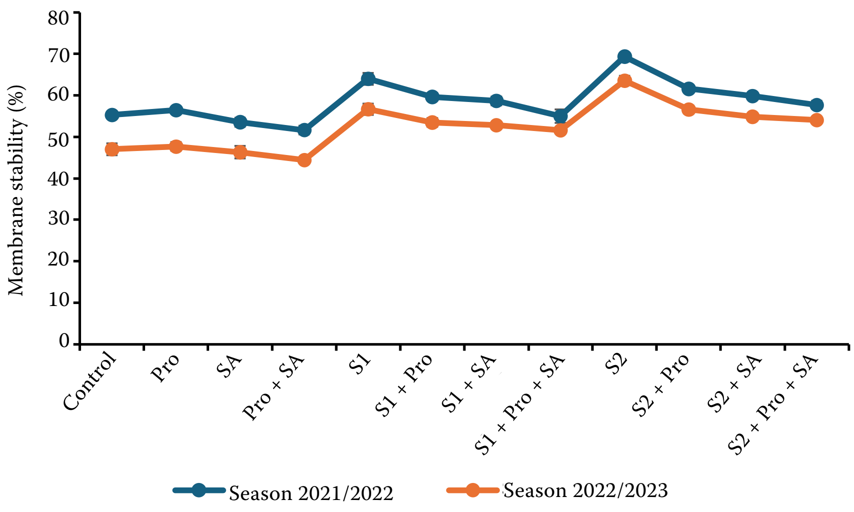

Figure 5. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on membrane stability in broad bean leaves under different SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

Antioxidant enzymes. Figure 6 shows the activity of antioxidant enzymes, i.e. CAT, POD, and SOD improved with rising salinity levels compared to unstressed broad bean plants in both seasons. The increase in antioxidant enzymes at the first level of SS was 39%, 143%, and 19% in the first season, respectively. At the second level of SS, the increases were 75%, 200%, and 45% in the first season, respectively. Pro and/or SA enhanced all antioxidant enzyme activities under unstressed and SSC conditions in both seasons. The combined foliar application of Pro and SA led to the highest enzyme activity during each season. The percentage increases were 21%, 38%, and 21% at the first salinity level and 23%, 39%, and 12% at the second SS level.

Relative water content (RWC). Figure 7 shows that RWC values decreased under SSC in both seasons. At the first salinity level, RWC decreased by 15% in the first and 17% in the second seasons. At the second salinity level, reductions were 31% in the first and 30% in the second seasons. Pro and/or SA mitigated the adverse salinity effects, resulting in increased RWC compared to normal and saline-stressed control plants. Pro and SA combined applications produced the highest RWC values during both seasons. The percentage increases were 16%, and 18% at the first salinity level, 35%, and 30% at the second salinity level in the first or second season, respectively.

Phenol content. A gradual increase in leaf phenol content was recorded with rising SS levels during both seasons (Figure 8). The increase in phenol content at the first salinity level was 8% in the first season and 51% in the second season. At the second salinity level, the increases were 51% in the first season and 82% in the second season. Pro and/or SA further increased leaf phenol content in broad

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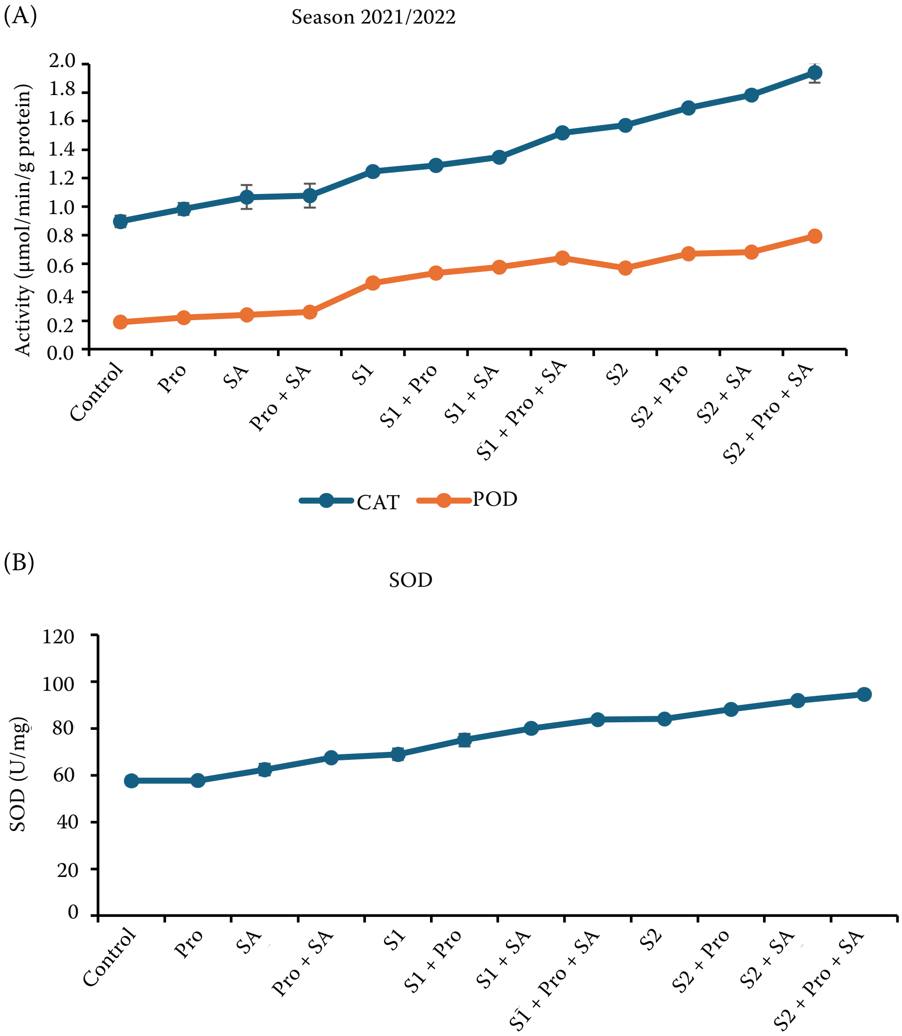

Figure 6. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro+SA), on antioxidant enzyme activity (A) CAT, POD, and (B) SOD in broad beans under different SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2022/2023 season
Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

beans compared to normal and salinity-stressed controls. Pro and SA combined application resulted in the highest leaf phenol content during both seasons. The percentage increases at the first salinity level were 13% in the first season and 21% in the second season, while at the second salinity level, the increases were 18% in the first season and 27% in the second season.

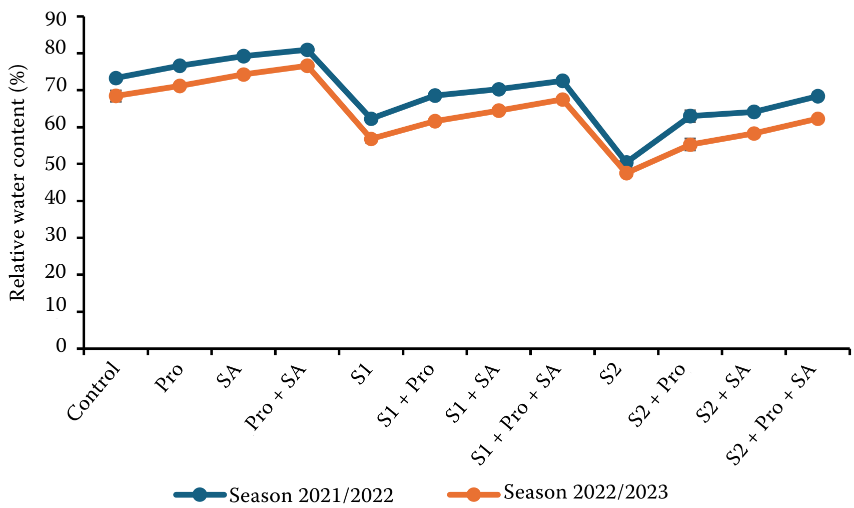

Figure 7. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro+SA), on the relative water content in broad bean in different SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons
Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; different letters on each bar indicate a significant contrast among treatments ($P < 0.05$)

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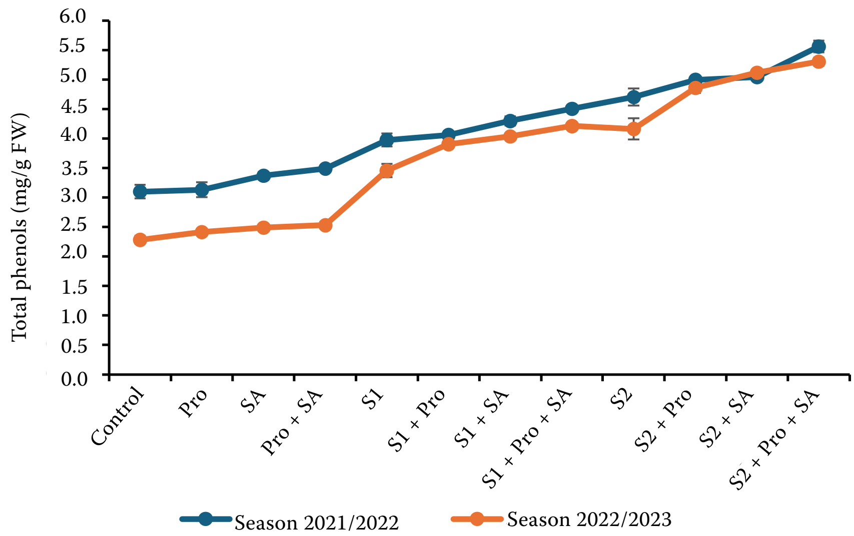

Figure 8. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on phenols content in broad bean leaves under different SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing in the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

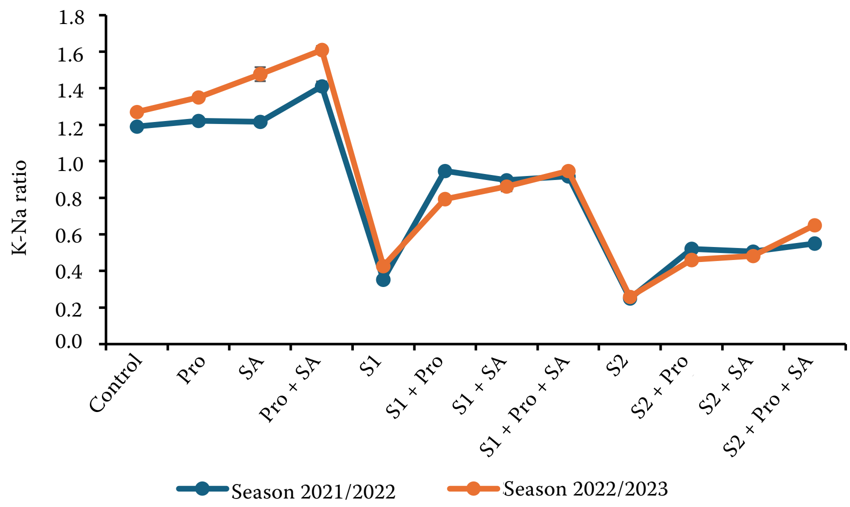

Figure 9. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on the potassium/ sodium ratio (K/Na ratio) in broad bean leaves under different SS levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing in the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

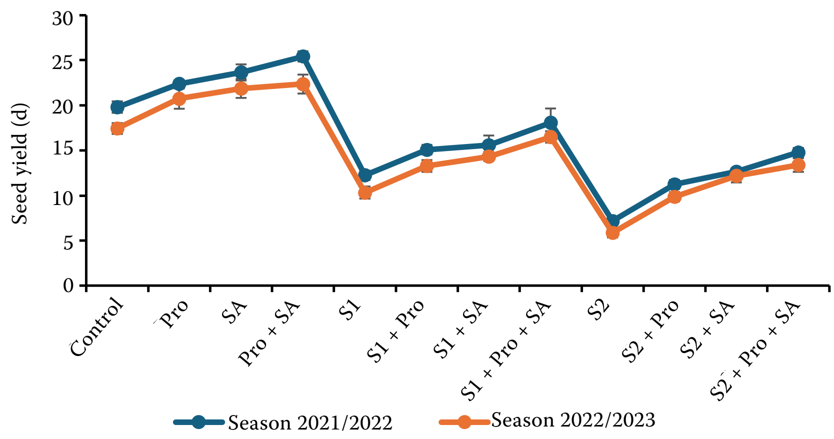

Figure 10. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on broad bean seed yield/plant under salinity levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at 60 days post-sowing during the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

Potassium/Sodium ratio (K/Na ratio). Figure 9 shows that the K/Na ratio declined as salinity levels increased in both seasons. The decrease in the K/Na ratio at the first SS level was 70% in the first season and 66% in the second season. At the second SS level, the reductions were 78% in the first season

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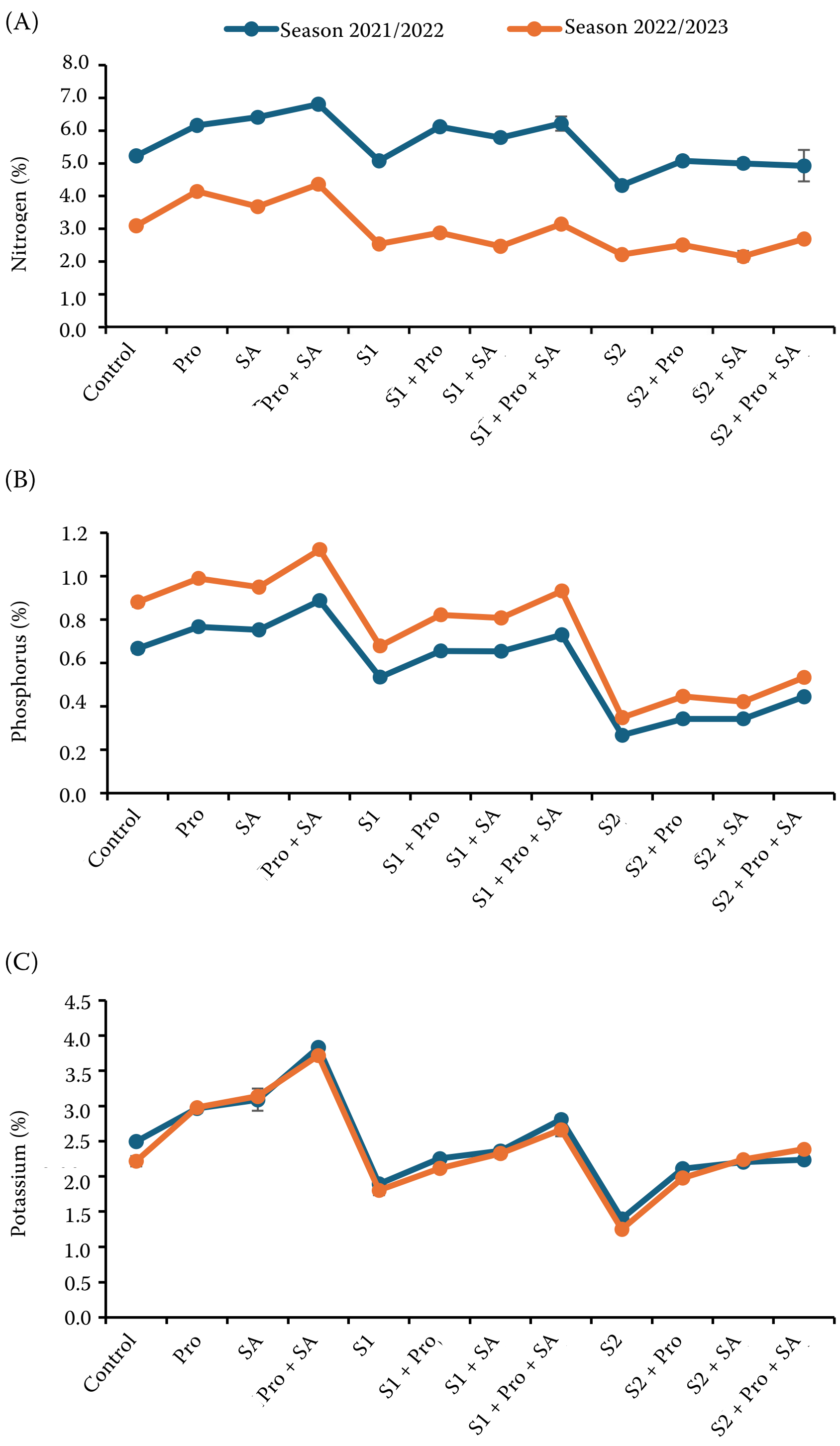

Figure 11. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on (A)nitrogen, (B) phosphorous and C) potassium percentages under salinity levels [4.69 ds/m (S1)and 6.25 ds/m (S2)] at harvest date in the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

and 79% in the second season. Pro and SA's application, individually or together, improved the K/Na ratio under both normal and SS conditions. The highest K/Na ratio values were obtained with the combined application of Pro or SA in both seasons. Percentage increases at the first salinity level were 159% in the first season and 122% in the second season, while at the second salinity level, the increases were 120% in the first season and 151% in the second season.

Seed yield (g/plant). Figure 10 showed that broad bean seed yield decreased with increasing SS levels. The reduction in seed yield at the first salinity level was 38% in the first and 40% in the second seasons. At the second salinity level, the reductions were 63% in the first and

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66% in the second seasons. However, the application of Pro and SA, either separately or in combination, improved plant seed yield under normal and SS conditions. The combined treatment caused the greatest increase in plant seed yield. The percentage increases at the first salinity level were 47% in the first season and 59% in the second season, while at the second salinity level, the increases were 106% in the first season and 127% in the second season.

Nitrogen, phosphorous and potassium percentages. The data shown in Figure 11 indicates that percentages of N, P, and K in broad bean seeds decreased with increasing SS levels. Conversely, whether used separately or together, Pro and SA increased percentages of N, P, and K under both normal or SSC compared to untreated plants. Application of Pro + SA resulted in the highest percentages of N, P, and K in both seasons.

Crude protein percentage. The crude protein percentage in broad bean seeds decreased with increasing salinity levels (Figure 12). At the first salinity level, the reductions were 2% in the first season and 18% in the second season. At the second SS level, the reductions were 17% in the first season and 28% in the second season. Pro and/or SA resulted in higher crude protein percentages under normal and SSC conditions than untreated plants. The combined application of Pro and SA achieved the highest crude protein percentage in broad bean seeds compared to the individual treatments. The percentage increases at the first salinity level were 2% in the first season and 23% in the second season, while at the second salinity level, the increases were 13% in the first season and 21% in the second season.

DISCUSSION

The decline in broad bean growth parameters and chlorophyll content under salinity stress is well-documented in various plant species (Nasrallah et al. 2022; El-Beltagi et al. 2024). The reduction in plant height and leaf area index under salinity stress conditions (SSC) can be attributed to factors such as limited cell division, restricted cell expansion, and inhibited apical growth (Balasubramaniam et al. 2023; Ramadan et al. 2023). These effects are primarily due to the accumulation of hydrogen peroxide (H_2O_2) due to salinity stress, which, along with other ROS, triggers lipid peroxidation. This process is indicated by elevated malondialdehyde (MDA) levels in plants exposed to SSC, including *Oryza sativa* (Frukh et al. 2020). Increased peroxidation of polyunsaturated fatty acids compromises membrane fluidity and increases permeability, leading to significant membrane damage (Gill & Tuteja 2010).

Salinity stress imposes several challenges on plants, such as hindered water absorption, oxidative damage, ionic imbalance, disrupted nutrient uptake, and decreased chlorophyll content and photosynthetic efficiency (El-Beltagi et al. 2024; Kapoor et al. 2024). Regarding leaf production per plant, Ahmed et al. (2019) reported that lettuce (*Lactuca sativa* L.) decreased leaf number with increasing salinity levels. The reduced growth and chlorophyll content in broad bean plants under SSC may also be linked to decreased root nodule formation. Abiotic stressors like salinity stress can lead to stomatal closure, reduced chlorophyll synthesis, and activation of chlorophyllase enzymes, which disrupt chloroplast structure and pigment-

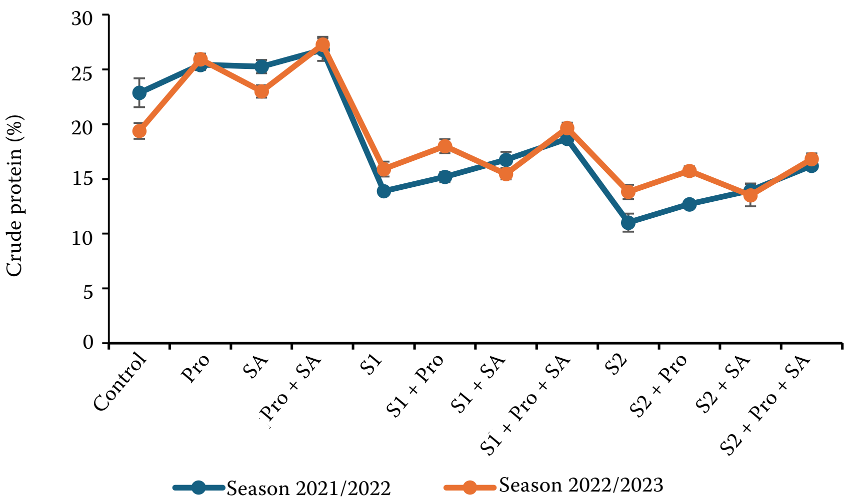

Figure 12. The effect of proline (Pro) and salicylic acid (SA), both separately and together (Pro + SA), on crude protein percentage in broad bean seeds under different SS levels [4.69 ds/m (S1) and 6.25 ds/m (S2)] at harvest date in the 2021/2022 and 2022/2023 seasons

Values are represented as mean ± standard deviation; vertical bars represent the standard deviation of the means; letters that differ on both bars indicate significant contrast among the treatments ($P < 0.05$)

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protein complexes (Hayat et al. 2012). Changes in chlorophyll levels are also associated with oxidative stress, Rubisco enzyme inactivation, and alterations in chlorophyll ultrastructure (Rady 2011).

The results for growth parameters and chlorophyll content underscore the beneficial effects of applying Pro and SA in mitigating salt stress. Various studies have reported that certain concentrations of Pro or SA can regulate several aspects of plant growth or development under SSC, leading to increased biomass and productivity (Wu et al. 2017; Koo et al. 2020). For instance, utilizing pro-enhanced growth in two alfalfa cultivars under SSC, with greater salt stress tolerance (SST) and higher dry weight associated with higher endogenous Pro accumulation. Farissi et al. (2014) indicated that salinity increases Na^+ and Cl^- levels while reducing essential nutrients such as Ca^{2+}, K^+, Mg^{2+}, NO_3^-, and S, leading to overall nutrient deficiency. Several studies have linked the positive impact of Pro or SA on plant SST to enhanced nutrient assimilation (Abdelhamid et al. 2013). Additionally, Pro or SA helps scavenge ROS, reducing oxidative damage and maintaining cellular redox balance (Okuma et al. 2008; Jogawat 2019).

Moreover, these treatments protect chloroplasts and thylakoid structures during drought stress (Aldesuquy et al. 2018) while minimizing photoinhibition (Hare & Cress 1997). Their contribution to sustaining chlorophyll levels, maintaining leaf turgor, and improving stomatal conductance is closely associated with enhanced plant drought resistance (Panda et al. 2021).

Salinity stress profoundly affects plant physiology, increasing endogenous proline levels, which function as osmoprotectants. Proline helps plants mitigate osmotic stress by stabilizing cellular proteins and membranes, neutralizing free radicals, and maintaining redox homeostasis (Xu et al. 2017; El Moukhtari et al. 2020; Sagervanshi et al. 2021; Jangra et al. 2022). Similar increases in proline have been observed in multiple species, such as pearl millet (Khan et al. 2020) and *Pisum sativum* (Ahmed et al. 2020; El-Beltagi et al. 2024).

The role of proline in plant defence against SS is further strengthened by SA pretreatment, which increases abscisic acid (ABA) levels in seedlings, thereby maintaining higher proline concentrations (Kuznetsov & Shevyakova 1999). The observed enhancement in membrane stability under SSC likely results from adaptive modifications in membrane composition and structure (Mansour 2013). Membrane stability is closely linked to SST across various plant species (Yang et al. 2009; Kholova et al. 2010).

The observed increase in antioxidant enzyme activity, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), under salinity stress aligns with findings from Khan et al. (2020) and El-Beltagi et al. (2024), who reported similar enzyme activity elevations in pearl millet and pea plants, respectively. Plants employ various defence mechanisms to counteract abiotic stress, with enhanced antioxidant enzyme activity playing a crucial role (Mohamed et al. 2023; El-Waraky et al. 2024).

Additionally, the results indicate that SA or Pro foliar application further enhanced antioxidant enzyme activity under salinity stress conditions (SSC). SA and Pro are believed to stimulate antioxidant enzyme activity, thereby reducing the harmful effects of ROS accumulation caused by SSC (El-Beltagi et al. 2022a, 2022b; Shalaby et al. 2023; Hadid et al. 2024).

The decrease in relative water content (RWC) with increasing SS level is due to osmotic effects that restrict water uptake by plants and affect their water capacity (de Morais 2018). Silva et al. (2021) noted a 13% reduction in RWC with higher SS levels in irrigation water. Both water uptake and loss influence relative water content through transpiration. In contrast, applying SA or Pro externally increased the RWC of broad beans. Relative water content indicates the equilibrium between water uptake and water loss through transpiration (Wu et al. 2017; de Freitas et al. 2019). Salicylic acid or Pro may help reduce membrane damage caused by SS, thereby minimizing water loss and maintaining an optimal water status by lowering the transpiration rate (Fairoj et al. 2022).

In this study, total phenolic content in broad bean leaves increased under SSC as a defence mechanism against its harmful effects. To counteract the negative impacts of SS, plants produce a range of secondary metabolites, including phenolic compounds, to combat oxidative damage or scavenge ROS (Orsini et al. 2016; Santander et al. 2022). SA or Pro treatments mitigated SS effects by enhancing the total phenolic content and reducing Na^+ and Cl^- levels (Ghanem et al., 2021; Gao et al. 2023). Additionally, the phenolic amount in rosemary leaves also improved in response to salinity or SA and Pro treatments, supporting previous findings (Bagherifard et al. 2015; Ghanem et al. 2021). Higher antioxidant activities observed

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in plants exposed to high SS levels and/or treated with SA at 100–300 ppm are linked to an increased phenolic composition (El-Esawi et al. 2017). By inhibiting lipid peroxidation, phenolic compounds contribute to maintaining the integrity and functionality of membranes under SSC (Castillo et al. 2022).

The study demonstrated a significant decrease in the K/Na ratio in broad bean leaves under SSC. Potassium (K) is crucial for normal plant metabolic functions. Still, high sodium (Na) levels in growth medium compete with K for uptake, decreasing the K/Na ratio and subsequently decreasing plant growth (Hussain et al. 2015). Research indicates that the balance between Na and K affects light-driven reactions by influencing the stacking of grana in chloroplasts (Tränkner et al. 2018). The increase in the K/Na ratio in broad bean leaves treated with Pro or SA underscores its beneficial effects under stress.

Treatment with Pro or SA helps mitigate SS effects by decreasing Na^+ and Cl^- uptake and translocation while enhancing K^+ assimilation in plants. However, the effectiveness of Pro or SA in these processes can vary depending on factors, i.e. concentration, application method, plant species, specific stress conditions, and plant developmental stage (Samy et al. 2015; El Moukhtari et al. 2020).

The study also revealed a significant decline in broad bean seed yield as salinity stress levels increased. Plants are particularly vulnerable to the adverse effects of SS during the reproductive stage (Balasubramaniam et al. 2023). Factors contributing to this decline include impaired water and nutrient uptake, osmotic stress, ion toxicity, and oxidative stress (Zhao et al. 2021).

However, Pro or SA's foliar application mitigated SS's negative impact on broad bean seed yield. Proline application has been reported to enhance grain yield in *Triticum aestivum* (Rady et al. 2019) and *Zea mays* under salinity SSC (Alam et al. 2016). In general, Exo-Pro improves plant growth and productivity under SSC, though the exact mechanisms likely linked to hormonal regulation are not yet fully elucidated (El Moukhtari et al. 2020).

The yield improvement resulting from SA application is closely associated with its ability to stimulate plant growth, increase chlorophyll pigment content, enhance osmolyte accumulation (such as proline), and boost the activity of ROS-scavenging enzymes and phenol content (Zivcak et al. 2016; Urmi et al. 2023).

The study revealed a significant decrease in N, K, and P percentages in broad bean seeds under SSC. These results align with previous research (Zhu et al. 2020; Kafi et al. 2021). High SS levels may adversely affect the growth or activity of soil microorganisms, which are crucial for the conversion and availability of essential nutrients. Factors such as high leaching, N losses as nitrate (NO_3^-), decreased nitrification rates due to high salt concentrations, and the chloride ions toxic effects on microbial activity further contribute to reduced nutrient availability (Feigin 1985). Excessive Na^+ levels compete with K^+ for uptake, leading to a diminished K/Na ratio (Hussain et al. 2015). Principal component analysis indicates a strong negative correlation between Na accumulation or nutrient (K, P) acquisition in salt-stressed plants (Ashraf et al. 2023). Additionally, phosphorus (P) uptake is often reduced due to the presence of chloride (Cl^-) and sulfate (SO_4^{2-}) ions (Ehtaiwesh 2022). The diminished phosphorus (P) availability in saline environments is also linked to ionic effects that lower P activity and solubility in the soil solution (Maas & Grattan 1999).

Several studies have linked the positive effects of Exo-Pro or SA on plant tolerance to SS with increased nutrient assimilation. Abdelhamid et al. (2013) found that applying Exo-Pro boosted levels of P, K, nitrate (NO_3^-), or nitrite (NO_2^-) in *Phaseolus vulgaris* under varying salinity levels. Alam et al. (2016) indicated that Exo-Pro enhances the uptake of N, P, and K^+ in *Zea mays* under SSC. Besides improving nutrient uptake, Exo-Pro stimulates the enzyme activity involved in nutrient assimilation under saline stress. Applying SA to plants increased all Na ratios (K/Na, Ca/Na, Mg/Na), as salicylic acid mitigated SS effects and improved nutrient uptake (Youssef et al. 2017).

The study revealed a significant reduction in the crude protein percentage in broad bean seeds under SSC. This finding aligns with previous reports, such as those by Goharrizi et al. (2020), who documented decreased protein content under SSC. ROS are known to harm proteins (Ahmad et al. 2010). Moreover, salt stress adversely affects rhizobial activity by decreasing the number and biomass of nodules, hindering leghemoglobin synthesis, or reducing nodule respiration. This results in lower nitrogenase activity and reduced nitrogen fixation rates (Monica et al. 2013).

The positive impact of Exo-Pro on the activity of nitrogenase under saline stress has been well-documented, improving nitrogen fixation in legumes and other nitrogen-fixing plants (El Moukhtari et al. 2020). Besides Pro, SA has also been shown to en-

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hance protein levels. For example, El Tayeb (2005) reported increased amino acid concentrations in maize plants following SA treatment. Similarly, despite the general tendency for SS to decrease protein levels in leaves, SA has been found to counteract this effect and elevate protein levels (Sahar et al. 2011).

Salicylic acid and Pro offer protective effects against various stresses, i.e. salinity. Still, their combined impact on broad beans and other plants under SS has not been extensively studied. Evidence suggests that combining SA and Pro can more effectively alleviate the detrimental salinity effects than their individual applications. For instance, Urmi et al. (2023) indicated that the joint application of Pro and SA significantly improved photosynthetic pigments, RWC, membrane stability index, nutrient uptake, plant growth, and yield in rice. This combined treatment enhances stress tolerance by boosting osmoprotectants, up-regulating antioxidant enzyme activity, reducing oxidative stress or improving nutrient transport. Additionally, both SA and Pro play a role in maintaining chlorophyll content, which is vital for salinity tolerance (Panda et al. 2021).

A comparative heatmap analysis was conducted to evaluate various parameters measured in this study, including plant height, leaf area, dry weight, chlorophyll pigments (*a*, *b*, total), endogenous proline, antioxidant enzymes (CAT, POD, SOD), leaf total phenols, membrane stability (EC %), and seed biochemical analysis (crude protein and nutrient content, NPK) under two salinity levels (S1 and S2) (Figure 13). This analysis highlighted a clear distinction in plant growth and physio-biochemical responses among salinity-stressed plants, both with and without treatment with Pro or SA, individually or in combination. Remarkably, plants treated with a combination of Pro or SA showed the most significant alleviation of SS, demonstrating enhanced tolerance across all measured parameters.

Figure 14 summarizes the detrimental impacts of two different SS levels, S1 and S2 (4.69 ds/m and 6.25 ds/m), on broad bean plants. These impacts include reductions in plant growth, chlorophyll pigment content,

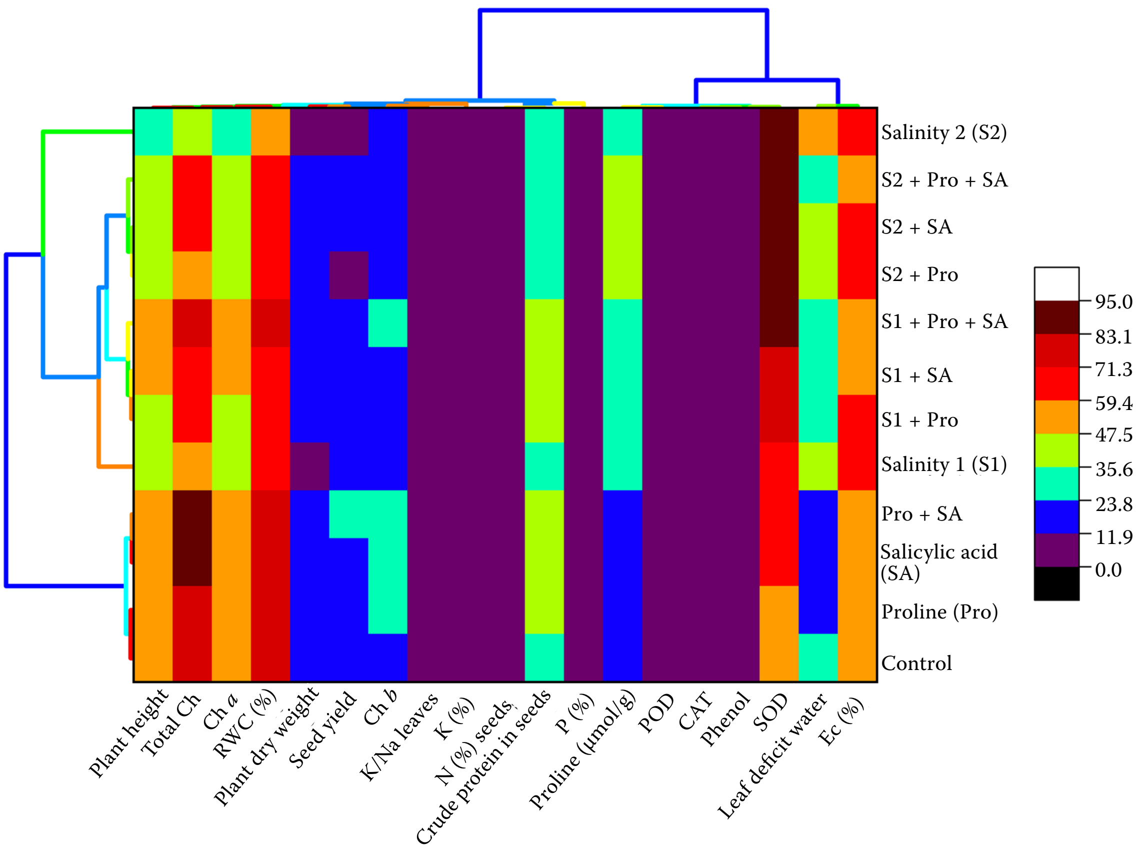

Figure 13. Heatmap analysis of the growth, physiological or biochemical attributes in broad bean salinity stressed (4.69 ds/m and 6.25 ds/m) treated with proline, salicylic and acid their combination, plant growth (plant height, leaf area, plant dry weight), Ch *a*, Ch *b*, total Ch, membrane stability index (MSI), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), relative water content (RWC), seed nutrient amount percentage (NPK), endogenous Pro content as well as leaf phenols content

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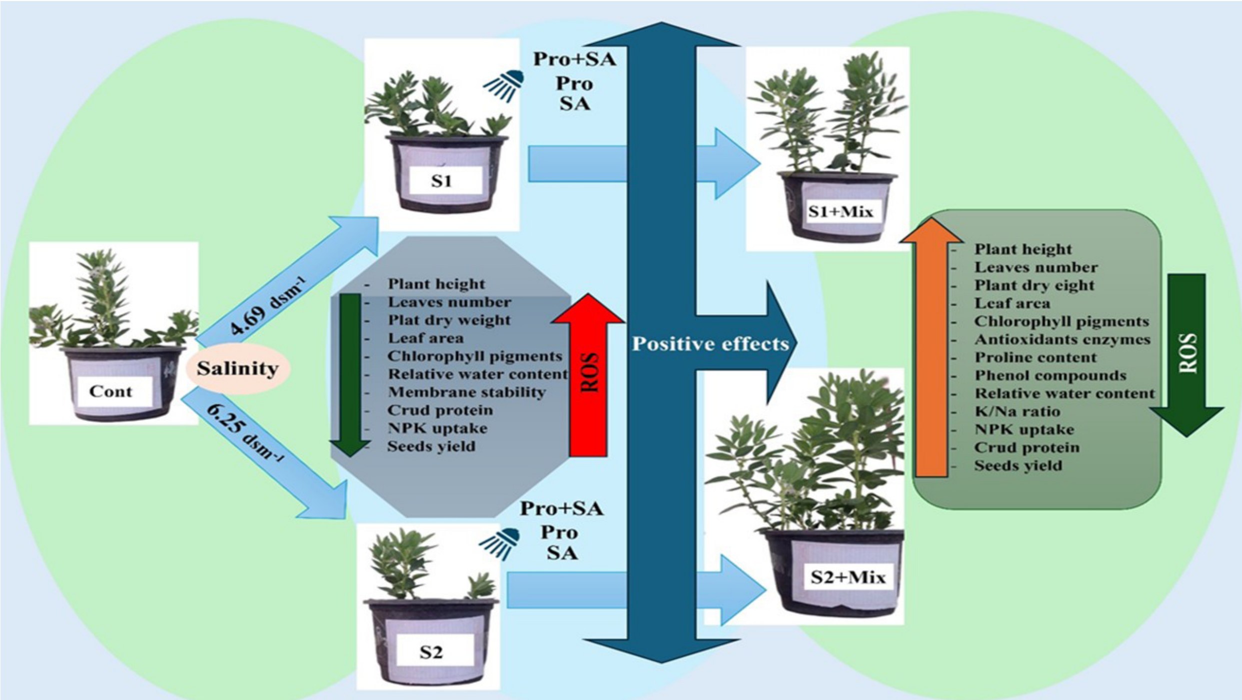

Figure 14. A graphical abstract outlining the adverse effects of two different SS levels, S1 and S2 (4.69 ds/m and 6.25 ds/m), on broad bean plants

The salinity stress impacts various parameters, including plant growth (plant height, leaf area, number of leaves per plant, plant dry weight), chlorophyll pigment content, membrane stability, seed crude protein, K/Na ratio, seed yield, and seed nutrient content (N, P, K), while increasing in reactive oxygen species (ROS); application Pro and/or SA via foliar spray mitigate these negative impacts by enhancing physio-biochemical parameters or antioxidant enzyme activity while decreasing ROS

membrane stability, seed crude protein, K/Na ratio, seed yield, and seed nutrient content (N, P, and K). Salinity stress also increased ROS. However, applying SA and/or Pro mitigates the harmful effects of salt stress on broad beans by inducing oxidative or osmotic stress while also regulating physiological traits, biochemical parameters, and antioxidant enzyme activity.

In conclusion, this study demonstrated that SS significantly reduced plant growth, seed yield characteristics, chlorophyll, RWC, membrane stability, K/Na ratio, and percentages of N, P, K, or crude protein in broad bean seeds. Concurrently, SS increased antioxidant enzyme activity (CAT, POD, SOD), leaf phenol content, and Endo-Pro levels. The application of Pro or SA effectively reduced SS's harmful effects. However, their combined application was more effective than their individual use. The combination of Pro and SA provided a more pronounced alleviation of salinity-induced oxidative stress by enhancing the accumulation of Endo-Pro or phenols and up-regulating the activities of antioxidant enzymes. This combined treatment notably improved chlorophyll pigments, RWC, seed nutrient content, crude protein, and broad bean seed yield. Overall, the results indicate that applying SA and/or Pro reduces the adverse effects of salinity-induced oxidative or osmotic stress on broad bean plants. This mitigation occurs by modulating physio-biochemical parameters or antioxidant enzyme activity, ultimately improving plant growth or productivity.

Acknowledgement: The authors would like to extend their sincere appreciation to the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (KFU241386), for supporting this research work.

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Received: July 28, 2024
Accepted: April 17, 2025
Published online: May 4, 2025