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Ameliorating Effect of Salicylic Acid on Physiological and Biochemical Characteristics of *Satureja spicigera* (C. Koch) Boiss. under NaCl Stress

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ABSTRACT

Creeping savory is a wild plant that is used for comestible consumption, preparation of beverages, and production of sanitary ware and herbal drugs. To investigate the effects of salinity stress and salicylic acid on antioxidant enzymes, photosynthetic pigments, relative water content, proline, and soluble protein content in *S. spicigera* a factorial experiment was conducted based on a Completely Randomized Design (CRD) and three replications. The experiment was implemented at the greenhouse of Agriculture and Natural Resources Research and Education Center of Kermanshah, Iran (2019). Experimental treatments were four levels of salinity (0-50-100-150 mM NaCl) and two levels of salicylic acid (0 and 2 mM). Results showed that increasing salinity levels caused a significant reduction in relative water content, leaf fresh weight, leaf dry weight, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content. Salinity drastically enhanced the antioxidant activities (SOD, POD, and CAT), and cell proline content. Salicylic acid considerably decreased proline content under salt stress conditions, but improved antioxidant activities of SOD, POD, and CAT, and enhanced chlorophyll a, chlorophyll b, total chlorophyll, carotenoid content, protein content, relative water content, and leaf fresh weight under salt stress. Salicylic acid reduced the destructive effect of salinity on some morphological, physiological, and biochemical characteristics in creeping savory.

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1. Introduction

Creeping savory (*Satureja spicigera* (C. Koch) Boiss.) is a medicinal oil-bearing plant. It grows in the north and north-west of Iran, and is somewhat drought tolerant (Yousefi *et al*., 2023). The important main essential oil compounds of *S. spicigera* are thymol and carvacrol (Yousefi *et al*., 2023). *S. spicigera* is used as a seasoning to prepare food, beverages, sanitary ware and herbal drugs.

Salinity stress can lead to an increase in ionic toxicity, osmotic stress, changes in physiological and biochemical processes, and an increase in oxidative stress by the production of ROS (Khan *et al*., 2014), subsequently, it reduces the growth of plants. The production of large amounts of ROS (O^{-}_2, $^{\cdot}O$, H_2O_2 and HO_2) causes the toxic effects of salt-induced oxidative stress that harm plant cell structure and function. An antioxidative defense system helps plants tolerate oxidative stress by scavenging ROS (Polash *et al*., 2019). In this defense system, SOD converts superoxide into H_2O_2, then CAT (mainly in peroxisomes) and, or POD (in chloroplast) convert H_2O_2 into H_2O and oxygen. NaCl stress has increased the activities of SOD, POD, and CAT in some plants such *Satureja khuzestanica* (Saadatfar and Hossein Jafari, 2022), *Triticum asetivem* (Afridi *et al*., 2019), and *Brassica carinata* (Husen *et al*., 2018).

NaCl stress affects photosynthetic pigments. Salt stress has decreased photosynthetic pigments in *Satureja hortensis* (Mohammadi *et al*., 2017), *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022), and *Nigella sativa* L. (Zarei *et al*., 2019).

Osmotic adjustment (OA) is one of the main ways to protect plants under osmotic stress. Proline has a prominent effect on cell OA and the high accumulation of proline under osmotic stress helps to absorb water in

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the cell (Zhang *et al*., 2012). In addition, proline plays an essential role in reducing electrolyte leakage by inhibiting reactive oxygen species against environmental stress (Kaur and Asthir, 2015). Several proteins accumulate in plants (salt stress proteins and stress-associated proteins) in response to salinity stress (Athar *et al*., 2022) that play a crucial role in OA (Chowdhury *et al*., 2017). Plant's tissues (depending on salt concentration and plant tolerance to salt) typically respond to salt stress by degrading proteins or producing salt stress-related proteins (Wang *et al*., 2015). In hyperosmotic stress, the cell protein content usually increases, and in hyperionic stress, cellular protein content decreases (Wang *et al*., 2015).

Accumulation of proline under salt stress conditions has been reported in some plants such as *S. hortensis* (Mohammadi *et al*., 2017), *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022), and *Thymus vulgaris* (Harati *et al*., 2015). Severe NaCl stress has declined the content of protein in *Amaranthus cruenus* (Menezes *et al*., 2017), *T. vulgaris* (Harati *et al*., 2015), and cotton (Dong *et al*., 2014).

The decrease in plant growth under salinity conditions is usually due to the effect of salinity on photosynthesis and its side processes, which vary according to the plant variety and environmental conditions. The physiological responses of plants to salinity conditions can cause a decrease in the photosynthetic pigments and, as a result, a decrease in plant production (Hasanuzzaman and Fujita, 2013). Chlorophyll and carotenoid content increases or remain unchanged in plants resistant to salt stress while decreasing in sensitive species (Ashraf and Harris, 2013). There are some reports that photosynthetic pigments has been decreased in *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022), *S. hortensis* (Mohammadi *et al*., 2017), *N. sativa* L. (Zarei *et al*., 2019), and *A. cruentus* under salt stress (Menezes *et al*., 2017).

RWC is an important feature that can be used to compare sensitive and tolerant plants in salinity stress. This index has decreased under salt stress in different plants such as *Lemon verbena* (Ghanbari *et al*., 2023) and *Capsicum annuum* (Kaya *et al*., 2020b).

Salicylic acid (SA) is a phenolic compound that plays a vital role in regulating plant physiological processes such as photosynthesis, nitrogen metabolism, proline (Pro) metabolism, antioxidant defense system, and plant-water relations under stress conditions (Khan *et al*., 2014) and thereby it regulates the growth and development of plants and their response to biotic and abiotic stress factors. The role of salicylic acid in the tolerance to salt stress reported in some crops, including *Brassica juncea* (Nazar *et al*., 2015), *Vicia radiata* (Khan *et al*., 2014) and *Medicago sativa* (Palma *et al*., 2013).

SA application has decreased the activity of SOD in *B. carinata* plants under salt stress (Husen *et al*., 2018), and enhanced the activities of CAT and POD in *T. vulgaris* (Harati *et al*., 2015) and *Brassica parachinensis* (Kamran *et al*., 2020). It also has reduced the proline content in some plants such *N. sativa* L. (Zarei *et al*., 2019), *Lallemantia royleana* (Rostami, 2018), and *B. carinata* (Husen *et al*., 2018) and caused accumulation of protein in *C. annuum* (Kumar *et al*., 2022) and *T. vulgaris* (Harati *et al*., 2015) under salinity conditions.

The amounts of Chl a, Chl b, and total Chl in some plants such as *N. sativa* L. (Zarei *et al*., 2019) and *Dianthus superbus* (Ma *et al*., 2017) increased by the application of SA under salinity. SA has also increased leaf fresh and dry weight in tomato seedlings (Souri and Tohidloo, 2019), winter wheat (Khalvandi *et al*., 2021), and *Solanum melongena* L. (Mady *et al*., 2023).

S. Spicigera is a procumbent soft perennial plant, often spread, with inflorescences arising from trailing sinuous stems. Stems recurved-pubescent on two opposite sides, hairs few or absent, leaves linear-oblanceolate, 8-20 (-25) x 2-3 (-5) mm, mucronate, glabrous, green, bearing short axillary shoots. Inflorescence oblong to linear, 3-10(-15) cm, Verticillasters, usually approximate, pedunculate, laxly (2-) 6-flowered. Calyx manifestly sub-bilabiate, 3-4(-5) mm, pubescent, lower 2 teeth 1-1.5 x tube, upper teeth only 1/2-3/4 x lower pair. Corolla 6-8 mm, white, exserted at calyx, limb ample. The stamens long-exerted from corolla; Nutlets 1.2 mm, broadly oblong-ovoid; *Fl. 8-9; S. spicigera* grows in eroded dry banks and rocky places at 20-1500 m altitude (Davis, 1982).

So far, no information published about the effect of salinity stress and salicylic acid on the creeping savory. In this research, the effects of salinity stress and the moderating role of salicylic acid on the physiological, biochemical, and photosynthetic traits of *Satureja spicigera* were investigated.

2. Materials and methods

2.1. Experimental design and treatments

A greenhouse factorial experiment (3 replicates) was carried out in a controlled environment at the Agriculture and Natural Resources Research and Education Center of Kermanshah (47°, 04" E; 34°,15" N), Iran in 2019 based on a completely randomized design (RCB). Factor A was four levels of salinity (0, 50, 100, and 150 mM NaCl) and factor B was two levels of salicylic acid (0 and 2 mM).

2.2. Seeds cultivation

The seeds of the creeping savory were obtained from the Research Institute of Forests and Rangelands of Iran (RIFR). Seeds were disinfected with 0.5% sodium hypochlorite, washed with distilled water, and then dried with blotting paper. They were planted in a tray, in a mixed soft bed of coco peat and peat moss (1:1) and watered by sprinkling every day during the germination period. The seedlings were watered every two days until reaching the 6-leaf stage. The equal size and healthy seedlings were transferred to the plastic pots (30 cm height and 17 cm diameter, one seedling per pot), at the stage of 6 to 8 leaves. The pots were filled with a 1:1:1 mixture of farm soil, sand, and rotten cow manure (4.5 kg, PH= 7.03, clay-loam, EC= 0.70 dS/m, p= 138 ppm, O.C. = 1.75% and Total N= 0.28%). The plants were kept under 17 h/d light photoperiod by 300 mMOL/m2×s and 7 hours of darkness (Hernández-Adasme *et al*., 2023), and relative humidity of 50-60%. During the establishment of the seedlings and before the implementation of salt treatments (2 weeks), the pots were irrigated once every three days with farm well water in an equal amount of 2500 ml for each pot.

2.3. Preparation of sodium chloride and salicylic acid treatments

Merck NaCl (CAS #: 7647-14-5, EC Number: 231-598-3, Molar Mass: 58.44 g/mol) was used to prepare NaCl treatments after modifying purity. The concentrations of 0, 50, 100, and 150 mM NaCl (2.2, 6.5, 9.1, and 13 dS/m) were prepared by adding double distilled water (DDW). The control plants were irrigated with double distilled water.

Salicylic acid (HOC_6H_4COOH; CAS #: 69-72-7; Merck; Germany) was used to prepare SA 2 mM (0.276 g/l). To prepare 10 L of SA 2 mM, 2.76 g of SA was well dissolved in 2 L warm DDW, and a few mL of ethanol, then it was well mixed (Ma *et al*., 2017). The final volume reached 10 L by DDW.

2.4. Implementation of treatments

Eight treatments consisting of irrigation (250 ml to each pot, once every three days) by 0, 50, 100, and 150 mM NaCl concentrations (Kumar *et al*., 2022) and foliar spraying with100 ml of SA (0 and 2 mM) were implemented as follows: T1 (distilled water); T2 (distilled water + 2 mM SA); T3 (50 mM NaCl); T4 (50 mM NaCl + 2 mM SA); T5 (100 mM NaCl); T6 (100 mM NaCl + 2 mM SA); T7 (150 mM NaCl); and T8 (150 mM NaCl = 2 mM SA). In order to adapt the plants to salinity and to avoid osmotic shock, in two steps (one week), T3 to T8 pots were irrigated by 20 mM NaCl (250 ml), and then salt treatments were performed. The plants were watered once, after every 4 NaCl treatments (12 days), with distilled water to remove the accumulated salts in the pots. Foliar spraying was done once every three days (8 times) by 100 ml SA (2 mM) twelve days after the start of salinity treatments (Andalibi *et al*., 2021; Ma *et al*., 2017). The SA control plants were sprayed with 100 ml DDW.

2.5. Studied traits

Some morphological, physiological, photosynthetic and biochemical traits include: LFW, LDW, RWC, Pro content, SP content, Chl a, Chl b, total chlorophyll, carotenoid content, and the enzymatic activities of SOD, POD, and CAT were studied.

2.6. Collection of samples and measurement of traits

In order to the measurement of photosynthetic pigments, proline, protein and the activity of antioxidant enzymes, the healthy and active leaves of the plant separated and after freezing in liquid nitrogen, they were stored at -20 °C.

2.6.1. Measurement of leaf fresh weight (LFW), leaf dry wight (LDW), and relative water content (RWC)

To determine the RWC (%), 30 young leaves were selected from each plant, and separated, and immediately weighed (LFW) in the laboratory with a scale (Sartorius BP210D, Germany; 0.0001 g); then they were placed in DDW 16 to 18 hours (for complete dehydration) in a laboratory environment with an approximate temperature of 22 °C. The leaf surface water was dried with filter paper and the samples were

reweighed leaf turgor weight (LTW). The leaves were placed in an oven at 70 °C for 48 hours and the LDW was measured. The means of LFW and LDW were calculated (mg). RWC was calculated from the following formula (Equation 1) (Bian and Jiang, 2009):

(1) RWC= (FW–DW)/(TW–DW)100

2.6.2. Measurement of chlorophyll and carotenoid contents

Chlorophyll a, b, and carotenoid content were measured by the standard method of Lichtenthaler and Welburn (1983). 25 mg of fresh leaves were powdered in a Chinese mortar with liquid nitrogen and then wholly homogenized with 2 ml of 96% ethanol in the dark condition. Samples were shaken well and centrifuged for 10 minutes (10000 rpm, 4 °C). Supernatant was transferred into the microtubes and was read by a Bio Tek Powerwave (XS2) Microplate Reader, USA at 663, 646, and 470 nm. Amounts of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids content (mg g^{-1} FW) were calculated by following formulas (Equation 2-5):

(2) Chl a = 13.36 (A664.2) - 5.19 (A648.6)

(3) Chl b = 27.43 (A648.6) - 8.12 (A664.2)

(4) Chl t = 5.24 (A664.2) + 22.24 (A648.6)

(5) Car = [1000 (A470) - 2.13(Chl a) - 97.64(Chl b)]/209

2.6.3. Measurement of antioxidant activity, proline and total protein

2.6.3.1. Preparation of extraction buffer

The extraction buffer (200 ml) was prepared according to the method of Ramachandra Reddy et al. (2004). 2.428 g Tris with 0.2 g PVP was well dissolved in 40 mL DDW (pH=8) and the final volume was 200 mL. The container was covered with aluminum foil and stored in a refrigerator (4°C).

2.6.3.2. Preparation of crude leaf extract

Based on the method of Ramachandra Reddy et al. (2004), leaf samples were crushed entirely in liquid nitrogen. The 250 mg of crushed leaves were transferred to a 2 ml microtube, and then 1 ml of extraction buffer was added. Samples were mixed by vortex (twice, 30 seconds, in 2-hour intervals, whereas the samples were kept in the refrigerator between each step), then the samples were kept in the refrigerator for 12 hours, and again were mixed (30 seconds). Mixtures were centrifuged (15 min., 4°C, and 13,000 rpm), and then the supernatant phase was separated and stored at -20 °C (Ramachandra Reddy *et al*., 2004).

2.6.3.3. Measurement of SOD activity

The enzymatic activity rate of Superoxide dismutase (SOD, EC 1.15.1.1) was measured (Beauchamp and Fridovich, 1971) based on the ability of SOD to stop the photochemical regeneration of Nitrotetrazolium Blue chloride (NBT) by superoxide radicals in the presence of riboflavin at light condition. After the completion of the reaction, the optical absorbance of samples was read at 560 nm (enzymatic unit equivalent to 50% inhibition) by a Bio Tek PowerWave XS2 Microplate spectrophotometer, USA. The rate of enzymatic activity (µ mole min^{-1} mg of soluble protein) was calculated using the following formula (Equation 6):

$$SOD\ (\mu mol\ g^{-1}\ FW) = \frac{100 - \left[\frac{(OD_{cont} - OD_{sample})}{OD_{cont}} \times 100\right]}{50} \quad (6)$$

OD_{cont}: absorbance of control at 560 nm
OD_{sample}: absorbance of samples at 560 nm

2.6.3.4. Measurement of POD activity

Enzymatic activity of peroxidase (POD; E.C. 1.11.1.7) was measured by the method of Chance and Maehly (1995) with modifications. The absorbance of the solutions was read for 15 min at 30 s intervals at a wavelength of 470 nm by Bio Tek Gen 5 software in a Bio Tek PowerWave XS2 Microplate spectrophotometer, USA. The rate of POD enzymatic activity was calculated using the Beer-Lambert law (0.0266 Mcm^{-1}) and was expressed in terms of H_2O_2 consumption (µ mole min^{-1} mg of soluble protein).

2.6.3.5. Measurement of CAT activity

Enzymatic activity of catalase (CAT; E.C. 1.11.1.6) was measured by the method of Sinha (1972) with some modifications. After completion of the reactions, the OD of the samples was read at 570 nm by a Bio Tek PowerWave XS2 Microplate reader, USA. The rate of CAT enzymatic activity was calculated using the Beer-

Lambert law (0.0394 Mcm^{-1} extinction coefficient) and was expressed regarding H_2O_2 consumption (µ mole min^{-1} mg of soluble protein).

2.6.3.6. Measurement of soluble proteins

Soluble protein concentration (mg/g FW) was measured based on the method of Bradford (1976). The 1 µl of the crude leaf extract was added to 200 µl of Coomassie Brilliant Blue. After 15 minutes, the OD of samples was read at 595 nm by Bio Tek Gen 5 software in a Bio Tek PowerWave XS2 Microplate Spectrophotometer, USA. The concentration of soluble protein was obtained according to the absorption of the samples and using the Bovine Serum Albumin (BSA) standard curve.

2.6.3.7. Estimation of free proline content

Proline content was measured based on (Bates *et al*., 1973). The OD of plant samples and proline standard was read at 520 nm by a Bio Tek PowerWave XS2 Microplate Spectrophotometer, USA. The standard curve was prepared based on the OD of proline standard. The OD of each sample was put into the standard equation and proline concentration was reported as µg/g FW.

2.7. Statistical analysis

Analysis of variance (factorial) and mean comparison were performed using IBM SPSS Statistics 26 software. The means (±SD) were compared using Duncan's Test ($p<0.05$), and the significant differences in mean were displayed using bars, and different letters.

3. Results and discussion

Significant differences were observed (Table 1) for Chl a, Chl b, Chl t, Car, proline, soluble protein, RWC, LFW, and enzymatic activities of SOD, POD, and CAT (P= 0.01) and for LDW (P= 0.05). SA treatments showed significant differences for the traits of LFW, Chl t, RWC, and SOD activity (P= 0.01) and for Chl a, Car, Proine content, SP, POD and Cat antioxidant activity (P= 0.05). SA did not show a significant difference for the LFW, and Chl b. The interaction effect of NaCl × SA (Table 1) was significant for Chl b, Chl t, and SOD activity (P= 0.05) and for Chl a, Car, Pro content, SP, RWC, enzymatic activities of POD, and CAT (P= 0.01). NaCl × SA was not significant to LFW and LDW.

3.1. Chlorophyll a

An increase in salinity caused a significant decrease in Chl a (Table 2). The highest amount of Chl a (13.12 mg/g FW) was observed in the NaCl 0 mM + SA 2 mM, and the lowest Chl a (6.17 mg/g FW) was observed in the 150 mM NaCl (Fig. 1a). SA increased the amount of Chl a in all salinity treatments as well as in the NaCl control treatment. This increase was much higher in high salinity treatments (100 and 150 mM NaCl). SA increased the amounts of chl a by 39.85% in the 100 mM NaCl and 64.02% in the 150 mM NaCl.

3.2. Chlorophyll b

An increase in salinity caused a significant decrease in chlorophyll b (Table 2). The highest amount of Chl b (4.27 mg/g FW) was observed in the 0 mM NaCl + 2 mM SA and the lowest (2.71 mg/g FW) was observed in 150 mM NaCl (Fig. 1b). SA has increased the amount of Chl b by 16.62, 2.19, 2.86, and 21.71% in the 0, 50, 100, and 150 mM NaCl, respectively.

3.3. Total chlorophyll

An increase in salinity caused a gradual decrease in total Chl (Table 2). The highest amount of total Chl (17.40 mg/g Fw) was observed in the 0 mM NaCl + 2 mM SA and the lowest (8.88 mg/g FW) was observed in 150 mM NaCl (Fig. 1c). SA in salinity treatments and NaCl control treatment caused an increase in the amount of total Chl. SA increased total Chl up to 26.61, and 51.13% in the 100 and 150 mM NaCl treatments, respectively.

3.4. Carotenoid

An increase in salinity caused a decrease in the leaf carotenoids (Table 2). The highest amount of carotenoid (3.87 mg/g FW) was observed in the control salt treatment, and the lowest (1.68 mg/g FW) was observed in the 150 mM NaCl (Fig. 1d). SA increased the amount of carotenoid by 48.24% in 100 mM and 62.50% in 150 mM NaCl respectively. SA caused a non-significant reduction in carotenoid levels in the salt control and 50 mM NaCl (low salinity stress).

3.5. Proline

By increasing of salinity stress, the proline content of the leaf was increased (Table 2). The highest content of proline (13.00 µg/g) was observed in the 150 mM NaCl and the lowest proline (2.17 µg/g) was observed

in the 100 mM NaCl + 2 mM SA (Fig. 2a). SA caused an increase in proline by 22.33% in the NaCl control treatment but the application of SA decreased Proline content 7.19, 86.71, and 46.97%, respectively in the 50, 100, and 150 mM NaCl.

3.6. Total protein

Increasing salinity up to 100 mM increased the content of leaf-soluble protein, but in the treatment of 150 mM, its amount was decreased (Table 2). The highest leaf-soluble protein (1.07 mg/g) was observed in the 50 mM NaCl + 2 mM SA and the lowest soluble protein (0.558 mg/g) was observed in the 150 mM NaCl (Fig. 2b). The use of SA in all salinity treatments increased the content of leaf-soluble protein. SA application (2 mM) increased leaf protein content in the 0, 50, 100, and 150 mM NaCl by 135.18, 29.12, 7.81, and 42.26%, respectively.

3.7. RWC

Relative water content decreased by 11.18, 22.42, and 27.92% respectively, in the 50, 100, and 150 mM NaCl. The highest RWC (91.70%) was observed in the treatment of 0 mM NaCl (control) and the lowest RWC (66.08%) was observed in the 150 mM NaCl (Fig. 2c). Application of 2 mM SA reduced the RWC by 5.59% in the control NaCl, but it increased RWC by 9.16, 3.01, and 33.41%, respectively in the 50, 100, and 150 mM NaCl (Fig. 2c).

3.8. LFW and LDW

Leaf fresh weight and leaf dry weight significantly reduced in all salinity treatments compared to the control (Table 2). SA significantly increased LFW in all salinity treatments (Fig. 2d) whereas it had no significant effect on LDW (Table 1; Fig. 3a). The highest LFW was observed in the treatment of 50 mM NaCl + 2 mM SA and the lowest was observed in the 150 mM NaCl (Fig. 2d).

Table 1. Analysis of variance of photosynthetic pigments, proline content, protein content, RWC, LFW, LDW, and SOD, POD, and CAT activities in *S. spicigera* under different NaCl and SA treatments

S. O.V.	df	Chl a	Chl b	Car	Chl t	Pro	LFW
Salt	3	22.25**	1.07**	2.70**	32.24**	0.05**	15.76**
SA	1	25.66*	0.43^{ns}	1.02**	32.76**	0.07*	10.86**
Salt×SA	3	4.53**	0.23*	0.71**	5.03*	0.03**	3.74^{ns}
Error	6	0.39	0.04	0.01	0.52	0.0001	2.886
CV (%)		6.70	6.51	3.81	5.75	14.72	15.78
S. O. V.	df	RWC	SP	SOD	POD	CAT	LDW
Salt	3	351.89**	179800**	7.69**	4.91**	5.49**	1.23*
SA	1	264.04**	483700*	2.06**	8.46*	13.59*	0.03^{ns}
Salt×SA	3	199.04**	67180**	0.15*	1.01**	3.60**	0.05^{ns}
Error	6	19.076	1.42	0.02	0.05	0.2	0.34
CV (%)		5.40	0.19	8.01	8.87	11.56	29.23

* and **= significant differences at the level of 0.05 and 0.01, respectively and Ns = no significant difference

Table 2. Means comparison of leaf photosynthetic pigments, Pro content, SP, LFW, LDW, RWC, and SOD, POD and CAT activities in *S. spicigera* under different NaCl or SA treatments (Duncan's test; a= 0.05)

Treatments		Means±SD					
		LFW (mg)	Chl a (mg/g FW)	Chl b (mg/g FW)	Car (mg/g FW)	Chl t (mg/g FW)	Pro (µg/g FW)
Salt (mM)	0	11.86±1.39^{ab}	12.19±0.1^{a}	3.67±0.02^{a}	3.87±0.1^{a}	15.86±0.1^{a}	2.3±0.01^{d}
	50	12.31±0.62^{a}	10.43±0.1^{b}	3.2±0.1^{b}	3.01±0.1^{b}	13.63±0.2^{b}	8.0±0.20^{c}
	100	8.80±0.62^{c}	7.78±0.1^{c}	3.38±0.1^{ab}	1.99±0.01^{c}	11.16±0.1^{c}	10.0±0.61^{b}
	150	10.09±0.01^{bc}	6.17±0.9^{d}	2.71±0.4^{c}	1.68±0.3^{c}	8.88±1.3^{d}	13.0±0.63^{a}
SA (mM)	0	10.09±1.93^{b}	9.14 ±2.46^{b}	3.24 ±0.40^{a}	2.64±0.91^{b}	12.4±2.8^{b}	8.11±0.04^{a}
	2	11.84±2.28^{a}	11.21±1.33^{a}	3.51±0.51^{a}	3.05± 0.36^{a}	14.72±1.7^{a}	3.80±0.03^{b}
Mean		10.76±2.07	9.14±2.46	3.24±0.4	2.64±0.92	12.38±2.79	5.96±0.40
Treatments		LDW (mg)	SP (mg/g FW)	RWC (%)	SOD activity (µ mole min^{-1} mg protein)	POD activity (µ mole min^{-1} mg protein)	CAT activity (µ mole min^{-1} mg protein)
Salt (mM)	0	2.32 ±0.50^{a}	0.43±0.04^{d}	91.70±0.52^{a}	0.73±0.01^{c}	1.14±0.1^{c}	2.17±0.11^{d}
	50	2.45±0.53^{a}	0.83±0.03^{b}	81.42±5.68^{b}	1.75±0.03^{b}	1.16±0.1^{c}	3.26±0.12^{c}
	100	1.53±0.49^{b}	0.99±0.03^{a}	71.1±2.13^{cd}	1.98±0.12^{b}	2.14±0.3^{b}	3.95±0.16^{b}
	150	1.69±0.24^{ab}	0.56±0.22^{c}	66.08±7.62^{d}	3.64±0.17^{a}	3.05±0.2^{a}	4.69±0.38^{a}
SA (mM)	0	2.07±0.46^{a}	0.70±0.02^{b}	77.7±10.87^{b}	1.44±0.98^{b}	1.87±0.84^{b}	3.52±0.99^{b}
	2	2.12±0.44^{a}	0.99±0.01^{a}	84.54±6.23^{a}	2.02±1.09^{a}	3.06±0.99^{a}	5.02±1.35^{a}
Mean		2.85±0.52	0.84±0.02	80.90±9.83	2.02±1.09	1.87±0.81	3.52±0.99

The common letters indicate no significant differences

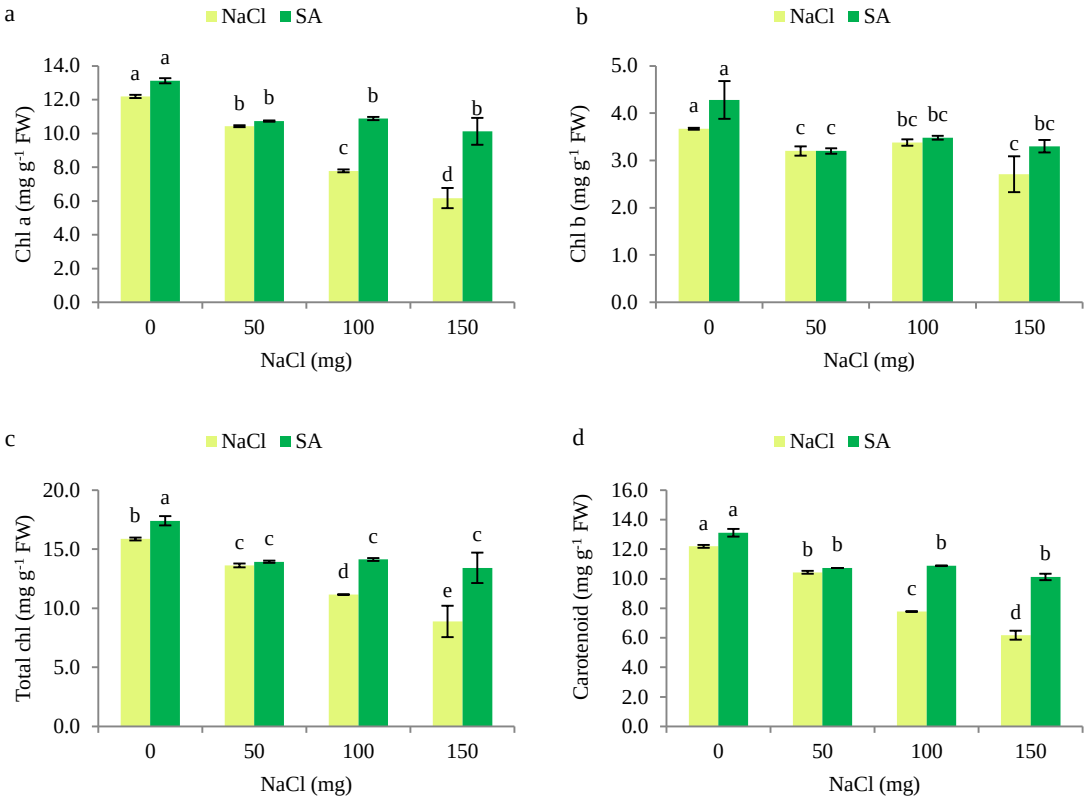

Figure 1. The content of Chl a (a), Chl b (b), total Chl (c), and carotenoid (d) (mg g^{-1} FW) of *Satureja spicigera* plants under different treatments of NaCl×SA. Columns with the same letters are not significantly different based on Duncan Mean ± SD (P= 0.05).

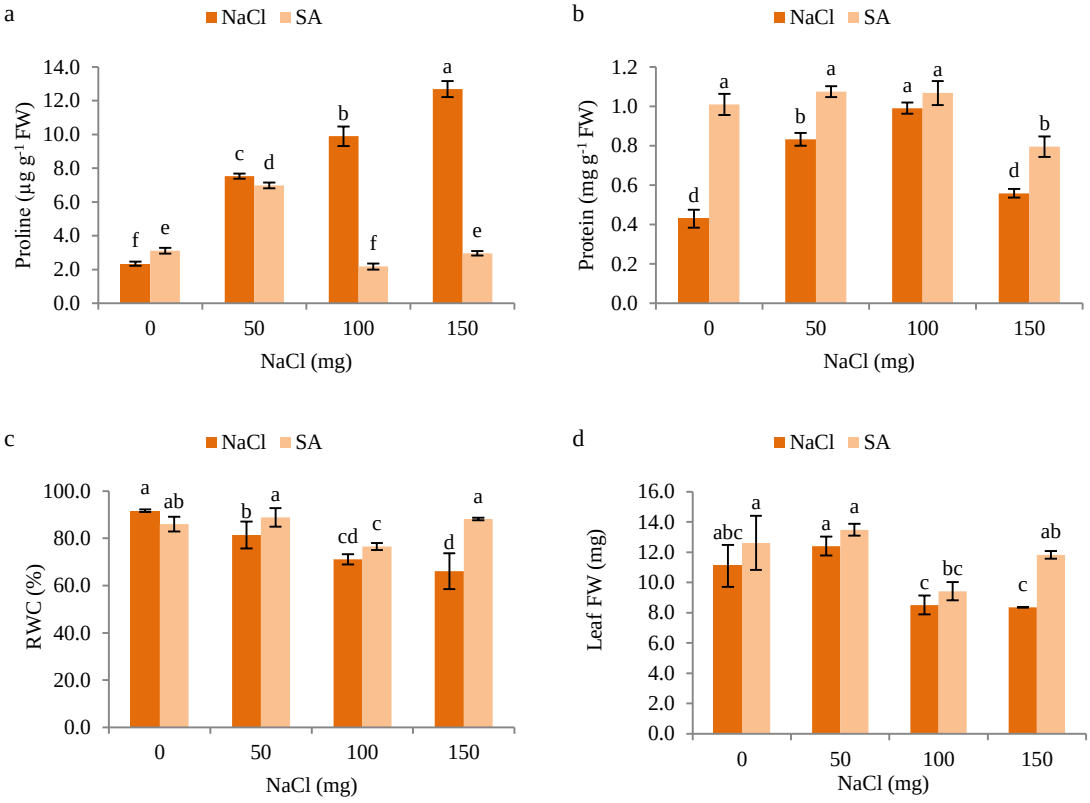

Figure 2. The proline content (a), protein content (b), RWC (c) and leaf fresh weight (d) of *Satureja spicigera* plants under different treatments of NaCl×SA. Columns with the same letters are not significantly different based on Duncan Mean ± SD (P= 0.05).

3.9. SOD activity

Increasing salinity stress enhanced SOD activity (Table 2). Maximum activity of SOD (3.64 µmol/min mg protein) was observed in the 150 mM NaCl + 2 mM SA and the SOD minimum activity (0.46 µmol/min mg protein) was observed in the 0 mM NaCl (Fig. 3b). The foliar spraying with 2 mM SA increased the activity of SOD in all NaCl treatments. SA application increased the activity of SOD by 36.99, 54.29, 18.69, and 20.88%, respectively in the 0, 50, 100, and 150 mM NaCl.

3.10. POD activity

Increasing the NaCl levels improved peroxidase activity (Table 2). The foliar spraying with 2 mM SA was increased the POD activity by 32.46, 175.00, 80.84, and 0.20%, respectively in the 0, 50, 100, and 150 mM NaCl. The highest activity of POD (3.87 µmol/min mg protein) was observed in the 100 mM NaCl+ 2 mM SA and the lowest it (1.14 µmol/min mg protein) was observed in the 0 mM NaCl (Fig. 3c).

3.11. CAT activity

The activity of CAT was enhanced significantly by increasing NaCl stress (Table 2). The foliar spraying with 2 mM SA increased the CAT activity by 79.72, 66.26, and 71.90%, in the 0, 50, and 100 mM NaCl respectively, but SA decreased CAT activity (15.14%) in the 150 mM NaCl. The highest activity of CAT (6.79 µmol/min mg protein) was observed in the treatments of 100 mM NaCl+ 2 mM SA, and the lowest it (2.17 µmol/min mg protein) was observed in the 0 mM NaCl (Fig. 3d).

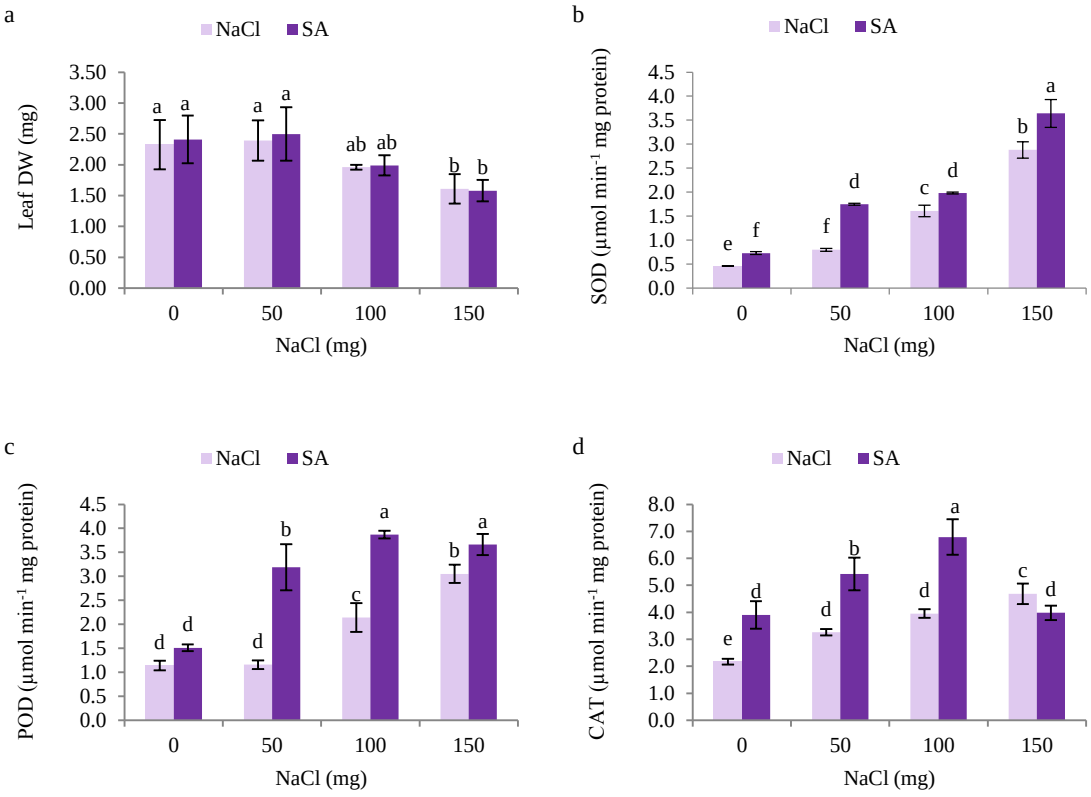

Figure 3. Leaf dry weight (a) and the enzymatic activity of SOD (b), POD (c), and CAT (d) of *Satureja spicigera* plants under different treatments of NaCl×SA. Columns with the same letters are not significantly different based on Duncan Mean ± SD (P= 0.05).

Relative water content is a valuable trait for investigating plant water status. In the current study, RWC decreased significantly with increasing salinity intensity. Salinity stress disturbs the ionic balance between the soil and the plant (Balti *et al.*, 2021), increases the osmotic stress (Kumar *et al.*, 2022), and reduces the turgor pressure (Wang *et al.*, 2023). These processes can reduce water absorption by root which causes RWC to decrease. Similar to these results, RWC has decreased under different salt concentrations in

some medicinal plants or crops such as *L. verbena* plants (Ghanbari *et al*., 2023), *C. annum* (Kaya *et al*., 2020b), *A. cruentus* (Menezes *et al*., 2017) and *Oryza sativa* (Jini and Joseph, 2017). The application of SA significantly increased the RWC of leaves under salt stress conditions in the current research. In some previous studies, under different salinity levels, RWC has improved by SA application in different species such *Lantana camara* (Dehestani Ardakani *et al*., 2021), *C. annum* (Kaya *et al*., 2020b), maize (Tahjib-Ul-Arif *et al*., 2018), rice (Jini and Joseph, 2017), and *Citrus sinensis* (Khoshbakht and Asgharei, 2015) (Fig. 3c).

Chlorophyll concentration is an index of plant tolerance to salinity. Anjum et al. (2014) stated that osmotic stress causes damage to the chloroplast structure, Chl oxidation, and reduction of Chl, followed by a reduction in photosynthesis due to the induction of oxidative stress. In the present study, the increase in salinity caused a significant decrease in Chl a, Chl b, and total Chl. Similar to our finding, salinity has caused a significant decrease in photosynthetic pigments in *S. hortensis* (Mohammadi *et al*., 2017), *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022), *L. camara*, (Dehestani Ardakani *et al*., 2021), *Linum usitatissimum* L. (Dubey *et al*., 2020), and *N. sativa* L. (Zarei *et al*., 2019). SA significantly increased the amount of Chl a, Chl b, and total Chl in all salinity treatments and in the control treatment. An increase in the amount of chlorophyll by SA has been reported in other plants such St. John's wort (Kwon *et al*., 2023), *Portulaca oleracea* (Panahyan Kivi *et al*., 2020), *N. sativa* L. (Zarei *et al*., 2019), and *D. superbus* (Ma *et al*., 2017).

Carotenoids are essential in reducing oxidative stress and regulating ROS cellular homeostasis in plants (Ashraf, 2009). In this study, the increase in salinity caused a decrease in the leaves carotenoid content. In some previous studies, carotenoid content was decreased under salinity stress in *C. annuum* (Kumar *et al*., 2022), *N. sativa* L. (Zarei *et al*., 2019), and *S. hortensis* (Fabriki Ourang and Mehrabad Pourbenab, 2016). In the present study, SA significantly increased carotenoids at 100 and 150 mM NaCl treatments. Application of exogenous SA in *N. sativa* L. caused an increase in carotenoids under salt stress (Zarei *et al*., 2019).

The proline content was augmented significantly by increasing salinity stress in the present study. Similar to our results, the proline content was increased by salinity levels in *S. hortensis* (Mohammadi *et al*., 2017), *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022), *B. carinata* (Husen *et al*., 2018) and *T. vulgaris* (Harati *et al*., 2015). SA significantly decreased proline content under severe salt stress conditions (100 and 150 mM NaCl). Similar to this finding, under salt stress conditions, the application of SA has reduced proline content in St. John's wort plants (Kwon *et al*., 2023), *N. sativa* L. (Zarei *et al*., 2019), *L. royleana* (Rostami, 2018), and *B. carinata* (Husen *et al*., 2018).

By increasing salinity up to 100 mM, the amount of soluble protein in leaves increased significantly, but in the treatment of 150 mM, its amount showed a significant decrease. Exactly like this finding, 100 mM NaCl has induced a significant increase in soluble proteins in *Broussonetia papyrifera*, and the concentration of the soluble proteins has decreased at 150 mM NaCl in this plant (Zhang *et al*., 2013). Also, the content of protein in *A. cruenus* (Menezes *et al*., 2017) and *T. vulgaris* (Harati *et al*., 2015) has increased at low salinity treatments and declined at severe NaCl stress.

Low and mild salt stress induces the accumulation of proteins in the cell. Accumulated proteins in plants under salt stress may be the result of sequential expression of existing proteins or may be synthesized de novo (Qasim *et al*., 2003). At high salinity levels, the synthesis of proteins decreases due to the reduction of water and nutrients available to the plant, and some proteins are also decomposed into smaller molecules. It should be considered that salinity-mediated changes in protein profiles may be due to changes in regulation of mRNAs transcription. Also, salt concentration can stimulate or inhibit the translation of mRNAs and thus increase or decrease the accumulation of proteins in the cell (Zhang *et al*., 2013). In oxidative stress (induced by salinity stress), many free radicals are produced. These free radicals have a high affinity to bonding with proteins, enzymes and nucleic acids and cause their destruction and denaturation (Peltzer *et al*., 2002). The denaturation of proteins directly reduces their accumulation in the cell (Bishnoi *et al*., 2006) and the destruction of nucleic acids and enzymes reduces the biosynthesis of de novo proteins (Zhang *et al*., 2013).

The use of SA in all salinity treatments increased the amount of leaf-soluble protein in our study. In line with this, SA has caused accumulation of protein under

salinity conditions in *C. annuum* (Kumar *et al*., 2022), *C. roseus* (Abdolmohammadi and Omidi, 2017), and *T. vulgaris* (Harati *et al*., 2015).

The increased antioxidant activity enables plants to resist potential oxidative damage caused by salinity (Hasanuzzaman *et al*., 2021). In the present research, increasing the NaCl up to 150 mM significantly enhanced the activity of the SOD, POD, and CAT. In line to these findings, NaCl has increased the activities of SOD, POD, and CAT in many medicinal and agricultural plants such as *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022) and *B. carinata* (Husen *et al*., 2018). Also, NaCl has improved POD and, or CAT activities in *C. annuum* (Kumar *et al*., 2022) and *Amarantus tricolor* (Sarker and Oba, 2020). The effect of exogenous SA on antioxidant enzymatic activity are varying depending on plant tolerance to abiotic stress (Zhang *et al*., 2011) and SA concentrations (Wang *et al*., 2022). In the present study, 2 mM SA enhanced SOD activity in *S.spicigera* plants under salt stress. Like to this finding, SA has increased SOD enzymatic activity in some plants such as *Vitis vinifera* (Aazami *et al*., 2023), *T. aestivum* (Alam *et al*., 2022), and Watermelons (Moustafa-Farag *et al*., 2020). Foliar spraying with 2 mM SA significantly enhanced POD and CAT activity up to 100 mM NaCl. SA application significantly has enhanced the activities of CAT and POD in *B. parachinensis* (Kamran *et al*., 2020), and *T. vulgaris* (Harati *et al*., 2015) in salt stress conditions. Also, under salt stress conditions application of exogenous SA effectively has improved the activity of antioxidant enzymes (POD and or CAT) in *D. superbus* (Ma *et al*., 2017), *N. sativa* (Zarei *et al*., 2019) and *L. royleana* (Rostami, 2018). These findings are in line with our results.

Leaf fresh weight and leaf dry weight significantly reduced in all salinity treatments compared to the control. NaCl stress has reduced the Leaf fresh weight and leaf dry weight in *S. hortensis* (Mohammadi *et al*., 2017) and *S. khuzestanica* (Saadatfar and Hossein Jafari, 2022). SA significantly increased the Leaf fresh weight in all salinity treatments, although it increased Non-significantly leaf dry weight. In line with our finding, at high salinity levels, the application of SA has improved leaf fresh weight and leaf dry weight in *L. camara* (Dehestani Ardakani *et al*., 2021), maize (Kaya *et al*., 2020a), and mustard (Kohli *et al*., 2019).

4. Conclusion

Salt stress declined leaf RWC, photosynthesis, and subsequently growth, and enhanced leaf proline and protein content and antioxidant activity in *S. spicigera*. The exogenous application of 2 mM SA under salt stress conditions ameliorated RWC, photosynthetic pigments, and antioxidant activity, and subsequently reduced salt mediated-oxidative damage.

Abbreviation

C: control; Car: carotenoid; CAT: catalase; Chl: chlorophyll; DW: distilled water; DDW: double distilled water; LFW: leaf fresh weight; LDW: leaf dry weight; LTW: Leaf turgor weight; OA: osmotic adjustment; OD: optical density; POD: peroxidase; Pro: proline; ROS: reactive oxygen species; RWC: relative water content; SA: salicylic acid; SOD: superoxide dismutase; SP: soluble protein

Conflict of interests

The authors declared no potential conflicts of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

The data supporting the results are available by the author [B. Y.].

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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