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Effects of Salicylic Acid on Photosynthetic Pigments, Osmolytes, and Antioxidant Enzyme Activities in White Savory (*Satureja mutica* Fisch.) Exposed to Various Salt Levels

Borzou Yousefi[1]*, Roya Karamian[1]

1 Department of Biology, Faculty of Science, Bu-Ali Sina University, Hamadan, Iran

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ABSTRACT

White savory (*Satureja mutica* Fisch.) is an oil-bearing plant with applications in traditional medicine, pharmaceutical industries, and food additives in homemade dishes. The current research comprised a greenhouse experiment in a factorial arrangement based on a completely randomized design (CRD). It included four salinity levels (0, 50, 100, and 150 mM NaCl), two salicylic acid (SA) levels (0 and 2 mM), and three replicates. By increasing the NaCl concentration, the content of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid significantly declined. Increasing NaCl up to 100 mM caused a significant increase in proline and soluble protein content. The amount of proline at 150 mM NaCl showed no significant change compared to 100 mM NaCl, but the soluble protein sharply decreased at 150 mM NaCl. The enzymatic activities of superoxide dismutase, catalase, and peroxidase significantly increased in response to higher NaCl concentrations. Saturated water deficiency increased significantly, and leaf fresh and dry weights decreased substantially at 100 and 150 mM NaCl. SA enhanced chlorophyll a, chlorophyll b, total chlorophyll, carotenoid content, and leaf fresh and dry weight, depending on the NaCl treatments. SA applications considerably boosted peroxidase and catalase activities despite the presence of NaCl at any concentration. Also, SA significantly improved superoxide dismutase activity at 50 and 100 mM NaCl but could not counter its decrease when the NaCl level was 150 mM. SA significantly reduced saturated water deficiency and proline content despite any of the NaCl treatments. SA mitigated the adverse effects of NaCl on *S. mutica* by improving antioxidant activity, photosynthetic pigments, and physiological characteristics.

Abbreviation: Control (C), Carotenoid (Car), Catalase (CAT), Chlorophyll (Chl), Dry weight (DW), Fresh weight (FW), Turgor weight (TW), Peroxidase (POD), Reactive oxygen species (ROS), Salicylic acid (SA), Saturated water deficiency (SWD), Superoxide dismutase (SOD)

Introduction

White savory (*Satureja mutica* Fisch. & C. A.) is a wild edible and medicinal plant that grows scattered in rangelands in the northeast, north, and northwest of Iran (Gohari et al., 2011). *S. mutica* has a low water requirement and is resistant to water deficit conditions (Karimi et al., 2022). White savory is an essential oil-bearing perennial, relatively woody plant, about 45 cm tall, with long flowering stems, short hairs on its leaves, and white-colored flowers (Jamzad, 2012). Important compounds in white savory essential oil are thymol and carvacrol (Yousefi and Safari, 2022). White savory is used in

*Corresponding author's email: borzooyoosefi@yahoo.com

traditional medicine to treat rheumatic pain, migraine, toothache, and diarrhea (Mazandarani and Monfaredi, 2017). Its application in food additives can benefit homemade foods and food industries. White savory essential oil has applications in the medicine and food industries (Sagdic and Ozcan, 2003). White savory is usually irrigated in most arid and semi-arid regions and rainfed in temperate zones with an annual rainfall of about 450 mm and more at altitudes of 800-1650 (Yousefi and Safari, 2022).

Salinity stress can lead to osmotic and oxidative stress (Khan et al., 2014) and ultimately cause a decrease in the growth of plants (Montanari et al., 2008). Production of large amounts of reactive oxygen species (ROS: O^{-2}, $\cdot O$, H_2O_2, and HO_2) causes toxic effects of salt-induced oxidative stress, which is harmful to plant cell structure and function. Multiple mechanisms play a role in avoiding osmotic effects under salt stress (Ma et al., 2020). Scavenging free radicals by antioxidant enzymes (SOD, POD, and CAT) is one of the most essential defense mechanisms against oxidative stress (Gill and Tuteja, 2010). Antioxidant enzyme activities (SOD, POD, CAT, and APX) provide plants with resistance to salinity stress by eliminating ROS (Polash et al., 2019). In an enzymatic defense mechanism against ROS agents, first, SOD converts superoxide into H_2O_2. Then CAT (mainly in peroxisomes) and POD (in chloroplasts) convert H_2O_2 into H_2O and oxygen.

Osmotic adjustment is a primary way to protect plants under osmotic stress. High proline accumulation under osmotic stress helps to absorb water in the cell and maintain osmotic regulation (Zhang et al., 2012). Also, proline plays a vital role in maintaining protein content and membrane structure, upholding cell function, and reducing electrolyte leakage by inhibiting reactive oxygen species against environmental stress (Kaur and Asthir, 2015). Proline accumulation under salt stress conditions has been reported in many plants, such as cotton (Dong et al., 2014), and *Jerusalem artichoke* (Huang et al., 2013). 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 osmotic adjustment (Qun et al., 2017; Chowdhury et al., 2017). Plant tissues usually respond to salt stress by degrading proteins or producing salt-stress-related proteins (Wang et al., 2015). Cell protein changes under salt stress conditions depend on stress intensity and species tolerance to salt stress. Usually, cell protein content increases in response to hyperosmotic stress (Wang et al., 2015), and cellular protein content decreases in response to hyperionic stress (Badr, 2015).

A decrease in plant growth under salinity conditions usually results from the effect of salinity on photosynthesis and its marginal processes, which vary according to plant variety and environmental conditions. Plant physiological responses to salinity conditions can cause a decrease in photosynthetic pigments and, as a result, decrease biomass production (Hasanuzzaman and Fujita, 2013). Previous research shows that chlorophyll content increases or remains unchanged in plants resistant to salt stress while it decreases in salt-sensitive species (Ashraf and Harris, 2013).

Salicylic acid is a phenolic compound that regulates plant physiological processes such as photosynthesis, nitrogen metabolism, proline metabolism, antioxidant defense system, and plant-water relations under stress conditions (Khan et al., 2014; Miura and Tada, 2014). Salicylic acid modulated the activity of SOD and H_2O_2 metabolizing enzymes (such as CAT, POD, and APX) in plants exposed to osmotic stress (Saruhan et al., 2012). Molecular studies have shown that salicylic acid can regulate many aspects of plants at the gene level (Jumali et al., 2011) and thus can improve plant tolerance to abiotic stresses such as salinity (Nazar et al., 2015; Khan et al., 2014). Salicylic acid enhances tolerance mechanisms to salt stress in many crops, including *Brassica juncea* (Nazar et al., 2015), *Vicia radiata* (Khan et al., 2014), and *Medicago sativa* (Palma et al., 2013).

This study involved an attempt to cultivate this plant in semi-saline soils. Since no information is available about the effects of salicylic acid on salt-affected *S. mutica* plants, we evaluated the effects of salinity stress and the moderating role of salicylic acid on some physiological, biochemical, and photosynthetic traits of *S. mutica*.

Materials and Methods

Plant materials

In this experiment, *S. mutica* seeds were obtained from the Research Institute of Forests and Rangelands (RIFR) in Iran. The seeds originated from Aladagh Mountain (37°, 04" E; 57°, 29" N) in North Khorasan, with an altitude of 1610 m, average rainfall of 347.71 mm, and average annual temperature of 13.3 ºC. Seed purity was 93% and seed germination was 38.4%. Seeds were disinfected with 0.5% sodium hypochlorite, washed with distilled water, and then dried on sterile paper. The seeds were planted in trays, containing a mixed soft bed of coco peat and peat moss (1:1). Irrigation occurred through water sprinkling every day during the germination period. The seedlings were watered every two

days until they reached their six-leaf stage. Unique healthy seedlings, with similar size, were transferred to plastic pots (one seedling per pot) after 4 weeks, by which time the seedlings had 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^{-1}, p= 138 ppm, O.C.= 1.75% and total N= 0.28%). The plants were kept under 17 h d^{-1} light photoperiod by 300 mMOL m^{-2} s^{-1} and 7 h of darkness (Hernández-Adasme et al., 2023). Relative humidity was 50-60% in the greenhouse. During seedling establishment and before implementing salt treatments (2 weeks), the pots were irrigated once every three days with groundwater. Each pot received 2500 mL of water per irrigation session.

Experimental design

A greenhouse factorial experiment was based on a completely randomized design (CRD), with four levels of salinity (0-50-100-150 mM NaCl), two levels of salicylic acid (0 and 2 mM), and three replications in a controlled environment at RIFR, Kermanshah, Iran.

Merck NaCl (CAS#: 7647-14-5, EC value 231-598-3, molar mass: 58.44 g mol^{-1}) was used for preparing NaCl treatments after modifying purity. Concentrations of 0, 50, 100, and 150 mM NaCl (2.2, 6.5, 9.1, and 13 dS m^{-1}) were prepared by adding double-distilled water. Control plants were irrigated by double-distilled water.

Salicylic acid (HOC_6H_4COOH; CAS #: 69-72-7; Merck; Germany) was used for preparing SA (2 mM) (0.276 g L-1). To prepare 10 L of SA (2 mM), 2.76 g of SA was dissolved in 2 L warm double-distilled water, and a few mL of ethanol. Then, the solution was mixed thoroughly (Ma et al., 2017). Final volume reached 10 L by double-distilled water.

Eight treatments consisted of irrigation (250 mL for each pot, once every three days) at 0, 50, 100, and 150 mM NaCl concentrations (Kumar et al., 2022) and two foliar sprays of salicylic acid at 0 and 2 mM SA (Heidarian and Roshandel, 2021). Treatment groups were 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). To acclimatize the plants to salinity and avoid osmotic shock, T3 to T8 pots were irrigated by 20 mM NaCl (250 mL) in two steps (one week), and then salt treatments were performed. The plants were watered with distilled water once after every 4 NaCl treatments (12 days) 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 beginning the salinity treatments (Ma et al., 2017; Andalibi et al., 2021). SA control plants were sprayed with 100 mL double-distilled water. Healthy plant leaves were separated and after freezing in liquid nitrogen, they were stored in -20 °C until further analysis to measure photosynthetic pigments, proline, protein, and antioxidant enzyme activities.

Measurement of leaf-related traits

To determine the leaf FW (g), leaf DW (g), and SWD (%), 30 young leaves were selected from each plant. The leaves were separated and immediately weighed (leaf FW) on a digital balance (Sartorius BP210D, Germany). Then, the leaves were placed in double-distilled water for 16 to 18 h to allow complete dehydration in a laboratory environment at approximately 22 °C. Leaf surface water was dried with filter paper and the samples were reweighed (leaf TW). Then, the leaves were placed in an oven at 70 °C for 48 h, and the leaf DW was measured (Vaieretti et al., 2007). Average values of leaf FW and leaf DW were calculated (g). The SWD (%) was calculated according to the following formula (Hellmuth, 1970).

$$SWD = 100 - [\frac{FW - DW}{TW - DW}] \times 100$$

Measurement of chlorophyll and carotenoid contents

Chlorophyll a, b, and carotenoid content were measured according to a standard method (Lichtenthaler and Welburn, 1983). Twenty-five mg of fresh leaf sample were powdered in a Chinese mortar with liquid nitrogen, and then wholly homogenized with 2 mL of 96% ethanol in dark conditions. Samples were shaken well and centrifuged for 10 min (10000 rpm, 4 °C). After obtaining the supernatant, it was transferred to microtubes and read by a spectrophotometer (Bio Tek Powerwave (XS2) Microplate, USA) at 664.2, 648.6, and 470 nm. Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content (mg g-1 FW) were calculated according to the following formulae (Lichtenthaler and Welburn, 1983).

$$Chl\ a = 13.36\ (A_{664.2}) - 5.19\ (A_{648.6})$$
$$Chl\ b = 27.43\ (A_{648.6}) - 8.12\ (A_{664.2})$$
$$Chl\ t = 5.24\ (A_{664.2}) + 22.24\ (A_{648.6})$$
$$Car = \frac{[1000\ (A_{470}) - 2.13\ (Chl\ a) - 97.64\ (Chl\ b)]}{209}$$

Measurement of antioxidant activity, proline, and total protein

An extraction buffer (200 mL) was prepared according to Ramachandra Reddy et al. (2004). Accordingly, 2.428 g of Tris was mixed with 0.2 g PVP and dissolved well in 40 mL of DDW (pH=8). The final volume reached 200 mL. The containers were covered with aluminum foil and stored in the refrigerator (4 °C). Leaf samples were crushed entirely in liquid nitrogen. Then, 250 mg of crushed leaf was transferred in 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 in the refrigerator were vortexed between each step. The samples were kept in the refrigerator for 12 h and mixed again (30 s). The mixtures were centrifuged (15 min, 4 °C, and 13,000 rpm). The supernatant phase was separated and kept at -20 °C (Ramachandra Reddy et al., 2004).

Measurement of SOD activity

Superoxide dismutase activity rate (SOD, EC 1.15.1.1) was measurable according to 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 with exposure to light. The samples were transferred to the Bio Tek PowerWave XS2 Microplate spectrophotometer, USA, after the completion of the reactions, and its optical absorbance was read at 560 nm wavelength (enzymatic unit equivalent to 50% inhibition) by a Bio Tek Gen 5 software. The enzymatic activity rate was calculated using the following formula:

$$SOD(\mu mo\,\mathrm{lg}^{-1}FW) = \frac{100 - \left[\frac{\left(OD_{cont} - OD_{sample}\right)}{OD_{cont}} \times 100\right]}{50}$$

OD cont: absorbance of control at 560 nm
OD sample: absorbance of samples at 560 nm (Beauchamp and Fridovich, 1971).

Measurement of POD activity

Enzymatic peroxidase activity (POD; E.C. 1.11.1.7) was measurable according to Chance and Maehly (1995) with modifications. Absorbance values of the solutions were 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. POD enzymatic activity rate was calculated using the Beer-Lambert law (0.0266 M cm-1) and was expressed in terms of H2O2 consumption (μM min^{-1} mg of soluble protein).

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. The OD of each sample was read by Bio Tek PowerWave XS2 Microplate spectrophotometer, USA, at 570 nm, after completing the reactions. CAT enzymatic activity rate was calculated using the Beer-Lambert law (0.0394 M cm^{-1} extinction coefficient) and was expressed regarding H_2O_2 consumption (μM min-1 mg of soluble protein).

Measurement of soluble proteins

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

Estimation of free proline content

Proline content was measured based on a relevant method (Bates et al., 1973). The standard curve was illustrated according to Bates et al. (1973). The OD of plant samples and proline standard was read at 520 nm by Bio Tek Gen 5 software in a Bio Tek PowerWave XS2 Microplate Spectrophotometer, USA. Then, the OD of each sample entered the standard equation and was reported as μg g^{-1} FW.

Statistical analysis

Analysis of variance (factorial) and mean comparisons were performed using IBM SPSS Statistics 26 software. The means (±SD) were compared using the LSD test ($p<0.05$). Significant differences in mean values were indicated by dissimilar letters.

Results

Among NaCl treatments, significant differences were observed ($p\leq0.01$) in all of the investigated traits, i.e., Chl a, Chl b, total Chl, carotenoid, proline content, protein, SWD, leaf fresh weight, leaf dry weight, and the enzymatic activities of SOD, POD, and CAT (Table 1). Between SA treatments, significant differences were observed ($p\leq0.01$) in Chl a, total Chl, proline, and soluble protein, SWD, leaf fresh weight, and antioxidant activities of SOD, CAT, and POD. Different SA levels

caused significant differences ($p \leq 0.05$) in leaf dry weight, carotenoid, and Chl b (Table 1). The interaction of NaCl × SA (Table 1) significantly affected leaf dry weight ($p \leq 0.05$), Chl a, Chl b, total Chl, carotenoids, proline content, soluble protein, SWD, leaf fresh weight, and enzymatic activities of SOD, POD, and CAT ($p \leq 0.01$).

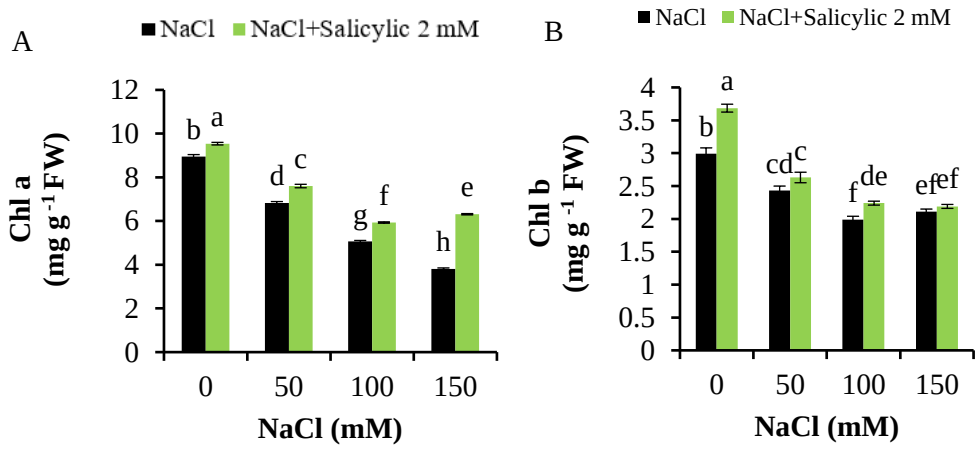
Table 1. Analysis of variance of photosynthetic pigments, proline content, protein content, SWD, Leaf FW, Leaf DW, and enzymatic activities of SOD, POD, and CAT in *S. mutica* under various NaCl and SA treatments.

S. V.	df	Chl a	Chl b	Chl t	Carotenoid	Leaf FW	Leaf DW
Salt	3	21.77**	1.93**	39.65**	1.85**	9.38**	1.96**
SA	1	3.39**	0.20*	5.24**	0.43*	8.75**	0.18*
Salt × SA	3	2.85**	0.23**	0.26**	0.42**	6.05**	0.12*
Error	6	0.01	0.02	0.01	0.01	1.00	0.03
CV (%)		0.81	2.5	0.68	7.53	9.60	10.88
S. V.	**df**	**Proline**	**protein**	**SWD**	**SOD**	**POD**	**CAT**
Salt	3	0.1**	386655**	553.63**	0.73**	1.71**	8.28**
SA	1	0.01**	55713.4**	463.75**	0.41**	0.28**	1.57**
Salt × SA	3	0.1**	42113.3**	185.37**	0.70**	0.27**	0.64**
Error	6	0.002	2356.30	8.72	0.01	0.02	0.02
CV (%)		24.96	5.99	18.02	11.45	12.46	7.14

* and **= significant differences at the level of 0.05 and 0.01, respectively. Ns= no significant difference. CAT= catalase, Chl= chlorophyll, DW= dry weight, FW= fresh weight, POD= peroxidase, SOD= superoxide dismutase, SWD= saturated water deficiency.

Chlorophyll a

Chlorophyll a decreased by 23.80, 43.46, and 57.43% in response to 50, 100, and 150 mM NaCl compared to the control, respectively. The highest amount of Chl a (9.54 mg g^{-1} FW) occurred in response to 50 mM NaCl + 2 mM SA, and the lowest (3.81 mg g^{-1} FW) in response to 150 mM NaCl. Salicylic acid increased the Chl a by 6.59, 11.44, 17.19, and 65.62%, respectively, in response to the 0, 50, 100, and 150 mM NaCl treatments (Fig. 1A).



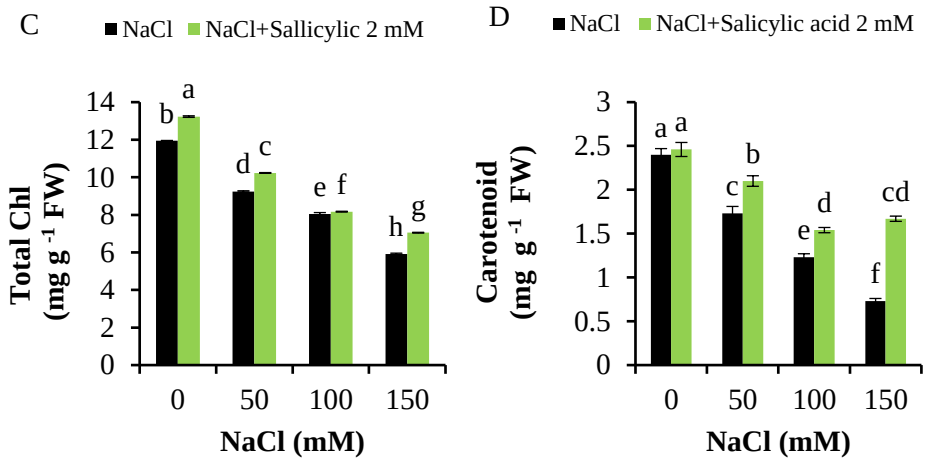


Fig. 1. Mean comparison of interaction effect of NaCl × SA (salicylic acid) for Chl a (A), Chl b (B), Total chl (C), and carotenoid (D) According to Least significant test (LSD), means with the same letter do not have a significant difference (p≤0.05). Chl= chlorophyll and FW= fresh weight.

Chlorophyll b

Chlorophyll b decreased by 18.73, 33.45, and 29.43% in response to 50, 100, and 150 mM NaCl treatments, respectively, compared to the control treatment. The maximum amount of Chl b (3.69 mg g-1 FW) occurred in response to the 50 mM NaCl + 2 mM SA and the lowest Chl b (1.99 mg g-1 FW) in the 100 mM NaCl. Salicylic acid increased the amount of Chl b by 23.41, 8.23, 12.56, and 3.79% at 0, 50, 100, and 150 mM NaCl, respectively (Fig. 1B).

Total chlorophyll

Total leaf chlorophyll decreased by 22.61, 32.58, and 50.42%, respectively, at 50, 100, and 150 mM NaCl compared to the control group. Salicylic acid increased the amount of total Chl by 10.80, 10.71, 1.49, and 19.09% in response to the 0, 50, 100, and 150 mM NaCl treatments, respectively (Fig. 1C).

Carotenoid content

Carotenoid content decreased by 2.92, 48.75, and 69.58% at 50, 100, and 150 mM NaCl treatments, respectively, compared to the control group (Table 2). The highest carotenoid content (2.46 mg g-1 FW) occurred in response to the 50 mM NaCl + 2 mM SA and the lowest (0.73 mg g-1 FW) in 150 mM NaCl. Salicylic acid increased the carotenoid content by 2.50, 21.39, 25.20, and 128.77% in response to the 0, 50, 100, and 150 mM NaCl, respectively (Fig. 1D).

Table 2. Comparison of means for leaf photosynthetic pigments, proline content, and soluble protein in *S. mutica* under different NaCl and SA treatments (LSD test; p<0.05).

Treatments		Means ± SD					
		Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Chl t (mg g^{-1} FW)	Car (mg g^{-1} FW)	Proline (µg g^{-1} FW)	protein (mg g^{-1} FW)
SA (mM)	0	6.38±1.93^{b}	8.82±2.26^{b}	1.60±0.06^{b}	1.60±0.06^{b}	9.0±0.05^{a}	0.86±0.03^{a}
	2	7.13±1.73^{a}	9.75±0.03^{a}	1.86±0.05^{a}	1.86±0.05^{a}	1.50±0.08^{b}	0.81±0.13^{b}
NaCl (mM)	0	8.96±0.14^{a}	11.9±0.04^{a}	2.4±0.12^{a}	2.4±0.12^{a}	1.3±0.02^{c}	0.68±0.04^{c}
	50	6.82±0.11^{b}	9.24±0.08^{b}	1.73±0.13^{b}	1.73±0.13^{b}	7.3±0.04^{b}	1.02±0.05^{b}
	100	5.93±0.05^{c}	8.17±0.04^{c}	1.54±0.05^{c}	1.54±0.05^{c}	14.0±0.03^{a}	1.23±0.09^{a}
	150	3.81±0.07^{d}	5.9±0.08^{d}	0.73±0.05^{d}	0.73±0.05^{d}	14.0±0.02^{a}	0.51±0.06^{d}
	Mean	6.75±1.83	2.53±0.55	9.29±2.33	1.73±0.57	4.28±0.50	0.84±0.24
	LSD	0.06	0.07	0.04	0.06	0.08	0.03

The common letters indicate no significant differences. Car= carotenoid, Chl= chlorophyll, FW= fresh weight.

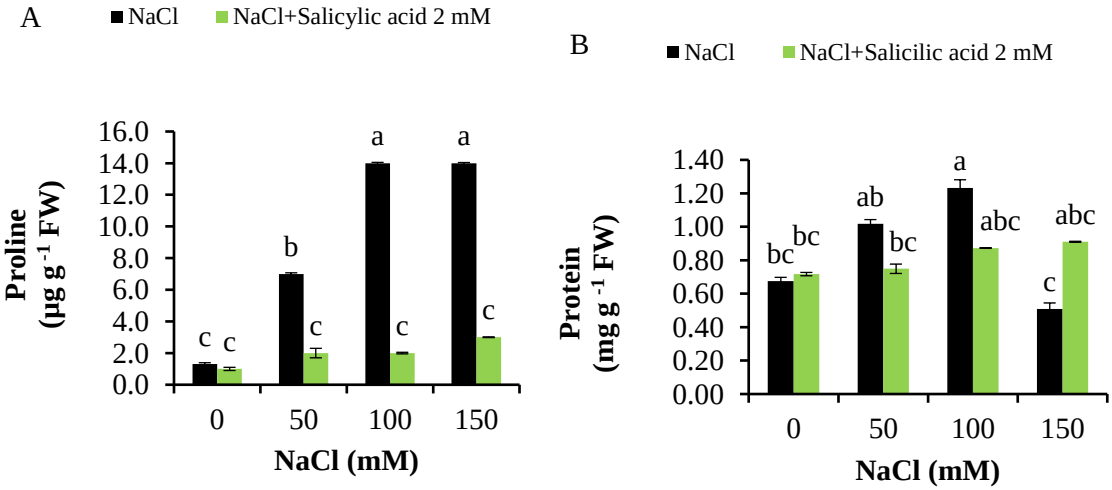
Table 2 (continue). Comparison of means for leaf FW, leaf DW, SWD, and the activities of SOD, POD, and CAT in *S. mutica* under different NaCl and salicylic acid (SA) treatments (LSD test; $p<0.05$).

Treatments		Means ± SD					
		Leaf FW (mg)	Leaf DW (mg)	SWD (%)	Activity (µmole min^{-1} mg protein)		
					SOD	POD	CAT
SA (mM)	0	9.83±1.01^{b}	1.53±0.42^{b}	20.79±13.18^{a}	0.75±0.02^{a}	1.08±0.05^{b}	1.82±0.05^{b}
	2	11.04±2.15^{a}	1.71±0.66^{a}	11.99±6.04^{b}	0.50±0.02^{b}	1.30±0.07^{a}	2.33±0.91^{a}
NaCl (mM)	0	9.91±1.19^{ab}	1.90±0.24^{a}	8.34±0.88^{b}	0.19±0.01^{d}	0.58±0.07^{d}	0.29±0.02^{d}
	50	10.43±0.52^{a}	1.92±0.05^{a}	8.44±0.88^{b}	0.49±0.02^{c}	1.00±0.07^{c}	1.11±0.02^{c}
	100	10.31±0.18^{a}	1.11±0.11^{b}	34.49±2.67^{a}	0.64±0.02^{b}	1.25±0.11^{b}	2.43±0.07^{b}
	150	8.67±1.01^{b}	0.93±0.06^{b}	31.87±4.52^{a}	1.68±0.01^{a}	1.50±0.09^{a}	3.46±0.21^{a}
	Mean	10.43±1.76	1.62±0.55	16.39±11.07	0.62±0.45	1.19±0.53	2.08±1.12
	LSD	0.61	0.11	1.10	0.04	0.07	0.07

The common letters indicate no significant differences. CAT= catalase, DW= dry weight, FW= fresh weight, POD= peroxidase, SOD= superoxide dismutase, SWD= saturated water deficiency.

Proline content

By increasing the NaCl up to 100 mM, the proline content increased sharply. Increasing the NaCl from 100 mM to 150 mM did not cause a further increase in proline content (Table 2). The proline content increased by 483.46, 976.92, and 976.92% in response to the 50, 100, and 150 mM NaCl treatments, respectively, compared to the control. The highest amount of leaf proline content (14.00 µg g^{-1}) occurred in response to 150 mM NaCl and the lowest (1.00 µg g^{-1}) in response to the 0 mM NaCl + 2 mM SA (Fig. 2A). SA application decreased proline by 23.08, 71.43, 271.43, and 257.14% at 0, 50, 100, and 150 mM NaCl concentrations, respectively.



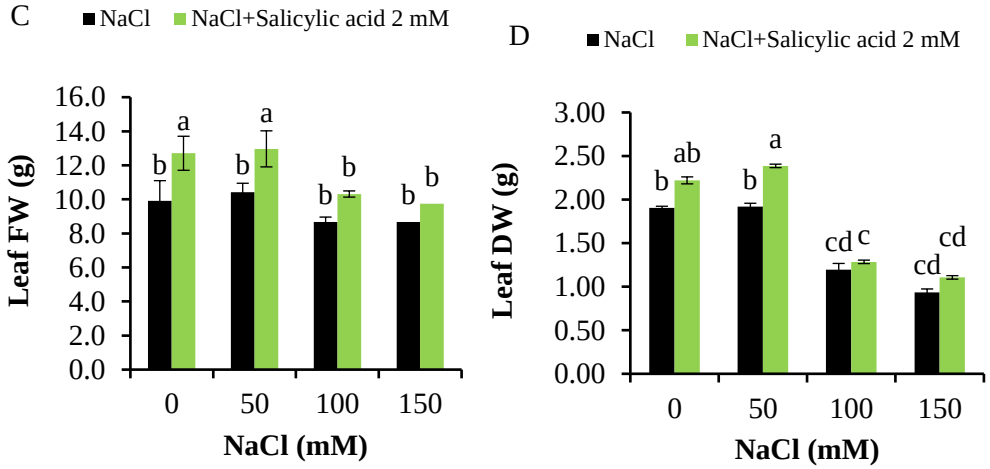


Fig. 2. Mean comparisons of interaction effects of NaCl × SA (salicylic acid) for proline content (A), protein (B), leaf fresh weight (C), and leaf dry weight (D). According to the Least Significant Difference test (LSD), means with the same letter do not have a significant difference ($p<0.05$). FW= Fresh weight and DW= dry weight.

Soluble protein content

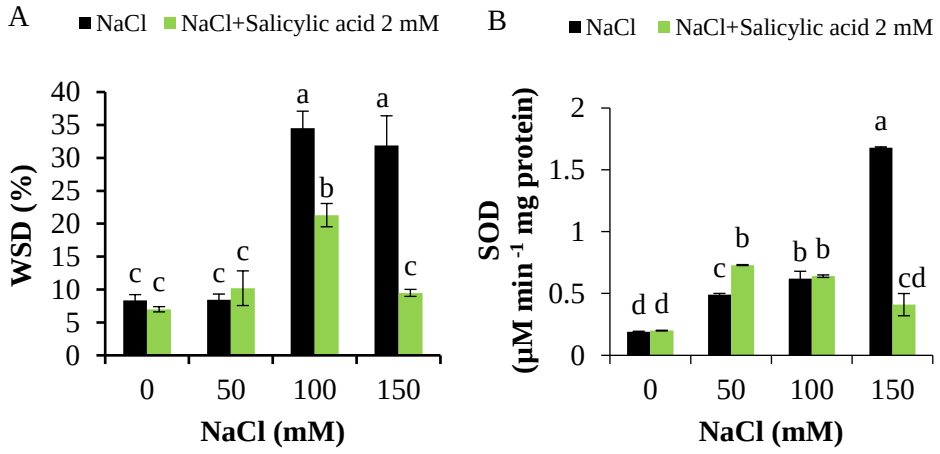
As NaCl increased to 100 mM, the amounts of soluble protein increased but decreased at 150 mM NaCl (Table 2). The leaf soluble protein increased by 50.00 and 80.88% in response to the 50 and 100 mM NaCl concentrations, respectively, but soluble protein decreased by 25.00% in the 150 mM NaCl, compared to the control. The highest soluble protein was observed in the 100 mM NaCl concentration (1.23 mg g^{-1}) and the lowest (0.51 mg g^{-1}) in the 150 mM NaCl (Table 2). Applying 2 mM SA reduced the soluble protein content by 26.47, 29.27, and 78.43% in response to 50, 100, and 150 mM NaCl, respectively (Fig. 2B).

Leaf fresh weight and leaf dry weight

The highest leaf fresh weight occurred in response to the 50 mM NaCl + SA 2 mM (12.97 g) and the lowest (8.67 g) in the 150 mM NaCl (Fig. 2C). The highest leaf dry weight occurred in response to the 50 mM NaCl + SA 2 mM (2.39 g) and the lowest (0.93 g) in the 150 mM NaCl (Fig. 2D). Applying 2 mM SA significantly increased leaf fresh weight and leaf dry weight at concentrations of 0 and 150 mM NaCl, but SA had no significant effect on these traits at 100 and 150 mM NaCl treatments (Fig. 2D).

Saturated water deficiency

Applying 50, 100, and 150 mM NaCl treatments increased the saturated water deficiency of cells by 1.67%, 315.60%, and 283.95%, respectively, compared to the non-NaCl control group (Table 2). The lowest SWD (7.00%) occurred in response to the 0 mM NaCl + 2 mM SA and the highest (31.87%) in 150 mM NaCl. Salicylic acid (2 mM) reduced the saturated water deficit by 38.26 and 70.23% in response to 100 and 150 mM NaCl concentrations, respectively, but increased the SWD by 20.81% at 50 mM NaCl (Fig. 3A).



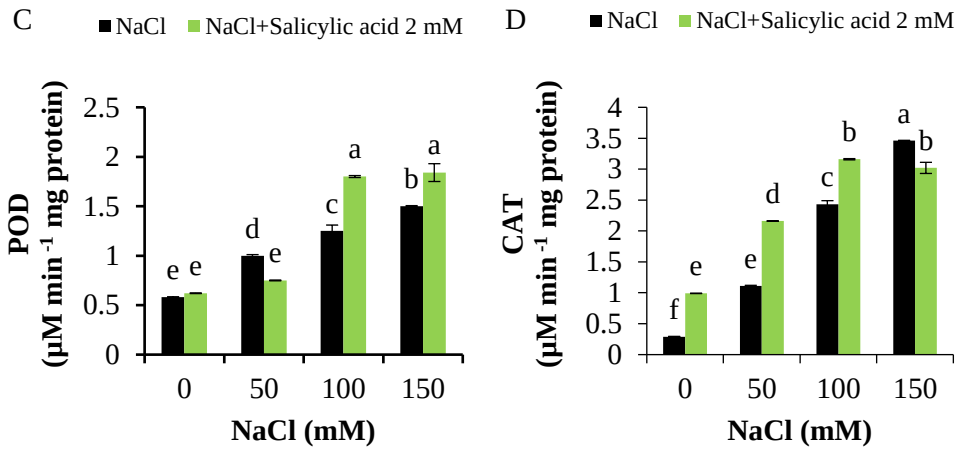


Fig. 3. Mean comparisons regarding the interaction effect of NaCl × SA (salicylic acid) for saturated water deficiency (A), the enzymatic activities of superoxide dismutase (B), peroxidase (C), and catalase (D). According to the Least Significant Difference test (LSD), means with the same letter do not have a significant difference ($p<0.05$). SWD= saturated water deficiency, SOD= superoxide dismutase, POD= peroxidase, and CAT= catalase.

SOD activity

SOD enzymatic activity increased when using higher NaCl concentrations (Table 2). Maximum SOD activity (1.68 µmol min^{-1} mg protein) occurred in response to the 150 mM NaCl treatment, and the minimum SOD activity (0.19 µmol min^{-1} mg protein) in the control (Fig. 3B). Foliar spraying 2 mM SA increased the SOD enzymatic activity by 5.26, 48.98, and 3.23% in response to the 0, 50, and 100 µM NaCl, respectively. Applying 2 mM SA caused a severe decrease (175.60%) in SOD activity at 150 mM NaCl (Fig. 3B).

POD activity

By increasing the salinity, the POD activity increased. Peroxidase activity increased by 72.41, 115.52, and 158.62% in response to the 50, 100, and 150 mM NaCl concentrations, respectively, compared to the control (Table 2). The maximum POD activity (1.98 µmol min^{-1} mg protein) occurred in response to 100 mM NaCl + 2 mM SA, and the minimum POD activity (0.58 µmol min^{-1} mg protein) in 0 mM NaCl (Fig. 3C). Salicylic acid caused a significant increase in POD activity by 44.00 and 22.67% in response to the 100 and 150 mM NaCl, respectively (Fig. 3C).

CAT

The increase in salinity caused a substantial rise in catalase enzyme activity. Catalase activity increased by 382.76, 737.93, and 1093.10% in response to the 50, 100, and 150 mM NaCl concentrations, respectively, compared to the control (Table 2). The highest CAT enzymatic activity (3.46 µmol min-1 mg protein) occurred in response to the 150 mM NaCl, and the lowest CAT enzymatic activity (0.29 µmol min^{-1} mg protein) in the control (Table 2). Salicylic acid increased the CAT enzymatic activities by 241.38, 94.59, and 30.04% in response to the 0, 50, and 100 mM NaCl, respectively. However, CAT activity decreased (12.79%) in response to the SA application when using 150 mM NaCl (Fig. 3D).

Discussion

Chl concentration in leaf samples under stress conditions is an index of plant tolerance to salinity. Anjum et al. (2011) stated that osmotic stress causes damage to the chloroplast structure, Chl oxidation, and reduction of Chl, followed by a decrease 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 findings, salinity decreased photosynthetic pigments significantly in *Satureja hortensis* (Mohammadi et al., 2023), *Satureja khuzestanica* (Saadatfar and Jafari, 2022), *Lantana camara* (Dehestani Ardakani et al., 2021), *Linum usitatissimum* L. (Dubey et al., 2020), and *Nigella sativa* L. (Zarei et al., 2019).

SA significantly increased Chl a, Chl b, and total Chl in all salinity treatments and the control. Similar results were reported about other plants such as St. John's wort (Kwon et al., 2023), *Portulaca oleracea* (Panahyan et al., 2020), *Nigella sativa* L. (Zarei et al., 2019), and *Dianthus. superbus* (Ma et al., 2017).

Carotenoids play an essential role in reducing oxidative stress and regulating ROS cellular

homeostasis in plants (Ashraf, 2009). The reduction of carotenoids has been seen in salt-sensitive plants, while carotenoid content increased in salt-resistant plants (Gomathi and Rakkiyapan, 2011). An increase in salinity caused a decrease in the carotenoid content in our study. The carotenoid content decreased under salinity stress in *Capsicum annuum* (Kumar et al., 2022), *Nigella sativa* L. (Zarei et al., 2019), and *Satureja hortensis* (Fabriki ourang and Mehrabad-Pourbenab, 2016). SA significantly increased carotenoid levels in all NaCl-treated plants. Similar to our results, SA caused an increase in carotenoids in *Nigella sativa* L. under salt stress conditions (Zarei et al., 2019).

In salt stress conditions, organic osmolytes accumulation, such as soluble proteins and proline, can help to maintain cell turgor pressure, regulate cell water absorption, and moderate water retention (Mittal et al., 2012). It helps to scavenge free radicals and maintain the stability of the cell wall structure and proteins (Saadia et al., 2012). In the present study, the proline content increased significantly in response to higher salinity stress up to 100 mM NaCl but stopped at 150 mM NaCl. Similar to our results, in *Satureja hortensis* (Mohammadi et al., 2023), *Satureja khuzestanica* (Saadatfar and Jafari, 2022; Aryan et al., 2018), *Brassica carinata* (Husen et al., 2018) and *Thymus daenensis* (Harati et al., 2015) the proline content increased significantly in response to salinity levels. SA significantly decreased the proline contents under all studied salt stress treatments (0, 50, 100, and 150 mM NaCl). Similar to our results, SA application significantly reduced the proline content under salt stress conditions in some plants such as St. John's wort (Kwon et al., 2023), *Nigella sativa* L. (Zarei et al., 2019), *Lallemantia royleana* (Rostami, 2018), and *Brassica carinata* (Husen et al., 2018).

As NaCl increased to 100 mM, the soluble protein increased gradually but decreased at 150 mM NaCl. Similar to our results, the protein content increased at low salinity treatments but declined at severe NaCl stress conditions in *Amaranthus cruenus* (Menezes et al., 2017) and *Thymus vulgaris* (Harati et al., 2015). In our study, using 2 mM SA increased the amount of leaf-soluble protein at 150 mM NaCl. Similar to our results, 1 mM SA caused protein accumulation under salinity conditions in *Capsicum annuum* (Kumar et al., 2022) and *Thymus vulgaris* (Harati et al., 2015).

An increased antioxidant activity enables plants to resist potential oxidative damage caused by salinity (Ahmad and Umar, 2011). In the present research, increasing the NaCl up to 150 mM significantly enhanced the SOD, POD, and CAT activities. Similar to our findings, NaCl levels increased the SOD, POD, and CAT activities in many medicinal and agricultural plants such as *S. khuzestanica* (Saadatfar and Jafari, 2022), *Triticum asetivem* (Afridi et al., 2019), and *Brassica carinata* (Husen et al., 2018). Also, NaCl stress enhanced POD and/or CAT activities in *Capsicum annuum* (Kumar et al., 2022), *Helianthus annuus* (Mehak et al., 2021), *Sorghum bicolor* (Punia et al., 2021), *Amarantus tricolor* (Sarker and Oba, 2020), and *Brassica oleracea* (Sahin et al., 2018).

Foliar spraying with 2 mM SA at 0, 50, and 100 μM NaCl increased SOD enzymatic activity by 5.26, 48.98, and 3.23%, respectively. However, the application of 2 mM SA at the 150 mM NaCl concentration caused a severe decrease (175.60%) in SOD activity. Salicylic acid at 100 and 150 mM NaCl caused a significant increase in POD activity and significantly increased CAT enzymatic activity at 0, 50, and 100 mM NaCl. In line with our results, SA application significantly mitigated the salinity-induced increase in SOD activity in *Brassica carinata* plants (Husen et al., 2018). Also, it increased CAT and POD activities in *Thymus vulgaris* (Harati et al., 2015) and *Brassica parachinensis* significantly (Kamran et al., 2020). Also, the application of exogenous SA effectively improved antioxidant enzyme activities (POD and, or CAT) in *Dianthus superbus* (Ma et al., 2017), *Capsicum annuum* (Mahdavian et al., 2007), *Nigella sativa* (Zarei et al., 2019), and *Lallemantia royleana* (Rostami, 2018) under salt stress conditions.

The leaf fresh weight increased insignificantly in response to 50 and 100 mM NaCl, but these traits significantly reduced in 150 mM salinity treatments compared to the control. Leaf dry weight increased insignificantly at 50 mM NaCl but decreased significantly at 100 and 150 mM NaCl. A low concentration of salt stress can promote growth and yield due to root growth and root activity under low salt stress. These effects facilitate water absorption and nutrients by roots, thus improving dry matter production (Zhang et al., 2023). Low salt stress induces plant growth in some plants, such as *Vigna radiate* (Ogunsiji et al., 2023) and buckwheat (Zhang et al., 2023). Intense NaCl stress reduced the leaf FW and leaf DW. Similar to our results, severe NaCl stress reduced the leaf FW and leaf DW in *Satureja hortensis* (Mohammadi et al., 2023) and *Satureja khuzestanica* (Saadatfar and Jafari, 2022). In *Vigna radiata* plants, leaf weight and plant biomass decreased significantly (52.2% and 53.3%) in response to 100 and 200 mM NaCl

treatments (Ogunsiji et al., 2023). In the present study, using 2 mM SA significantly increased the leaf FW in all salinity treatments. In line with our findings, the application of SA improved FW and DW in *Lantana camara* (Dehestani Ardakani et al., 2021), wheat (Saddiq et al., 2021), maize (Kaya et al., 2020a), mustard (Kohli et al., 2019), and *Brassica carinata* (Husen et al., 2018) in severe salinity conditions. Also, salicylic acid increased leaf fresh weight and leaf dry weight in some plants such as tomato seedlings (Souri and Tohidloo, 2019), winter wheat (Khalvandi et al., 2021), and *Solanum melongena* L. (Mady et al., 2023).

SWD increased drastically in response to increasing salinity intensity. Similar to our results, SWD increased under different salt concentrations in some medicinal plants or crops such as *Lemon verbena* (Ghanbari et al., 2023), *Capsicum annum* (Kaya et al., 2020b), *Amaranthus cruentus* (Menezes et al., 2017), *Oryza sativa* (Jini and Joseph, 2017), and *Solanum lycopersicum* (Görgényi Miklósné Tari et al., 2015). Applying 2 mM SA reduced the SWD considerably under salt stress conditions. In some previous studies, under different salinity levels, SWD decreased by SA application in species such as *Lantana camara* (Dehestani Ardakani et al., 2021), *Capsicum annum* (Kaya et al., 2020b), maize (Tahjib-Ul-Arif et al., 2018), rice (Jini and Joseph, 2017), *Solanum lycopersicum* (Görgényi Miklósné Tari et al., 2015), and *Citrus sinensis* (Khoshbakht and Asgharei, 2015).

Conclusion

In recent years, some agricultural factors such as row spacing, fertilizer requirements, and water requirements have assisted optimization in cultivating and domesticating white savory plants. Due to the unavailability of sufficient information about climatic conditions and soil properties, we studied the effect of salt stress and salicylic acid on white savory to optimize its cultivation in semi-saline soils. Salt stress reduced photosynthetic pigments, protein, leaf fresh weight, and dry weight but enhanced antioxidant activity, saturated water deficiency, and proline content. SA mitigated the adverse effects of salinity on these traits under salt stress conditions. Since this plant grows in the northern regions of Iran and humid conditions, we suggest conducting future studies on more types of arable soil and finding suitable climatic conditions to optimize the treatments.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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