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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF COTTON TO TIMES AND TYPE OF STRESS MODULATOR IN SALINE CONDITIONS

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ABSTRACT. Among the different agronomic techniques used to reduce the negative effects of salinity, external applications of stress modulators are considered as an efficient approach for salinity stress alleviation. An experiment was conducted as a factorial arrangement based on a complete randomized block design in 3 replications to evaluate the foliar application effects of different stress modulators on the physiological and biochemical responses of cotton cultivated in a saline condition. The involved factors included foliar application time (flowering and flowering+bolling stages) and 4 stress moderator types (control, Salicylic acid, Glycine Betaine, and Sodium Nitroprusside Application times had no significant impacts on the plant physiological attributes. Foliar application of salicylic acid further increased the activities of enzymatic or non-enzymatic antioxidants in cotton as compared to the other osmotic modulators. Salicylic acid spraying enhanced the contents of Chlorophyll a (76.4%), Chlorophyll b (47.7%), carotenoids (73.3%), proline (90.8%), catalase (82.6%), superoxide dismutase (74.5%), and guaiacol peroxidase (98.1%) in comparison to the control treatment. Overall, The modulatory effectiveness of the enzymes in reducing salinity stress by augmenting their antioxidant activities could be classified as salicylic acid > glycine Betaine > sodium nitroprusside.

1. INTRODUCTION

Environmental stress accounts for 71% of crop yields. It is estimated that about 20% of crop yield loss is caused by salinity stress. About 7 million lands under agricultural crops in Iran are influenced by salt stress impacts since this country has got the highest percentage of saline lands in the world after India and Pakistan [1]. Salinity stress is one of the major environmental stresses affecting plant growth by decreasing

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water uptakes, disturbing nutrient uptakes, reducing ion toxicities, and producing oxygen free radicals [2, 3]. One of the biochemical changes that occur under such environmental stresses as salinity is the production of Reactive Oxygen Species (ROSs) through electron-chloroplast transfer chain closure and NADP+/NADPH ratio reduction[4]. Oxygen-free radicals prevent photosynthesis by damaging cell membranes and eventually killing the plant. Plants use both antioxidant and non-antioxidant enzymatic systems to encounter the oxidative stress induced by the accumulation of oxygen free radicals [5]. Synthesis and accumulations of glutathione peroxidase, superoxide dismutase, catalase, peroxidase, and glutathione are their enzymatic mechanisms of alleviating oxidative stress and tocopherol, ascorbic acid, carotenoid, and glutathione synthesis and accumulations are their non-enzymatic reduction mechanisms [2]. It has been reported that plant antioxidant activities and the negative effects of stresses can be increased and decreased by using some modulators, respectively[6-8]. The effects of SNP [9], SA, GB, and putrescine [10] on salinity stress tolerance in cotton has been already documented.

SNP is an NO-releasing compound and one of the stress-reducing agents. Today, NO is classified in the group of plant growth regulators. SNP moderating role in environmental stresses, such as salinity, drought, heavy metals, and mineral deficiency, has been reported in many studies [10]. It is believed to have the dual toxic or protective roles, depending on its concentration, as well as plant type, tissue, and age, and the stress type applied to the plant [9]. It reduces leaf water depletion, ion leakage, and transpiration by stimulating stomach closure and thereby increases stress tolerance [11]. The external application of SNP has been evidenced to decrease stress tolerance by reducing hydrogen peroxide and malondialdehyde, enhancing antioxidant enzymatic activities, regulating proteins at post-translational levels, and increasing cell division. Its external application in cotton augmented the activities of oxygen-reducing enzymes and decreased H2O2 accumulation under salinity stress. Growth and photosynthesis include net photosynthesis rate and transcription [12]. It has been reported that foliar application of 0.05 M SNP enhances yield and yield components, increases the contents of pigments, total soluble sugars, proline, total free amino acids, phenols, and soluble proteins, besides antioxidant activities and activities of antioxidant enzymes [10].

GB is a quaternary ammonium and the most common organic solution that accumulates in plant cells in response to stress. It acts as a cytoplasmic osmolyte and protects plant enzymes and membranes against decay. GB accumulation in response to stress has been proven in many crops, including sugar beet, spinach, barley, and wheat [6]. Nevertheless, natural GB production is not enough to protect plants under severe stresses. In such circumstances, the external application of GB may be a

useful solution to overcome environmental stresses in plants. Various studies have reported the relationship between stress tolerance and external GB use [8]. The use of GB can naturally improve stress tolerance in those plants that are unable to synthesize it [13]. GB not only acts as a protective protein for maintaining normal intracellular osmotic pressure, but also stabilizes the quaternary structures and activities of enzymes and protein complexes, thus keeping membrane integrity under salt, drought, and cold stress conditions [6]. It can also effectively protect Photosynthesis System II (PSII) under salinity stress [14]. The modulatory effects of external GB consumption on reducing the impacts of drought stress [15], salinity [16] and cold stress [7] has been evidenced in cotton.

Salicylic acid (SA) or ortho-hydroxy benzoic acid belongs to a group of phenolic compounds that have been identified as important signal molecules for modulating plant responses to environmental stresses [17]. Increased photosynthetic activity, decreased transpiration, water retention in tissues, enhanced cytosolic content [4], augmented antioxidant enzyme activities [5], and free proline accumulation are the physiological effects of external SA application. In cotton, it has been reported that salinity significantly decreases its growth and yield, but the foliar application of 200 ppm of SA reduces salinity stress effects by enhancing the activities of antioxidant enzymes [18]. In addition to alleviating the impacts of salinity stress by SA application, Hameed and Ali (2016) reported that 1 mM of foliar application in cotton increased heat stress tolerance by enhancing the activities of antioxidant and non-antioxidant enzymes [19]. The positive benefits of SA intakes in lowering copper-induced oxidative stress [20], plant pests [21], and drought stress [18] have been also evidenced.

Although under stress conditions, plant cells reduce the negative effects of oxygen free radicals by accumulating antioxidant and non-antioxidant enzymes, it is well known that the external applications of these stress modulators can increase the productions of enzymatic and non-enzymatic antioxidants and enhance salt tolerance in plants. In environmental stress conditions, such as salinity stress, the response of crops to the type of stress modulator may be different. Although, in many studies, the effect of only one modulator has been reported by researchers, but determination of the most suitable stress modulator was not intended by researchers. Therefore, in this research, the effects of some modulator times and types on the physiological and biochemical traits of cotton under saline conditions were investigated.

2. Materials and Methods

The experiments were conducted as a factorial arrangement in a randomized complete block design with three replications. Factors were application time (flowering and flowering+bolling stages) and stress types of moderators (control, SA (2 mM), GB (100 mM), and SNP (100 µM). In control, No stress modifiers were used, and water was sprayed. The experiments were carried out at a private farm located at 57◦ 44' East latitude and 36◦ 13' North longitude with a height of 990 m above sea level during 2017-2018. According to the soil analysis results (Table 1), the studied soil was of a loamy-clay type with a pH of 7.2, EC of 10.5 ds/m, and total N, P, and K contents of 0.02%, 110and 4 mg/kg, respectively. The data on the rainfalls and mean temperatures during the cotton growing stages are presented in Table 2.

Table 1. Physicochemical properties of soil on experimental site

Manganese ($mg\ kg^{-1}$)	Sodium	Zinc	Copper	Iron	Phosphorus PPm	Potash	Nitrogen (%)	Sand %	Clay	Silt	EC (dS m^{-1})	$pH_{(1:5)}$
7	40.5	0.55	0.46	2.42	4	110	0.02	63	13	24	10.5	7.2

Table 2. Average temperature and total rainfall in growing season in two years.

Month	Average temperature (◦C)		Total rainfall (mm)	
	2017	2018	2017	2018
January	5.9	5.76	15	1.6
February	6.5	9.99	49.9	20
March	12.5	17.1	21.4	2
April	18.9	17.9	29.4	32.2
May	26.9	23.3	14.2	17.8
June	30.86	29.52	0	0.7
July	31.02	2.8	2.8	0.0
August	29.86	30.4	0.0	0.0
September	25.7	26.3	0.0	6.5
October	19.1	17.5	0.0	35.1
November	13.7	12.5	2.0	0.6
December	7.21	6.59	0	1.2

The experimental filed went under fallow and wheat cultivations during the first and second years. Seed-bed preparation included moldboard plowing in the autumn and harrowing and leveling in the spring. According the soil analysis results (Table 1), 100 kg P_2O_5 ha^{-1} as triple super phosphate and 100 kg K_2O ha^{-1} as potassium sulfate, together with one-third of 150 kg N as urea, were uniformly broadcasted before

sowing. Another N fertilizer was top-dressed at the first hand-weeding (about 40 days after emergence) and early flowering stages. Cotton seeds were obtained from Agricultural Jihad in Sabzevar and sown on April, 15th, 2017 and April, 20th, 2018. Each plot contained four 5-m-long rows spaced 50 cm from each other, along which the plants were planted at the equal distances of 20 cm (10 plants.m^{-2}). After completing the planting stage and at the 5-6 leaf stages, the plants were thinned in the rows at a distance of 20 cm from each other to obtain an optimum density. Irrigations were done every 10 days during the growing season according to the local custom. Weeding (weed removal) was done manually. Triton 100 X was utilized as a surfactant to better absorb foliar applications of the modulators.

Plant sampling

All the assays were performed on the 4th extended leaf, 30 days after spraying from 5 randomly plants.

The amounts of photosynthetic pigments were determined by using Arnon method [22]. The contents of Chlorophyll and carotenoids were measured via spectrophotometry at 645, 663, and 470 nm and expressed in mg/g fresh weight (mg g^{-1}F.W).

$$\text{Chlorophyll a} = (19.3 \times A_{663} - 0.86 \times A_{645})\ V/100W$$
$$\text{Chlorophyll b} = (19.3 \times A_{645} - 3.6 \times A_{663})\ V/100W$$
$$\text{Carotenoides} = 100(A_{470}) - 3.27(\text{chl. a}) - 104(\text{chl. b}) / 227$$

Proline concentration

The method applied by Bates et al. [23] was employed to measure leaf proline content. To this aim, 0.2 g of the leaf tissue was weighed and thoroughly gelled in 3 ml of 3% sulfosalicylic acid in Chinese mortar. Upon centrifuging the homogenates at 18,000 rpm for 15 min, 2 ml of the filtered extract was transferred to the gut tubes and 2 ml of ninhydrin reagent and 2 ml of glacial acetic acid were added to all the tubes. Then, they were placed in 100°C water for 1 hour. After cooling the tubes, 4 ml of toluene was added to each tube. The tubes were vortexed for 15-20 s to allow formations of two separate phases. The top phase was read at 520 nm by the spectrophotometer (Uv/Vis model T70+ , German). Proline concentration in mg/g of fresh leaf tissue was determined using the standard curve. The unit was expressed as mg/g fresh weight.

Enzyme Extraction

For enzyme extraction, 0.5 g (fresh weight) of the frozen leaf tissue was grounded with mortar in liquid N2 to obtain a fine powder, which was then homogenized in 5 mL of an extraction buffer containing 50 mM cold buffered monophosphate (pH 7.8), 0.1 mM EDTA, 0.3% TritonX-100, and 4% polyvinylpolypyrroidone. The homogeneous material was centrifuged for 15 min at the gravity of 15,000 g at 4°C. The upper transparent phase was applied to measure enzyme activity [24].

Catalase activity measurement

CAT (E.C. 1.11.1.6) activity was determined using the method of Aebi [25]. For a total volume of 3 mL, 30 µl of the extract was applied to 50 mM buffered monophosphate (pH 7.0) and 2% H_2O_2. Enzyme activity was measured at 240 nm for 2 min using a spectrophotometer (Cary 100) and expressed as changes in the absorbance units (ΔA) min^{-1} mg $protein^{-1}$

$$(Unit)/_{min} = 2(1 - \frac{\Delta Asample}{\Delta Acontrol})$$

Superoxide dismutase activity measurement

Superoxide dismutase (SOD; E.C. 1.15.1.1) activity in the leaf extracts was measured using the inhibition measurement of nitroblue tetrazolium (NBT) photochemical reduction [26]. The reaction mixture had a final volume of 3 ml and contained 2.25 ml of 50 mM potassium phosphate buffer (pH=7.8), 200 µL of EDTA (0.66 mM), 300 µL of methionine (10 mM), 150 µL of NBT (66 µM), 50 µL of riboflavin (3.35 µM), and 50 µL of the enzyme extract. The reaction was carried out in a transparent glass cuvette at 25°C under the same white light intensity for all the samples. A special cuvette for each sample was placed in the dark as a blank (zero reaction time control). After shining the light for 15 min, the light absorption at 560 nm was read and the absorption difference was set to zero for 15 min. NBT inhibitory ability in each sample was determined by the enzymatic extract of each sample as compared to the light absorption difference of 0 and 15 minutes for the control reaction (without enzyme) according to the following formula: One unit of superoxide dismutase activity equivalent to 50% inhibition of nitrobutetrazolium-to-formamazone photobleaching. Finally, the enzyme activity was reported based on superoxide dismutase unit/min/g fresh weight.

Ascorbate peroxidase activity measurement

Ascorbate peroxidase (APX; E.C. 1.11.1.1) activity was determined as described by Nakano and Asada [27]. The 3-mL reaction mixture contained 312.5 mM sodium phosphate buffer (pH=7), 0.5 mM sodium ascorbate, 0.1 mM ethylene diamine tetraacetic acid, 2.2 1 mM hydrogen peroxide, and 50 µL of the enzyme extract. The reaction was started by adding hydrogen peroxide to the reaction mixture and then, changes in the light absorption relative to the control reaction without hydrogen peroxide were recorded. The conversion rate of ascorbate to dehydroascorbate was measured for 60 s at 290 nm and finally, the enzyme activity was determined by using ascorbate extinction coefficient of 18 mM^{-1} cm^{-2} expressed as the change in µM ascorbate consumed per min/g fresh weight.

Glutathione peroxidase (GPX) measurement

Guaiacol peroxidase (EC 1.11.1.7) activity was measured according to the method of Fielding and Hall [28]. In this approach, 3 mL of the reaction mixture containing 100 mM potassium phosphate buffer (pH=7), 20 mM guaiacol, 10 mM H_2O_2, and 50 mM enzyme extract was allowed to increase absorption via guaiacol oxidation at 470 nm, which was measured for 3 min. Enzyme activity was expressed as the change in µM ascorbate consumed per min/g fresh weight by using the extinction coefficient of 18 mM^{-1} cm^{-2}.

Hydrogen peroxide (H_2O_2) extraction and measurement

Hydrogen peroxide (H_2O_2) was calculated with minor modifications using Sagisaka's test [29]. First, 0.5 mg of fresh leaf was homogenized with 5 mL of 0.1% TCA (w/v) in an ice bath and centrifuged at 18000×g for 15 min. The supernatant (0.5 mL) was added to 0.5 mL of 10 mM K-phosphate buffer (pH=7) and 1 mL of 1 M KI. The absorptions in the samples were determined with a spectrophotometer at 390 nm. The H_2O_2 concentration was expressed as nmol H_2O_2 mg $protein^{-1}$

Sodium and potassium measurements

Hamada and El-Enany [30] method was used to measure leaf sodium and potassium content. In summary. 0.5 g dry matter of leaves washed and then 10 ml of concentrated nitric acid was added and kept at room temperature for 48 hours. In order to remove all vapors, the specimens were placed on a heated oven thermostat for 2 hours. After leaving acidic vapors and viewing a colorless solution, 100 ml of

distilled water was added to each sample. Using Whatman filter paper, the samples were get smooth and sodium and potassium values were measured by Flame Photometer (Jenway, Models PFP7, UK).

Membrane stability index measurement

Membrane stability index was measured based on the electrical conductivity of ions leaking from the leaf cells into deionized water. For this purpose, 0.1 g of the fresh leaves were immersed in the test tubes containing 20 ml of deionized water. Then, one sample was kept at 40°C and another sample at 100°C for 30 min. The electrical conductivities of the samples were measured and recorded by using a conductivity meter after they reached room temperature. The membrane stability index was calculated based on the following formula:

$$\text{Membrane stability index} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] * 100$$

where C_1 and C_2 represent the electrical conductivities of the samples at 40 and 100°C, respectively.

Statistical analysis

The experiment was arranged as a factorial arrangement based on completely randomized block design with three replicates. In a combined analysis of data, the interaction of year, stress modulator type, and application times was non-significant; therefore, the data were combined for both years and presented with the interaction of application times and stress modulator type. Data were subjected to analysis of variance (ANOVA) by using the software of SAS (SAS 9.1, USA). The means were compared using the least significant difference test ($P \leq 0.05$).

3. Results and discussions

Chlorophyll pigments

Spraying time affected the amount of chlorophyll pigments and spraying at the flowering compared to the flowering+bolling stage had more additive effects on chlorophyll pigments namely chlorophyll a, chlorophyll b, and total chlorophyll, while the contents of carotenoids at the flowering+bolling stage were higher than those of foliar applications at the flowering stage. Among the stress modulators,

foliar application with GB had more positive effects on chlorophyll pigment content, which was not significantly different from the case of foliar application with SA (Table 3).

TABLE 3. Means of chlorophyll pigments content as affected by time and type of stress modulator application

	chlorophyll *a* (mg. g^{-1}FW)	chlorophyll *b* (mg. g^{-1}FW)	Total chlorophyll (mg. g^{-1}FW)	Cartrenoid (mg. g^{-1}FW)
		Application times		
flowering	1.56±0.22 a	1.45±0.14a	3.01±0.47 a	0.76±0.08 a
flowering+bolling stage	1.35±0.28 b	1.20±0.19 b	2.55±0.33b	0.84±0.13 a
		Stress modulator type		
Control	0.74±0.13 c	0.86±0.12 c	1.59±0.22 c	0.53±0.12 d
GB	1.81±0.38 a	1.68±0.26 a	3.49±0.63 a	1.10±0.20 a
SNP	1.59 ±0.38b	1.24±0.26 b	2.83±0.61 b	0.70±0.12 c
SA	1.70±0.40 ab	1.52±0.25 ab	3.23±0.63 a	0.86±0.11 b

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at $p \leq 0.05$, Similar letter within the same columns not significant difference based on FLSD.

Both in the foliar applications at the flowering and flowering+bolling stages, spraying with GB produced more chlorophyll a contents compared to those produced by the other stress modulators although the difference between GB and SA in spraying at the flowering+bolling stage was less than that of spraying at the flowering stage. Foliar application with SNP at the flowering+bolling stage also decreased chlorophyll a content compared to that at the flowering stage. Similar changes in chlorophyll b content were observed, except that foliar application with SNP at the flowering+bolling stage increased chlorophyll b content compared to its application at the flowering stage (Table 4).

TABLE 4. Interaction effect of time and type of modulator application on photosynthetic pigment

Time of spraying	Modulator type	chlorophyll a (mg. g^{-1}FW)	chlorophyll b (mg. g^{1} FW)	Total chlorophyll (mg. g^{-1}FW)	Caratenoid (mg. g^{-1} FW)
Flowering	Control	0.73±0.20 c	0.92±0.17 b	1.65±0.36 d	0.55±0.17 c
	GB	1.76±0.47 a	1.61±0.22 a	3.37±0.62 ab	0.58±0.09 c
	SNP	1.35±0.49 b	0.82±0.27 b	2.17±0.67 c	0.83±0.22 b
	SA	1.57±0.54 ab	1.45±0.34 a	3.02±0.82 b	0.81±0.14 b
Flowering+bolling	Control	0.73±0.19 c	0.79± b	1.53±0.30 d	0.51±0.19 c
	GB	1.84±0.67 a	1.75±0.50 a	3.60±1.17 a	0.86±0.10 b
	SNP	1.83±0.62 a	1.66±0.41 a	3.49±0.99 ab	1.34±0.39 a
	SA	1.84±0.65 a	1.59±0.40 a	3.43±1.05 ab	0.93±0.18 b

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at $p \leq 0.05$, Similar letter within the same columns not significant difference based on FLSD.

Twice foliar applications at the flowering+bolling stage produced more carotenoid contents resulted from all the stress modulators compared to once foliar application at the flowering stage. This increase of carotenoid content was higher for SNP application than for the other modulators, while foliar application with SA had the lowest enhancement (Table 4). Studies have shown that photosynthetic pigment is one of the important determinants of photosynthetic efficiency and plant growth [31]. Chloroplast and photosynthetic system degradations, chlorophyll photooxidation, reaction with ROSs, degradation of chlorophyll precursors, inhibition of new chlorophyll biosynthesis, activation of chlorophyll-degrading enzymes, such as chlorophyllase, and hormonal abnormalities are the reasons for lowered chlorophyll contents in saline conditions [32]. Consistent with these results, the reductions of chlorophyll a, b, and total chlorophyll contents, as well as carotenoids of plants exposed to salinity stress, were reported by El-Beltagi et al. [18]. It was also observed that salinity significantly reduced chlorophyll a and b contents, whereas SA treatment decreased the trend of chlorophyll content reduction [20]. SA increasing role of chlorophyll content has been attributed to stimulations of the enzymes related to chlorophyll biosynthesis or inhibition of photosynthetic system disturbance, thereby alleviating chlorophyll degradation. Foliar application of SA in saline conditions augmented the contents of chlorophyll a, b, total chlorophyll, and carotenoids, which is in agreement with the results of this research. The enhancing effects of SA on photosynthetic capacity could be ascribed to its stimulating effects on Rubisco activity and pigment content [17]. The external application of GB in maize under salinity stress had an additive effect on chlorophyll pigments as one of the effective factors in increasing photosynthetic capacity under salinity conditions [3]. Liu et al., [12] evidenced that chlorophyll a content in cotton decreased by 25.27% in salinity conditions, However, 0.1 mM SA foliar application increased chlorophyll a content by 2.10% compared to no salinity stress and 36.46% compared to salinity stress conditions. Foliar spraying with SNP had greater positive effects on the contents of chlorophyll a, So that it increased by 27.68% and 70.64% compared to those of non-stress and stress conditions, respectively.

Antioxidant enzymes

Foliar application time had no significant effects on CA enzyme activity (Table 5). Cultivation in a saline condition significantly decreased CA enzyme activity and the external applications of the stress modulators significantly enhanced CA enzyme activities compared to the control treatment, showing that foliar application with SA had more positive additive effects than those of the other modulators. There was no statistically significant difference between the external applications of SA and SNP

(Table 5). Although foliar applications with GB and SNP at the flowering+bolling stage augmented CA enzyme activity compared to its application at the flowering stage, the external SA application caused a 21% decrease in its activity (Table 6). Foliar application time had a significant effect on SOD activity and foliar application at the flowering+bolling stage enhanced SOD enzyme activity up to 26.89% as compared to its application at the flowering stage (Table 5). Foliar application with SA among the other modulators in this study had more additive impacts on SOD enzyme activity though showing no statistically significant difference with the external application of GB (Table 5). Foliar application at the flowering+bolling stage for all the modulators had higher SOD enzyme activities compared to foliar treatments at the flowering stage. The enhancement rates of SOD enzyme activity with twice spraying at the flowering+bolling stage were 39.39, 36.08, and 18.56% higher for GB, SA, and SNP compared to its once application at the flowering stage, respectively (Table 6).

TABLE 5. Means of antioxidant enzymes as affected by time and type of stress modulator application

	CAT (units/mg proteins)	SOD (units/mg proteins)	APX (units/mg proteins)	GPX (units/mg proteins)
		Application times		
flowering	66.8±4.65 a	35.8±2.66 b	54.5 ±0.64a	0.51±0.04 b
flowering+bolling stage	65.7 ±4.01a	45.8±2.16 a	54.1±0.78a	0.58±0.31 a
		Stress modulator type		
Control	53.24±2.2 c	23.10±1.02 c	50.54±2.15 c	0.27±0.02 c
GB	67.94±2.41 b	46.80±2.06 a	55.62±0.28 b	0.65±0.04 a
SNP	69.72±3.02 ab	44.15±3.31 b	54.71±0.42 b	0.66±0.05 a
SA	74.24±5.98 a	48.75±3.35 a	56.12±1.41 a	0.61±0.02 b

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at p≤0.05, Similar letter within the same columns not significant difference based on FLSD.

TABLE 6. Interaction effect of time and type of modulator application on antioxidant enzymes

Time of spraying	Modulator type	CAT (units/mg proteins)	SOD (units/mg proteins)	APX (units/mg proteins)	GPX (units/mg proteins)
Flowering	Control	56.1±1.61 b	22.7±3.91d	51.8±1.59 b	0.25±0.02 c
	GB	60.3±5.93 ab	39.1±1.51 c	54.9±0.63 a	0.78±0.06 a
	SNP	68.0±6.31 a	40.4±3.44 b	55.1±0.56 a	0.61±0.04 ab
	SA	82.9±7.36 a	41.3±3.82 b	56.0±1.40 a	0.67±0.08 a
Flowering+bolling	Control	50.4±4.38 b	23.5±3.60 d	49.2±1.88 b	0.28±0.03 c
	GB	75.6±8.12 a	54.5±2.02 a	56.3±0.54 a	0.51±0.02 b
	SNP	71.4±7.12 a	47.9±1.52 ab	54.3±0.45 a	0.71±0.03 a
	SA	65.5±1.51 a	56.2±2.45 a	56.1±1.14 a	0.54±0.05 b

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at p≤0.05, Similar letter within the same columns not significant difference based on FLSD.
Superoxide dismutase (SOD), catalase (CA) , Ascorbate peroxidase(APX), guaiacol peroxidase (GA).

Foliar application time had no significant effects on APX activity, but the external applications of the stress modulators significantly increased APX activity, while the additive effects of external SA application was greater than those of the other modulators (Table 5). APX activity responses to the modulator times and types were different. Although there was no significant difference between foliar applications with SA at the flowering+bolling and flowering stages, foliar applications with SNP and GB at the flowering+bolling stage decreased and increased APX activities compared to its application at the flowering stage, respectively (Table 6).

Gaiacol peroxidase activity was affected by foliar application time and its application at the flowering+bolling stage created more gaiacol peroxidase activity compared to its application at the flowering stage (Table 5). Although the external applications of the stress modulators enhanced guaiacol peroxidase activity, no significant differences were observed between the modulators in this respect (Table 5). Foliar applications with SA and GB at the flowering+bolling stage increased guaiacol peroxidase activities and foliar application with SNP decreased its activity compared to foliar application at the flowering stage (Table 6).

CAT, POX, and SOD are the antioxidant enzymes that protect cells from oxidative stress caused by oxygen free radicals. As can be seen in the table, applications of the external stress modulators have increased the enzymatic activities, while catalase and superoxidase have provided the most and least reactions to their applications, respectively. The enhanced peroxidase activities under salinity stress in canola [6] and cotton [9] leaves reduced the damaging effects of salinity stress. SA induced the activities of these antioxidant enzymes in D. superbus under saline conditions [20]. The increase in growth caused by SA consumption has been linked to an increase in antioxidant activities, which protect plants from oxidative damage [19]. Studies have shown that SA plays an important role in modulating the activities of antioxidant enzymes against salinity stress, while making plants more capable of withstanding salinity damage [4, 18-20]. No difference in SOD enzyme activity was observed in the cotton mutants capable of synthesizing GB and in those mutants lacking this ability [2]. However, under salinity stress, SOD activities were increased by both types of mutants and were significantly higher in transgenic mutants after 14 days of salinity stress. Nawaz and Ashraf [3] reported that foliar application of GB enhanced SOD activity in maize, especially during the vegetative time, under salinity stress conditions. This increase in SOD activity could protect the photosynthetic system from the oxidative damage caused by salt stress. In wheat, it was reported that foliar application of GB led to a significant increase in SOD activity in saline conditions. Its spraying at a concentration of 50 mM enhanced SOD activity by 24%, but it was not statistically significantly different from the case of spraying with 75 mM [33]. In

soybean, it was shown that salinity stress significantly augmented the activity of CAT enzyme and pre-treatment with GB significantly enhanced its activity in salinity stress conditions, while increasing GB concentrations led to little change in its activity in non-saline conditions[34]. In mungbean, it was reported that salinity stress significantly enhanced GPX activity. Under this condition, the external use of Pro or GB augmented the activity of GPX enzyme, the highest activity of which was observed 48 hours after GB consumption [35]. Enhanced salinity tolerance has been also reported with increasing SOD activities with external GB applications in sompe crops [6, 8, 14-16].

Membrane stability index, proline content and content of sodium and potassium

Membrane stability index was not affected by foliar application time and the interaction between the stress modulator times and types. The external application of GB enhanced membrane stability index, which was not significantly different from that induced by SA foliar application. The external application of SNP augmented membrane stability index by 3.61% compared to the control treatment, which was statistically similar to that yielded by SA foliar application (Table 7).

TABLE 7. Means of membrane stability index, proline content and content of sodium and potassium as affected by time and type of stress modulator application

	EL (%)	Proline (mg. g^{-1}FW))	Na (mg. g^{-1}dW)	K (mg. g^{-1}FW)
Application times				
flowering	45.57±1.01 a	60.17±4.9 b	54.49±2.53 a	42.50±2.27 a
flowering+bolling stage	46.14±2.39 a	63.33±6.6 a	54.00±2.61 a	45.16±3.39 a
Stress modulator type				
Control	47.3±3.51 a	37.17±6.06 d	56.17±1.66 a	34.17±2.81 c
GB	43.9±0.88 b	67.83±3.44 b	55.66±1.09 a	44.48±1.86 b
SNP	46.5±0.96 a	59.00±6.41 c	54.62±2.13 a	43.50±2.22 b
SA	45.58±1.06 ab	83.00±4.72 a	50.54±1.7 b	53.17±2.10 a

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at $p \leq 0.05$, Similar letter within the same columns not significant difference based on FLSD.

The least electrolytic leakage indicating membrane stability index was obtained with GB foliar application. GB has been reported to not only act as a protective protein in maintaining intracellular osmotic pressure, but also stabilize quaternary structures and the activities of complex enzymes and proteins, which help keeping membrane integrity under stress conditions caused by salt, cold, and frost [6, 7]. It has been

reported that in cotton mutants capable of GB synthesis under saline stress, electrolytic leakage is lower than that induced in the mutants lacking GB synthesis capability [2].

Proline accumulation was influenced by foliar application time. Foliar application effects at the flowering+bolling stage were higher than those at the flowering stage. Under control conditions, the accumulated proline content was 123% lower than that caused by SA spraying, which had the highest proline content. Foliar applications of all the stress modulators produced more proline contents (82.48, 58.73, and 123% for GB, SNP, and SA, respectively) compared to those of the control (Table 7). The additive effects on proline contents were similar at those of the different foliage times, except for SNP, with which foliar application at the flowering+bolling stage caused a greater increase in proline content compared to that produced by its application at the flowering stage (Table 8).

TABLE 8. Interaction effect of time and type of modulator application on proline and potassium content

Time of spraying	Modulator type	Proline (mg. g^{-1}FW))	K (mg. g^{-1}FW)
Flowering	Control	38.3±1.6 d	36.6±3.41 c
	GB	67.0±1.07 b	48.6±1.22 b
	SNP	54.0±2.53 c	42.0±2.07 b
	SA	81.3±2.97 a	42.6±1.58 b
Flowering+bolling	Control	36.0±1.38 d	31.6±2.07 c
	GB	68. 7±2.57 b	40.3±1.83 b
	SNP	64.0±2.22 b	45.0±2.07 b
	SA	84.7±2.43 a	63. 7±1.95 a

Values are mean ± SE (n = 6). Letter denotes significant differences between treatments at $p \leq 0.05$, Similar letter within the same columns not significant difference based on FLSD.

In saline conditions, proline accumulation is inhibited as a defense mechanism in plants. Proline is a non-toxic preservative for osmotic regulation under salinity and other environmental stresses [6, 13]. On the other hand, proline accumulated in plants enhances antioxidant capacity and neutralizes hydroxyl free radicals [6]. In this investigation, the external applications of the stress modulators increased proline contents in cotton. According to our findings, SA foliar application in cotton was reported to augment proline content in a saline condition. This increase in proline content enabled the plant to be more resistant to stress through osmotic adjustment. In addition, proline acts as an energy source that can help improve salinity tolerance [18].

Sodium and potassium contents were not affected by spraying time and the stress moderator effects on them were not significant although spraying with the stress modulators slightly decreased and increased their contents compared to the control treatment, respectively. The decreasing effect on sodium uptake by foliar application with SA was not statistically significant with that triggered by its application with GB (Table 7). While foliar application with SA at the flowering+bolling stage decreased Na^+ content, its application with GB increaseditsuptake compared to that obtained by foliar application at the flowering stage. As with sodium content, potassium content was enhanced with the external applications of SNP and SA at the flowering+bolling stage and foliar application with GB reduced potassium content compared to when it was applied at the flowering stage (Table 8).

4. Conclusion

Overall, the results of this experiment revealed that in saline conditions, external applications of the stress modulators had additive effects on the enzymatic and non-enzymatic antioxidants. Among the modulators used in this experiment, the external application of SA had more improvement effects on the studied physiological and biochemical traits. Although the external applications of GB were not statistically different from those of SA in most of the studied traits, the external application of SNP increased the traits compared to the control treatment; however, this increase was less than those caused by SA and GB applications. The external applications of the stress modulators did not have great effects on the uptakes of ions, such as sodium and potassium, although their uses decreased and increased sodium and potassium uptakes, respectively. The results of our investigation demonstrated that applications of the stress modulators could reduce the effects of salinity stress on cotton growth and development under saline conditions by enhancing the activities of antioxidant enzymes. Furthermore, the external applications of SA and GB at the flowering+bolling stage and SNP application at the flowering stage could augment the activities of antioxidant enzymes and result in salt stress toleration in cotton.

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