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Salicylic Acid and α-Tocopherol Ameliorate Salinity Impact on Wheat

Saleha Saeed, Abd Ullah,* Sami Ullah,* Mohamed S Elshikh, Javaria Noor, Sayed M. Eldin, Fanjiang Zeng, Fazal Amin, Mohammad Ajmal Ali, and Iftikhar Ali*

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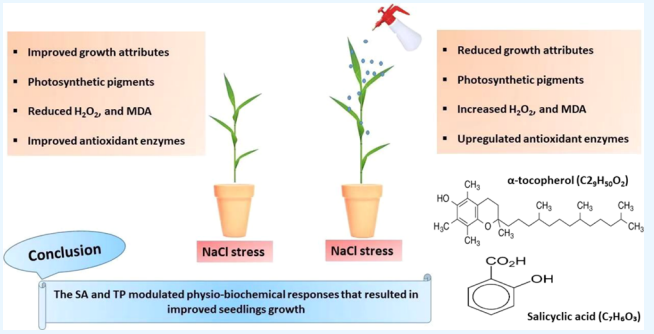
ABSTRACT: *Background:* Soil salinity negatively impacts agricultural productivity. Consequently, strategies should be developed to inculcate a salinity tolerance in crops for sustainable food production. Growth regulators play a vital role in regulating salinity stress tolerance. *Methods:* Thus, we examined the effect of exogenous salicylic acid (SA) and alpha-tocopherol (TP) (100 mg/L) on the morphophysio-biochemical responses of two wheat cultivars (Pirsabak-15 and Shankar) to salinity stress (0 and 40 mM). *Results:* Both Pirsabak-15 and Shankar cultivars were negatively affected by salinity stress. For instance, salinity reduced growth attributes (i.e., leaf fresh and dry weight, leaf moisture content, leaf area ratio, shoot and root dry weight, shoot and root length, as well as root−shoot ratio), pigments (chlorophyll a, chlorophyll a, and carotenoids) but increased hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and endogenous TP in both cultivars. Among the antioxidant enzymes, salinity enhanced the activity of peroxidase (POD) and polyphenol oxidase (PPO) in Pirsabak-15; glutathione reductase (GR) and PPO in Shankar, while ascorbate peroxidase (APOX) was present in both cultivars. SA and TP could improve the salinity tolerance by improving growth and photosynthetic pigments and reducing MDA and H_2O_2. In general, the exogenous application did not have a positive effect on antioxidant enzymes; however, it increased PPO in Pirsabak-15 and SOD in the Shankar cultivar. *Conclusions:* Consequently, we suggest that SA and TP could have enhanced the salinity tolerance of our selected wheat cultivars by modulating their physiological mechanisms in a manner that resulted in improved growth. Future molecular studies can contribute to a better understanding of the mechanisms by which SA and TP regulate the selected wheat cultivars underlying salinity tolerance mechanisms.



1. INTRODUCTION

Globally, wheat (*Triticum aestivum L.*) is the first domesticated cereal crop and is cultivated by an estimated 80 million farmers, feeding 2.5 billion people worldwide.[1] However, because of recurring salinity, land degradation, fluctuating temperatures, and other causes, these countries' output is declining. As a result, the fundamental issue ahead is to conserve and protect natural resources from depletion to ensure long-term production sustainability. According to the findings of a recent study that analyzed data from studies published between 1980 and 2015, salinity on a worldwide scale resulted in yield decreases of up to 21% in wheat (*Triticum aestivum L.*).[2] The high concentration of minerals (ions: K^+, Mg^{2+}, Ca^{2+}, Na^+, NO_3^-, HCO_3, SO_4, and Cl^-) in water and soil is known as salinity.[3]

Salinity (environmental stress) limits the growth and development of plants.[4,5] Salinity stress in plants (xylem) disrupts water flow to nearby elongating cells, suppressing cell elongation. Salinization generally causes a high accumulation of salt ions, including Na^+ and Cl^- ions, leading to ionic toxicity, osmotic imbalance, impaired nutrient acquisition, metabolic disruption, excessive reactive oxygen species (ROS) production, and oxidative stress damage.[4,6,7] Plants have developed complex mechanisms to respond and adapt to stress conditions, including salinity stress, via their cellular, physiological, and molecular mechanisms.[8−1011] For instance, several physiological processes have been shown to relate to salinity tolerance, including osmotic adjustment, ionic homeostasis, nutrient uptake, antioxidant enzyme and metabolite systems, and hormone regulation.[12−1415] Salinity stress affects the production of ROS and antioxidant-mediated metabolism in plants. Excessive ROS such as $O_2^{\cdot-}H_2O_2$ and OH can cause degradation of protein, lipids, and macromolecules, causing metabolic arrest in the cell. Consequently, increased ROS-metabolizing enzymes and/or

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metabolites may enable plants exposed to salinity to efficiently scavenge ROS as well as minimize ROS-induced damage.[16] Metabolites such as proline, proteins, glycine betaine, and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APOX) play a promising function in protecting cells from acute cellular damage by scavenging ROS.[17−1920] As a result of modulating ion homeostasis, adjusting osmotic pressure, and enhancing glyoxalase activity, the antioxidant defense system reinforces plant growth under salinity stress.[21,22] There are a number of complex metabolic pathways, physiological traits, and molecular and genetic networks that contribute to the plants' ability to adapt to salinity conditions. Among several salinity alleviation strategies, one of the important strategies is to apply the compounds exogenously.

The majority of the processes outlined above can be modulated in plants exposed to salinity by supplying them with different exogenous growth regulators, including phytohormones, polyamines, osmoprotectant molecules, antioxidants, and trace elements, in order to improve salinity tolerance.[5]

Salicylic acid (SA) is a phenolic molecule that regulates plant growth and development both in optimal and stressful conditions.[23−252627] Plants have been found to be protected from abiotic stresses by salicylic acid since it regulates a variety of physiological processes, including photosynthesis, proline metabolism, N metabolism, glycine betaine biosynthesis, antioxidant mechanism, and plant-water status. Therefore, SA has been implicated in plants' resistance to various abiotic stresses, including ozone, metals, UV-B radiation, extreme temperatures, droughts, and salinity.[16]

Alpha-tocopherol (α-Toc) is a nonenzymatic lipid-soluble antioxidant. It stabilizes electron transport, photosynthetic reactions, and membrane integrity, thereby maintaining the source-sink relationship and increasing plant performance.[28,29] Moreover, the application of foliar administration of tocopherol is also an effective way of improving plant growth under salinity stress.[30] α-Tocopherol also reduces ROS in chloroplasts, improving plants' photosynthetic efficiency.[5] Furthermore, it functions as a protective agent by quenching free radicals produced as a result of salinity stress as well as by increasing the production of other antioxidants.[31] It is therefore evident that SA and TC protect plants from the damage caused by salinity. Researchers have demonstrated that SA and TP have beneficial effects on plant growth and physiology under stressful conditions, including salinity, in significant research studies. Stress tolerance varies, however, depending on the type, duration, and intensity of stress and the type and cultivar of plants. Moreover, the combined application to wheat seedlings under salinity is still poorly understood.

Thus, we applied exogenous salicylic acid and alpha-tocopherol on wheat cultivars (Pirsabak-15 and Shankar) subjected to controlled and salinity stress (40 mM) conditions aiming to investigate (1) how exogenously applied SA and TP affect wheat tolerance to salinity by regulating the physio-biochemical characteristics, (2) which mode of application (individual or combined) is most efficient, and (3) which growth regulator is most effective (SA or TP).

2. MATERIALS AND METHODS

2.1. Plant Materials and Experimental Conditions. We obtained seeds of two wheat cultivars (Pirsabak-15 and Shankar), primitive cultivars/landraces from diverse places with rainfall less than 1000 m, from the cereal crop research center Pirsabaq Nowshera (CCRIP), KP, Pakistan. We surface sterilized the seeds with 70% ethanol and rinsed them with distilled water after 3 min. The experiment was conducted in July 2021, inside a greenhouse of the Department of Botany, University of Peshawar, Pakistan, under optimum conditions. The seeds were planted in earthen pots filled with dirt and silt (pH 6.9, bulk density of 1.55 g cm^{-3}, and EC 0.288 ds/m) in a 2:1 ratio (18 cm lower inner diameter, 18 cm upper inside diameter, 20 cm height, and 2 cm thickness). The pots were organized in a completely randomized design (CRD) and were rain-protected. A total of 10 seeds were planted in each pot. Each treatment was repeated three times. A standard pot experiment was followed, and no pest or disease concerns were identified during the trial period.

2.2. Treatment Details. Salt and growth regulators were applied at tillering. Plants were divided into groups treated with a control (0 mM) or salinity stress (40 mM). Salicylic acid and tocopherol were sprayed onto leaves (100 mg/L) with a sprinkler. Using a pressure pump, exogenous salicylic acid and tocopherol were applied to the leaves. A total of three foliar applications were applied at three-day intervals. Harvesting was done after 50 days of sowing. According to standard procedures, three plants were randomly selected from each treatment for the assessment of their growth attributes. Next, we immediately froze the leftover plants in liquid nitrogen and subsequently stored them at −80 °C in order to evaluate their physio-biochemical and enzymatic properties.

2.3. Growth Attributes. Using a measuring tape, we measured the shoot height and root length. We measured the leaf fresh weight and leaf, shoot, and root dry weight of the seedlings using an electric balance. The following formulas were used to calculate the leaf area index, leaf area ratio, leaf moisture content, and root−shoot ratio.

$$\mathrm{LAI} = \frac{\mathrm{Leafarea}}{\mathrm{Landarea}} \tag{1}$$

$$\mathrm{Leafarearatio} = \frac{\mathrm{leafarea}}{\mathrm{finalplantdryweight}} \tag{2}$$

$$\mathrm{Root-shootratio} = \frac{\mathrm{Rootdrymass}}{\mathrm{shootdrymass}} \tag{3}$$

$$\%\mathrm{moisturecontent} = \frac{\mathrm{Wetweight} - \mathrm{dryweight}}{\mathrm{Dryweight}} \tag{4}$$

2.4. Leaf Photosynthetic Pigment. The approach developed by Lalay et al.[32] was used to evaluate the different forms of chlorophyll (Chl a and Chl b). A standard method[33] was used to determine the carotenoid (CAR) contents. The following equations were used to calculate the quantities:

$$\mathrm{Chla} = \{12.7(\mathrm{OD663}) - 2.69(\mathrm{OD645})\} \times \frac{V}{1000} \times W \tag{5}$$

$$\mathrm{Chlb} = \{22.9(\mathrm{OD645}) - 4.68(\mathrm{OD663})\} \times \frac{V}{1000} \times W \tag{6}$$

$$\mathrm{Carotenoid} = \mathrm{DA480} + (0.114 \times \mathrm{DA663}) - (0.638 \times \mathrm{DA645}) \tag{7}$$

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Table 1. Effect of Salicylic Acid and Tocopherol Foliar Spray on Leaf Fresh Weight (LFW), Leaf Dry Weight (LDW), Leaf Moisture Content (LMC; %), Leaf Area Ratio (LAR), and Leaf Area Index (LAI) of Wheat (*Triticum aestivum*) Cultivars under Salinity Stress[a]

cultivars	treatments	LFW (mg)	LDW(mg)	LMC (%)	LAR (cm^2)	LAI
Pirsabak-15	control	90.0 ± 0.01^{ab}	30.0 ± 0.06^{bd}	30.0 ± 4.77^{a}	75.2 ± 0.37^{bc}	0.90 ± 0.02^{a}
	salinity	30.0 ± 0.23^{e}	26.0 ± 1.00^{cd}	10.0 ± 17.9^{d}	58.9 ± 0.98^{c}	0.17 ± 0.04^{f}
	salinity + SA	70.0 ± 0.08^{b}	40.0 ± 1.76^{bc}	18.0 ± 9.14^{bc}	34.8 ± 1.02^{e}	0.36 ± 0.03^{d}
	salinity + TP	90.0 ± 0.40^{ab}	44.0 ± 1.66^{b}	26.6 ± 7.62^{b}	38.4 ± 1.08^{de}	0.23 ± 0.02^{e}
	salinity + SA + TP	100 ± 0.13^{a}	48.0 ± 1.29^{a}	16.6 ± 0.98^{c}	39.7 ± 0.97^{de}	0.89 ± 0.03^{ab}
Shankar	control (0 mM NaCl)	90.0 ± 0.21^{ab}	38.6 ± 2.0^{c}	17.2 ± 6.09^{bc}	98.5 ± 0.76^{b}	0.62 ± 0.03^{b}
	salinity (40 mM NaCl)	50.0 ± 0.02^{d}	18.9 ± 1.98^{d}	12.2 ± 6.32^{cd}	53.9 ± 1.70^{cd}	0.32 ± 0.03^{de}
	salinity + SA	50.0 ± 0.11^{d}	10.1 ± 1.2^{e}	29.8 ± 9.76^{sb}	84.7 ± 0.91^{b}	0.42 ± 0.05^{b}
	salinity + TP	60.0 ± 0.07^{c}	15.0 ± 0.96^{de}	30.0 ± 8.32^{a}	136 ± 2.15^{a}	0.60 ± 0.02^{b}
	salinity + SA + TP	70.0 ± 0.09^{b}	19.8 ± 0.05^{d}	30.4 ± 0.78^{a}	89.4 ± 1.92^{bc}	0.44 ± 0.01^{c}

[a]Values are presented as mean ± standard deviation. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

Table 2. Effect of Salicylic Acid and Tocopherol Foliar Spray on Shoot Height (SH), Shoot Dry Weight (SHDW), Root Length (RL), Shoot Moisture Content (SHMC; %), and Root/Shoot Ratio (RSR) of Wheat (*Triticum aestivum*) Cultivars under Salinity Stress[a]

cultivars	treatments	SH (cm)	SHDW (mg)	RL (cm)	SHMC (%)	RSR
Pirsabak-15	control	35.6 ± 0.68^{a}	460 ± 0.10^{a}	13.3 ± 0.29^{a}	410 ± 0.76^{a}	0.56 ± 0.51^{b}
	salinity	19.3 ± 1.29^{d}	110 ± 0.1^{c}	6.83 ± 0.47^{d}	80.2 ± 3.66^{a}	0.13 ± 0.16^{cd}
	salinity + SA	20.8 ± 0.48^{cd}	90.0 ± 0.09^{cd}	7.06 ± 0.54^{cd}	120 ± 2.11^{e}	0.31 ± 0.15^{de}
	salinity + TP	31.3 ± 0.87^{b}	120 ± 0.09^{c}	8.60 ± 2.11^{c}	190 ± 2.43^{c}	0.43 ± 0.23^{c}
	salinity + SA + TP	26.3 ± 0.73^{c}	140 ± 0.07^{bc}	7.23 ± 1.88^{d}	240 ± 1.45^{bc}	0.29 ± 0.15^{d}
Shankar	control (0 mM NaCl)	28.4 ± 1.39^{c}	171 ± 0.04^{b}	10.9 ± 0.36^{b}	268 ± 1.67^{b}	0.66 ± 0.29^{a}
	salinity (40 mM NaCl)	18.8 ± 0.88^{e}	80.4 ± 0.08^{d}	8.06 ± 1.22^{c}	110 ± 1.20^{f}	0.21 ± 0.98^{e}
	salinity + SA	20.9 ± 1.18^{cd}	90.0 ± 0.07^{cd}	6.83 ± 0.47^{d}	170 ± 1.29^{cd}	0.34 ± 0.67^{d}
	salinity + TP	27.5 ± 0.67^{c}	80.0 ± 0.05^{d}	8.30 ± 2.26^{c}	120 ± 1.58^{e}	0.46 ± 0.90^{bc}
	salinity + SA + TP	22.1 ± 0.00^{cd}	90.0 ± 0.04^{cd}	9.66 ± 0.23^{bc}	160 ± 1.60^{d}	0.36 ± 0.06^{cd}

[a]Values are presented as mean ± standard deviation. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

where V is the extract level (in mL), W is the weight of the fresh leaves, and DA is the optical density at the specified wavelength.

2.5. Determination of Biochemical Parameters. The total proline content (TPC) was estimated according to a standard technique.[34] The measurement of soluble protein was done according to the following procedure.[35]

$$\text{Protein}\%\left(\frac{W}{W}\right) = C_p \times V \times \frac{\text{DF}}{\text{Wt}} \tag{8}$$

where Wt is the weight of the leaves, V is the volume of the buffer lysis, DF is the dilution factor, and C_p is the protein concentration (mg L^{-1}) (mg). Using a standard method,[5] the sugar content of the leaves was calculated. Using the method described in refs 26 and 36, the phenolic content of the leaves was measured. Similar to this, the flavonoid activity was measured using the methodology described in refs 37 and 38. Phenolic and flavonoid content's optical densities (ODs) were measured at 730 and 430 nm, respectively.

2.6. Determination of Hydrogen Peroxide (H_2O_2) and Malondialdehyde. A standard technique[39] was used to measure the H_2O_2 activity. At wavelengths of 420 and 390 nm, respectively, the ODs of sugar and H_2O_2 were measured. The MDA content was determined using the technique described in ref 40. The OD was measured at 530 nm. Meantime, alpha-tocopherol was calculated using the technique described in ref 41. According to the following formulas, the MDA content was calculated:

$$\text{MDA(nmol)} = \frac{D(\text{A532nm} - \text{A600nm})}{1.56} \times 105 \tag{9}$$

2.7. Determination of Antioxidant Enzymatic Assays. The determination of superoxide dismutase (SOD) activity at 560 nm was performed using a spectrophotometer.[42] Moreover, the Shomali et al.[43] method was used to determine the activity of peroxidase (POD) and glutathione reductase (GR) at 420 and 340 nm, respectively. Ascorbate peroxidase (APOX) enzyme quantification was performed using a standard technique.[33]

$$\text{EA} = \Delta A \times \text{Totalassay}\frac{\text{Volume}}{\Delta t} \times \in \times i \times \text{enzymesamplevolume} \tag{10}$$

where Δt is the incubation duration, E is the substrate's absorbance coefficient, and ΔA is the change in absorbance.

2.8. Statistical Analysis. In order to calculate the mean and standard error from the gathered data, Microsoft Excel 2010, US, was utilized. Co-Stat Window ver. 6.3 was used to conduct an analysis of variance (ANOVA) to discover significant variations between treatments. Standard methods were used to compute the mean and standard error, and a least significant difference (LSD) test was run at the 0.05 significance level, and the results are displayed in letters (AE). R Studio 8.1 was used to carry out the correlation study.

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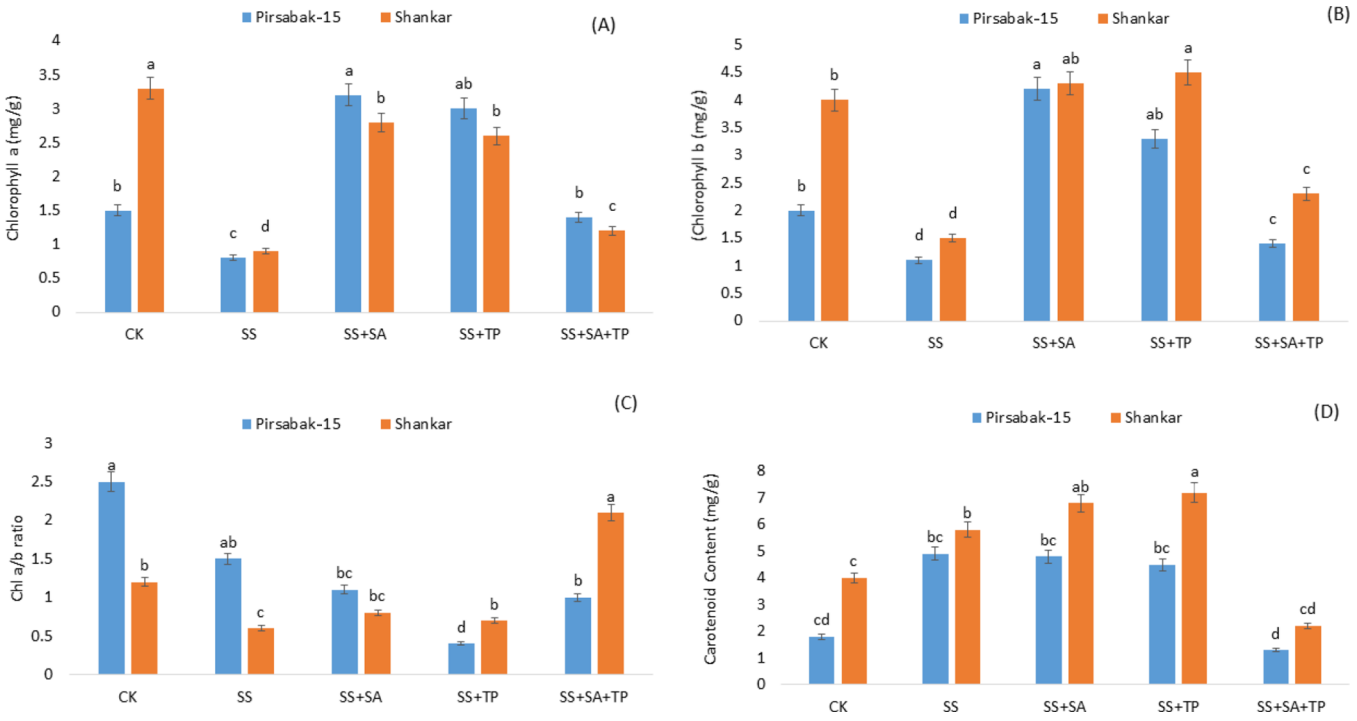

Figure 1. Effect of salicylic acid and tocopherol foliar spray on (A) chlorophyll a, (B) chlorophyll b, (C) chlorophyll a/b ratio, and (D) carotenoid content of *Triticum aestivum L.* under induced salinity stress. The bar represents standard deviation (SD) of mean, $n = 3$. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

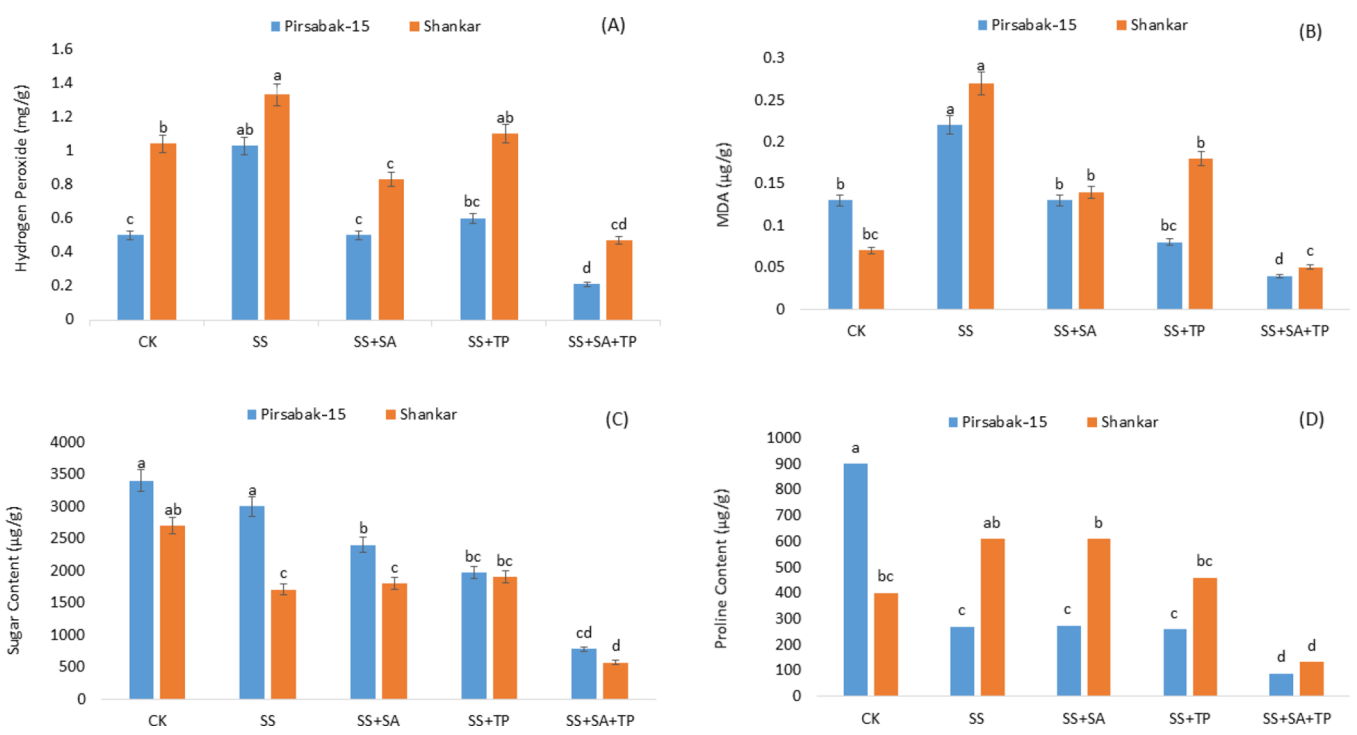

Figure 2. Effect of salicylic acid and tocopherol foliar spray on (A) malondialdehyde, (B) proline, (C) sugar, and (D) hydrogen peroxide content of *Triticum aestivum L.* under induced salinity stress. The bar represents standard deviation (SD) of mean, $n = 3$. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

3. RESULTS

3.1. Ameliorative Effect of SA and Tocopherol on Growth Parameters of Wheat under Salinity Stress. Salinity stress adversely impacted the growth features of both cultivars. For instance, salinity significantly declined leaf fresh weight and dry weight, leaf moisture content, leaf area ratio, and leaf area index, compared to controlled conditions. However, exogenous application of salicylic acid (SA) and tocopherol (TP) under salinity improved the aforementioned leaf growth features compared to their untreated peers (Table 1). A

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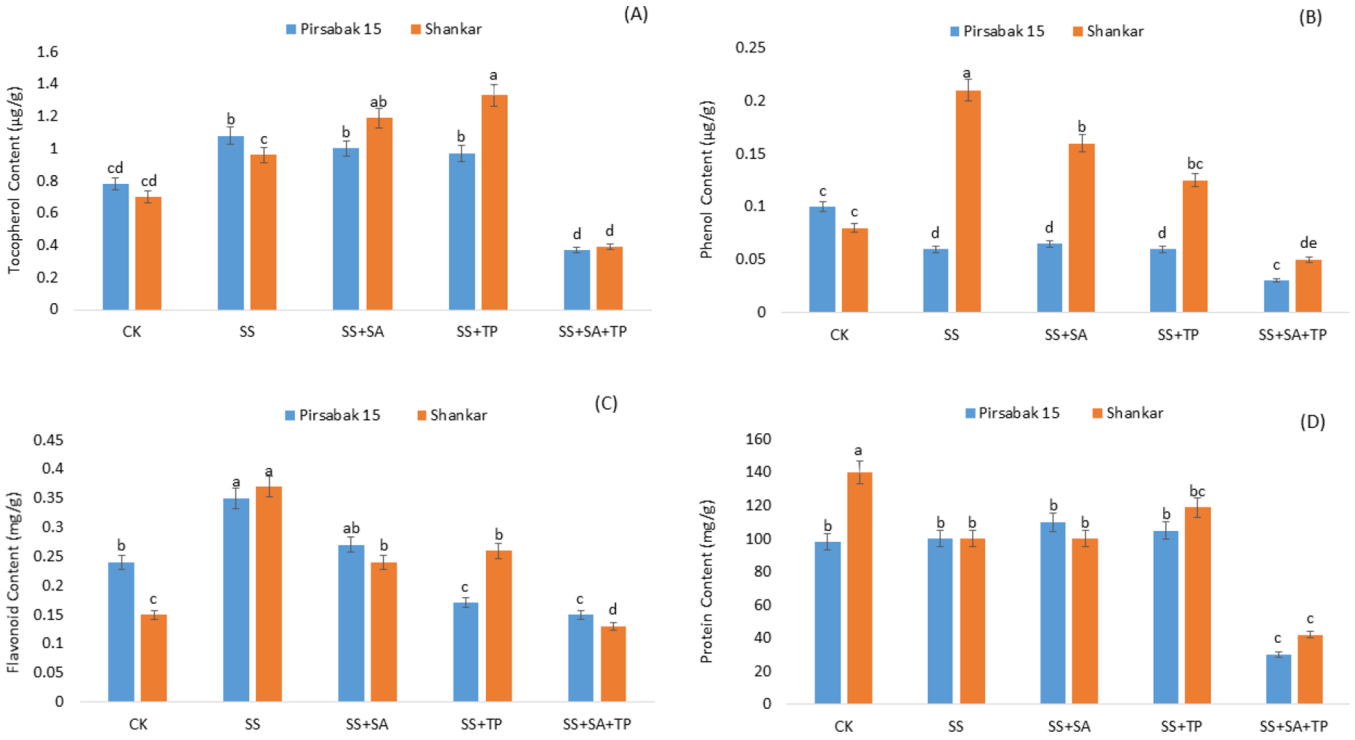

Figure 3. Effect of salicylic acid and tocopherol foliar spray on (A) tocopherol, (B) phenol, (C) flavonoid, and (D) protein content of *Triticum aestivum L.* under induced salinity stress. The bar represents standard deviation (SD) of mean, $n = 3$. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

combination of SA and TP resulted in a greater positive effect on leaves' fresh and dry weight as well as leaf area index than either agent alone. Moreover, shoot height, shoot dry weight, root length, and root−shoot ratio were inhibited significantly under salinity stress when compared with the controlled conditions. However, compared with their untreated counterparts, exogenous application of SA and TP improved root and shoot growth under salinity. Among the treatment groups, the highest shoot height of both cultivars was reported for TP application alone, followed by combined application of TP and SA. The highest shoot dry weight of both cultivars and root length of Shankar and shoot moisture content of Pirsabak-15 were reported under salinity and combined application of SA and TP (Table 2). Overall, findings indicated that both individual and combined application of SA and TP reduced the salinity-induced damaging effects on growth attributes (Tables 1 and 2).

3.2. Impact of SA and Tocopherol on Photosynthetic Pigments under Salinity Stress. Salt stress alone and in combination with individually applied SA and TP substantially decreased the concentrations of photosynthetic pigments [chlorophyll a (Chl a), chlorophyll b (Chl b), Chl a/b ratio, and carotenoids (Car)] in Pirsabak-15 cultivar and those of Chl b and Car in Shankar cultivar, compared to their controlled conditions (Figure 1A−D). However, exogenous SA and TP increased Chl a, Chl b, and Car in Pirsabak-15 and Chl a and Chl b in Shankar cultivar subjected to salinity, compared to their untreated peers under salinity and controlled conditions. Besides, individual application of SA and TP in salinity-stressed seedlings increased Car in Shankar cultivar, compared to controlled and untreated salinity-exposed seedlings (i.e., no SA and TP). Moreover, the combined application of SA and TP under salinity reduced Chl a, Chl b, and Car, compared to their controlled and untreated salinity-stressed peers (Figure 1A,B,D).

3.3. Effect of SA and TP on MDA and H_2O_2 Concentrations in Wheat Cultivars under Salinity Stress. Increased concentrations of H_2O_2 and MDA indicate salinity-induced oxidative stress damages in plants. In our study, salinity stress significantly increased H_2O_2 and MDA concentrations compared to control (Figure 2 A,B). However, the individual and combined effects of SA and TP reduced their concentrations under salinity stress compared to their untreated peers.

3.4. Effect of SA and TP on Biochemical Changes in Wheat Cultivars under Salinity Stress. In response to salt stress, sugar content decreased in both cultivars, whereas proline decreased in Pirsabak-15 but increased in Shankar cultivar, compared to their control (Figure 2C,D). However, the lowest sugar and proline contents observed in salinity-stressed seedlings were supplied with combined SA and TP application (Figure 3A). Moreover, endogenous TP concentration increased in both cultivars following salinity stress. Individual application of SA and TP had no significant effect on TP in Pirsabak-15 but did increase it in Shankar cultivar. The combined treatment of SA and TP significantly decreased endogenous TP, compared to all other treatments.

However, combined SA and TP severely declined the endogenous TP level. The phenol contents decreased in Pirsabak-15 but increased in Shankar under salinity alone and with individual application of exogenous SA and TP (Figure 3B). Both cultivars had increased flavonoids when exposed to salinity as compared with the control. SA and TP individually and combined decreased flavonoids in both cultivars, compared to their untreated peers (Figure 3C). Moreover, protein contents remained unchanged in Pirsabak-15 but reduced significantly in Shankar subjected to salinity stress alone and in

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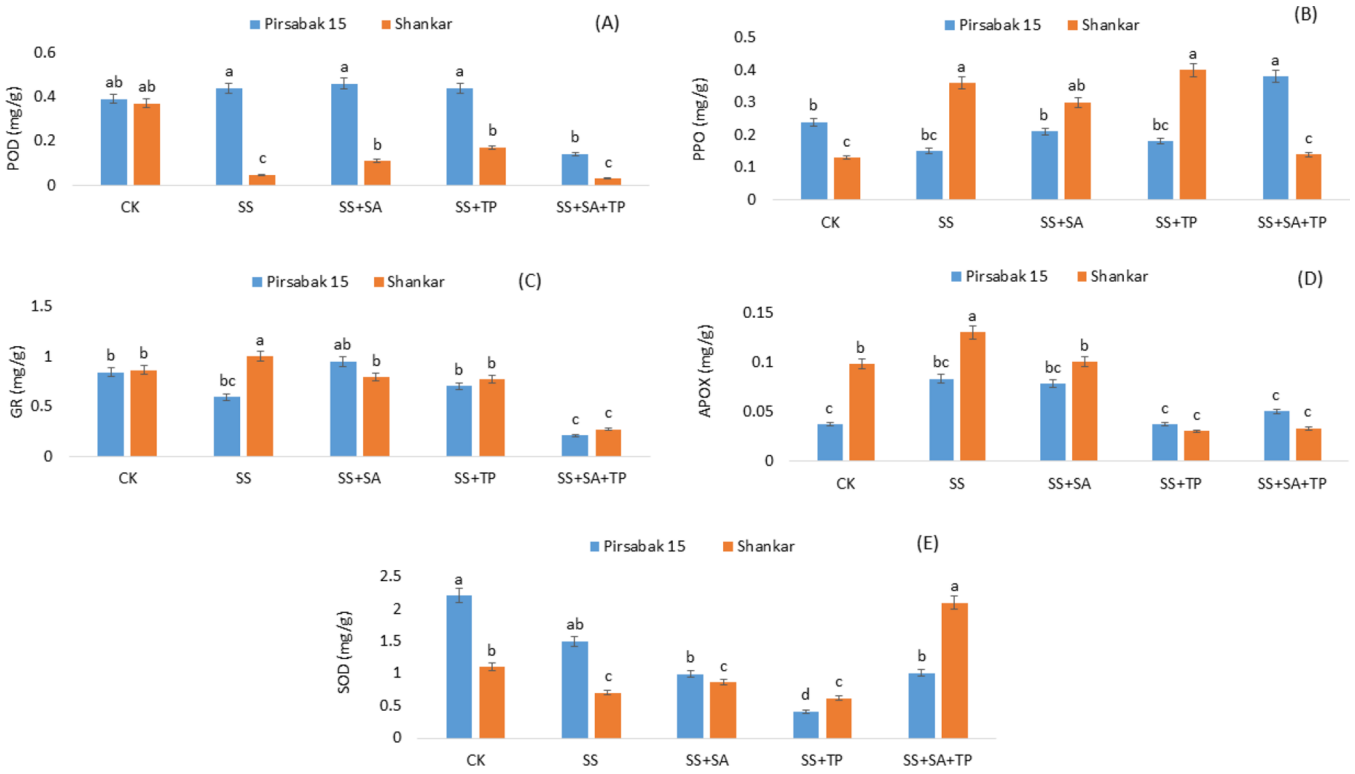

Figure 4. Effect of salicylic acid and tocopherol foliar spray on (A) peroxidase, (B) polyphenol oxidase, (C) glutathione reductase, (D) ascorbate peroxidase, and (E) superoxide dismutase content of *Triticum aestivum L.* under induced salinity stress. The bar represents standard deviation (SD) of mean, $n = 3$. Based on Tukey LSD, different letters indicate significantly different values at $P < 0.05$. Control and salinity groups represent 0 and 40 mM of NaCl stress.

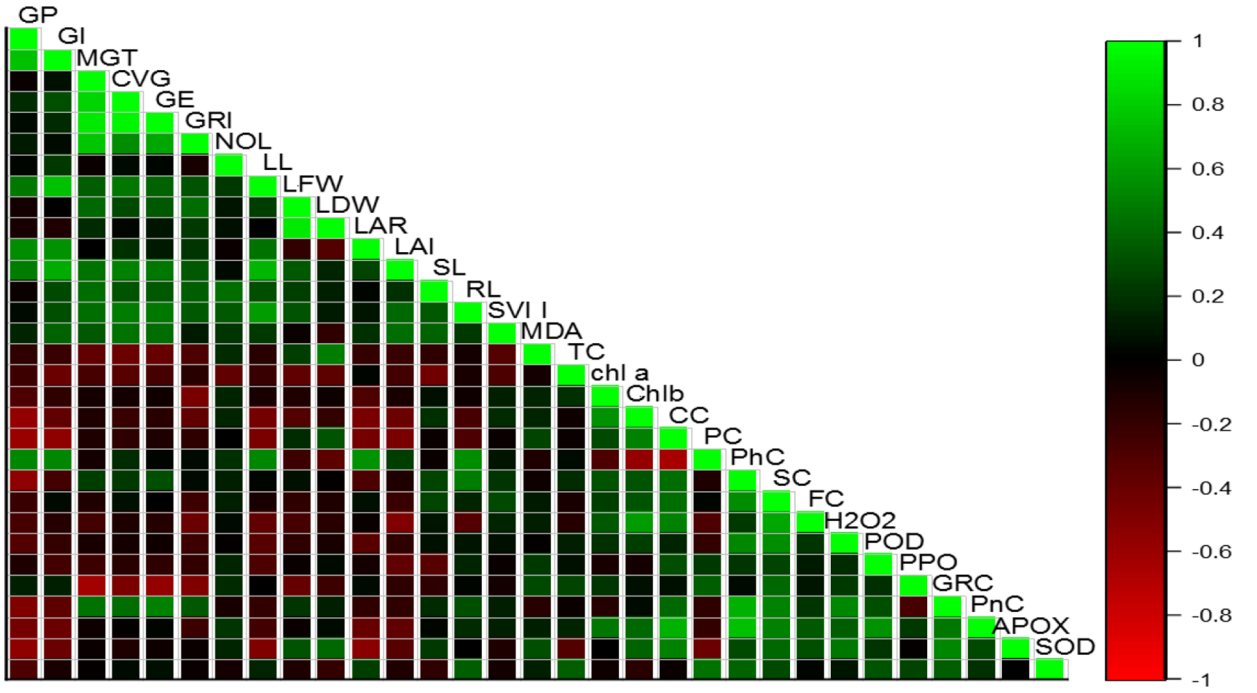

Figure 5. Correlation between various germination attributes of *Triticum aestivum L.* var. Pirsabak-15 under salinity stress: leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline content (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxidase dismutase (SOD).

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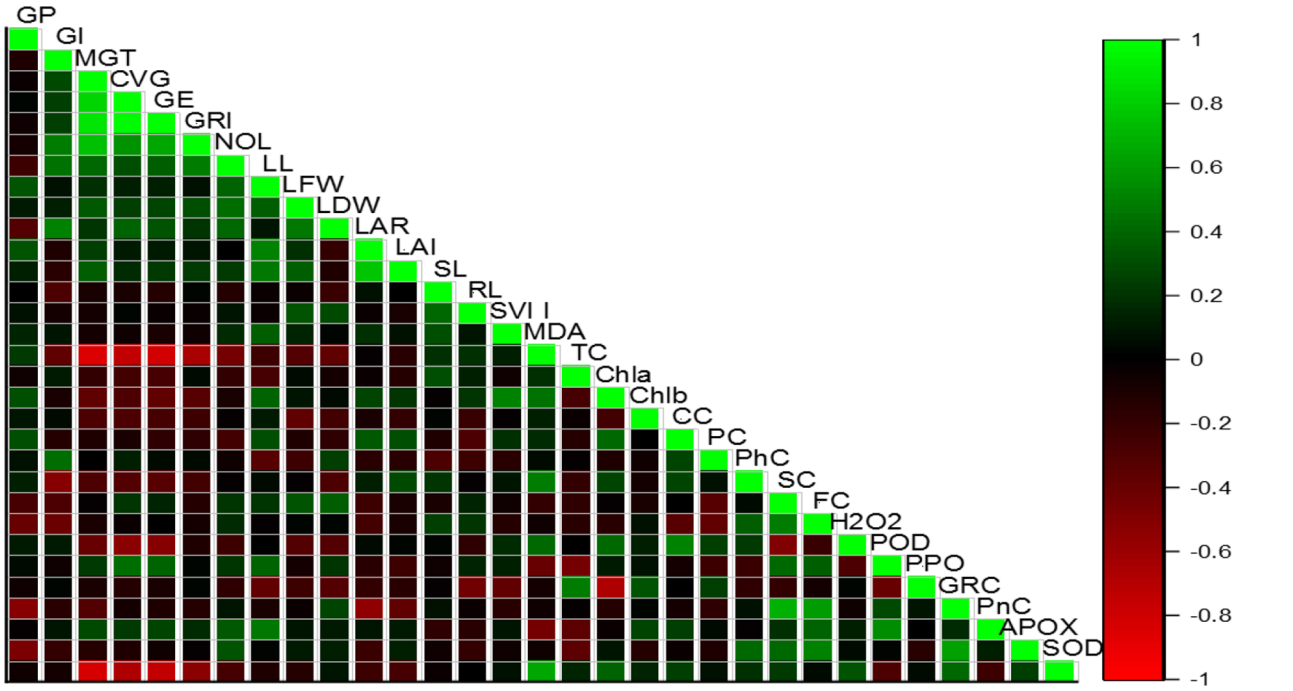

Figure 6. Correlation between various germination attributes of *Triticum aestivum L.* var. Shankar under induced salinity stress: leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline content (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxide dismutase (SOD).

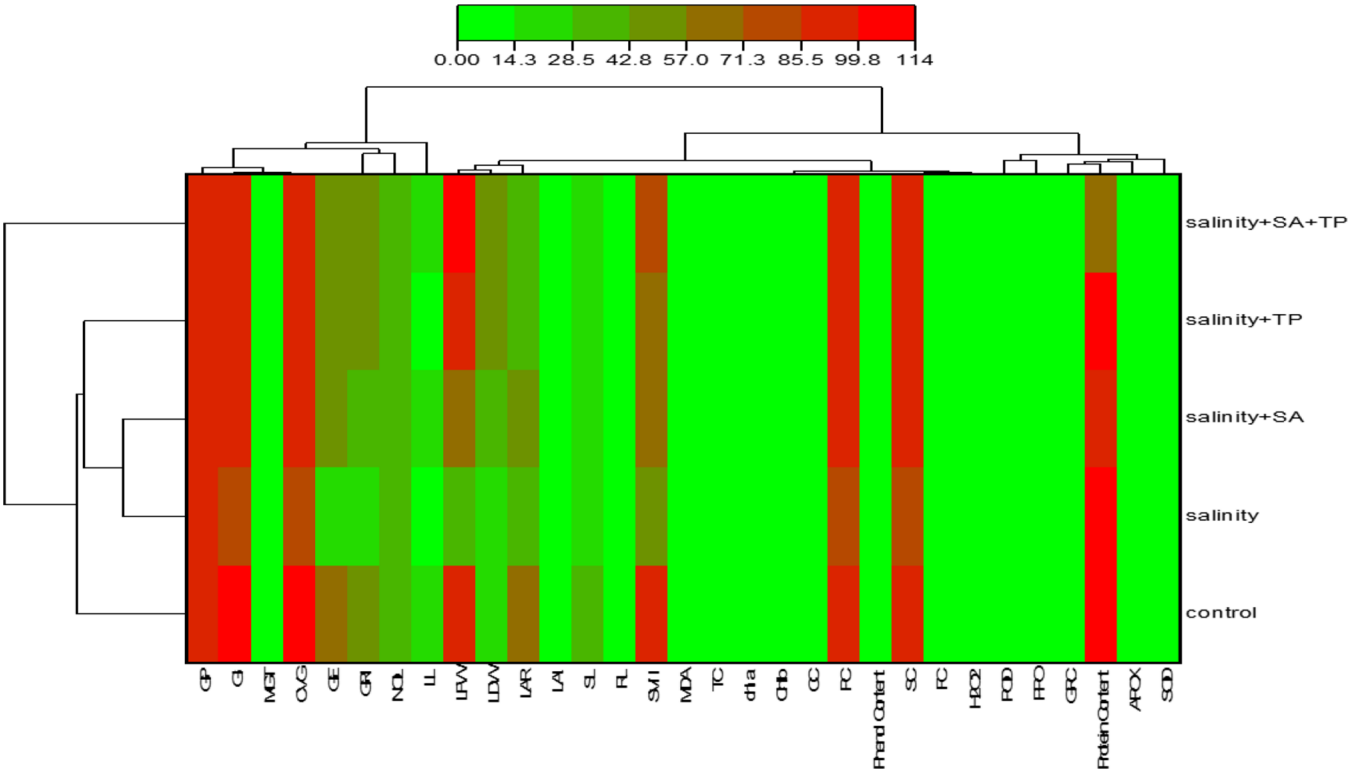

Figure 7. Heatmap histogram correlation between various germination attributes of *Triticum aestivum L.* var. Pirsabak-15 under induced salinity stress: leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline content (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxide dismutase (SOD).

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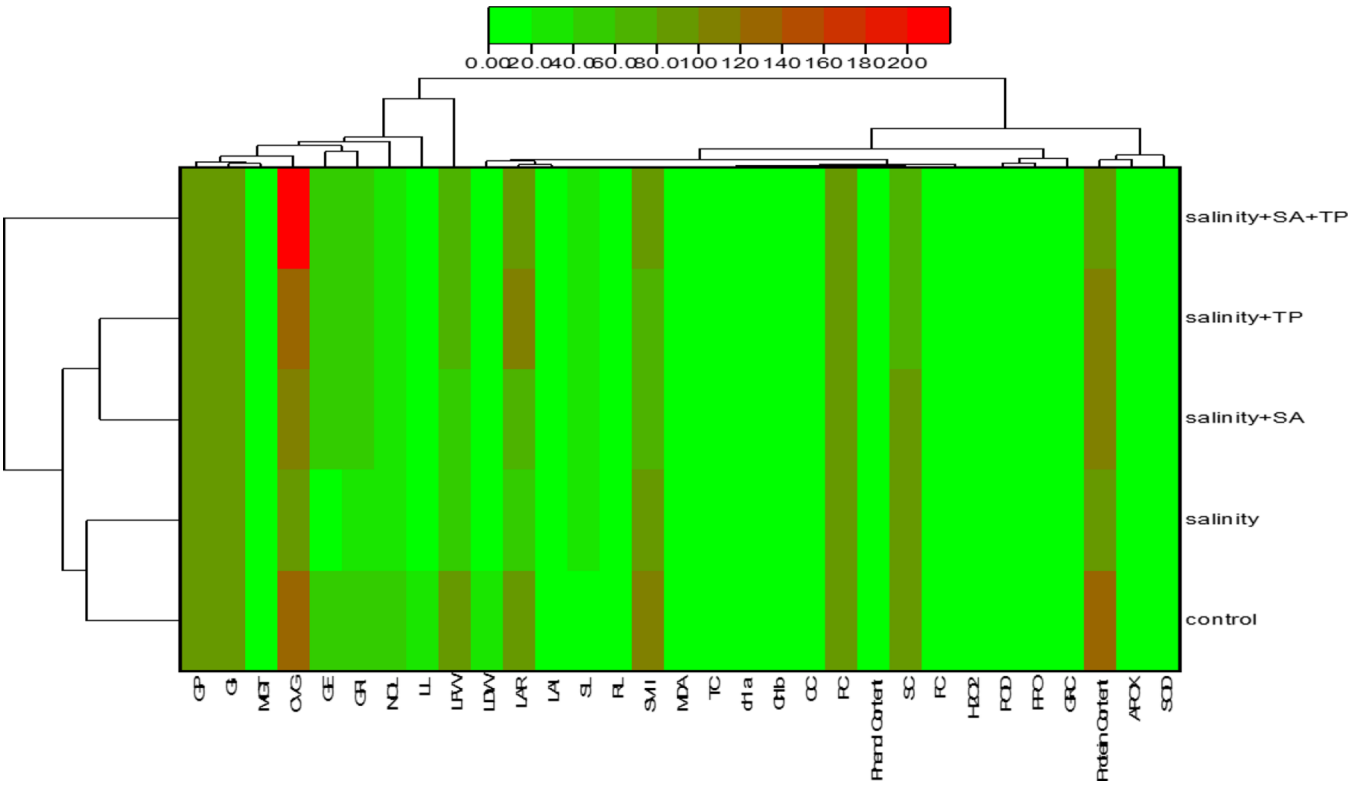

Figure 8. Heatmap histogram correlation between various germination attributes of *Triticum aestivum L.* var. Shankar under induced salinity stress: leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline content (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxide dismutase (SOD).

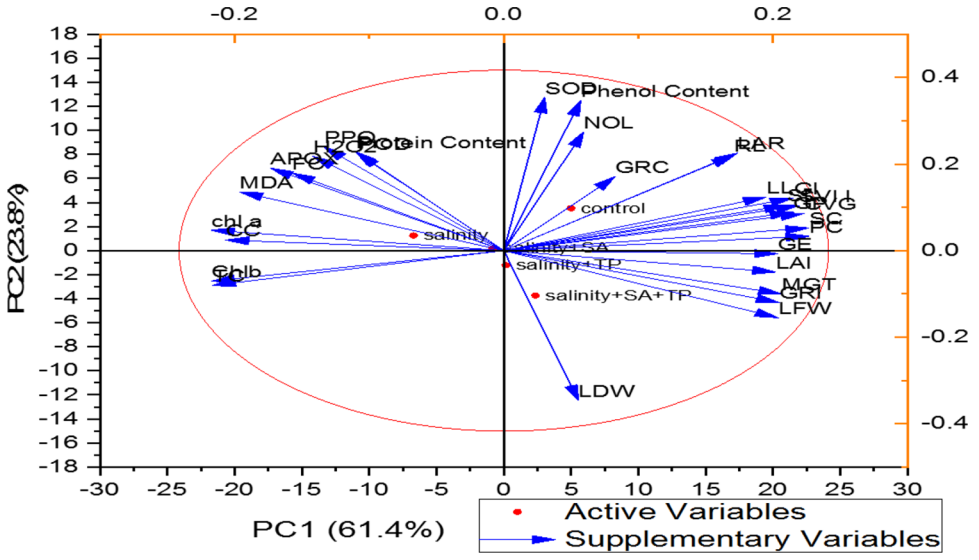

Figure 9. Loading plot of PCA on various germination attributes of *Triticum aestivum L.* var. Pirsabak-15 under induced salinity stress: leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxide dismutase (SOD).

combination with SA and TP. Specifically, SA and TP combined had a more negative effect on phenol, flavonoids, and protein compared to their individual applications (Figure 3C,D).

3.5. Effect of SA and TP on Enzymatic Antioxidant Mechanism in Wheat under Salinity Stress. The upregulation of antioxidant enzymes indicates that plant tissues are under stress. In our study, salinity stress increased the activities of peroxidase (POD) and polyphenol oxidase (PPO) activity in Pirsabak-15, glutathione reductase (GR) and PPO in Shankar, whereas ascorbate peroxidase (APOX) activity in both

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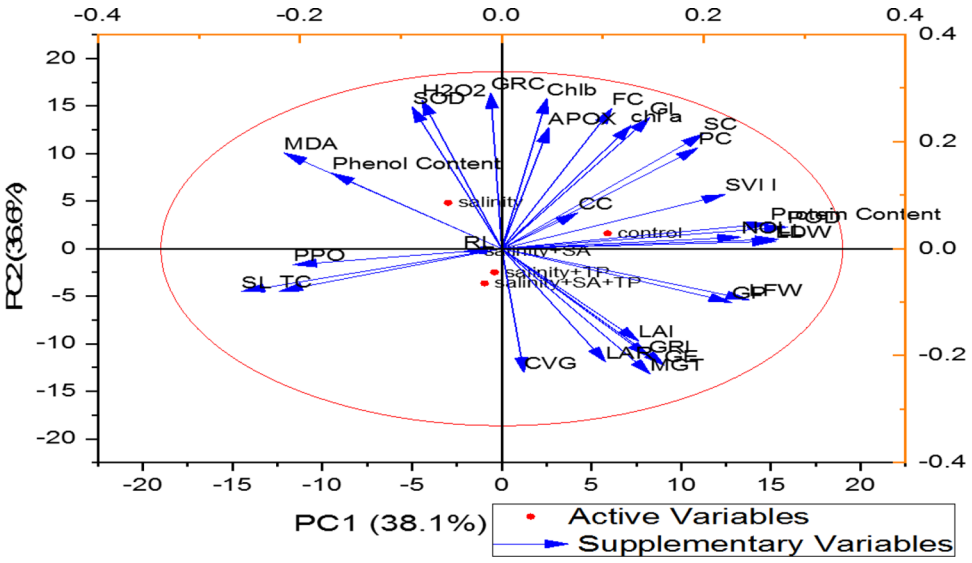

Figure 10. Loading plot of PCA on various germination attributes of *Triticum aestivum L.* var. Shankar under induced salinity stress: leaf length (LL), leaf fresh weight (LFW), leaf dry weight (LDW), leaf area ratio (LAR), leaf area index (LAI), shoot length (SL), root length (RL), malondialdehyde content (MDA), tocopherol content (TC), chlorophyll a content (Chl a), chlorophyll b content (Chl b), carotenoid content (CC), proline content (PC), phenolic content (PhC), sugar content (SC), flavonoid content (FC), hydrogen peroxide (H_2O_2), peroxidase (POD), polyphenol oxidase (PPO), glutathione reductase (GRC), protein content (PnC), ascorbate peroxidase (APOX), and superoxide dismutase (SOD).

cultivars, compared to their controlled conditions (Figure 4 A–D). Salinity stress decreased PPO and GR in Shankar and SOD (Figure 4E) in both cultivars, compared to the control (Figure 4). Under salinity stress, individual application of SA and TP further increased POD in Pirsabak-15 and PPO and SOD in both cultivars. The effect of combined application of TP and SA was more severe, as the reduction in activities of POD, GR, and PPO in both cultivars was higher, compared to their individual application. However, combined SA and TP did increase PPO in Pirsabak-15 and SOD in the Shankar cultivar (Figure 4).

3.6. Correlation, Heat Map Correlation, and Principal Component Analysis of Different Parameters Measured for Wheat Cultivars under Salinity Stress. The correlation analysis was carried out to quantify the relationship between the attributes measured on wheat plants grown in the saline soil with or without the application of salicylic acid and alpha-tocopherol (Figures 5 and6). The correlation matrix reported the correlation among 31 measured traits across the five treatments. These coefficients compute a linear connection and are sensitive to outliers. Generally, the growth traits, such as leaf fresh and dry weight, shoot and root length, leaf area index, and leaf area, were positively correlated with negatively correlated biochemical parameters such as H2O2, SOD, POD, PPO, APOX, Chl a, Chl b, CC, and MDA. In addition, the other parameters and especially the enzymatic activities increased under stress conditions due to the scavenging nature and production of ROS in cells.

Additionally, a heatmap histogram was shown between the variables of the various treatments investigated in this investigation (Figure 6). These factors are yielding outcomes that are consistent with what the correlation analysis revealed. Two distinct clusters were formed between treatments; the first cluster was composed of control, salinity, salinity + SA, salinity + TP, and salinity + SA + TP for Pirsabak-15, while for Shankar, clusters were formed (Figures 7 and 8).

Under generated salt stress, the principal component analysis (PCA) was used to link the morphophysiological characteristics and antioxidant enzymes with one another. PCA was performed on the experimental data set, which included control, treatment, and 22 morphological and 17 physio-biochemical variables (Figures 9 and 10). The findings show that the respected treatments were all successfully distributed over the whole data set. The distribution of all the data set's elements clearly demonstrates that salt stress had a considerable impact on several morpho-physio-biochemical characteristics in all the experimental treatments, whether or not salicylic acid and alpha-tocopherol were applied topically. The PCA plot demonstrates that all variables are dispersed, indicating that the salinity had a significant impact on these parameters. The findings indicated that the first two main components accounted for 57% of the data set's overall volatility.

4. DISCUSSION

4.1. Salicylic Acid- and Alpha-Tocopherol-Dependent Changes in Growth Parameters under Salinity Stress. Salinity stress has been linked to impaired growth, development, metabolism, and yield in several crop plants, including wheat. However, exogenous application of several growth regulators such as jasmonic acid (JA), salicylic acid (SA),[13,26,44−46] naphthyl acetic acid (NAA),[47] abscisic acid (ABA),[48] and α-tocopherol (TP)[49] have been reported to minimize the negative impacts of salinity on morpho-physio-biochemical attributes and yields in several crop plants.[50] In the present study, growth attributes (leaf fresh and dry weight, leaf area, shoot and root length, their fresh and dry weight, and root−shoot area) were severely inhibited in salinity-exposed wheat plants, compared to controlled conditions.

Decreased growth under saline conditions is directly related to impaired photosynthetic activity and stunted vegetative growth. Besides, there is a link between decreased growth and a reduction in photosynthesis and the uptake of essential ions.[13,51] Salinity-induced impairments in growth features have been reported in several studies,[5,7,30,44,52] which supports our findings. However, the foliar application of SA and TP alleviated the adverse effects of salinity by improving these growth

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characteristics. This indicates that SA and TP stimulate a number of signaling factors involved in the growth stimulation of wheat under salinity stress. Photosynthesis is also associated with enhanced levels of tocopherol, which is linked with improved growth.[26,52,53] There has also been evidence that SA promotes the growth and productivity of plants under abiotic stress, including salinity,[16,26] which supports our findings. Moreover, another study reported that SA application increases biomass in wheat under salinity stress, which has been mainly attributed to enhanced antioxidant mechanisms and reduced MDA levels, which is consistent with our findings.[24] Moreover SA application has also been reported to control stomatal opening under stress, leading to a decrease in transpiration and water loss, as well as the maintenance of turgor, and controlling the growth and productivity of plants under stress conditions.[25,54] Furthermore, wheat seedlings treated with exogenous Toc showed better growth parameters than those treated without the substance. Previous studies reported that TP can improve tolerance of plants subjected to stress conditions including salinity, which corroborates our findings.[55,56] SA and TP induced improvements in nutrient uptake, water status, photosynthetic pigments, and other physiological features supporting better growth of plants grown in saline environments, confirming what we observed in our experiments.

4.2. Exogenous Application of SA and TP Improved Physio-Biochemical Attributes under Salinity Stress. In the present study, salinity stress significantly impaired photosynthetic pigments (chlorophyll a, b, carotenoid, chlorophyll a/b ratio, and total chlorophyll), compared to controlled conditions, which is in agreement with previous studies.[13,57,58] A high degree of salinity decreased photosynthetic pigment production, and this pernicious effect can be attributed to the increases in chlorophyllase activity, the decreased rate of photosynthetic activity, as well as the reduction of CO_2 assimilation and movement of stomata.[59] As a result of salt stress, cells produce more oxygen radicals, which leads to oxidation, resulting in pigment degradation.[8] Salinity stress has the potential to reduce the plant's overall photosynthetic rate at any stage in the process of photosynthesis. Exogenous applications of SA and TP significantly altered the level of photosynthetic pigments under salinity stress compared to that of unfertilized plants. It has been shown that SA enhances the growth and photosynthesis of cotton plants (*Gossypium hirsutum L.*) under salt stress, thus helping them to withstand stress.[60] Similarly, exogenous application of SA enhances growth performance and photosynthetic efficiency while reducing oxidative stress in red mung bean (*Vigna angulariz*) under salinity.[61]

Through the decrease of ROS and the enhancement of antioxidant systems, SA minimizes the harmful effects of salinity on chlorophyll. This improved plant growth by stimulating cell division and elongation and hindering chlorophyll oxidase enzymes, thereby preventing chlorophyll degradation and improving photosynthesis.[62] Moreover, TP protects the chloroplast structure from salinity-induced photoinhibition.[63] α-Tocopherol is also a powerful antioxidant that protects chlorophyll from oxidative bursts induced by salinity.[30,55] Exogenous SA and TP increased the effectiveness of photosynthetic pigments, which had previously been supported by other findings as well.[64] TP is thought to play an important role in preserving membrane lipids and protecting PSII from photoinactivation during the xanthophyll cycle of chloroplasts, thereby preserving their chlorophyll content.[65]

A variety of osmolytes are stored by plants to respond to oxidative stressors, primarily proline and sugar.[7,66,67] An increase in proline content under salt stress has been associated with salinity stress tolerance in many plants due to its osmoprotective properties. Several compounds are used as stress indicators, including Pro, MDA, and H_2O_2.[68] During salinity stress, the proline levels in Pirsabak-15 decreased but increased in the Shankar cultivar, indicating the persistence of salinity stress tolerance in Shankar cultivars.

Our findings are consistent with previous studies on soybean,[58] mung bean,[14] and Chufa.[20] In both cultivars, SA and TP alone had no significant effect on proline content, compared to their untreated counterparts; however, combined application of SA and TP significantly reduced proline levels. Shankar cultivars treated with salinity alone and with individual application of SA and TP may have benefited from the accumulation of proline to protect their photosynthetic apparatus and to eliminate excess ROS to stabilize their membranes, proteins, and enzymes.[69,70] There have been similar findings reported for soybeans[71] and fava beans,[53] which support the findings of this study. Furthermore, both cultivars were found to have increased endogenous TP concentrations following salinity stress. The individual application of SA and TP did not have a significant effect on TP in the Pirsabak-15 cultivar but did increase it in the Shankar cultivar.

It has been found that the phenol content of Pirsabak-15 decreased, but that of Shankar increased under salinity. Both cultivars had increased flavonoids when exposed to salinity, as compared with the control. Phenolic compounds are not only the most abundant secondary metabolites but also excellent antioxidants capable of scavenging excess ROS generated by stress. They have been shown to play an important role in protecting plants against both biotic and abiotic stresses.[72,73] Flavonoids, which are phenolic compounds, are also reported by Tohidi et al.[74] to possess antioxidant properties. In plants, flavonoids act as antioxidants and provide protection against a wide range of environmental stresses. Under stress conditions, flavonoids assist in counterbalancing ROS production and repairing damage caused by them. As powerful antioxidants, flavonoids enable plants to cope with oxidative stresses by quenching free radicals, thus preventing oxidative stress on the cells. A flavonoids-mediated suppression of ROS generation occurs through four mechanisms, including (i) inhibiting singlet oxygen, (ii) recycling of other antioxidants, (iii) chelating transition metals, and (iv) inhibiting ROS-producing enzymes (cyclooxygenase, lipoxygenase, monooxygenase, and xanthine oxidase).[43,75,76] However, in our study, we observed a decrease in the phenol and flavonoid content after both SA and TP applications compared to their untreated peers, which indicated that neither SA nor TP had a positive impact on phenol and flavonoids.

Moreover, endogenous TP concentration increased in both cultivars following salinity stress. The biosynthesis and accumulation of tocopherol are considered to be one of the most important responses of plants to oxidative stress caused by a range of abiotic stresses. They are capable of scavenging ROS and lipid peroxidation as well as preventing membrane lipid peroxidation.[77,78] Tocopherol protects PSII from the effects of oxygen singlets.[79,80] Accordingly, we suggest that tocopherols may have important mechanisms of action such as a non-enzymatic antioxidant in cultivars under salinity stress and individual applications of SA and TP to cope with stress.

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Furthermore, the protein content of Pirsabak-15 remains unchanged, whereas that of Shankar subjected to salinity stress alone and in combination with salinity stress and TP is significantly reduced. According to other studies, proteins function as osmotins and contribute to the tolerance of salt stress.[35] In addition, plants are capable of upregulating small molecules of proteins, which can be used to store and mobilize N during a period of stress relief.[81] It has been suggested that these proteins may also play a role in osmotic adjustment.[82] Previous studies by Sh[63] and Orabi and Abdelhamid[53] indicated that spraying TP on flax (*Linum usitatissimum L.*) and fava bean (*Vicia faba*) significantly increased their protein content when exposed to salt stress. Compared to their individual applications, SA and TP combined had more negative effects on phenol, flavonoids, and proteins, indicating that combined SA and TP had no positive effects. As a result of salt-induced osmotic stress, ion toxicity, and nutrient imbalance, several biomolecules are destroyed, and metabolic, physiological, and biochemical processes in cellular components are disrupted, which is responsible for the overproduction of ROS as well as severe damage to the cellular components. This results from damage to the cell membranes, mitochondria, chloroplasts, and peroxisomes.[83] Increased concentrations of MDA and H_2O_2 indicate salinity-induced oxidative stress damages in plants. We found that salinity stress increased MDA and H_2O_2 concentrations significantly compared with controls. The same findings were reported for mung bean,[14] tiger nuts,[20] maize,[84] barley,[85] as well as canola.[86] In contrast, the application of SA, TP, and SA + TP resulted in a greater decrease in H_2O_2, indicating their protective benefits against salt stress. The findings of Kostopoulou et al.[87] confirm our findings that TP application can lower H_2O_2 levels in citrus plants exposed to salt stress. Furthermore, Farouk[88] showed that plants treated with TP (100 mg/L) showed decreased levels of H_2O_2 when exposed to salt stress.

Under stressful conditions, such as salt stress, plants have an antioxidant defense system that is specifically designed to scavenge ROS. In this study, salinity and the application of SA and TP affected the antioxidant enzymes in a different manner. For instance, salinity stress increased peroxidase (POD) and polyphenol oxidase (PPO) activities in Pirsabak-15, glutathione reductase (GR) and PPO activities in Shankar, while ascorbate peroxidase (APOX) activities were increased in both cultivars. Individual applications of SA and TP in Pirsabak-15 increased the levels of POD, PPO, and SOD under salinity stress. Overall, the exogenous application had no positive effect on the antioxidant enzymes. In both cultivars, combined applications of SA and TP decreased POD and GR, PPO and APX in Shankar and SOD in Pirsabak-15, whereas increased PPO in Pirsabak-15 and SOD in Shankar cultivar (Figure 4), indicating their positive effects on PPO and SOD for reducing the ROS-induced damages under salinity stress. A number of plant species have been shown to benefit from the addition of exogenous SA and TP by decreasing ROS generation and oxidative damage. As an example, *B. juncea*[89] and rapeseed[83] showed decreased H_2O_2 and MDA levels when supplied with exogenous TP applications, confirming our findings. TP-induced enhancement of antioxidant mechanisms has also been reported in salt-stressed sunflower plants.[55,90] Exogenous plant growth regulators may have prevented cell membrane damage caused by overproduction of ROS by altering the enzymatic and nonenzymatic machinery.

5. CONCLUSIONS

The results of our study confirm the ameliorative effects of exogenous applications of AS and TP on wheat seedlings under salt stress. For instance, under salinity stress, we observed a reduction in the growth and physio-biochemical characteristics of our selected wheat cultivars. However, exogenous SA and TP mitigated the salinity-induced impairments in both cultivars by reducing the oxidative stress (H_2O_2 and MDA concentrations), enhancing photosynthetic pigments and antioxidant enzyme activity, nonenzymatic antioxidants and osmolytes, and growth attributes, compared to their untreated peers. Hence, we suggest that SA and GABA application can assist in reducing salinity damage to seedlings of our selected wheat cultivars in salinity-affected regions worldwide, including Pakistan. Future molecular studies with different levels of SA and TP application could provide a deeper understanding of their role in modulating salinity tolerance mechanisms in wheat so that they can determine what concentration is beneficial and how it can stabilize their growth in salinity-prone areas.

AUTHOR INFORMATION

Corresponding Authors

Abd Ullah − *Xinjiang Key Laboratory of Desert Plant Root Ecology and Vegetation Restoration, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China; Cele National Station of Observation and Research for Desert-Grassland Ecosystems, Cele 848300, China*; Email: Abdullahbotany123@gmail.com

Sami Ullah − *Department of Botany, University of Peshawar, Peshawar 25120, Pakistan*; Email: samibotany@uop.edu.pk

Iftikhar Ali − *Center for Plant Sciences and Biodiversity, University of Swat, Charbagh 19120, Pakistan; Department of Genetics and Development, Columbia University Irving Medical Center, New York, New York 10032, United States; School of Life Sciences & Center of Novel Biomaterials, The Chinese University of Hong Kong, Hong Kong, SAR, China*; orcid.org/0000-0003-2319-2175; Email: iftikharali@uswat.edu.pk

Authors

Saleha Saeed − *Department of Botany, University of Peshawar, Peshawar 25120, Pakistan*

Mohamed S Elshikh − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Javaria Noor − *Department of Botany, Islamia College Peshawar, Peshawar, KP 19650, Pakistan*

Sayed M. Eldin − *Center of Research, Faculty of Engineering, Future University in Egypt, New Cairo 18939, Egypt*

Fanjiang Zeng − *Xinjiang Key Laboratory of Desert Plant Root Ecology and Vegetation Restoration, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China; Cele National Station of Observation and Research for Desert-Grassland Ecosystems, Cele 848300, China*

Fazal Amin − *Department of Botany, University of Peshawar, Peshawar 25120, Pakistan*

Mohammad Ajmal Ali − *Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c02166

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Author Contributions

S.S. and A.U. contributed equally to this work and share the first authorship. Conceptualization: S.U. and A.U.; formal analysis: F.A.; investigation: S.S.; methodology: S.U. and A.U.; software: F.A., J.N., F.Z., S.M.E., and M.A.A.; supervision: S.U., I.A., and A.U.; writing—original draft: S.S., S.M.E., M.A.A., M.S.E., and I.A.; writing—review and editing: F.A., A.U., J.N., F.Z., M.S.E., and I.A.

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