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Dissecting the osmotic and oxidative stress responses in salt-tolerant and salt-sensitive wheat genotypes under saline conditions

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Abstract: Salinity represents a significant abiotic stress that markedly influences plant growth through osmotic stress induction. Plants commonly undergo osmotic adaptation when subjected to prolonged periods of saline stress. The current experiments were conducted on five wheat (*Triticum aestivum* L.) genotypes with contrasting salt tolerance capacities – Mirbashir 128, Gobustan, Gyzyl bughda, Fatima, and Zirva 80 under salinity stress caused by 150 mmol NaCl. The relative water content and osmotic potential were found to decrease significantly in salinity-sensitive genotypes (Fatima and Zirva 80) compared to salinity-tolerant ones (Mirbashir 128, Gobustan, and Gyzyl bughda) when treated with 150 mmol NaCl. Salinity also caused the accumulation of soluble sugars and proline, the amounts of which were observed to be higher in salinity-tolerant genotypes than sensitive ones, while lipid peroxidation was higher in salinity-sensitive genotypes. In salinity-tolerant genotypes, 150 mmol NaCl caused increased antioxidant enzyme activities and accumulation of flavonoids, including anthocyanins, confirming the rapid development of the stress reactions in these plants. Differences in the osmoregulation indicators and antioxidant responses between salinity-tolerant and sensitive plants are assumed to be related to their salinity-tolerance traits. This investigation provides pivotal foundational insights for enhancing the salt tolerance of wheat genotypes, thereby potentially enhancing both yield and quality in diverse wheat cultivars thriving in saline environments.

Keywords: ascorbate; malondialdehyde; osmolytes; reactive oxygen species; water transport

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The mechanism for developing osmotic stress tolerance in plants is triggered by salinity stress. Osmotic stress, in turn, causes numerous physicochemical changes in plants (decrease in cell turgor, narrowing of the plasma membrane, and physical changes in the cell wall (Zhao et al. 2021). In plants, osmotic signalling pathways contribute to reducing the stress effects by regulating processes ranging from gene expression to activating enzymes, which carry out the biosynthesis of osmolytes and water transport (Yang and Guo 2017). Osmotic adjustment (OA) is one of the main mechanisms in the plant cell that ensures the maintenance of cell turgor by adapting plants to conditions with low water potential. OA occurs by limiting the absorption of toxic ions such as Na^+ or reducing their concentration in the cytoplasm by accumulating them in the vacuole, as well as by synthesising osmolytes (proline, soluble sugars, polyols, and glycine betaine) (Zhao et al. 2021). Due to the synthesis of osmolytes in the cell, the osmotic potential indicator decreases (Tasmina et al. 2017). The synthesis of proline and sugars under stress conditions can be assessed as their participation in osmotic adjustment. Thus, sugars and proline ensure the stability of the structure and functions of macromolecules by regulating the osmotic balance between the cytosol and the vacuole in the cell (Zulfiqar et al. 2020). Proline has the properties of enzyme protection, scavenging free radicals, and stabilisation of intracellular pH (Spormann et al. 2023).

Ion stress and osmotic stress induced by salinity result in a metabolism imbalance and the toxic accumulation of reactive oxygen species (ROS) that causes oxidative damage in plants (Hasanuzzaman et al. 2021). ROS are formed due to salinity stress in many plant organelles, including peroxisomes, mitochondria, chloroplast and the apoplast. Plant cells respond using effective regulatory mechanisms to scavenge them upon the accumulation of ROS. Thus, a series of downstream adaptive responses are activated (Seleiman et al. 2020). ROS operate as essential signalling molecules at low levels. ROS production and scavenging balance are regulated by control mechanisms (Raja et al. 2017). Under salinity stress, oxidative stress is regulated by some proteins (Hasan et al. 2023a). They activate ROS scavengers or mediate the gene expression of ROS-responsive genes (Dvorak et al. 2021). Some authors say salinity stress stimulates ROS-scavenging enzymes and antioxidants (Laus et al. 2022). Thus, ascorbate peroxidase and catalase are known to be activated by salinity stress, improving plant tolerance to oxidative stresses (Hasan et al. 2023b).

Catalase is a heme-containing enzyme that converts hydrogen peroxide directly into water and O_2. APX reduces hydrogen peroxide in water by using ascorbate (AsA) as a donor. Catalase is mostly found in peroxisomes (it scavenges hydrogen peroxide produced during photorespiration and β-oxidation of fatty acids), as well as mitochondria and cytosol (Dumanovic et al. 2021). Because ascorbate peroxidase is more abundant in the cell than catalase and has a higher affinity for hydrogen peroxide, it neutralises more hydrogen peroxide. Besides, APX is also present in mitochondria (Laus et al. 2022).

In response to stress-induced oxidative damage, secondary metabolic pathways are activated in plants, thereby initiating the synthesis of secondary metabolites (carotenoids, tocopherols, tocotrienols, and phenolic compounds) (Hamed et al. 2014). Secondary metabolites are important in plants' adaptation to environmental changes (Shoeva et al. 2017). Phenolic compounds, as a structural component of the cell wall, participate in the regulation of growth and development processes, and in plant responses to all types of abiotic stresses (Mnich et al. 2020).

Previously, we studied the response to stress in five wheat genotypes (*Triticum aestivum* L.) with contrasting drought tolerance based on the photochemical reactions of photosynthesis and some physiological and biochemical indicators (Ibrahimova et al. 2021) and classified these genotypes according to their drought tolerance. In this paper, we present the results of a study on the osmotic regulation mechanisms and antioxidant responses of different wheat genotypes under salinity stress. The primary objective is to understand the impact of salinity on both tolerant and susceptible wheat cultivars and to elucidate the underlying mechanisms associated with salinity tolerance in these genotypes. This focus aims to provide deeper insights into how different genotypes respond to salinity stress, contributing to the development of strategies for improving wheat resilience under saline conditions.

MATERIAL AND METHODS

Experimental setup. Mirbashir 128 (MIR; tolerant), Gobustan (GOB; tolerant), Gyzyl bughda (GYZ; tolerant), Fatima (FAT; sensitive), and Zirva 80 (ZIR; sensitive) genotypes of winter wheat (*Triticum aestivum* L.) differing in drought tolerance provided by GeneBank of the Azerbaijan Research Institute of Crop Husbandry have been used in the research.

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A 10% SAVO solution (Biochemie, 124 Bohumin, Czech Republic) containing 5% NaClO was used for the seed sterilisation. After 15 min the seeds were washed three times with distilled water. The seeds germinated on wet filter paper (Whatman R3) in Petri dishes under a 14/10 h (day/night) regime with 250 µmol/m^2/s light intensity at 24/18 °C. The 7-day-old seedlings with similar sizes were transferred to 7 L plastic trays with a pre-aerated Reid York nutrient solution. This nutrient solution was used in our experiment, with a pH range of 5.5–6.5. Its composition per litre of distilled water includes macronutrients such as 210 mg of NH_4NO_3, 540 mg of KNO_3, 240 mg of KH_2PO_4, 300 mg of $Ca(NO_3)_2$, and 100 mg of $MgSO_4$. It also contains micronutrients, including 12 mg of Fe-EDTA, 0.2 mg of $MnSO_4$, 0.05 mg of $ZnSO_4$, 0.01 mg of $CuSO_4$, 0.02 mg of H_3BO_3, and 0.01 mg of Na_2MoO_4. Aeration was performed regularly six times a day for 2 h. The nutrient solution was changed every 3 days. Plants were grown at a temperature of 24/18 °C, relative humidity of 55–60%, and light intensity of 150 µmol/m^2/s. In our previous study (Rastogi et al. 2020), from 0 to 250 mmol NaCl concentrations were tested, and 150 mmol NaCl was found to impact the plant significantly; therefore, this study used 150 mmol NaCl concentration. Fourteen-day treatment with salt started after the emergence of the 3rd leaf (BBCH-13), and 150 mmol NaCl was added only once. At the same time, clean water was used daily, which contributed to maintaining water balance after evaporative loss in plant seedlings. Finally, all the plants were harvested at 28 days.

Determination of leaf relative water content, malondialdehyde and soluble sugar content. The Gravimetric method determined leaves' relative water content (RWC) (Barr and Weatherley 1962). After the determination of the fresh weight (F_W) of leaves, they were placed in containers filled with distilled water and stored in the dark at 4 °C for 24 h. Then, the leaves' water-saturated weight or turgor weight (TW) was determined. To determine the dry weight of the leaves, they were placed in a thermostat (80 °C) for 48 h. After the leaves were completely dried, their dry weight (D_W) was determined. RWC of leaves was calculated by the following formula:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

Malondialdehyde (MDA) was determined spectrophotometrically by the reaction of thiobarbituric acid (TBT) (Kumar and Knowles 1993). Precipitation was performed (at 1 000 × *g* for 10 min) after crushing 0.5 g of leaves in 5% trichloroacetic acid (TCA). Then, 4 mL of a mixture of 0.5% TBT and 20% TCA was added to the supernatant and heated in a water bath for 30 min. The mixture was cooled using an ice bath and precipitated at 1 000 × *g* for 15 min. A Hitachi 557 spectrophotometer (Hitachi High-Tech Corporation, Tokyo, Japan) was used to measure optical density at 532 and 600 nm. The anthrone-sulphuric acid method (Fales 1951) was applied to determine sugars.

Determination of proline content and osmotic potential. The Bates method (Bates et al. 1973) was used to determine proline content. The psychrometric method measured the osmotic potential (YS) of pre-frozen in liquid nitrogen and thawed leaves. A microvoltmeter Wescor HR-33 with measuring chamber C-52 (Wescor Inc., 370 West 1700 South Logan, USA) was used in the experiments.

Enzyme extraction and activity determination. Before adding the homogenisation buffer, 0.05 mol/L Na_2HPO_4/NaH_2PO_4 (pH 7.0), 1.0 g of the leaves were ground in liquid nitrogen. Precipitation was carried out at 16 000 *g* for 20 min at 4 °C. The activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was determined in the obtained supernatant using the spectrophotometric method (290 nm). The applied method is based on decreased optical density due to the oxidation of ascorbic acid in the presence of H_2O_2 (Nakano and Asada 1981). A decrease in the optical density at 240 nm due to the decomposition of H_2O_2 was measured to determine catalase activity (EC 1.11.1.6) (Allen et al. 1986).

Quantification of anthocyanins and flavonoids. In experiments, anthocyanin and flavonoid accumulation was studied using a multifunctional portable, non-invasive chlorophyll fluorescence technique – Multiplex-3® sensor (Force-A, Paris, France). Multiplex-3 is a portable, multi-parameter sensor.

The dynamics of the accumulation of anthocyanins and flavonoids were monitored for 10 days after 3-day exposure of plants to stress. Measurement of flavonoids was carried out by logarithmic calculation of the observed fluorescence ratio in the far-red range after illumination with a wavelength of 375 nm (UV light) and 635 nm (red light) based on the Beer-Lambert law. The determination of anthocyanins (red-coloured anthocyanins) was performed based on the calculation of the logarithm of the fluorescence ratio obtained with excitation under 516 nm (green light) and 635 nm (red light) light (Mbarki et al. 2018).

The anthocyanin (ANTH) index, which estimates green-light absorbing components like anthocyanins

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and flavonoids, was determined by taking the logarithm of the ratio between red-light induced fluorescence (FRFR) and green-light induced fluorescence (FRFG), also adjusted with a correction factor (kG):

$$\text{Anthocyanin (ANTH) index} = \log[\text{FRFR}/(\text{kG} \times \text{FRFG})].$$

Similarly, the flavonoid (FLAV) index was calculated by taking the logarithm of the ratio between far-red fluorescence induced by red light (FRFR) and far-red fluorescence induced by UV light (FRFUV), adjusted with a correction factor (kUV) to prevent negative values:

$$\text{Flavonoid (FLAV) index} = \log[\text{FRFR}/(\text{kUV} \times \text{FRFUV})]$$

These adjustments were made to ensure accurate fluorescence measurements (Mbarki et al. 2018).

Statistical analysis. All the obtained data were analysed using a one-way analysis of variance (ANOVA) with MINITAB 17.0 software (State College, USA), presenting the treatment mean ± standard error for three samples (n = 3). The Fisher's *LSD* (least significant difference) test indicates that bars marked with different letters show significant differences at the $P \leq 0.05$ level. For each treatment, three biological replicates were conducted, with each replicate involving a minimum of three plants to evaluate various parameters under identical experimental conditions.

RESULTS

Morphological indicators (length and weight of roots and shoots) of the studied wheat genotypes changed under salinity, and these changes were different for tolerant and sensitive genotypes. The phenotypic changes in plants treated with 150 mmol NaCl have been presented in Table 1. The root length displayed a slight decrease of about 2.17, 10.4 and 6.25% in tolerant Mirbashir 128, Gobustan, and Gyzyl bughda, however, in sensitive Zirva 80 and Fatima, the observed decrease was about 52.6% and 60% compared to the control plants. The stem length in tolerant genotypes, Mirbashir 128, Gobustan, and Gyzyl bughda, decreased by about 24, 16.6 and 32% respectively, while in the sensitive Fatima and Zirva-80 genotypes, the observed decrease was 46% and 56%, respectively (Table 1).

The effect of salt stress on fresh and dry weights of seedlings (roots and shoots) was clearer. Thus, in the tolerant genotypes, Mirbashir 128, Gobustan, and Gyzyl bughda the shoot fresh weight decreased in 41.4, 39.1 and 48%, respectively, and root fresh weight decreased by 44.2, 44.1 and 51.2%, respectively. Moreover, a decrease in shoot and root dry weight was observed in tolerant genotypes upon treatment with 150 mmol NaCl. The intensity of decrease in case of shoot dry was about 40.5, 33.6 and 53.8% in Mirbashir 128, Gobustan, and Gyzyl bughda, while in case of root dry weight was the decrease was about 31.2, 20 and 25%, respectively. However, in the salt-sensitive genotypes Fatima and Zirva-80, the fresh weight decreased by 66.5% and 61%, and the dry weight by 66.2% and 59.5%, respectively, compared to the control plants.150 mmol NaCl caused a decrease in osmotic potential in the leaves and roots of the studied genotypes (Figure 2A, B). As seen in the figure, there was a decrease in osmotic potential

Table 1. Effect of 150 mmol NaCl on morphological parameters of *Triticum aestivum* L. genotypes

Genotype	Treatment	Shoot length	Root length	Shoot fresh biomass	Shoot dry biomass	Root fresh weight	Root dry weight
		(cm)		(mg/plant)			
Mirbashir 128	0 mmol NaCl	25 ± 4^a	9.2 ± 0.7^a	25.45 ± 2.5^a	1.85 ± 0.18^a	2.8 ± 0.3^a	0.16 ± 0.02^a
	150 mmol NaCl	19 ± 4^b	9 ± 0.6^a	14.9 ± 1.1^b	1.1 ± 0.08^b	1.56 ± 0.14^b	0.11 ± 0.01^b
Qobustan	0 mmol NaCl	24 ± 3^a	9.6 ± 1.1^a	24.8 ± 1.2^a	1.84 ± 0.08^a	2.72 ± 0.18^a	0.15 ± 0.01^a
	150 mmol NaCl	20 ± 3^b	8.6 ± 0.9^b	15.1 ± 0.9^b	1.22 ± 0.05^b	1.52 ± 0.12^b	0.12 ± 0.01^b
Qyzyl bughda	0 mmol NaCl	25 ± 3^a	8 ± 0.8^a	26.58 ± 1.4^a	1.95 ± 0.1^a	2.83 ± 0.29^a	0.16 ± 0.02^a
	150 mmol NaCl	17 ± 4^b	7.5 ± 0.6^a	13.8 ± 1.2^b	0.9 ± 0.07^b	1.38 ± 0.14^b	0.12 ± 0.01^b
Fatima	0 mmol NaCl	24 ± 3^a	9.5 ± 1.1^a	24.28 ± 1.7^a	1.8 ± 0.08^a	2.75 ± 0.15^a	0.15 ± 0.008^a
	150 mmol NaCl	13 ± 2^b	4.5 ± 1.2^b	8.55 ± 0.6^b	0.61 ± 0.05^b	0.92 ± 0.11^b	0.07 ± 0.01^b
Zirva-80	0 mmol NaCl	25 ± 2^a	8.5 ± 1.2^a	23.55 ± 1.4^a	1.75 ± 0.1^a	2.68 ± 0.33^a	0.13 ± 0.01^a
	150 mmol NaCl	11 ± 2^b	3.4 ± 0.7^b	9.28 ± 0.7^b	0.71 ± 0.06^b	0.97 ± 0.24^b	0.08 ± 0.01^b

Bars labelled with different letters indicate significant differences at the $P \leq 0.05$ level

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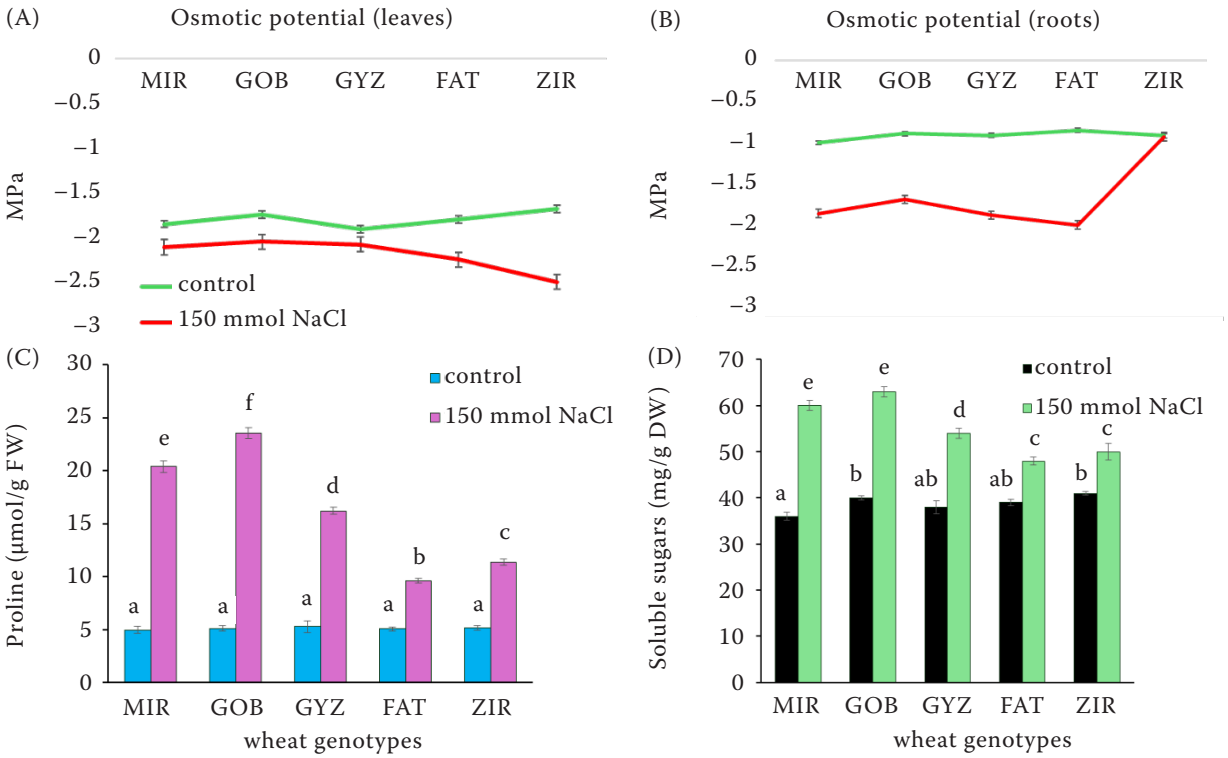

Figure 1. Effect of salt on osmotic potential (A, B), proline (C), and soluble sugar (D) content in wheat genotypes: Mirbashir 128 (MIR), Gobustan (GOB), Gyzyl bughda (GYZ), Fatima (FAT), and Zirva-80 (ZIR). FW – fresh weight; DW – dry weight

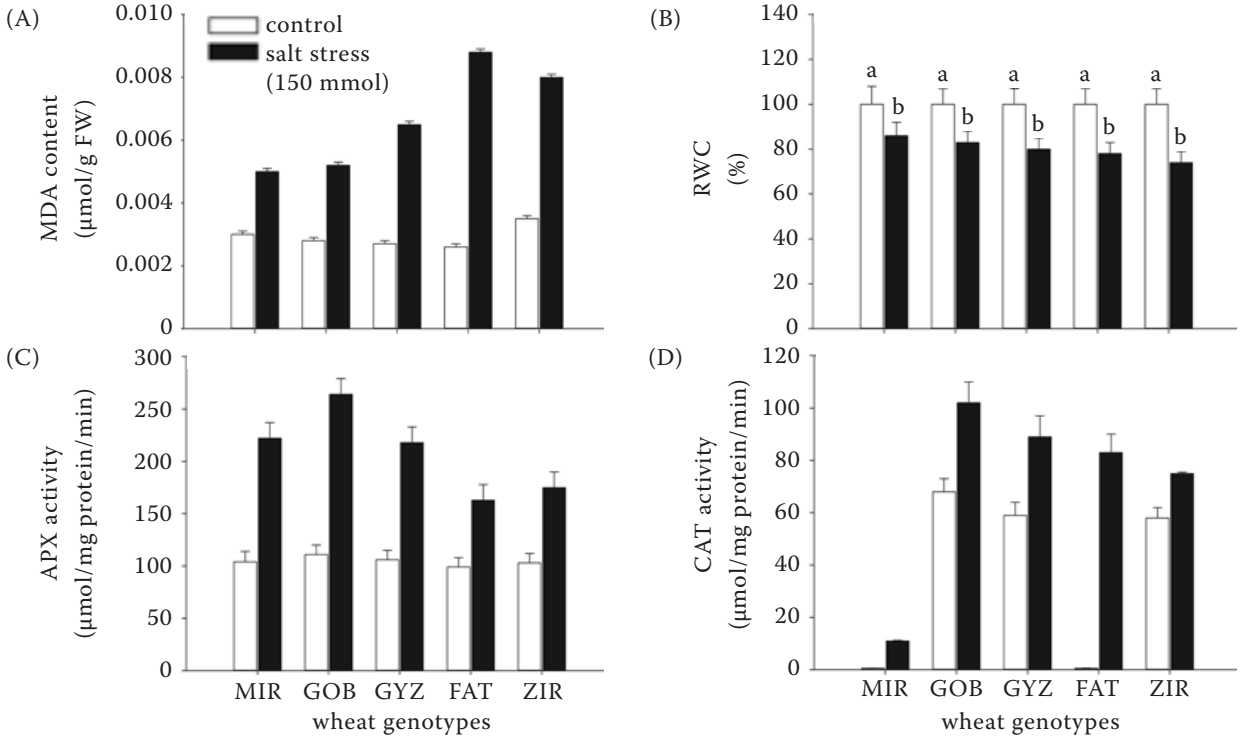

Figure 2. Effect of salt on (A) malondialdehyde (MDA); (B) relative water content (RWC); (C) ascorbate peroxidase (APX), and (D) catalase (CAT) in wheat genotypes: Mirbashir 128 (MIR), Gobustan (GOB), Gyzyl bughda (GYZ), Fatima (FAT), and Zirva 80 (ZIR)

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in roots more than in leaves. In tolerant genotypes Mirbashir 128, Gobustan, and Gyzyl bughda, leaf osmotic potential decresed by 73.5, 77.7 and 90.5% while in case of susptible geneotypes Fatima and Zirva-80 a susbstantial decrease of 141.1% and 251.7% was observed. Moreover, in the case of root osmotic potential, Fatima and Zirva-80 showed a significant decrease of about 431.1% and 386.3%, respectively. A moderate decrease of about 227.4% occurred in the Gyzyl bughda genotype. In our experiments, 150 mmol NaCl caused a sharp increase in the content of proline and soluble sugars in the studied genotypes (Figure 2C, D). The tolerant genotypes Mirbashir 128 and Gobustan displayed an increase of about 310% and 358.4% in proline content, respectively. However, Gyzyl bughda showed a moderate accumulation of about 206.8% increase in proline content. Less proline accumulation was observed in Fatima and Zirva-80, corresponding to about 89.9% and 119.9%, respectively. Again, the tolerant genotypes displayed a higher accumulation in the case of soluble sugars. Mirbashir 128, Gobustan, and Gyzyl bughda genotypes displayed 66.6, 57.5 and 42.10% increase in soluble sugars while 23% and 21.9% increase in soluble sugars were recorded in genotypes Fatima and Zirva-80, respectively. Additionally, a significant positive correlation was oberved between proline content flavonoids and relative water content at ($P < 0.001$), while soluble sugars content showed a significant positive correlation at ($P < 0.05$). A positive but non-significant correlation ($P \geq 0.05$) of proline with APX, CAT, and MDA was also observed during the study (Figure 3B).

During the current investigation, we observed an increase in the MDA content in all studied genotypes exposed to the NaCl effect (Figure 2A). In the Fatima and Zirva-80 genotypes, MDA content increased by about 238.4% and 128.5%, respectively, compared to that in control, while in the cultivars Mirbashir 128, Gobustan, MDA increased by about 66.6, 85.7 and 140%, respectively, which was not more than 1.6-fold in comparison to their respective control plants. MDA content displayed a strong positive correlation at $P > 0.01$ with soluble sugar and relative water content. However, MDA also positively correlated with CAT, MDA, proline and APX at a significant level of $P < 0.05$ (Figure 3C).

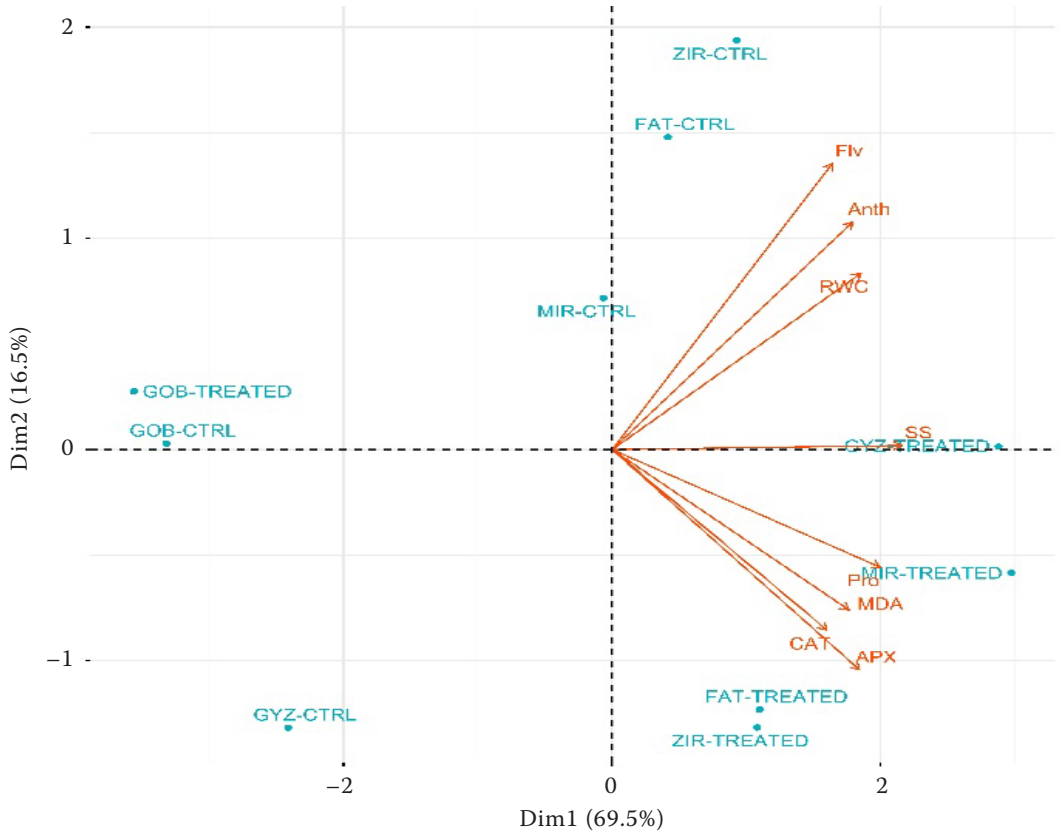

Figure 3. Principal component analysis (PCA) plot of the genotypes and the parameters. Flv – flavonoid; Anth – anthocyanin; RWC – relative water content; SS – soluble sugar; Pro – proline; MDA – malondialdehyde; CAT – catalase; APX – ascorbate peroxidase

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150 mmol concentration of NaCl caused a decrease in water content in the tissues of the studied genotypes. As seen in Figure 2B, among the genotypes, Mirbashir 128 had the highest (86.0%), and Zirva-80 had the lowest (74.0%) relative water content. Intermediate values (83, 80, and 78%) of RWC were found for other genotypes, Gobustan, Gyzyl bughda, and Fatima, respectively. Correlation analysis revealed a strong correlation between RWC and soluble sugars at $P < 0.01$. Moreover, a positive but non-significant correlation of MDA was observed with parameters like flavonoids, anthocyanins, MDA, APX and CAT.

The APX and CAT activities increased in leaves of the genotypes exposed to 150 mmol NaCl (Figure 2C, D). The tolerant genotypes Mirbashir 128, Gobustan, and Gyzyl bughda displayed an increase of about 113.4, 137.8 and 105.6% respectively, in the APX aktivity, while the genotypes Fatima and Zirva-80 displayed a slight increase of about 64.6% and 69.9% in the APX activity in comparison to that of their respective control plants. Similarly, geneoptypes Mirbashir 128, Gobustan, and Gyzyl bughda displayed higher activities of about 74.6, 50 and 50.8% in CAT activity, while in case of Fatima and Zirva-80 genotypes this value increased by 36% and 29.3%, respectively. From the correlation plot, we observed a strong correlation of APX with proline (0.93 at $P < 0.001$). CAT and soluble sugar also displayed a significant positive correlation (0.78 and 0.77 at $P < 0.01$). MDA showed a positive correlation with APX at $P < 0.05$ level. However, flavonoids, anthocyanins and RWC displayed a positive but non-significant correlation with APX. Meanwhile, CAT displayed a strong correlation with MDA and RWC at $P < 0.01$ level, while flavonoid, anthocyanin and proline displayed a positive but non-significant correlation with CAT.

Anthocyanin content in Mirbashir 128, Gobustan, and Gyzyl bughda genotypes increased in response to a 150 mmol concentration of NaCl (Table 1). The increase in anthocyanin content observed in these genotypes was about 114.2, 22.2 and 59.2%, respectively. However, in genotypes Fatima and Zirva-80, a substantial decrease of about 43.7% and 58.5%, respectively, was observed in anthocyanin content. Moreover, higher accumulation of 33.3% and 32.3% of flavonoids occurred in Mirbashir 128 and 32.3% Gobustan genotypes, respectively, at 150 mmol concentration of NaCl. In the Gyzyl bughda genotype exposed to stress, flavonoids remained at the same level as in the control variant, while in the Fatima and Zirva-80 genotypes, a sharp decrease of about 75% and 82.9% in the amount of flavonoids was observed (Table 2). Both anthocyanins and flavonoids strongly correlated with proline and soluble sugar at $P < 0.001$. Moreover, these secondary metabolites displayed a positive but non-significant correlation with MDA and CAT.

PCA, heatmap and correlation analysis. Principal component analysis (PCA) carried out during the present study revealed that the first two PCA axes (Dim 1 and Dim 2) accounted for 86% of the overall variation in the treatments and other variables, with contributions of 69.5% and 16.5%, respectively (Figure 3).

Principal component analysis also revealed a close correlation among treatments MIR-TREATED, GYZ-TREATED, FAT-TREATED, and ZIR-TREATED.

Table 2. Changes in anthocyanin (log FERR/G-ANTH) and flavonoid (log FERR/UV-FLAV) content under salt stress (150 mmol)

Genotype	Treatment	Anthocyanin (log FERR/G-ANTH)	Flavonoid (log FERR/UV-FLAV)
Mirbashir 128	0 mmol NaCl	0.014 ± 0.003^{b}	0.27 ± 0.014^{b}
	150 mmol NaCl	0.030 ± 0.003^{a}	0.36 ± 0.012^{a}
Gobustan	0 mmol NaCl	0.036 ± 0.007^{b}	0.34 ± 0.018^{b}
	150 mmol NaCl	0.044 ± 0.005^{a}	0.45 ± 0.028^{a}
Gyzyl bughda	0 mmol NaCl	0.027 ± 0.001^{b}	0.39 ± 0.009^{a}
	150 mmol NaCl	0.043 ± 0.005^{a}	0.38 ± 0.022^{b}
Fatima	0 mmol NaCl	0.032 ± 0.002^{a}	0.32 ± 0.019^{a}
	150 mmol NaCl	0.018 ± 0.003^{b}	0.08 ± 0.008^{b}
Zirva-80	0 mmol NaCl	0.041 ± 0.002^{a}	0.41 ± 0.017^{a}
	150 mmol NaCl	0.017 ± 0.002^{b}	0.07 ± 0.005^{b}

Bars labelled with different letters indicate significant differences at the $P \leq 0.05$ level

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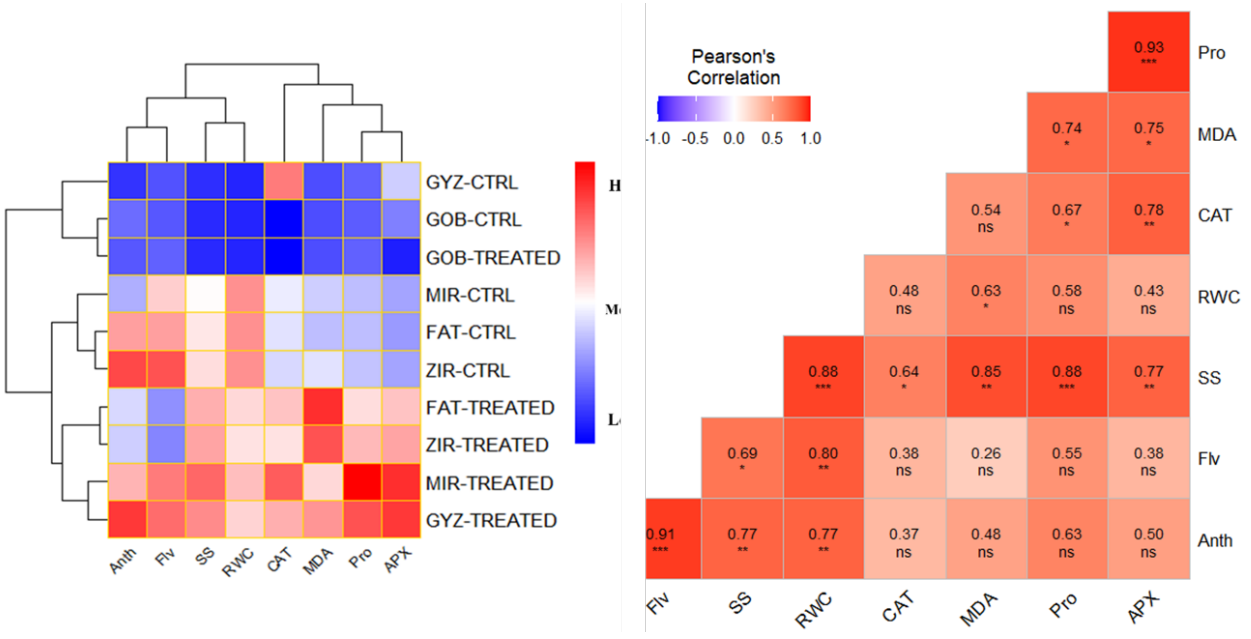
Within these treatments, parameters including CAT, APX, MDA, proline, and SS were more correlated. Conversely, other parameters, excluding a few such as RWC, anthocyanin content, and flavonoid content, were concentrated and pronounced in the treatments closely related to the second cluster.

The heatmap clustering analysis partitioned the treatments into three main distinct clusters based on the correlation observed among various antioxidant and physiological parameters (Figure 4A). The first cluster comprised treatments GYZ-CTRL, GOB-CTRL, and GOB-TREATED, while the second cluster included MIR-CTRL, FAT-CTRL, and ZTR-CTRL. The third cluster included FAT-TREATED, ZIR-TREATED, MIR-TREATED, and GYZ-TREATED. Within the first cluster, the treatments GOB-CTRL and GOB-TREATED exhibited stronger correlations compared to GYZ-CTRL. Notably, in GYZ-CTRL and MIR-TREATED, there was a significant increase in CAT activity compared to the other treatments. Similarly, within the second cluster, FAT-CTRL and ZIR-CTRL showed greater correlation compared to MIR-CTRL, where the anthocyanin content was lower than in the former two treatments. Proline displayed a significant positive correlation with APX, soluble sugar, CAT, and MDA. However, anthocyanin displayed a significant positive correlation with flavonoids, soluble sugar, and RWC. Soluble sugar was positively correlated with RWC, MDA, proline, and APX. No significant positive correlation was found between proline and anthocyanin, proline and flavonoid, and proline and RWC (Figure 4B).

DISCUSSION

Plants, being sessile, face various abiotic stresses that limit growth and productivity. Salt stress is particularly harmful, affecting plant survival. This study evaluated five *Triticum aestivum* L. genotypes under 150 mmol NaCl to assess changes in growth, osmotic adjustment, antioxidants, and water potential. Salt stress reduced growth and biomass in all genotypes, with tolerant ones showing less reduction. Fatima and Zirva-80 were the most affected. The growth decline is linked to ion toxicity, nutrient imbalance, reduced carbon fixation, and impaired photosynthesis (Pour-Aboughadareh et al. 2021). Accumulation of proline and sugars and in plants is considered to be



ns $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Figure 4. Diagram shows the relationship and concentration of various parameters within the treatments, highlighting how they cluster together based on similarities in wheat genotypes: Mirbashir 128 (MIR), Gobustan (GOB), Gyzyl bughda (GYZ), Fatima (FAT), and Zirva-80 (ZIR). (A) Cluster heatmap showing the division of different genotypes and parameters in different clusters, colour coding from red to blue represents the higher and lower values, and (B) correlation plot showing the correlation between different studied parameters. Flv – flavonoid; Anth – anthocyanin; RWC – relative water content; SS – soluble sugar; Pro – proline; MDA – malondialdehyde; CAT – catalase; APX – ascorbate peroxidase; ns – not significant

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a product of osmotic regulation and maintains the redox potential of the cell and protects cellular macromolecules from the effects of NaCl. In addition, the accumulation of sugars under stress conditions also allows plants to maintain their storage reserves (Radi et al. 2013). 150 mmol NaCl caused the accumulation of proline and soluble sugars in all studied cultivars. When we measured the changes in proline contents of the five wheat genotypes, we observed that the proline content of the Gobustan and Mirbashir 128 tolerant seedlings was higher than that of the other three studied genotypes considered as sensitive. The growth was higher in the Mirbashir 128 and Gobustan genotypes. In the Fatima and Zirva-80 genotypes, osmolytes were collected in a lower concentration, and the osmotic potential was more negative in these genotypes. Accumulation of Pro under salt stress has been correlated with stress tolerance in many plant species, and its concentration is generally higher in salt-tolerant than salt-sensitive plants (Ashraf and Foolad 2007). Proline has been widely reported to be a multifunctional amino acid that acts in different processes (Ingrisano et al. 2023). It stabilises cellular membranes, including the thylakoid membranes in chloroplasts, by interacting with lipid bilayers and preventing leakage and damage. This membrane stabilisation helps preserve the structural integrity and functionality of the photosynthetic apparatus, including the light-harvesting complexes and electron transport chain components. By accumulating osmolytes, the osmotic adjustment system assists plants in avoiding ion toxicity and maintaining water intake under both conditions. Compatible solutes like sugars and proline, glycerol, and glycine betaine participate in the osmoregulation process and ensure plant growth under stress conditions (Singh et al. 2020). Compatible solutes aid in osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilisation of enzymes and proteins, as noted by Ashraf and Foolad (2007) and Ibrahimova et al. (2021).

RWC is a critical indicator of a plant's water status. In this study, all genotypes exposed to 150 mmol NaCl showed reduced RWC, with 14–26% losses. However, salt-tolerant genotypes retained higher RWC than sensitive ones, consistent with prior findings (Hussein et al. 2017). Salt and drought stress also reduce water potential, impacting cell division, elongation, mesophyll and epidermal cell volume, leaf area, turgor potential, and assimilate synthesis (Nassar et al. 2020). Turgor is essential for physiological processes like stomatal regulation, photosynthesis, and leaf expansion, and its reduction under stress decreases RWC. Garg and Singla (2009) associated reduced RWC under salinity stress with root damage impairing water uptake. Water potential (Ψ) reflects the tissue's ability to hold water, determined by opposing components: turgor pressure (positive) and osmotic potential (negative). Our experiments showed that 150 mmol NaCl reduced osmotic potential, with leaf potential dropping from –3.21 MPa to –5.91 MPa and root potential from –2.15 MPa to –4.28 MPa. Similar declines were reported in wheat under NaCl stress, with leaf osmotic potential decreasing from –1.2 MPa to –2.5 MPa in tolerant cultivars (Sairam et al. 2002) and from –1.3 MPa to –2.4 MPa within seven days (Bouthour et al. 2015). The higher osmotic potential in roots than in leaves creates a gradient essential for water uptake from hypertonic solutions. Increased Na^+ ion concentration further lowers osmotic potential, enhancing water absorption and diluting toxic ions to prevent cell damage (Hmidi et al. 2018, Ibrahimova et al. 2021).

The increase in enzymatic activities was found to depend on the decrease in oxidative damage. A positive correlation was observed between higher tolerance levels to abiotic stresses and the expression of many antioxidant enzymes (Caverzan et al. 2016). Enzymes such as ascorbate peroxidase and catalase scavenge hydrogen peroxide by different mechanisms (Abdulmajeed et al. 2021, Alabdallah et al. 2021). Catalase and ascorbate peroxidase play crucial roles in detoxifying reactive oxygen species by converting H_2O_2 to water, with APX acting in the cytosol, mitochondria, peroxisomes, and apoplastic space. These enzymes were selected for this study due to their high affinity for H_2O_2. Studies have reported changes in CAT and APX activities under salinity stress in wheat (Bouthour et al. 2015, Ibrahimova et al. 2021). In this study, 150 mmol NaCl increased CAT and APX activity across all genotypes, with higher activity observed in the stress-tolerant Gobustan and Mirbashir 128 genotypes. These genotypes also showed lower malondialdehyde levels, indicating reduced lipid peroxidation. Feki et al. (2017) found that tolerant genotypes exhibited higher CAT, APX, and superoxide dismutase activity, while sensitive genotypes showed lower enzyme transcript levels, higher MDA and H_2O_2 levels, and reduced dry biomass. APX activity has been reported to increase significantly after prolonged salt exposure, and CAT activity varies across genotypes (Abdel Latef 2010). Athar

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et al. (2009) observed that 150 mmol NaCl reduces photosynthetic activity and K^+/Na^+ ratios, impairing growth in both tolerant and sensitive genotypes.

Salinity-induced ionic and osmotic stresses can affect plants' synthesis of secondary metabolites (Mbarki et al. 2018). Flavonoids and anthocyanins, important secondary metabolites, act as antioxidants by scavenging toxic ions generated during oxidative stress (Mbarki et al. 2018). In this study, 150 mmol NaCl triggered the accumulation of these compounds, with higher levels observed in salt-tolerant genotypes (Mirbashir 128, Gobustan) and lower levels in sensitive genotypes (Fatima, Zirva-80). Previous studies have shown that anthocyanin accumulation varies under stress. It was observed in tomatoes, cabbage, rice, and wheat under salinity stress (Cheng et al. 2013) and intolerant rice genotypes exposed to NaCl (Chunthaburee et al. 2016). Transgenic tobacco also showed higher flavonoid accumulation under salinity compared to wild-type plants (Naing et al. 2017). However, other studies report conflicting results. For example, Daneshmand et al. (2010) found no difference in anthocyanin levels between salt-sensitive and tolerant potato samples. Similar disparities were reported for drought stress, with anthocyanin synthesis increasing in drought-tolerant rice but decreasing in sensitive cultivars. It can be concluded that proline plays a key role in stress response, showing positive correlations with antioxidant enzymes (APX, CAT), MDA, and soluble sugars, highlighting its role in enhancing antioxidant defences and managing oxidative stress (Figure 5).

Its negative correlations with root and leaf osmotic pressure suggest protective effects against osmotic stress. Intolerant genotypes' higher APX and CAT activity under salinity stress reflects early activation of antioxidant defences. These indicators can serve as markers for identifying salt tolerance and studying plant responses to salinity stress.

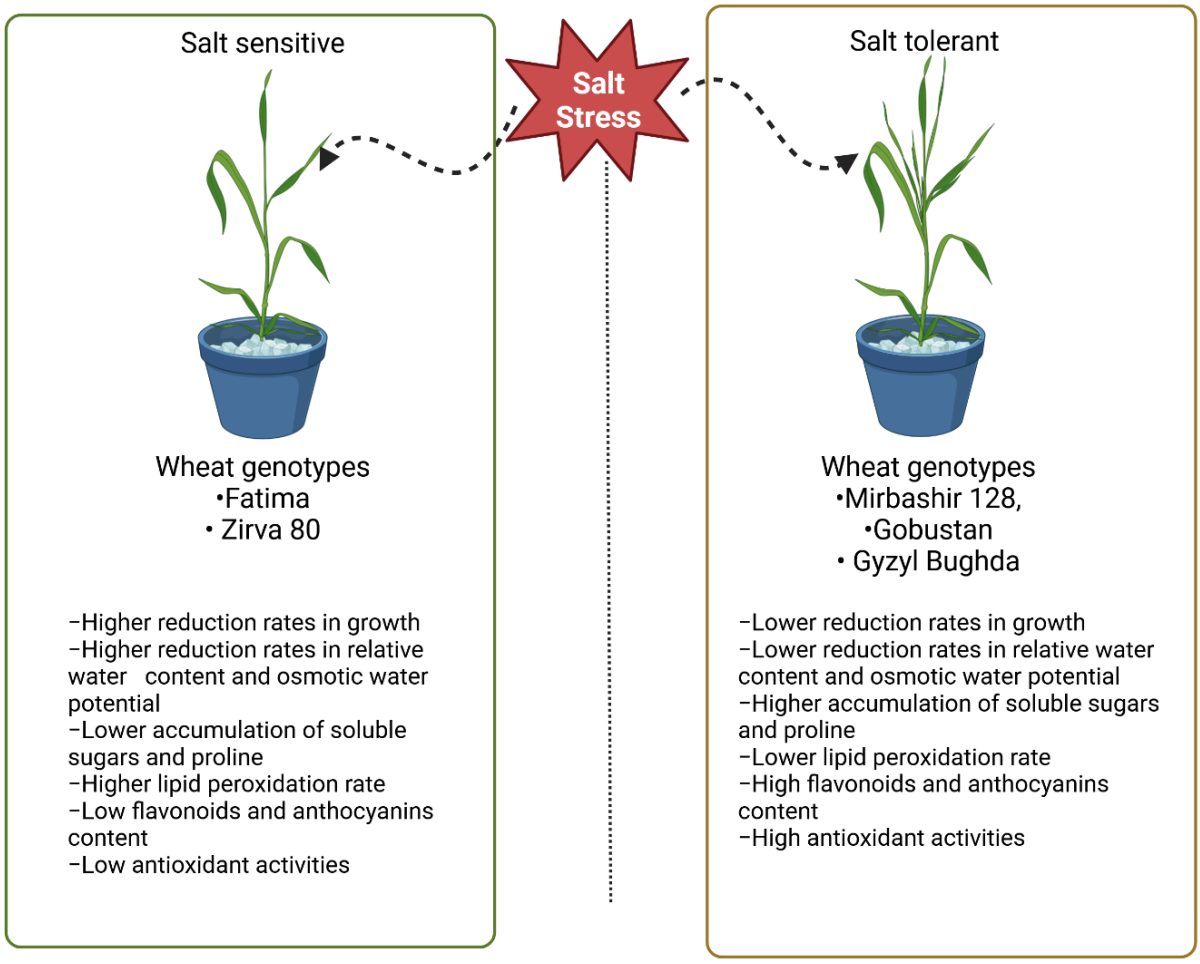

Figure 5. A schematic model figure illustrates the differences between salt-sensitive and salt-tolerant wheat genotypes and their underlying morphological and physiological mechanisms

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