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EVALUATION OF THE USABILITY OF ANCESTRAL HULLED WHEAT T. BOEOTICUM IN COPING WITH GLOBAL CLIMATE CHANGE BASED ON CELLULAR ANTIOXIDANT RESPONSES

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Abstract
Salinity is the second important factor after soil erosion that reduces crop yields by restricting plant growth and development. Misuse of fertilizers, excessive irrigation and industrial pollution are the main causes of widespread soil salinity, which poses a serious threat to agricultural productivity and food security for both humans and animals. Ancient wheats, unchanged for centuries, are seen as a promising solution for improving crop production on saline lands. Despite their importance for food security and local culture, these species are often neglected and undervalued in commercial production. In this study, we tried to predict the usability of this wheat in the reclamation of saline soils by measuring the tolerance of T. boeoticum to salt stress and the effectiveness of GB application in coping with stress. As salt stress, $CaCl_2$ (Control-25-50-100-150-200 mM) and $CaCl_2$ + Glycine-betaine (GB, 1 mM) were applied. The enzymatic and non-enzymatic responses of wheat roots and leafy stems to salt stress were analyzed separately to understand the specific reactions of each plant part. According to the results of the study, calcium chloride salt causes high levels of stress in T. boeoticum Boiss at concentrations above 100 mM. Combined application of glycine-betaine and calcium chloride significantly mitigated salt stress damage in wheat at 50, 100, and 150 mM salt concentrations. However, at 200 mM salt concentration, T. boeoticum Boiss suffered severe damage, resulting in approximately 80% reduction in stem length, leaf length, and chlorophyll content compared to the control. However, when 1 mM GB is applied together with 200 mM salt stress, the damage to wheat can be compensated by approximately 90% compared to the control. It has been observed that T. boeoticum Boiss variety has high antioxidant activity capacity in terms of both enzymatic and non-enzymatic antioxidant capacity. According to these results, it is predicted that ancient hulled wheat T. boeoticum Boiss can be used effectively in bringing saline/sodic areas into agriculture.

Keywords: Hulled wheat, glycine-betaine, $CaCl_2$, antioxidant activity, salt stress

1. INTRODUCTION

The primary impact of global climate change on grains is due to the disruption of water supply. The reduction in precipitation resulting from climate change, along with alterations in precipitation patterns (such as imbalanced and irregular precipitation) and extreme temperature increases, have necessitated the implementation of human-made irrigation techniques for crops. As a result of excessive and unregulated irrigation, groundwater levels declined rapidly, soil salinity and sodicity began to increase rapidly, and as a consequence, inefficiency occurred due to the excessive salinization of the soil. Soil salinization is the second leading cause of soil degradation, second only to soil erosion. It severely hinders plant growth and development, impacting crop yields. This issue

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has plagued agricultural activities for over 10,000 years (Yang & Guo, 2018; Shahid et al., 2018). The misuse of fertilizers, excessive irrigation, and industrial pollution are the primary causes of widespread soil salinization, posing a significant threat to agricultural productivity and food security for both humans and animals (Ouhibi et al., 2014; Hogue et al., 2022). Salinity problems can occur in all climatic conditions and can be caused by both natural and human-induced effects. Soil salinization is a dynamic phenomenon affecting over 100 countries worldwide, with no continent entirely free from salt-affected soils (Shahid et al., 2018). Recent regional studies have revealed the extent to which soil salinity has become widespread and its environmental impacts. These include the Aral Sea Basin in Central Asia, India, Pakistan, China, Syria and Iraq. The Euphrates Basin in Turkey, the Murray-Darling Basin in Australia and the San Joaquin Valley in the United States serve to illustrate the significance of the issue (Qadir et al., 2014; Shahid et al., 2018).

Soil salinization, a global issue affecting over 100 countries, results in the loss of approximately 2,000 hectares of arable land daily, leading to a 10-25% reduction in crop yields. In severe cases, it can cause soil desertification and complete crop failure (Shahid et al., 2018; Akram et al., 2021). Developing crops that can thrive in saline conditions is crucial to combat this problem. High concentrations of ions like sodium, potassium, chloride, and calcium in the soil induce osmotic and ionic stress in plants, hindering their water and nutrient uptake (Mahajan et al., 2008; Ismail et al., 2014, 2020). Soil salinity, defined as the concentration of soluble salts in soil water, primarily involves ions such as sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+), chloride (Cl^-), sulfate (SO_4^{2-}), bicarbonate (HCO_3^-), and nitrate (NO_3^-) (Tanji, 1990). The misuse of fertilizers, excessive irrigation, and industrial pollution are the primary causes of widespread soil salinization, posing a significant threat to agricultural productivity and food security for both humans and animals (Ouhibi et al., 2014; Hogue et al., 2022).

Ancient wheats, such as Einkorn (*Triticum monococcum* L. and *Triticum boeoticum* Boiss.), emmer (*Triticum turgidum* L. *spp. dicoccum* Schrank), and spelt (*Triticum aestivum spp. spelta*), have remained unchanged for over a century (Dinu et al., 2018; Temizgul et al., 2024). A defining characteristic of ancient wheat is its protective hull, which needs to be removed before milling. This hull has earned these wheats the name "hulled wheat" (Longin et al., 2015). Einkorn and emmer wheat played a pivotal role in the development and spread of agriculture. For millennia, they served as essential food sources until being gradually replaced by more productive polyploid wheat varieties during the Neolithic period (Nesbitt & Samuel, 1996). In this study, we utilized *T. boeoticum* Boiss, an ancient hulled wheat species.

Hulled wheat, a prime example of an underutilized species, holds significant importance for food security and cultural heritage, yet remains largely unknown and undervalued in commercial agriculture. Often overlooked by researchers and policymakers, many underutilized species are at risk of extinction due to a myriad of factors, including agricultural, genetic, economic, and cultural pressures (Padulosi et al., 2002). Today, emmer wheat occupies less than 1% of global wheat cultivation (Zaharieva et al., 2010). Previous research has indicated that emmer wheat has the potential to thrive in marginal areas under organic farming practices (Konvalina & Moudrý, 2007).

Reactive oxygen species are an inevitable consequence of aerobic reactions that are partially reduced or activated by the presence of oxygen. The term Reactive Oxygen Species is a compound name that refers to a variety of highly active compounds. Superoxide (O_2), hydroxyl (OH^-) and peroxyl (ROO^-) are examples of oxygen radicals. hydrogen peroxide (H_2O_2), singlet oxygen ($^{1/2}$ O_2) and ozone (O_3) are non-radical types of ROS (Ibrahimova et al., 2021). Reactive oxygen species are

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important for plants. They have a dual role in plants: a small amount acts as a signal that triggers abiotic stress responses for adaptation, while their overproduction causes oxidative damage. In severe cases, oxidative damage occurs in membranes (lipid peroxidation), proteins, nucleic acids, RNA and DNA, even leading to oxidative obliteration of the cell (Rahman et al., 2016). Chloroplasts, mitochondria, membranes or structural organelles of the cell, apoplast and nucleolus are the sites of ROS production. However, the peroxisome is also considered as a potent source of ROS, as the electron transport chain (ETC) and photochemical reactions are the majority of ROS-generating processes (Ghiyasi et al., 2008; Hasanuzzaman et al., 2017; Hassan et al., 2018).
Plants have an antioxidant defense system in which enzymatic and non-enzymatic antioxidants are present in their cellular organelles to scavenge various ROS to a certain extent. When ROS production is higher than the scavenging capacity of the antioxidant system, oxidative damage occurs. The antioxidant defense system consists of some well-known non-enzymatic antioxidants such as ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, flavonoids, etc. Contains. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST) and peroxiredoxin (PRX) are known enzymatic antioxidant compounds (Guo et al, 2015; Zou et al., 2016). Generally, in the antioxidant defense system, SOD provides frontline protection against ROS by converting O_2 to H_2O_2. CAT and APX then scavenge the H_2O_2 to H_2O. Glutathione peroxidase and GST also scavenge H_2O_2 to H_2O with the aid of GSH (McCord, 2000).
This study aimed to assess the salt tolerance of *T. boeoticum* Boiss, a Turkish landrace of emmer wheat, and its potential for cultivation in saline soils. To achieve this, we investigated the effects of glycine-betaine (1 mM), a known stress-mitigating agent, under various salt concentrations (0-25-50-100-150-200 mM $CaCl_2$) and combination with $CaCl_2$ + GB (1 mM). We analyzed the enzymatic and non-enzymatic responses of this hulled wheat to salt stress, focusing separately on the roots and leaf stems.

2. MATERIALS AND METHODS

2.1 Plant cultivation and stress practices

T. boeoticum Boiss. seeds were used as experimental material. The seeds were sown in 15 cm x 6 cm culture containers and grown in a controlled environment chamber maintained at 25 ± 1°C, 70 ± 5% relative humidity, and a 14/10-hour light/dark cycle. Before sowing, seeds were sterilized by a two-step process: a brief wash with liquid soap followed by a 5-minute treatment with ethyl alcohol and a 3-minute treatment with 5% calcium hypochlorite solution. Subsequently, the seeds were rinsed thoroughly with sterile distilled water. Twenty seeds were sown per container. Plants were grown for 10 days under non-stress conditions. Salt stress was then imposed for 15 days using 0, 25, 50, 100, 150, and 200 mM $CaCl_2$ solutions. Control plants were grown in Hoagland's solution (Hoagland & Arnon, 1950) for 25 days, with frequent replenishment to prevent dehydration. The nutrient solution in the culture containers was renewed daily. To investigate the effect of glycine betaine (GB), 1 mM GB was applied exogenously along with the salt stress treatments. After 15 days of stress treatment, plant roots and leaves were harvested, frozen in liquid nitrogen, and stored at -20°C for subsequent analysis. The experiment was conducted with three replicates.

2.2 Plant Growth Measurements

After 15 days of stress treatment, several growth parameters were measured:

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Fresh Weight: The fresh weight of roots and shoots was determined separately. Plants were removed from the containers, roots were washed with water to remove residual salts, and then both roots and shoots were weighed on an analytical balance.
Dry Weight: To determine dry weight, plant samples were dried in an oven at 65°C for 48 hours and then weighed.
Plant Height: The total height of each plant in the container was measured using a ruler.
Leaf Length: The length of the first fully expanded leaf from the stem was measured for each plant.

2.3 Enzyme Extraction and Protein Quantification

Crude enzyme extracts were prepared following the method of Yilmaz et al. (2017) with minor modifications. Briefly, 0.5 g of plant tissue was ground in a pre-cooled mortar and pestle with 2 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 1% polyvinylpolypyrrolidone (PVPP) and 0.1% EDTA. The homogenate was centrifuged at 15,000 x g for 20 minutes at 4°C, and the supernatant was collected and stored at -20°C until further use. For the extraction of ascorbate peroxidase (APX), 0.5 g of fresh tissue was ground in liquid nitrogen and homogenized in 2 mL of 50 mM Tris-HCl buffer (pH 7.2) containing 2% PVP, 1 mM EDTA, and 2 mM ascorbate (Akbulut & Cakir, 2010). The crude extract was stored at -20°C. Protein concentrations in the crude extracts were determined spectrophotometrically at 595 nm using the Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard protein. Protein concentrations were expressed as µg/mL.

2.4 Determination of chlorophyll and carotene content

Chlorophyll and carotene content were determined following the method of Yilmaz et al. (2020). Briefly, 100 mg of fresh leaf tissue was extracted with 7 mL of dimethyl sulfoxide (DMSO) in a water bath at 65°C until the tissue was completely decolorized. The extract was then transferred to a new tube and diluted to a final volume of 10 mL with DMSO. The absorbance of the extract was measured at 647 nm, 663 nm, and 470 nm against a DMSO blank. Chlorophyll content was calculated as mg/g fresh weight using the following formula:

$$Chl\ a\left(\frac{mg}{gr}fw\right) = (12{,}25 * A663) - (2{,}79 * A647)$$

$$Chl\ b\left(\frac{mg}{gr}fw\right) = (21{,}50 * A647) - (5{,}1 * A663)$$

$$Total\ Chl\left(\frac{mg}{gr}fw\right) = (7{,}15 * A663) + (18{,}71 * A647)$$

$$Carotene\left(\frac{mg}{gr}fw\right) = \frac{(1000 * A470) - (1{,}82 - Chl\ a) - (85{,}02 * Chl\ b)}{198}$$

2.5 Determination of enzyme activities

2.5.1 Catalase (CAT)

Catalase activity was measured according to the method of Duman et al. (2011) with slight modifications. Briefly, 50 µL of crude enzyme extract was added to 2 mL of 20 mM sodium phosphate buffer (pH 7.5) containing 15 mM H_2O_2. The decrease in absorbance at 240 nm was monitored for 3 minutes at 25°C using a Shimadzu UV-1800 spectrophotometer. A buffer-only

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solution served as a control. The molar extinction coefficient (ε) of H_2O_2 is 40 $mM^{-1}cm^{-1}$. Catalase activity was calculated using the following formula and expressed as units per milligram of protein.

$$SA\left(\frac{unite}{mg}protein\right) = \frac{\Delta Abs/min}{40} * \frac{\text{crude enzyme vol}}{\text{cuvette vol}} * \frac{1}{\text{prot cons}} * 1000$$

2.5.2 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured according to the method of Duman et al. (2011) with minor modifications. A reaction mixture containing 20 mM sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, 10 mM methionine, 0.1 mM nitroblue tetrazolium (NBT), and 0.005 mM riboflavin was prepared in a light-proof amber bottle. A standard SOD solution (0.1 mg mL^{-1}) was used to prepare standard tubes containing 10-500 ng mL^{-1} SOD. The reaction mixture (3 mL) was added to glass tubes, followed by 20 µL of the enzyme extract. Control tubes containing only the reaction mixture were also prepared. One control tube was wrapped in aluminum foil to serve as a dark control, while the other was exposed to light. The tubes were incubated under a fluorescent lamp (500 lumens) at a distance of 20 cm for 15 minutes. After incubation, the absorbance of the samples and standards was measured at 560 nm against a blank. The experiment was repeated three times, with each repetition performed in duplicate. The percentage inhibition of NBT reduction was calculated, and one unit of SOD activity was defined as the amount of enzyme required to inhibit NBT reduction by 50%. The percentage inhibition value was calculated in accordance with the formula presented below.

$$Inh\% = \frac{\text{Light Control Abs.} - \text{Sample Abs}}{\text{Light Control Abs.}} * 100$$

A logarithmic graph was plotted with enzyme concentration on the x-axis and % inhibition on the y-axis. A new graph was generated by taking the logarithm of the standard SOD enzyme concentrations and plotting them against the original % inhibition values. The SOD concentrations in the samples were determined using the equation of the line obtained from this graph and expressed as units per milligram of protein.

2.5.3 Ascorbate peroxidase (APX)

To determine ascorbate peroxidase (APX) activity, the method of Akbulut & Çakır (2010) was followed with minor modifications. A reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM ascorbate, 10 mM H_2O_2, and 50 µL of enzyme extract was prepared in a total volume of 1 mL. The reaction was initiated by adding H_2O_2. The decrease in ascorbate concentration was monitored at 290 nm for 3 minutes at 25°C using a quartz cuvette. A buffer-only solution was used as a blank. The enzyme activity was calculated based on the decrease in H_2O_2 concentration, using the extinction coefficient (ε) of H_2O_2 (2.8 $mM^{-1}cm^{-1}$ at 240 nm). The results were expressed as units per milligram of protein.

$$SA\left(\frac{unite}{mg}protein\right) = \frac{\Delta Abs/min}{2{,}8} * \frac{\text{crude enzyme vol}}{\text{cuvette vol}} * \frac{1}{\text{prot cons}} * 1000$$

2.5.4 Glutathione reductase (GR)

Glutathione reductase (GR) activity was measured according to the method of Misra & Gupta (2006). A reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.1 mM NADPH, and 1 mM oxidized glutathione (GSSG) was prepared. The reaction was

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initiated by adding 50 µL of crude enzyme extract. The decrease in absorbance at 340 nm was monitored for 5 minutes at 25°C. A buffer-only solution was used as a blank. The molar extinction coefficient (ε) of NADPH at 340 nm is 6.2 $mM^{-1}cm^{-1}$. The specific activity of GR was calculated and expressed as units per milligram of protein.

$$SA\left(\frac{unite}{mg}protein\right) = \frac{\Delta Abs/min}{6,2} * \frac{\text{crude enzyme vol}}{\text{cuvette vol}} * \frac{1}{\text{prot cons}} * 1000$$

Glutathione S-Transferase (GST)

Gluathione-S-transferase (GST) activity was measured following the method of Yilmaz et al. (2020). A reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.1 mM NADPH, 1 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) was prepared. After adding 50 µL of crude enzyme extract, the mixture was incubated for 5 minutes to allow for non-specific activity to cease. The change in absorbance at 340 nm was monitored for 5 minutes at 25°C using a 2 mL quartz cuvette. The molar extinction coefficient (ε) of NADPH at 340 nm is 6.2 $mM^{-1}cm^{-1}$. GST activity was calculated and expressed as units per milligram of protein.

$$SA\left(\frac{unite}{mg}protein\right) = \frac{\Delta Abs/min}{6,2} * \frac{\text{crude enzyme vol}}{\text{cuvette vol}} * \frac{1}{\text{prot cons}} * 1000$$

2.6 Lipid Peroxidation Analysis (MDA) (LPO)

Lipid peroxidation (LPO) in the tissues was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS). TBARS are products of lipid peroxidation, formed as a result of oxidative stress. Direct measurement of reactive oxygen species (ROS) is challenging due to their short half-lives. Therefore, the extent of oxidative damage is often estimated by measuring secondary products like TBARS (Janero, 1990; Jardine et al., 2002).

The TBARS assay, as described by Madhava & Sresty (2000) with minor modifications, was used to quantify malondialdehyde (MDA), a major product of lipid peroxidation. Briefly, 0.5 g of fresh tissue was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA) solution, and the homogenate was centrifuged at 12,000 x g for 5 minutes. To 1 mL of the supernatant, 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added. The mixture was heated in a boiling water bath for 30 minutes, cooled, and centrifuged at 12,000 x g for 15 minutes. The absorbance of the supernatant was measured at 532 nm and 600 nm using a spectrophotometer. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to correct for nonspecific absorbance. The amount of MDA was calculated using the molar extinction coefficient of MDA (155 $mM^{-1}cm^{-1}$). The results were expressed as nmol of MDA per gram of fresh weight.

2.7 Determination of the Proline Accumulation

Proline content was determined according to the method of Temizgul et al. (2016). Briefly, 0.25 g of fresh tissue was homogenized in 5 mL of 3% sulphosalicylic acid on ice. The homogenate was centrifuged at 5,000 x g for 10 minutes at 4°C. Two mL of the supernatant was mixed with 2 mL of acid ninhydrin and 1 mL of 3% sulphosalicylic acid. The mixture was incubated in a boiling water bath for 1 hour, followed by rapid cooling. Four mL of toluene was added to the mixture, and the tubes were left in the dark for 1-2 hours to allow toluene to extract the colored complex. The absorbance of the toluene layer was measured at 520 nm. A standard curve was prepared using 0.01

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µM to 1.5 mM proline. The proline content of the samples was calculated using the equation of the standard curve and expressed as nmol/g fresh weight.

2.8 Statistical Evaluation of Results

In order to ascertain the primary influence exerted by the variables examined in this study, as well as the interaction effects between them, a series of statistical analyses were conducted on the data obtained from a range of analytical and measurement procedures. These analyses employed One-Way (using SPSS 28.0), Two-Way (using SPSS 28.0), and Three-Way Analysis of Variance (ANOVA) (GraphPad Prism version 10.0.0). A three-way analysis of variance (ANOVA) was conducted between salt doses and plant parts. Furthermore, To determine statistical significance among different treatment levels, Duncan's multiple range test, Least Significant Difference (LSD) test, and Tukey's test were applied at a significance level of $P \leq 0.05$. All experiments were conducted in triplicate. Data are presented as mean ± standard deviation. Different lowercase letters (a, b, c, d, e) within tables and figures indicate statistically significant differences among means.

3. RESULTS

3.1 The effect of salt application on morphological indicators

The morphological changes observed in hulled wheat in response to salt applications are presented in Tables 1 and 2. A regular increase in wet weight was observed in the roots of hulled wheat until 100 mM applications. The increase in fresh root weight in hulled wheat exhibited significant variation depending on the applications ($p \leq 0.05$). In *T. boeoticum*, GB application resulted in an approximately 36% increase in fresh weight. From the application of 100 mM $CaCl_2$, a decrease in the fresh weight of wheat roots is observed (Table 1). The wheat stem also exhibits a decrease in fresh weight from the application of 150 mM $CaCl_2$ (Table 2).

The results indicate that an increase in dry weight of approximately 30-166% is observed in both roots and stems, depending on the application of salt (Tables 1 and 2). The application of GB results in a twofold increase in dry weight gain in both roots and trunk. The dry weight (DW) to fresh weight (FW) ratio increased by approximately 120% in the 100 mM salt application that was supplemented with 1 mM glycine-betaine (GB). The length of roots exhibited a decreasing trend with increasing salt doses. From an initial salt application of 100 mM, there was a noticeable reduction in plant height, with a 20% reduction observed at 200 mM. Furthermore, the length of the leaves of the plants was also found to be reduced in response to the increasing doses of salt. It can be observed that both plant height and leaf length increase in response to exogenous GB applications (Figure 1 and 2). It was observed that the contents of chlorophyll a and b decreased following the application of 150 mM of salt. The application of GB results in a twofold increase in the content of chlorophyll a and b. The content of carotene also decreases after the application of 150 mM salt (Table 2; Figure 1 and 2).

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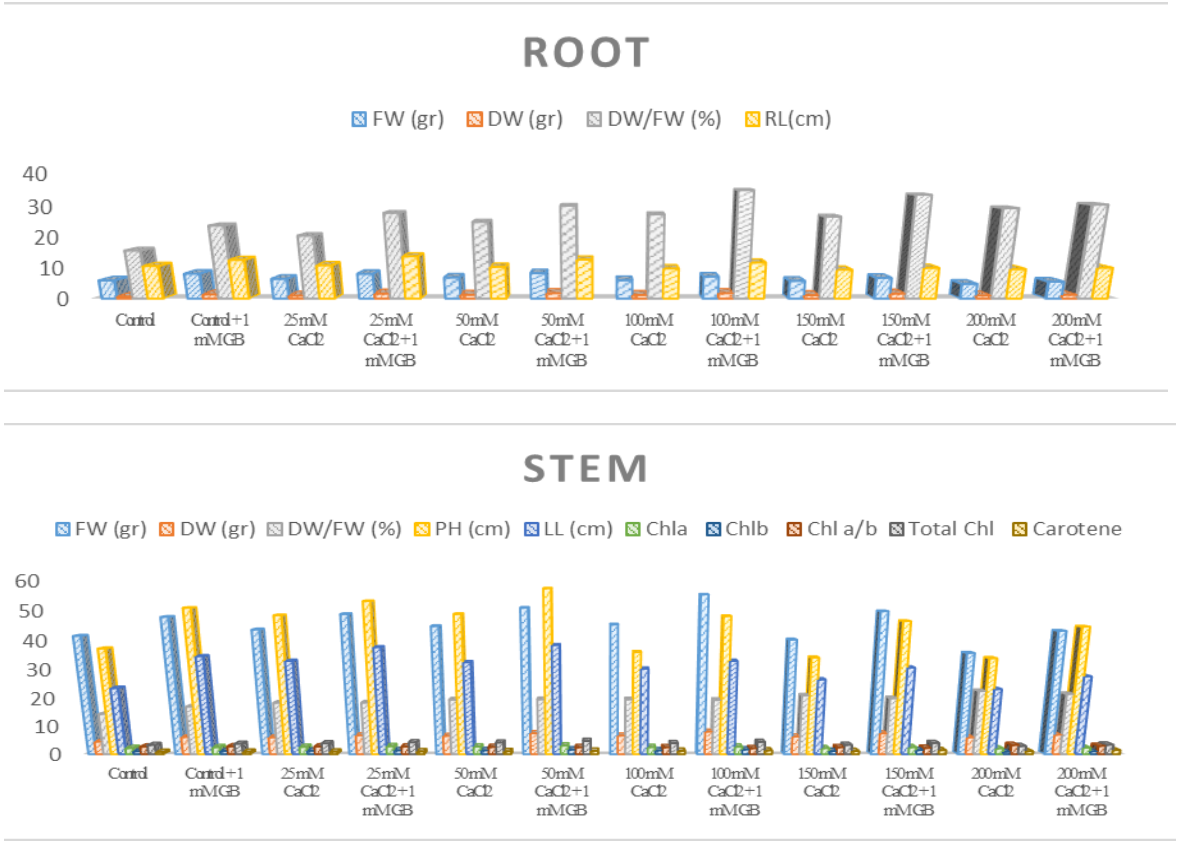

Figure 1. Changes in morphological parameters in roots and stems due to salt stress

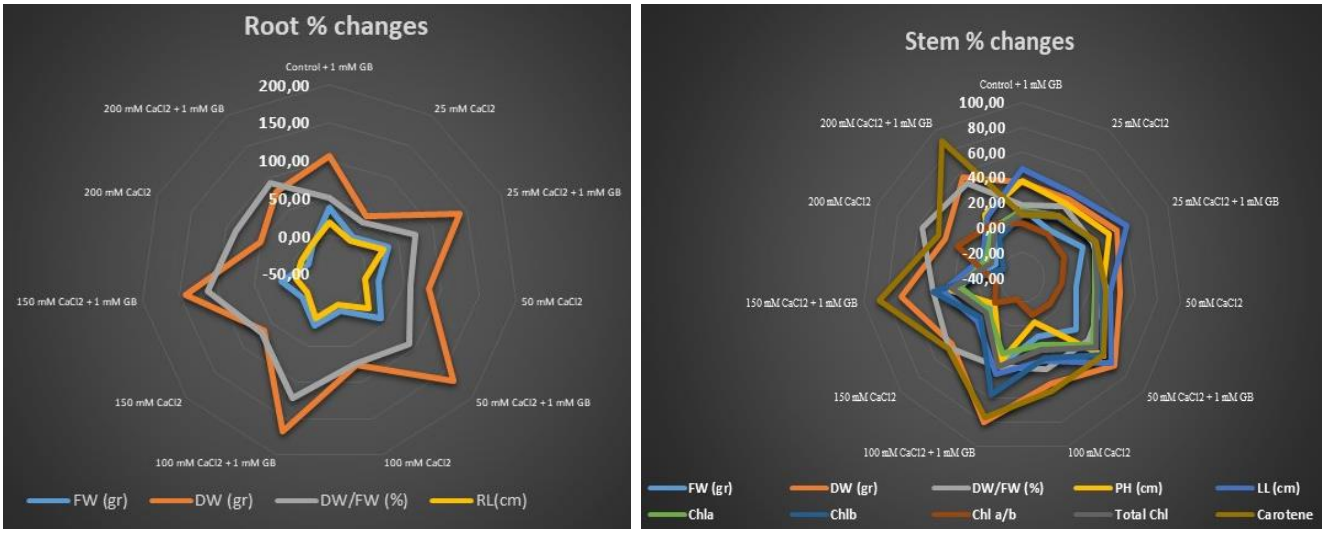

Figure 2. The percentage change of morphological parameters in roots and stems due to salt stress compared to the control

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Table 1. The morphological changes in the roots of T. boeoticum in response to the application of salt applications

Applications	FW (gr)	DW (gr)	DW/FW (%)	RL(cm)
Control	6.01	0.67	15.76	10.75
Control + 1 mM GB	8.23	1.39	23.73	12.74
25 mM $CaCl_2$	6.53	0.96	20.54	10.97
25 mM $CaCl_2$ + 1 mM GB	8.18	1.62	27.76	13.92
50 mM $CaCl_2$	6.97	1.24	24.92	10.47
50 mM $CaCl_2$ + 1 mM GB	8.42	1.79	30.02	12.84
100 mM $CaCl_2$	6.07	1.18	27.31	9.93
100 mM $CaCl_2$ + 1 mM GB	7.25	1.79	34.86	11.87
150 mM $CaCl_2$	5.87	1.11	26.55	9.51
150 mM $CaCl_2$ + 1 mM GB	6.86	1.63	33.39	10.13
200 mM $CaCl_2$	4.86	1.01	29.24	9.75
200 mM $CaCl_2$ + 1 mM GB	5.59	1.21	30.36	10.16

3.2 The effect of salt applications on total protein content

While salt applications have been found to increase the total protein content in the roots of wheat by between 10 and 25%, they also cause an increase in the total protein content of wheat stems by between 30 and 60%. The increase in protein content observed in exogenous GB applications reaches 45% in roots and 130% in stems (Table 3 and 4). The application of GB results in a significant increase in the protein content accumulated by wheat in 200 mM salt applications, particularly at high salt doses.

Table 2. The morphological changes observed in T. boeoticum stems in response to salt applications

Applications	FW (gr)	DW (gr)	DW/FW (%)	PH (cm)	LL (cm)	Chla (mg gr^{-1} fw)	Chlb (mg gr^{-1} fw)	Chl a/b	Total Chl (mg gr^{-1} fw)	Carotene (mg gr^{-1} fw)
Control	41.39	4.48	14.30	37.06	23.40	2.20	1.06	2.71	3.27	0.77
Control + 1 mM GB	47.81	6.11	16.86	50.87	34.42	2.50	1.17	2.81	3.68	0.85
25 mM $CaCl_2$	43.53	6.01	18.24	48.36	32.84	2.62	1.25	2.75	3.88	0.92
25 mM $CaCl_2$ + 1 mM GB	48.76	6.75	18.29	53.13	37.54	2.86	1.38	2.73	4.25	1.01
50 mM $CaCl_2$	44.68	6.56	19.37	48.76	32.35	2.74	1.39	2.58	4.14	1.04
50 mM $CaCl_2$ + 1 mM GB	50.88	7.51	19.48	57.36	38.25	3.11	1.63	2.50	4.75	1.20
100 mM $CaCl_2$	45.21	6.66	19.45	35.85	30.14	2.56	1.35	2.48	3.92	1.20
100 mM $CaCl_2$ + 1 mM GB	55.32	8.06	19.23	48.06	32.82	2.70	1.67	2.12	4.38	1.37
150 mM $CaCl_2$	40.10	6.32	20.80	33.97	26.37	2.17	1.16	2.48	3.33	1.13
150 mM $CaCl_2$ + 1 mM GB	49.73	7.48	19.85	46.38	30.50	2.52	1.47	2.25	3.99	1.45
200 mM $CaCl_2$	35.52	6.01	22.36	33.73	23.04	2.08	0.83	3.31	2.91	1.09
200 mM $CaCl_2$ + 1 mM GB	43.09	6.96	21.34	44.51	27.52	2.36	1.04	2.99	3.40	1.47

3.3 Effects of salt application on antioxidant enzyme activities

Tables 3 and 4 present the antioxidant enzyme responses occurring in the roots and stems of hulled wheat, respectively, in response to salt applications. The highest enzyme activities in the roots were observed for SOD and CAT in the 100 mM salt application, while GR, GST and APX were observed in the 50 mM salt application. In stems, all enzymes exhibited the highest enzymatic activity in the 100 mM salt application (Tables 3 and 4). In comparison to the control, antioxidant enzyme activities in the roots exhibited an increase of 7-50%, while in the stem, the increase was 12-102%. The exogenous application of GB resulted in a two-fold increase in antioxidant enzyme activities in roots and stems. In general, antioxidant enzyme activities in *T. boeoticum* exhibited a decline relative to the control at salt concentrations of 150 mM and above. The application of exogenous GB can tolerate losses in antioxidant enzyme activity due to high salt concentrations in roots and stems (Figure 3; Figure 5a, b).

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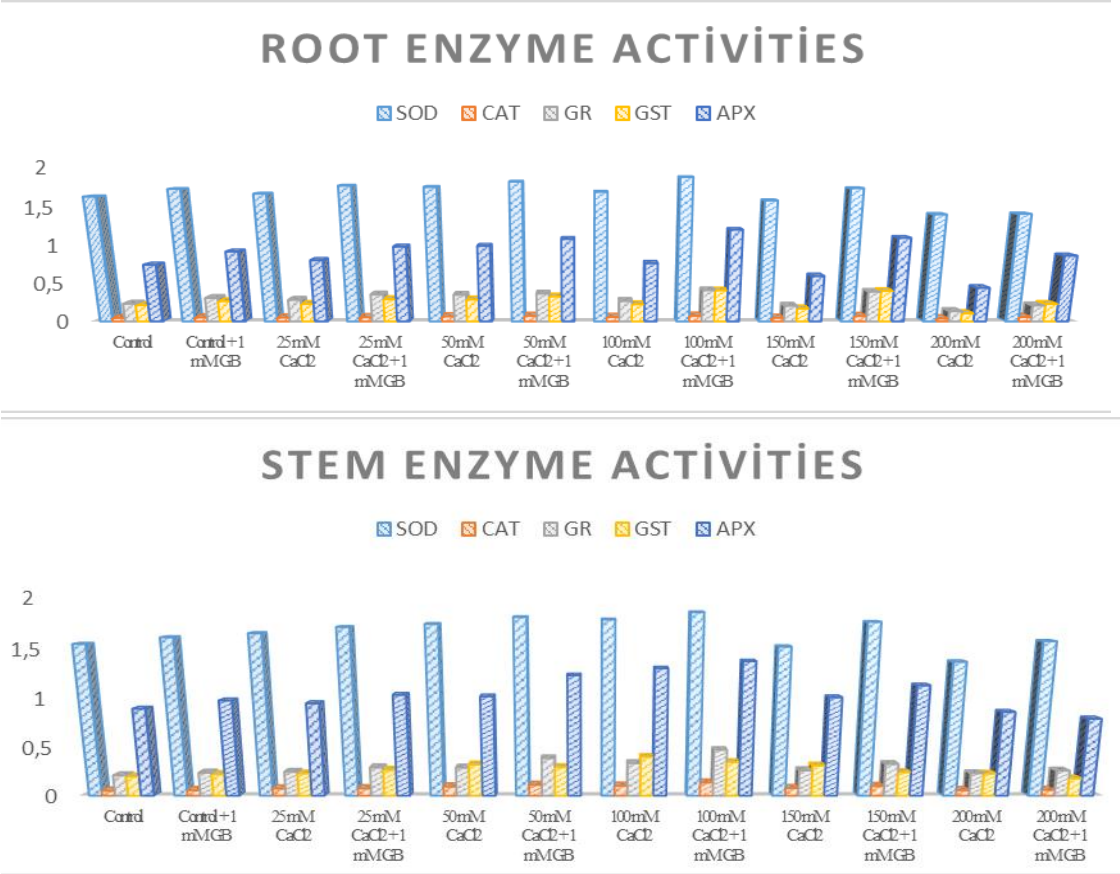

Figure 3. Antioxidant enzyme activities in roots and stems due to salt stress

Table 3. Effects of salt applications on antioxidant enzyme activities and proline and MDA content in T. boeoticum roots

Applications	TPC (μg ml^{-1} protein)	SOD (U ml^{-1} protein)	CAT (U ml^{-1} protein)	GR (U ml^{-1} protein)	GST (U ml^{-1} protein)	APX (U ml^{-1} protein)	PRO (nmol gr^{-1} fw)	MDA (nmol gr^{-1} fw)
Control	607.36	1.62	0.04	0.24	0.21	0.75	42.70	10.11
Control + 1 mM GB	845.47	1.72	0.05	0.31	0.27	0.92	51.85	8.74
25 mM $CaCl_2$	664.95	1.66	0.05	0.29	0.23	0.81	46.92	12.36
25 mM $CaCl_2$ + 1 mM GB	845.47	1.76	0.05	0.36	0.29	0.99	59.50	9.74
50 mM $CaCl_2$	709.31	1.75	0.06	0.36	0.29	1.01	74.89	18.69
50 mM $CaCl_2$ + 1 mM GB	880.88	1.81	0.07	0.37	0.33	1.08	109.87	10.81
100 mM $CaCl_2$	767.66	1.68	0.06	0.27	0.23	0.77	89.74	30.21
100 mM $CaCl_2$ + 1 mM GB	884.40	1.87	0.07	0.41	0.41	1.20	182.85	19.04
150 mM $CaCl_2$	753.66	1.58	0.05	0.20	0.17	0.61	106.05	60.92
150 mM $CaCl_2$ + 1 mM GB	830.28	1.73	0.07	0.39	0.41	1.10	216.64	21.38
200 mM $CaCl_2$	617.49	1.40	0.04	0.13	0.11	0.45	119.12	103.43
200 mM $CaCl_2$ + 1 mM GB	608.89	1.41	0.06	0.21	0.24	0.87	164.48	38.35

3.4 Effect on Proline Accumulation

The degree of proline accumulation in stems in response to different salt applications was determined to be higher than in roots. The percentage increase in proline levels in the stems compared to the control was found to range from 10 to 180% in roots and 100 to 400% in stems,

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respectively (Figure 5c and 5d). It was observed that GB applications resulted in approximately twofold increases in proline accumulation.

Table 4. Effects of salt applications on antioxidant enzyme activities and proline and MDA content in T. boeoticum stems

Applications	TPC (μg ml^{-1} protein)	SOD (U ml^{-1} protein)	CAT (U ml^{-1} protein)	GR (U ml^{-1} protein)	GST (U ml^{-1} protein)	APX (U ml^{-1} protein)	PRO (nmol gr^{-1} fw)	MDA (nmol gr^{-1} fw)
Control	338.09	1.54	0.05	0.21	0.19	0.89	43.99	9.77
Control + 1 mM GB	654.61	1.61	0.05	0.23	0.22	0.98	83.92	8.49
25 mM $CaCl_2$	436.74	1.65	0.07	0.24	0.23	0.95	43.84	11.09
25 mM $CaCl_2$ + 1 mM GB	707.80	1.71	0.07	0.29	0.27	1.03	105.31	9.80
50 mM $CaCl_2$	532.01	1.74	0.09	0.29	0.33	1.02	96.43	14.67
50 mM $CaCl_2$ + 1 mM GB	735.69	1.80	0.11	0.38	0.29	1.22	150.13	10.45
100 mM $CaCl_2$	503.61	1.78	0.10	0.34	0.40	1.29	176.39	22.88
100 mM $CaCl_2$ + 1 mM GB	779.45	1.85	0.13	0.48	0.35	1.37	177.71	13.16
150 mM $CaCl_2$	448.42	1.51	0.07	0.26	0.31	1.01	212.52	44.76
150 mM $CaCl_2$ + 1 mM GB	649.40	1.76	0.10	0.33	0.24	1.13	236.46	16.29
200 mM $CaCl_2$	343.11	1.36	0.05	0.23	0.23	0.86	219.50	106.25
200 mM $CaCl_2$ + 1 mM GB	589.47	1.56	0.06	0.26	0.18	0.79	281.32	35.97

3.5 Effect on lipid peroxidation (LPO) (MDA)

The calculated MDA amounts in hulled wheat vary between 10.11 and 103.43 nmol g^{-1} fw in the roots and 9.77 and 106.25 nmol g^{-1} fw in the stems (Table 3 and 4), depending on the salt applications. In the 200 mM salt application, an increase in MDA was observed in the roots and stems, with values ranging from 20 to 920% and 13 to 987%, respectively, in comparison to the control. The application of exogenous GB was found to reduce the MDA amounts that occur in 200 mM salt application to 0-279% in roots and 0-268% in stems (Figure 5).

4. DISCUSSION

4.1 Effects of Salt Stress on Plant Growth

Salt stress can lead to ion toxicity, disrupting the plant's nutritional balance and impairing physiological processes. This ultimately results in significant yield reduction (Taha et al., 2021). Additionally, salt stress induces oxidative stress in plants by affecting enzymatic activities, photosynthesis, membrane integrity, ionic homeostasis, hormonal balance, and water and nutrient uptake (Hussain et al., 2021; Ibrahimova et al., 2021). Studies by Guo et al. (2015) and Zou et al. (2016) have shown a reduction in root and shoot length and dry weight in wheat under 100 mM salt stress. Our study also revealed a decrease in the fresh weight of *T. boeoticum* roots with increasing salt concentrations (2-20%). Notably, a significant reduction in fresh weight of both roots and stems was observed at 200 mM salt stress (19.16% and 14.19%, respectively). The accumulation of excessive Na^+, Ca^{2+}, and Cl^- ions in plant tissues can hinder the uptake of essential nutrients, leading to disruptions in various plant processes. Guo et al. (2015) reported a decrease in potassium, calcium, and zinc uptake and an increase in sodium and chloride uptake in salt-sensitive wheat. Our findings of reduced fresh and dry weights of roots and stems, coupled with elevated $CaCl_2$ applications, suggest that the influx of high concentrations of Ca^{2+} and Cl^- ions into plant cells may contribute to water loss and cellular damage (Table 1 and 2).

Plants employ a strategy to reduce sodium toxicity by sequestering Na^+ ions in root vacuoles via the tonoplast pathway (Neubert et al., 2005). This mechanism helps to limit Na^+ transport to the shoots. Additionally, plants optimize K^+ uptake, which not only restricts Na^+ entry but also facilitates Na^+ efflux under salt stress. Calcium (Ca^{2+}) ions play a crucial role in this process by acting as stress

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signals, maintaining the K^+/Na^+ ratio in the cytosol, and ensuring plant survival in saline conditions (Wakeel et al., 2011). The observed 50% increase in stem dry and fresh weight and 30% increase in plant growth with 100 mM $CaCl_2$ application suggests that Ca^{2+} ions are transported to the stem rather than being retained in the roots. Conversely, the significant reduction in stem biomass, approximately 50% at 200 mM salt, indicates that salt accumulation in the roots hinders shoot growth. While plant height decreased by about 9% at 200 mM salt, the application of GB mitigated this effect, resulting in a 20% increase in plant height (Figure 2).

Salinity stress is a polygenic trait, meaning it is controlled by multiple genes. The release of Na^+ and the uptake of K^+; the maintenance of an optimal K^+/Na^+ ratio; osmotic regulation; and the enhancement of antioxidant enzyme activities are vital for plants under salt stress (Rahman et al., 2016). To improve plant yield under salt stress, various strategies have been employed, including genetic engineering and the application of osmoprotectants like glycine-betaine and proline. While these methods can be effective, they often require significant time and resources (Hassan et al., 2018). In our study, we observed that calcium chloride, particularly at concentrations of 100 mM and above, induced significant stress in spelt wheat. However, the application of exogenous glycine-betaine as an osmoprotectant mitigated this stress to a large extent. These findings suggest that the addition of glycine-betaine to the growth medium from the seedling stage onward can be a promising approach to cultivate wheat in saline soils.

The maintenance of ionic homeostasis in cells is of critical importance for plants to adapt to the presence of excess ions during salt stress. An appropriate K^+/Na^+ ratio is necessary to maintain low sodium and high potassium levels in the cytoplasm, which prevents cellular damage and nutrient deficiency under salt stress (Niu et al., 1995; Yang & Guo, 2018).

4.2 Effect on Photosynthesis (Chlorophyll and Carotene Contents)

Optimal photosynthetic activity is crucial for plant survival under suitable environmental conditions (Badawy et al., 2021). However, salt stress can significantly hinder photosynthesis. The accumulation of ions like Na^+, Ca^{2+}, and Cl^- within chloroplasts and the subsequent decrease in plant water potential disrupt the photosynthetic process (Hasanuzzaman, 2013). Guo et al. (2015) reported that salinity stress induces stomatal closure, reduces CO_2 absorption, and lowers transpiration rates in wheat. Additionally, salt stress can lead to a decline in photosynthetic pigment concentration, further compromising photosynthetic efficiency and productivity. In this study, we observed a significant decrease in chlorophyll a content, particularly at 200 mM salt concentration. However, the application of 1 mM glycine-betaine as an osmoprotectant mitigated this decline and led to a 7% increase in chlorophyll a content.

Salt stress disrupts the delicate balance between reactive oxygen species (ROS) and antioxidants, leading to oxidative stress. This can result in ionic toxicity, reduced leaf growth, impaired photosynthesis, and premature leaf senescence. Moreover, salt stress can negatively impact PS-II efficiency, stomatal conductance, intercellular CO_2 concentration, and electron transport, all of which contribute to reduced photosynthetic rates (Seleiman et al., 2022). The accumulation of ions in plant tissues can disrupt the K^+/Na^+ balance and trigger ROS production, damaging photosystems I and II (Sarker & Oba, 2019). This, in turn, can lead to a decrease in chlorophyll a content and alterations in the chlorophyll a/b ratio, further impairing photosynthetic efficiency. Additionally, salt stress can reduce stomatal density and induce stomatal closure, limiting CO_2 uptake and hindering photosynthesis (Levy et al., 2013; Charfeddine et al., 2019).

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Salt stress can significantly inhibit PS-II, a crucial component of the photosynthetic apparatus. Studies have shown that salt stress can alter the structure of chloroplasts, leading to a loose arrangement of granum thylakoids (Zhu et al., 2021). While halophytes often increase chloroplast number to cope with salinity, *T. boeoticum* exhibited a significant decrease in chlorophyll b content at higher salt concentrations, indicating severe damage to PS-II. However, the application of glycine-betaine mitigated this decline, suggesting its protective role in chlorophyll biosynthesis. During salt stress, reduced photosynthetic activity can lead to decreased oxygen production and increased ROS accumulation. While low levels of ROS play signaling roles, excessive ROS can damage cells. Glycine-betaine, when applied with calcium ions, can help regulate ROS levels and mitigate oxidative stress. Interestingly, salt stress also induced an increase in carotene content in *T. boeoticum*. Carotenoids, as non-enzymatic antioxidants, can protect photosystems from oxidative damage. The combination of glycine-betaine and calcium chloride further enhanced carotene accumulation, providing additional protection against salt stress.

In conclusion, salt stress significantly impairs photosynthetic capacity by reducing stomatal conductance, photosynthetic rate, chlorophyll content, and key enzyme activities. *T. boeoticum* showed tolerance to salt stress up to 100 mM $CaCl_2$, but higher concentrations caused significant damage. However, the combined application of glycine-betaine and calcium chloride mitigated the negative effects of salt stress, preserving photosynthetic activity and even increasing chlorophyll content. These findings highlight the potential of *T. boeoticum* as a valuable genetic resource for developing salt-tolerant wheat cultivars.

4.3 Effect of Salt Stress on Protein Concentrations

Plants employ a range of osmoprotectants, including proline, glycine-betaine (GB), dimethylsulfoniopropionate (DMSP), choline, polyols, and sugars like trehalose, sorbitol, and mannitol, to mitigate the adverse effects of ionic, oxidative, and osmotic stress. Many of these osmolytes are synthesized in response to multiple stresses, such as drought and cold, and their production can vary between species and tissues (Parvanova et al., 2004; Yancey, 2005; Pirzad et al., 2011; Sailaja et al., 2014; Yang & Guo, 2018; Temizgul, 2024). Numerous studies have demonstrated the efficacy of exogenous osmoprotectant application in alleviating salt and metal stress in plants (Hasanuzzaman et al., 2014; Aamer et al., 2018; Dustgeer et al., 2021). In our study, we observed a significant increase in total protein content, ranging from 1 to 57%, depending on the salt stress intensity. Notably, the combined application of glycine betaine and salt stress resulted in a remarkable 130% increase in total protein content (Tables 3 and 4). This suggests that exogenous glycine betaine enhances the plant's defense against reactive oxygen species (ROS) by inducing the production of both enzymatic and non-enzymatic antioxidants in hulled wheat.

4.4 Effects of Salt Stress on Enzymatic and Non-enzymatic Antioxidant Defense System

Plants possess an antioxidant defense system comprising both enzymatic and non-enzymatic antioxidants, which scavenge reactive oxygen species (ROS) to prevent oxidative damage. Superoxide dismutase (SOD) is a crucial first line of defense, converting superoxide radicals into hydrogen peroxide (H_2O_2). H_2O_2 is further detoxified into water and oxygen by catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). The AsA-GSH cycle, involving ascorbate (AsA), glutathione (GSH), and enzymes like APX, dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR), plays a vital role in H_2O_2 detoxification. Additionally, non-enzymatic antioxidants such as carotenoids, flavonoids, and

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phenolic acids contribute to ROS scavenging. Salt stress induces oxidative stress by increasing ROS production. While excessive ROS can be detrimental, low levels of ROS act as signaling molecules, triggering adaptive responses in plants. The antioxidant defense system is crucial in mitigating oxidative damage and maintaining ROS homeostasis. Studies have shown a strong correlation between antioxidant activity and salt tolerance in wheat species (Zhu, 2002; Munns & Tester, 2008; Al-Ashkar et al., 2019; Hassan et al., 2020; Van Zelm et al., 2020; Mittler et al., 2022). Salt stress can lead to increased activity of antioxidant enzymes like SOD, CAT, and APX, as well as enhanced production of antioxidants like ascorbate. Several studies have reported similar findings (Agati et al., 2012; Di Ferdinando et al., 2012; Liu et al., 2014; Hussain et al., 2019). Athar et al. (2007) observed increased ascorbate production and catalase activity in salt-tolerant wheat varieties. Zeeshan et al. (2020) and Dong et al. (2017) demonstrated increased activity of SOD, POD, CAT, and APX in response to salt stress. Ahanger et al. (2019) and Mandhania et al. (2006) also reported upregulation of antioxidant enzymes to counteract ROS accumulation under salt stress. In our study, we observed increased SOD activity in *T. boeoticum* under salt stress, particularly in the stems (Figure 3). The combination of 100 mM $CaCl_2$ and GB led to the highest increase in SOD activity. Similarly, the activities of CAT, GR, GST, and APX were significantly upregulated in salt-stressed plants, indicating the activation of the antioxidant defense system to mitigate oxidative damage (Figure 5a, b).

4.5 Effects of Salt Stress on Proline Accumulation

It is well-established that plants subjected to osmotic stress employ osmoregulation, accumulating sugars, polyols, amino acids, and quaternary ammonium compounds to mitigate stress effects (Farooq et al., 2015). Osmoregulation triggers antioxidant defense mechanisms, regulating the plant's water balance (Bose et al., 2014). Proline, an osmoprotectant, plays a crucial role in osmotic adjustment, ROS detoxification, and stabilization of photosystem II (Szabados & Savouré, 2010). Previous research has shown that exogenous glycine betaine (GB) application enhances antioxidant enzyme activities (SOD, APX, CAT), significantly improving wheat's salt tolerance (Raza et al., 2006; Temizgul, 2024). In our study, *T. boeoticum* exhibited a significant 1- to 5-fold increase in proline accumulation under increasing salt stress (Figures 4 and 5c, d). Other studies have demonstrated that GB (10-30 mM) and proline (60 ppm) applications enhance germination, mineral uptake, chlorophyll content, and reduce oxidative stress in wheat (Akhter et al., 2007; Hendawey, 2015). Rao et al. (2013) reported that increased proline and GB production activate antioxidant enzymes, mitigating salt stress. Exogenous osmoprotectants, including GB and proline, can enhance proline and potassium accumulation, improve the K/Na ratio, and stabilize protein and lipid structures (Duman et al., 2011; Temizgul, 2024). These compounds can enhance antioxidant activities, photosynthetic efficiency, and membrane stability, ultimately detoxifying ROS and promoting plant recovery under salt stress. Our study's highest proline accumulation was observed in the GB-supplemented 150 mM $CaCl_2$ treatment, confirming the synergistic effect of GB on proline accumulation and salt stress tolerance.

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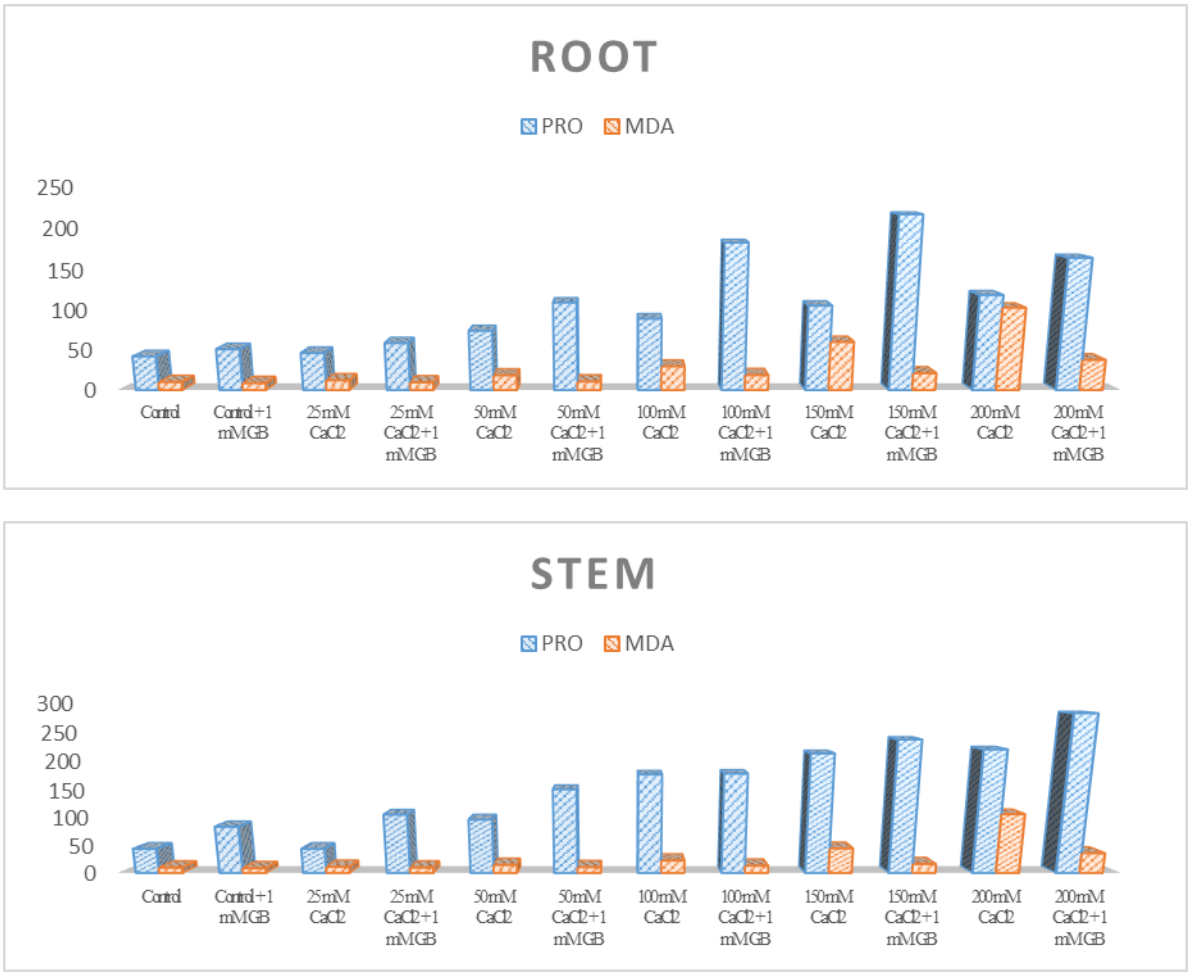

Figure 4. Changes in root and stem proline and MDA accumulation due to salt stress

4.6 Effects of Salt Stress on Lipid Peroxidation (LPO; MDA)

Salt stress can lead to increased production of reactive oxygen species (ROS) in wheat plants, resulting in cellular damage to lipids, proteins, and nucleic acids (Hasegawa et al., 2000; Mittler, 2002; Parida & Das, 2005; Hasanuzzaman et al., 2013; Temizgul, 2024). Salt-sensitive wheat varieties often exhibit higher levels of hydrogen peroxide and lipid peroxidation compared to salt-tolerant varieties under saline conditions (Hasanuzzaman et al., 2011). Zou et al. (2016) reported a significant increase in malondialdehyde (MDA) content in wheat seedlings exposed to 100 mM NaCl for 5 and 10 days, with increases of 35% and 68%, respectively. In our study, we observed a progressive increase in MDA accumulation with increasing salt concentrations, reaching a 987% increase at 200 mM $CaCl_2$ compared to the control (Figure 5).

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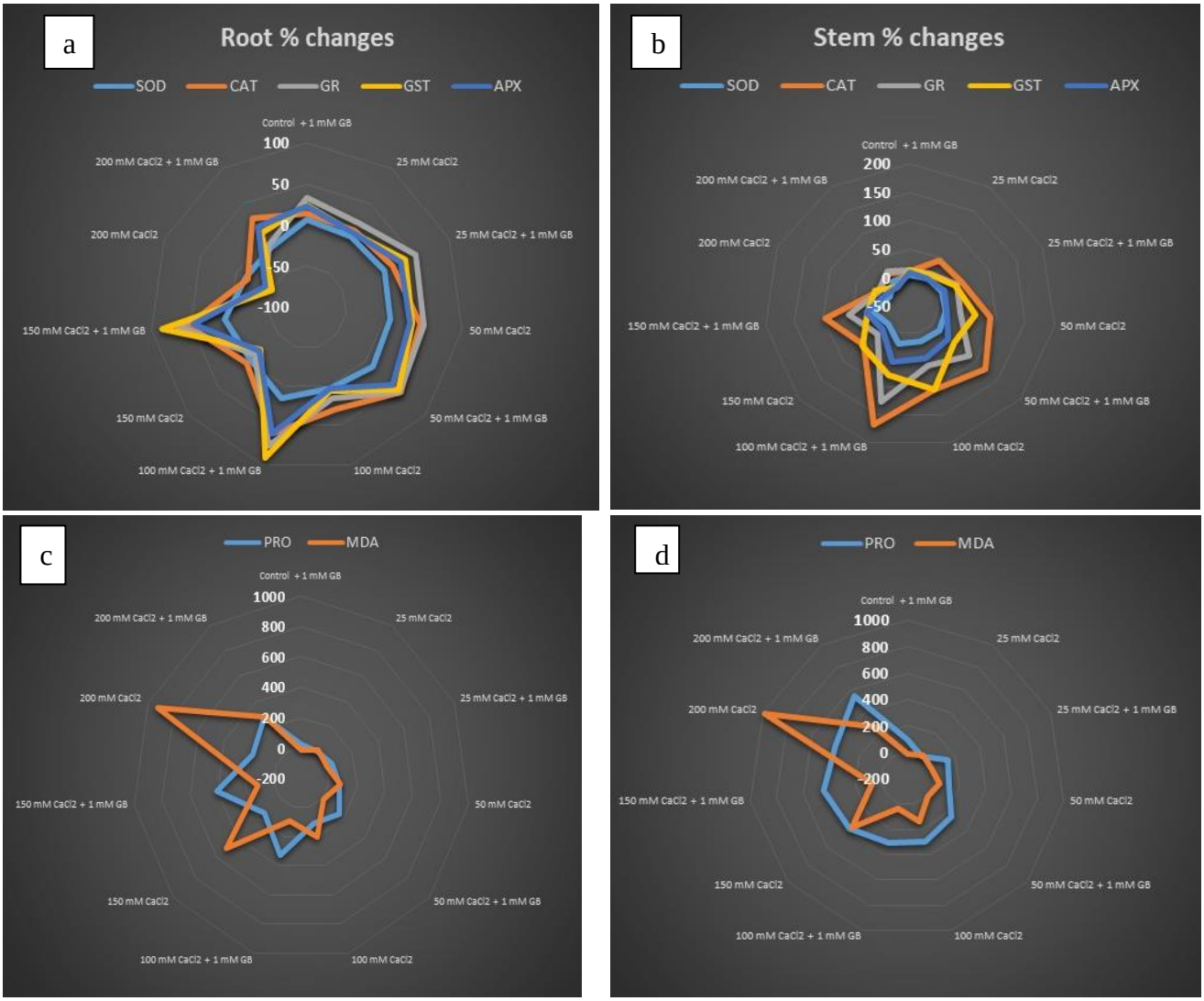

Figure 5. The percentage changes in antioxidant enzyme activities and proline and MDA content in roots and stems due to salt stress compared to the control

Ionic homeostasis plays a crucial role in regulating ion fluxes to maintain low Na^+ and high K^+ concentrations within the cell (Hasegawa et al., 2000; Farooq et al., 2015). The regulation of intracellular Na^+ and K^+ ions is essential for maintaining cell membrane potential and volume. To achieve this, plants employ various strategies, including active transport of excess Na^+ out of the cell and accumulation of Ca^{2+} in the plasma and tonoplast membranes. Additionally, plants utilize specific transporters to selectively uptake K^+ ions (Amtmann & Sanders, 1998; Blumwald, 2000).

5. CONCLUSIONS

Developing salt-tolerant wheat varieties through strategic agronomic practices or incorporating ancestral hulled wheat varieties, which possess inherent salt resistance, into breeding programs, can significantly enhance crop production in saline environments. Given Türkiye's status as a global wheat diversity center, it is highly probable that salt-tolerant varieties exist that could outperform modern wheat in sodic/saline soils, even if they may not yield as much under optimal conditions.

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Harnessing these varieties is crucial for ensuring food security in increasingly saline agricultural lands.

Wheat, a staple food globally, faces a significant threat from salinity stress. Hulled wheat, an ancient ancestor of modern wheat, offers valuable genetic resources. While modern wheat breeding has focused on optimizing yield and nutritional quality under ideal conditions, it may have inadvertently compromised other traits like salt tolerance and disease resistance. As climate change and human activities degrade the environment, it becomes imperative to explore and reintroduce wild relatives and landraces of wheat with robust salt tolerance.

In addition to genetic strategies, the exogenous application of osmoprotectants, phytohormones, and advanced seed treatments can improve salt tolerance in wheat. These approaches can mitigate the adverse effects of salinity stress, thereby boosting wheat productivity and ensuring food security. Ancient wheat species, such as hulled wheats, offer numerous health benefits, including improved nutritional profile and potential tolerance for individuals with gluten sensitivities. This growing recognition has fueled increased interest in ancient wheat-based products, particularly among consumers seeking healthier and more natural food options. *T. boeoticum*, as an ancient wheat species, has the potential to meet this rising demand.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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