International Journal of Horticultural Science and Technology
Journal homepage: https://ijhst.ut.ac.ir

Comparative Analysis of Salinity Stress on the Molecular, Biochemical, and Phenotypic Changes of Different Cabbage species (*Brassica*)

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ARTICLE INFO

Article history:

Received: 7 February 2025,
Received in revised form: 21 May 2025,
Accepted: 26 May 2025,

Article type:

Research paper

Keywords:

Antioxidant,
Brassica oleracea,
Enzyme activity,
Gene expression,
Protein content,
Salinity stress

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ABSTRACT

This study examined the antioxidant defense capacity of various cabbage species in response to salt stress. Seedlings were exposed to salinity levels of 0, 50, 100, and 150 mM for eight days. Results indicated that germination rates, fresh weight, and dry weight decreased with increasing salinity. Notably, Brussels sprouts maintained the highest germination percentage, even at 150 mM. Interestingly, both root and seedling lengths increased under salt stress across all cultivars. Gene expression analysis showed a significant upregulation ($P<0.05$) of glutathione reductase (GR) and ascorbate peroxidase (APX) activities under salinity. Other biological indices, including root and shoot growth, also exhibited increases, reflecting adaptive responses to salt tolerance. Although germination rates declined with higher salinity, the expression of the analyzed antioxidant genes rose by approximately 41.09% at 100 mM but dropped by 13.97% at 150 mM across all cultivars. Overall, the findings underscore the varied responses of Brassica cultivars to salt stress, with Brussels sprouts demonstrating notable resilience and enhanced antioxidant gene expression under high salinity conditions.

Abbreviations: Ascorbate Peroxidase (APX), Catalase (CAT), Complementary DNA (CDNA), Deoxyribonucleic Acid (DNA), Glutathione Disulfide (GSSG), Glutathione Reductase (GR), Hydroxyl Radicals (OH), Messenger RNA (mRNA), Peroxidase (POD), Quantitive PCR (qPCR), Reactive Oxygen Species (ROS), Ribosomal RNA (rRNA), Superoxide Dismutase (SOD)

Introduction

World crop productivity is significantly suppressed by salinity stress (Munns et al., 2015). Salt stress causes the production of reactive oxygen species (ROS) in the cell, inhibition of enzyme activity, reduction of plant production and photosynthesis rate, and reduction of plant growth (Li et al., 2013). Oxidative stress is brought on by the ionic imbalance that salinity creates, which raises the levels of hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and reactive oxygen species (superoxide (O^{2-}) (Polash et al., 2019).

Free radicals can cause significant damage to proteins, lipids, photosynthetic pigments, nucleic acids, and cell membranes (Ahmad and Ali, 2010). To counteract these harmful effects, cells and organelles possess protective mechanisms (Mansoor et al., 2022; Mostofa et al., 2021). In plants, two primary antioxidant defense systems mitigate oxidative damage: the enzymatic antioxidant system,

which includes enzymes such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and guaiacol peroxidase; and the non-enzymatic system, composed of molecules such as carotenoids, flavonoids, and anthocyanins (Jbir-Koubaa et al., 2015; Saxena et al., 2020). Among these, peroxidase is a key antioxidant enzyme found across many organisms (Margis et al., 2008; Zámocký and Ali, 2010).

Cabbage (Brassica oleracea L.) is one of the most widely cultivated vegetables worldwide (Embaby and Lotfy, 2015). In this study, three Brassica species—cabbage, broccoli, and Brussels sprouts—were examined. The antioxidant defense system in these plants not only detoxifies reactive oxygen species (ROS) but also maintains the balance of ROS production under salt stress, thus protecting plant tissues from oxidative injury (Joshi et al., 2011; Saxena et al., 2011).

Understanding the types of damage caused by salinity and the corresponding defense mechanisms, as well as identifying salt-tolerant cultivars, is critical for developing strategies to enhance plant resilience and productivity under stress conditions (Raza et al., 2023). Glutathione reductase (GR), for example, catalyzes the conversion of glutathione disulfide (GSSG) into sulfhydryl glutathione (GSH), a vital compound that sustains the cellular reducing environment and protects against oxidative stress (Dorion et al., 2021). The antioxidant system comprises not only key enzymes such as POX, CAT, and SOD, but also a range of vitamins, minerals, and phytochemicals. SOD, in particular, is localized in the cytosol, mitochondria, peroxisomes, and chloroplasts, where it catalyzes the dismutation of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) (Shehzad et al., 2023; Faizan et al., 2023). Accordingly, this study compared the antioxidant defense capacity of several cabbage species with varying levels of salinity tolerance to elucidate the physiological and biochemical mechanisms underlying plant adaptation to salt stress. The functional differences among these three Brassica species, particularly in terms of enzyme production, may also reflect their high antioxidant and nutritional value, supporting their role as potential cancer-preventive dietary components.

This research presents a novel approach to understanding and enhancing plant salinity tolerance by integrating molecular, biochemical, and phenotypic analyses. Through a comparative study of Brassica cultivars, the work not only identifies salt-tolerant species but also establishes a foundation for improving the nutritional and antioxidant profiles of crops. These findings pave the way for the development of biofortified Brassica cultivars with enhanced resilience to abiotic stress and greater health benefits, addressing the dual challenges of food security and nutritional quality in the context of a changing climate.

Materials and Methods

Cultivation of seeds and salt treatments

In this experiment, fully randomized design experiment, three salinity levels (50, 100, and 150 mM sodium chloride) were applied, with three replicates for each cabbage cultivar. Seeds of three *Brassica* species, i.e., lunar cabbage (*Brassica oleracea* Gongylodes), broccoli (*Brassica oleracea* Italica), and Brussels sprouts (*Brassica oleracea* var. *gemmifera*), were obtained from the Pakan Seed Company of Isfahan. The experiment was conducted using a germinator to maintain a stable temperature. For each cultivar, fifty seeds were placed on 18 cm plates lined with filter paper and subjected to salt stress under optimal growth conditions (25 °C and 16 h of light). The total duration of the experiment was 8 d. Sodium chloride (NaCl) was used as the salt source for the salinity treatments, while the control group was grown without added salt under the same environmental conditions. After 8 d, samples were harvested for measurement of multiple parameters, including germination rate and percentage, fresh and dry weight, root and shoot length, activities of antioxidant enzymes (ascorbate peroxidase, glutathione reductase, and guaiacol peroxidase), gene expression, protein profiles analyzed by SDS-PAGE, and levels of malondialdehyde and hydrogen peroxide.

Germination percentage and germination rate

Fifty healthy seeds were selected and placed on filter paper inside Petri dishes. Each Petri dish received an appropriate amount (5 mL) of saline solution with varying salinity levels. The dishes were then incubated at a constant temperature of 25 ± 1 °C in a germinator for 8 d. Throughout the experimental period, the solution levels in the Petri dishes were monitored every 2 d to maintain a volume of approximately 2.5 mL.

Germination percentage and germination rate were calculated using the following equations:

$$Germination\ Percentage = \frac{\Sigma G}{N} \quad (1)$$

$$Germination\ Rate = \frac{\Sigma Ni}{D} \quad (2)$$

Where:

G = Number of germinated seeds

N = Total number of seeds

Ni = Number of germinated seeds on day i

D = Number of days until n

Preparing an enzymatic extract

Two grams of seeds from each cultivar were cleaned with deionized water and blended with 10 mL of 0.2

M, pH = 7 phosphate buffer. The extract was filtered, centrifuged for 30 min at 12,000 rpm, and the supernatant was utilized to prepare the enzyme extract. (Dinajpur et al., 2023; Singh et al., 2010).

Peroxidase and glutation reducetase enzymes activity

In the presence of peroxide, H_2O_2 reacts quickly to form a quinoneimine chromogen, which has a maximum absorption at 510 nm and a vivid pink color. Two different amounts of aminoantipyrine and phenol were used: 0.0025 and 0.17 M, respectively (Solra et al., 2022; Sarika et al., 2015). Moreover, the oxidation of NADPH using the Foyer and Noctor method was used to measure the activity of the GR enzyme (Foyer and Noctor, 2011).

The ascorbate peroxidase and guaiacol peroxidase enzymes' activities

The reaction mixture included 0.1 mM EDTA, 0.5 mM ascorbic acid, 0.15 mM H_2O_2, 50 mM buffer consisting of potassium phosphate (pH = 7), and 50 µL of enzyme extracted. After the enzymatic reaction began and the ascorbic acid began to oxidize, the absorbance at 290 nm decreased 2 min later, and this was measured for comparison with the reaction's beginning (Raven, 2000; Haida et al., 2019). Ultimately, the peroxide reduction method was used for measuring the activation of the guaiacol peroxidase enzyme (Platonova and Belous, 2020; Tonami et al., 2004).

RNA extraction, cDNA synthesis, and QPCR

Total RNA was isolated and purified from various Brassica tissues based on the RNeasy plant mini kit protocol (Clontech, United States). CDNA synthesis was carried out in accordance with the manufacturer's instructions using the AddBio kit (South Korea). We used specific primers to improve the conditions for doing Realtime-RT-PCR (Table 1). We heated the samples at 95 degrees Celsius for 10 min at first, and then we repeated the process 40 times. Each time, we heated the samples at 95 degrees Celsius for 20 s, then at 55 degrees Celsius for 30 s, and finally at 72 degrees Celsius for 30 s. Melting point was estimated between 65 °C and 95 °C using 0.3 °C ramp rate in order to determine the melting curve. The process was optimized for 1 min at 95 °C. The reference gene for the relative gene expression analysis was the 18S rRNA gene. The 2-ΔΔCt technique was used in expression changes analysis in accordance with Livak and Schmittgen's instructions (Livak and Schmittgen, 2001). All gene expression analyses were performed in triplicate using Biofact 2x SYBR Green Master Mix (South Korea) on an ABI StepOne real-time PCR system.

Table 1. Primer sequences and their properties (primers in the table below were designed by the authors).

Primer name	Sequence	Amplicon length	Target gene	Accession number
FBRasrr	5'- GTATGGTCGCAAGGCTGAAAC-3'	135bp	*18S rRNA*	KX709367.1
RBRasrr	5'- GAGCTCTCAGTCTGTCAATCC-3'			
FAPXBr	5'- AGCAGTTCCCTACCATCTCTC-3'	121bp	*APX*	AB901371.1
RAPXBr	5'- GGTGGCTGGGGCTTGTCCTC-3'			
FPOXBr	5'- TCTTCACTTCCACGACTGCTTC-3'	125bp	*POX*	JQ321595.1
RPOXBr	5'- CCACTGGAAATCCTCGAGCC-3'			
FGrBr	5'- TACTCACGCACTAACATACC-3'	112bp	*GR*	NM_001302025.1
RGrBr	5'- CAAAGACAGTGTTCGCAAAG-3'			

Protein profile analysis by SDS-PAGE

The protein profile was examined using the SDS-PAGE technique. Under these circumstances, the reducing agent (in the case of reducing electrophoresis) and the impact of SDS totally denature proteins (Rosenberg, 2010; Lin et al., 2020).

Measurement of lipid peroxidation

Using the method developed by Heath and Packer in 1968, the amount of malondialdehyde (MDA). produced was utilized to determine the extent of membrane lipid peroxidation (Heath and Packer's, 1968). After that, a reading of the intensity of light absorption at 532 nm wavelength was made.

Hydrogen peroxide (H_2O_2) concentration measurement

In accordance with the interaction of H_2O_2 with potassium iodide (KI), the concentration of hydrogen peroxide was calculated, and the

samples' absorbance values were measured at 390 nm (Junglee et al., 2014).

Statistical analysis

There were three fully randomized replications in the experiment. The statistical program SPSS(v19) examined the outcomes. The analysis of variance operated through ANOVA. Duncan's test was used for the comparison of mean values.

Results

The effect of salinity on the germination rate of cabbage species

Analysis of variance revealed that the effects of salinity stress on the germination rate of Brussels sprouts were significant at the 1% level ($P < 0.01$). As shown in Figure 1a, although every seed germinated within the initial few days under no-salt conditions, the germination rate declined over time. Under salt stress, no increase in germination rate was observed on the 4th day at 50 and 100 mM concentrations; however, by the 6th and 8th days, a rise in germination was noted. At 150 mM, germination remained completely inhibited until the 6th day, with seeds only beginning to germinate on the 8th day.

For broccoli, the impact of salinity stress on germination rate was not statistically significant on the second day of the experiment. However, on the 4th and 8th days, it became significant at the 1% level ($P < 0.01$), and on the 6th day, it was significant at the 5% level ($P < 0.05$). Figure 1b illustrates a progressive increase in germination rate in the control group over time. Under salinity treatments, germination in broccoli increased beginning on the second day at 50 mM, on the 6th and 8th days at 100 mM, and only on the 8th day at 150 mM.

In lunar cabbage, salinity stress significantly affected germination rate on the 4th, 6th, and 8th days at the 1% level ($P < 0.01$). As shown in Figure 1c, germination speed increased at 50 mM salinity on the 6th and 8th days, and at 100 mM salinity on the 8th day. At 150 mM, however, germination speed remained unchanged across all observed days.

The effect of different salinity levels on the *percentage* of germination was statistically significant at the 1% level ($P < 0.01$) in both broccoli and Brussels sprouts, according to the variance analysis. However, for lunar cabbage, the differences in germination percentage were not statistically significant. As indicated in Figure 1d, the highest germination percentage was observed in Brussels sprouts. In this cultivar, only the 150 mM salt concentration reduced germination percentage.

This study highlights the differential germination responses of *Brassica* cultivars to salinity stress and provides valuable insight into cultivar-specific tolerance mechanisms. It highlights how temporal dynamics and concentration-specific responses influence both germination rate and percentage. These findings suggest that optimizing the germination environment and selecting salinity-resilient cultivars are critical strategies for ensuring successful and sustainable crop production on saline soils. The study supports a targeted approach to mitigating salinity stress and improving crop establishment and productivity under adverse conditions.

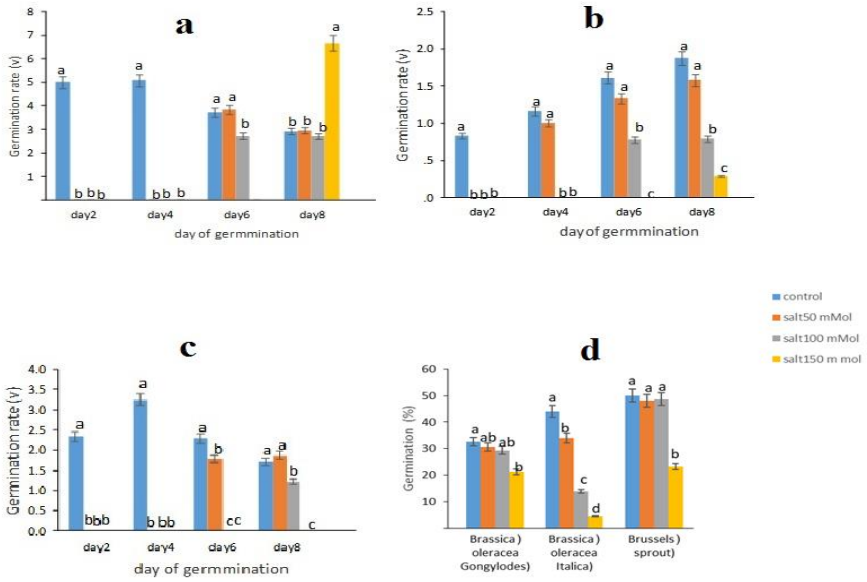

Fig. 1. Examination of (**a**) rates of germination of Brussels sprouts are affected by different salinity concentrations (0, 50, 100, and 150 mM); (**b**) broccoli seeds; (**c**) lunar cabbage seeds; and (**d**) proportion of germination of various types of cabbage seeds. The average ± standard deviation is the presented rate. Averages with similar letters have no statistically significant differences from each other.

Table 2 presents the analysis of variance (ANOVA) for germination percentage among different cabbage species, highlighting significant differences at the 1% level for Brussels sprout and broccoli, while kohlrabi showed no significant variation.

Table 2. Analysis of variance (ANOVA) for germination percentage in cabbage species.

Source	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout *(Brassica oleracea* var. gemmifera)	1475.667	3	491.889	86.804	0.000
Broccoli *(Brassica oleracea* var. italica)	2921.000	3	973.667	78.946	0.000
Kohlrabi *(Brassica oleracea* var. *gongylodes*)	222.333	3	74.111	2.814	ns 0.108

*, ns, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

Consequences of salinity on the length of roots and shoots in various cultivars of cabbage

The findings demonstrated that salt stress significantly affected shoot length in Brussels sprouts, broccoli, and lunar cabbage at the 1% level ($P < 0.01$). As illustrated in Figure 2a, a consistent decline in shoot length was observed across all cabbage types as salinity concentration increased. In lunar cabbage, all three salt concentrations caused a comparable reduction in shoot length. The 150 mM concentration had the most pronounced inhibitory effect on shoot elongation in both Brussels sprouts and broccoli. Analysis of variance further revealed a statistically significant effect of salt stress on root length in Brussels sprouts and lunar cabbage at the 5% level ($P < 0.05$). Figure 2b shows a general downward trend in root length across several cabbage species as salinity levels increased. This reduction was nearly uniform among all cultivars studied. Table 3 presents the ANOVA results for shoot and root lengths across the different cabbage species, indicating significant differences at both the 1% and 5% levels depending on the trait and cultivar.

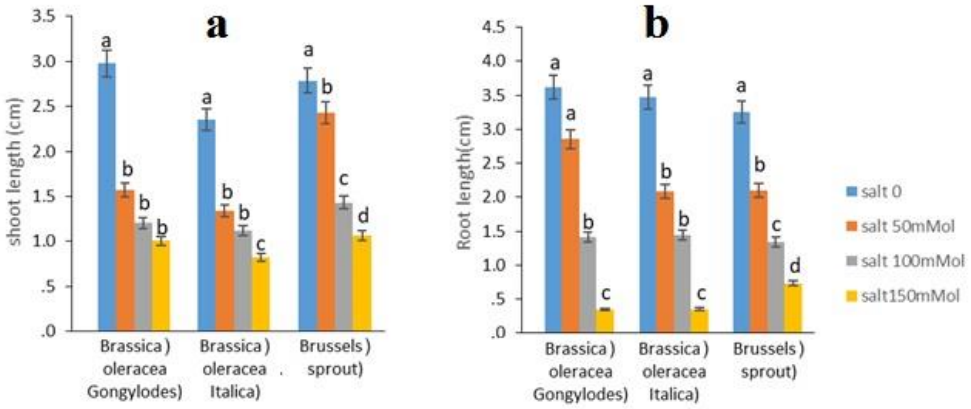

Fig. 2. Analysis of how different salt concentrations (0, 50, 100, and 150 mM) affected two variables: the length of the roots and the length of the shoots in several cabbage species. The data is the standard deviation ± average. Averages with similar letters do not statistically differ from one another.

The impact of salt on the weight of the fresh shoot and root in various cabbage

Salinity stress significantly affected the fresh weight of shoots across several cabbage species at the 1% significance level ($P < 0.01$). As shown in Figure 3a, shoot fresh weight consistently declined with increasing salinity concentration. Among the treatments, plants exposed to 50 mM salt exhibited the greatest reduction in shoot fresh weight compared to the control group. Similarly, the effect of salinity stress on root fresh weight in broccoli, lunar cabbage, and Brussels sprouts was also statistically significant at the 1% level ($P < 0.01$). Root fresh weight decreased progressively in all cultivars as salinity levels increased. Figure 3b indicates that Brussels sprouts exhibited the lowest root fresh weight among the cabbage species studied. Table 4 presents the analysis of variance (ANOVA) for fresh shoot and root weight in different cabbage species, showing highly significant differences ($P < 0.01$) across all traits and cultivars.

Table 3. Analysis of variance (ANOVA) for shoot length and root length in cabbage species.

Cultivar	Trait	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	Shoot Length	3.603	3	1.201	13.241	0.002
	Root Length	22.977	3	7.659	88.341	0.000
Broccoli (*Brassica oleracea* var. italica)	Shoot Length	7.109	3	2.370	22.396	0.000
	Root Length	19.203	3	6.401	28.330	0.000
Kohlrabi (*Brassica oleracea* var. gongylodes)	Shoot Length	3.987	3	1.329	28.785	0.000
	Root Length	15.329	3	5.110	17.175	0.001

*, ns, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

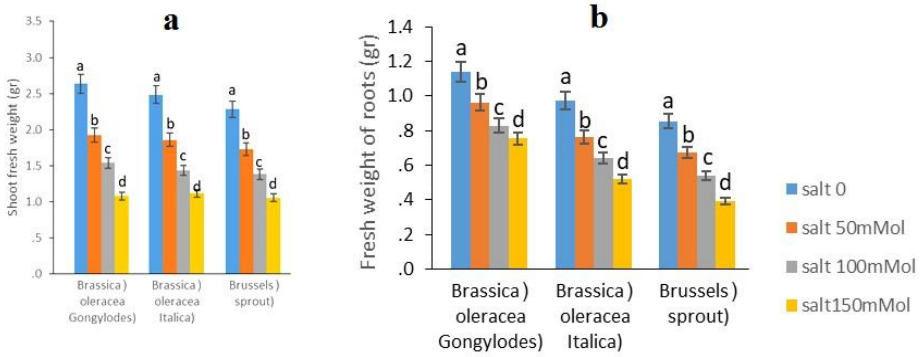

Fig. 3. A comparison of the detrimental impacts of different salt concentrations (0, 50, 100, and 150 mM) on the fresh shoot and fresh root weights of several cultivars of cabbage. Similar-letter averages indicate no statistically significant differences.

Table 4. Analysis of variance (ANOVA) for fresh shoot and root weight in cabbage species.

Cultivar	Trait	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	Fresh Shoot Weight	2.476	3	0.825	370.916	0.000
	Fresh Root Weight	0.349	3	0.116	263.667	0.000
Broccoli (*Brassica oleracea* var. italica)	Fresh Shoot Weight	3.143	3	1.048	131.488	0.000
	Fresh Root Weight	0.335	3	0.112	298.222	0.000
Kohlrabi (*Brassica oleracea* var. gongylodes)	Fresh Shoot Weight	3.906	3	1.302	334.557	0.000
	Fresh Root Weight	0.256	3	0.085	134.702	0.000

*, ns, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

Effects of salinity on the dry weight of the shoot and root in different species of cabbage

Salinity significantly affected the dry weight of shoots at the 1% significance level, as confirmed by variance analysis. As illustrated in Figure 4a, there was notable variation among cabbage species in their response to different salinity concentrations. Among them, Brussels sprouts exhibited the highest

sensitivity to salinity, with a more pronounced reduction in shoot dry weight, particularly at the 150 mM concentration. Similarly, the effect of salinity on root dry weight was also significant at the 1% level. Figure 4b presents the average effect of salinity treatments on root dry weight across different cabbage species, showing a consistent decline in root dry weight as salinity concentration increased.

Table 5 presents the analysis of variance (ANOVA) results for dry shoot and root weight in different cabbage species, revealing highly significant differences ($P < 0.01$) across all traits and cultivars.

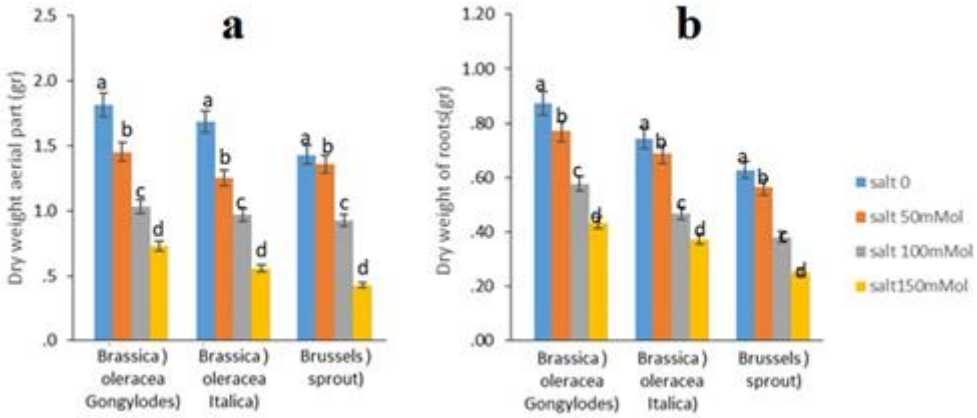

Fig. 4. Comparison of the impact on the dry weight of the (**a**) shoot and (**b**) roots of different cultivars of cabbage at different salinity concentrations (0, 50, 100, and 150 mM). Averages with similar letters do not statistically differ from each other.

Table 5. Analysis of variance (ANOVA) for dry shoot and root weight in cabbage species.

Cultivar	Trait	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	Dry Shoot Weight	1.933	3	0.644	462.890	0.000
	Dry Root Weight	0.267	3	0.089	119.805	0.000
Broccoli (*Brassica oleracea* var. italica)	Dry Shoot Weight	2.025	3	0.675	710.418	0.000
	Dry Root Weight	0.279	3	0.093	192.385	0.000
Kohlrabi (*Brassica oleracea* var. gongylodes)	Dry Shoot Weight	2.027	3	0.676	540.647	0.000
	Dry Root Weight	0.348	3	0.116	309.037	0.000

*, ^{ns}, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

Effect of salinity on glutathione reductase (GR), ascorbate peroxidase (APX) and peroxidase (POX) gene expression in different cabbage species

In the absence of salinity in the environment, *GR* gene expression was highest in brook cabbage and lowest in lunar cabbage, as shown in Figure 5a. The lunar cabbage exhibited the highest amount of *GR* gene expression at a salinity concentration of 100 mM, and the lowest expression at a salinity value of 150 mM. Gene expression in lunar cabbage was found to be increased in sample treated with a 100 mM concentration comparison to those treated with a control and two other salinity concentrations ($P < 0.05$). Control plants in this variety exhibited the least amount of gene expression. As compared to the control, the treatments did not significantly lower the expression of any gene in broccoli ($P < 0.05$). Comparing the three concentrations of gene expression in Brussels sprouts to the control showed the same rise in gene expression. The results demonstrated how different cultivars responded to salt conditions in terms of the expression of the glutathione reductase enzyme gene. Broccoli, lunar cabbage, and Brussels sprouts showed a significant ($P < 0.05$) response to salinity on the expression of the *APX* gene. Brussels sprouts at a concentration of 100 mM exhibited the highest expression of the *APX* gene under saline

conditions (Fig. 5b, average 10.033). Additionally, at the 5% level ($P < 0.05$), there was a significant influence of salinity on the expression of the *POX* gene in various cultivars of cabbage. Lunar cabbage exhibited the lowest average gene expression (11.495) in the salinity treatment at a dose of 50 mM. Figure 5c showed that there was no significant difference ($P > 0.05$) in the *POX* gene expression level in the examined species at a salinity concentration of 150 mM. The application of salt treatment in all three concentrations was unable to raise this gene's expression in treated groups compared to the control ($P < 0.05$).

Table 6 presents the analysis of variance (ANOVA) for the expression of *GR*, *PCX*, and *POX* genes in different cabbage species, demonstrating significant differences ($P < 0.05$) across all measured traits.

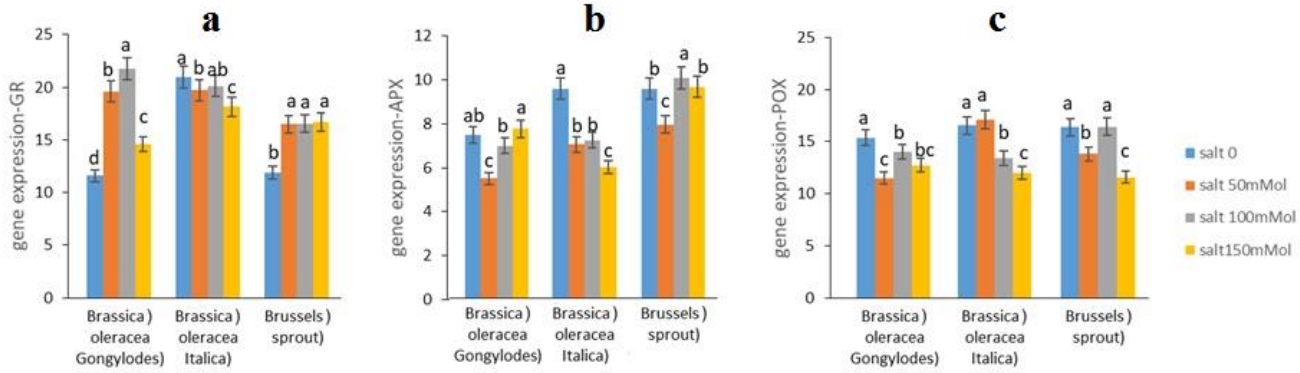

Fig. 5. An investigation of the effects of various quantities of salt (0, 50, 100, and 150 mM) on the expression of the genes (**a**) *GR*; (**b**) *APX*; and (**c**) *POX* in many cabbage species. Averages with similar letters do not statistically differ from one another.

Table 6. Analysis of variance (ANOVA) for gene expression (*GR*, *PCX*, and *POX*) in cabbage species.

Cultivar	Gene	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	*GR*	50.168	3	16.723	13.630	0.002
	PCX	8.001	3	2.667	2.918	0.001
	POX	44.134	3	14.711	4.067	0.050
Broccoli (*Brassica oleracea* var. italica)	*GR*	12.443	3	4.148	4.148	0.003
	PCX	20.364	3	6.788	5.572	0.023
	POX	25.095	3	8.365	8.895	0.006
Kohlrabi (*Brassica oleracea* var. gongylodes)	*GR*	193.657	3	64.552	64.552	0.002
	PCX	9.054	3	3.018	0.340	0.007
	POX	54.018	3	18.006	4.252	0.045

*, ^{ns}, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

Impact of salt on the activity of the enzyme's glutathione reductase, guaiacol peroxidase, and ascorbate peroxidase in cabbages

The analysis of variance revealed that the ascorbate peroxidase enzyme activity in various cabbage species was significantly affected by different salinity levels. Figure 6a presents a comparison of the average ascorbate peroxidase activity under various salt treatments. All cabbage species showed an increase in enzyme activity when the salt concentration was raised to 150 mM. Among them, Lunar cabbage exhibited the highest ascorbate peroxidase activity at 150 mM salinity. With the exception of broccoli, all species displayed nearly identical activity levels at this concentration. In broccoli, enzyme activity showed only a slight increase under salinized conditions compared to the control. The analysis of variance also demonstrated that guaiacol peroxidase enzyme activity in several cabbage species was significantly influenced by salinity. Among the tested species, Brussels sprouts exhibited the highest level of guaiacol peroxidase activity. These findings indicate that guaiacol peroxidase is one of the enzymes activated by salinity in cabbage species, with its activity increasing as the salinity concentration rises. Figure 6c shows the comparison of average glutathione reductase enzyme activity across various salt treatments. Different cabbage types exhibited elevated glutathione reductase activity in response to increasing salinity. Broccoli showed the highest

activity at 50 and 100 mM salt concentrations, while Lunar cabbage displayed the highest enzyme activity at 150 mM. However, there was no significant difference in enzyme activity between the 100 and 150 mM salinity treatments.

Table 7 presents the ANOVA results for the activity of antioxidant enzymes (ascorbate peroxidase, guaiacol peroxidase, and glutathione peroxidase) in different cabbage species, highlighting significant differences ($P < 0.05$) among treatments.

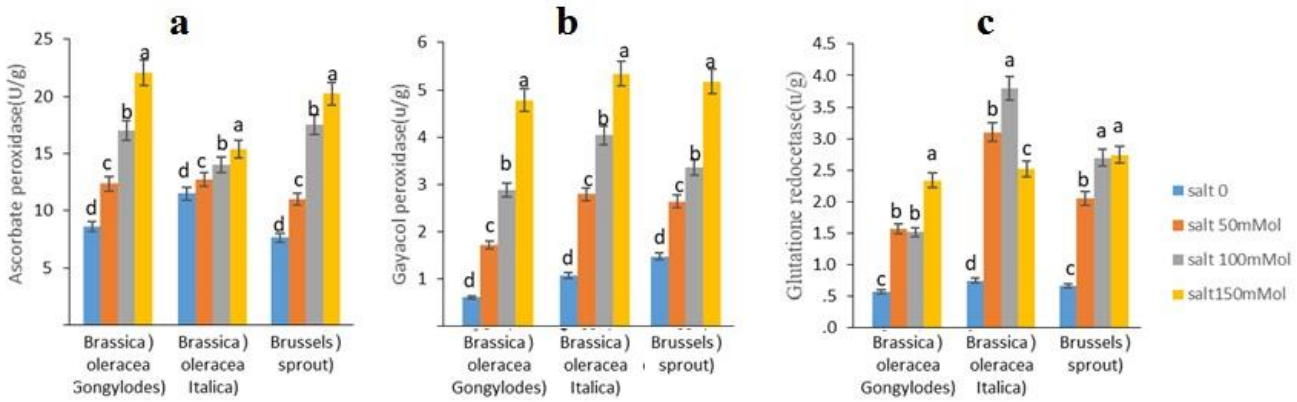

Fig. 6. Comparing the effects of varying salinity concentrations (0, 50, 100, and 150 mM) on the activity of the enzyme's ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase in several cabbage species. Similar-letter averages do not differ from one another significantly.

Table 7. Analysis of variance (ANOVA) for enzymatic activities in cabbage species.

Cultivar	Enzyme	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	Ascorbate Peroxidase (APX)	300.503	3	100.168	71.322	0.000
	Guaiacol Peroxidase (GPX)	21.669	3	7.223	37.537	0.000
	Glutathione Peroxidase (GSH-Px)	8.420	3	2.807	42.163	0.000
Broccoli (*Brassica oleracea* var. italica)	APX	787.733	3	262.578	194.208	0.000
	GPX	29.814	3	9.938	123.4058	0.000
	GSH-Px	15.357	3	5.119	18.374	0.001
Kohlrabi (*Brassica oleracea* var. gongylodes)	APX	307.043	3	102.348	52.473	0.000
	GPX	28.589	3	9.530	487.670	0.000
	GSH-Px	4.723	3	1.574	5.260	0.027

*, ^{ns}, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

The effects of salinity on the protein content of several species of cabbage

According to the results of the variance analysis, the salt levels varied significantly affected the protein content of several cabbage species. Figure 7 shows the comparison results of the average protein content under different salt concentrations. Under salt stress, the protein content of many cabbage species increased in comparison to the control. Compared to other cabbage species, the amount of protein in lunar cabbage rose greater at a salt concentration of 50 mM. Other species, with the exception of broccoli (51.617µg g^{-1}), exhibited comparable protein levels at 100 mM. The maximum protein content was found in lunar cabbage at 150 mM salinity, with an average of 96.102 µg g^{-1}.

Table 8 presents the ANOVA results for protein content in different cabbage species, demonstrating statistically significant differences ($P < 0.05$) among treatments.

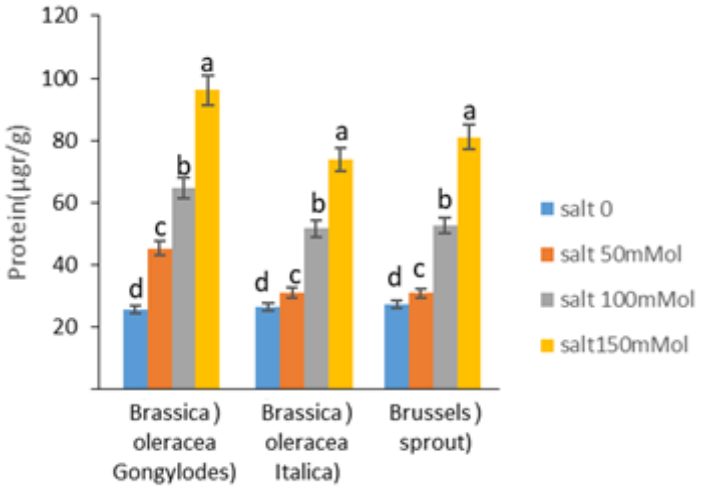

Fig. 7. A comparison between the protein content of various cabbage species at varying salt concentrations (0, 50, 100, and 150 mM). Similar-letter averages do not differ from one another statistically significantly.

Table 8. Analysis of variance (ANOVA) for protein content in cabbage species.

Cultivar	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	5519.425	3	1839.808	10.889	0.003
Broccoli (*Brassica oleracea* var. italica)	4240.050	3	1413.350	14.223	0.001
Kohlrabi (*Brassica oleracea* var. gongylodes)	8164.051	3	2721.350	48.779	0.000

*, ^{ns}, and ** indicate non-significant, significant at the 5% level, and significant at the 1% level, respectively.

The effects of salinity on various cabbage species' protein profiles

The protein patterns of three cabbage species grown under different salinity treatments were examined. Figure 8 displays images of the separated protein bands. A prominent main band with a high molecular weight of 72 kDa was consistently observed in all species across all treatments. This high molecular weight band pattern indicates that all species showed a strong increase in 72 kDa protein production under varying salinity conditions. Additionally, low molecular weight proteins, as small as 10 kDa, were detected in Brussels sprouts and Lunar cabbage. As salinity levels increased, protein concentrations also rose, with the highest levels observed at 100 and 150 mM. These findings confirm that protein band patterns can undergo notable changes under salt stress conditions.

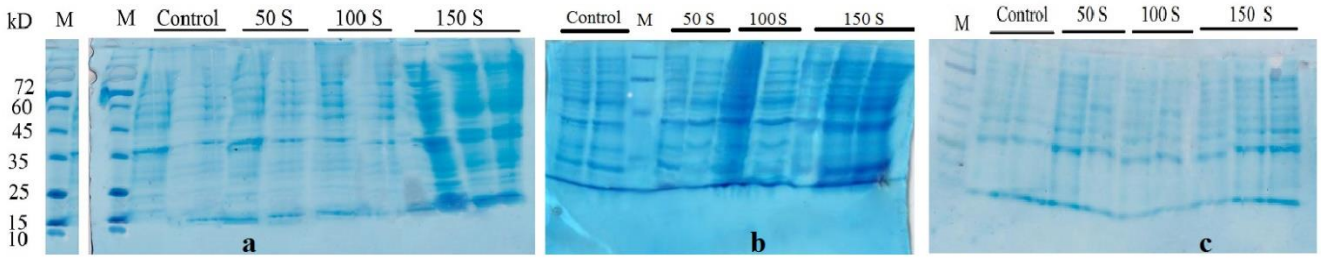

Fig. 8. Protein profile of (**a**) Brussels sprouts, (**b**) broccoli, and (**c**) lunar cabbage.

In the control treatment of Brussels sprouts, a 35 kDa protein band was observed, which also appeared in the other salinity treatment groups, showing increased intensity under the 150 mM treatment. Consistent with the results of the total protein assay, the 150 mM treatment group exhibited more protein

bands than the other groups. At this salinity level, broccoli displayed a greater number of bands and increased band thickness compared to other treatments. Overall, however, there was no considerable difference in protein banding patterns among the various salinity conditions.

The influence of salt on the amount of malondialdehyde in different species of cabbage

The analysis of variance revealed significant differences in malondialdehyde (MDA) levels among different cabbage species under varying salt concentrations ($P < 0.05$). Figure 9a presents a comparison of the average MDA content across salinity treatments. In all cabbage types, MDA levels increased under salt stress, peaking at 150 mM. However, there were no significant differences among the cabbage species in terms of this response. The analysis of hydrogen peroxide content is shown in Figure 9b, which compares the average levels under different salinity concentrations. Hydrogen peroxide levels increased with rising salinity. Among the treatments, broccoli exposed to 150 mM salt exhibited the lowest average hydrogen peroxide concentration (11.66).

Table 9 presents the ANOVA results for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content in different cabbage species, indicating significant differences ($P < 0.05$) among treatments.

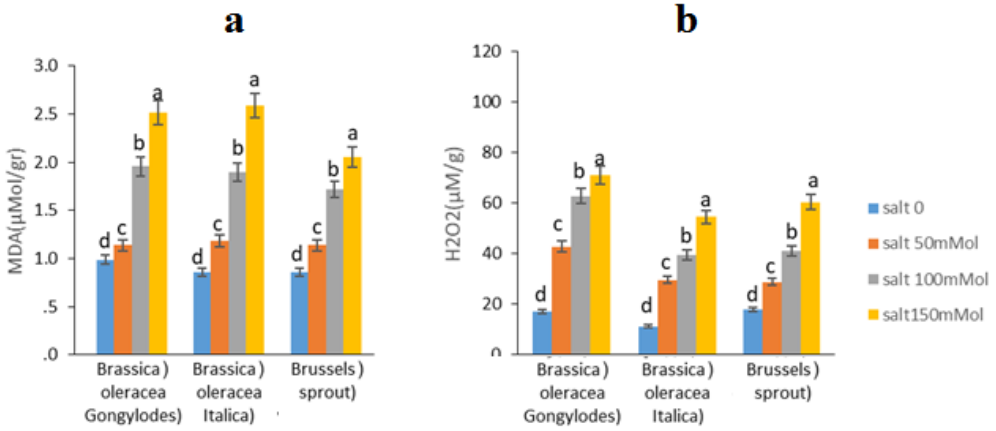

Fig. 9. A comparison of the effects of varying salt concentrations (0, 50, 100, and 150 mM) (**a**) on the levels of malondialdehyde and (**b**) hydrogen peroxide in various kinds of cabbage. Similar-letter averages do not differ from one another significantly.

Table 9. Analysis of variance (ANOVA) for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content in cabbage species.

Cultivar	Parameter	Type III Sum of Squares	Degrees of Freedom (D.F.)	Mean Square	F-Statistic	Significance (Sig.)
Brussels Sprout (*Brassica oleracea* var. gemmifera)	MDA Content	33.879	3	11.293	1.160	0.038
Broccoli (*Brassica oleracea* var. italica)	MDA Content	42.881	3	14.294	1.203	0.026
Kohlrabi (*Brassica oleracea* var. gongylodes)	MDA Content	61.398	3	20.466	1.179	0.030
Brussels Sprout (*Brassica oleracea* var. gemmifera)	H_2O_2 Content	2995.833	3	998.611	14.749	0.001
Broccoli (*Brassica oleracea* var. italica)	H_2O_2 Content	2975.000	3	991.667	9.288	0.006
Kohlrabi (*Brassica oleracea* var. gongylodes)	H_2O_2 Content	5230.729	3	1743.576	74.393	0.000

The correlations between *GR*, *APX*, and *POX* gene expression and *GR*, *APX*, and *POX* enzymes appear in Figure 10. The findings demonstrated a strong positive (dark blue R = 1) and strong negative (dark red R = -1) correlation between the enzymes *GR*, *APX*, *POX*, and gene expression (Fig. 10).

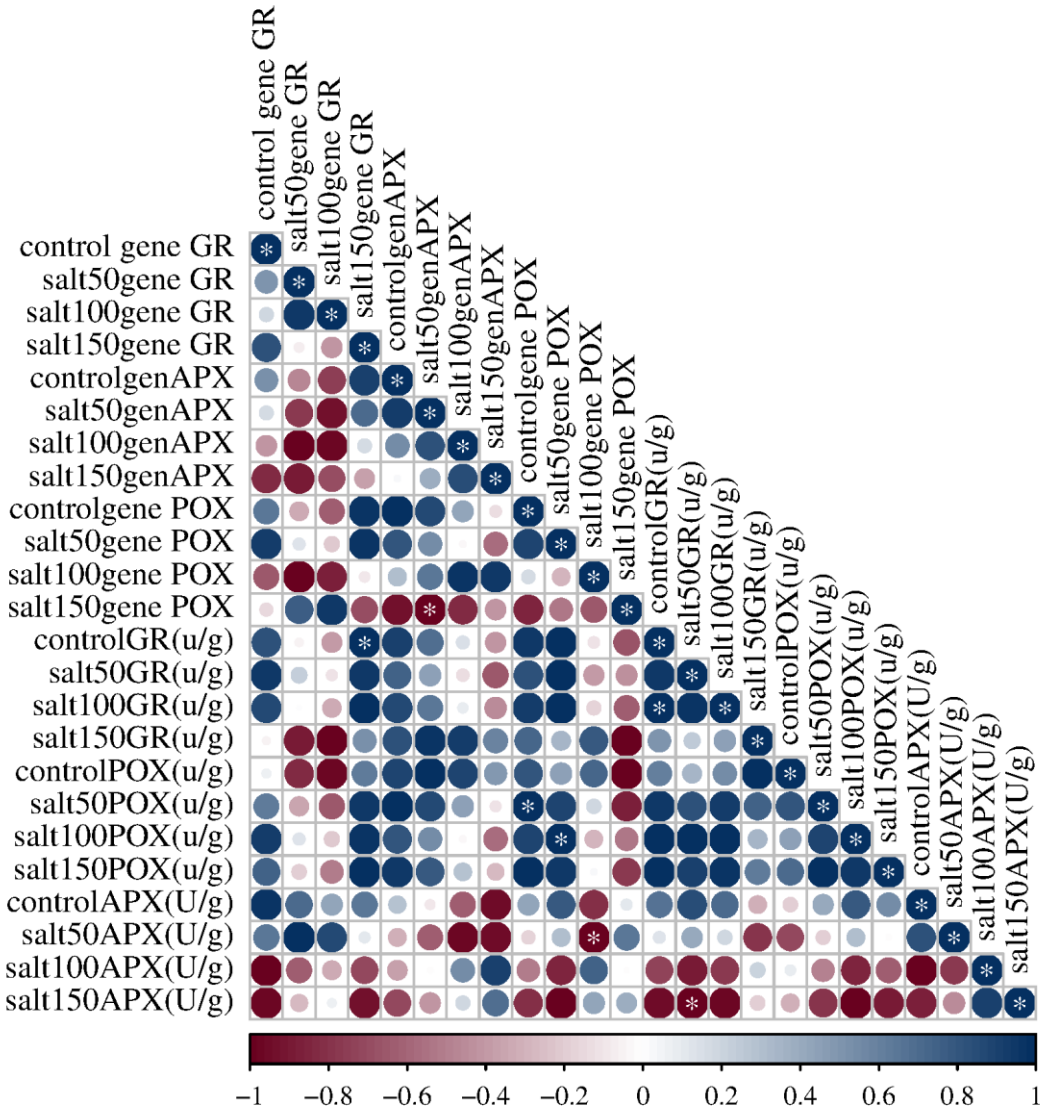

Fig. 10. *Correlation heatmap of gene expression and enzymatic activities under salt stress.* The heatmap depicts the correlations between gene expression levels (GR, APX, and POX) and their corresponding enzymatic activities across different salinity treatments. Color and circle size represent the strength and direction of the correlations: dark red indicates strong negative correlations, while dark blue represents strong positive correlations. The intensity of the color and the size of the circles are proportional to the absolute values of the correlation coefficients. Statistically significant correlations ($P < 0.05$) are marked with an asterisk (*), highlighting key relationships that may reflect regulatory or compensatory interactions within the antioxidant defense system. These correlations provide insights into the coordinated transcriptional and enzymatic responses to salt stress in *Brassica napus*.

This study elucidates the molecular, biochemical, and physiological mechanisms underlying salinity tolerance in cabbage species, with a particular focus on key antioxidant genes—*GR*, *APX*, and *POX*—and their associated enzymatic activities. The elevated activity of APX and GR in Lunar cabbage at 150 mM salinity underscores its enhanced antioxidative defense response. Additionally, the appearance of unique stress-induced protein bands, such as the prominent 72 kDa band in Brussels sprouts, reflects distinct adaptive strategies under salt stress. These findings offer valuable molecular markers for salinity resilience and provide a foundation for breeding or engineering *Brassica* crops capable of thriving in saline environments, thereby contributing to sustainable agriculture and global food security.

Discussion

This study investigated the effects of different salinity levels on the physiological, biochemical, and genetic characteristics of three well-known cabbage cultivars: Lunar cabbage, broccoli, and Brussels sprouts. Soil salinity is a global issue, affecting more

than one billion hectares across 100 countries (Singh, 2022). The presence of excessive salt in the soil disrupts plant physio-biochemical processes, thereby impairing growth, development, and overall productivity. Salinity significantly influences multiple stages of plant growth, including germination, vegetative development, and reproductive dynamics (Hailu and Mehari, 2021). Furthermore, it induces ion toxicity, osmotic stress, nutrient deficiencies (notably N, Ca, K, P, Fe, Zn), oxidative stress, and limits water uptake from the soil (Huang et al., 2023).

Among the studied cultivars, Lunar cabbage, broccoli, and Brussels sprouts demonstrated notable resistance to salt stress and showed potential as sources of antioxidants. Their resilience can largely be attributed to their ability to mitigate oxidative damage through antioxidant defense mechanisms, a key determinant of salinity tolerance. This observation is consistent with findings by Jamil et al. (2005), who reported that salinity treatments significantly affected germination percentage, germination rate, shoot and root lengths, shoot and root fresh weights, leaf area, and the number of leaves in *Brassica napus*, *Brassica oleracea*, and *Brassica oleracea* var. *botrytis*. In our study, germination percentage and rate decreased as salinity levels increased, aligning with these earlier results.

Further supporting evidence comes from Gürsoy et al. (2022), who found that salinity significantly reduced germination percentage, germination speed, seedling length (SL), root length (RL), seedling fresh weight (SFW), root fresh weight (RFW), relative water content (RWC), total chlorophyll (Total Chl), and carotenoids (Crt). Their study also showed that chitosan treatment alleviated the detrimental effects of salt stress. Similarly, Mosavikia (2020) examined the effects of chitosan nanoparticles and pyridoxine seed priming on the salt tolerance of milk thistle seedlings. The results demonstrated that salt stress at 0, 50, 100, and 150 mM significantly reduced germination percentage (by 49.12%), seed length (by 50.07%), and chlorophyll a and b levels. However, priming with chitosan and pyridoxine improved salt tolerance by enhancing physiological traits, including proline content, antioxidant enzyme activity, and photosynthetic pigmentation, findings that align with our observations. Odat et al. (2021) also reported that salinity caused a significant reduction in vetch (*Vicia sativa*) hypocotyl and radicle dry weight, as well as overall seedling length, compared to control plants treated with water. Their study highlighted the benefits of chitosan seed priming in improving germination and early growth metrics in *Vicia sativa* under salt stress conditions.

Regardless of the salt concentration, chitosan significantly improved key growth parameters, including hypocotyl length, dry weight of hypocotyl leaves, and radicle dry matter across all treatment levels. These findings suggest that chitosan effectively mitigates salinity-induced stress in plants. Functioning as an elicitor, chitosan triggers a variety of defense and stress tolerance mechanisms that help plants cope with adverse conditions. In our study, chitosan seed priming played a significant role in enhancing early growth metrics, supporting the idea that it can reduce salt-induced damage through multiple physiological pathways.

Zhang et al. (2021) similarly reported that exogenous application of chitosan under salt stress conditions increased plant biomass and overall growth, while reducing sodium accumulation and enhancing potassium uptake in leaves. These effects collectively contributed to improved salt tolerance, consistent with our findings. In their study on lettuce, chitosan treatments led to increased resilience to salinity stress.

Morovvat et al. (2020) investigated the effects of foliar applications of salicylic acid (0.5 g L^{-1}), chitosan (2 g L^{-1}), and a combination of both on plant physiological traits and performance under reduced irrigation. Their results showed that decreased irrigation led to a reduction in yield and physiological indices. However, both salicylic acid and chitosan improved biological yield under stress conditions. Notably, the highest yield was recorded at 45,717 kg ha^{-1} under non-stress conditions and 45,683 kg ha^{-1} in plants treated with a combination of 2 g L^{-1} chitosan and 0.5 g L^{-1} salicylic acid.

Chitosan is known to promote plant growth by influencing multiple developmental processes and providing protection against a variety of plant diseases. Through its interaction with primary and secondary messengers in the stress signal transduction system, chitosan can reduce the harmful effects of environmental stresses. Attia et al. (2021) examined the anti-stress potential of chitosan dissolved in various organic acids and applied as a foliar spray on tomato plants (*Solanum lycopersicum* L.) under salt stress. Their study investigated how a chitosan–organic acid mixture affected protein content, enzyme activity, and other physiological characteristics, further supporting the use of chitosan as a stress alleviator under saline conditions.

According to the findings, salt stress inhibited plant growth and reduced the production of essential components such as pigments, carbohydrates, proteins, and potassium. In contrast, tomato plants (*Solanum lycopersicum*) grown under saline conditions exhibited elevated levels of antioxidant enzyme activity, proline, ascorbic acid, total phenols, hydrogen peroxide (H_2O_2), sodium (Na^+), and malondialdehyde (MDA). Application of chitosan to non-stressed plants improved their appearance and enhanced the production of defense-related compounds, pigments, and nutritional content. These observations are consistent with the results of our study, which similarly demonstrated that chitosan

treatment enhanced the visual health and metabolic activity of non-stressed plants. Furthermore, chitosan application reduced the concentrations of MDA, H_2O_2, and Na^+, thereby mitigating the harmful effects of salinity on tomato plants.

Plants, whether stressed or not, responded differently to chitosan in terms of the levels and activities of specific enzymes such as peroxidase, polyphenol oxidase, and superoxide dismutase. Salt stress altered protein profiles by affecting the expression of proteins of various molecular weights, but these changes were partially or fully reversed following chitosan treatment. These findings are in agreement with our own results.

In addition, the study reported that, in non-saline conditions, broccoli exhibited the highest expression of the *GR* gene, while Lunar cabbage showed the lowest expression. These findings support those of Abdelaal et al. (2019), who demonstrated that salinity significantly increased lipid peroxidation—measured by elevated MDA levels—as well as superoxide and hydrogen peroxide accumulation compared to non-saline conditions. Lipid peroxidation serves as a reliable biomarker for cellular damage under salt stress. The increase in MDA content and elevated lipid peroxidation observed in our study further confirm the presence of oxidative stress in plants exposed to salinity. This is consistent with the findings of Liu et al. (2022) and Liu et al. (2014), who reported increased MDA levels in *Solanum lycopersicum* and *Torreya grandis* under salt stress.

According to Idrees et al. (2011), plants have evolved a defense mechanism known as antioxidant metabolism to counteract harmful compounds called reactive oxygen species (ROS). Zhang et al. (2023) also noted that enzymatic antioxidants play a critical role in plant defenses against various abiotic stresses by scavenging ROS. Multiple studies (Hussain et al., 2023; Gedeon et al., 2022; González-García et al., 2021) have shown that *Catharanthus roseus* exhibits an upregulation of its antioxidant system under salt stress conditions. Similarly, Brenes et al. (2020) reported that plants subjected to plant growth-promoting rhizobacteria (PGPR) combined with salinity stress showed increased expression levels of several ROS pathway genes—*APX*, *CAT*, *GR*, and *DHAR*—relative to untreated controls, as confirmed by RT-PCR data.

In sweet peppers, the negative effects of soil salinity are less severe than in many other species, largely due to the increased activity of key antioxidant enzymes such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and glutathione reductase (GR). This enzymatic response constitutes a natural defense mechanism, enhancing the plant's ability to adapt to salinity stress by scavenging ROS and mitigating both oxidative and osmotic damage. Our findings align with those of Foyer et al. (1994) and ALKahtani et al. (2020). Importantly, our data demonstrated that chitosan pretreatment enhanced the expression of *POX*, *APX*, and *GR* in salt-stressed cabbage cultivars. The stimulation of these enzymes in chitosan-treated plants contributes to improved resilience under salinity stress, underscoring the compound's role as an effective elicitor of antioxidant responses.

It is well established that a plant species' susceptibility or tolerance to salt is determined by its genetic and biochemical makeup. Plants adapted to saline environments often display distinct patterns of protein synthesis and accumulation. Salinity may lead to an increase or decrease in total soluble protein compared to control conditions, and in some cases, specific proteins may disappear entirely (Yildiz, 2007). Salt-tolerant and salt-sensitive genotypes exhibit clearly differentiated protein profiles (Witzel et al., 2014). The use of protein banding patterns as indicators offers a cost-effective approach to identifying salt-tolerant cultivars. In our study, the increase in protein bands during salt stress was shown to depend on the salinity concentration. This finding aligns with observations by Mohammad (2007), who reported that *Vigna radiata* (mung bean) exposed to salt stress exhibited an increase in its protein banding profile.

Conclusions

The findings of this study demonstrated that salt stress induces an increase in protein content across all three *Brassica* species examined, including *Brassica oleracea* var. *Brussels sprout* and *Brassica oleracea* var. *gongylodes*. Notably, the protein profile revealed that most of the stress-responsive proteins were of low molecular weight. Antioxidant enzyme activity, particularly ascorbate peroxidase (APX) and glutathione reductase (GR), was found to correlate with elevated levels of malondialdehyde (MDA), a key marker of oxidative stress. Interestingly, enzymatic activity levels exceeded the corresponding gene expression levels for APX and GR, suggesting that the stress response was primarily driven by the activation of pre-existing enzymes rather than de novo protein synthesis. This study further uncovers the complex interaction between gene expression and enzymatic activity of APX and GR under salt stress, highlighting the essential role of post-translational modifications in modulating the oxidative stress response. Moreover, the identification of low molecular weight proteins that respond to salinity stress offers promising candidates as molecular biomarkers, potentially guiding targeted breeding strategies. These insights contribute to the development of *Brassica* cultivars with enhanced salinity tolerance by optimizing molecular and biochemical traits. Ultimately, this work supports the advancement of sustainable

agricultural practices through improved crop adaptability in saline environments.

Author contributions
All authors read and approved the final manuscript.

Funding
This research received no external funding.

Conflict of Interest
The authors indicate no conflict of interest in this work.

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