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Chitosan pre- and post-treatment modulates molecular and physiological responses to salinity in *Brassica Napus L*

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Salt stress affects plant growth and development to a great extent in establishing biochemical imbalances and inducing oxidative damage. The effort was undertaken to assess the relative effectiveness of chitosan pre- and post-treatments in relieving salt stress in *Brassica napus L.*, a moderately salt-sensitive oilseed crop, with respect to the expression patterns of *P5CS*, *PIP*, and *APX* genes along with proline and protein accumulation. One factorial experiment was performed under controlled conditions, where the plants were exposed to varying NaCl concentrations (50, 100, 150, and 200 mM), following either the pre-treatment of chitosan (50 mg/L) or post-treatment of chitosan (50 and 100 mg/L). Gene expression analysis by means of qRT-PCR showed a significant ($p < 0.05$) upregulation under pre-treatment of *P5CS* and *APX*, thereby enhancing osmoprotection and antioxidant defense; however, under post-treatment, the response of *P5CS* and *APX* was only moderate. Post-treatment levels of proline and total protein were markedly higher as well. Interestingly, proline content increased by approximately 743% in pre-treated plants at 200 mM of NaCl compared to the control ($p < 0.05$), indicating enhanced stress tolerance. Besides, the plants subjected to pre-treatment were perfectly grown at 200 mM NaCl whereas those subjected to post-treatment were unable to survive at 150 mM. These findings reveal that the salt tolerance mechanism is better gained by chitosan pre-treatment due to osmotic adjustment enhancement and antioxidative mechanism. This study presents the potential of chitosan to be developed as a strategic biostimulant in promoting crop resistance to saline conditions.

Keywords Chitosan pre-treatment, Chitosan post-treatment, Salt stress, *Brassica Napus L.*(Canola), Oxidative stress, Proline accumulation, Gene expression (*P5CS*, *APX*, *PIP*)

Salt stress is among the imperiling abiotic conditions affecting agricultural productivity worldwide, especially in the case of salt-sensitive crops like *Brassica napus L.* (canola or rapeseed)¹. The salinity hampers some essential physiological and biochemical processes such as the uptake of water, nutrient balancing, and carbon assimilation, finally leading to the reduction in growth and yield of the plant^{2–4}. Osmotic stress sets in immediately after salt exposure in the initial phase, once the salinity reaches high levels, ion toxicity takes place; both of these malpractices lead to a disruption of ionic homeostasis, thereby affecting cellular functioning^{5–8}. Plants produce osmolites like proline and glycine betaine, etc., which help them to keep osmotic balance in place⁹. An excessive quantity of sodium (Na⁺) and chloride (Cl⁻) ions interferes with enzymatic activity, nutrient uptake, and cellular hydration; hence, their ejection toward regions of lower excess occurs through selective ion transporters^{10,11}. Additionally, salt stress triggers oxidative stress accompanied by an overproduction of reactive oxygen species (ROS) such as H₂O₂, O₂[−], and ·OH, which exacerbate cellular components unless depleted by antioxidant mechanisms^{12,13}. Indeed, stress conditions induce the reprogramming of gene expression and the activation of defense signaling pathways¹⁴.

Chitosan, a biopolymer derived from chitin, has gained attention as a biostimulant capable of enhancing plant resilience to environmental stresses, including salinity¹⁵. Chitosan functions as a natural defense elicitor by triggering the expression of stress-responsive genes and stimulating various defense signaling pathways in

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plants¹⁶. Additionally, it plays a crucial role in promoting plant growth by modulating key cellular processes, including ion homeostasis and antioxidant defense mechanisms, particularly under stress conditions¹⁷. Chitosan mediates stress signaling pathways, leading to the upregulation of osmoprotectants, aquaporins, and antioxidant enzymes¹⁸. *P5CS* (Δ^1 -pyrroline-5-carboxylate synthetase) is one of the most crucial genes involved in the salt stress response that is important for the synthesis of proline, an osmoprotectant that stabilizes cellular structures under stress¹⁹. The other important genes include *PIP* (plasma membrane intrinsic protein), which controls the transportation of water through cellular membranes so as to balance the quantity of cellular water in cells under stress conditions; and *APX* (ascorbate peroxidase), which is a vital enzyme that scavenges for reactivity oxygen species (ROS), sustaining the activities of cells under oxidative damage due to salt stress²⁰. Chitosan has been extensively studied for several decades for alleviating salt-related stress. However, limited research has been conducted to compare its effects on gene expression in rapeseed using different strategies of chitosan application, including pre-treatment and post-treatment. Knowledge of how various modes of chitosan application affect the expression pattern of *P5CS*, *PIP*, and *APX* genes may help to improve its usage for enhancing salt tolerance in rapeseeds. This study intends to explore the expression pattern of these genes under salt stress conditions with respect to the different applications of chitosan as pre-treatment or post-treatment; thus, it will assist in developing advanced management strategies for salinity in crop production.

Materials and methods

Plant material

The current study performed investigations using the German hybrid rape seed cultivar of Rohan, which was provided by the company Arman Sabz from Adina in Isfahan.

Experimental procedures: pre-treatment

A completely randomized factorial design was used with three biological replicates per treatment. Plastic pots (14 cm diameter) were filled with a 1:1 (v/v) mixture of cocopeat and perlite. Seeds were sown and after 8 days of germination, seedlings were irrigated with Hoagland solution for 15 days. Chitosan pre-treatment (50 mg/L) was applied via foliar spray for 4 consecutive days. After this, plants were subjected to NaCl salinity stress at concentrations of 0 (control), 50, 100, 150, and 200 mM for 4 days. Leaf sampling was performed at three time points: 0, 10, and 20 days after salt treatment initiation. Each condition included three biological replicates ($n = 3$). Gene expression analysis was carried out using qRT-PCR (Fig. 1).

Experimental procedures: post-treatment

This experiment was also conducted in a completely randomized factorial design with three replicates per treatment. Seeds were sown in plastic pots (14 cm diameter) filled with a 1:1 (v/v) cocopeat and perlite mix and irrigated three times daily with distilled water until germination. After germination, plants were irrigated daily with distilled water and weekly with Hoagland solution. At the three-leaf stage, plants were subjected to four salinity treatments (0, 50, 100, and 150 mM NaCl) and three chitosan levels (0, 5, and 10 mg/L). Chitosan was applied via foliar spray every other day for 4 weeks. NaCl was also applied consistently throughout this period. For gene expression analysis, leaf samples were collected 48 h after the final treatment. All treatments included three biological replicates ($n = 3$) (Fig. 1).

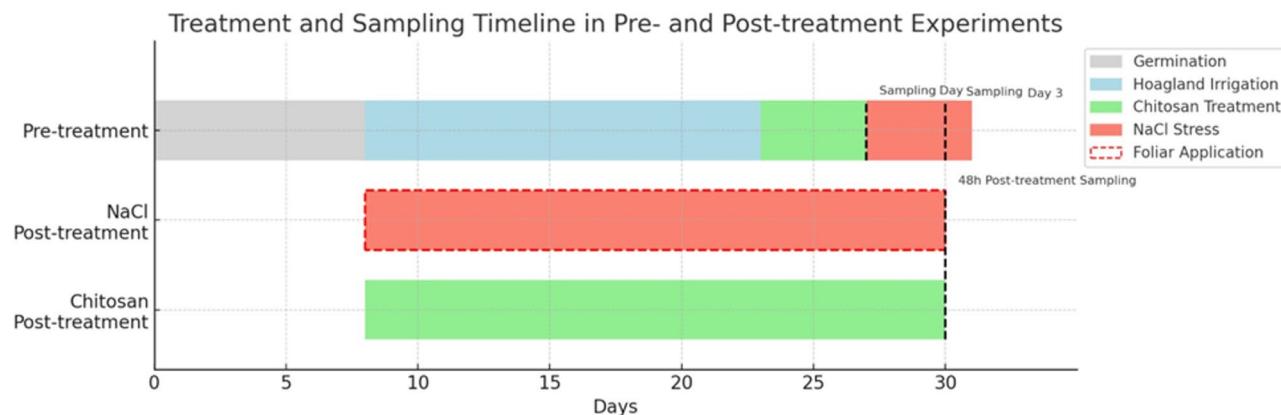


Fig. 1. Timeline of experimental treatment and sampling for both pre- and post-treatment periods.

Germination took place in the first 8 days, after which Hoagland nutrient irrigation was started. During the pre-treatment stage, chitosan was applied, at a concentration of 50 mg/L, for 4 days; then, the NaCl treatments (50 to 200 mM) were applied. The post-treatment design called for spraying the plants with chitosan (0, 5, and 10 mg/L concentrations) and NaCl (0, 50, 100, and 150 mM) every 2 days for a duration of 4 weeks. The gene expression study was done 48 h after the final treatment.

RNA extraction and the synthesis of cDNA

Total RNA was isolated from precipitation with lithium chloride according to previous methods²¹²². First, cDNA synthesis was performed according to the manufacturer's protocol using the GeneALL kit (Cat. No. 601–602). A 0.2 mL vial containing 2.5 μ L of RNA, 0.5 μ L of dNTP mix, 0.5 μ L of oligo dT, and 0.5 μ L of random hexamer was placed in a PCR machine and incubated at 70 °C for 5 min. The vial was then removed and placed on ice for 2 min. Afterward, 0.5 μ L of reverse transcriptase, 0.5 μ L of 2X buffer, and 0.5 μ L of RNasin were added to each vial. The reaction mixture was then subjected to PCR cycling conditions: 25 °C for 10 min, 42 °C for 50 min, and 80 °C for 10 min. The cDNA products were frozen and stored at last for further analyses at –20 °C.

Real-time PCR

In this study, the real-time PCR runs were performed using an ABI Step One Real-Time PCR system (Applied Biosystems) along with a BioFACT™ kit. This was performed according to the manufacturer's instruction manual. Following completion of PCR amplification and establishment of the threshold line, Cycle Threshold (Ct) values from the amplification curves were exported from the Gene Rotor software to an Excel spreadsheet for analysis. Data normalization was done using the expression of the ribosomal 16 S RNA gene in the GenEx software environment. SPSS V20 was used for statistical analysis and graphical representation, as detailed in Table 1²³. Primers were designed by means of Gene Runner v6.5.5 \times 64, a program commonly used for primer design considerations and mainly based on the sequences of the target genes. The primers were subsequently optimized for specificity and efficiency toward the following target genes: P5CS, PIP, and APX, along with a reference gene, 18 S rRNA. They were synthesized on an ABI 394 synthesizer (ABI, USA) following standard protocols for oligonucleotide synthesis in order to yield the desired quality of primers for the purpose of RT-PCR amplification with recommended purity and sequence fidelity.

Reference for primer sequences, annealing temperature, and accession numbers for the NCBI GenBank for ORF-1ab targets is provided in Table 1.

Formula1: $\Delta CT = CT$ (a target gene*) – CT (a reference gene**).

* = PIP, APX, P5CS.

** = mCtRNA.

Preparation and activity assay of ascorbate peroxidase (APX)

Plant tissues were powdered with liquid nitrogen to a fine powder, and 50 mM Tris-HCl buffer solution (pH 7.4) containing 0.6 M KCl, 7 mM MgCl₂, 3 mM EDTA, 1 mM DTT, and 5% PVP was added to 0.25 g of tissue material. The homogenized mixture was filtered, and the resulting solution was centrifuged at 4 °C at 30,000 g for 35 min. The supernatant obtained was subsequently used for the measurement of enzyme activity for APX, following the protocols established by^{24,25}. Enzyme activity assays were conducted using 50 mM Tris-HCl buffer (pH 7.0) with 20 mM MgCl₂ and other essential components, with absorbance measured at 535 nm as per the adaptations by^{26,27}.

Ascorbate peroxidase (APX) activity was determined following the method described by²⁸. The reaction mixture contained 2 mL of 0.05 M phosphate buffer (pH 7.0) and 20 μ L of 5 mM hydrogen peroxide, maintained in an ice bath. Subsequently, 100 μ L of the enzyme extract was added, and the absorbance change at 290 nm was recorded. The reaction was initiated by adding 10 μ L of 50 μ M ascorbate solution. The enzyme activity was expressed as the change in absorbance per minute per milligram of protein.

Determination of total protein content

Plant sample protein total was determined by the Bradford assay²⁹. In brief, 20 μ L of the sample extracts was added to microplate wells, followed by 200 μ L of Bradford reagent freshly prepared. The mixture was kept at room temperature for a color development time of 10 min. Absorbance was read at 595 \pm 20 nm in an ELISA microplate reader. A standard curve was prepared from a series of dilutions of protein.

BSA (bovine serum albumin) being used as the standard protein. By applying the linear regression equation generated from the standard curve, the protein concentration in the samples was calculated (formulae 2):

Formula2: $y = 0.0136x + 0.0196$.

where y represents the absorbance and x corresponds to the protein concentration (μ g/mL).

Gen names	Primer names	Primer sequences (forward/reverse)	Product length, nt	melting temperatures (°C)	NCBI Accession No
18 S rRNA	FBRasr	5'-GTATGGTCGCAAGGCTGAAAC-3'	135 bp	59.8	KX709367.1
	RBRasrr	5'-GAGCTCTCAGTCTGCAATCC-3'		57.8	
APX	FAPXBr	5'- AGCAGTTCCCTACCATCTCTC-3'	121 bp	58.2	AB901371.1
	RAPXBr	5'- GGTGGCTGGGGCTTGTCTC-3'		59.8	
PIP	FHMPIP2	5'-CCGGTATCTCTGGTGGTCAC-3'	128 bp	56.9	NM_001316255.1
	RHMPIP2	5'-CCCAACTCCACATATCGCAC-3'		57.6	
P5Cs	FZKPCS	5'-TTACACAAGGTGATCACTGATG-3'	143 bp	57.8	XM_009118014.22.2
	RZKPCS	5'-GCTTGTGCTTCTCTTGGG-3'		58.3	

Table 1. A list of the primer sequences utilized in this research.

Determination of proline content

Proline was analyzed based on³⁰ procedure with some alterations. Tidy powders from the plant specimens were prepared and 0.1 g powder was sampled and homogenized in 10 mL 3% sulfosalicylic acid. Homogenate was filtered through paper filter, and to the equal amount of same solution of the filtrate 2 mL of filtrate was mixed with equal volumes of ninhydrin reagent and glacial acetic acid in a test tube. The resulting mixture was then incubated in a water bath at 100 °C for 1 h. The tubes were allowed to cool, and 4 mL of toluene was added using a fume hood. The tubes were shaken hard for 17–20 s so that the phase could separate. The upper organic phase containing the pink complex was decanted, and absorbance at 520 nm was read using a spectrophotometer.

Quantification of mineral components

The mineral composition of the plant materials was analyzed by³¹ but with minor modifications. The aerial parts of the plants are oven-dried at 70 °C for 48 h; 0.1 g of dry matter was weighed, powdered, and ashed in an electric furnace at 160 °C for 14 h. Ash was cooled and dissolved in 3–5 mL of distilled water, filtered, and placed in special plastic tubes. Concentration of mineral elements was measured using an atomic absorption spectrophotometer.

Statistical analyses

Following the acquisition of experimental data, preliminary statistical assessments were conducted to prepare the dataset for analysis of variance (ANOVA). To decipher complex multivariate relationships, Principal Component Analysis (PCA) was employed alongside Pearson correlation analysis. Additionally, a flower plot was utilized to visually represent the degree of overlap among multiple samples relative to a core reference sample, facilitating an intuitive comparative analysis. Given the heterogeneity in measurement scales, data were standardized by transforming all variables to a mean of zero and a standard deviation of one, ensuring uniformity in statistical comparisons. Post-hoc pairwise comparisons were performed using Duncan's Multiple Range Test to determine significant differences among treatment groups. Each treatment included three biological replicates ($n=3$). Statistical analyses were executed using SPSS (Version 20), ClustVis (<https://bii.cs.ut.ee/clustvis/>), and SRplot (<https://www.bioinformatics.com.cn/en>), with the results systematically interpreted and presented. Significance levels of $p < 0.05$ were considered statistically significant in all analyses.

Results

Based on the experimental findings, it is evident that Pre-Treatment with chitosan significantly enhanced the plant's ability to tolerate salinity stress, allowing growth even at high salt concentrations of up to 200 mM NaCl. In contrast, Post-Treatment did not support growth at 200 mM NaCl, with the plants failing to survive at this concentration, though they did manage to grow at 150 mM NaCl. This suggests that Pre-Treatment, by priming the plants before stress exposure, has a more robust impact on improving salinity tolerance. The chitosan treatment in the Pre-Treatment phase likely induced osmotic regulation and ion homeostasis mechanisms that better equipped the plants to handle higher salinity levels. Therefore, Pre-Treatment appears to be a more effective strategy for promoting growth under salt stress conditions in *Brassica napus* L., as compared to Post-Treatment, which has a limited effect when applied after the onset of stress.

Gene expression

*Effect of Chitosan Pre-Treatment on the expression of PIP, APX, and P5CS genes in rapeseed (*Brassica Napus* L.) under salinity stress*

Expression of selected stress-responsive genes were evaluated in *Brassica napus* L. by quantitative real-time PCR (qRT-PCR) with respect to chitosan priming treatment in salinity conditions. The expression levels of *PIP* were stimulated significantly in both roots ($P < 0.05$) and the shoots ($P < 0.01$) but more in roots with treatment duration. Chitosan priming induced *APX* gene expression ($P < 0.01$), with constant high expression on day 20 after treatment in roots and shoots. *P5CS* gene expression was also induced significantly ($P < 0.05$ for shoots, $P < 0.01$ for roots), showing a time increase especially in shoots. From these results, we infer the chitosan priming had promoted the expression of stress-responsive genes and thus could serve as a means to improve salinity tolerance through antioxidant defense, proline biosynthesis, and water transport in rapeseed. The evidence shows that priming chitosan can effectively modulate stress-responsive gene expression for water transport, *PIP*; antioxidant defense, *APX*; and osmolyte biosynthesis, *P5CS* in rapeseed plants under salinity stress. The temporality of these genes would suggest a gradual and continuous transcriptional response, which indicates the potential of chitosan as a biochemical elicitor-primer to augment stress resilience in plants.

Ascorbate peroxidase activity pre-treatment

The peroxidase activity was marked by considerable impacts of chitosan pre-treatment under salt stress condition ($p < 0.01$). Peroxidase activity in rapeseed plants increased significantly, reaching the highest level on day 20. Minimum activity was recorded in the control plants. Comparison between patterns of gene expression and enzyme activity showed that peroxidase gene expression increased 10 days after the last treatment. Immediately following the last treatment, peroxidase activity was also high in harvested plants. This seems to suggest that the increase in enzyme activity at this point was independent of gene expression mechanisms and probably suggests a post-transcriptional regulatory mechanism.

Ascorbate peroxidase activity post-treatment

The activity of ascorbate peroxidase was highly influenced ($p < 0.05$) by salt-induced stress, whereby it greatly increased the enzyme activity. There was maximum activity at the salinity of 150 mM. Chitosan application

significantly influenced ($p < 0.05$) the value of APX activity. It had no effect at 5 mg/L and showed effects at 10 mg/L.

There was a statistically significant interaction between salt stress and chitosan. The maximum activity of the enzyme APX obtained was in plants treated with 150 mM NaCl plus 5 mg/L chitosan while the lower activity was noted in plants treated with 50 mM NaCl plus 5 mg/L chitosan. Moreover, the application of chitosan to the plant stressed with either 50 or 100 mM NaCl showed increased APX activity than the one subjected to salt stress. However, with an addition of 5 mg/L chitosan to 150 mM NaCl treatment, a higher enzyme activity was recorded as compared to salt stress alone while that with 10 mg/L reduced APX activity. From this result, it could be inferred that post-treatment with chitosan had an effect on APX activity under saline conditions, with different responses observed at different concentrations of different levels of salt. The results may suggest the likelihood of such chitosan inhibiting the oxidative stress responses in rapeseed plants under salinity stress conditions.

Pre-treatment with chitosan significantly increased peroxidase activity in rapeseed under salt stress, with the highest activity observed on day 20, independent of gene expression. Post-treatment with chitosan also enhanced ascorbate peroxidase activity, particularly at 150 mM NaCl, indicating its role in oxidative stress modulation.

Pre-treatment proline content

The analysis of variance demonstrated a statistically significant effect of chitosan Pre-Treatment on proline accumulation in canola plants at the 1% significance level ($p < 0.01$). In the aerial parts, proline levels remained comparable to the control on days 0 and 10 post-treatment, whereas a significant increase was observed on day 20. In contrast, root proline content exhibited a gradual increase over time, peaking on day 20. While control plants displayed higher proline levels in the aerial parts than in the roots, chitosan-treated plants exhibited a slight shift, with proline accumulation being more pronounced in the roots. Interestingly, these findings did not align with the expression pattern of the P5CS gene, which was significantly upregulated in the aerial parts compared to the roots, suggesting potential post-transcriptional or regulatory mechanisms influencing proline biosynthesis.

Post-treatment proline content

The results indicate that proline contents were significantly influenced by both chitosan and salt ($p < 0.01$). Proline tends to slightly increase as salt concentration is raised. Additionally, a significant increase in proline content was recorded as chitosan was applied to canola plants.

Effect of pre-treatment on mineral content in *Brassica Napus L*

The findings showed that chitosan pretreatment had highly significant effects ($p < 0.01$) on sodium and potassium levels in canola under salt stress. On the twentieth day post-treatment, sodium was most abundant in the roots, while the amounts in the aboveground parts were insignificant, indicating that sodium was retained in the roots as a protective mechanism. Furthermore, chitosan minimally impacted lowering Na^+ levels in roots. In comparison, potassium levels peaked in the roots but thereafter declined gradually with time. At the level of molecular change, these changes are associated with altered expression of *PIP*, *APX*, and *P5CS* genes, which are important in ion transport, oxidative stress response, and phytochelatin biosynthesis, respectively. Upregulation of *PIP* (plasma membrane intrinsic proteins) indicates the increased regulation of water and ion transport under chitosan treatment. The increased expression of *APX* (ascorbate peroxidase) would also indicate improved antioxidant defense toward salt-induced oxidative damage. Induction of the *P5CS* (phytochelatin synthase) may also contribute to ion and metal detoxification homeostasis. This indicates that chitosan has induced both physiological changes and expression changes in genes that will improve Na^+ and potassium (K^+) homeostasis under salt such that it boosts the salt tolerance of canola.

Effect of post-treatment on mineral content in *Brassica Napus L*

The results show that Na^+ and K^+ concentrations in canola were significantly affected by salt, chitosan application, and their interaction. Higher salinity caused the accumulation of Na^+ to a larger extent, being the highest at 150 mM NaCl, while K^+ concentration reduced, indicating ionic imbalance due to salt stress. Chitosan alone suppressed Na^+ accumulation and regulated K^+ content, with the most efficient effect at the 10 mg/L level. Under the treatment conditions of combined salt with chitosan, chitosan limited Na^+ uptake at intermediate salinity levels, but not at higher salinity levels, suggesting that chitosan could have a vital role in enhancing salt tolerance through regulation of Na^+ and K^+ homeostasis, possibly via enhanced ion selectivity and osmotic adjustment capacity.

Pre-treatment effects of salinity stress and chitosan on protein content in *Brassica Napus L*

The analysis of variance showed that chitosan pretreatment significantly affected the protein content of *Brassica napus L.* at the 1% level ($p < 0.01$). over time, both aerial and root tissues showed increased protein levels post-treatment. In the aerial parts, they appeared to increase more noticeably after 20 days of treatment. In roots, the increase was gradual. This indicates that chitosan appears to modulate protein accumulation under stress with separate temporal responses in various plant parts.

Post-treatment effects of salinity stress and chitosan on protein content in *Brassica Napus L*

This study revealed that protein content in canola plants was significantly influenced by salinity stress and the applied concentrations. There was a remarkable increase in protein levels upon salinization as compared to the control group. The effect of chitosan alone was statistically significant ($p < 0.01$), but its effect on protein content is limited. However, under saline stress, application of chitosan to canola plants lessened the protein increase induced by salinity. Chitosan inhibited the synthesis of stress-related proteins at all concentrations and thus can

have a role in modulating protein production under salt stress. These findings highlight the complex interplay between salinity, treatment with chitosan, and protein metabolism in canola.

Comprehensive visualization of physiological and molecular responses to stress: a flower plot-based multi-parametric analysis

The flower plot was constructed to visualize the comparative analysis of different physiological and molecular parameters against a particular state or condition, with the innermost core (500) being the reference. Each petal represents one variable such as gene expression levels (*PIP*, *APX*, *PCS*), protein content, antioxidant enzyme activity (APX), ion concentrations (K, Na), and proline accumulation for different tissues of the plant (root, stem). The color gradient of each petal denotes relative variation, wherein red shades indicate upregulation probably in connection with stress adaptation and green shades symbolize stable or downregulated values. This visualization rather successfully shows differing metabolic adjustments under the condition of stress, implying a sophisticated regulatory network modulating tolerance in plants. With incorporation of these multiple parameter data sets within a single plot, the holistic assessment of physiological and biochemical changes, induced by stress, becomes practical, thereby rendering it a useful asset to the realms of plant stress physiology and omics (Fig. 2).

The flower plot comprehensively presents the physiological and molecular responses in plants under stress, with 500 as the core value base. Each petal corresponds to a different parameter, for example, gene expression levels (*P5CS*, *APX*), protein content, antioxidant enzyme activity (APX), ion concentrations (Na, K), and proline

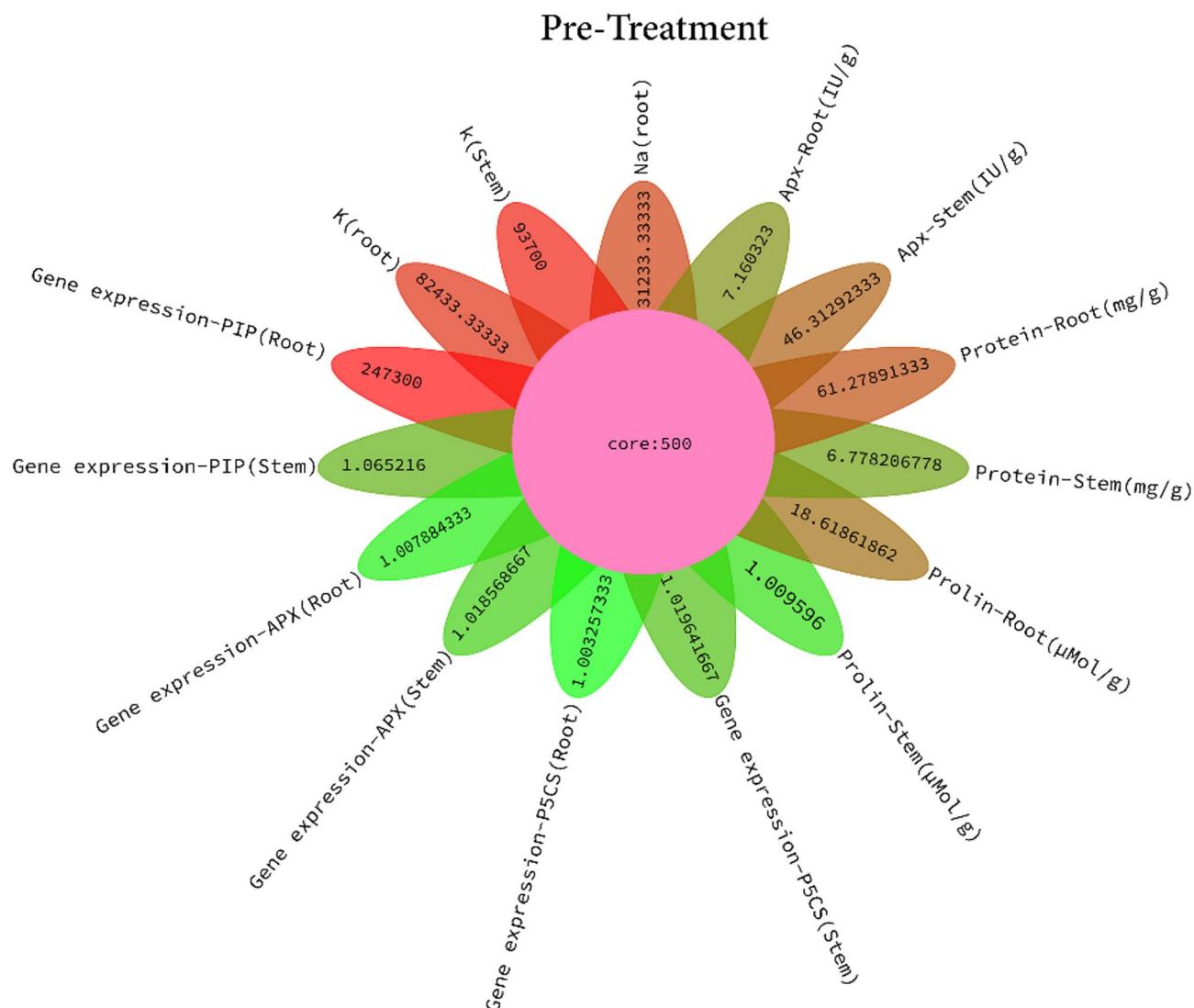


Fig. 2. Flower diagram comparative of physiological and molecular responses of *Brassica napus* L. under salinity stress, along two conditions prestreatment. The central core (500) is representing reference sample while each petal is distinct for a particular parameter, as in gene expression (*P5CS*, *APX*), protein content, antioxidant activity (APX), ion concentrations (Na, K), and proline accumulation. Colour gradient symbolizes the relative changes: red for upregulation, green for downregulation or stability; useful to show major stress related metabolic adjustments ($n=3$).

accumulation measured in various plant tissues. The color gradient of the petals shows the relative differences—that is, red shades would indicate high upregulation, particularly in the fields of proline content (31.08 mg/g FW) and *P5CS* expression (16.66)—implying a very significant role of these components in osmotic and stress regulation. Green colors, on the other hand, refer to lower or stable levels of expression, such as *APX* gene expression (0.09) and thus show a differential antioxidant response. The combination of multiple-dimensional datasets all in one form captures the total interpretation of the underlying aspect of changes in physiology and biochemistry induced by stress in plants, thus rendering valuable data into the intricate regulatory networks governing plant resilience under adverse conditions (Fig. 3).

Principal component analysis (PCA) of physiological and molecular responses in the pre-treatment

The PCA biplot illustrated a comprehensive visualization of variance concerning physiological and molecular responses under pre-treatment conditions with PC1 and PC2 explaining a considerable amount of total variance. Initial observations were segregated in space, indicating that control (time 0) and salt stress conditions were totally distinguished, with salt time 10 sitting toward the positive PC1 axis signifying major salinity-induced changes (Fig. 4). Nutrient-related variables, especially potassium in roots and stems, were strongly associated with the negative PC1 axis, implying a depletion under salt stress. On the other hand, gene expression markers (*P5CS*, *PIP*, *APX* in root and stem) and stress-responsive metabolites (proline and protein content) were located toward the positive PC1 and PC2 axes, emphasizing their upregulation as coping responses to salinity. The co-localization of enzymatic activity (*APX*-Root) with gene expression parameters lends extra credence to the activation of oxidative defense and osmoprotective pathways. Thus, such findings highlight salt stress-induced

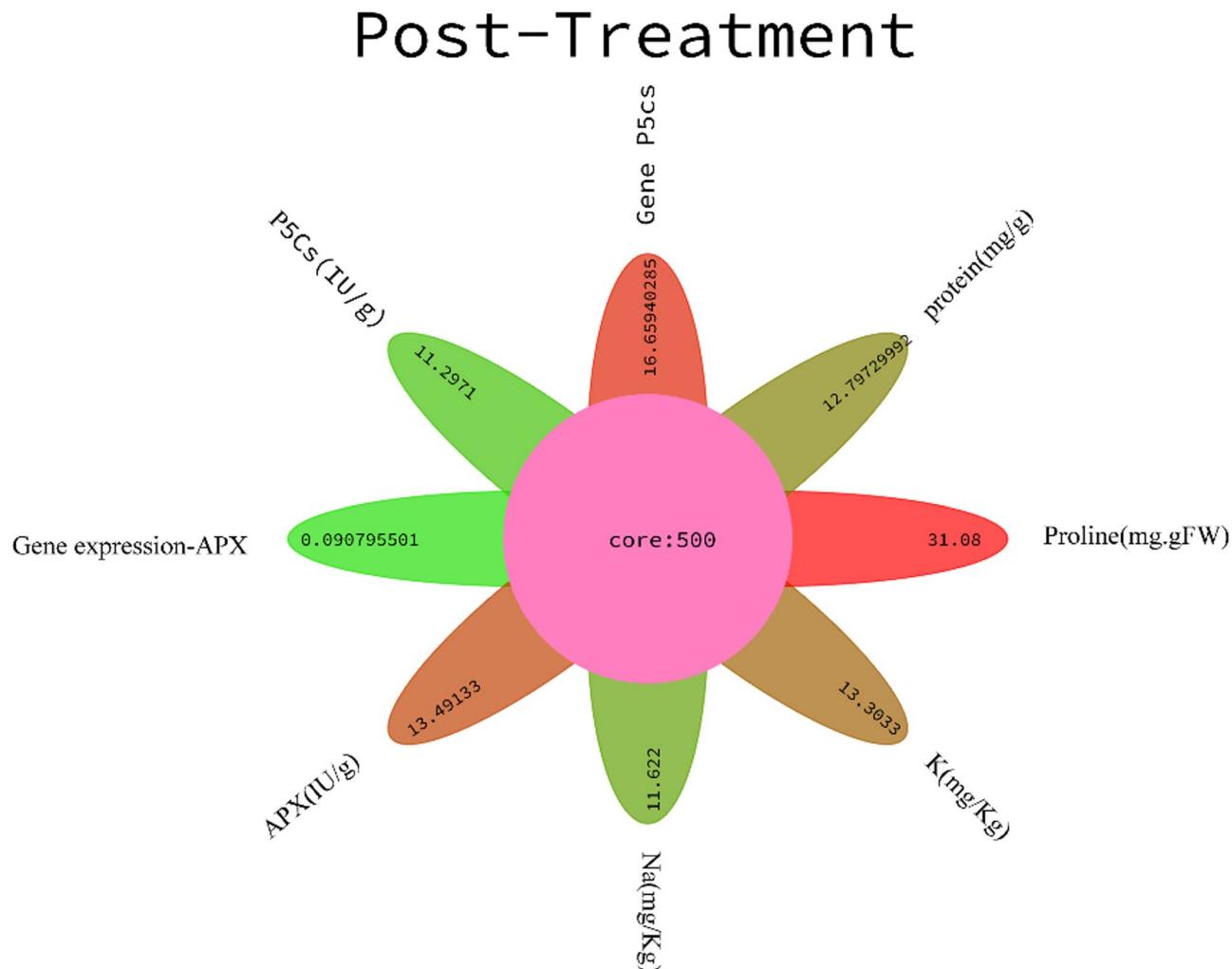


Fig. 3. The flower plot represents physiological and molecular responses of stress conditions. Core (500) serves as a reference, while petals include parameters such as gene expression (*P5CS*, *APX*), protein content, antioxidant activities, ion concentrations (Na, K), and proline accumulation. A color scale from red to green indicates upregulation and downregulation, respectively, of each parameter, highlighting metabolic adjustments in the Post-Treatment phase ($n=3$).

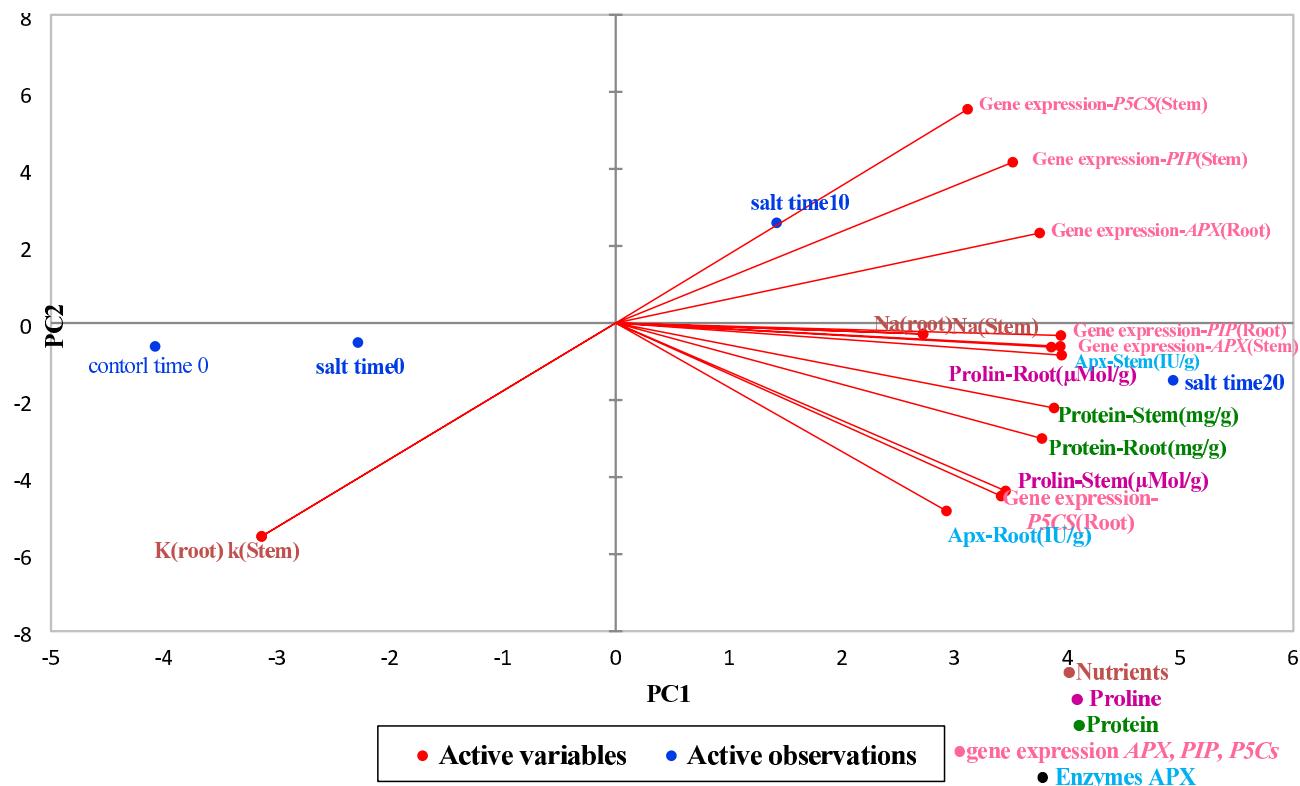


Fig. 4. Principal Component Analysis (PCA) biplot illustrating the distribution of active variables and observations under pre-treatment conditions. The plot represents the relationships between physiological (nutrient content, protein levels, and proline accumulation) and molecular (gene expression of *P5CS*, *PIP*, and *APX*) parameters in response to initial salt stress exposure. Active variables (red vectors) indicate the direction and magnitude of each parameter's contribution to principal components, while active observations (blue points) represent different experimental conditions. The clustering pattern highlights metabolic and transcriptional differences between control and salt-treated samples, providing insights into early-stage stress responses ($n=3$).

transcriptional and metabolic reprogramming that comprise crucial regulatory elements in salinity tolerance mechanisms (Fig. 4).

Principal component analysis (PCA) of physiological and molecular responses in the post-treatment

The PCA biplot of post-treatment conditions gives an extensive portrayal of variance in physiological and molecular responses to various treatments for PC1 and PC2, accounting for an important share of total variance. The spatial arrangement of observations shows that control, chitosan, and NaCl-treated groups are visibly separated; Chitosan 10 and Chitosan 5 + NaCl 150 show the most pronounced shifts along the PC1 axis, indicating altered metabolic and transcriptional activities due to treatment. Such nutrient related variables like Na, K are highly related to NaCl treatments which are beneficial for their important role in ionic regulation under salt stress (Fig. 5). So, upregulation of gene expression markers *APX*, *PIP*, *P5CS* in co-treatments of chitosan-NaCl indicates activation of antioxidative and osmoprotective responses. In addition, stress-related metabolites (proline and protein level) are also brought closer to PC2, as they are considered to play a very important role in salinity adaption. Results indicated that application of chitosan, more so with NaCl co-treatment, influences greatly the physiological and molecular responses leading to improvement in the resilience to abiotic stresses by transcriptional reprogramming and metabolic adjustment (Fig. 5).

Correlation matrix of physiological and molecular variables under pre-treatment conditions

To visualize the relationships between physiological and molecular variables regarding stress responses, the correlation coefficient plot is viewed as a gradient gradient color circle, where dark blue circles represent strong positive correlations, while dark red circles represent strong negative correlations. Statistically significant comparisons are marked with an asterisk (*) (Fig. 6). As stated, the levels of gene expression for *PIP*, *P5CS*, and *APX* are significantly positively correlated, suggesting a possible co-regulated transcriptional control under the imposed treatment conditions. On the contrary, enzyme activity for *APX* (IU/g) has a positive correlation with gene expression for *APX*, indicating that the two defense mechanisms have a direct joining between transcriptional and enzymatic antioxidant defense. There also exists moderate positive correlation values for

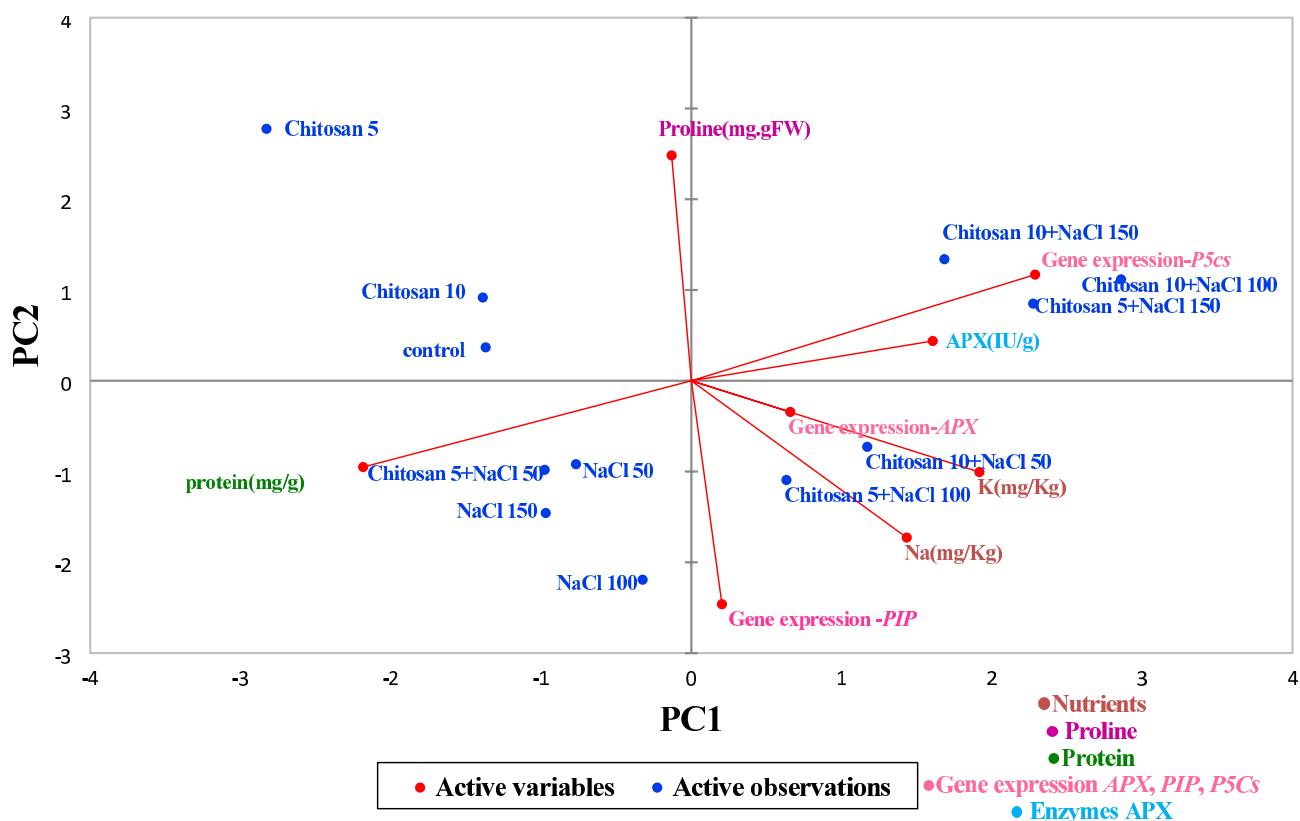


Fig. 5. Principal Component Analysis (PCA) biplot illustrating the distribution of active variables and observations following post-treatment conditions. The plot highlights the relationships between physiological parameters (such as proline and protein content) and molecular markers (including gene expression of APX, PIP, and P5CS) in response to various treatments. Active variables (red vectors) indicate the contribution of each parameter to the principal components (PC1 and PC2), while active observations (blue points) represent different treatment conditions. The distinct clustering of treatment groups, particularly Chitosan 10 and Chitosan 5 + NaCl 150, reflects significant treatment-induced changes in metabolic and molecular responses, providing insights into stress adaptation mechanisms ($n=3$).

Na^+ and K^+ , reflecting their roles to play in ionic homeostasis during stress adaptation. In addition, proline level (mg/g FW) shows a strong direct relationship with both P5CS expression and APX activity, hence upholding its dual functions in osmoprotection and antioxidant defense. Also, proline accumulation (in mg/g) has been significantly positively correlated with total protein content (in mg/g), suggesting a role in adaptive metabolic responses (Fig. 6). The collective picture of such findings is that cooperative regulation of an array of antioxidative enzyme systems, osmoprotectants, and ion transport mechanisms proves crucial for stress resilience, thereby granting a molecular insight into the diverse adaptive strategies followed under different treatment conditions (Fig. 6).

Correlation matrix of physiological and molecular variables under post-treatment conditions
The result from correlation coefficient analysis illuminates a strong positive relationship in gene expression levels of PIP, P5CS, and APX, which implies a tightly regulated action in the control for water transport via flow into proline biosynthesis and then to antioxidative defense pathways (Fig. 7). The significant positive correlation found between APX enzymatic activity (IU/g) and APX gene expression indicated a transcriptional regulation on the enzymatic response under conditions of stress. This study also evaluates Ion homeostasis, where moderate positive correlation exists in Na^+ and K^+ concentrations; hence, they play vital roles in osmotic balance. So, the positive correlation between proline accumulation and P5CS gene expression indicates the significance of proline biosynthesis in stress adaptation. Furthermore, the strong positive correlation between proline content and protein amount indicates a functional link between osmoprotective mechanisms and metabolic adjustment due to treatment. Therefore, this shows that these treatments will trigger a whole-system response post-treatment regarding the co-ordinated regulation of antioxidant defenses, osmoregulatory metabolites, and ion transport-all increasing stress tolerance as well as helping to maintain physiological resilience (Fig. 7).

Discussion

Plant growth and productivity are restricted by soil salinity as one of the potential environmental restrictions. This study has been designed to verify how chitosan affects the physiological parameters and expression level of

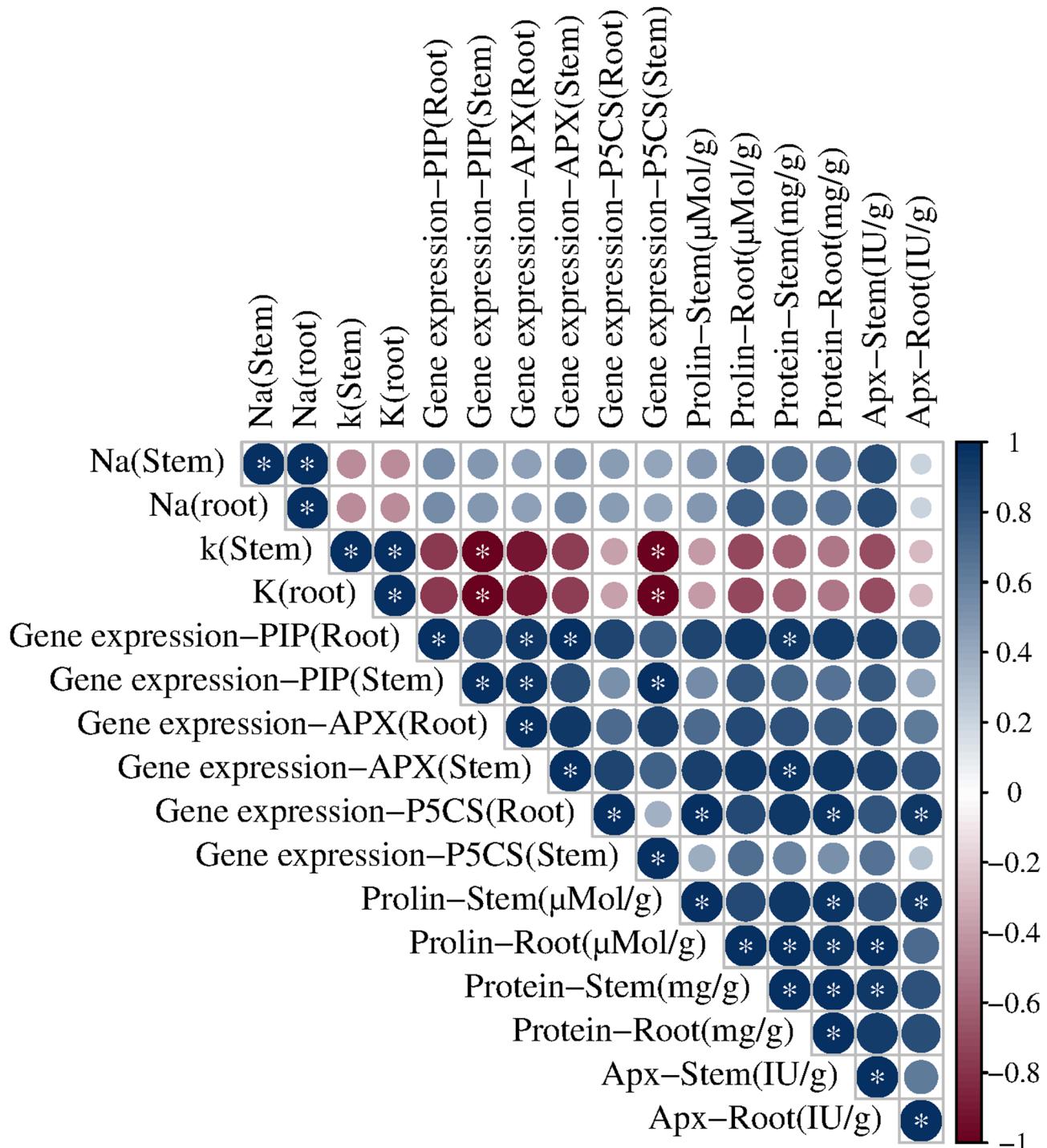


Fig. 6. Correlation coefficient plot depicting the relationships between gene expression levels (*PIP*, *P5CS*, and *APX*), enzymatic activity (APX IU/g), ion content (Na, K), and metabolic markers (proline and protein) in pre-treatment conditions. The correlation strength and direction are conveyed by the size and intensity of the circle color, with large dark blue circles representing strong positive correlations and dark red ones for strong negative correlations. Statistically significant correlations are denoted by asterisks (*). The seen patterns highlight the interrelationship of antioxidative defense mechanisms, osmoprotective responses, and ion homeostasis in pre-treatment conditions ($n=3$).

three key genes (*P5CS*, *PIP*, and *APX*) in *Brassica napus* L. under salt stress. According to our data, the chitosan plays an enhancing role in salinity stress alleviation through increased drought resistance, osmoregulation, and intrinsic antioxidant defenses^{32,33}. Aquaporins, encoded by *PIP* genes, play an essential role in water transport across membranes, and their regulation is a critical aspect of plant response to drought and salinity stress.

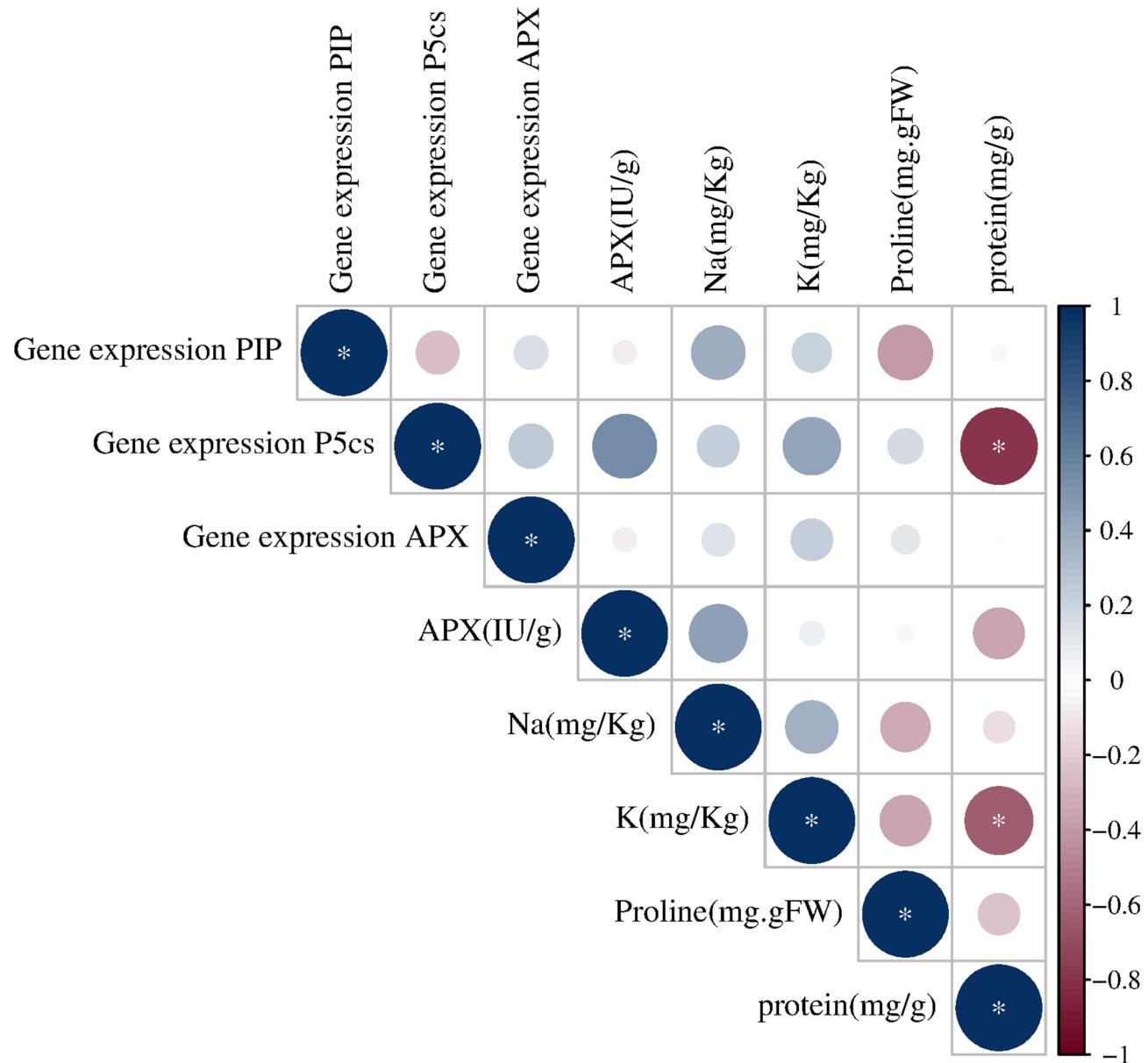


Fig. 7. Correlation coefficient plot showing relationships amid gene expression levels (*PIP*, *P5CS*, *APX*), enzyme activities (APX IU/g), ion contents (Na, K), and metabolic markers (proline, protein) in post-treatment conditions. The bigger and more intense the color of the circle, the stronger the correlations in that particular direction, with dark blue indicating strong positive correlations and dark red, strong negative correlations. Statistically significant correlations are indicated by asterisks (*). The interrelations provided emphasize the regulatory network underlying stress adaptation concerning antioxidant responses, osmolyte accumulation, and ion homeostasis after a treatment.

The significant increase in *PIP* expression in chitosan-treated plants under salt stress observed in our study is consistent with the findings of Ibraheem et al.^{34,35}, who reported increased expression of aquaporin genes in rice following chitosan treatment. Similarly, Stassinos et al.³⁶ showed that *PIP* expression levels were significantly upregulated in *Brassica napus* L. cultivars subjected to high saline conditions, especially in salt-tolerant varieties. Increased osmotic water uptake provides for osmotic adjustment through proline accumulation under stress. The key enzyme in proline biosynthesis is encoded by the *P5CS* gene and *P5CS* gene expression is strongly induced by salinity stress^{37,38}. Our results revealed higher *P5CS* expression in chitosan-primed plants subjected to salt stress, being in line with the observations of Cherif et al. (2020) in *Rosmarinus officinalis*³⁹, where application of chitosan enhanced the levels of proline under stress. Similar results were reported by Saadat et al.⁴⁰, who demonstrated upregulation of proline biosynthesis genes in chitosan-treated saline condition of common beans seeds. Again, research work by Ghorbanpour et al.⁴¹ proved that chitosan, in fact, acts to modulate the proline biosynthesis pathways in various plant species for osmotic balance and stress alleviation. The antioxidant defense system is one other factor that protects things in a salt stress environment. In this regard, the *APX* gene carries

instructions for making the enzyme ascorbate peroxidase, a very important enzyme capable of neutralizing ROS by degrading hydrogen peroxide. Our results confirmed an appreciable enhancement of APX expression in chitosan-treated plants under salinity. In agreement with this result, Bigham Soostani et al.³⁴ reported chitosan-induced APX upregulation in *Brassica napus* L. grown under salt stress. Additional bringing more insight, Liptay and Zariffa⁴² found higher plants treated with chitosan. Similarly, Khan et al.⁴³ observed increased activities of antioxidant enzymes, including APX, in chitosan-treated maize under salt stress. On the contrary, other researchers like Shahbazi et al.⁴⁴, Zhou et al.⁴⁵ and Mohammadi et al.⁴⁶ reported variable responses that might be attributed to differences in plant species, nature of experimental conditions, or chitosan formulations. It is also worth noting that while our study focused primarily on PIP, P5CS, and APX, chitosan has been reported to influence the expression of other key genes involved in salt stress response, including NHX1, HKT1, and SOS1, which regulate ion transport and Na⁺/K⁺ homeostasis⁴⁷, contributing to improved ionic balance and reduced sodium toxicity. Salinity stress significantly alters protein metabolism in plants, leading to either an increase or decrease in total protein content depending on species-specific responses and stress intensity. In the present study, salinity induced an increase in total protein content in *Brassica napus* L., consistent with ious reports on quinoa⁴⁸ and tomato⁴², where protein accumulation was attributed to osmotic adjustment mechanisms. This adaptation likely involves stress-responsive proteins that mitigate cellular damage and contribute to metabolic adjustments under salinity stress. Conversely, studies on rice⁴⁹ and *Achillea fragrantissima*⁵⁰ have reported a reduction in protein content under saline conditions, suggesting species-dependent differences in protein synthesis and degradation pathways.

In the present study, Principal Component Analysis (PCA) and correlation matrix analysis were employed as the main statistical tools to explore the complex dataset. PCA was used to reduce the dimensionality of the data and identify the major sources of variation, thereby facilitating the interpretation of multiple physiological and biochemical parameters. The correlation matrix, on the other hand, provided an integrated overview of the relationships among the measured variables, revealing potential interactions and patterns relevant to stress responses. These approaches are widely applied in plant stress physiology for their effectiveness in capturing complex biological relationships. Furthermore, recent literature has demonstrated the role of chitosan in enhancing plant tolerance to abiotic stress by modulating physiological traits and metabolic pathways. For example, Tabassum et al.⁵¹ reported that chitosan improves antioxidant enzyme activities and mitigates oxidative damage under stress conditions. Similarly, Rafique et al.⁵² showed that chitosan influences the expression of stress-responsive genes, consistent with our findings. These studies support the relevance of our experimental design and highlight chitosan's potential as a biostimulant for improving plant resilience under saline environments.

Chitosan application modulated protein accumulation, supporting its role as a bio-stimulant in enhancing stress tolerance. Pre-treatment with chitosan resulted in a significant increase in total protein content over 20 days, aligning with findings by⁵³, who demonstrated that chitosan application in wheat increased protein levels by enhancing nitrogen assimilation and metabolic activity. This increase in protein content may be linked to the upregulation of genes involved in protein biosynthesis and stress response⁵⁴, as well as the regulation of key enzymes in glycolysis and other metabolic pathways⁵⁵. Additionally, the protective role of chitosan-based treatments has been reported in *Vitis vinifera*, where foliar application of chitosan-salicylic acid nanocomposites increased protein levels under salt stress⁵⁶. The observed increase in protein content following chitosan treatment may be an adaptive response aimed at stabilizing cellular structures and maintaining metabolic homeostasis. Chitosan-induced protein accumulation could result from enhanced ribosomal activity and increased synthesis of stress-protective proteins⁵⁷. Furthermore, chitosan-mediated upregulation of stress-responsive proteins in wheat⁵⁸ and rice⁵⁹ suggests a conserved mechanism by which chitosan enhances stress tolerance through protein metabolism. As protein synthesis plays a crucial role in cellular adaptation to abiotic stress, chitosan pre-treatment appears to be a promising approach for mitigating salinity-induced damage by sustaining protein biosynthesis and maintaining cellular function. Pre-treatment with chitosan exhibits a more pronounced effect on protein accumulation in *Brassica napus* L. under salinity stress compared to post-transcriptional regulation. By priming metabolic pathways before stress exposure, pre-treatment enhances protein biosynthesis, stabilizes enzymatic functions, and mitigates the detrimental effects of salinity-induced oxidative stress. In contrast, post-transcriptional mechanisms primarily modulate protein expression after stress onset, limiting their efficacy in preventing early cellular damage. These findings underscore the significance of pre-treatment strategies in reinforcing stress adaptation through the upregulation of stress-responsive proteins and the maintenance of cellular homeostasis.

Salinity stress significantly disrupts ion homeostasis in plants, primarily by increasing sodium (Na⁺) accumulation while simultaneously reducing potassium (K⁺) uptake. The results of this study indicate that exposure to 150 mM NaCl leads to a substantial increase in Na⁺ content and a corresponding decline in K⁺ levels in *Brassica napus* L., consistent with previous findings^{47,60}. This imbalance is largely attributed to the antagonistic relationship between Na⁺ and K⁺, where the influx of Na⁺ through non-selective cation channels disrupts K⁺ transport, leading to impaired cellular functions^{61,62}. The observed decline in K⁺ concentration suggests that salinity stress compromises K⁺ retention mechanisms, which are critical for enzymatic activity, osmoregulation, and photosynthetic efficiency⁶³.

K⁺ is an essential element involved in various physiological and biochemical processes, including protein synthesis, enzyme activation, and stomatal regulation. However, under saline conditions, the high Na⁺ levels in the rhizosphere hinder K⁺ uptake by outcompeting it at the transport sites, ultimately leading to K⁺ deficiency^{64,65}. This is in agreement with previous reports on other plant species, such as soybean⁶⁶ and faba bean⁶⁷, where elevated salinity resulted in a marked reduction in K⁺ content in aerial tissues. The depletion of K⁺ in salt-stressed plants has been linked to the depolarization of plasma membranes and the activation of Na⁺-permeable channels, further exacerbating ion toxicity^{68,69}. The detrimental effects of Na⁺ accumulation on plant metabolism are further amplified by its interference with essential nutrient transport, leading to osmotic stress, oxidative

damage, and metabolic dysfunction. Our findings align with earlier studies that demonstrated a decrease in K^+ / Na^+ ratio under salt stress conditions, which is a key indicator of salt tolerance in plants^{70,71}. The reduced K^+ availability negatively impacts protein synthesis and ATPase activity, both of which are crucial for maintaining cellular homeostasis^{72,73}. Given the critical role of K^+ in mitigating salt stress, strategies aimed at enhancing K^+ uptake and retention, such as the application of K^+ fertilizers or the development of salt-tolerant cultivars with improved K^+ selectivity, could be effective in alleviating the adverse effects of salinity in *Brassica napus* L.

Based on the results, Pre-Treatment demonstrated a more substantial effect on maintaining potassium (K^+) homeostasis and reducing sodium (Na^+) accumulation under salinity stress. Pre-Treatment strategies, particularly with osmoprotectants or ionic regulators, enhanced the selective uptake of K^+ while restricting Na^+ influx, thereby mitigating ionic imbalance. In contrast, post-transcriptional regulation played a crucial role in modulating stress-responsive genes; however, its impact on immediate ion transport mechanisms was less pronounced compared to Pre-Treatment. The ability of Pre-Treatment to prime the cellular defense system before exposure to stress conditions suggests that it is a more effective strategy for sustaining ionic equilibrium and improving salt tolerance in *Brassica napus* L.

Conclusion

This study provides a comparative evaluation of chitosan pre-treatment and post-treatment in *Brassica napus* L. under salt stress, focusing on key molecular and biochemical responses. Our findings reveal that pre-treatment was more effective in enhancing stress tolerance, as evidenced by the upregulation of *P5CS*, *PIP*, and *APX* genes. The increased expression of *P5CS* in pre-treated plants correlated with higher proline accumulation, supporting osmotic adjustment and stress resilience. Similarly, elevated *APX* expression indicated a more robust antioxidant defense system, mitigating oxidative damage. Additionally, the enhanced expression of *PIP* genes in pre-treated plants suggests improved water homeostasis under saline conditions. The observed increase in total protein content further supports the role of chitosan pre-treatment in stabilizing cellular functions. Notably, pre-treated plants exhibited sustained growth even under high salinity conditions of up to 200 mM NaCl, whereas post-treated plants failed to survive at this concentration and only managed to grow at 150 mM NaCl. These findings underscore the superior efficacy of chitosan pre-treatment in enhancing salt stress tolerance in *Brassica napus* L., highlighting its potential application for improving crop resilience in saline environments.

Data availability

Data are provided within the manuscript information files.

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Additional information

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