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PACLOBUTRAZOL DEPENDENT SALT TOLERANCE IS RELATED TO *CLC1* AND *NHX1* GENE EXPRESSION IN SOYBEAN PLANTS

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ABSTRACT

Paclobutrazol (PBZ) enhances plant resistance to salt stress in two ways: directly, by straight clearance of reactive oxygen species; and indirectly by enhancing antioxidant enzyme activity, photosynthetic efficiency, and metabolite content, and by regulating transcription factors associated with stress. However, the regulatory effects of PBZ under salt stress in soybean are still not well explained and need further investigation. With this aim, the combination effect of salinity (250 mM NaCl) and three different doses of PBZ (5, 10 and 20 ppm) on physiological, biochemical and molecular traits of soybean (*Glycine max* L.) leaves were studied in soil experiments. Furthermore, physiological parameters (relative growth rate, relative water content), chlorophyll, malondialdehyde (MDA), hydrogen peroxide (H_2O_2) content and as well as enzymatic antioxidants (SOD, POX, APX, CAT and GST), ion content (Na, Cl) and soybean Na^+/H^+ antiporter *GmNHX1* and chloride channel *GmCLC1* gene expressions were investigated. The results showed that PBZ caused a reduction in salt-induced damages and an increase in biomass yield, water status, and chlorophyll. Moreover, PBZ regulated enzymatic antioxidants and alleviated the oxidative damages under salinity. In this study, for a first time it was determined that PBZ increased both *GmNHX1* (ABA dependent or independent) and *GmCLC1* (ABA independent) gene expressions and reduced Na and Cl concentrations in soybean under salinity. In conclusion, PBZ plays a role as a regulator and stimulant in salt stress response by mostly regulating ion balance in soybean leaves.

Key words: *GmNHX1*, *GmClC1*, paclobutrazol, soybean, salinity

INTRODUCTION

In recent years, PBZ is extensively used in agriculture for regulating plant development and increasing crop yield under stress conditions in soybean, sorghum, maize [Kamran et al. 2020]. Sustaining the endogenous cytokinin concentration, maintaining water status, improving nutrient uptake, carbohydrate and chlorophyll biosynthesis, and promoting antioxidant capacity and nitrogen metabolism have been attributed to PBZ [Forghani et al. 2018, Tesfahun and Yıldız 2018, Kamran et al. 2020, Kuandykova et al. 2020]. In many studies, PBZ application in plants exposed to environmental stress renders tolerance to abiotic stresses by decreasing lipid peroxidation, increasing the amount of proline, and reducing the membrane damages [Fletcher et al. 2000, Aly and Latif 2011, Jungklang and Saengnil 2012].

Soybean is one of the three major crops worldwide, along with wheat and rice, because of its nutritional value. With increasing salinity levels, soybean production can be reduced by as much as 40% [Papiernik et

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al. 2005]. Salt tolerance mechanisms in soybean can be classified into four major categories: (a) maintenance of ion homeostasis; (b) adjustment in response to osmotic stress; (c) restoration of oxidative balance; and (d) other metabolic and structural adaptations [Phang et al. 2008]. Inclusion and exclusion of Na^+ and Cl^- in roots and leaves of soybean cultivars is related to salt tolerance capability. Soybean Na^+/H^+ exchangers (*NHXs*) are tonoplast-localized Na^+/H^+ antiporters that function in sequestering of excessive cytosolic Na^+ into the vacuole for ameliorating it's toxic effects to the metabolic activities in cytosol. Similarly, Cl^- homeostasis is a determining factor of salt tolerance in soybean and the tonoplast-localized chloride channel (*ClCs*) is very important to increase salt tolerance [Wei et al. 2016].

The present study was undertaken to evaluate the role of paclobutrazol in modulation of soybean salt tolerance in detail. Many reports have revealed the positive effects of paclobutrazol in reducing the plant stress damages by increasing proline and antioxidant enzymes, nitrogen metabolism and yield. However, little is known about how it exactly works, which mechanisms it stimulates and what is the defence strategy which is most effective. To clarify these issues, growth, water status, Na^+ and Cl^- content, enzymatic antioxidants, *GmNHX1* and *GmClC1* gene expressions were determined comparatively in soybean plants grown under salinity and PBZ treatment.

MATERIALS AND METHODS

Experimental design and plant material

Soybean (*Glycine max* L. Merr.) SA88 seeds were obtained from a commercial provider (Agrova, Adana, Turkey). The seeds were sown in plastic pots (width 20 cm, length 12 cm, height 18 cm), each of which contained 30–40 seeds and a mixture of soil : clay : clay-loam in the ratio of 2 : 1 : 1, pH: 6.5. Seeds were placed in the dark for 5 days for germination. After germination, seedlings were grown in a growth room at 25°C (16 h day/8 h night photoperiod), light intensity of 350 µmol m^{-2} s^{-1} and watered with Hoagland solution, 25 ml per pot, for 8 days [Hoagland and Arnon 1950].

The pots were divided into eight treatments: control (1), NaCl 250 mM (2), PBZ; P1 5 ppm (3), PBZ2 10 ppm (4), PBZ3 20 ppm (5), NaCl + PBZ1 5 ppm (6), NaCl + PBZ2 10 ppm (7), and NaCl + PBZ3 20 ppm (8). Each one variant contained three pots (totally 40 plants). The pots were watered with 25 ml Hoagland solution for 10 days. Then, the seedlings were harvested on the 10th day and the samples were preserved at –80°C. All PBZ (5, 10, 20 ppm) concentrations were applied with the Hoagland solution in soil medium (Fig. 1).

Physiological parameters

Relative growth rate. The relative growth rate (RGR) of shoots was calculated from the dry mass data taken at initial and final harvests, using the formula given by Hunt et al. [2002]. For dry weight (DW) calculations, shoots and roots were dried in the oven at 70°C for 48 h and then weighed.

Relative water content. The relative water content (RWC) was calculated according to Smart and Bingham [1974].

Total chlorophyll content. The chlorophyll content of leaves was measured in accordance with the method specified by Lichtenthaler and Buschmann [1987].

Na^+ and Cl^- ion content. The Na^+ content was determined by flame photometry according to Mathis [1956]. The Cl^- concentration was obtained by wet oxidation of dried leaf tissue with nitric and perchloric acids in accordance with the method adapted by Johnson and Ulrich [1959].

Biochemical parameters

Hydrogen peroxide content. Hydrogen peroxide concentration was estimated spectrophotometrically according to the method of Alexieva et al. [2001] and was calculated using a standard curve, prepared with known amount of hydrogen peroxide.

Malondialdehyde content. Malondialdehyde content was estimated following the original method of Kramer et al. [1991] as a parameter reflecting biomembrane integrity deterioration.

Enzymatic antioxidant activity

SOD enzyme activity. The superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm [Beauchamp and Fridovich 1973].

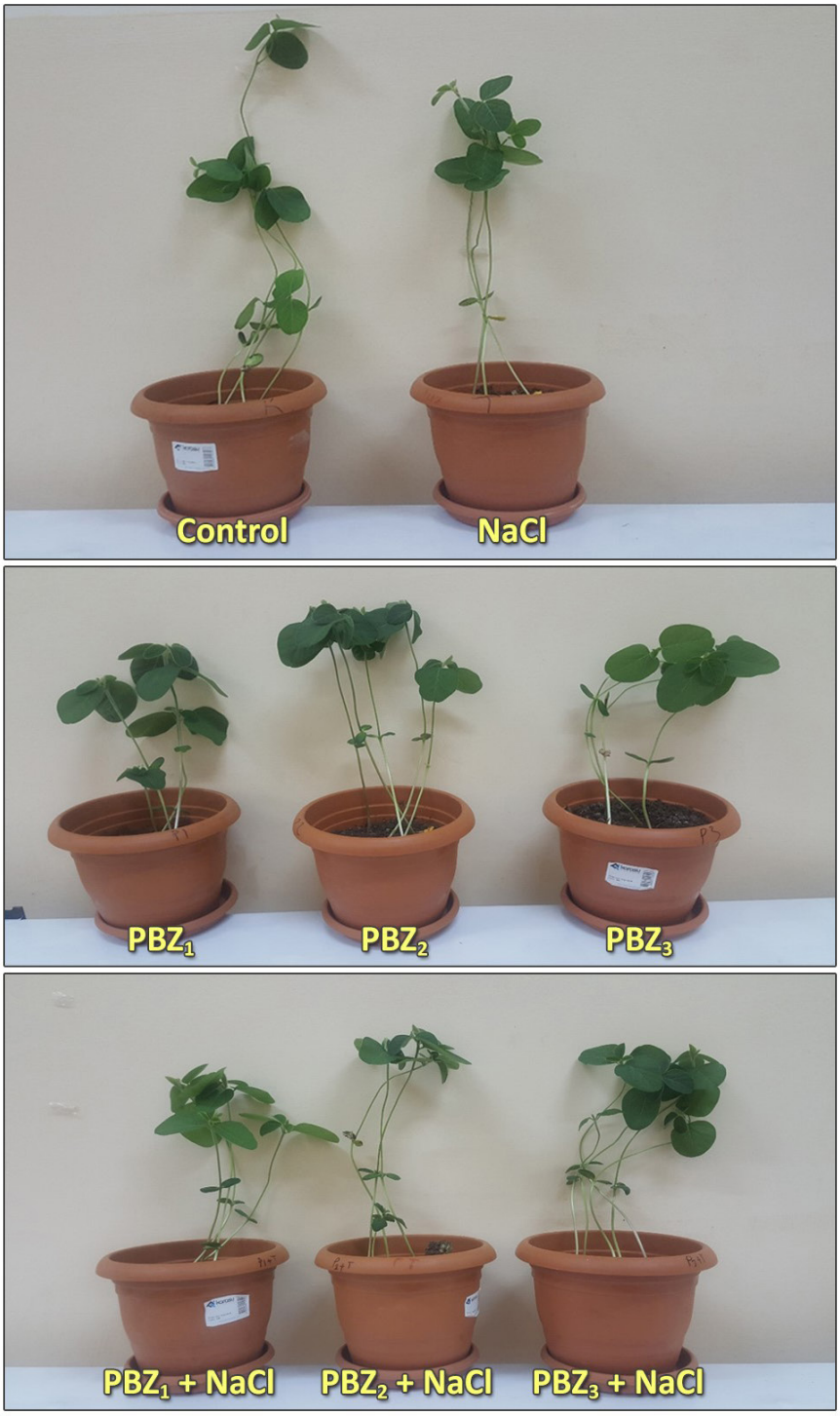


Fig. 1. Phenotypic traits of soybean (*Glycine max* L.) plants grown under NaCl stress and treated with paclobutrazol (PBZ). Control (1), NaCl 250 mM (2), PBZ; PBZ_1 5 ppm (3), PBZ_2 10 ppm (4), PBZ_3 20 ppm (5), NaCl + PBZ_1 5 ppm (6), NaCl + PBZ_2 10 ppm (7), NaCl + PBZ_3 20 ppm (8)

CAT enzyme activity. The catalase (CAT; EC 1.11.1.6) activity was assayed by monitoring the degradation of H_2O_2 at 240 nm over 2 min against a supernatant-free blank [Bergmeyer 1955].

POX enzyme activity. The peroxidase POX (EC 1.11.1.7) activity was based on the method described by Herzog and Fahimi [1973].

APX enzyme activity. The ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada [1981].

GST enzyme activity. Glutathione-*S*-transferase (GST; EC 2.5.1.18) activity was determined by the method of Habig et al. [1974] by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using reduced glutathione (GSH) as substrate.

Gene expression analyses of *GmClC1* and *GmNHX1.* The expression patterns of *GmClC1* and *GmNHX1* genes in salt and PBZ treated plants were evaluated with qRT-PCR analysis. In order to isolate total RNAs from the leaf tissues of soybean plants, Trizol Reagent (Thermo Fisher Scientific, US) was used by following the procedure recommended by the manufacturer. The integrity and quality of extracted RNAs were determined with NanoDrop 2000D UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA synthesis was carried out by using 1 µg of DNAse I treated total RNA with Revert Aid TM First Strand cDNA Synthesis Kit (Thermo Scientific, USA). BioRad CFX96 (BioRad, Hercules, CA) and Sso Advance Universal SYBR Green Supermix (Biorad) was used for the amplification of target gene according to the manufacturer's protocol. The EF-1α gene (LOC107826390) was used as an internal reference gene to normalize the expression of target gene. The relative expression level of target genes was calculated by using Ct method [Poku et al. 2019]. All reactions were repeated three times with triple biological replicates. Primer design was made using Acs number NM_001358090, NM_001249565, NM_001250237 (Tab. 1).

Statistical analysis

The experiment was conducted in a completely randomized design and measurements were performed with 6 replicates (n = 6). Statistical variance analysis of the data was performed using one way ANOVA and differences among treatments were compared using Tukey's posthoc analysis with least significant differences at the 5% level.

RESULTS AND DISCUSSION

In the present study, salt treatment increased more than twice the root length while did not influence the shoot length (Tab. 2). PBZ application also increased root length and the most increase was 80% by PBZ_2 (10 ppm), while a decrease in shoot length was detected (by 28.3%). However, the combination of PBZ and salt treatment decreased the length of roots (by 5.7%, by 13.6%, and by 22.1% respectively) and shoots (by 23.8%, by 20.8%, and by 26.6% respectively) as compared to the plants grown under salinity conditions only (Tab. 2). Similarly, the height of shoot was significantly decreased in PBZ treated barley plants under salt stress [Özmen et al. 2003]. In our study, PBZ application alone increased root length and decreased shoot length of soybean (Tab. 2). In parallel with these results, Yan et al. [2013] observed also that uniconazole, like paclobutrazol promoted root activity, root bleeding sap and improved root growth in soybean plants. PBZ significantly decreased soybean plant height by provoking a typical retardant inhibition of GAs biosynthesis [Fletcher et al. 2000].

The results indicated that PBZ individual treatments had no effect on RWC but increased significantly RGR (up to 76.5% after PBZ_3) as compared to the respective controls. However, the treatments with PBZ (5, 10, 20 ppm) increased RGR (~5.1, 4.3, 6 fold) and partially compensated the water loss due to salinity in combine-treated plants as compared to the salt-treated only (Tab. 2). The results of this study demonstrate a marked reduction in relative water content under saline conditions (Tab. 2). Furthermore, the application of PBZ improved RWC in the leaves of the salt-stressed plants. Our results are in agreement with Fletcher and Nath [1984] who stated that PBZ plays a role to decrease water loss and increases tolerance of plants to water stress. In recent years, it was found that PBZ can regulate hormone balance by inhibiting GA_3 biosynthesis and inducing ABA to maintain the water balance in plants [Fletcher et al. 2000]. From

Table 1. Genes used in this study. Gene identification number (gene ID number), forward (OR), reverse and primer sequences, expected amplicon length (bp)

NCBI accession number	Gene name	Primer name	Primer sequence	qPCR product lenght
NM_001358090	Glycine max actin-3 (ACTIN)	GmActin F	5′- CCCCCTCAACCCAAAGGCCA-3′	83 bp
		GmActin R	5′-TGGCCACATACATGGCAGGCA-3′	
NM_001249565	Glycine max chloride channel (CLC1)	GmCLC1 F	5′-TGGTGGAAGAGGTTGAAGAAAGGGA-3′	106 bp
		GmCLC1 R	5′-GCTCCAACAAGAGCAAGTGGATTGG-3′	
NM_001250237	Glycine max sodium/ hydrogen exchanger 1 (NHX1)	GmNHX1 F	5′-ACCATGCCTCCGTGGTCTCCA-3′	97 bp
		GmNHX1 R	5′-TCGTTCATCCACCGATTCTCCTCA-3′	

Table 2. Effects of paclobutrazol treatment on root and shoot length, relative water content, relative growth regulation and chlorophyll content of soybean (*Glycine max* L.) leaves under salt stress. Control (1), NaCl 250 mM (2), PBZ; PBZ1 5 ppm (3), PBZ2 10 ppm (4), PBZ3 20 ppm (5), NaCl + PBZ1 5 ppm (6), NaCl + PBZ2 10 ppm (7), NaCl + PBZ3 20 ppm (8). Columns with different letters represent significantly different ($P < 0.05$) values

	Root lenght	Shoot lenght	RWC	RGR	CHL
Control	$20.3167^{h} \pm 0.306$	$30.7500^{a} \pm 0.200$	$88.3429^{b} \pm 0.306$	$0.0416^{f} \pm 0.001$	$44.8578^{f} \pm 0.075$
NaCl	$44.3500^{a} \pm 0.361$	$30.8333^{a} \pm 0.577$	$74.8435^{h} \pm 0.030$	$0.0288^{g} \pm 0.001$	$34.7424^{h} \pm 0.108$
PBZ_1	$35.4833^{e} \pm 0.643$	$28.2133^{b} \pm 0.158$	$86.7472^{d} \pm 0.032$	$0.0459^{f} \pm 0.004$	$44.6657^{g} \pm 0.010$
PBZ_2	$36.5833^{d} \pm 0.473$	$22.0433^{f} \pm 0.093$	$87.8314^{c} \pm 0.030$	$0.0629^{e} \pm 0.003$	$57.8014^{e} \pm 0.020$
PBZ_3	$24.4500^{g} \pm 0.557$	$25.2833^{c} \pm 0.252$	$89.0562^{a} \pm 0.124$	$0.0734^{d} \pm 0.006$	$101.3925^{a} \pm 0.041$
PBZ_1 + NaCl	$41.8167^{b} \pm 0.208$	$23.4833^{de} \pm 0.643$	$81.3932^{e} \pm 0.300$	$0.1437^{b} \pm 0.006$	$71.3072^{b} \pm 0.003$
PBZ_2 + NaCl	$38.3167^{c} \pm 0.404$	$24.4000^{cd} \pm 1.00$	$78.6523^{f} \pm 0.058$	$0.1179^{c} \pm 0.003$	$61.3162^{d} \pm 0.001$
PBZ_3 + NaCl	$34.5500^{f} \pm 0.529$	$22.6100^{ef} \pm 1.00$	$76.0275^{g} \pm 0.072$	$0.1693^{a} \pm 0.010$	$61.5466^{c} \pm 0.020$

these findings, it could be suggested that PBZ treatment probably contributed for higher ABA levels in soybean grown under salinity as compared to solely salt-treated plants, which provides better water content in combine-treated ones.

In the present study, Table 2 shows that 10 and 20 ppm of PBZ doses significantly increased the chlorophyll content as compared to the control, while 5 ppm did not change the chlorophyll level. On the other hand, PBZ (5, 10, 20 ppm) increased chlorophyll content (~2.1, 1.76, 1.77 fold) under salinity as compared to the salt treatment alone (Tab. 2). Salt stress conditions decreased chlorophyll content, while PBZ (5, 10, 20 ppm) applied alone significantly increased it (Tab. 2). Likewise, PBZ treatment enhanced chlorophyll content in *Triticum aestivum* [Nouriyani et al. 2012]. Besides, Özmen et al. [2003] reported also that exogenous PBZ application increased chlorophyll and carotenoid contents in two barley cultivars subjected to salinity.

Figure 2 demonstrates that, MDA value was increased significantly (by 37.6%) after salt treatment

as compared to the control. PBZ_1 application also increased MDA values (by 28.3%) as compared to the control group. When PBZ was applied to salt treated plants, it prevented MDA increase in leaves, and there was a decrease by 10%, 18.7% and 14% respectively as compared to NaCl-treated only soybean (Fig. 2). As shown in Figure 3, hydrogen peroxide was increased under salt stress by 50.32%. PBZ_1 treatment also increased H_2O_2 level by 20.7%. However, PBZ_2 application decreased hydrogen peroxide content by 5.3% and PBZ_3 did no change it as compared to the control. In this study, three different PBZ applications diminished H_2O_2 values by 30.3, 25.4 and 46.9% respectively as compared to salt treatment alone (Fig. 3).

Regarding the MDA (Fig. 2) and H_2O_2 (Fig. 3) results, we found that salinity induced oxidative damage in soybean leaves although SOD, APX and CAT enzyme activities were increased significantly (Fig. 4a, 4b, 4e). Similarly, Agha et al. [2021] showed that salt treatmant increased MDA and CAT enzyme activity in soybean (*Glycine max* L.). From the data obtained in our model system, it could be suggested that soybean plants enhanced their antioxidant defence particularly to alleviate damages but this activation was not efficient enough to remove ROS produced by salt treatment. Both 250 mM NaCl, and 10 ppm PBZ applications increased SOD activity by ~2.8 fold, and ~1.8 fold respectively as compared to the control (Fig. 4a). PBZ_3 treatments led to ~1.7 fold decrease in SOD activity under salt stress, as compared to the salt treatment alone (Fig. 4a).

The results indicated that PBZ (5, 10 ppm) treatment increased APX activity (81% and 2.1 fold), while 20 ppm PBZ treatment decreased it insignificantly (by 5%) as compared to the control. Combination of PBZ (5 ppm) and salt treatment decreased APX (35.3%) as compared to the salt stress. While PBZ (10, 20 ppm) applications additionally increased (by 51.1% and by 15.7% respectively) this activity under salinity compared to the salt treatment alone (Fig. 4b). Our results showed that all single treatments did not alter considerably GST activity (Fig. 4c). On the other hand, combination of PBZ (5, 10, and 20 ppm) and salinity decreased GST activity by 42.3%, by 19.5%, and by 10.2% respectively. Figure 3d shows that salt treatment significantly decreased (by 68%) the POX activity, while PBZ treatments had no effect (5 ppm), or slightly increased (10 and 20 ppm) this enzymatic activity as compared to the control. However, PBZ (5, 10, 20 ppm) treatment increased POX activity (~2.1, 2.1, 2.17-fold respectively) under salinity conditions as compared to the salt treatment alone (Fig. 4d).

Salt treatment alone increased CAT activity by 42.4% as compared to the control, while the single application of the three PBZ concentrations significantly decreased CAT (Fig. 4e). However, PBZ treatments increased CAT activity (by 75.6, by 48.2, and by 3.4% respectively) under salinity conditions compared to the single salt application. A number of studies showed that paclobutrazol minimizied the adverse effects of salinity by increasing antioxidative enzymes in plants [Fletcher and Hofstra 1990, Ozmen et al. 2003, Srivastava et al. 2010, Sofy 2016, Sofy et al. 2020, Khunpon et al. 2019]. Interestingly, in our study paclobutrazol treatment induced APX, POX and CAT enzyme activities significantly but reduced SOD and GST under salinity as compared to salt treatment alone (Fig. 4a–4c). From these facts, it could be suggested that under salt stress conditions, PBZ plays role as an activator of H_2O_2 scavenging enzymes while inhibiting SOD enzyme activity, which leads to lower H_2O_2 level in combine treated plants as compared to the salt treated only. These results correlate also with the reduced lipid peroxidation production (lowest MDA with PBZ_2, 10 ppm) under salinity in comparison with salt treatment alone. The results obtained on the enzymatic antioxidants are in agreement with the report of Forghani et al. [2020] who showed that PBZ induced CAT and GR activity but did no change the SOD activity in sorghum plants cultured on Hoagland solution containing sodium chloride.

Significant downregulation of *GmCLC1* gene was observed in salt-stressed plants, while PBZ (5, 20 ppm) treatments induced *GmCLC1* transcript level in soybean (Fig. 5a). However, PBZ (20 ppm) treatment greatly upregulated *GmCLC1* gene expression (~4.18 fold), while other two concentrations had no noticeable effect on transcript level under the salinity condition when compared to salt stress only. *GmNHX1* gene expression was downregulated almost equally after all treatments with exception of the combination of PBZ (20 ppm) and NaCl (Fig. 5b). PBZ (20 ppm) treatment upregulated (~1.8 fold) *GmNHX1* gene ex-

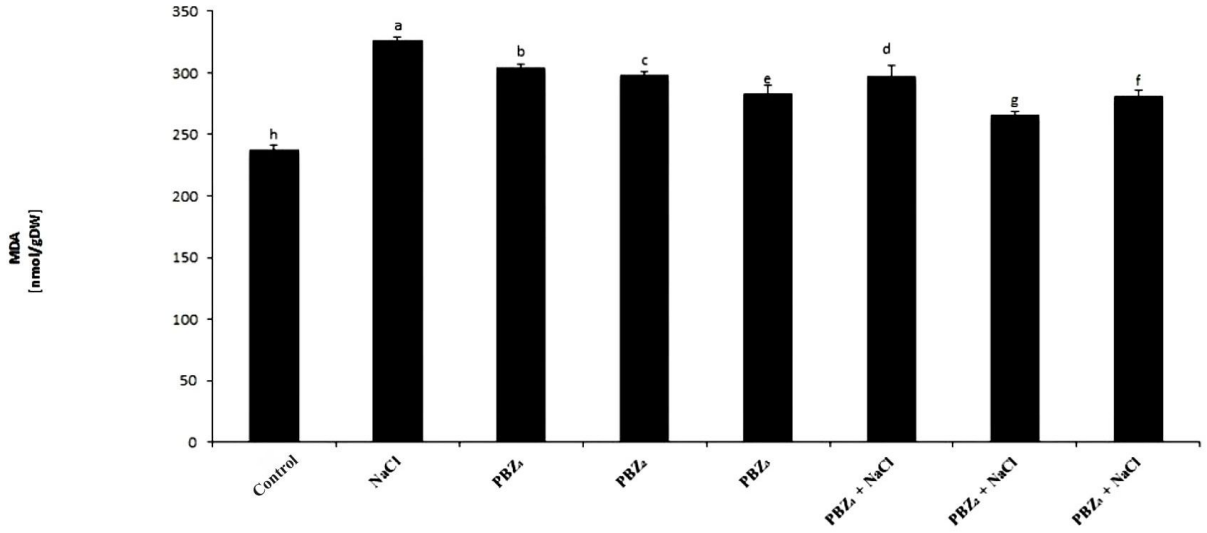


Fig. 2. Effects of paclobutrazol treatment on MDA content of soybean (*Glycine max* L.) leaves under salt stress. Control (1), NaCl 250 mM (2), PBZ; PBZ_1 5 ppm (3), PBZ_2 10 ppm (4), PBZ_3 20 ppm (5), NaCl + PBZ_1 5 ppm (6), NaCl + PBZ_2 10 ppm (7), NaCl + PBZ_3 20 ppm (8). Columns with different letters represent significantly different ($P < 0.05$) values

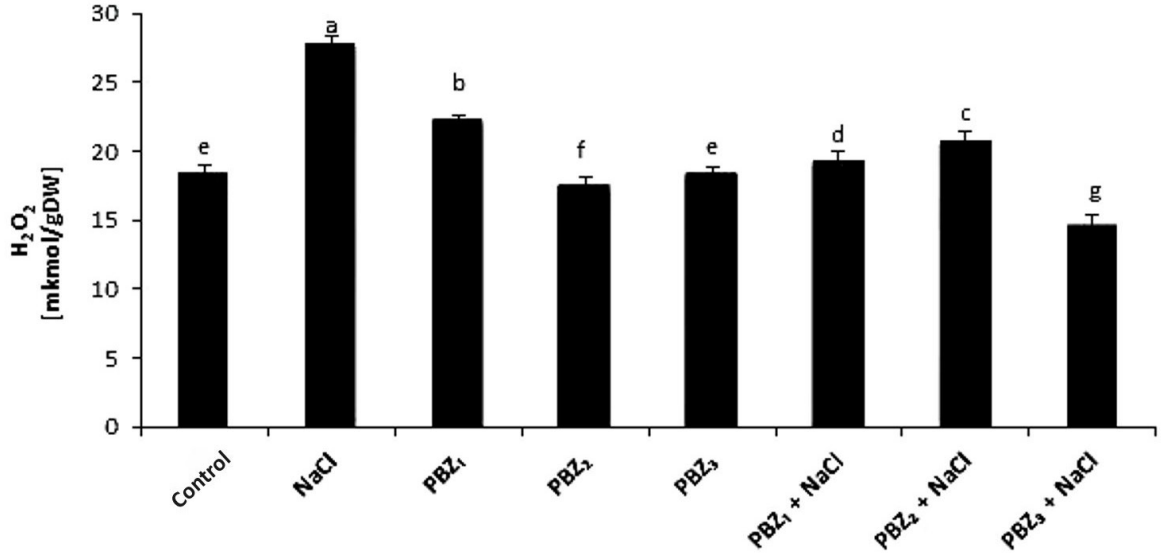
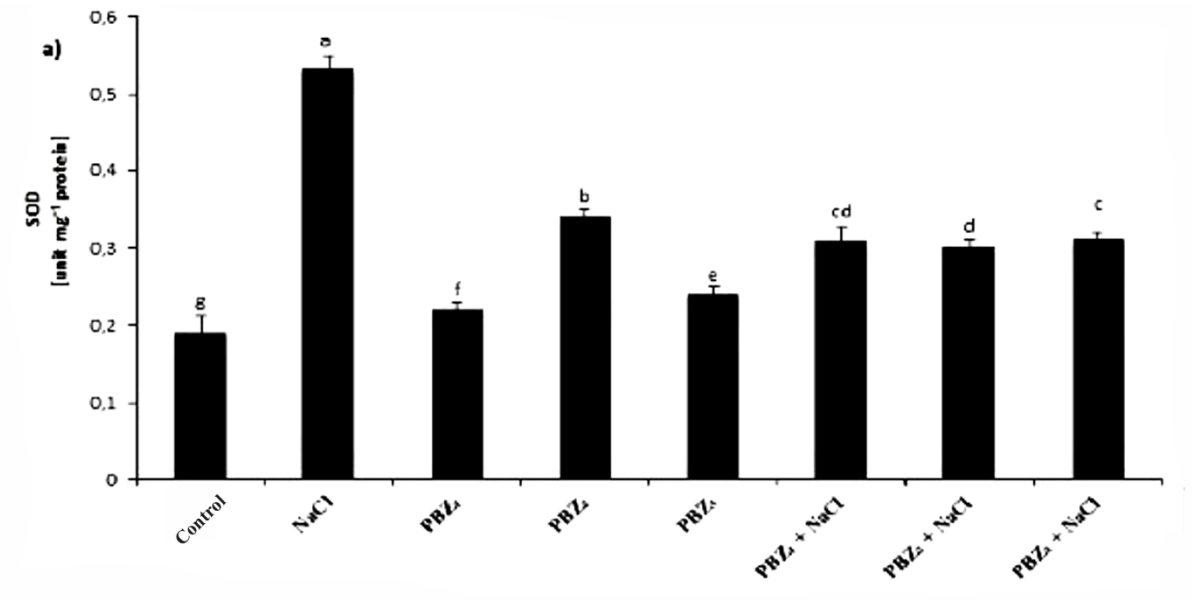


Fig. 3. Effects of paclobutrazol treatment on H_2O_2 content of soybean (*Glycine max* L.) leaves under salt stress. Control (1), NaCl 250 mM (2), PBZ; PBZ_1 5 ppm (3), PBZ_2 10 ppm (4), PBZ_3 20 ppm (5), NaCl + PBZ_1 5 ppm (6), NaCl + PBZ_2 10 ppm (7), NaCl + PBZ_3 20 ppm (8). Columns with different letters represent significantly different ($P < 0.05$) values

pression under the salinity condition when compared to salt stress only.

Both plant tonoplast-located ion transporters – Na^+/H^+ antiporters namely Na^+/H^+ exchangers (NHXs) and chloride channels (CLCs) have function in mitigation of toxic effects of Na^+ and Cl^- resp. by ion sequestering from cytoplasm into the vacuole. The *GmNHX1* and *GmClC* genes were isolated from soybean and upregulation by salinity of both gene expressions was established [Li et al. 2006]. However,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SOD
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0,5
0,4
0,3
0,2
0,1
0
a
g
f
b
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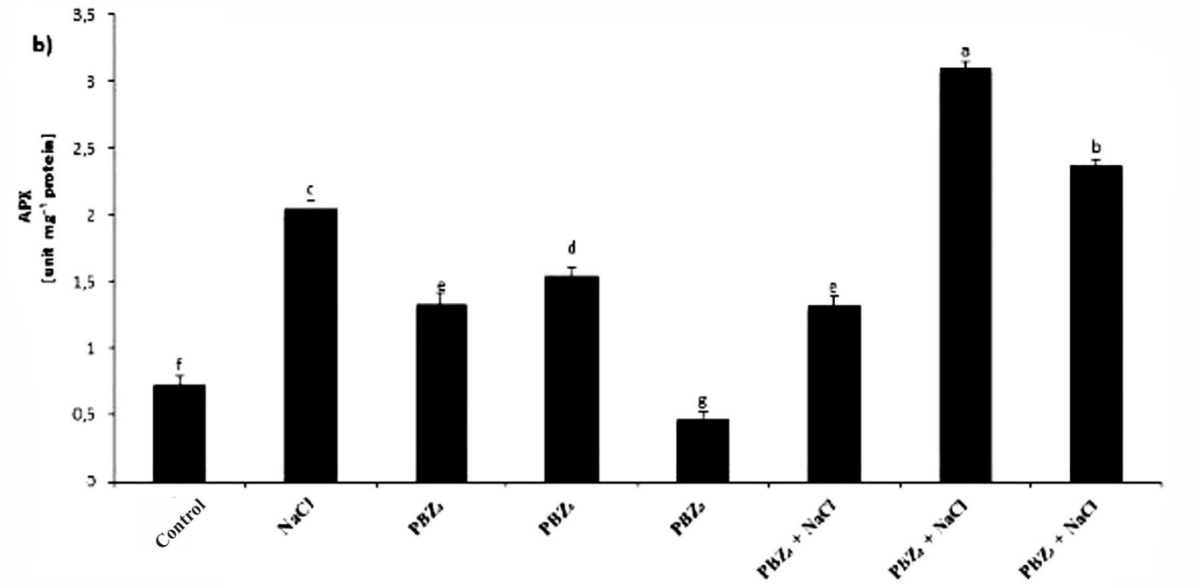
cd
d
c
Control
NaCl
PBZ1
PBZ2
PBZ3
PBZ1 + NaCl
PBZ2 + NaCl
PBZ3 + NaCl


b)
APX
[unit mg-1 protein]
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3
2,5
2
1,5
1
0,5
0
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c
e
d
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e
a
b
Control
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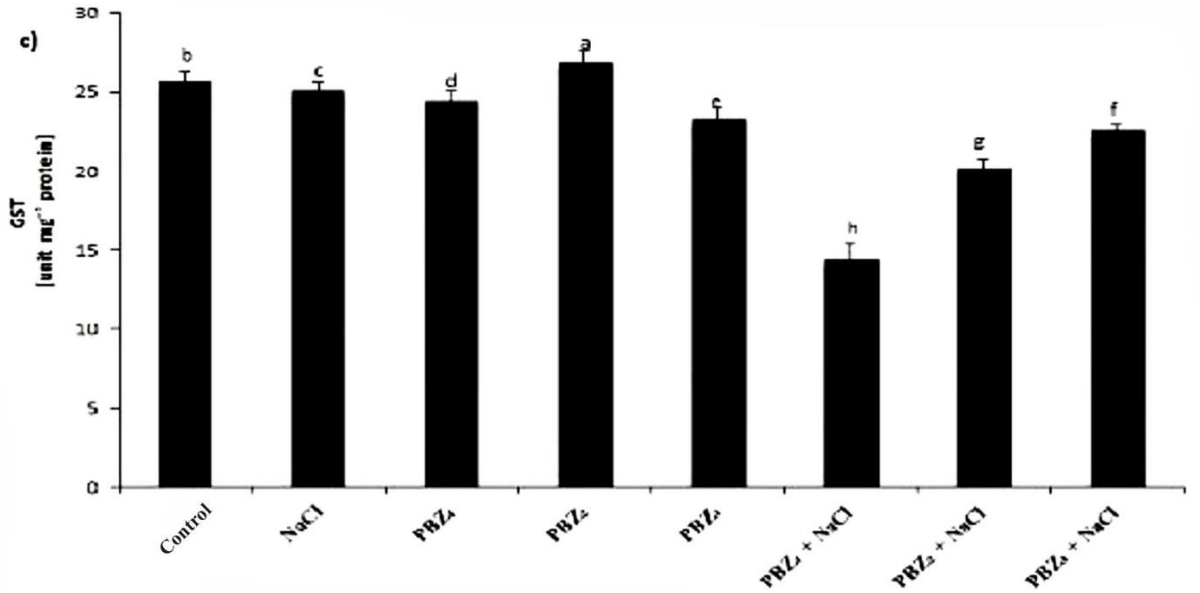
PBZ1
PBZ2
PBZ3
PBZ1 + NaCl
PBZ2 + NaCl
PBZ3 + NaCl


c)
GST
[unit mg-1 protein]
30
25
20
15
10
5
0
b
c
d
a
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g
f
Control
NaCl
PBZ1
PBZ2
PBZ3
PBZ1 + NaCl
PBZ2 + NaCl
PBZ3 + NaCl

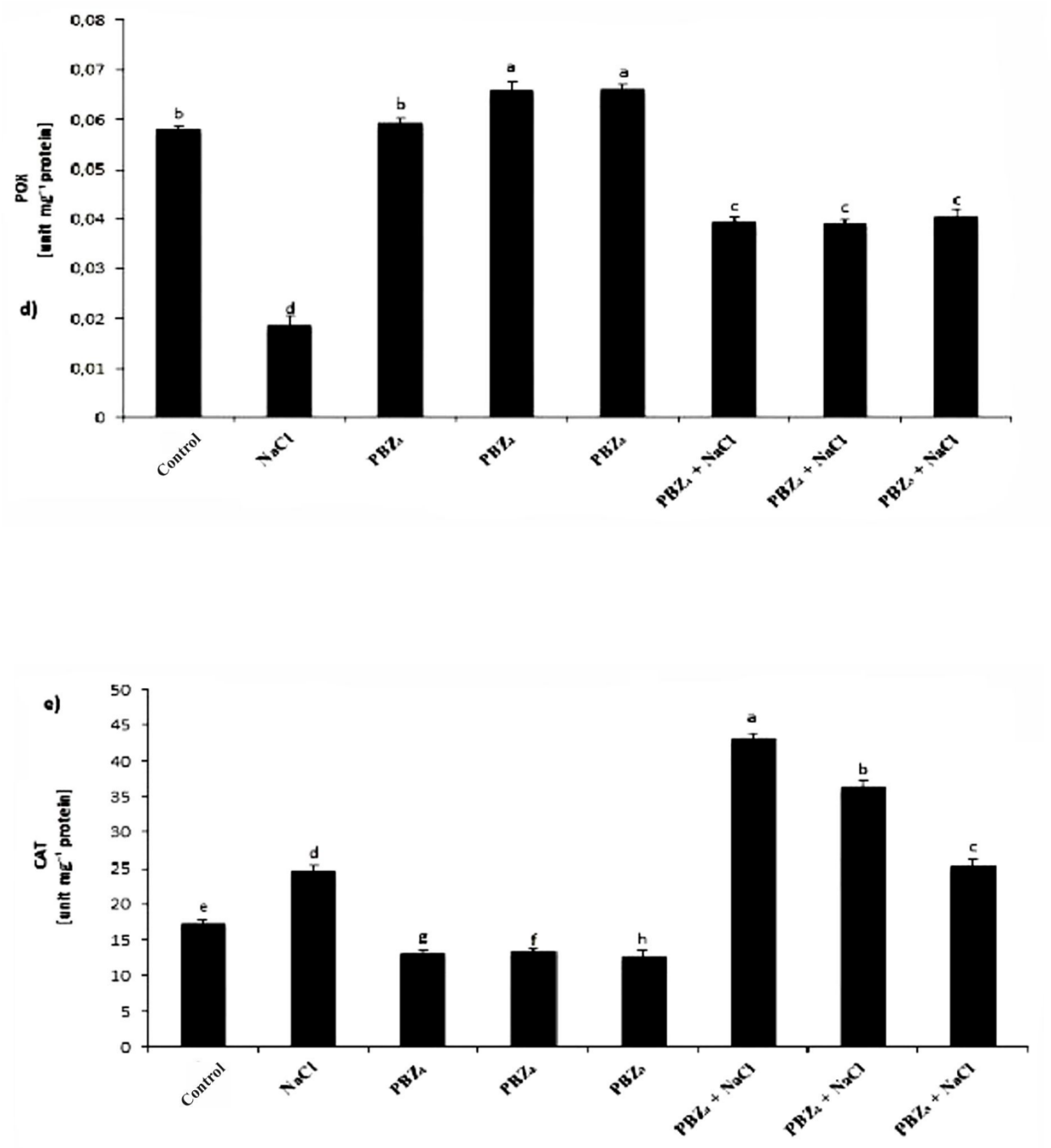
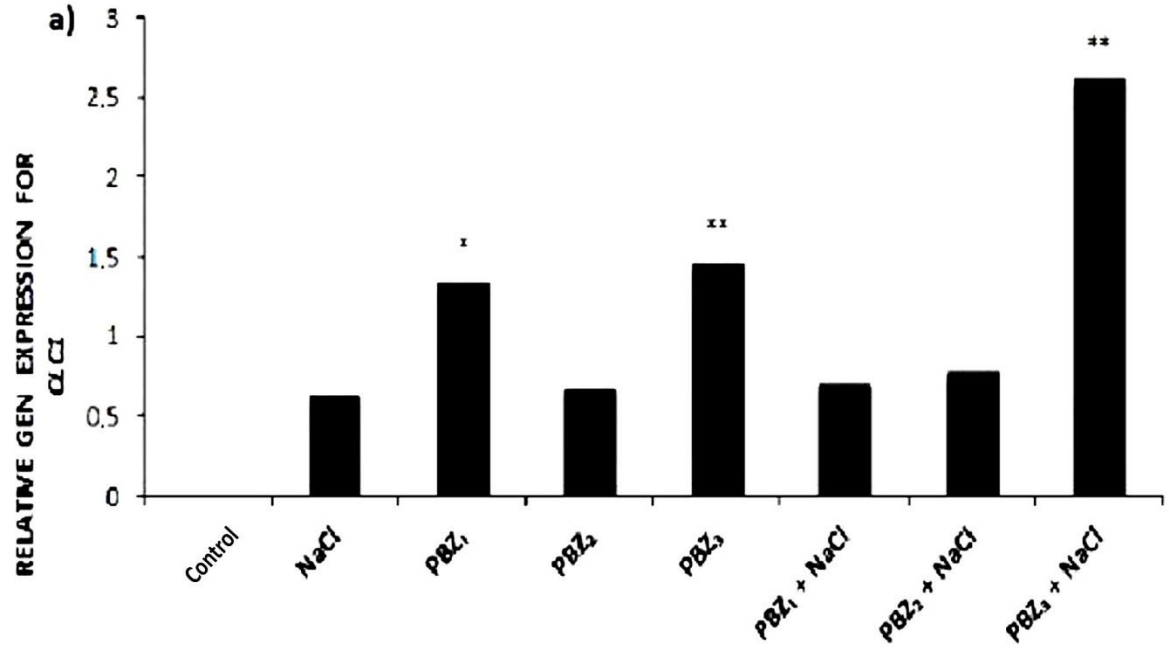


Fig. 4. Effects of paclobutrazol treatment on SOD (a), APX (b), GST (c), POX (d), CAT (e) antioxidant enzyme activities of soybean (*Glycine max* L.) leaves under salt stress. Control (1), NaCl 250 mM (2), PBZ; PBZ_1 5 ppm (3), PBZ_2 10 ppm (4), PBZ_3 20 ppm (5), NaCl + PBZ_1 5 ppm (6), NaCl + PBZ_2 10 ppm (7), NaCl + PBZ_3 20 ppm (8). Columns with different letters represent significantly different ($P < 0.05$) values


a)
RELATIVE GEN EXPRESSION FOR CLCI
3
2.5
2
1.5
1
0.5
0
*
**
**
Control
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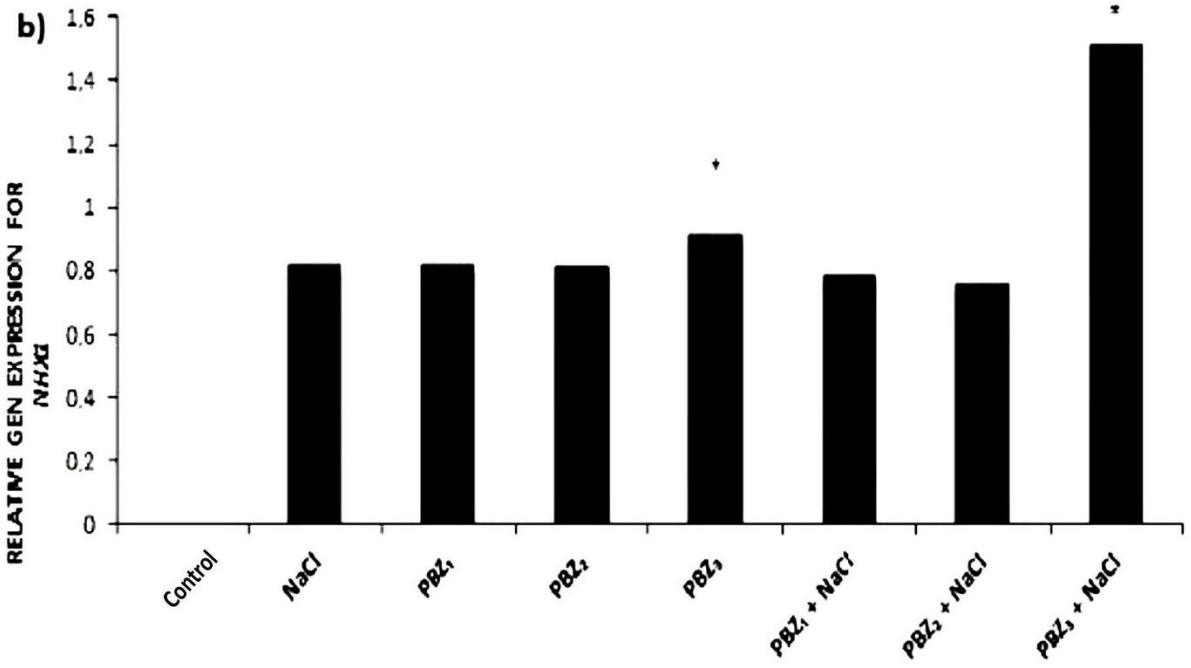
PBZ1
PBZ2
PBZ3
PBZ1 + NaCl
PBZ2 + NaCl
PBZ3 + NaCl


b)
RELATIVE GEN EXPRESSION FOR NHX1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
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PBZ2
PBZ3
PBZ1 + NaCl
PBZ2 + NaCl
PBZ3 + NaCl

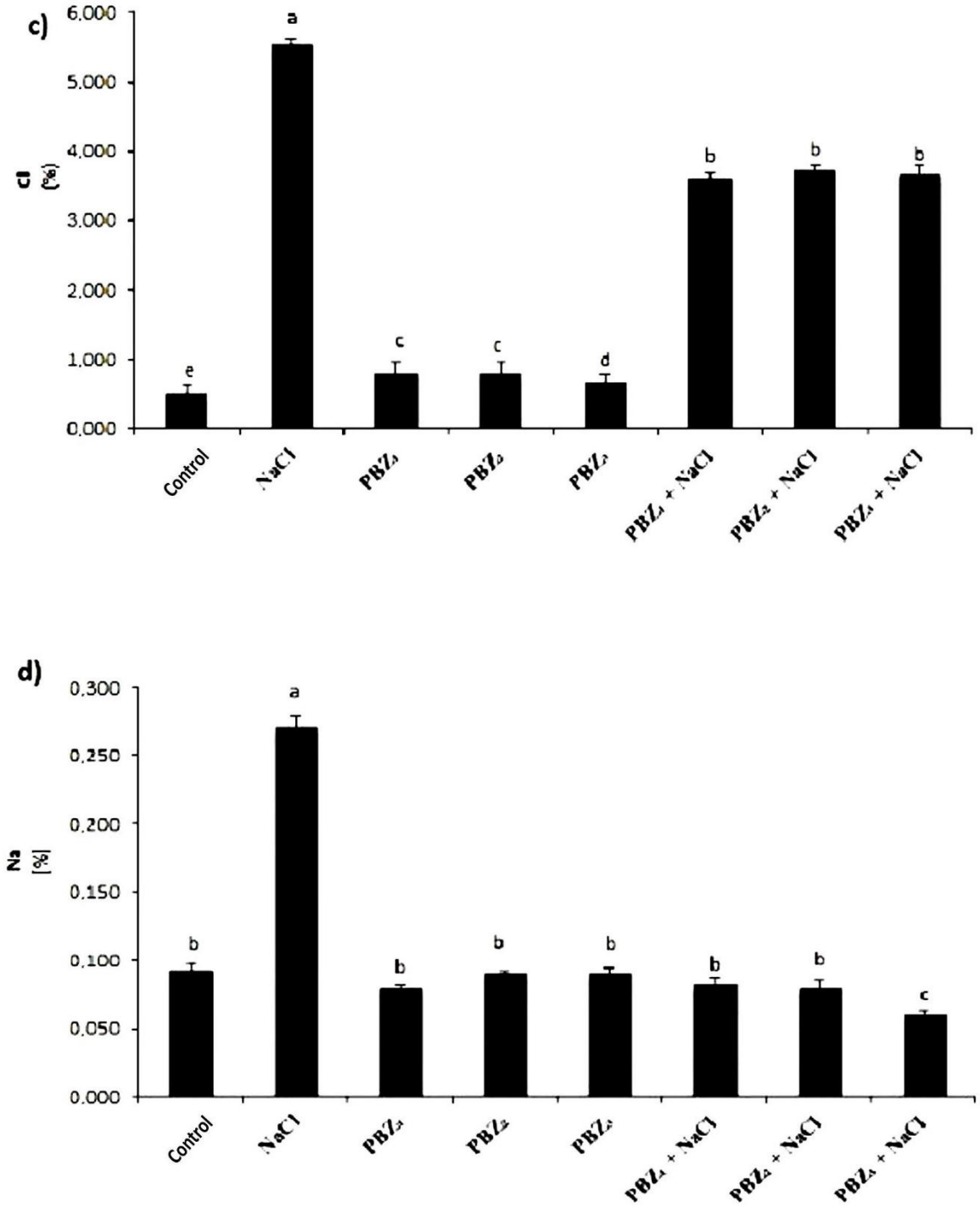

Fig. 5. Effects of paclobutrazol treatment on relative *GmCLC1*(a), *GmNHX1* (b) gene expression determined by q RT-PCR and Cl^- (c), Na^+ (d) ion concentration in leaves of soybean (*Glycine max* L.) under salt stress. Control (1), NaCl 250 mM (2), PBZ; PBZ_1 5 ppm (3), PBZ_2 10 ppm (4), PBZ_3 20 ppm (5), NaCl + PBZ_1 5 ppm (6), NaCl + PBZ_2 10 ppm (7), NaCl + PBZ_3 20 ppm (8). Columns with different letters represent significantly different ($P < 0.05$) values

in our study, *GmNHX1* and *GmClC1* gene expressions were downregulated under salinity, while PBZ_3 under the same conditions upregulated these genes (Fig. 5a, 5b). These results correlate with the data of toxic ions (Na^+ and Cl^-) accumulation. Similarly to our results, PEN (pencanozole – a triazole compound) application induced *NHX1* gene expression, while decreased toxic Na^+ in cytosol under stress conditions [Shaki et al. 2018]. To our knowledge, nowadays there is no any information available concerning triazole (paclobutrazol in partiqular) effects upon *CLC1*s gene expression. Our study for a first time revealed that PBZ is able to unduce gene expression of tonoplast-localized Cl^- channels in soybean that in turn leads to reducing the Cl^- accumulation in shoots under salinity conditions. It has been demonstrated that *GmNHX1* is induced with ABA dependent or independent pathway, while *GmClC1* is ABA independent [Phang et al. 2008]. Consequently, although, it has been known that PBZ could protect plants from salt stress by increasing ABA level, this study firstly revealed that PBZ can also be able to induce ABA independent pathway signal mechanism in soybean plants.

Figure 5c shows that salt treatment increased the Cl^- concentration in leaves as compared to the control (~11.2 fold). However, applied alone PBZ (5, 10, 20 ppm) had no effect on Cl^- concentration while the combination with NaCl treatment decreased Cl^- concentration by 1.5, 1.4, 1.5 fold respectively as compared to the salt treatment alone (Fig. 5c). Our results showed that after salt treatment, Na^+ concentration was increased ~2.9 fold in soybean plants as compared to the control (Fig. 5d). On the other hand, PBZ (5, 10, 20 ppm) application completely alleviated salt-induced accumulation of Na^+ and its concentration reached the control level. In addition, single applications of PBZ did not alter significantly sodium concentration. The toxic effects of the NaCl are also manifested through an unbalanced nutrient uptake, which leads to disruption of normal plant growth and development [Keutgen and Pawelizik 2008]. In our study, salt stress increased Na^+ and Cl^- concentration as compared to control (Fig. 5d, 5c). This increase caused a decrease of RGR, RWC, chlorophyll content and provoked oxidative damage. However, PBZ application under salinity stress decreased Cl^- and Na^+ ion concentrations in leaves as compared to salt treatment (Fig. 5c, 5d). The diminished by PBZ application toxic effects of NaCl stress resulted in better phenotype (Fig. 1). It is evident that PBZ treatment alleviated the negative consequenses of soil salinity.

CONCLUSION

It is clear that all doses of PBZ treatment mitigated salt induced damage in soybean, which is withnessed by better phenotypic trait as compared to fitness of salt-treated only plants. The most effective dose was PBZ_2 (10 ppm), which induced APX acitivity, accompanied by lowest MDA. *NHX* and *ClC1* gene expressions were most induced by PBZ_3 (20 ppm). This study demonstrated that PBZ could be used as a protectant to increase salt tolerance in soybean plants. The expressions of tonoplast located antiport genes were remarkable but not the only reason for increasing salt tolerance. However, the question how exactly PBZ triggers signaling systems and modulates gene expression in plants under salinity remains open.

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