Expression of gibberellin synthesis genes and antioxidant capacity in common bean (*Phaseolus vulgaris* L. cv. Sadri) seeds induced by chitosan under salinity

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Abstract

Effects of chitosan on the expression of gibberellin synthesis genes, activity of antioxidant enzymes, and biochemical traits of common beans under salinity stress were analyzed using a factorial experiment based on a completely randomized design with four replications. Four salinity levels (0, 50, 100 and 150 mM) and four chitosan levels (0, 0.25, 0.50, and 0.75% by weight-volume) were applied. Expression of gibberellin synthesis genes (*GA3OX1*, *GA3OX2*, *GA3OX3*, *GA3OX4*, *GA3OX5*, and *GA3OX6*) was assessed using qRT-PCR along with some biochemical indices. Salinity stress increased the activity of peroxidase and malondialdehyde (MDA) content. Chitosan 0.75% pretreatment increased peroxidase activity while decreasing MDA content. Phosphate content in seeds pretreated with 0.75% chitosan and no salinity increased by 57% compared to the control. With increasing salinity, the activity of superoxide dismutase, ascorbate peroxidase, and glutathione reductase increased while catalase activity decreased. Increases by 14%, 46%, and 34% were observed in the activity of superoxide dismutase (in 0.25% chitosan pretreatment), and ascorbate peroxidase and glutathione reductase (in 0.75% Chitosan pretreatment), respectively under 150 mM salinity stress. Under no salinity, the activity of catalase enzyme increased by 39% in 0.75% chitosan pretreatment compared to the control. *GA3OX1* gene expression in 0.75% chitosan priming under no salinity was higher than the other genes. With increasing salinity, gene expression decreased so that the lowest gene expression was non-primed seeds under 150 mM salinity. However, with further increase of salinity, expression of *GA3OX4*, *GA3OX5*, and *GA3OX6* genes reached zero. The study suggests that seed priming with 0.75% chitosan can reduce the adverse effects of salinity and improve seedling growth in common bean, as chitosan enhances *GA3OX* genes under salinity, which are necessary for germination and seedling establishment.

Keywords: germination, hormone, qRT-PCR, priming, salt.

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Introduction

Legumes are among the crops that grow all over the world and have adapted to different climatic conditions from temperate to tropical and from wet to dry. These plants have a high nutritional value and are one of the most important plant sources of protein, as they are the second most important human food source after cereals, and play an important role in animal nutrition as protein supply (Parsa and Bagheri, 2013). Due to

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their ability to mutualize with nitrogen-fixing bacteria, these plants can provide much of the nitrogen requirement (Khajehpour, 2015). Common bean (*Phaseolus vulgaris* L.) is one of the most important crops in the legume family in the world, which is considered as an important source of protein and calories, and is also a complement to rich carbohydrate sources such as rice, corn, and cassava (Mola and Belachew, 2015). The grain protein of this plant is two to three times higher than that of cereals and 10 to 20 times higher than starchy plants. Therefore, in countries where the production of animal protein is limited, this plant can play an essential role in providing the protein needed by humans (Ghamari and Ahmadvand, 2015).

Salinity stress is one of the most important factors that reduce crop yield in arid and semi-arid regions (Doraki et al., 2019). Therefore, increasing knowledge about environmental problems makes it important to look for easy and feasible alternatives to overcome the harmful effects of salinity on plants (Latef et al., 2021). Salinity stress refers to conditions that ions (such as sodium and chlorine) accumulate in the seed growth medium, disrupting the natural growth of seedlings (Ashraf and McNeilly, 2004). Increasing salinity stress leads to accumulating various types of reactive oxygen species (ROS) in cells and damaging membrane lipids, proteins, and nucleic acids with negative effects on germination of bean seeds, which ultimately decreases germination indices (Ghanbari et al., 2019).

Priming is a type of seed preparation technique that has been approved by many farmers for better establishment and growth of crop bases even in adverse environmental conditions (Bose et al., 2018). Priming actually activates pre-germination metabolism, which involves a wide range of physiological functions that activate DNA repair pathways and ROS modification systems, as well as helping to maintain genome integrity (Paparella et al., 2015).

Chitosan is a polymer produced by fungi, insects, and crustaceans, which has positive effects on the growth and productivity of many plants and has antimicrobial activities (Malerba and Cerana, 2016). Priming with chitosan is a new trend to achieve highly resistant seeds and improve germination and seedling growth under various conditions of abiotic stress (Maroufi et al., 2011). Evaluation of the effect of different levels of chitosan on the activity of antioxidant enzymes in *Aloe vera* plants under stress showed that chitosan improves the activity of antioxidant enzymes (Taheri et al., 2017). When plants are exposed to biotic and abiotic stresses, the oxidative damage appears as secondary stress, so plants activate their antioxidant systems. Under such conditions improving plant growth depends upon the effective function of antioxidant enzymes to overcome oxidative stress (Kheirizadeh Arough et al., 2016). Abdalla et al. (2011) reported the effect of chitosan stimulation on the activity of antioxidant enzymes under stress in safflower.

Chitosan in medicinal plants increases the production of secondary metabolites by expressing genes involved in the metabolite biosynthesis pathway (Boonlertnirun et al., 2010). In a study on the effect of chitosan on the expression pattern of some genes related to pathogenicity in wheat infected with powdery mildew, qPCR results showed that after infection with the pathogen, all three genes (*PR1*, *PR2*, *PR5*) in plants treated with chitosan had a significant increase compared to control plants. The results showed that the use of chitosan can play an important role in increasing plant resistance to disease (Rahneshan et al., 2020). Chitosan increases rice seedling growth through the gene expression network between the nucleus and chloroplasts (Chamnanmanoontham et al., 2015). Due to the effect of chitosan on the expression of plant genes, it has been reported that these substances are able to enhance resistance to some adverse environmental factors (Demirevska et al., 2019). Also, increased expression of phenylalanine ammonia lyase gene and protein increased accumulation of secondary metabolites in chitosan treated plants (Nag and Kumaria, 2018). Gibberellins are widely involved in plant various biological activities such as seed germination and GA3OX genes are the key enzymes for the transformation of gibberellins (Hu et al., 2021).

There is no report on the effect of chitosan on *GA3OX* expression in common beans under

salinity in the literature; therefore, the aim of this study was to investigate the effect of different concentrations of chitosan on the expression of gibberellin synthesis genes, activity of antioxidant enzymes, and biochemical traits under salinity stress in common beans.

Materials and Methods

Experimental design and treatments

A factorial experiment was conducted based on a completely randomized design with four replications. Experimental factors included four salinity levels (0, 50, 100, and 150 mM) and four chitosan levels including 0 (distilled water), 0.25%, 0.50%, and 0.75% by weight-volume, all of which being dissolved in 1% acetic acid.

Seeds were first immersed in priming solutions and distilled water for 12 hours. After priming, the seeds were washed several times with distilled water and dried at laboratory temperature. Then, standard germination test was performed on them. Germination test was performed by paper towel in three replications of 50 seeds at 25 ℃ for seven days. In this method, filter paper (Boeco-Germany size 58 * 58) was used and 50 seeds were placed in a row on a layer of filter paper and saline solution (sodium chloride) with different levels was added to each filter paper and then another filter paper was placed on the seeds. The bottom edge of the papers was folded 3-4 cm wide and wrapped in a tube from the side edge and moved vertically into the germinator.

Measurement of antioxidant enzymes activity

In order to determine the activity of antioxidant enzymes in beans, seedlings were grown inside the germinator at 25 ℃ by paper towel method. After opening the initial leaves of each treatment, several seedlings were randomly selected and placed in aluminum foil until extraction of the enzyme extract and were transferred to the freezer at -70 ± 2 ℃. To prepare the enzymatic extract, 0.5 g of the sample was weighed from each treatment and homogenized inside a porcelain mortar (previously stored in the refrigerator) using liquid nitrogen, then 1.5 ml of cold phosphate buffer (pH = 7) containing 0.5 mM EDTA was added to the mortar. The homogenates were then transferred to 2 mm microtubes and centrifuged at 15,000 rpm at 4 ℃ for 15 minutes. The resulting supernatant was divided into three parts to prevent the harmful effects of sequential freezing and thawing of the samples and then stored at -20 ℃ until the measurement of antioxidant enzymes activity (Sairam et al., 2002).

Catalase (CAT) activity was determined by the method of Aebi (1984). Briefly, A 20 µl of the supernatant from the enzyme extraction stage was added to 1.5 ml of reaction mixture containing 30 µl water, 50 µl of 1 M Tris-HCl buffer (pH 8.0), 5 mM EDTA, and 900 µl of 10 mM H_2O_2. The light absorbance was recorded at 240 nm wavelength for 60 sec.

The peroxidase (POX) activity was measured according to the method of Macadam et al. (1992). A 450 µl of hydrogen peroxide solution and 450 µl of guaiacol solution were mixed at low temperature (ice bath), and 100 µl of enzyme extract was added and the absorption changes at 470 nm were recorded by a spectrophotometer. In blank instead of enzymatic extract, 100 µl of phosphate buffer (pH = 7.8) was used. Enzymatic activity was calculated using Lambert-Beer's law and the extinction coefficient of the product of guaiacol peroxidase reaction (26.6 mM cm^{-1}). Enzyme activity was finally expressed in unit mg $protein^{-1}$ min^{-1}.

Assay of superoxide dismutase (SOD) activity was done by Giannopolitis and Ries method (1977). The basis of measuring the activity of SOD enzyme is inhibiting the radical reaction of superoxide with nitroblutrazotolium and preventing the formation of superoxide-nitroblutrazole by this enzyme. The blank sample was placed in the dark for 15 minutes and the control samples and the enzyme extract were placed on a shaker in a light chamber with two 20 W fluorescent lamps for 15 minutes and 100 rpm. Then, the absorbance was recorded at 560 nm. The difference between the adsorption of each extract in the light duration of 15 minutes and the adsorption of the enzyme extract in the same light time actually indicate that SOD inhibits the spontaneous reaction and formation of formazan.

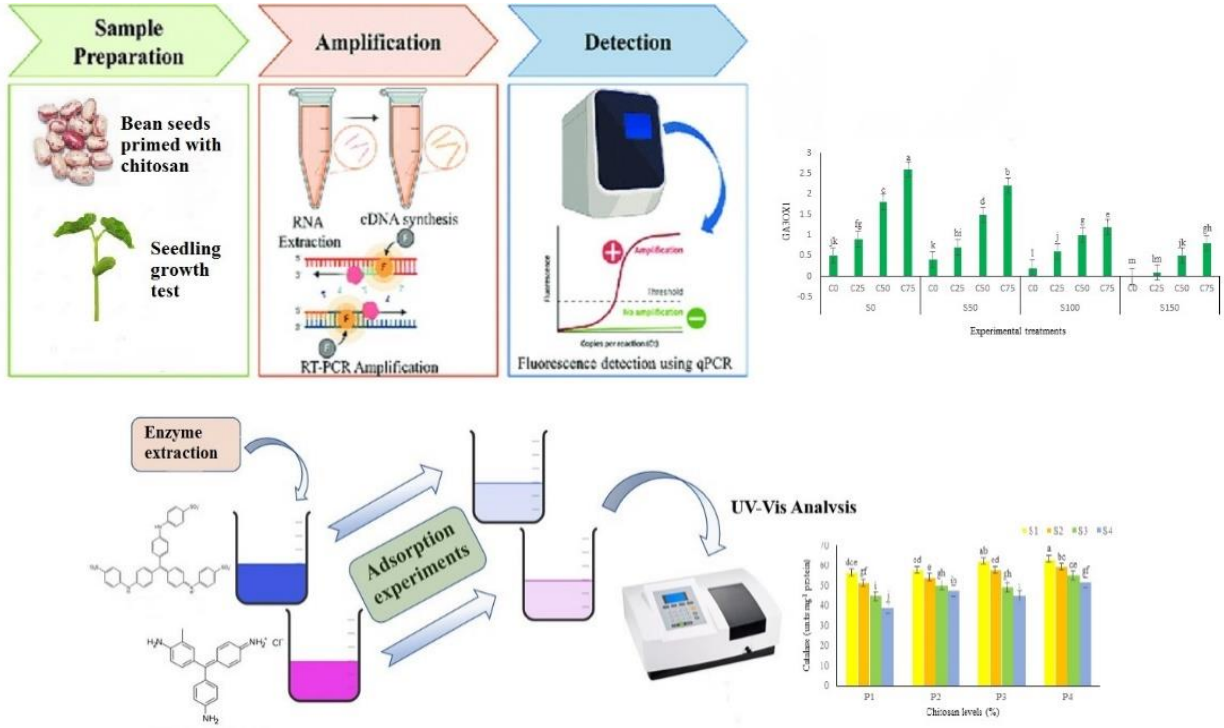

Fig. I. Workflow of our experiment representing the procedure of the study

The ascorbate peroxidase (APX) activity was determined by Nakano and Asada method (1981) with slight changes. The reaction medium was prepared with phosphate buffer (pH = 7.8), 0.1 mM EDTA, 50 mM ascorbate, 0.3 mM hydrogen peroxide, and 10-20 µl of enzyme extract. The reaction was started by adding H_2O_2. Reduction in adsorbate due to ascorbate oxidation was recorded at 290 nm and 20 seconds after the addition of hydrogen peroxide by the spectrophotometer. The amount of this enzyme was calculated based on the changes in the absorption unit $min^{-1}mg\ prot^{-1}$.

Glutathione reductase (GR) activity was measured according to Esterbauer and Grill (1978) with slight modification. The experimental mixture was 100 mM Tris HCl (pH = 7.8), 2 mM EDTA, 0.1 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and 50 µmol of enzyme extract with a total volume of 2 ml. The reaction was started by adding NADPH and then the adsorption amount was recorded at 340 nm. GR activity was expressed in terms of unit uptake per second per milligram of protein (Fig. I).

Antioxidant capacity assay in the shoot tissue extracts was measured using the stable DPPH radical following the method described by Hatano et al. (1988). Fresh tissues (1.0 g) were ground into fine powder and extracted with 10 mL ethanol (90%) by constant shaking at room temperature for 48 h. The extract was centrifuged at 13,000 rpm for 10 min and the supernatant was used for estimating antioxidant activities. The alcoholic solution of DPPH radical (0.5 mL, 0.2 mM) was added to 100 ml of the sample extract, mixed vigorously, and kept standing in dark for 40 min. Then, absorbance was measured at 517 nm, and the capacity to scavenge DPPH radical was calculated using the following formula:

Scavenging (%) $D = [(A_0 - A_1) /A0)] \times 100$

where A_0 and A_1 are the absorbance of the control reaction and the absorbance of the sample at 517 nm, respectively. Inhibitory concentration at 50% (extract concentration that cause 50% scavenging of DPPH radical IC50) was also determined.

Measurement of malondialdehyde (MDA)

The amount of MDA was measured using the method of McCue and Shetty (2002). In the test tubes, 200 ml of homogeneous tissue was mixed with 800 ml of distilled water. Then, 500 ml of 20% trichloroacetic acid was mixed with 1 ml of 10 mM

Table 1
Forward and reverse primers used in Real Time PCR

Gene	Primer Sequence	Product Size, bp	NCBI Acc. No. or Ref
GA3OX1	5′ GCTTGCACCAACCCACTTTC 3′ 5′TGGCACGAAGGATTCACCAT 3′	195	XM-003522590.5
GA3OX2	5′ GCTCGGGACAACTTGGGTAA 3′ 5′ AGGCTCTTCGATCTCCTGGT 3′	318	XM-003527750.5
GA3OX3	5′ TGGTGAACCATGCCATACCC 3′ 5′ AGGCTGCGCATGTCTTTTTG 3′	387	XM-003543614.5
GA3OX4	5′ TCCACCGCTTTTGTTCCTCA 3′ 5′CGAGCTCGGATATCACCGTT 3′	223	XM-003544576.4
GA3OX5	5′AACCTGCAACCCGCTTATGT 3′ 5′TCGCTCGCATCTCTTCCTTC 3′	377	XM-003546191.5
GA3OX6	5′ATCGCCATTCTTCCCCAAGC 3′ 5′GCTCAGGACAACGAGGGTAG 3′	278	XM-003550114.5
Actin	GAAGTTCTCTTCCAACCATCC 3′′ 5 TTTCCTTGCTCATTCTGTCCG 3′ ′5	175	(26)

thiobarbituric acid. The test tubes were transferred to a 100 ℃ incubator for 30 minutes. It was then centrifuged at 13,000 g for 10 minutes and the absorbance was read at 532 nm.

Measurement of phosphate reserves

One g of grain was digested in 20 ml of concentrated nitric acid, and then 10 ml of 72% perchloric acid was added to warm the solution and lose the color to the last degree. The digest was cooled and transferred to a 100 ml flask. Then, 5 ml of sulfuric acid, 5 ml of ammonium vanadate, and 5 ml of ammonium molybdate were added to the solution and kept for 3 minutes. The amount of phosphorus in the sample was determined by photometric method (Warraich et al., 2002).

RNA Extraction, cDNA Synthesis and Real-time PCR Gene Expression Analysis

To extract the total RNA from the samples, the RNX-plus extraction kit of Sinagen Company (Iran) was used. The quality of RNA extracted in 1% agarose gel was evaluated. To determine the purity and concentration of RNA samples, a nanodrop spectrophotometer (Thermoscientific, 2000c) was used. RNA samples were then treated with DNase I (RNase-free, Fermentase) and used as an RT-RNA reaction model. After standardizing the concentration of RNA samples, the cDNA of each sample was made (Thermo scientific) according to the cDNA synthesis kit instructions (Mahmoodi Jaraghili et al., 2016). Relevant sequences for primer design were obtained using the accession number of the relevant genes on the NCBI database and were designed by observing all the basic principles in primer design and determining the size of 100 to 400 bp for PCR primer product (Table 1) using Primer3web program (https://primer3.ut.ee/). Gene expression was assessed using Real Time PCR Light Cycler 96 Roche (Fig. I). In order to standardize the data, the samples were normalized with the actin housekeeping gene, because it has the lowest variation across all tissues and was selected as the normalization gene for all samples (Livak and Schmittgen, 2001). Gene expression ratio was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analysis of data was performed using SAS 9.4 software after normality test, and the means were compared with the least significant difference (LSD) test at 5% probability level.

Results

Antioxidant enzymes activity

The highest CAT activity (63.535 unit mg^{-1} protein) was obtained in pretreatment with 0.75% chitosan and 0 mM salinity (control) while the lowest (38.875 unit mg^{-1} protein) was in control (distilled water) priming and 150 mM salinity (Fig. II). Increasing salinity severely decreased CAT activity, but priming with chitosan compensated for the reduction of enzyme catalectic action. There was

11% increase in the CAT activity after priming with the highest concentration of chitosan regarding control at non-saline conditions, but under sever salinity the increase reached 32% in comparison with the control.

The highest POX activity (70.656 unit mg^{-1} protein) was observed in 0.75% chitosan pretreatment, and the lowest (52.788 unit mg^{-1} protein) was recorded in the control (distilled water), which was 34% greater than control (Fig. III). POX activity increased with increasing salinity up to 100 mM and then decreased at 150 mM, but the difference was not significant. The highest POX activity was observed at 100 mM salinity (61.925 unit mg^{-1} protein), and the lowest activity (59 unit mg^{-1} protein) was recorded in the control (data are not shown).

The highest SOD activity (182.125 unit mg^{-1} protein) was achieved in the pretreatment with 0.25% chitosan and 150 mM salinity, which was not significantly different from other concentrations of chitosan, and its lowest quantity (156.8 units mg^{-1} protein) was recorded in control priming (distilled water) and 0 mM salinity (Fig. IV). Under severe saline conditions, SOD activity increase was 9% compared with the control, but under non-saline condition the amount of increase was 11%.

The highest APX (32.05 unit mg^{-1} protein) and GR activities (73.925 unit mg^{-1} protein) were related to pretreatment with 0.75% chitosan and 150 mM salinity, that were 60% and 48% greater than control, respectively. The lowest activities (17.215 and 48.525 unit mg^{-1} protein, respectively) were observed in control priming (distilled water) and without salinity (Figs. V & VI).

Antioxidant capacity based on DPPH increased as the concentration of chitosan raised, and the highest capacity was observed at 0.75% of chitosan (Fig. VIIa). Salinity also increased the antioxidant capacity, and the highest values were observed at 100 and 150 mM (Fig. VII, b).

MDA content

Priming with chitosan decreased the amount of MDA approximately by 16% and the highest MDA (0.918 mM g^{-1} FW) was observed in control

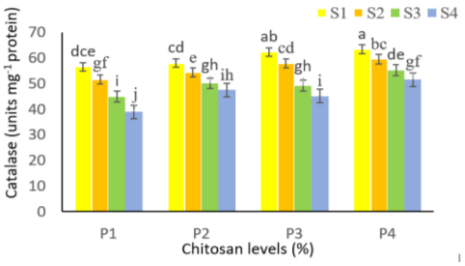

Fig. II. Mean comparison for the interaction effect of chitosan and salinity on catalase activity in common bean; P1: chitosan 0, P2, chitosan %0.25, P3: chitosan %0.50, P4: chitosan %0.75, S1: 0 salinity; S2: 50 mM salinity; S3: 100 mM salinity; S4: 150 m salinity; different letters in each column indicate significant difference (P≤ 1%).

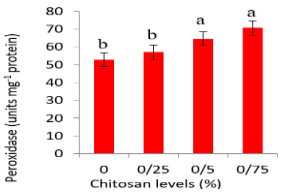

Fig. III. Mean comparison for the simple effects of chitosan on peroxidase activity in common bean; different letters in each column indicate significant differences (P≤ 1%).

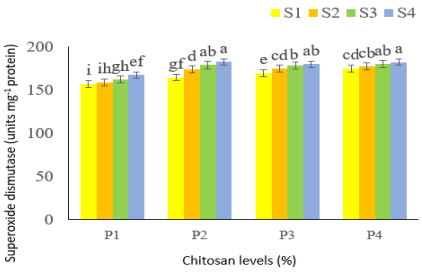

Fig. IV. Mean comparison for the interaction effect of chitosan and salinity on superoxide dismutase activity in common bean; P1: chitosan 0, P2: chitosan %0.25, P3: chitosan %0.50, P4: chitosan %0.75, S1: 0 salinity, S2: 50 mM salinity, S3: 100 mM salinity, S4: 150 mM salinity; different letters in each column indicate significant difference (P≤1%).

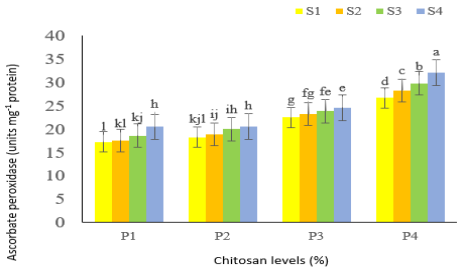

Fig. V. Mean comparison of the interaction effect of chitosan and salinity on ascorbate peroxidase activity in common bean; P1: chitosan 0, P2: chitosan %0.25, P3: chitosan %0.50, P4: chitosan %0.75, S1: no salinity, S2: 50 mM salinity, S3: 100 mM salinity, S4: 150 mM salinity; different letters in each column indicate significant difference (P≤ 1%).

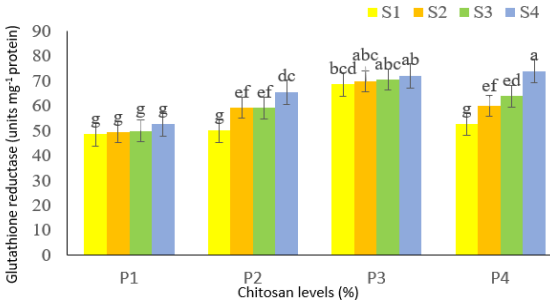

Fig. VI. Mean comparison of the interaction effect of chitosan and salinity on glutathione reductase activity in common bean; P1: chitosan 0, P2: chitosan %0.25, P3: chitosan %0.50, P4: chitosan %0.75, S1: 0 salinity, S2: 50 mM salinity, S3: 100 mM salinity, S4: 150 mM salinity; different letters in each column indicate significant difference (P≤0.01).

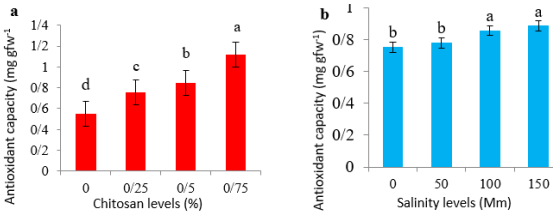

Fig. VII. Mean comparison of the simple effects of chitosan (a) and salinity (b) on antioxidant capacity in common bean; different letters in each column indicate significant differences (P≤0.01).

(distilled water), but the lowest content of MDA (0.763 mM g^{-1} FW) was related to priming with 0.75% chitosan (Fig. VIII, a). Salinity increased MDA content and the highest amount was recorded at 150 mM salinity (0.93 mM g^{-1} FW), but the lowest value (0.758 mM g^{-1} FW) was observed (Fig. VIII, b). This was 22% greater than control.

Phosphate content

The highest phosphate content (0.054 µmol g^{-1} FW) was obtained by 0.75% chitosan pretreatment under 0 mM salinity (control) while the lowest content (0.02325 µmol g^{-1} FW) was observed in the control (distilled water) under 150 mM salinity (Fig. IX). Increasing salinity levels decreased the amount of phosphate reservoirs, and priming in contrast raised the phosphate content.

Expression of gibberellin synthesis genes

The highest relative expression of gibberellin synthesis genes was observed in priming treatment with 0.75% chitosan and salinity level of

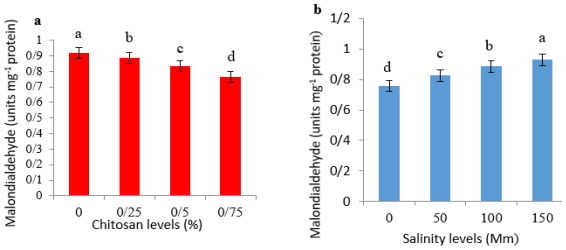

Fig. VIII. Mean comparison of the simple effects of chitosan (a) and salinity (b) on malondialdehyde content in common bean; different letters in each column indicate significant differences (P≤0.01).

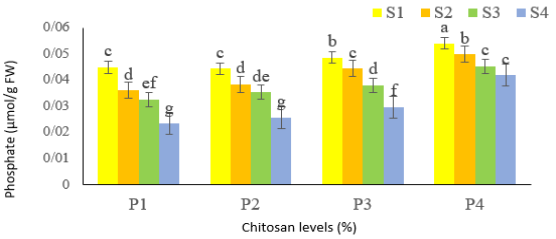

Fig. IX. Mean comparison for the interaction effect of chitosan and salinity on phosphate content in common bean; P1: chitosan 0, P2: chitosan %0.25, P3 chitosan %0.50, P4: chitosan %0.75, S1: 0 salinity, S2: 50 mM salinity, S3: 100 mM salinity, S4: 150 mM salinity; different letters in each column indicate significant difference (P≤0.01).

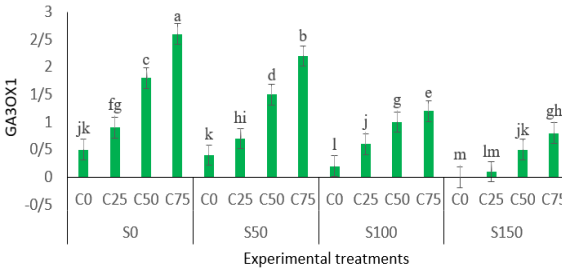

Fig. X. Expression pattern of gibberellin synthesis gene *GA3OX1* due to priming and salinity; C0: chitosan 0, C25: chitosan %0.25, C50: chitosan %0.50, C75: Chitosan %0.75, S0: 0 salinity, S50: 50 Mm salinity, S100: 100 Mm salinity, S150: 150 Mm salinity; different letters in each column indicate significant differences (P≤0.01).

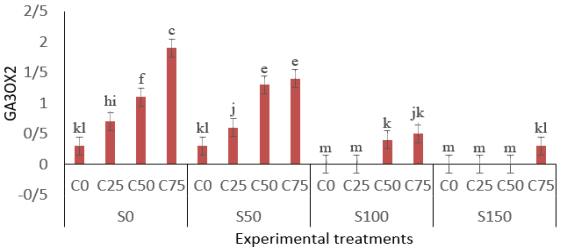

Fig. XI. Expression pattern of gibberellin synthesis gene *GA3OX2* due to priming and salinity; C0: chitosan 0, C25: chitosan %0.25, C50: chitosan %0.50, C75: chitosan %0.75, S0: 0 salinity, S50: 50 Mm0 salinity, S100: 100 Mm0 salinity, S150: 150 Mm 0 salinity; different letters in each column indicate significant differences (P≤0.01).

0 mM. With increasing salinity, the expression of gibberellin synthesis genes decreased. The lowest amount was in the control treatment (distilled water) and salinity of 150 mM (Figs. X, XI, & XII). There were no detectable *GA3OX4*, *GA3OX5*, and *GA3OX6* expression based on the qRT-PCR results.

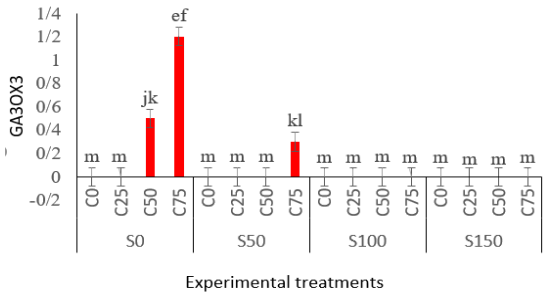

Fig. XII. Expression pattern of gibberellin synthesis gene *GA3OX3* due to priming and salinity; C0: chitosan 0, C25: chitosan %0.25, C50: chitosan %0.50, C75: chitosan %0.75, S0: 0 salinity, S50: 50 Mm salinity, S100: 100 Mm salinity, S150: 150 Mm salinity; different letters in each column indicate significant differences (P≤0.01).

Discussion

Results showed that the activity of antioxidant enzymes, except catalase, under salinity stress conditions was higher than in normal conditions. According to the findings of the study, chitosan treatment increased the activity of antioxidant enzymes, phosphate content, and expression of gibberellin synthesis genes in the plant. In the present study, the activity of catalase showed a significant decrease in stress conditions compared to the control, which is consistent with the results of Borzouei et al. (2012), Doraki et al. (2016), and Fidalgo et al. (2004). This decrease may be due to increased activity of ascorbate peroxidase and polyphenol oxidase enzymes under salinity stress, which causes the decomposition of hydrogen peroxide (Arora et al., 2002). Decreased activity of catalase may lead to accumulation of hydrogen peroxide and decrease the activity of some enzymes in the Calvin cycle such as ribulose monophosphate kinase and bisphosphatases (Asada, 2000). Decreased activity of this enzyme in the Calvin cycle produces reactive oxygen species by decreasing the ratio of $NADPH^{+}$/NADP in chloroplasts (Mittler, 2002). In addition, depletion of this enzyme may be due to inhibition of new enzyme synthesis (Fidalgo et al., 2004). Pretreatment with chitosan increased the activity of catalase. Other similar results have been obtained in chitosan-sprayed tomatoes under salinity stress, indicating an increase in catalase activity (Gu et al., 2012).

Salinity stress increases the activity of peroxidase enzyme in beans thus reduces the destructive effects of salinity stress in this plant (Azizi et al., 2019). Increased peroxidase activity under salinity stress can suggest the protective role of this enzyme. Also, considering the important role of POX in removal of H_2O_2, reduction of malondialdehyde, and the preservation of integrity of cell membranes, the increase of this enzyme under stress seems quite reasonable. Comparison of the means showed that with increasing salinity, activities of superoxide dismutase and ascorbate peroxidase increased. The results of this experiment are consistent with research conducted on wheat (Esfandiari et al., 2008) and chickpeas (Doraki et al., 2016). Increasing SOD enzyme keeps superoxide radicals at a lower level and reduces oxidative damage caused by salinity stress (Borzouei et al., 2012). Under stress conditions, the generated hydrogen peroxide causes membrane degradation and lipid peroxidation. Increased activity of catalase and peroxidase enzymes leads to its decomposition (Malecka et al., 2012). It seems that the increase in the activity of antioxidant enzymes is due to the effect of chitosan stimulation on genes involved in the biosynthesis of antioxidant enzymes (Yang Feng et al., 2009). Lack of adequate increase in the activity of antioxidant enzymes leads to a decrease in the plant's ability to withstand damage caused by salinity stress. Reports have shown that the activity of antioxidant enzymes such as POX and APX increases under salinity stress by chitosan (Ray et al., 2016).

Chitosan improves the selective absorption of ions, and the transport of ions was damaged by stress through having antioxidant effect on membrane (Amiri et al., 2017). The antioxidant activity of chitosan is mediated by a variety of mechanisms (Park et al., 2004). Chitosan promotes growth and development by increasing the activity of key enzymes in nitrogen metabolism (nitrate reductase, glutamine

synthetase, and protease) and improving nitrogen transport (Mondal et al., 2012). It also increases plants' resistance by activating a number of compounds such as phyto-auxins and enzymes like chitinases under salinity stress (Mahdavi and Rahimi, 2013). Chitosan can neutralize OH^- and O^{2+} free radicals and has been shown to have protective properties against DNA (Harish Prashanth et al., 2007). The mechanism of neutralization of free radicals by chitosan may be related to its specific structure, which consists of a large number of available amine and hydroxyl groups that react with ROS (Sun et al., 2004). Oxygen free radicals produced during stress damage cell membranes, nucleic acids, and cell proteins due to their high affinity for proteins and lipids (Bradford and Hsiao, 1882). Jabeen and Ahmad (2013) in their study on the activity of antioxidant enzymes in response to salinity stress in *Carthamus tinctorius* (L.) and *Helianthus annuus* (L.) reported an increase in stress resistance in plants in relation to the antioxidant properties of chitosan. Musapour et al. (2016) stated that chitosan pretreatment increased antioxidant activity. Chitosan seems to be able to control salinity by employing mechanisms that are responsible for protecting the plant from oxidative stress. Chitosan compensates for salinity damage by increasing osmotic regulators such as reducing membrane lipid peroxidation and reducing cell membrane permeability. In fact, chitosan with its protective role can stabilize membranes. Also, by regulating the osmotic pressure of the cell, it increases the absorption of water and essential nutrients by the plant and as a result, plant growth under stress conditions increases (Guan et al., 2009). It could be argued that stimulants such as chitosan may activate new genes that trigger enzymes and eventually different biosynthetic pathways, leading to the formation of secondary metabolites. In general, chitosan seems to increase plant resistance to stress and stimulate plant growth by increasing the activity of antioxidant enzymes and scavenging oxygen species (Harish Prashanth et al., 2007). Safikhana et al. (2018) showed that chitosan could protect plants against salinity stress damage by increasing the activity of antioxidant enzymes.

There are some reports that confirm the role of chitosan in increasing antioxidant capacity under salinity (Golkar et al., 2019). DPPH free radical scavenging activity can play an important role as an antioxidant in plants against salinity stress. The amount of DPPH decomposed in a certain time indicates the activity of radical scavenging by antioxidants (Perez et al., 2007).

Damage to cell membranes as a result of environmental stresses such as salinity is associated with an increase in the amount of malondialdehyde, which can be directly related to an increase in H_2O_2 (Qian et al., 2015). In the present study, the MDA content decreased at high concentrations of chitosan, which is consistent with the results of other studies (Guan et al., 2009; Safikhana et al., 2018; Golkar et al., 2019; Perez et al., 2007; Qian et al., 2015; Ali et al., 2021). It appears that chitosan can reduce lipid oxidation by chelating metal ions or combining them with lipids (Sui et al., 2002) and by eliminating free radicals directly or by antioxidant enzymes, to prevent the oxidation of fats and prevent the increase of malondialdehyde. Reducing the amount of malondialdehyde with chitosan in this study indicates a reduction in damage to cell membranes and an increase in the stability of plant cell membranes.

Phosphate is a constituent of all plant tissues, especially in young flowers and seeds. The presence of this element is essential for cell division and development of meristematic tissues. Phosphorus is an ingredient in many compounds such as nucleic acids, phospholipids, and coenzymes such as adenosine triphosphate. Phosphate is an inorganic compound and a salt of phosphoric acid. Salinity reduces phosphate and thus reduces the energy in the seed for seedling growth and emergence. Salinity destroys DNA, leading to disruption of the transcription process and ultimately the failure to synthesize the essential enzymes (amylase and antioxidants) required for the germination process. Without proper enzymatic activity, seed stores are not hydrolyzed and, as a result, the molecules needed to synthesize energy carriers such as ATP will not be available (MacDonald, 1999). Increasing the amount of phosphate indicates the positive effect

of seed priming and can increase the ratio of ATP to ADP, which will increase the energy level in the seed. In their study, Dzung et al. (2011) reported that chitosan consumption increases phosphorus concentration.

The results of evaluation of gene expression using real time-PCR showed that the relative expression of gibberellin synthesis genes in primed seeds treated under salinity stress compared to the control follows an increasing trend, so that in the treatment without priming, expression of gibberellin synthesis genes was minimal. In this study, priming with chitosan 0.75% under salinity stress significantly increased the relative expression percentage of *GA3OX1* gene compared to the relative expression of *GA3OX2-6* genes which indicates the importance of *GA3OX1* transcription factor in response to salinity stress. Increased expression of this gene probably confirms that the seed maintains the expression level of *GA3OX1* gene to counteract salinity and has the ability to inhibit salinity factors. However, further increase in salinity reduced the expression of gibberellin synthesis genes.

The expression of *GA3OX4*, *GA3OX5*, and *GA3OX6* was reduced to zero. This reduction could also be due to the destruction of the gene-producing structures of gibberellin synthesis by toxic radicals (Shim et al., 2003), and priming with chitosan had no effect on them. Chitosan acts as a stress signaling molecule and induces the expression of salinity-resistant genes that activate the immune system and tolerate stress (Linh et al., 2020). However, the expression levels of *GA3OX2* and *GA3OX3* increased compared to other genes under similar conditions. Our results showed that all three genes are involved in the response to salinity stress in beans. The effect of chitosan depends on factors such as elicitor concentration, treatment method, and duration of exposure to chitosan (Esmaeilzadeh Bahabadi et al., 2013).

Chitosan identifies genes by stimulating cellular signals and molecular interactions between plant receptors at the cell membrane or cytoplasmic level. As a result, the signal received by plant cells stimulates the expression of pathway-related genes and causes the synthesis of secondary metabolites in plants (Zhao and Sakai, 2003). Secondary metabolites are induced as a defense mechanism against stress (Golkar et al., 2019) and increase plant resistance to environmental stresses. Chitosan probably induces a signal for the synthesis of the hormone gibberellin and enhances plant growth through some signaling pathways related to auxin biosynthesis through the tryptophan-dependent pathway (Sopalun et al., 2010). Increased gene expression in chitosan treatment has been reported in lemongrass (Mahdavian Fard et al., 2021), which was consistent with the results of this study. Chitosan can positively regulate osmotic pressure and thus eliminate the adverse effects of stress (Hidangmayum et al., 2019). In this study, combining biochemical responses and analysis of gibberellin synthesis gene expression may help to understand the mechanism of chitosan effects on beans at the molecular level.

Conclusion

Based on the results of the present study, chitosan pretreatment increased the activity of some antioxidant enzymes and phosphate content therewith reducing malondialdehyde levels. According to the comparison results, the concentration of 0.75% of chitosan pretreatment can be considered as a more effective treatment for bean seedlings under salinity stress and can be used to reduce the adverse effects of salinity stress. Also, the expression level of gibberellin synthesis genes was low in salinity conditions and priming increased the expression of these genes.

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