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Effect of Salicylic Acid and Calcium Chloride on Lipid peroxidation and Scavenging Capacity of Radical of red bean (Phaseolus calcaratus L.) under Salt Stress

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ABSTRACT

Soil salinity is one of the critical challenges for development of culture area of agricultural crops. In the present study a pot experiment was conducted in factorial based on completely randomized design aimed to investigate the impact of exogenous application of salicylic acid (SA 0, 0.75 and 1.5 mM) and calcium chloride (CaCl2 0, 50 and 100 mM), solely or in combination, on plant growth, photosynthetic pigments (total chlorophyll (Chl), carotenoids, anthocyanin), and some metabolic parameters (reducing sugars, proline, lipid peroxidation and scavenging ability on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical) of red bean exposed to salt stress (0, 25 and 75 mM NaCl). Results showed that exogenous application of SA or calcium (Ca) alone improved plant performance under NaCl stress. Growth slowed down under salinity. Malondialdehyde (MDA), DPPH radical, anthocyanin, and proline content were increased under salinity stress. However, application of SA and Ca enhanced the growth parameters, improved the Chl, carotenoids, and reducing sugars content, and significantly reduced MDA and DPPH radical in plants. Therefore, induced tolerance to salinity as the result of SA and Ca application may be related to the regulation of antioxidative responses. Furthermore, the beneficial effect of SA and Ca were achieved by applications of 0.75 mM SA and 50 mM CaCl2, which are recommended to improve red bean performance under saline conditions. In conclusion, exogenous application of SA and Ca improved salinity stress tolerance through the regulation of antioxidant system.

Introduction

Salinity restriction induces in plants oxidative stress that makes a significant decrease in photosynthesis activities and leaf Chl content (Zafar et al., 2018; Trabelsi et al., 2019); which depends on plant species tolerance to salinity (Ashraf et al., 2010). The accumulation of secondary metabolites is one mechanism that preserves plants from high NaCl stress. These metabolites are anti-oxidants that perform in osmotic regulation (Winkel Shirley, 2002). For example, carotenoids are increased and show antioxidant properties when plants are irrigated with saline water (de Pascale et al., 2001). The generation of anthocyanins might enhance antioxidant properties, which is necessary for conserving plants in response to reactive oxygen species (ROS) (Rouholamin et

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al., 2015). Proline is one of the foremost essential osmoprotectants in plants that plays an important role as an osmoticum for improving plant tolerate to salinity stress (Bartels and Sunkar, 2005). Under saline conditions, plants show an increase in their proline content (Lee et al., 2001). In plants, Ca is an important secondary messenger functions in transduction of signals related to environmental stimuli and hormones (Kudla et al., 2010; Seifikalhor et al., 2019). Increase in Ca level can also modulate sucrose-induced sugar uptake and improve anthocyanin accumulation (Xu et al., 2014). SA is a phenolic phytohormone that plays an important role in response to salinity and drought (Shakirova et al., 2003; Shahmoradi and Naderi, 2018). The role of SA in inducing salt tolerance had been reported in many plant species (Arfan et al., 2007; Aliniaeifard et al., 2016a). It has been reported that exogenous SA can increase plant tolerance to abiotic stresses (Erasalan et al., 2007).

Ca is an ion that prevents salinity toxicity and top Ca levels will maintain the cell membrane from the adverse effects of salinity (Abdel Latef, 2011; Seifikalhor et al., 2019). Hence, the utilization of Ca^{+2} can be an effective way to promote plant growth and development under biotic stress conditions (Naeem et al., 2018; Lastochkina et al., 2020). Exogenous utilization of Ca^{+2} enhances tolerance to salinity stress via ever-changing stress-induced ROS metabolism, growth performance, and photosynthetic effectiveness (Ahmad et al., 2018; Mukhtar et al., 2016; Acosta- Motos et al., 2017). Development of most of the plants retards under harsh saline conditions, because of their sensitivity to salt stress. It has been shown that supplemental Ca improves the growth and also the photosynthetic capacity in Senna (Arshi et al., 2006). Moreover, Navarro et al. (2000) discovered that the growth-enhancing impact of supplemental Ca in salt-exposed plants was because of the ameliorative effect of Ca on root hydraulic conductivity and water uptake. The Na/Ca ratio is considered very important in response of glycophyte and halophyte plants to high salinity concentrations (Munns and Tester, 2008).

Since there is not any study on the SA and $CaCl_2$ under salt stress on the red bean. Present study was conducted (1) to examine the toxic effects of salt stress on different morphological and physiological characteristics of red bean, (2) to determine whether SA and $CaCl_2$ could decrease phytotoxic effects of NaCl on red bean plants.

Materials and methods

Culture condition and treatments

The seeds of red bean Sayad cultivar were sterilized with sodium hypochlorite (1%) for 5 min and washed several times by distilled water. A pot experiment was designed and implemented under greenhouse conditions in Payame Noor University of Kerman. Plastic pots, with 1 kg capacity, were filled with sieved soil. Five red bean (*Phaseolus calcaratus* L.) seeds were sown in the May 2019 in each pot and pots were replicated three times per concentration of chemical used for the experiment. Seeds germinated after 72 h at 25 °C, thereafter the seedlings were transplanted to the pots containing sand, clay, and humus (3:1:2) under a light density of 100 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C and 16 h photoperiod. The experiment was conducted in factorial format based on completely randomized design (CRD). Fourteen days after emergence of seedlings when the plants had 4 leaves, sodium chloride (0, 25, and 75 mM), SA (0, 0.75 and 1.5 mM) and $CaCl_2$ (0, 50 and 100 mM) were applied for 10 days during vegetative growth of the plants. A foliar spray of SA was applied twice to the plants, using an atomizer. The leaves were harvested 24 days after treatment. The following 15 treatments were used for the present study: T_1: 0 mM NaCl (control); T_2: 25 mM NaCl; T_3: 75 mM NaCl; T_4: 0 mM NaCl +

0.75 mM SA; T_5: 0 mM NaCl + 1.5 mM SA; T_6: 25 mM NaCl + 0.75 mM SA; T_7: 25 mM NaCl + 1.5 mM SA; T_8: 75 mM NaCl + 0.75 mM SA; T_9: 75 mM NaCl + 1.5 mM SA; T_{10}: 0 mM NaCl + 50 mM Ca; T_{11}: 0 mM NaCl + 100 mM Ca; T_{12}: 25 mM NaCl + 50 mM Ca; T_{13}: 25 mM NaCl + 100 mM Ca; T_{14}: 75 mM NaCl + 50 mM Ca; T_{15}: 75 mM NaCl + 100 mM Ca.

Biomass measurement

The ultimate harvest was performed after 24 days of treatment. The plant samples were initially washed with tap water and then rinsed with deionized water three times. Length of shoots and roots were measured using a ruler. Roots and shoots were weighed (FW) and then dried in an oven at 70 °C for 48 h, and weighed again to have the dry biomass (DW).

Pigment analysis

Fresh leaf tissues were ground with a mortar and pestle under liquid N_2. Acetone (80% v/v) was added to extract pigment; after centrifugation of the supernatant for 10 min at 10,000 rpm, OD was measured at 470, 646.8 and 663.2 nm using a spectrophotometer (Lichtenthaler, 1987). The extinction coefficients and the equations reported by Lichtenthaler (1987) were used to calculate the amounts of Chl *a*, *b* and carotenoids.

To determine the concentration of anthocyanins, 0.1 g fresh leaves were taken and were extracted in 15 mL glass centrifuge tubes containing 10 mL of acidified methanol (methanol: HCl, 99: 1, v:v) and kept overnight in the dark. The samples were brought up to volume, and the absorbance was determined at 550 nm. Anthocyanin concentrations were calculated using an extinction coefficient of 33000 mol^{-1} cm^{-1} (Wanger, 1979).

Determination of proline and reducing sugars

Proline was extracted and quantified based on the method of Bates et al. (1973). Fresh leaf samples (about 0.1 g fresh weight, FW) were homogenized in 3 mL 3% sulfosalicylic acid and the homogenate was centrifuged at 1700 × g for 10 min. A 0.5 mL aliquot of the supernatant was transferred to a tube containing 0.5 mL acetic acid and then 1 mL acid ninhydrin was added. The mixture was boiled for one hour. The reaction was stopped in an associate degree ice bath. The reaction mixture was extracted with 1 mL toluene, mixed completely by the vortex. The optical density of the upper toluene phase was determined at a wavelength of 520 nm, using toluene as the blank, and L-proline as a standard.

Reducing sugars were determined by the anthrone reagent method using glucose as the standard according to Jeffries et al. (1988). For the measurement of reducing sugars, DNS (dinitro salicylic acid) 1%, sodium 1.6% and 25% sodium-potassium tartrate were slowly dissolved on the heater. Then 2 mL of the extracted plant extract was mixed with 1 mL of the made dye and placed in a boiling water bath at 100 °C for 10 min, then the whole solution was mixed with 10 mL of distilled water and the samples were read in 546 nm and the amount of reducing sugars was calculated with the help of the relevant standard curve.

Lipid peroxidation

Lipid peroxidation was measured consistent with the MDA content. Leaf tissue (0.2 g) of plants were homogenized in 10 mL of 0.1% (w/v) trichloroacetic acid (TCA) and then centrifuged at 10000 g for 15 min. supernatant (1 ml) was then vortexed with 4 mL of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA), and the solution was heated for 30 min at 95 °C. The samples were cooled on ice for 5 min and re-centrifuged for 10 min at 10000 g. The non-specific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for the MDA measurement (Heath and Packer, 1969), and at 455 nm for other aldehydes (Meirs et al., 1992). For the MDA

and aldehyds calculation, an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used at 455 nm as the average of the extinction coefficient obtained for five other aldehyds (propanal, butanal, hexanal, heptanal, and propanal dimethyl acetal).

Scavenging ability on DPPH radical

Methanolic extract of leaves was subjected to the free radical scavenging activity assay using the method described by Shimada et al. (1992). Each extract (0.2 mg mL^{-1}) in methanol (2 mL) was mixed with 2 mL of a freshly prepared methanolic solution containing 100 ppm of DPPH radicals. The mixture was shaken vigorously and remained for 30 min in the dark. Then the absorbance was measured at 517 nm. The percentage of DPPH scavenging activity was measured consistent with the following formula:

$$\text{Inhibition of DPPH radical (\%)} = \frac{[\text{Absorbance of control-Absorbance of sample}] \times 100}{\text{Absorbance of control}}$$

Statistical design and analysis

Analysis of variance (ANOVA) using SPSS software (Version 17) was used for statistical analysis. The data were presented as the mean of three replicates ± standard error (SE). Tukey's HSD post hoc test was applied for multiple comparisons of the means. The mean values at $p < 0.05$ were treated as significant point. Figures were drawn using Excel software (2013).

Results

Effect of exogenous application of SA and Ca on plant growth under salinity condition

The red bean plant growth was significantly affected by salinity treatment (25 and 75 mM NaCl), compared to the control plants (Fig. 1; Table 1). The salinity treatment at 75 mM reduced shoot lengths, with an average reduction of 12%, while this impact did not exceed than 5% at 25 mM NaCl (Fig. 1). Root length was found to be NaCl dose-dependent, whereas similar decreases of shoot length were observed for both NaCl concentrations compared to the controls. Similarly, data presented in Figure 2 indicated that salinity stress significantly ($p<.05$) reduced the fresh and dry weight of shoot and root (Fig. 2).

Application of SA (0.75 and 1.5 mM) alone or with NaCl (25 and 75 mM) significantly improved growth parameters. The maximum increase in shoot and root length was recorded by 19% and 43%, respectively, under combined application of NaCl (75 mM) and SA (1.5 mM), as compared to the plants treated with NaCl (75 mM) alone. Similarly, the fresh weight of shoot and root were improved by 22% under NaCl (75 mM) and SA (0.75 mM) application as compared to their respective controls (Fig. 1, 2; Table 1).

Similar to SA, the application of $CaCl_2$ to red bean plants grown under salinity conditions enhanced the growth parameters. The application of 50 and 100 mM $CaCl_2$ alone and with 25 and 75 mM NaCl significantly increased growth of red bean. The largest effects in all measured growth parameters were observed in plants grown at 50 mM $CaCl_2$ alone and minimum were obtained with 75 mM NaCl treatment (Fig. 1, 2; Table 1).

Table 1. Values for mean square from analysis of variance for shoot and root length, shoot and root fresh and dry weight of *Phaseolus calcaratus* L. treated with salicylic acid (SA) and Calcium Chloride (Ca), singly or in combination, to salt stress.

Source	df	Shoot length	Root length	Shoot fresh weight	Root fresh weight	Shoot dry weight	Root dry weight
Salinity (NaCl)	2	12.610*	1.473*	0.342*	0.136*	0.020*	0.008*
Salicylic Acid (SA)	2	12.000*	1.330*	0.264*	0.029*	0.016*	0.002*
Calcium Chloride (Ca)	2	22.013*	1.863*	0.549*	0.055*	0.032*	0.003*
NaCl * SA	4	4.500*	0.895*	0.047*	0.032*	0.003*	0.002*
NaCl * Ca	4	4.013*	1.183*	0.058*	0.031*	0.003*	0.002*
Error	30	0.052	0.018	0.000	0.000	0.000	0.000
Total	45						

ns = non-significant

*$P \leq 0.05$

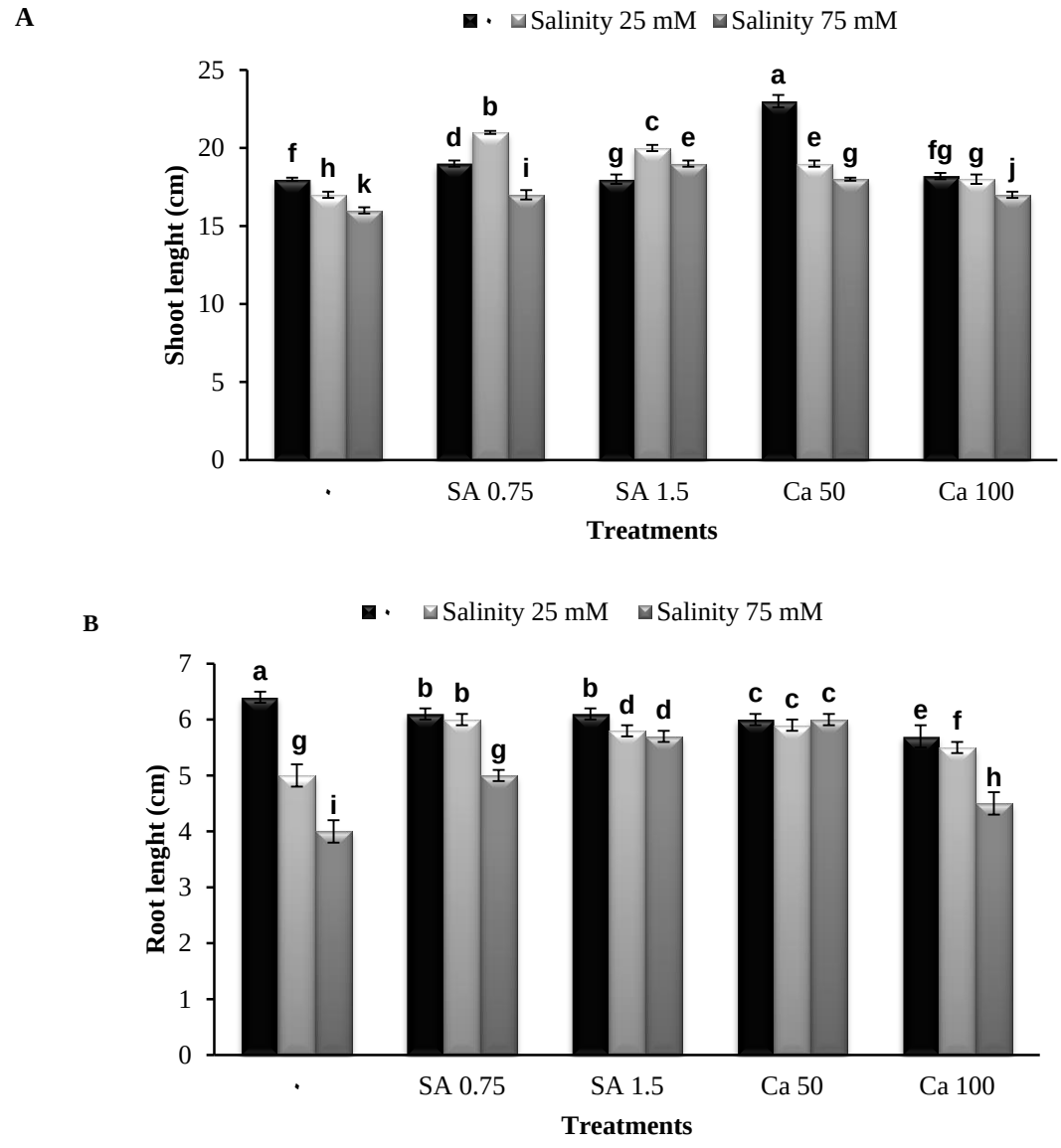
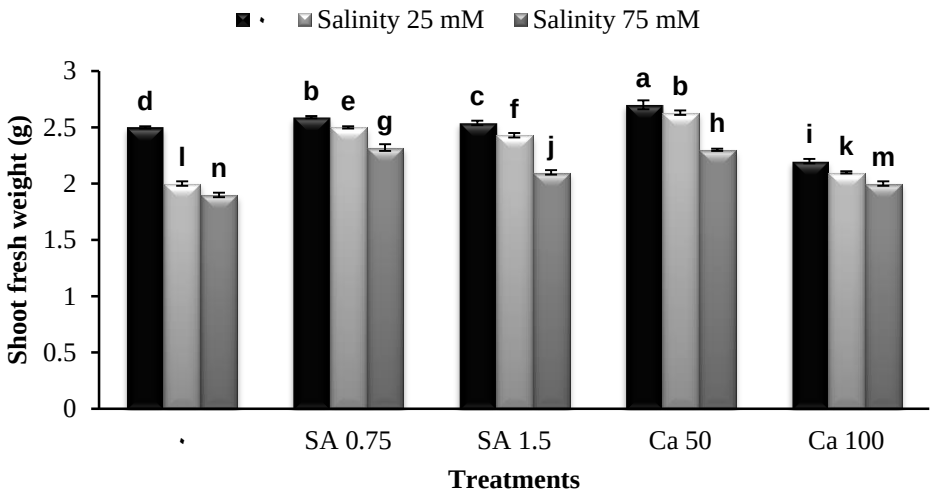
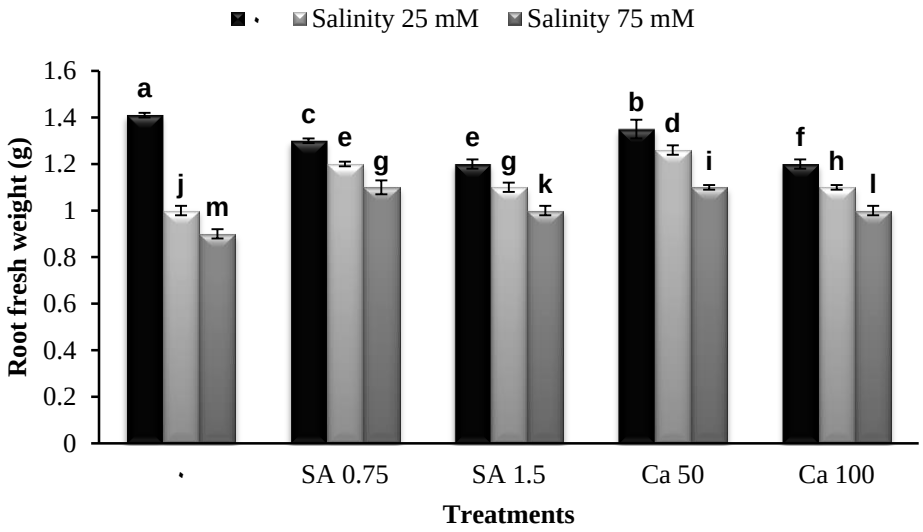


Fig. 1. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on shoot (A) and root (B) length of *Phaseolus calcaratus* L under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

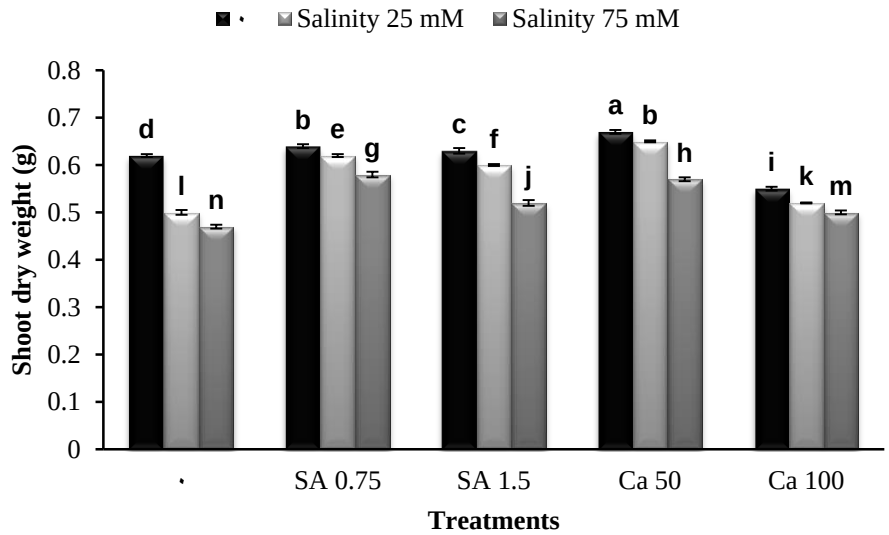
A


Salinity 25 mM
Salinity 75 mM
Shoot fresh weight (g)
0
0.5
1
1.5
2
2.5
3
d
l
n
b
e
g
c
f
j
a
b
h
i
k
m
SA 0.75
SA 1.5
Ca 50
Ca 100
Treatments

B


Salinity 25 mM
Salinity 75 mM
Root fresh weight (g)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
a
j
m
c
e
g
e
g
k
b
d
i
f
h
l
SA 0.75
SA 1.5
Ca 50
Ca 100
Treatments

C


Salinity 25 mM
Salinity 75 mM
Shoot dry weight (g)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
d
l
n
b
e
g
c
f
j
a
b
h
i
k
m
SA 0.75
SA 1.5
Ca 50
Ca 100
Treatments

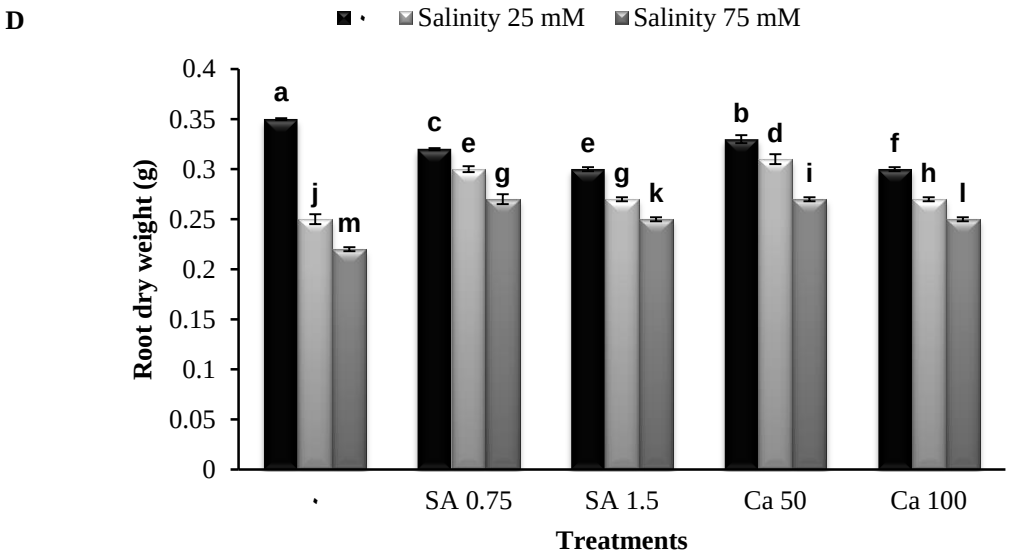


Fig. 2. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on shoot (A, C) and root (B, D) fresh and dry weights of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Effect of exogenous application of SA and Ca on photosynthetic pigments under salinity condition

Chl (a, b and total) and carotenoids contents were significantly decreased in plants grown in salinity stress as compared to control (Table 2). The carotenoids and total Chl content decreased by 70% and 45% respectively, in 75 mM NaCl, as compared to control. However, foliar application of SA and Ca significantly ($p < 0.5$) improved Chla, Chlb, total Chl, and carotenoid contents. Maximum total chlorophylls and carotenoids concentrations were obtained in the plants treated by sole application of SA (0.75 mM) and Ca (50 mM) and minimum concentrations were obtained with 75 mM NaCl treatment. Application of 0.75 mM SA along with NaCl 25 and 75 mM significantly enhanced total Chl by 67% and 92% while carotenoids by 44% and 136%, respectively, as compared to the rest of the treatments. Also, the combined application of Ca (50 mM) and NaCl (25 and 75 mM) significantly increased total Chl by 75% and 74% and carotenoids by 100% and 233% respectively, in comparison with the control treatments (Table 2, 3).

Table 2. Values for mean square from analysis of variance for Chla, Chlb, total Chl, carotenoid, MDA and other aldehyde contents of *Phaseolus calcaratus* L. treated with salicylic acid (SA) and calcium chloride (Ca) under salt stress.

Source	df	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	MDA	Other aldehyde
Salinity (NaCl)	2	0.385*	1.688*	3.618*	0.131*	0.0009*	0.047*
Salicylic Acid (SA)	2	1.985*	6.812*	16.131*	0.152*	0.0005*	0.036*
Calcium Chloride (Ca)	2	2.624*	5.537*	15.720*	0.606*	0.0008*	0.032*
NaCl * SA	4	0.128*	0.809*	1.478*	0.049*	0.0003*	0.013*
NaCl * Ca	4	0.245*	1.234*	1.900*	0.149*	0.0004*	0.008*
Error	30	0.006	0.002	0.014	0.001	0.0000	0.000
Total	45						

ns = non-significant

*$P \leq 0.05$

Table 3. Effect of salicylic acid (SA), Calcium Chloride (Ca) on Chlorophyll a, Chlorophyll b, total Chlorophyll, and carotenoid contents of *Phaseolus calcaratus* L under salt stress (NaCl). Values with similar letters are not significantly different at $p < 0.05$.

Treatments	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoid (mg/g FW)
0 mM NaCl (control)	2.52 ± 0.01^{g}	3.91 ± 0.04^{g}	6.43 ± 0.05^{g}	1.11 ± 0.01^{c}
25 mM NaCl	2.20 ± 0.02^{j}	2.38 ± 0.02^{j}	4.58 ± 0.04^{j}	0.65 ± 0.02^{j}
75 mM NaCl	1.44 ± 0.02^{l}	2.05 ± 0.02^{k}	3.49 ± 0.04^{l}	0.33 ± 0.02^{l}
0 mM NaCl + 0.75 mM SA	3.01 ± 0.01^{b}	4.67 ± 0.04^{b}	7.68 ± 0.05^{b}	1.15 ± 0.01^{b}
0 mM NaCl + 1.5 mM SA	2.90 ± 0.1^{e}	4.1 ± 0.04^{d}	7 ± 0.14^{e}	0.98 ± 0.02^{e}
25 mM NaCl + 0.75 mM SA	3.08 ± 0.1^{ab}	4.58 ± 0.01^{c}	7.66 ± 0.11^{ab}	0.94 ± 0.01^{f}
25 mM NaCl + 1.5 mM SA	3.0 ± 0.01^{ce}	4.15 ± 0.03^{d}	7.15 ± 0.04^{ce}	0.85 ± 0.02^{h}
75 mM NaCl + 0.75 mM SA	2.67 ± 0.03^{f}	4.04 ± 0.03^{e}	6.71 ± 0.06^{f}	0.78 ± 0.03^{i}
75 mM NaCl + 1.5 mM SA	2.50 ± 0.02^{h}	4 ± 0.02^{f}	6.5 ± 0.04^{h}	0.65 ± 0.02^{k}
0 mM NaCl + 50 mM Ca	3.09 ± 0.04^{a}	4.75 ± 0.08^{a}	7.84 ± 0.12^{a}	1.23 ± 0.04^{a}
0 mM NaCl + 100 mM Ca	2.30 ± 0.2^{i}	3 ± 0.08^{h}	5.3 ± 0.28^{i}	0.85 ± 0.05^{gh}
25 mM NaCl + 50 mM Ca	3.20 ± 0.1^{a}	4.84 ± 0.06^{a}	8.04 ± 0.16^{a}	1.3 ± 0.04^{a}
25 mM NaCl + 100 mM Ca	2.50 ± 0.1^{i}	3.1 ± 0.09^{h}	5.6 ± 0.19^{i}	0.93 ± 0.04^{g}
75 mM NaCl + 50 mM Ca	3.0 ± 0.01^{d}	3.1 ± 0.06^{h}	6.1 ± 0.07^{d}	1.1 ± 0.01^{d}
75 mM NaCl + 100 mM Ca	2.20 ± 0.01^{k}	2.86 ± 0.02^{i}	5.06 ± 0.03^{k}	0.88 ± 0.02^{g}

Effect of exogenous application of SA and Ca on lipid peroxidation under salinity condition

Salt stress-induced lipid peroxidation in red bean leaves. Indeed, increasing concentration of NaCl (25 and 75 mM) escalated the MDA and other aldehyde production in red bean (Fig. 3). Leaves showed higher values of MDA and other aldehydes under NaCl stress (75 mM), as compared to their respective controls. The values of MDA and other aldehyde were respectively, increased by 22% and 34% in leaf under 75 mM NaCl, as compared to the control. The addition of SA and Ca considerably reduced the production of MDA and other aldehydes, in leaves at all levels of NaCl. MDA and other aldehyde showed 9% and 18% decrease in leaves under 75 mM NaCl, respectively when plants treated with 0.75 mM SA, as compared to their respective controls. Application of Ca to plants slightly decreased the level of MDA and other aldehydes, in leaves at all levels of NaCl. The maximum decrease in MDA and other aldehyde was observed by 12% and 12% in leaves, respectively, under combined application of NaCl (75 mM) and Ca (50 mM), as compared to NaCl treatment alone (Fig 3. A, B; Table. 3).

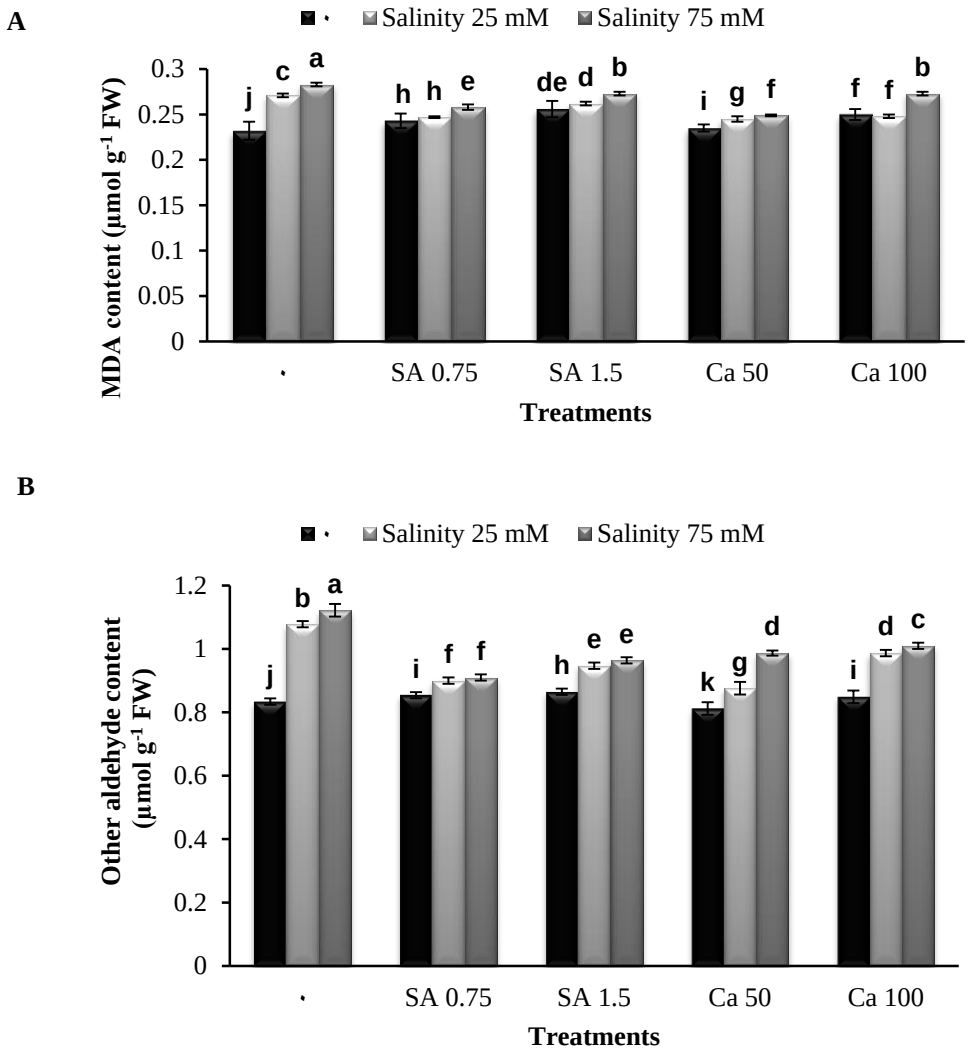


Fig. 3. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on MDA (A) and other aldehyde (B) contents of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Effect of exogenous application of SA and Ca on reducing sugars content under salinity condition

In our study, red bean plants exposed to NaCl treatment showed a decrease in their leaf reducing sugars content. 25 and 75 mM of NaCl respectively decreased leaf sugar concentration by 16% and 20% as compared to control (Fig. 5; Table. 4). Application of SA and Ca alleviated this adverse effect of salinity on sugar level. The SA (1.5 mM) increased the reducing sugars content of leaves by 58% under NaCl 75 mM, compared to NaCl treatment alone. The maximum increase (94%) in reducing sugars content detected by Ca 50 mM application as compared to the respective control (Fig. 4).

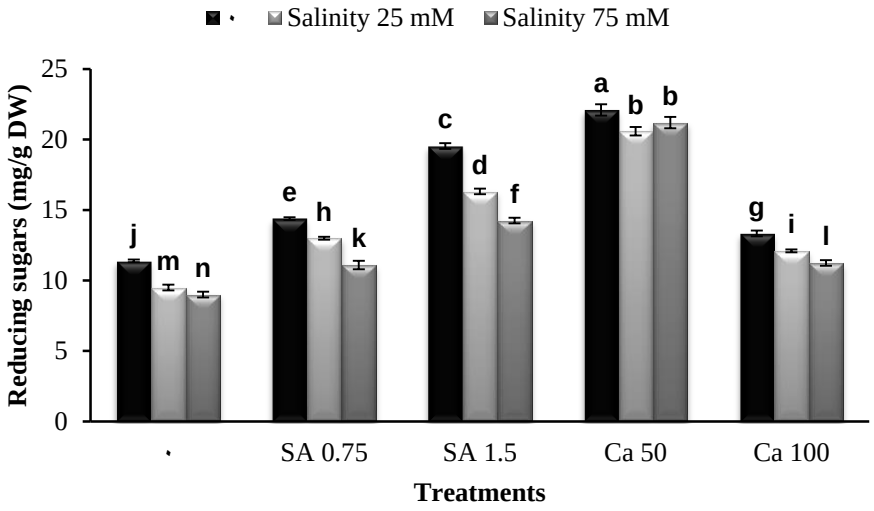


Fig. 4. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on reducing sugars content of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Effect of exogenous application of SA and Ca on proline content under salinity condition

Proline content in red bean leaves increased respectively, in 25 and 75 mM of NaCl exposed plants by 15% and 24% as compared to the control. SA and Ca treatments increased the proline concentration in comparison with the control plant. The level of proline in plants treated with SA and Ca improved in red bean plants under salinity. The maximum increase of 55% in proline content detected under 1.5 mM SA as compared to the respective control. The Ca (100 mM) increased the proline content of leaves by 13% under NaCl 75 mM, compared with NaCl treatment alone (Fig. 5; Table. 4).

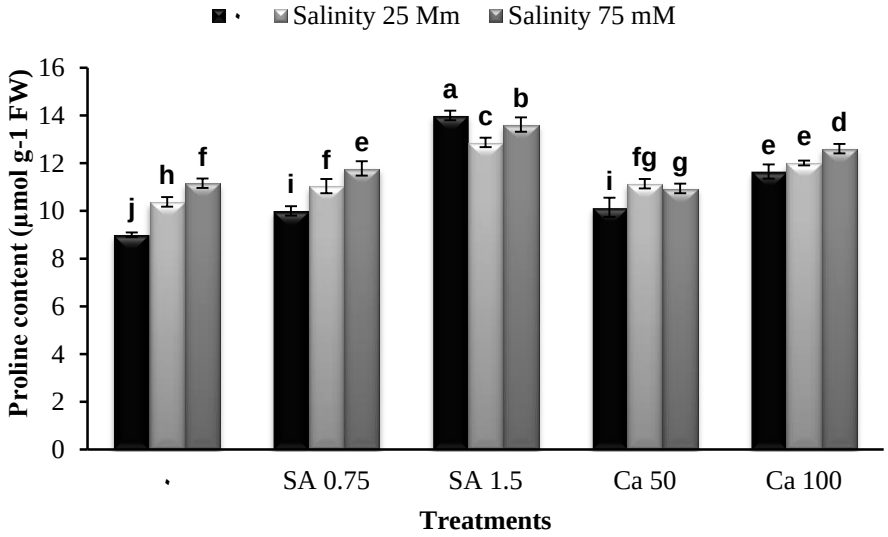


Fig. 5. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on proline content of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Table 4. Values for mean square from analysis of variance for reducing sugars, proline, Anthocyanin, DPPH radical scavenging activity of *Phaseolus calcaratus* L. treated with salicylic acid (SA) and calcium chloride (Ca) under salt stress (NaCl).

Source	df	Reducing sugar	Proline	Anthocyanin	% inhibition of DPPH radical
Salinity (NaCl)	2	25.759*	1.885*	0.087*	19.819*
Salicylic Acid (SA)	2	102.972*	27.172*	0.104*	85.118*
Calcium Chloride (Ca)	2	323.748*	8.668*	0.052*	86.372*
NaCl * SA	4	1.920*	1.866*	0.118*	34.109*
NaCl * Ca	4	0.659*	0.547*	0.083*	27.800*
Error	30	0.055	0.051	0.002	0.049
Total	45				

ns = non-significant

*$P \leq 0.05$

Effect of exogenous application of SA and Ca on anthocyanin content under salinity condition

Anthocyanin content in red bean leaves significantly increased under 25 and 75 mM of NaCl stress by 18% and 91% as compared to the control, respectively. Application of 0.75 and 1.5 mM SA along with NaCl 75 mM significantly reduced anthocyanin content by 14% and 15%, respectively, as compared to NaCl alone. The Ca (50 and 100 mM) reduced the anthocyanin content of leaves by respectively 14% and 10% under 75 mM NaCl as compared with NaCl alone (Fig. 6; Table. 4).

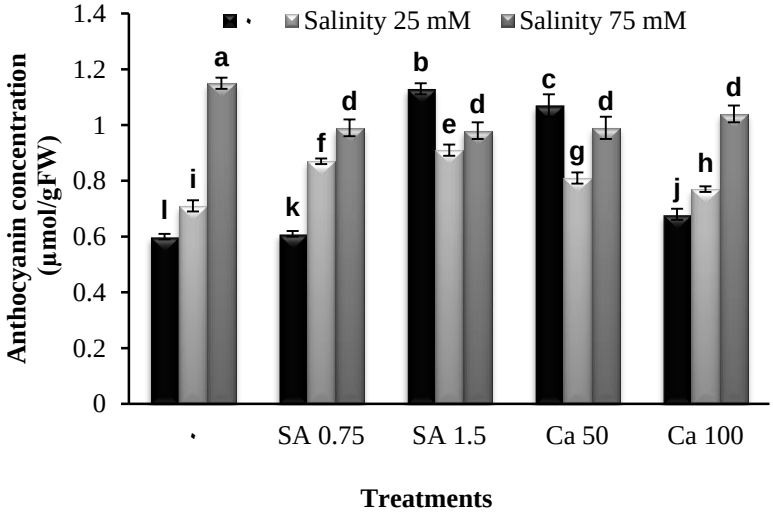


Fig. 6. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on anthocyanin concentration of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Effect of exogenous application of SA and Ca on antioxidant activity under salinity condition

Our result showed that significant changes in DPPH radical scavenging activity occurred in response to salinity. DPPH radical scavenging activity in red bean leaves significantly increased in 25 and 75 mM of NaCl salt-stressed plants respectively by 130% and 196% as compared to the control. Application

of SA and Ca alleviated this adverse effect of salinity on DPPH radical scavenging activity. SA (0.75 mM) reduced the DPPH radical scavenging activity of leaves by 43% and 52% respectively under 25 and 75 mM NaCl, compared with NaCl treatment alone. Also, the Ca (50 and 100 mM) reduced the DPPH radical scavenging activity of leaves by 50% and 44% under NaCl 75 mM, compared with NaCl treatment alone (Fig. 7; Table. 4).

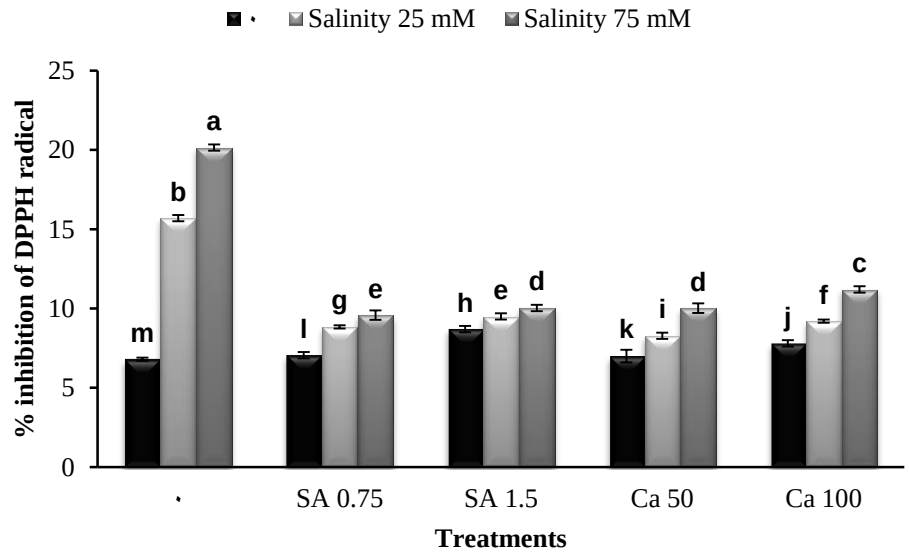


Fig. 7. Effect of salicylic acid (SA; 0, 0.75 and 1.5 mM) and calcium chloride (Ca; 0, 50 and 100 mM) on DPPH radical scavenging activity of *Phaseolus calcaratus* L. under three levels of salinity (0, 25, 75 mM). Values with similar letters are not significantly different at $p < 0.05$.

Discussion

The reduction of plant growth under salinity conditions could be a common phenomenon in mesophytes (Hernández, 2019). The results of this study indicated that salinity stress decreased the plant growth of red bean plants. However, foliar application of Ca and SA considerably improved the growth and biomass production in red bean plants by altering the biochemical and physiological processes. It is well-documented that salinity stress reduces growth and biomass production, which can result in impaired photosynthetic activity and nutrient imbalance (Ashraf and Ali, 2008) as reported in absentia (Akhtar et al., 2013), olive (Trabelsi et al., 2019; Larbi et al., 2020; Aliniaeifard et al., 2016b) and Wheat (Zafar et al., 2016).

The importance of Ca on tolerance of plants to salinity has been already reported (Tuna et al., 2007; Seifikalhor et al., 2019; Tanveer et al., 2020). In this study, application of $CaCl_2$ to red bean plants grown under salinity conditions improved the measured growth parameters. The most important effects in all measured growth parameters were determined in plants grown at 50 mM $CaCl_2$ alone. Similar results were found in olive trees (Larbi et al., 2020), tomato (Tuna et al., 2007) and strawberry (Kaya et al., 2002).

The application of $CaCl_2$ alone and with NaCl significantly improved fresh and dry weight of the shoot. Similar results were reported by Mohammad et al. (1998) and Tuna et al. (2007). The appliance of 10 mM of Ca to olive plants grown at 200 mM NaCl enhanced all measured parameters except shoot dry weight (Larbi et al., 2020).

In this study, the effect of SA on the growth parameters of red bean plants considerably enhanced compared with control. The positive effect of SA is additionally attributed as a plant

growth regulator because of its ability to increase nutrient uptake, enzymatic activities, protein synthesis, photosynthetic activity, protect against biotic and abiotic stresses, and increase in antioxidant capacity of plant (Sahu, 2013; Blokhina et al., 2003; Aliniaeifard et al., 2016a)). The promoting effect of SA on a rise of carbon dioxide assimilation, photosynthetic rate, mineral uptake, and nutrient movement may be a result of improvement different nutrient in leaves (Szepesi et al., 2005; Magda et al., 2013). It is worth noting that our results are in consistent with previous reports (Abd El-Hameid Asmaa et al., 2017; El-Khallal et al., 2009; Delavari et al., 2010).

In this study, salinity considerably reduced Chl contents, which can result in inhibition in their synthesis (Ashraf and Ali, 2008). The decrease in Chl contents under saline conditions in red bean plants caused a discount in crop productivity. Similar results have additionally been reported (Larbi et al., 2020; Akhtar et al., 2013; Radi et al., 2013). This means that a decrease in Chl contents in plants that grown in saline conditions is also the result of the activation of chlorophyllase enzyme, which causes the decomposition of leaf Chl (Tsuchiya et al., 1999). However, some reports showed that a decrease in leaf Chl contents in plants due to the reduction in synthesis of 5-aminolinolic acid, a necessary precursor of protochlorophyllide, which converted to Chl when exposed to light (Radi et al., 2013). Moreover, uptake of chloride in high amounts additionally reduced leaf Chl contents as a result of high concentrations of chloride in leaves act as a bleaching agent (Tavakkoli et al., 2010). In this study, the application of Ca and SA lessened the adverse effects of salinity on Chl and carotenoid in red bean plants. This confirms previous findings about Ca application (Larbi et al., 2020). Application of Ca helps maintain the stability of the chloroplast membrane to prevent Chl decomposition (Naeem et al., 2018). This study showed that the appliance of SA increased the Chl contents but it was not as effective as Ca, which is indicative of prominent role of Ca than SA for pigment maintenance. Studies have shown that application of SA improved the photosynthetic pigments of plants under stress conditions, which may provide the ability of the plant to tolerate environmental stresses by osmotic stress (Abd El-Hameid Asmaa et al., 2017; Ananieva et al., 2004).

Salinity will induce changes in the levels of pigments like Chl and carotenoids so that carotenoids are necessary for the integrity of the photosystem and will scavenge reactive oxygen species generated under salinity conditions (De Pascale et al., 2001; Ahmad et al., 2005).

MDA quantification, a product of lipid peroxidation, is an indicator of membrane damages resulting in electrolyte leakage under salinity conditions (Katsuhara et al., 2005). MDA is usually used to indicate oxidative damage in fatty acids (Azevedo Neto et al., 2008), and its accumulation under salinity stress has been determined in plants like cotton (Meloni et al., 2003), sugar beet (Bor et al., 2003), cowpea (Cavalcanti et al., 2004), maize (Azevedo Neto et al., 2006) and rice (Demiral and Tu¨rkan, 2006). In the present study, severe NaCl salinity stress increased the MDA and other aldehyde production in red bean. Increased lipid peroxidation, which measured by MDA accumulation, could result from the generation of ROS under salinity conditions (Bor et al., 2003). Indeed, NaCl-treated plants supplemented with SA or Ca showed a major decrease of MDA content in red bean leaf, as compared to plants treated solely by salinity. It can be hypothesized that the application of SA and Ca includes a role in the induction of antioxidant enzyme activity resulting in a decrease in ROS and lipid peroxidation on a long term basis (Gunes et al., 2007).

The accumulation of proline was a typical response to salt stress and was shown to be associated with increasing NaCl concentrations

(Aghaei et al., 2009). It has been reported as a biochemical marker for enhanced NaCl tolerance in potato plants (Martinez et al., 1996). Most studies have shown an increase in proline levels by SA (Janda et al., 2007). So that Application of SA increased the amount of proline in salt-stressed plants (Abd El-Hameid Asmaa et al., 2017). SA has also been reported to protect plants against salinity (Misra and Saxena, 2009; Al-Whaibi et al., 2012; Heidarian and Roshandel, 2021). The synthesis of proline in the presence of salinity and SA, is regulated by activation of pyrroline-5-carboxylate reductase (P-5-CR) and γ-glutamyl kinase and inhibition of proline oxidase and proline dehydrogenase (Misra and Saxena, 2009).

The exogenous Ca induces anthocyanin synthesis by regulating the expression of related structural and regulatory genes (Michailidis et al., 2017; Zhu et al., 2019).

Sugars play a significant role in osmoregulation under abiotic stress conditions (Fallon and Phillips, 1989). Total soluble carbohydrates are necessary solutes that accumulate within the cytoplasm under NaCl stress and contribute to plant survival (Rejšková et al., 2007). Increasing the availability of sugars also promotes the synthesis of anthocyanins (Hiratsuka et al., 2001).

DPPH activity is used to determine the total antioxidant potential. The results of the present research showed that DPPH radical scavenging activity in red bean leaves was significantly increased in salt-stressed plants compared to the control. About DPPH-free radical scavenging activity in response to salinity, SA-treated plants in comparison with controls are more potent in controlling ROS production. Also, SA treatment increased DPPH radical scavenging activity in NaCl-stressed safflower plants compared with controls (Shaki et al., 2017).

The results obtained from the current study illustrate that the application of SA and Ca improved the NaCl tolerance of red bean through the protection of photosynthetic pigments and osmolyte accumulation, which can be responsible for improved plant growth under saline condition.

Conclusion

Salinity stress affected plant growth, pigment system and other physiological attributes in the current study. Our results indicated that SA or Ca application to red bean plants mitigate the harmful effects caused by NaCl stress. SA (0.75 mM) and Ca (50 mM) showed to be effective in improving growth and physiological response of plant under NaCl stress. Due to low cost and high efficiency of SA and Ca, they can be used for optimal production of agricultural products in saline conditions.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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