Effect of calcium chloride and salicylic acid on antioxidative responses of lentils (*Lens culinaris* Medik.) under salt stress

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Abstract

Salinity causes harmful physiological and morphological effects in plants. The present study was conducted to investigate the effect of calcium chloride ($CaCl_2$ 0, 50, and 100 mM) and salicylic acid (SA 0, 0.75, and 1.5 mM) on the physiological characteristics of lentils (*Lens culinaris* Medik.) under salinity stress (0, 25 and, 75 mM NaCl). Results showed that the adverse effects of salinity were improved by calcium and salicylic acid. Also, root and shoot lengths, dry and fresh weights, carotenoids, and chlorophylls decreased under salt stress while proline, inhibition of DPPH radical, malondialdehyde (MDA), and anthocyanin increased. However, calcium and salicylic acid increased dry and fresh weights, the root and shoot lengths, reducing sugars, carotenoids, and chlorophylls, and significantly reduced inhibition of DPPH radical and malondialdehyde in lentils. Calcium and salicylic acid were found to promote salinity tolerance, which could be related to regulating antioxidant responses. According to these findings, the most appropriate concentrations for improving the physiological parameters of lentils under salt stress and regulating their antioxidant system are recommended as 50 mM calcium and 1.5 mM salicylic acid.

Keywords: malondialdehyde, photosynthetic pigments, proline, reducing sugars

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Introduction

Soil salinity is one of the main abiotic stresses in semi-arid and arid regions. It involves the abundance of salts of sodium chlorides, calcium and magnesium sulphates and bicarbonates in soil and water (Hoang et al., 2014). Increased salinity reduces chlorophyll and photosynthetic activities in plants, which have different NaCl tolerance (Trabelsi et al., 2019; Zafar et al., 2018).

Plants' protection strategies in response to salinity involve a number of chemicals and compounds. Carotenoids, as secondary metabolites, play an essential protective role in osmotic regulation against salinity stress (Winkel Shirley, 2002; Tashakorizadeh et al., 2022). Anthocyanins protect the plant against reactive oxygen species produced under physiological stress conditions (Rouholamin et al., 2015). Proline increases in saline conditions and has a protective and vital role in plants' resistance to salt stress (Bartels and Sunkar, 2005).

Salicylic acid and calcium chloride are among recommended treatments to increase plants'

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growth under abiotic stresses. SA as a phytohormone has a vital role in reducing drought, and salinity stresses as the healing effects of salicylic acid against abiotic stresses have been confirmed in various plants (Shakirova et al., 2003; Arfan et al., 2007; Erasalan et al., 2007). Calcium is involved in transmitting environmental signals and hormones. Increased calcium level causes the accumulation of anthocyanins in plants (2021b; Xu et al., 2014). Increased Ca levels in cell membranes were shown to protect the plant against the adverse effects of salt stress (Mukhtar et al., 2016; Abdel Latef, 2011; Acosta- Motos et al., 2017; Naeem et al., 2018; Ahmad et al., 2018). Calcium chloride is known to be capable of improving concentration of photosynthetic pigments and growth parameters in plants (Mahdavian, 2021). Moreover, the influential role of calcium on plant growth is achieved through water uptake and electrical conduction of roots under salinity conditions (Navarro et al., 2000). Therefore, the right ratio of Na^{+1} to Ca^{+2} is necessary for the plant to cope with salinity stress (Munns and Tester, 2008).

Legumes are among the main foods of humans in many countries as they are a source of protein, carbohydrates, minerals, vitamins, and also a wide range of compounds with antioxidant properties. Among legumes, lentils (*Lens culinaris* Medik.) have a major role in human diet because of their protein, fiber, minerals, vitamins, and phenolic compound contents (Zhang et al., 2015). A valuable plant species with protein contents of more than 20% and favorable characteristics such as the ability to grow under poor agricultural conditions, lentils have played an important role in the diet of low-income people in developing countries as (Tadayyon et al., 2011).

While lentils are able to survive in relatively poor soils and in adverse environmental conditions, like many other types of legumes, lentils are also classified as a salinity-sensitive plant (Ouji et al., 2015). In previous studies, different lines and cultivars of lentils have shown different levels of tolerance to salinity and were able to grow in soils containing up to about 250 mM sodium chloride (Kumawat and Gothwal, 2018; Kumawat et al., 2017; Singh et al., 2017; Ouji et al., 2015).

The study of salinity tolerance of 162 types of lentils in hydroponic environment showed that salinity tolerance is related to factors such as restriction of movement of Na^+ and Cl^- along with an increase in the thickness of the epidermis and an increase in vascular bundles, a decrease in the absorption of essential nutrients such as calcium and potassium, a decrease in the production of H_2O_2, an increase K^+ accumulation, proline accumulation, antioxidant enzyme activity, seedling growth, biomass amount and seedling survival (Singh et al., 2017).

Few studies have reported on the effects of salicylic acid and calcium chloride on lentils under NaCl. This research was therefore designed to assess the effects of high doses of salinity on the physiological parameters of lentils and determine whether SA and $CaCl_2$ can modify the toxic effects of salinity.

Materials and Methods

Design and treatments

After disinfection with sodium hypochlorite, 5 lentil seeds (*Lens culinaris* Medik.) were planted in 1 kg pots filled with clay, humus, and sand (1:2:3) under day/night temperatures of 25/20 ℃. The experiment was conducted in factorial format based on a completely randomized design (CRD). NaCl (0, 25, and 75 mM), $CaCl_2$ (0, 50, and 100 mM) and SA (0, 0.75, and 1.5 mM), individually or in combination, were applied to the seedlings for 10 days. Salicylic acid was used twice and the plants were ready to harvest 24 days after treatment. The 15 treatments of the study included:

T_1: 0 mM NaCl (control),

T_2: 25 mM NaCl,

T_3: 75 mM NaCl,

T_4: 0 mM NaCl + 1.5 mM SA,

T_5: 0 mM NaCl + 0.75 mM SA,

T_6: 25 mM NaCl + 1.5 mM SA,

T_7: 25 mM NaCl + 0.75 mM SA,

T_8: 75 mM NaCl + 1.5 mM SA,

T_9: 75 mM NaCl + 0.75 mM SA,

T_{10}: 0 mM NaCl + 100 mM Ca,

T_{11}: 0 mM NaCl + 50 mM Ca,

T_{12}: 25 mM NaCl + 100 mM Ca,

T_{13}: 25 mM NaCl + 50 mM Ca,

T_{14}: 75 mM NaCl + 100 mM Ca, and

T_{15}: 75 mM NaCl + 50 mM Ca.

Biomass measurement

After washing the plants with water, the lengths of roots and shoots were measured. Furthermore, to determine the dry weight, the roots and shoots were dried in an oven for 48 hours.

Pigment analysis

Lichtenthaler (1987) method was used to estimate the levels of carotenoids and chlorophylls. In this method, acetone was used to extract pigments after freshly weighed leaves were ground in a mortar, and the absorption of samples was read at 470, 646.8, and 663.2 nm by a spectrophotometer.

To measure the levels of anthocyanins in plant samples, 0.1 g of fresh leaves were weighed and ground in a mortar with 10 ml of acidified methanol, and the mixture was stored in the dark. Absorb samples at 550 nm were then read by a spectrophotometer (Wanger, 1979).

Proline and reducing sugars

To measure proline content, fresh leaf samples were thoroughly ground with 3% sulfosalicylic acid in a mortar. After centrifugation, ninhydrin reagent and glacial acetic acid were added to the resulting solution. Toluene was added to the samples and the absorbance was read at 520 nm spectrophotometrically (Bates et al., 1973).

Jeffries et al. (1988) method was used to measure reducing sugars. One (1) ml of the color mixture containing sodium 1.6%, DNS (dinitro salicylic acid) 1%, and 25% sodium-potassium tartrate was added to 2 ml of the plant extract and after heating for 10 minutes the absorbance was read at 546 nm.

Lipid peroxidation

To measure the amount of lipid peroxidation, the absorption of samples was measured to assay malondialdehyde at a wavelength of 532 nm (Heath and Packer, 1969); for other aldehydes, the absorbance was read at 455 nm (Meirs et al., 1992).

Scavenging ability on DPPH radical

Shimada et al. (1992) method was used to measure the free radical scavenging activity. For extraction purposes, the methanolic solution of leaf samples was used and the absorption of the samples was read at 517 nm.

Statistical Analysis

The data were presented as the mean of three replicates ± standard error (SE). The experiment was conducted in factorial format based on a completely randomized design (CRD). ANOVA was applied for data analysis by using SPSS software. All the data were analyzed using two-way analysis of variance using SPSS software. Tukey's HSD post hoc test was applied for multiple comparisons of the means ($P \leq 0.05$). Assumption of ANOVA is homogeneity of variances.

Results

Effect of Ca and SA on plant growth

Salinity treatment at concentrations of 25 and 75 mM showed a significant reduction compared to the control on lentil plant growth. Shoot lengths decreased by 34% and 8% at 75 mM and 25 mM, respectively, compared with control plants (Fig. I). A similar decrease in shoot length was also observed for root length. Salinity considerably decreased the dry weight of the roots and shoots (Fig. II).

NaCl (75 and 25 mM) with and without SA (1.5 and 0.75 mM) significantly increased growth parameters. The highest root and shoot length increases by 70% and 57%, respectively, were recorded under the combined application of SA (1.5 mM) and NaCl (75 mM) compared to NaCl-treated plants (75 mM) alone. Similarly, root and shoot dry weights improved by 200% and 135%

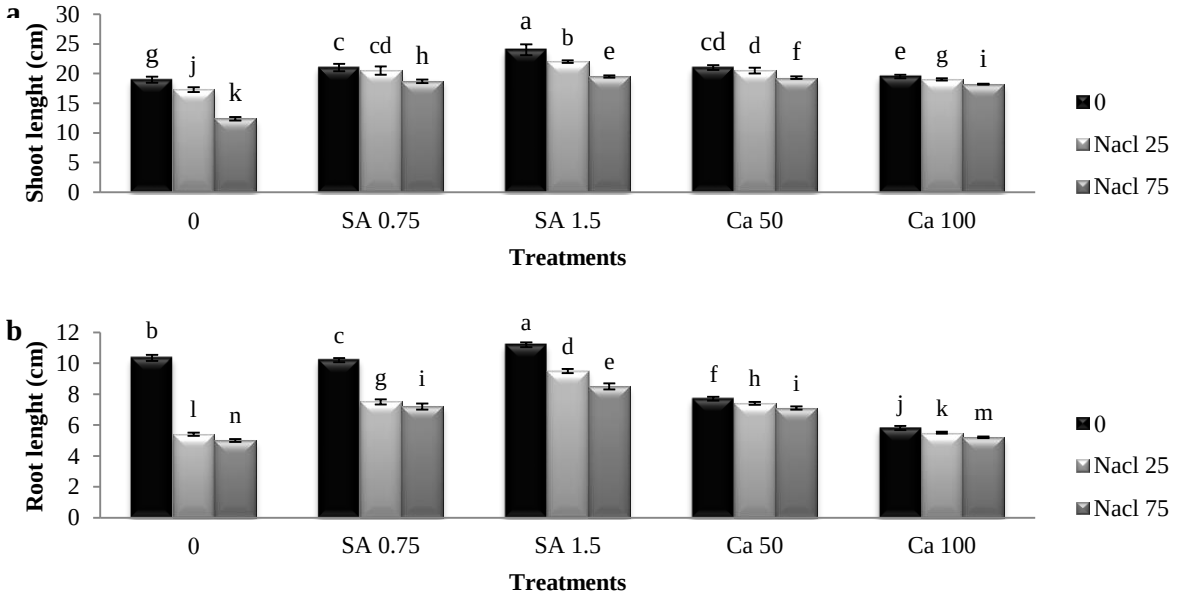

Fig. I. Effect of SA, Ca, and salt stress on the shoot and root lengths of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

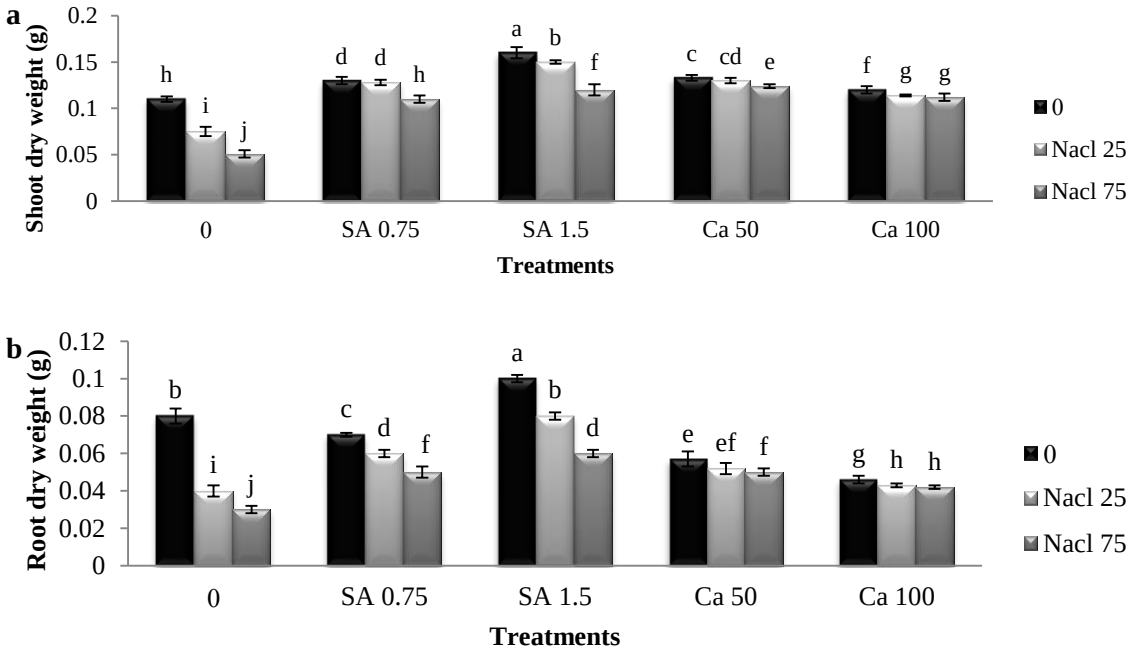

Fig. II. Effect of SA, Ca, and salt stress on the shoot and root dry weight of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

under SA (1.5 mM) and NaCl (75 mM), respectively, compared to their control (Figs. I & II).

$CaCl_2$ (100 and 50 mM) alone and with NaCl (75 and 25 mM) significantly increased all growth parameters. The lowest effect was observed in all growth parameters measured at 75 mM NaCl concentration alone while the biggest effect was obtained with 50 mM calcium treatment (Figs. I & II).

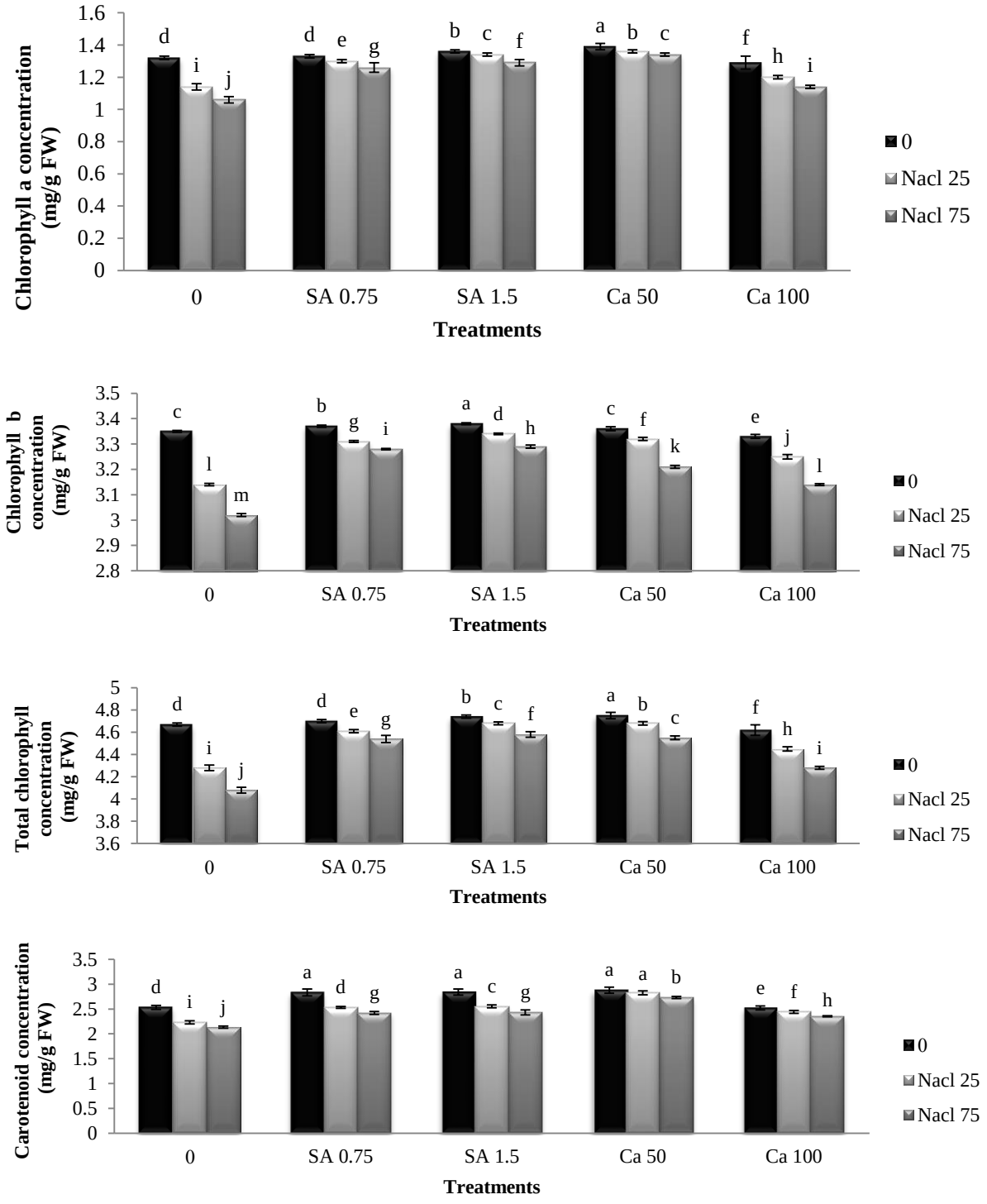

Fig. III. Effects of SA, Ca, and salt stress on Chla, Chlb, total Chl, and carotenoid contents of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

Photosynthetic pigments

The results of this research showed a significant reduction in carotenoid and chlorophyll contents of lentil leaves in saline conditions relative to control (Fig. III). The total chlorophyll and carotenoids in the leaves at a concentration of 75 mM NaCl decreased by 12 and 15%, respectively, compared to the control. However, calcium and SA increased the content of carotenoids and

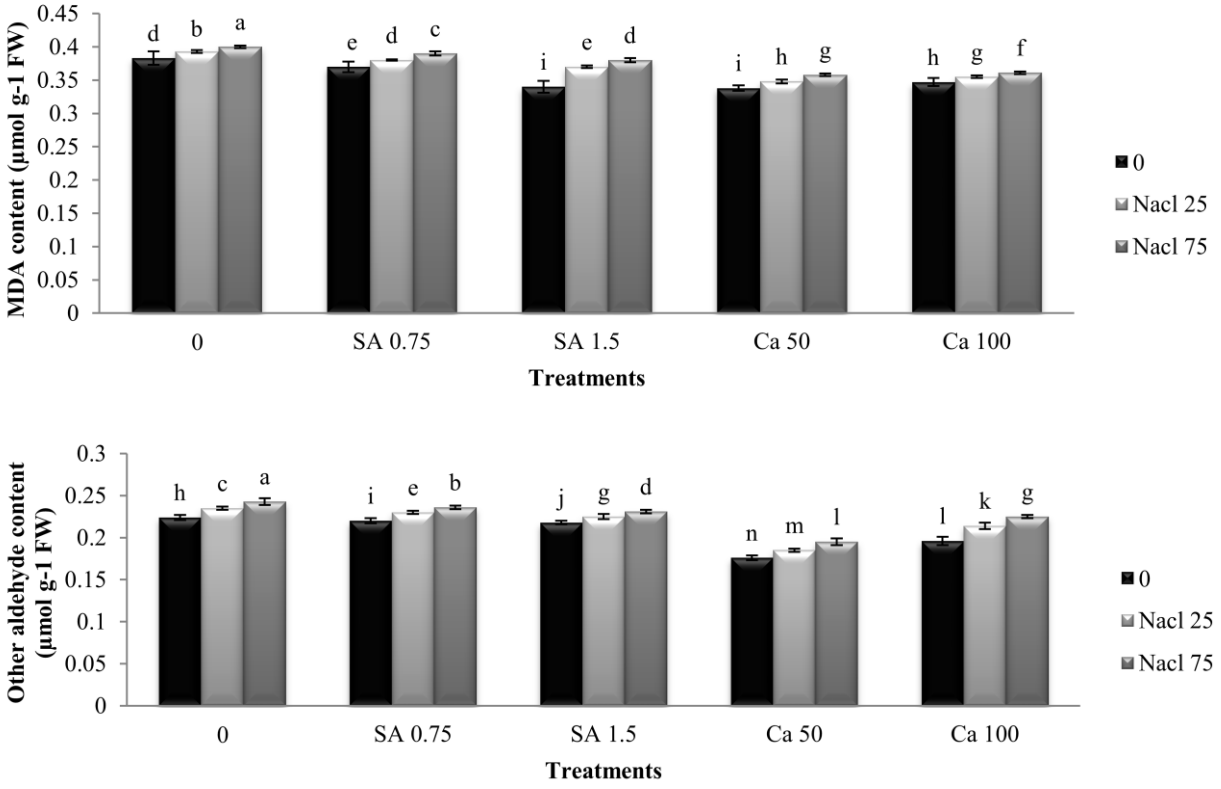

Fig. IV. Effects of SA, Ca, and salt stress on MDA and other aldehyde contents of *Lens culinaris* Medik; values with similar letters are not significantly different at $p<0.05$.

chlorophylls. The highest amount of chlorophyll and carotenoids was obtained in plants that were used only with calcium (50 mM) and SA (1.5 mM), and the least amount of photosynthetic pigments was observed in 75 mM salinity. SA (1.5 mM) treatment significantly increased total chlorophyll contents by 8 and 10% under 25 and 75 mM salinity conditions, respectively while it increased carotenoids by 12% under both salinity levels compared to salinity alone. Also, calcium (50 mM) under 25 and 75 mM salinity significantly increased carotenoids by 21% and 22%, respectively while increasing total chlorophyll by 8% and 10%, respectively, in comparison with salinity alone (Fig. III).

Lipid peroxidation

Salinity treatments with 25 and 75 mM NaCl showed a significant increase relative to control in the production of the malondialdehyde and other aldehydes in lentils (Fig. IV). Concentration of 75 mM NaCl increased the level of malondialdehyde and other aldehydes in leaves by 4% and 8%, respectively, compared to the control. Calcium and SA also significantly modulated the concentration of malondialdehyde and other aldehydes in leaves of the plants under all NaCl levels. Under 75 mM salinity and 1.5 mM SA treatment they were reduced by 5% and 3%, respectively, relative to controls. Also, NaCl (75 mM) and calcium (50 mM) significantly decreased the concentrations of MDA and other aldehydes by 10% and 19%, respectively, compared to plants treated under salinity alone (Fig. IV. A & B).

Reducing sugars content

Results showed a significant decrease in reducing sugar contents under NaCl. Sodium chloride 75 and 25 mM lowered the levels of reducing sugars by 17% and 10%, respectively, relative to the control (Fig. V). On the other hand, calcium and SA significantly modulated the deleterious effect of NaCl on sugar contents. For instance, 1.5 mM salicylic acid increased the amount of reducing sugars in plants by 20% under 75 mM salinity relative to plants treated with salinity alone. The

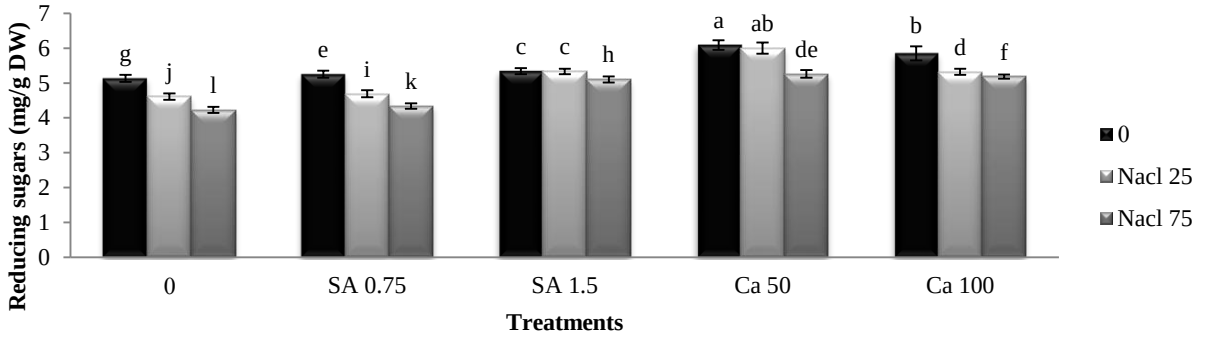

Fig. V. Effects of SA, Ca, and salt stress on reducing sugars content of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

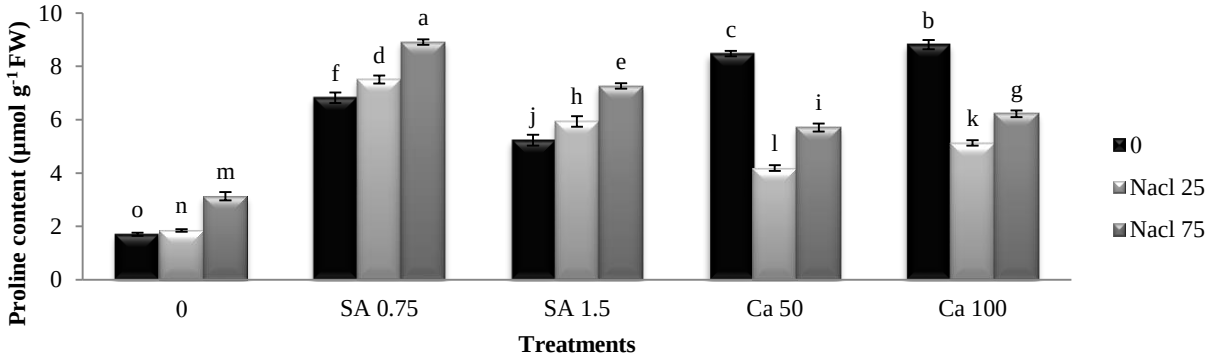

Fig. VI. Effects of SA, Ca, and salt stress on proline content of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

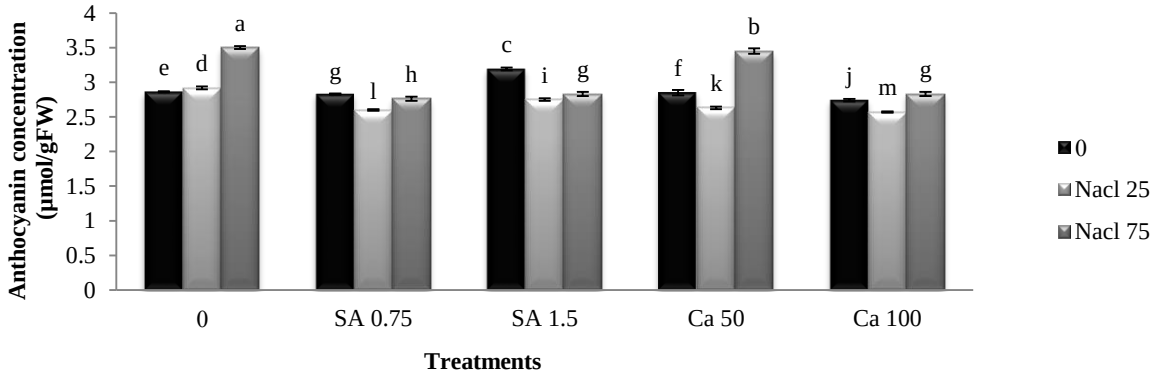

Fig. VII. Effect of SA, Ca, and salt stress on anthocyanin concentration of *Lens culinaris* Medik; values with similar letters are not significantly different at p<0.05.

highest increase in the amount of reducing sugars (18%) was obtained in the 50 mM calcium treatment compared to the corresponding control (Fig. V).

Proline Content

Sodium chloride 75 and 25 mM increased the amounts of proline in lentil leaves by 83% and 8%, respectively, relative to the control. Calcium and SA significantly modulated the deleterious effects of salinity in terms of proline levels. Under 75 mM salinity condition, maximum proline content (184%) was obtained in lentils treated with 0.75 mM SA, relative to plants treated with 75 mM salinity alone. Also, under 75 mM salinity, 100 mM calcium treatment significantly increased concentration of proline by 98% in comparison with the plants treated with NaCl alone (Fig. VI).

Anthocyanin content

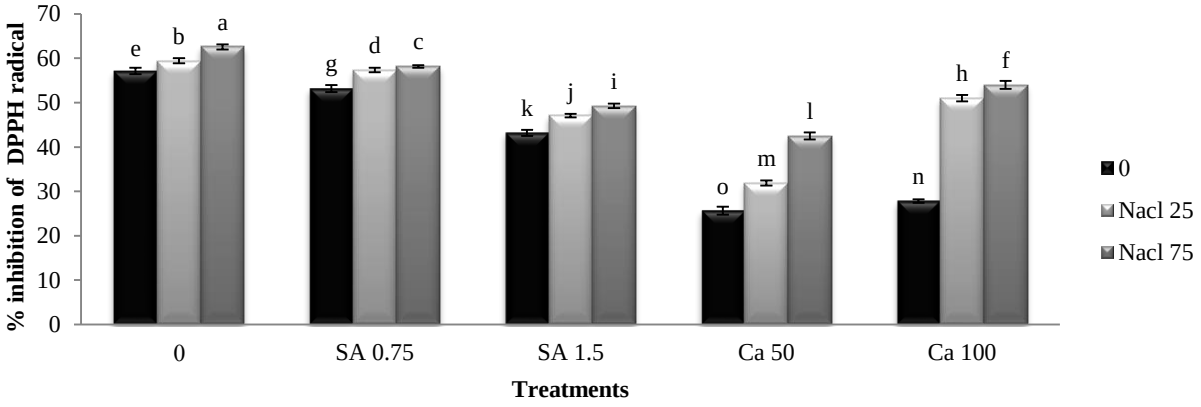

Fig. VIII. Effects of SA, Ca, and salt stress on DPPH radical scavenging activity of *Lens culinaris* Medik, values with similar letters are not significantly different at $p<0.05$.

Sodium chloride 75 and 25 mM increased anthocyanin contents by 75% and 20%, respectively, relative to the control. SA (1.5 and 0.75 mM) reduced anthocyanin concentration of plants by 19% and 21%, respectively, under 75 mM NaCl relative to plants treated with salinity alone. In lentils under 75 mM salinity stress, calcium treatments 50 and 100 mM decreased the anthocyanin content of leaves by 2% and 19%, respectively, compared to salinity alone (Fig. VII).

Antioxidant activity

Salinity levels 25 and 75 mM NaCl increased DPPH radical scavenging activity by 4% and 9%, respectively, relative to control. On the other hand, calcium and salicylic acid significantly modulated the deleterious effect of NaCl through DPPH radical scavenging activity. In comparison to plants treated with NaCl alone, salicylic acid 1.5 mM decreased the DPPH radical scavenging activity of plants by 21% and 20%, under sodium chloride 75 and 25 mM, respectively. Also, sodium chloride (75 mM) and calcium (100 and 50 mM) significantly reduced the DPPH radical scavenging activity by 13% and 32% in comparison with the plants treated with NaCl alone (Fig. VIII).

Discussion

Salinity reduces the growth of plants, especially mesophytes (Hernández, 2019). This investigation also showed that NaCl treatment reduced growth parameters in lentil plants. On the other hand, using SA and Ca significantly improved the plant growth. Various studies have also shown that reduced plant growth due to salinity can impair photosynthetic activities (Larbi et al., 2020; Trabelsi et al., 2019; Akhtar et al., 2013; Zafar et al., 2016).

Calcium increases the plan tolerance to salinity (Tanveer et al., 2020; Mahdavian, 2022). The results of the present study showed that calcium chloride improved the growth parameters of lentil. For instance, calcium increased the dry and fresh weights of the plants under study. Fifty (50) mM calcium chloride was found as the most effective treatment in this study. These are in agreement with the findings of previous studies (Larbi et al., 2020; Mohammad et al., 1998). For example, in their study on olives, Larbi et al. (2020) showed that 10 mM calcium concentration improved growth parameters of the plants under 200 mM salinity treatment.

Similarly, salicylic acid was found to have improved the growth parameters of lentils. Salicylic acid, as a plant growth regulator, is able to protect plants against abiotic stresses, e.g. salinity, by increasing enzymatic, photosynthetic, and protein synthesis activities (Sahu, 2013; Blokhina et al., 2003). SA improves nutrients in plants through increasing mineral uptake, nutrient mobility, and photosynthesis rate (Magda et al., 2013; Szepesi et al., 2005). The results of the present study are in agreement with previous studies (Abd El-Hameid Asmaa et al., 2017; Mahdavian, 2022).

The results showed a significant drop in photosynthetic pigment contents under salt stress. This is in agreement with the findings of previous studies (Akhtar et al., 2013; Larbi et al., 2020; Radi et al., 2013). Chlorophyll decomposition of leaves is the cause of reduced chlorophyll content under salinity conditions (Tsuchiya et al., 1999). Also, the decrease in the amount of chlorophyll can be due to the decrease in the synthesis of 5-aminolinolic acid (Radi et al., 2013).

Calcium and salicylic acid significantly modulated the deleterious effect of salinity on lentils, consistent with previous findings on plant treated with calcium (Larbi et al., 2020). One of the roles of calcium is to help maintain the integrity of the membrane and prevent the decomposition of chlorophyll (Naeem et al., 2018). Calcium had a more effective role in increasing chlorophyll content than salicylic acid. Various studies showed that salicylic acid can improve plants' tolerance under salinity stress (Mahdavian, 2022; Abd El-Hameid Asmaa et al., 2017).

Sodium chloride causes changes in the levels of carotenoids and chlorophylls while carotenoids are needed to maintain the integrity of the photosynthetic apparatus and scavenging ROS generated under salinity condition (Ahmad et al., 2005).

Malondialdehyde content is an index used to quantify membrane and oxidative damage (Katsuhara et al., 2005; Azevedo Neto et al., 2008). Studies have shown that the concentration of malondialdehyde increased under salinity conditions in plants such as cotton (Meloni et al., 2003), rice (Demiral and Tu¨rkan, 2006), maize (Azevedo Neto et al., 2006), cowpea (Cavalcanti et al., 2004), and sugar beet (Bor et al., 2003). The present study showed that malondialdehyde and other aldehydes increased under NaCl stress in lentils. Although the levels of reactive oxygen species were not assayed in this study, the increase in malondialdehyde content could be due to the increase in ROS production under salinity stress (Bor et al., 2003). On the other hand, calcium and salicylic acid treatments in this study reduced the concentration of malondialdehyde under salinity stress. Therefore, it is concluded that calcium and salicylic acid induce lipid peroxidation and the activity of antioxidant enzymes (Gunes et al., 2007).

Many studies have shown that proline levels increase significantly with increasing salinity levels (Aghaei et al., 2009; Martinez et al., 1996). Salicylic acid treatment can improve proline content under salinity stress, indicating that proline plays a vital role in protecting plants from salinity stress (Al-Whaibi et al., 2012).

Calcium utilization can increase anthocyanins by regulating the expression of regulatory and structural genes (Zhu et al., 2019; Michailidis et al., 2017). Soluble carbohydrates have been shown to accumulate in the cytoplasm of plants during salinity stress, indicating the role of sugars in regulating NaCl (Rejšková et al., 2007; Fallon and Phillips, 1989). Therefore, it can be concluded that the accumulation of sugars can increase anthocyanins in plants (Hiratsuka et al., 2001).

This study demonstrated that salt treatment improved the lentil plants' ability to scavenge DPPH radicals. Also, increasing the DPPH radical inhibition activity shows that SA treatment had a higher potential to inhibit ROS production in lentils under salt stress. Similar results were observed for the effect of salicylic acid on safflower plants under salt stress (Shaki et al., 2017). This research showed that calcium and SA improved salinity tolerance in lentils by osmolality accumulation and protection of photosynthetic pigments which can cause plant growth.

Conclusion

The present study showed that salinity stress affects growth and physiological parameters in lentils. Applying calcium or salicylic acid has an influential role in reducing salinity stress on lentil plants. Accordingly, applying 50 mM calcium and 1.5 mM salicylic acid is suggested for mitigating the adverse effects of salinity stress in these legumes.

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