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Modulation of the nutritional and biochemical status of hydroponically grown *Cucurbita pepo* L. by calcium nitrate under saline conditions

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Abstract: Salinity is one of the critical environmental factors that decreases the water availability and induces nutritional imbalance in crops. In order to study the effect of calcium nitrate [$(Ca(NO_3)_2$] in the nutrient solution under salinity conditions, an experiment was designed with different salinity (0, 50, and 100 mM) and $Ca(NO_3)_2$ (2, 3, and 4 mM) levels on *Cucurbita pepo* (zucchini). Based on the results, an increase in the salinity from 0 to 100 mM caused a decrease in the leaf potassium and calcium concentration, whereas the iron, magnesium and zinc concentrations increased. The most effective $Ca(NO_3)_2$ level in increasing the nutritional quality and yield of zucchini was 3 and 4 mM. Salinity at 50 and 100 mM significantly increased the leaf sodium concentration and leaf area as well as the leaf number per plant, while the application of both $Ca(NO_3)_2$ levels modulated the harmful effects of salinity. The amount of malondialdehyde (MDA), proline and hydrogen peroxide (H_2O_2) as well as the catalase (CAT) activity increased under the severe salinity conditions, whereas the application of 4 mM $Ca(NO_3)_2$ had the potential of removing the negative effects of severe salinity. The catalase activity increased along with the increase in the $Ca(NO_3)_2$ concentration, which was independent from the salinity level. However, the amount of proline, MDA and H_2O_2 decreased in plants fed with 3 and 4 mM $Ca(NO_3)_2$ compared to the control in the presence of salinity. These findings suggest that both the 3 and 4 mM concentrations of $Ca(NO_3)_2$ under 50 mM salinity could be used to improve the zucchini performance by maintaining the ion homeostasis and inducing the antioxidant defence system.

Keywords: antioxidant enzyme; $Ca(NO_3)_2$; leaf area index; NaCl; nutritional balance, zucchini

Different stages of the plant life cycle are almost always exposed to many adverse environmental factors in arid and semi-arid lands; the plant responses to the drought stress is different according to the plant species, kind of stress, duration and the intensity of the stressors. Salinity, which is the most severe environmental factor among external stressors, limits plant growth and development. Kaya et al. (2002) stated that about one-third of world's cultivated land is exposed to salt stress. In some regions near to Urmia Lake, north-western Iran, which is one of the largest hypersaline lakes in the world (Eimanifar, Mohebbi 2007), salinity of the agricultural lands is going to become a crisis. In this case, some solutions are proposed including the improvement of agricultural soils by leaching (Yang et al. 2019) or using ameliorative nutritional treatments (Akladious, Mohamed 2018). Today's

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application of fertilisers as a foliar spray (Zayed et al. 2011) or in combination with applications to the soil are considered more since they can enhance the nutrient absorption. Saline soils cause a reduction in the osmotic potential and water use efficiency by the plants (Acosta-Motos et al. 2017). In some crops, such as the tomato, an increase in the salinity improves the quality and taste of the fruit as well as carotenoid, but the glutathione peroxidase (GPX) and catalase (CAT) activities also increase along with the increase in the salinity level. Thus, a crop yield reduction is inevitable (Khanbabaloo et al. 2018). Plants grown in excess sodium chloride have a lower biomass, a lower membrane stability index (MSI), a lower fruit performance, and less chlorophyll in comparison with control plants. In addition to ionic toxicity (Ashraf, Harris 2004), salt stress causes oxidative damage by producing a variety of free radicals (Neill et al. 2002). In salt-stressed plants, the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), GPX and glutathione reductase (GR) increased when compared to the controls and the high activity of the mentioned enzymes in salt-tolerant genotypes are observed more than in salt-sensitive genotypes of maize (De Azevedo Neto et al. 2006).

Calcium, as an essential nutrient, has significant effects on the plant physiological reactions and improves the morphological and biochemical characteristics of salt-stressed plant (Munns 2002). Kaya and Higgs (2002) showed that the addition of 0.5 and 1 g $Ca(NO_3)_2$ to the soil led to an increase in the biomass, fruit performance, and leaf greenness as well as the membrane stability index. Also, the amount of Ca^{2+} and N^+ were similar to the levels in the control samples of cucumbers. The findings of Akladious and Mohamed (2018) showed that irrigation of peppers with saline water including 100 mM of sodium chloride in combination with a lower concentration of $Ca(NO_3)_2$ significantly increased the vegetative factors, leaf relative water content, chlorophyll, the amount of nutritional elements and phytochemicals. In another study, a 3.5 mM calcium and 100 mM NaCl treatment of *Cakile maritima* caused more growth and better tolerance at moderate salt stress levels. The effect of calcium was more pronounced in the least amount of malondialdehyde (MDA), electrolyte leakage (EL) and H_2O_2 which was accompanied with higher antioxidative enzyme activities (Amor et al. 2010).

Cucurbita pepo, as the most popular vegetable in the world, which is especially planted in the Mediterranean region in greenhouses (Rouphael et al. 2004), has been the topic of comprehensive experiments on the effect of salt stress and different fertilisers of different chemical compositions on the crop productivity and nutritional value (Colla et al. 2007). Salinity has negative effects on plants, but it is suggested that the severity of the salinity damage depends on the plant species. So, according to the literature, *Cucurbita pepo* is tolerant to moderate salt stress (Rouphael et al. 2006).

It is suggested that the application of a soilless system to grow *Cucurbita pepo* shows a higher fruit performance in the quality and quantity parameters including the ripening quotient, water-use efficiency and more nutrient uptake, such as N^+, Mg^{2+}, Na^+, Fe^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, than zucchini plants cultivated in the soil (Rouphael et al. 2004). Hence, the use of hydro culture in some vegetables, such as *Cucurbita pepo*, is economical. Since in soilless cultivations, salinity is an unavoidable problem and its control in the nutrient solution is difficult and also costly, and the limitations in providing fresh water from different sources including wells, effluents and recycled water, the present work was aimed at evaluating the physiological and biochemical characteristics of the product as well as the yield, nutritional value and element partitioning in *Cucurbita pepo* leaves in relation to the induced salinity and treatment with calcium nitrate [$Ca(NO_3)_2$] under hydroponic culture.

MATERIAL AND METHODS

Cultural conditions and treatments. A factorial experiment with a completely randomised design and three replications was undertaken to evaluate the effect of three different salt stress concentrations (S0 = 0, S1 = 50 and S2 = 100 mM) induced by NaCl and three treatment levels of calcium nitrate (Ca0 = 2, Ca1 = 3 and Ca2 = 4 mM) on the yield and plant quality of *Cucurbita pepo* cv. Dareen. *Cucurbita pepo* seeds with 99% purity and a 98% germination rate (Seminis, USA). Each seed was planted in a 12-litre black polyethylene pot and filled with sand and kept in a greenhouse, and was fed with the following nutrient solutions as the determined treatments until the fruit set and ripening. In total, 54 pots were used for the planting

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of the seeds, as 2 pots per plot were used for the 9 treatment combinations with three replications. The plants were irrigated with water containing Hoagland solutions composed of different nutrient concentrations (Table 1) until the plants were completely developed within a period of two weeks. The pH of the Hoagland solution in the stock tank was adjusted to 6.5 by means of potassium hydroxide or sulfuric acid. The NaCl concentration in the feeding solution was organised as S0 = 0 mM NaCl (control), S1 = 50 mM NaCl and S2 = 100 mM NaCl and the EC values were 0.18 (S0), 6 (S1) and 12 dS/m (S2).

The *Cucurbita pepo* plants were manually fed with 400 mL of the different treatment solutions for each pot three times in a week. To avoid any salt accumulation in the pots, they were leaching with 1 200 mL of tap water at the end of each week until the water was drained. The *Cucurbita pepo* plants were kept under greenhouse conditions with the following programme; temperature of 25–30 °C, 60–80% relative humidity, 14-hour light period and photosynthetic photon flux density of 400 μm/m^2/s. 90 days after sowing, the plants were harvested and were assessed for the physiological, biochemical and nutritional studies.

Assay of leaf number, leaf area and yield. The number of leaves was recorded. The leaf area was measured with a Delta-T Image Analysis System (Delta-T, LTD, Cambridge, UK) and the number of fruits per plants were measured and weighed at harvesting.

Nutrients assay. The plant tissues were kept for 24 h in an oven at 80 °C until dried, then 0.5 g of the dried ground leaves were digested with concentrated sulphuric acid to measure the amount of Fe^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, using atomic absorption spectrophotometry (Shimadzu, AA6300, Japan) (Hocking, Pate 1977). The concentration of Na and K were evaluated by means of flame photometry (Jeneway, model PFP7) according to the Na and K standard curves of the known concentration, following a previously described method (Ren et al. 2005).

Biochemical traits assay

Proline assessment. To measure the proline content in the *Cucurbita pepo* leaves, 0.2 g of fresh leaves were homogenised in 2 mL of 3% aqueous sulfosalicylic acid. The solution was centrifuged at 10 000 rpm for 30 min, then supernatant was removed and the pellet was washed with 3% aqueous sulfosalicylic acid. The supernatant was pooled, and the amount of proline was evaluated by means of a ninhydrin reagent and toluene extraction (Bates et al. 1973). To measure the amount of proline in each of the samples, the mentioned method was set up with a standard solution of proline with the determined ranges of the method (0–39 mg/mL).

Malondialdehyde assessment. Measuring the amount of malondialdehyde was carried out by means of 2-thiobarbituric acid reactive metabolites (Zhang et al. 2007). In the present protocol, after homogenising 1.5 mL of the extract in 2.5 mL of 5% tetrabutylammonium (TBA), the reaction was warmed to 95 °C for 15 min and quickly cooled on ice. Then, the reaction solution was centrifuged for 10 min at 5 000 rpm, and the absorption at 532 nm by the supernatant was recorded by means of a spectrophotometer. To correct the non-specific turbidity, the absorbance value measured at 600 nm was subtracted from the first absorption amount at 532 nm.

Hydrogen peroxide assessment. The H_2O_2 determination in the leaf samples was carried out following the Liu et al. (2014) method. In this method, 0.5 g of fresh leaves were homogenised in liquid nitrogen and a potassium phosphate buffer (pH 6.8). The extracted sample was centrifuged at 7 000 rpm for 25 min at 4 °C. One hundred millilitres (100 mL)

Table1. Concentrations of the nutrients used in the Hoagland solution

Stock solution			
Macronutrient (g/L)		**Micronutrient (mg/L)**	
$Ca(NO_3)_2{\cdot}4H_2O$	0.47	H_3BO_3	2.86
KNO_3	0.3	$MnCl_2{\cdot}4H_2O$	1.81
$MgSO_4{\cdot}7H_2O$	0.25	$ZnSO_4{\cdot}7H_2O$	0.22
$NH_4H_2PO_4$	0.06	$Na_2MOO_4{\cdot}2H_2O$	0.02
Iron (sequestrene 6% Fe)	0.1	$CuSO_4{\cdot}5H_2O$	0.08

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aliquots of the supernatants were added to 1 mL of a xylenol solution, mixed, and then it was left to rest for 30 min, based on the clarity of the colour, the amount of H_2O_2 in the samples was measured by a spectrophotometer (Shimadzu, Japan) at 560 nm.

Catalase extract preparation and assessment. To prepare the enzyme extract, the *Cucurbita pepo* leaf was ground (1 : 10, w/v) in an ice-cold mortar by adding 50 mM of the sodium phosphate buffer (pH 7.0) including 0.5 M of sodium chloride, 1 mM of ethylenediaminetetraacetic acid (EDTA), and 1 mM of sodium ascorbate. The reaction solution was purified by means of filtration through a Micra cloth. The homogenised leaf solution was then centrifuged at 10 000 rpm for 15 min. After centrifugation, the supernatants were collected to evaluate the catalase activity. The total catalase activity was recorded by means of a spectrophotometer following the protocol of Aebi et al. (1984), by monitoring the decline in absorbance at 240 nm as the H_2O_2 (e = 39.4 mM/cm) was consumed.

Statistics. The experimental data were analysed after confirming the homogeneity of variance of the experimental errors and their normality using SAS software (Ver. 9.4). The least significant difference (LSD) method was also used for comparison at a 5% probability level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Interaction between the salinity and calcium nitrate on the *Cucurbita pepo* leaf number per plant

The application of 2 and 3 mM $Ca(NO_3)_2$ in the control had the highest leaf number with 10.7 and 11 leaves per plant, respectively. The results also showed that 4 mM $Ca(NO_3)_2$ at a concentration of 100 mM NaCl had the lowest number of leaves per plant (2.7), but the difference between the treatment of 2 and 3 mM $Ca(NO_3)_2$ at 100 mM NaCl and the concentration of 2 mM $Ca(NO_3)_2$ were not significant at a concentration of 50 mM NaCl.

The interaction between the treatments showed that the response of the different levels of NaCl to the application of $Ca(NO_3)_2$ were different, so that increasing the $Ca(NO_3)_2$ concentration from 3 to 4 mM in the controls (without salinity) decreased the leaf number per plant, while, at the level of 50 mM NaCl using additive amounts of $Ca(NO_3)_2$ from 2 to 4 mM, significantly increased the number of leaves per plant. Finally, at 100 mM NaCl, the interaction between the different concentrations of $Ca(NO_3)_2$ and the leaf number was not significantly different (Figure 1). Also, studies on barley have shown that the number of leaves decreased along with an increase in the salt concentration in the nutrient solution, which was attributed to the excess salt in the leaf, which usually led to abscission of leaves (Munns 2002). Ca^{2+} has many functions in plant metabolism, including membrane stability, signal transduction as a secondary messenger and controlling the enzyme activity. There are some reports suggesting that Ca^{2+} can help the plant to cope with the adverse effect of salt stress. Most plants that are susceptible to salinity in the presence of low concentration of Ca^{2+} under salt stress, encounter severe damage to the shoots and roots (Grieve et al. 2012) which is due the excessive simultaneous entrance of Na^+ and other ions such as Cl^-. Under salinity, many elements are absorbed non-selectively and cause high toxicity in the plant (Assaha et al. 2017). Thus, it has been suggested that the non-selective absorption of elements by plant roots may be due to the fact that salinity reduces the pH gradient across the root tonoplast membrane and leads to the non-selective adsorption of elements, and ultimately leads to ionic toxicity, such as Na^+, or ionic deficiency, such as K^+. Maintaining the pH gradient across the root tonoplast membrane of the plant under salt stress plays a key role in plant resistance to salinity. In this case, the Ca^{2+} ion preserves the pH difference by acting on the tonoplast membrane. It has been suggested that Na^+ reduces the binding of Ca to the plasma membrane and reduces the entry of Ca into the cell, thereby causing more Ca^{2+}

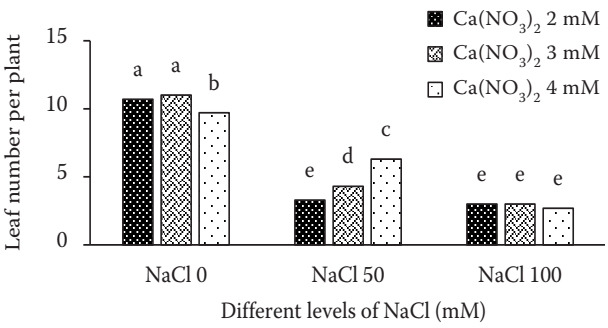

Figure 1. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf number per plant in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

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to be removed from the plant (Grieve et al. 2012). As mentioned above, it seems that under the control conditions and also at 100 mM NaCl, the effect of the ion toxicity is more pronounced either by $Ca(NO_3)_2$ or NaCl. However, the combination of 50 mM NaCl and different levels of calcium nitrate are able to control the ion toxicity.

The findings of Rus et al. (2001) demonstrated that the amount of Ca^{2+} in the plant shoots exposed to 100 mM salinity drastically decreased compared to the control. Changes in the amount of intra-cellular Ca^{2+} are an initial response to salt stress in all plants. This message is initially produced by the roots and, eventually, the Ca^{2+} concentration of the shoots decreases which is likely due to the inducible changes of Na^+ on the amount of Ca^{2+} in the cell. Oliveira et al. (2018) reported that the interaction between salt stress and calcium nitrate on the number of leaves in lettuce cultivars was significant as the leaf number decreased along with increasing the salinity level, while a foliar application of $Ca(NO_3)_2$ was able to moderate the effect of the salt stress on reducing the leaf number.

Interaction between the salinity and calcium nitrate on the *Cucurbita pepo* leaf area index

The application of 4 mM $Ca(NO_3)_2$ in the control treatment had the highest leaf area index. In the present work, the lowest leaf area was assigned to the 100 mM level of NaCl in combination with 2 mM calcium nitrate. The findings suggested that along with the increase in the salt concentration, the leaf area decreased significantly, whereas the application of calcium nitrate increased the leaf area index compared to the control (Figure 2). The main reason for a decrease in the vegetative traits could be related to a decrease in the cell division and elongation (Arif et al. 2019) due to the decreased water uptake and physiological processes and finally to ion toxicity in saline soils (Munns 2005). Plants under salinity stress, by reducing the leaf area, prevent water loss and, as a result, the plant leaves become smaller and thicker in these conditions. An increase in the level of salinity led to the physiological drought (second stress), which is the main factor in preventing turgidity, cell growth and division which ultimately reduces the leaf area in plants (Romanova et al. 2002). In general, the fastest response to salinity stress is to reduce leaf area, which is further stopped by the leaf growth and development by increasing in the intensity of the stress. At moderate level of stress, the growth and development of the leaves continued again after elimination of the salt stress (Zhu 2001). Researchers have shown that a foliar application of calcium and potassium has a significant role in increasing the leaf area in rice under salinity conditions (Sultan 2001).

According to the findings of some researchers, the major effect of Ca^{2+} is due to its controlling effect on sodium entrance in vessels (Hussain et al. 2013). It has been demonstrated that high levels of calcium in rhizosphere are necessary to maintain the high root ion uptake and accumulation of calcium and potassium (Song et al. 2013). The presence of calcium in the environment causes the preservation of the integrity of the cell membrane and its selective permeability, and reduces the toxicity of chlorine and sodium in plants (Giorgi et al. 2010). However, the effect of Ca is dependent to the plant species, calcium concentration and sodium source (Khoshgoftarmanesh et al. 2010). An adequate amount of this element under salinity can reduce the amount of the sodium uptake by reducing the ratio of sodium to potassium and providing better conditions for the plants to grow.

Na^+ may compete with Ca^{2+} for conjugating the binding sites on membranes, and an adequate level of Ca^{2+} can preserve the cell membrane from salt stress damage (Mozafari, Kalantari 2005). Calcium ions maintain the integrity of the plasma membrane in root and shoot cells which are essential for normal cell viability and function (Yarnia et al. 2009). Oliveira et al. (2018) concluded that the application of calcium nitrate at different levels of salinity stress improved the leaf area index in two lettuce cultivars. In fact, a decrease in the growth and develop-

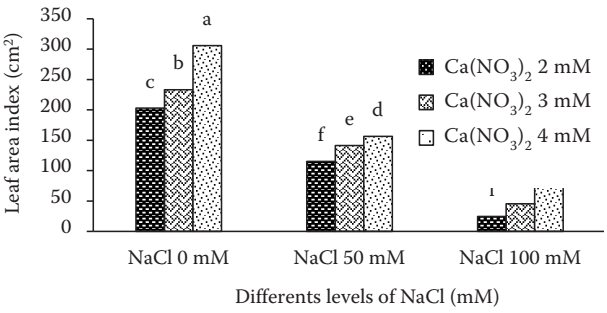

Figure 2. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf area index in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

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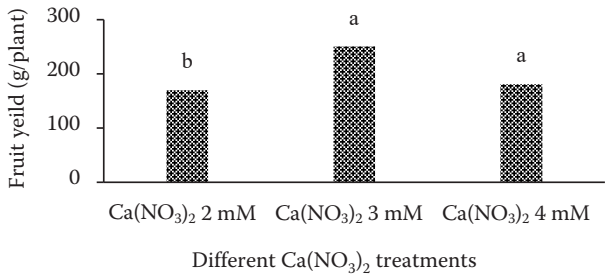

Figure 3. The effect of the different levels of $Ca(NO_3)_2$ on the fruit performance in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

ment of most plants under salinity may be attributed to a decrease in their leaf area (Munns 2002).

Interaction between the salinity and calcium nitrate on the *Cucurbita pepo* fruit performance

The results showed that although the concentration of 3 mM calcium nitrate had the highest (25.7 g per plant) fruit performance, it was not significantly different from the level of the 4 mM calcium nitrate (18.7 g per plant). The lowest fruit yield also was recorded in the controls (17.1 g per plant) (Figure 3). It can be concluded that the increase in the fruit performance can be related to the positive effect of 3 mM $Ca(NO_3)_2$ on improving the leaf number per plant and the leaf area index. Calcium has a key function in photosynthesis, hydrocarbon transport and nitrogen uptake processes in plant cells (Lester, Grusak 2004). Kaya and Higgs (2002) found that the application of $Ca(NO_3)_2$ in both non-salinity and salinity treatments significantly increased the fruit yield. The positive response of a foliage spray of calcium in improving the quantity and quality of the fruit as well as the performance of leafy vegetables, such as endives (Tzortzakis 2008) and spinach (Turhan et al. 2013), has been reported. In another study, high calcium levels in the nutrient solutions led to an increase in the fruit yield in tomatoes (Lolaei 2012).

Regarding the Ca^{2+} effect on the fruit performance parameters, the supplemental calcium in the nutrient solution eliminated the harmful effect of salinity in *Cucurbita pepo* by improving the factors affecting the crop performance especially in the 3mM $Ca(NO_3)_2$ treatment under 50 mM NaCl. The same results were previously documented by Parvin et al. (2015) and Kaya and Higgs (2002). Calcium treatments, especially at 3mM concentration, increased the vegetative factors of the plants under stress. It can be related to the various roles of calcium, such as the participation in the plant cell walls and membranes; so, it causes an improvement in the integrity of the cell membrane by conjugation with the various proteins and lipids on the membranes (Davis et al. 2003).

Interaction between the salinity and calcium nitrate on the nutritional elements in the *Cucurbita pepo* leaf

K^+ concentration. The results showed that as well as an increase in the salinity concentration, the leaf potassium content decreased so that the level of 100 mM NaCl was more effective in decreasing the leaf potassium concentration compared to 50 mM and the control levels, by 20.19% and 13.30%, respectively (Figure 4). Under salinity, high amounts of sodium not only disrupt the uptake of potassium by the root, but also disrupt the root membrane permeability. The amount of K^+ in the plant tissues decreases under sodium salinity or increases under the ratio of Na^+/Ca^{2+} (Maksimović et al. 2010). In fact, salt stress, by disrupting the mechanism of potassium uptake by the roots, has been able to reduce the potassium concentration of plant organs (Sheidaei et al. 2014). Our results agree with Keutgen, Pawelzik (2008), Zushi et al. (2009), and Nemati et al. (2011). However, the application of $Ca(NO_3)_2$ compensated the amount of potassium in the leaves. Both the 3 and 4 mM $Ca(NO_3)_2$ concentrations had the biggest effect

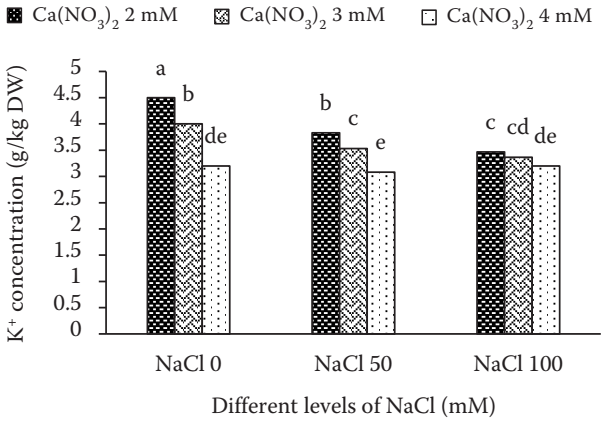

Figure 4. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf K^+ concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

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on the K^+ absorption and there was no significant difference between them. The potassium uptake is controlled by at least two mechanisms that transport potassium through the root cell membranes, both of which involve the high- and low-affinity K^+ transport system. Since soils often have concentrations of less than 1 mM potassium, systems with a high affinity for plant potassium nutrition is predominant (Assaha et al. 2017). Both systems are adjusted by Ca^{2+}. Under saline conditions, the method of potassium transport in the roots of plants activates the calcium, and an increase in the extracellular calcium raises the cytosolic potassium (Assaha et al. 2017). Lolaei (2012) suggested that the maximum concentration of potassium belonged to the plants treated with 100 mg/L calcium under control situations (no salinity). The amount of K^+ increased in *Vigna unguiculata* with the foliar application of calcium nitrate. Excess sodium causes cell membrane damage by dissipating the membrane potential and, subsequently, the Cl^- absorption down the chemical gradient facilitated. An anion carrier has been implicated in this passive flux. Similarly, sodium competes with potassium for the intracellular influx because the mentioned cations are transported via common carrier proteins (Murillo-Amador et al. 2006).

Na^+ concentration. The concentration of 2 mM calcium nitrate at 100 and 50 mM salinity levels had the highest leaf sodium concentration (2 and 1.7 g per kg DW), respectively. The lowest leaf sodium concentration was used for all three levels of 2, 3 and 4 mM $Ca(NO_3)_2$ in the control conditions. As well as increasing the $Ca(NO_3)_2$ level in the controls, the leaf sodium concentration did not show a significant decrease, while at 50 and 100 mM salinity levels, increasing the $Ca(NO_3)_2$ concentration significantly decreased the leaf sodium and both the highest and lowest leaf sodium levels were observed at 2 and 4 mM $Ca(NO_3)_2$, respectively (Figure 5).

The levels of Ca, Mg, and K in the root and shoot decreased with the application of sodium chloride, as demonstrated in earlier experiments (Khan et al. 1997), and the mechanisms of the difference in the nutritional elements under salt stress is still understood. It has been stated that plants growing under saline conditions have the excessive absorption of sodium and chlorine ions, which causes damage to the plant and the premature death of the leaves. At the cellular level, extreme levels of Na^+ and Cl^- cause ionic toxicity, cell membrane disfunction, loss of turgidity, cell dehydration, decreased leaf area and ultimately reduced growth (Cabrera, Perdomo 2003). These events can be attributed to the inhibitory effect of calcium on the sodium absorption and nitrate effect on the chlorine absorption (Massa et al. 2009). The results of Dkhil, Denden (2010) showed that, under saline conditions, the carbohydrates and phenols accumulated in the plant and the amount of sodium increases while the amount of potassium decreased.

Mg^{2+} concentration. By increasing in the concentration of $Ca(NO_3)_2$ from 2 to 3 and 4 mM, the concentration of the leaf Mg^{2+} was significantly reduced, so that the 3 and 4 mM levels of calcium

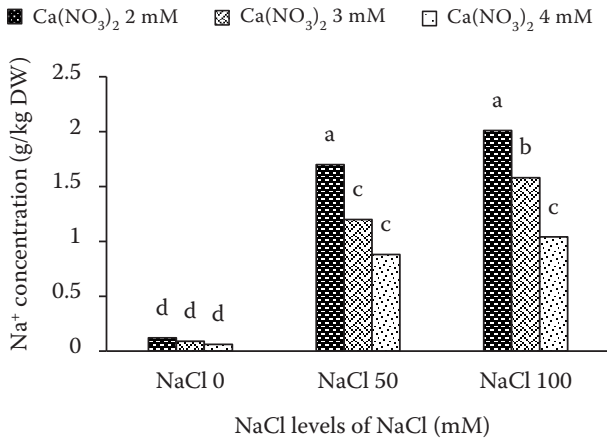

Figure 5. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf Na^+ concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

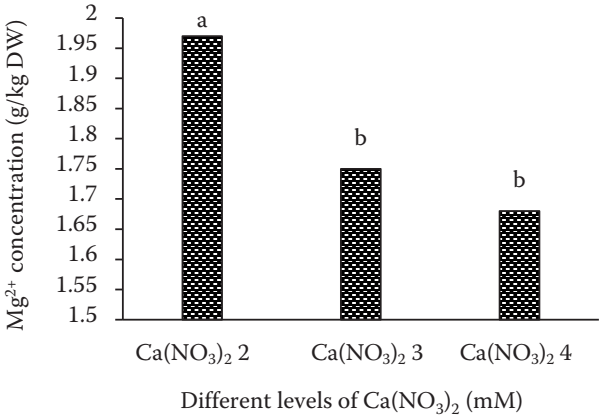

Figure 6. The effect of the different levels of $Ca(NO_3)_2$ on the leaf Mg^{2+} concentration in *Cucurbita pepo*
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nitrate with an average of 1.75 and 1.68 g/kg DW, respectively, reduced the Mg^{2+} concentration compared to the 2 mM level by 11.11% and 14.64%, respectively (Figure 6). The most important reason for the reduction in the level of Mg^{2+} in the leaf samples could be due to the antagonistic relationship between this element and the Na^{+} and Ca^{2+} cations. Marschner (1995) stated that the Mg^{2+} uptake can be intensively affected by other cations including potassium and calcium. Salinity led to the decreased absorption of macro elements in *Cucurbita pepo* plants, which can be due to the decrease in energy that encourages its translocation, the increase in Na ions that cause the membranes to depolarised (Zuazo et al. 2004) and the existence of the antagonistic effect between Na and other elements including K, Ca, Mg, and P (Wakeel 2013). These findings agree with Rahneshan et al. (2018). The application of 3 mM calcium nitrate improved the absorption of the nutrients which can be due to the ability of Ca^{2+} to alleviate Na^{+} toxicity (Achakzai et al. 2010).

Ca^{2+} concentration. The highest level of the leaf Ca^{2+} was allocated to the unstressed plants (3.63 g per kg DW) treated with 4 mM $Ca(NO_3)_2$, while the lowest concentration of the mentioned element was allocated to the level of 100 mM salinity (2.80 g per kg DW). However, the difference between the level of 100 and 50 mM salinity was not significant (Figure 7). This result indicates a negative relationship between the salinity and Ca^{2+} concentration as, at low levels of salinity, the concentration of Ca^{2+} ions in the plants increases due to the increase in the ionic strength and the decrease in the activity of Ca^{2+} ions in the soil, but at high salinity levels, there is a great deal of Na in the soil and its ability to compete with Ca^{2+} reduces its uptake by plants (Garg et al. 2009). There are some studies on the Ca^{2+} diminish (Khaled, Fawy 2011) which agree with our results. Treatment with 4 mM $Ca(NO_3)_2$, in addition to having the highest amount of Ca in the leaf, caused an increase in the content of this element at 50 and 100 mM salinity by 8.94% and 4.12%, respectively (Figure 7). This result indicated that the effect of 4 mM $Ca(NO_3)_2$ under 50 mM salinity was more than the 100 mM salinity. These findings are evidence for the claim that increases in the Ca levels lead to a decrease in the Na absorption by the root and its translocation towards the leaf (Zuazo et al. 2004). However, the difference between the 3 and 4 mM levels of $Ca(NO_3)_2$ in the control was not significant. The results of the various levels of calcium nitrate on the seed germination showed different responses (Salles et al. 2019). Shabala et al. (2006) suggested an extra mechanism of the calcium function in salinity-stressed plants: (1) the inhibition of Na -induced K efflux through outwardly directed (2) K permeable carriers. So, the two major threats imposed by salt stress are induced via the hyperosmotic and hyperionic status associated with the excess amount of Cl and Na absorption, leading to Ca and K deficiency and to other nutrient equilibrium imbalances (Marschner 1995).

Fe^{2+} concentration. The result of the salt stress effect on the level of Fe^{2+} in the leaves showed that the salinity levels of 100 and 50 mM with 788.3 and 743.5 mg/kg DW, respectively, had more Fe^{2+} compared with the control treatment (508.9 mg per kg DW). It should be noted that the difference between the 100 and 50 mM levels of NaCl on the leaf Fe^{2+} content was not significant and the increase in the salinity level from zero (control) to 50 mM significantly increased the leaf Fe^{2+} concentration (Figure 8A). The treatment with 2 and 3 mM $Ca(NO_3)_2$ had the highest concentration of the leaf Fe^{2+}, while the lowest concentration was observed at 4 mM $Ca(NO_3)_2$ (Figure 8B). The difference between the 2 and 3 mM concentrations of $Ca(NO_3)_2$ on the amount of the leaf Fe^{2+} was not significant. Therefore, it can be concluded that the concentration of 50 mM salinity in combination with 3 mM $Ca(NO_3)_2$ is the best composition for the Fe^{2+} ad-

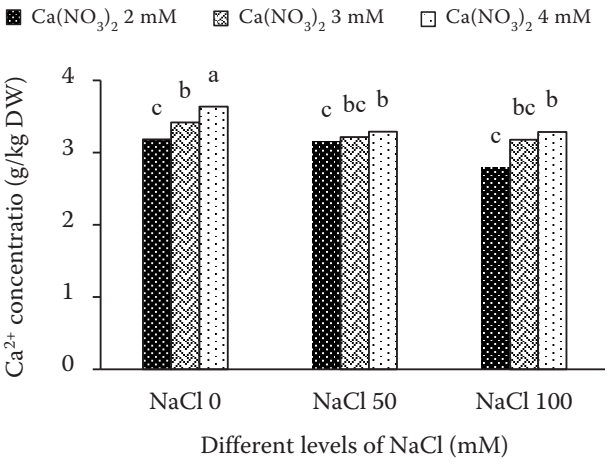

Figure 7. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf Ca^{2+} concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

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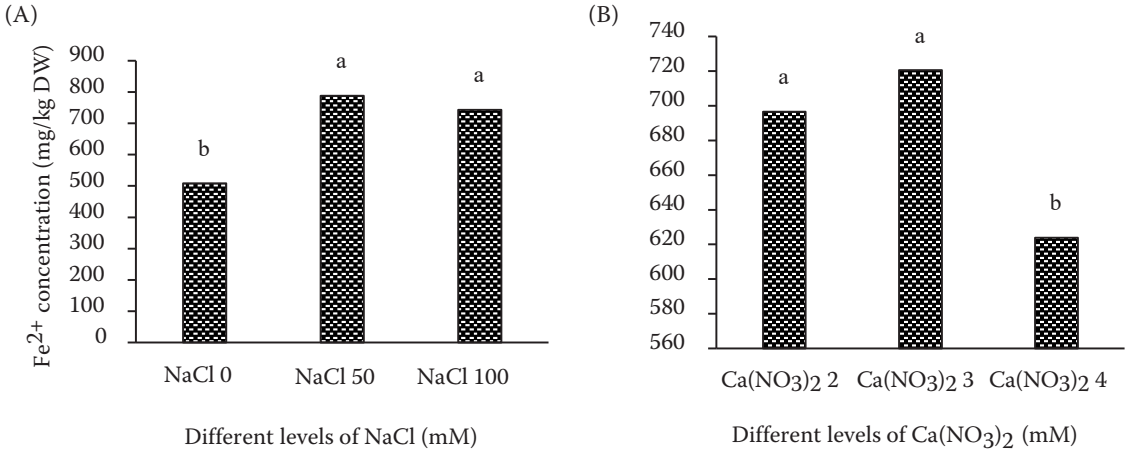

Figure 8. The effect of the salt stress (A) and $Ca(NO_3)_2$ (B) on the leaf Fe^{2+} concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

sorption, although the interaction between the salinity and $Ca(NO_3)_2$ were not significantly different. It is possible that an increase in the amount of calcium nitrate increased the concentration of Ca ions, where Ca^{2+} competed with Fe^{2+} for the uptake, and the iron uptake decreased.

An increase in the level of Fe^{2+} in the *Cucurbita pepo* leaf and mangoes under saline conditions was stated by Villora et al. (2000) and Zuazo et al. (2004), respectively. However, in other studies, the salinity reduced the concentration of Fe^{2+} in the leaves and other plant organs. Wakeel (2013) stated that, in saline and sodium soils, the amount of trace elements in the soil is low and the plants, in these circumstances, usually encounter a lack of these elements and as well as an increase in the levels of salt stress, the plant becomes Fe^{2+} deficient. Tunçtürk et al. (2011) observed that the effect of salt stress on the shoots' Fe^{2+} concentration was different depending on the cultivar. It seems that the increase in the amount of Fe^{2+} in the shoot with the application of NaCl in the soil is related to two factors: (1) an increase in the level of soluble Fe^{2+} in soil water due to the exchange process of Na^{+} with Fe^{2+} and Fe^{3+} which causes the release of Fe^{2+} into the soil solution, (2) a decrease in the plant dry matter after salinity, which increases the Fe^{2+} concentration of the shoots according to the concentration effect (Marschner 1995). The imbalance may be due to the effect of the salt stress on the nutrient utilisation, competition in the absorption, the disruption in the transport or the distribution of nutrients in the plants, or may be due to the insolubility of the nutrients which, thus, cause deficiency. In conclusion, it can be stated that one of the negative effects of salinity is to make a disruption in the element's equilibrium, such as zinc and iron.

Mn^{2+} concentration. The effect of various concentrations of salt stress on the Mn^{2+} concentration in the *Cucurbita pepo* leaves was significantly different at 100 and 50 mM salinity levels (61.1 and 56 mg/kg DW), respectively, and both of them had the highest concentration of the leaf Mn^{2+} in comparison with the controls. However, there was no significant difference between these two levels of salinity on the Mn^{2+} concentration, but its absorption was increased in the salinity conditions compared to the control, where the lowest concentration of Mn^{2+} (43.50 mg/kg DW) belonged to the control plants (Figure 9). While an increase in the

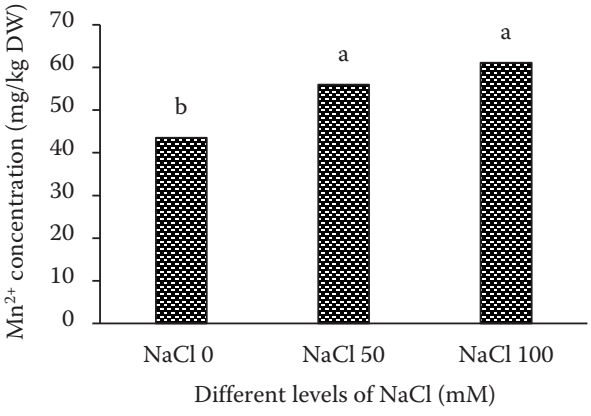

Figure 9. The effect of the different levels of salt stress on the leaf Mn^{2+} concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

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Mn^{2+} concentration has been demonstrated in some literature studies (Víllora et al. 2000; Achakzai et al. 2010) under salinity, a decrease in the Mn^{2+} concentrations has also been stated to occur in spinach and lettuce. Garg et al. (2009) reported that salt stress diminished the concentration of Mn in the stems and roots of chickpeas and ultimately caused a reduction in the plant growth and performance. The effect of salinity on the Mn^{2+} concentration was similar to the Fe^{2+} in our experiment which could be dependent on the species. However, in the present study, the application of calcium nitrate did not have any significant effect on the Mn^{2+} concentration in the *Cucurbita pepo* leaves.

Zn^{2+} concentration. The level of salinity had positive effects on the Zn^{2+} absorption and the highest content of Zn^{2+} was related to the 100 mM salinity in combination with 4mM $Ca(NO_3)_2$ (Figure 10). However, the 50 mM salinity level in combination with 3 and 4 mM $Ca(NO_3)_2$ treatment had the lowest amount of Zn^{2+} in comparison with similar treatments except for 2 mM calcium nitrate during salinity. Under salinity, an increase in the Zn^{2+} concentration was also reported in squash (Víllora et al. 2000). The existence of a high amount of salts in the nutrient solution can cause an ion imbalances or disruption in the ion homeostasis in the plant cells (Parida, Das 2005). The experiments showed that the concentration of Zn^{2+} increased significantly when 4 mM calcium was used in the controls, too.

It seems that different concentrations of $Ca(NO_3)_2$ may act as a source of salinity, but the positive effects of $Ca(NO_3)_2$ is more drastic under salinity levels. However, the threshold of salinity is another limiting factor in the effectiveness of Ca^{2+}. Also, it seems that calcium builds up in the cell wall, making it stronger and, thus, reduces the absorption of elements.

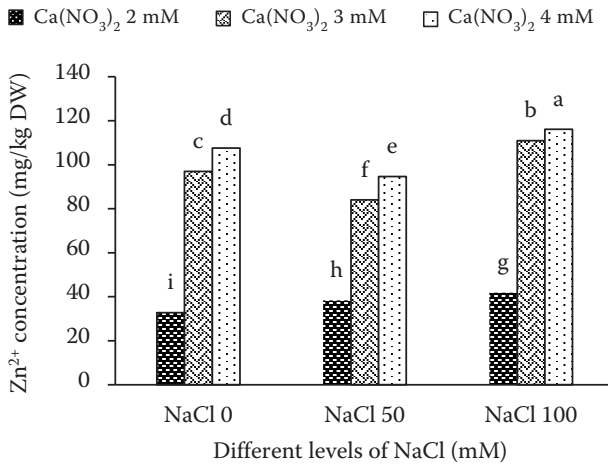

Figure 10. The interaction between the salt stress and $Ca(NO_3)_2$ on the leaf Zn^{2+} concentration in *Cucurbita pepo*
Different letters indicate significant differences at $P < 0.05$ among the treatments

The effect of the salinity and calcium nitrate on the biochemical traits in the *Cucurbita pepo* leaf

The effects of salinity on the amount of proline in the *Cucurbita pepo* leaves were significantly different as it increased at the 50 mM salinity level, but the increasing trend was not linear and it decreased at a high salinity level (100 mM). However, the control plants had the lowest proline content and it seems that, as the intensity of the salt stress increases, the plant's defences are gradually reduced and, at severe salinity stress, the plant metabolism can be rapidly disrupted and the plant cannot demonstrate a good defence mechanism. The tolerance threshold of the plant species to stress is individual and along with its increased stress intensity to a critical range, the defence responses of the plant can be decreased. Another reason could be that proline breaks down rapidly after stress which leads to support of the oxidative phosphorylation cycle in the mitochondria and provides the required adenosine triphosphate (ATP) to return to normal conditions (Satoh et al. 2002). However, using calcium nitrate causes a reduction in the proline content, where the most effective concentration was 3 mM. The presence of calcium in the salt conditions increases the activity of proline oxidase and decreases the proline content in the plant, which is consistent with the results of this experiment (Cheruth, Azooz 2009). It seems that under salinity in combination with calcium nitrate, the amount of proline was not increased. According to Shi et al. (2004), the activity of proline 5-caboxilase synthase in a growing medium having NaCl and $CaCl_2$ is less that the activity of proline.

Along with an increase in the salt stress level, the catalase enzyme activity increased. The use of Ca^{2+} in the controls as well as salinity levels causes an increase in the catalase activity. Catalase, as an antioxidant enzyme, is required to activate the defence responses against stress and scavenge free radicals. The high activity of this enzyme in treatments with calcium nitrate can be attributed to the structural role of calcium. Calcium binds to the cell wall pec-

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tin and increases the catalase activity by lignification of the cell wall (Lee et al. 2007).

One of the reasons for a diminish in the growth and photosynthetic capacity of the plant during extreme external factors, for example salt stress, is the disruption of the balance between the accumulation of active oxygen species (AOS) and the induction of oxidative stress. One of the active free radicals is H_2O_2 that causes oxidative damage by means of membrane intermittent permeability causing an increase in the lipid peroxidation (Upchurch 2008). The level of lipid peroxidation and hydrogen peroxide are major indicators showing the amount of oxidative stress in the plant when exposed to undesirable external factors (Siddiqui et al. 2013). The results indicated that the catalase enzyme had the biggest ability to scavenge hydrogen peroxide in the plant's leaves since it is positively correlated to the amount of H_2O_2 in the plant. Similar findings were reported by Akram et al. (2012) who found that, under saline conditions, a large amount of hydrogen peroxide and malondialdehyde accumulated in the plant leaves, and the catalase, peroxidase, and superoxide dismutase activity increased in sunflowers demonstrating an antioxidant enzymatic defence against stress.

The lipid peroxidation of the membranes estimated by the amount of MDA (Table 2), in the treatment with the 4 mM concentration of $Ca(NO_3)_2$, decreased by 22.4% by the severe salt stress of the control, while the lowest and the highest amount of MDA were allocated to the control in combination with all the $Ca(NO_3)_2$ treatments and the control level of 100 mM salinity. There was no significant difference in all the $Ca(NO_3)_2$ levels at 50 mM salinity. As shown in Table 2, there was no significant effect on the peroxidation of lipids in the controls either with calcium or without calcium. However, under severe salt stress, the level of malondialdehyde increased just slightly in the absence of Ca. There are some reports showing that the application of supplemental calcium led to an increase in salt stress resistance (Cachorro et al. 1993), and improvement in the growth (Martinez-Ballesta et al. 2000). It is demonstrated that there are at least two possible roles for calcium in tolerating salinity stress: (1) a pivotal signalling function in the salinity response leading to adaptation and a direct inhibitory effect on the Na entry system (Yokoi et al. 2002) and (2) Ca application reduced oxidative stress through the boosting of antioxidant enzymatic activity. This result suggests the importance of Ca for the prevention of oxidation and for the preservation of plant resistance under saline conditions. The best protection in term of antioxidative status occurred at 4 mM concentration of $Ca(NO_3)_2$ at both 50 and 100 mM levels of salinity. The findings of this work suggested that application of 4 mM calcium nitrate leads to an increase in the level of antioxidants independent of the salt in the CAT activity. Hernandez et al. (2003) concluded that the activity of antioxidative enzymes is related to the Ca concentration, salt concentration and plant species. Shi et al. (2004)

Table 2. The interaction between the salt stress and $Ca(NO_3)_2$ on the proline, malondialdehyde (MDA), H_2O_2 and catalase (CAT) in the *Cucurbita pepo* leaf

Treatments	Proline (nmol/g FW)	MDA (μmol/g FW)	H_2O_2 (μmol/g FW)	CAT (U/mg protein)
S0 Ca0	0.31^{c}	1.133^{d}	0.32^{e}	0.036^{d}
S0 Ca1	0.39^{c}	1.2^{d}	$0.41d^{e}$	0.055^{cd}
S0 Ca2	0.39^{c}	1.174^{d}	$0.48d^{e}$	0.090^{bc}
S1 Ca0	1.36^{a}	1.84^{bc}	0.55^{cd}	0.076^{cd}
S1 Ca1	0.47^{bc}	1.53^{bcd}	0.41^{de}	0.1^{bc}
S1 Ca2	0.47^{bc}	1.489^{cd}	0.47^{de}	0.134^{ab}
S2 Ca0	1.51^{a}	2.54^{a}	1.30^{a}	0.136^{ab}
S2 Ca1	0.62^{b}	2.075^{ab}	0.69^{bc}	0.142^{ab}
S2 Ca2	0.45^{bc}	1.97^{bc}	0.80^{b}	0.15^{a}

Values in the same column with different lower case letters are significantly different at $P \leq 0.01$; NaCl levels are noted as S0 = 0, S1 = 50, S2 = 100 mM and calcium nitrate treatments are noted as Ca0 = 2, Ca1 = 3, Ca2 = 4 mM

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studied the effect of salinity on the activity of antioxidant enzymes, H+-ATPase and H+-PPase in tomato plants and demonstrated that both salt, either as NaCl or $Ca(NO_3)_2$, inhibited the plant's growth and increased the antioxidative enzyme activities, the effect of NaCl being much heavier.

CONCLUSION

Salt stress decreased the growth parameters, such as the number of leaves per plant, the leaf area, and also the yield of *Cucurbita pepo* and the level of 100 mM salinity was more effective than the 50 mM in the worth effect of salinity. As the level of salt stress increased from zero to 100 mM, the amount of potassium and calcium in the plant leaves decreased and the concentration of Fe^{2+}, Mn^{2+} and Zn^{2+} increased in the leaves. It seems that Na^+ can cause degradation in the cell wall and disruption in the cell membrane permeability leading to an increase in some micronutrient absorption. The highest leaf Fe^{2+} and leaf Zn^{2+} concentrations were at the 2 and 3 mM levels, and the highest leaf Ca^{2+} concentration was at 4 mM level of $Ca(NO_3)_2$. The application of the 3 and 4 mM levels of calcium nitrate can lead to the increased economic performance of this product. Under salinity, the enhancement in the level of proline, malondialdehyde and hydrogen peroxide were observed. Also, the activity of catalase increased at a 100 mM salinity level indicating the destructive role of NaCl in the cellular dysfunction via the increase in the peroxidation of membrane lipids by the production of reactive oxygen species (ROS). However, it is suggested that calcium is able to modify the physical properties of the soil, improve the growth and antioxidative defence of *Cucurbita pepo* and to reduce the salinity damage. Appropriate concentrations of Ca^{2+} likely decrease the intensity of the stress; on the other hand, high levels of Ca^{2+} may have an inhibitory effect on the absorption of coupling factors including Fe, Mn, Cu, and Zn elements. Among the $Ca(NO_3)_2$ concentrations, the highest leaf K^+ concentration and fruit yield were observed at 3 and 4 mM $Ca(NO_3)_2$. So, it can be suggested that the threshold of salinity for *Cucurbita pepo* is 50 mM which can be mitigated with 3 mM $Ca(NO_3)_2$, however, using calcium nitrate without salinity cannot be advised as the positive effects of the $Ca(NO_3)_2$ application in moderate concentrations were seen in combination with the salinity. Also, using calcium nitrate in the nutrient solution in normal conditions not only improved the growth factors of *Cucurbita pepo*, but also caused an increase in the antioxidative status of plant, indicating that calcium nitrate acts like another source of salinity. However, calcium nitrate led to a significant reduction in the harmful effects of salt stress by reducing the oxidative stress via an increase in the ROS scavenging capacity of the antioxidative enzymes, although the effect of calcium nitrate was dose dependent. It seems that the adverse effect of high concentrations of $Ca(NO_3)_2$ under salinity stress is due to an increase in the relationship of inconsistency between the electrical conductivity of the nutrient solution and also due to severe burns and leaf fall in the foliar application of Ca^{2+} although we did not encounter this phenomenon. In conclusion, our study showed that the threshold of salinity for *Cucurbita pepo* is 50 mM which can be mitigated with both the 3 and 4 mM calcium nitrate application. The Ca application can cause an increase in the resistance of zucchini via an increase in the activity of various defence enzymes making the balance in the absorption of the elements as well as causing an increase in the leaf number and leaf area. However, the most critical element was the concentration of $Ca(NO_3)_2$ in the nutrient solution for *Cucurbita pepo*.

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