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Original article

Calcium supplementation improves *in vitro* salt tolerance of date palm (*Phoenix dactylifera* L.)

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Abstract:

Calcium (Ca^{2+}) supplementation activate many downstream responses in plants to regulate their development and growth under saline conditions. *In vitro* experiments were conducted for investigating the ameliorating effects of calcium under saline conditions in date palm (*Phoenix dactylifera* L) cultivar, Khalas. The plantlets were subjected to NaCl stress (0, 100 and 200mM) in combination with $CaCl_2$ (0, 5 and 10mM). Ionic concentrations of essential ions and growth characteristics were investigated. The number of leaves were improved with the $CaCl_2$ supplementation significantly. Similarly, the 10mM Ca^{2+} significantly enhanced the leaf dry weight. With increasing NaCl levels, the dry weight was affected significantly with the decreasing ionic ratios. However, the supplementation of $CaCl_2$ considerably improved these ionic ratios. With an increase in salinity, Na^+, K^+, Ca^{2+} and Mg^{2+} increased significantly, while K^+/Na^+ ratio were decreased with increasing salt levels. However, the $CaCl_2$ supplementation significantly improved K^+/Na^+ ratios. The transcript expression of *NHX1* and *HA1* genes was also investigated. The expression of *NHX1* and *HA1* was increased with increasing NaCl however, the addition of $CaCl_2$ remarkably reduced the expression of both genes. The expression of *NHX1* was more prominent in roots than shoots.

Keywords: Salt stress, date palm, Calcium, NaCl, *NHX1*, *HA1*, RT-PCR

Introduction

Excess amount of salts in the cultivated soils is one of the major abiotic constraints, limiting sustainable crop productivity (Safdar et al., 2019). Although it is difficult to estimate the crop losses exactly, ~950 Mha of surface land and 230 Mha of cultivated land have been affected directly or indirectly by soil salinity (Ruan et al., 2011). The growth of the affected plants has been adversely affected due to disturbance in the water uptake, which leads to an osmotic and ionic imbalance in plants (Munns and Gilliham, 2015). The deficiency of available water in the soil ultimately causes secondary effects like oxidative stress and eliciting Na^+ toxicity in plants (Flowers and Colmer, 2015). The excessive accumulation of Na^+ in the cellular compartments is deleterious to cellular metabolism. It also disturbs calcium (Ca^{2+}) ionic balance in the apoplast and cytosol (Larbi et al., 2020). Moreover, at higher salinity, the Na^+ cations also compete with intracellular K^+ for their

influx across the cells (Shabala, 2013). The ability of crop plants to detect and respond appropriately to these ionic modifications is a pre-requisite to their long-term survival under a harsh saline environment (Deinlein et al., 2014). Consequently, various plants have adopted diverse mechanisms to cope with the excessive salts in their surrounding environment (Munns and Gilliham, 2015). The accumulation of proline contents in the cellular compartments is a counter-defense strategy, which helps to adjust the osmotic balance, scavenges free radicals and stabilizes the extracellular structures during abiotic stresses (de Freitas et al., 2019). Calcium is ubiquitously an established secondary messenger and positive plant growth regulator in plants under abiotic and biotic stresses (Larbi et al., 2020). Furthermore, Ca^{2+} also maintain the apoplastic acidification by regulating H^+ effluxes and H^+-ATPase pump in the cell wall during abiotic stress (Yang et al., 2019). During extreme salinity, Na^+ may replace the existing Ca^{2+} in the cellular membranes; however, the salt-resistant plant species always maintain a low Na^+/Ca^{2+} and Na^+/K^+ ratios in the cytosol (Wang et al., 2018). Ultimately, the addition of nutrients such as Ca^{2+} and/or K^+ may also help to ameliorate the adverse effects of salinity (Larbi et al., 2020; Morgan et al., 2014). The role of Ca^{2+} as an ameliorating agent to mitigate the adverse effects of high salinity is well-established in many crop and plant species such as olive, rice, sesame, tomato, *Salicornia persica* and many others (Larbi et al., 2020; Roy et al., 2019; Souguir et al., 2019; Tanveer et al., 2020). Besides various suggested mechanisms of ionic fluxes, the exact phenomenon that how plants maintain low Na^+/Ca^{2+} and Na^+/K^+ ratios by preventing the accumulation of excessive Na^+ flux in the cytosol is still ambiguous (Morgan et al., 2014).

There are various types of genes expressed during salt stress, namely, "single-function genes" and "regulatory genes" (Agarwal et al., 2013). The single-function genes are responsible for producing osmolytes, transporter proteins, antioxidants, polyamines and lipid metabolites and others (Shao et al., 2008). Whereas, the regulatory genes regulate the expression of salt-stress tolerant genes (Agarwal and Jha, 2010). Such genes are directly or indirectly involved in different abiotic stress tolerance pathways (Isayenkov, 2019). Among the other general responses from the host plants, restricted Na^+ entry into the cells, Na^+ efflux from the cell and compartmentalization of Na^+ into the vacuole are the most important (Moshaei et al., 2014). Various antiporter and symporters (i.e. Na^+/H^+ antiporters embedded in plasma and vacuolar membranes) or cellular channels mediate these ionic fluxes. Plasma membrane (PM) H^+-ATPase and H^+-PPiase pumps jointly administer the active transport of Na^+ across the cytosol (Fan et al., 2018). The cellular survival under adverse saline conditions largely depends upon the activity of the plasma membrane sub-unit H^+-ATPase gene *HA1*. It has been known for active phosphate transport and maintain Na^+ homeostatis during salt stress in plant cells (Yao et al., 2020). Several Na^+/H^+-antiporter genes have been characterized from various glycophytes (El Mahi et al., 2019) and halophytes plants (Moshaei et al., 2014; Tiwari et al., 2019). A Na^+/H^+ antiporter *salt overlay sensitive* (SOS1) gene is involved in Na^+ extrusion and transport through the root meristem. Another member of the Na^+/H^+ family is the vacuolar Na^+/H^+ antiporter (*NHX1*) *NHX1*gene, which is upregulated at higher salinity in plants (Toranj et al., 2020). The *NHX1* is a Na^+/H^+ exchanger responsible for Na^+ sequestering from the cytosol into vacuoles, thereby inducing high salt tolerance in plants (Ahmad et al., 2020). The transgenic plants overexpressing *SOS1* and *NHX1* genes showed better salt tolerance with decreased Na^+ accumulation (Fan et al., 2018; Nguyen et al., 2019).

The date palm, (*Phoenix dactylifera*) L., is one of the most important crops of the oasis ecosystem in the Kingdom of Saudi Arabia. Date palm is an extremophilic plant and moderately tolerant to extreme abiotic stresses (Yaish and Kumar, 2015). It can adapt to the gradual increase in soil salinity (Al-Khateeb et al., 2019). However, prolonged exposure to extreme conditions can directly affect the quality and productivity of fruits and thus, it necessitates exploring the stress-responsive traits and mechanisms (Patankar et al., 2019). During this study, we tried to investigate the effect of Ca^{2+} supplementation on the response of date palm cultivar 'Khalas' to salinity. The aim was to investigate how additional Ca^{2+} supplies affect various stress-related genes and ionic concentrations in date palm under salinity.

Materials And Methods

These experiments were conducted at the Department of Agricultural Biotechnology, College of Agriculture and Food Science, King Faisal University, Saudi Arabia.

Preparation Of Plant Material

The explants from offshoots of date palm cultivar 'Khalas' were used to raise embryogenic calli for culturing as described by Al-Khateeb and Al-Khateeb (2015). The shoot tips were kept in an anti-oxidant solution before sterilization (Al-Khateeb, 2006). The explants were then cultured on the basal Murashige & Skoog (MS) media (Murashige and Skoog, 1962). The standard quantities of vitamins and other supplements were used as per requirement.

In Vitro Salt Treatment Of The Plantlets

Plantlets of the date palm cultivar, Khalas, were cultured in MS basal medium supplemented with 0, 100 and 200 mM sodium chloride and 0, 5 and 10 mM $CaCl_2$. A total of 9 treatments of both NaCl and $CaCl_2$ were used. The treatment combinations were replicated three times in a factorial completely randomized design.

The plantlets were grown in the MS medium with various salt treatments for eight (8) weeks. After treatment, the stressed plants were harvested. Shoots (leaves) and roots were separated, and fresh weights were recorded. The plantlets were washed two times with dH_2O. For removing the ions from the spaces of roots, these plantlets were washed with an isotonic solution of sorbitol for two minutes. The dry weight of roots and shoots was calculated after oven-drying them at 85°C for 48 hour (h).

Ion Determination

To analyze and quantify the potassium (K^+), sodium (Na^+), calcium(Ca^{2+}) and magnesium (Mg^{2+}), ~500 mg of freshly harvested leaves and roots from salt-treated and control treatment was used. The samples were homogenized with pestle and mortar and extracted in 25 ml of de-ionized water at 90°C for 4h. The Na^+ and K^+ contents were analyzed with a flame photometer (PFP7, Jenway, United Kingdom) while, Ca^{2+} and Mg^{2+} concentrations were determined with a GBS 905 atomic absorption spectrophotometer as described by Al-Khateeb et al. (2020).

RNA extraction and cDNA synthesis

Total RNA was isolated from the leaves using TRIZOL reagent (Invitrogen Inc., CA, USA) following the manufacturer's instructions. The purified RNA samples were then treated with DNaseI (ThermoFisher Scientific) to remove any traces of DNA in the samples. The integrity and quality of RNA were assessed by agarose gel electrophoresis. First-strand cDNA was synthesized using 1-2 µg of the total isolated RNA using a cDNA synthesis kit and subsequently used for further amplification (ThermoScientific).

Quantitative Reverse Transcription-Pcr (Qrt-Pcr) Amplification

The gene-specific primers were used to quantify *NHX1* and *HA1* genes by real-time PCR (RT-PCR) from date palm (Supplementary Table 1). The RT-PCR quantifications were performed in the CFX Connect™ RT-PCR Detection System (BIO-RAD) using the SYBR Green Master Mix kit (ThermoFisher Scientific). The PCR profile for amplification was: initial denaturation for 5 min at 95°C followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The melting curve of the primers was recorded and analyzed to check for any non-specific amplicons or presence of primer dimers. Each run included negative reaction control and 18S rRNA as the housekeeping gene to normalize the data. All the samples were analyzed with three experimental replicates and the data was averaged. The standard errors of the mean values were also recorded.

Statistical Analysis

All the obtained data were subjected to randomized complete block factorial design for analysis using Analysis of variance (Gomez and Gomez, 1984). For separating the treatment means, the least significant difference model at 5 percent probability was used. For all the analyses, the SAS Software Package (SAS, 2011) was used.

Results

In the present investigations, the effects of NaCl and $CaCl_2$ in different concentrations and combinations on growth and ionic concentrations were assessed. The data on the plant's growth attributes is presented in Table 1.

Effect Of Salinity On Growth Characters

During this study, the increasing salt concentrations progressively reduced the overall plant growth. The number of leaves were decreased significantly with increasing salt levels with little or no ameliorating effects of calcium (Table 1). The leaf dry weight was initially decreased with increasing salt levels; however, the addition of $CaCl_2$ to the medium improved the leaf dry weight compared to the control plants. On average, there was a 15% and 32% decrease in leaf dry weight under 100 and 200 mM NaCl treatments, respectively. When the medium was supplemented with 5 and 10 mM $CaCl_2$, only a 5% decrease was observed in leaf dry weight and no decrease was observed with 10 mM $CaCl_2$ under 100 mM NaCl stress. Similarly, this reduction declined to 7 and 14% compared with a 32% decrease with the same concentrations of $CaCl_2$ under the 200 mM NaCl stress. While the effect of salinity was more pronounced in roots and a decrease of 24% and 41% were recorded under the 100 and 200 mM salt stress, respectively. The addition of $CaCl_2$ improved the root dry weight at 100 mM NaCl; however, at higher salinity at 200 mM, no ameliorating effect of the $CaCl_2$ was observed. The data indicated that roots were more prone to increasing salt levels compared to leaves, hence were most affected. The total dry weight followed a similar trend where the addition of $CaCl_2$ improved the growth at 100mM NaCl as compared to 200mM NaCl concentrations. Total plant dry weight declined to 23 and 32% under 100 and 200 mM salt concentrations. However, with the application of 5 and 10 mM of $CaCl_2$ at 100 mM NaCl, the total plant dry weight reduction was 10 and 2%, respectively. Whereas, under 200 mM NaCl caused a reduction of total plant dry weight from 23% to 15 and 35% to 24% upon supplementing 5 mM and 10 mM $CaCl_2$, respectively.

The dry weight root/shoot ratio followed a similar pattern due to the ameliorating effect of calcium. However, at high salt levels (200 mM) there were no significant effects on the root/shoot ratio than lower salt treatments (Table 1). The ameliorating effect of Ca^{2+} was more pronounced at 100 mM salt levels compared to 200 mM, which resulted in a high total dry weight root/shoot ratio.

The Effect Of Salinity On Ionic Concentrations

The concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} were determined in leaves and roots of date palm plantlets under the NaCl induced stress. As expected, the leaves and root Na^+ concentrations were increased significantly with increasing salt stress (Figure 1). This increase was more pronounced in roots compared to leaves. However, with the supplementation of Ca^{2+}, the Na^+ concentrations decreased significantly in the treated plants, particularly under high salt stress levels of 200 mM NaCl (Figure 1). No significant ameliorating differences were observed between 5 and 10mM $CaCl_2$ at the 100 mM NaCl stress. However, supplementation of 10mM $CaCl_2$ produced superior effects to ameliorate Na^+ accumulation as compared to 5mM Ca^{2+} at 200mM NaCl. The K^+/Na^+ ionic ratios were also reduced under increasing salt stress. However, supplementation of 5 and 10 mM $CaCl_2$ improved the K^+/Na^+ ratios of leaves and roots (Table 2; Figure 1).

The K^+ concentrations were also determined in leaves and roots under the NaCl stress (Figure 2). The controlled plants maintained a low accumulation of K^+ in the root and leaves at 100 and 200 mM NaCl, re-

Table 1. Effect of different combinations and concentrations of NaCl and $CaCl_2$ stress on growth characteristics of date palm cultivar, Khalas.

NaCl (mM)	$CaCl_2$ (mM)	Number of leaves	Leaf Dry wt (g)	Root dry wt (g)	Total Dry wt (g)	Dry weight Root/shoot ratio
00	00	3.75BC*	1.01A	0.17A	1.17AB	0.18
00	05	5.00AB	1.03A	0.14AB	1.16AB	0.14
00	10	6.25A	1.09A	0.15AB	1.24A	0.14
100	00	3.00C	0.86AB	0.04EF	0.90BC	0.06
100	05	3.00C	0.96AB	0.09CD	1.05ABC	0.09
100	10	3.00C	1.02A	0.13BC	1.15AB	0.13
200	00	3.00C	0.68B	0.07DE	0.75C	0.10
200	05	3.75BC	0.94AB	0.05EF	0.99ABC	0.05
200	10	3.00C	0.86AB	0.03F	0.88BC	0.03

**Values followed by the same letter in a column do not differ significantly at 5% probability level according to Duncan's Multiple Range Test.*

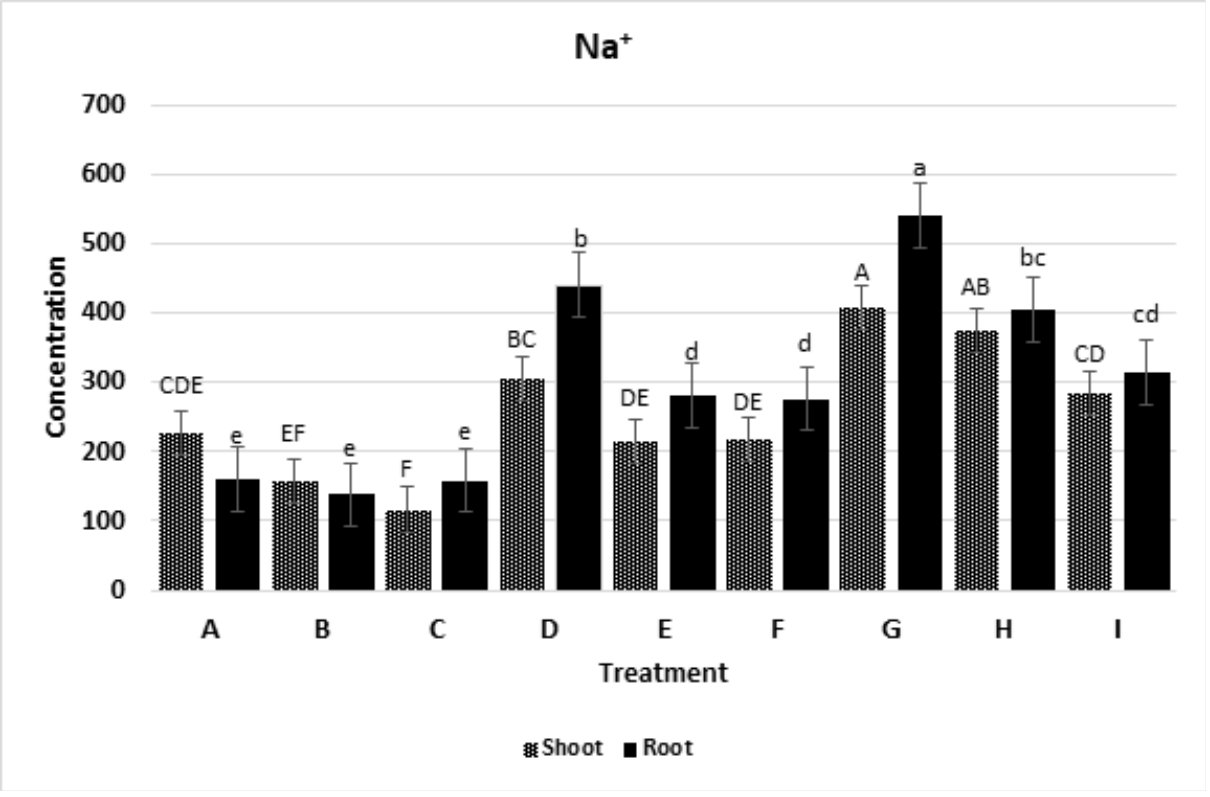


Figure 1. Effect of NaCl + CaC_{12} on Na^+ concentration (µmol.g-1 DW) in shoots and roots of date palm cultivar, Khalas**.** Bars= 0.05 A**:** (Na00**:**Ca0**),** B**: (**Na00**:**Ca05), C**:** (Na00**:**Ca10**),** D**:** (Na100**:**Ca00**),** E**: (**Na100:Ca05), F: (Na100**:**Ca10), G**: (**Na200:Ca00**),** H: (Na200:Ca05), I: (Na200:Ca10).

**Different letters over the bars show significant differences among treatment (p <0.01) separated by Duncan's Multiple Range Test. Error bar indicates means ± SE.*

spectively (Figure 2). However, the supplementation of 5 and 10 mM $CaCl_2$ improved the K^+ accumulation at 100mM and 200mM NaCl stress, respectively, compared to the control plants.

The Ca^{2+} concentration was determined in the shoots and roots under NaCl stress (Figure 2). The Ca^{2+} concentrations in the root were very low and did not change significantly in the control and salt-stressed plants. Supplementation of $CaCl_2$ substantially enhanced the concentration of Ca^{2+} in roots and shoots of both the control and the salt-stressed plants. This increase was more pronounced at higher NaCl levels (200 mM) supplemented with 5 and 10 mM $CaCl_2$. The accumulation of Ca^{2+} was higher in the leaves than roots at 100 mM NaCl supplemented with 5 and 10 mM $CaCl_2$. However, the supplementation of 5 and 10mM $CaCl_2$ produced similar results at 200 mM NaCl salt stress in both leaves and roots. Thus, at 200 mM NaCl, the accumulation of Ca^{2+} was highly significant with the supplementation of 5 and 10 mM $CaCl_2$ compared to the control plants.

The effects of salt stress on the concentrations of Mg^{2+} ions were also determined (Figure 4). No significant differences were observed in the leaf Mg^{2+} concentrations under all the salt treatments. The supplementation of $CaCl_2$ also produced similar results, and the plants maintained almost the same Mg^{2+} status at 100mM NaCl. However, the supplementation of 5 and 10 mM $CaCl_2$ significantly affected the accumulation of Mg^{2+} in the roots of the salt treated plants at 100 and 200 mM NaCl, respectively (Figure 4).

The ionic ratios of different ions were calculated for roots and shoots (Table 2). The overall K^+/Na^+ and Ca^{2+}/Na^+ ratios showed a similar trend. With an increase in salt stress levels, the K^+/Na^+ ratio decreased significantly compared to the control plants. However, the supplementation with 5 and 10mM $CaCl_2$ significantly improved the K^+/Na^+ ratio under 100 and 200mM NaCl, respectively. The maximum increase in the K^+/Na^+ ratio was recorded as 3.36 at 10 mM $CaCl_2$ compared with 5 mM concentrations. A similar trend was observed in the Ca^{2+}/Na^+ ratio with increasing salinity. However, with the supplementation of $CaCl_2$ to the growth medium, both in roots and shoots, these ratios improved significantly. Contrary to it, there was no significant improvement in the leaf and root K^+/Ca^{2+} ratios in all the $CaCl_2$ treated plants as compared to control (Table 2).

Expression of *NHX1* and *HA1* genes under salt stress

The transcript expression level of vacuolar *NHX1* and plasma membrane *HA1* genes were analyzed in the shoot and root tissues of the date palm cultivar 'Khalas'. The expression pattern of these genes was normalized using 18S-rRNA as an internal control.

Table 2. Effect of different combinations and concentrations of NaCl and $CaCl_2$ on the ionic ratios in date palm cultivar, Khalas.

Treatment		K^+/Na^+ ratio		Ca^{2+}/Na^+ ratio		K^+/Ca^{2+} ratio	
NaCl (mM)	CaC_{l2} (mM)	Leaf	Root	Leaf	Root	Leaf	Root
00	00	1.24	1.68	0.51	0.68	2.42	2.45
00	05	2.25	2.14	1.16	2.55	1.94	0.84
00	10	3.36	2.58	1.75	2.30	1.97	1.12
100	00	0.73	0.49	0.23	0.18	3.24	2.69
100	05	1.16	0.99	1.13	0.56	1.02	1.77
100	10	1.29	1.08	1.14	0.80	1.13	1.34
200	00	0.45	0.34	0.14	0.26	3.11	1.31
200	05	0.61	0.63	0.85	0.78	0.71	0.79
200	10	0.98	0.90	1.13	1.01	0.87	0.89

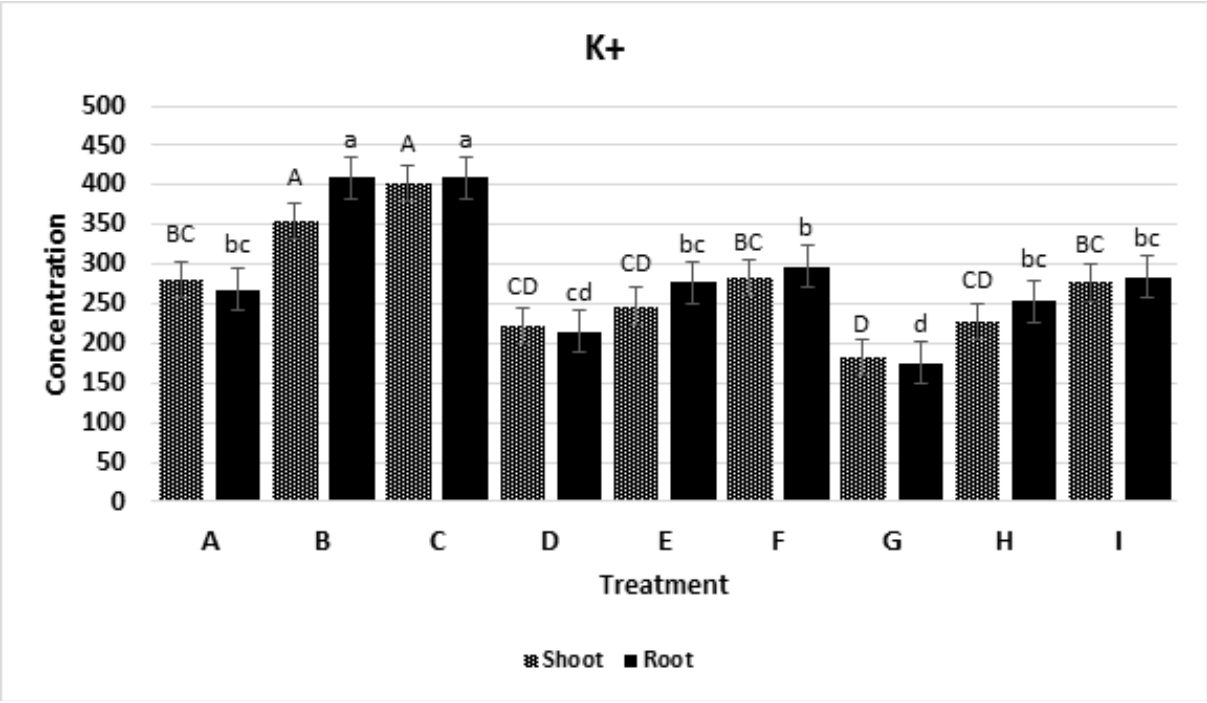


Figure 2. Effect of NaCl + $CaCl_2$ on K^+ concentrations (µmol.g-1 DW) in shoots and roots of date palm cultivar, Khalas. Bars= 0.05.

A: (Na00:Ca0), B: (Na00:Ca05), C: (Na00:Ca10), D: (Na100:Ca00), E: (Na100:Ca05),

(F; Na100:Ca10), G: (Na200:Ca00), H: (Na200:Ca05), I: (Na200:Ca10).

**Different letters over the bars show significant differences among treatment (p <0.01) separated by Duncan's Multiple Range Test. Error bar indicates means ± SE.*

The results showed that both gene transcripts were induced in the plants' root and shoot tissues under NaCl treatment. However, the *NHX1* transcript level was very low in shoots as compared to root tissues. In root tissues, the expression of *NHX1* was increasing with an increase in NaCl treatment (Figure 5). In the plants treated with 100 and 200 mM NaCl, the supplementation of 5 mM and 10 mM $CaCl_2$ reduced the expression of *NHX1* even at high NaCl concentrations. However, in shoots, the transcript expression levels of *NHX1* were not significant as compared to roots. Supplementation of the plants with 10 mM $CaCl_2$ produced the best results even at 100 and 200 mM NaCl levels in the root tissues.

The *HA1* transcripts were almost similarly induced in both shoot and root tissues. The reduction in the expression pattern was remarkably significant in the plants treated with NaCl and supplemented with $CaCl_2$. The transcripts of *HA1* significantly reduced the shoots and roots of the plants treated with 100 and 200 mM NaCl and supplemented with 10mM $CaCl_2$. A similar pattern was observed in both shoot and root tissues.

Discussion

Salinity induces adverse effects on overall plant growth and causes a nutrient imbalance in plants (Ashraf et al., 2020; Larbi et al., 2020). The plants respond to high soil salinity through various biochemical and physiological changes to alleviate the deleterious effects of salt stress. These responses may include the up-regulation of salt-responsive traits (Liu et al., 2019) and/or salt-adaptation mechanism (Al-Khateeb et al., 2019). During this study, the plant growth characteristics of date palm cultivar 'Khalas' were significantly affected at 100 and 200mM NaCl compared to

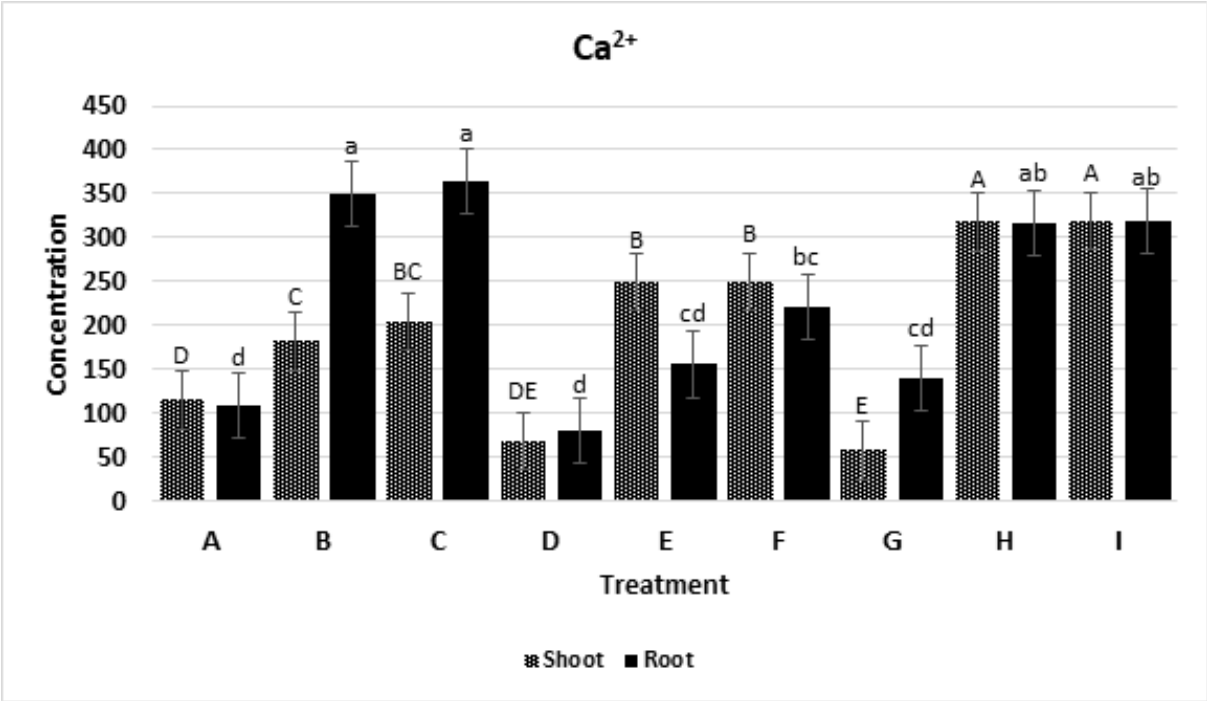


Figure 3. Effect of NaCl + $CaCl_2$ on Ca^{2+} concentration (µmol.g-1 DW) in shoots and roots of date palm cultivar, Khalas. Bars= 0.05

A: (Na00:Ca0), B: (Na00:Ca05), C: (Na00:Ca10), D: (Na100:Ca00), E: (Na100:Ca05),

F; Na100:Ca10), G: (Na200:Ca00), H: (Na200:Ca05), I: (Na200:Ca10)

**Different letters over the bars show significant differences among treatment (P <0.01) separated by Duncan's Multiple Range Test. Error bar indicates means ± SE.*

the control plants. The shoot, root and the total dry weights were decreased significantly with increasing salt concentrations. Similar reductions in the plant growth characteristics were observed in soybean (Arshi et al., 2010) and alfalfa (Al-Khateeb, 2006) under induced NaCl stress.

The supplementation of Ca^{2+} has been proved to play a significant role in mitigating the adverse effects of salt stress by promoting photosynthesis and biomass production (Ashraf et al., 2020). Han et al. (2019) observed that the addition of 10 mM $CaCl_2$ significantly improved the plant growth characteristics and adjusted the ionic imbalance by ameliorating Na^+ and enhancing Ca^{2+}, Mg^{2+} and K^+ in millet. Similarly, the plant growth, cellular nutrients, water uptake and ionic status of olive plants were improved with 10 mM $CaCl_2$ in the salt-stressed plants (Larbi et al., 2020). In our study, we also found that the supplementation of Ca^{2+} was very effective in reverting the toxicity of alleviated NaCl. The improvement in plant biomass due to supplementation of $CaCl_2$ under salt stress has also been reported from senna (Arshi et al., 2010), tomato (Ahmad et al., 2018), sesame (Souguir et al., 2019), rice (Roy et al., 2019) and many other crops.

The previous studies demonstrated that the roots store the excessive Na^+ and protect the above-ground parts from the deleterious effects (Sarabia et al., 2019; Mahajan et al., 2020). The present study corroborates these findings and found that the Na^+ accumulation was higher in roots than in shoots (Figure 1). Thus, the effect of NaCl was more pronounced in roots than in leaves with a higher accumulation of Na^+ ions and ultimately decreased K^+/Na^+ ratios. The accumulation of Na^+ has been shown as positively correlated with the increasing salinity in *Aeluropus littoralis* plants, which tend to excrete the excessive Na^+ to protect them from the drastic effects (Barhomi et al., 2007). Possibly, date palm uses this mechanism through their roots, which is supported by the accumulation of Na^+ in the roots as a start point to get rid of excessive salts. Our results

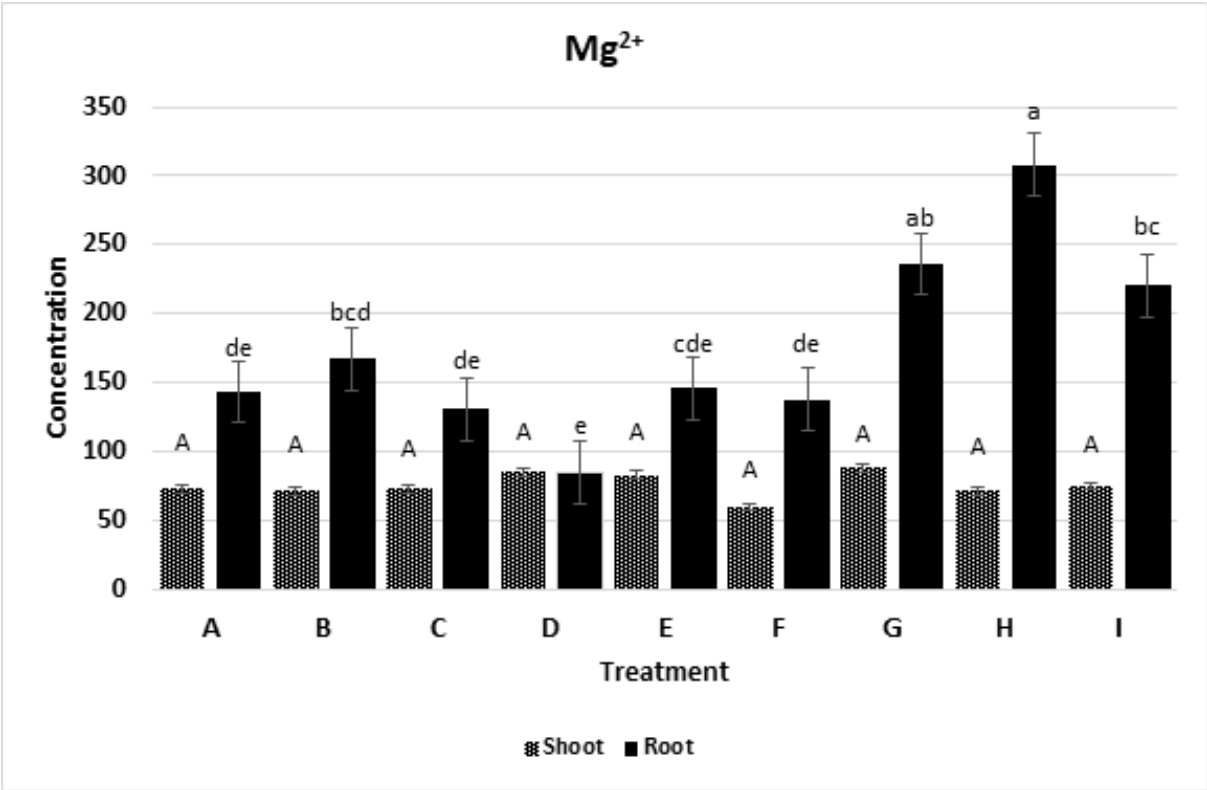


Figure 4. Effect of NaCl + $CaCl_2$ on Mg^{2+} concentrations (µmol.g-1 DW) in shoots and roots of date palm cultivar, Khalas. Bars= 0.05

A: (Na00:Ca0), B: (Na00:Ca05), C: (Na00:Ca10), D: (Na100:Ca00), E: (Na100:Ca05), (F; Na100:Ca10), G: (Na200:Ca00), H: (Na200:Ca05), I: (Na 200: Ca10).

**Different letters over the bars show significant differences among treatment (p<0.01) separated by Duncan's Multiple Range Test. Error bars indicates means ± SE.*

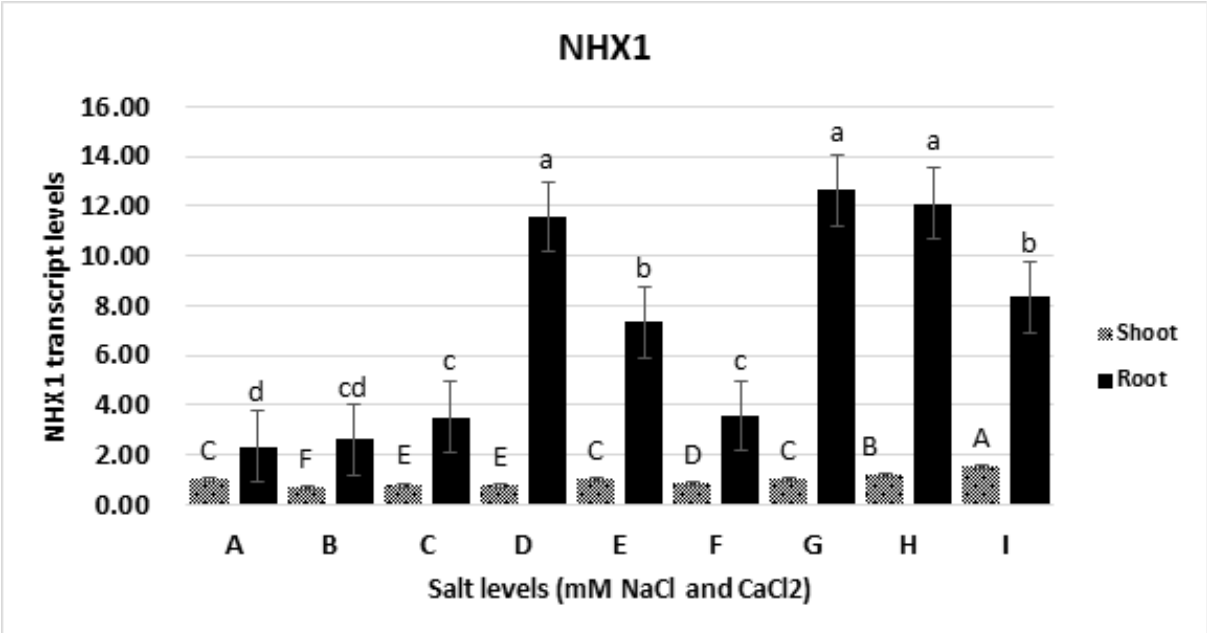
indicated that the accumulation of Na+ ions decreased with the addition of $CaCl_2$, the K^+/Na^+ ratios were improved at 100 and 200 mM NaCl (Table 2; Figure 1). Contrarily, the increase in salt concentrations negatively affected the accumulation of Ca^{2+} ions (Table 2). The accumulation of Ca^{2+} was, however improved with the supplementation of $CaCl_2$ even at higher salt levels. Ultimately, the Ca^{2+}/Na^+ ratios were also improved significantly in both leaves and root tissues (Table 2). The high Ca^{2+}/Na^+ ratio under the supplementation of $CaCl_2$ could be attributed to an increase in Ca^{2+} and a decrease in Na^+ concentration in both shoot and roots. Thus, the key aspect of salt tolerance in plants is the ionic homeostasis i.e. removal or compartmentalization of toxic ions and accumulation of essential ions (such as Ca^{2+}, Mg^{2+} and K^+). Thereby, the salt-adaptive or salt-resistant plants accumulate their excessive Na^+ ions in the roots and prevent their translocation to the leaves (Munns and Gilliham, 2015).

Plant cells translocate the excessive Na^+ into the apoplast through H^+-ATPase and Na^+/H^+ antiporters and thus, modulate the ionic homeostasis in the saline environment (Assaha et al., 2017). The up-regulation of *NHX1* gene has been reported in *Sesuviium portulacastrum* (Nikalje et al., 2018), *A. littoralis* (Moshaei et al., 2014), *A. marina* (Chen et al., 2010) and many other plants species. The expression of *NHX1* and *HA1* Na^+ ion transporter gene transcripts was substantially upregulated in our experiments. The overall response of roots was stronger at higher salinity during our experiments. Higher transcript levels of *NHX1* were also observed in the roots during this study. This indicates that Na^+ concentration in date palm tissues is regulated in roots rather than shoot, which is also sup-

ported by the inability of Ca^{2+} to be re-translocated in the plant tissues. Similar results have been observed in *Aleuropus littoralis* (Moshaei et al., 2014), *Arabidopsis thaliana* (Yokoi et al., 2002) and rice (Fukuda et al., 2011). The higher expression of *NHX1* in the roots has been explained by a higher accumulation of Na^+ ions in the root tissue of the date palm cultivar 'Khalas' in our experiment (Figures 1 and 5). Alternatively, this high response of both gene transcripts may be due to a high level of Na^+ concentration in the growing media. However, the magnitude of the *NHX1* response seems more related to the increase in Na^+ accumulation in the plant tissues than the elevated level of Na^+ in the growing media. Thus, the accumulation of Na^+ ions and the transcript level of *NHX1* might be correlated to each other. Moreover, when the plants were supplemented with $CaCl_2$, the expression of *NHX1* was also reduced significantly even at higher NaCl stress in roots. This response could attribute the role of *NHX1* in reducing the accumulation of Na^+ in root and shoot tissues.

The upregulation of vacuolar antiporter *NHX1* and plasma membrane related H^+-ATPase *HA1* genes indicated the possible induction of Na^+/H^+ antiporter activity. It is observed that the coordinated overexpression of H^+-ATPase and the Na^+/H^+ antiporter may administer the salt tolerance in plants (Jaarsma and de Boer, 2018; Moshaei et al., 2014). The supplementation of Ca^{2+} has been shown to regulate the ionic channels in the plasma membrane and modulate Na^+/K^+ transport and H-ATPase activity in the salt-stressed plants (Chen et al., 2017). Hence, the downregulation of both these genes might explain the ameliorating effects of Ca^{2+} supplementation in the date palm cultivar 'Khalas'.

The results in our study indicated that the exogenous application of $CaCl_2$ successfully mitigates the adverse effects of salinity in date palm. All the growth parameters showed a positive response even in higher NaCl levels. The ionic status of Ca^{2+}, K^+ and Mg^{2+} in the roots and shoots confirmed that Ca^{2+} supplementation inhibited the translocation of Na^+ from roots into the foliar parts in date palm. The supplementation of 10 mM $CaCl_2$ was an optimum dose for all parameters studied. Thus, it may be concluded that it is very important to select an appropriate fertilizer dosage when applied to plants under high salinity stress. A higher $CaCl_2$ supplementation might not be very cost-effective to improve date palm plants' nutritional and physiological status under saline conditions.



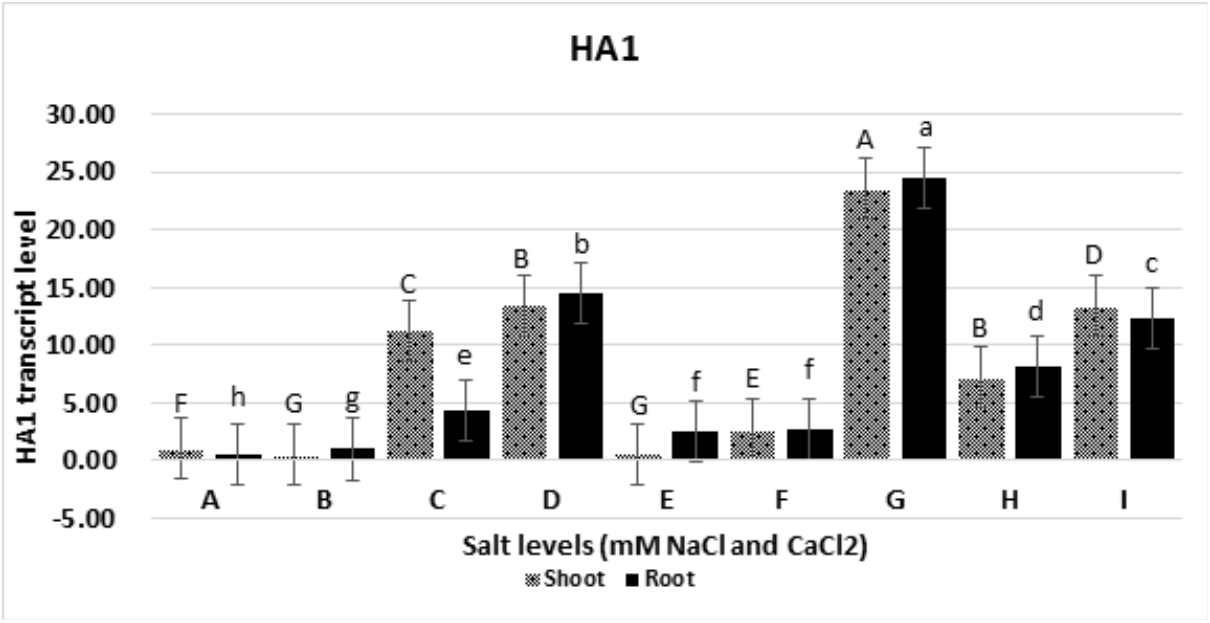


Figure 5. The relative expression of *NHX1* and *HA1* in the shoots and roots of date palm cultivar Khalas under different concentrations of NaCl and $CaCl_2$. A: (Na00:Ca0), B: (Na00:Ca05), C: (Na00:Ca10), D: (Na100:Ca00), E: (Na100:Ca05), (F; Na100:Ca10), G: (Na200:Ca00), H: (Na200:Ca05), I: (Na 200: Ca10). The data was normalized using 18s-rRNA as an internal control. Different letters over the bars show significant differences among treatment ($P < 0.01$) separated by Duncan's Multiple Range Test. Error bar indicates means + SE.

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Disclosure Statement

The authors declare no conflict of interests.

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