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Full Length Article

Effects of Exogenous $CaCl_2$ and Calcium Inhibitors on the Reactive Oxygen Species Metabolism and Ca^{2+} Transport of Tamina (*Vitis vinifera*) Grapevines under NaCl Stress

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Abstract

Soil salinity affects the growth and development of grapevines; however exogenous calcium application can mitigate the growth inhibition effects of salt stress to a certain extent. Therefore, this study was conducted to evaluate the effects of exogenous $CaCl_2$ and inhibitors {$LaCl_3$, CPZ (chlorpromazine), heparin, and EB (eosin)} on sand–cultured annual self–rooted Tamina grape seedlings in the presence of 200 mmol L^{-1} NaCl. Results showed that salt stress inhibited the shoot length and total biomass of plants by 14.1 and 17.5%, respectively compared with control plants. Salt stress obviously increased the electrolyte leakage of roots and leaves and O_2^- production rate and H_2O_2 contents in grapevine leaves while it decreased the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) after ten days of treatment. In the NaCl + $CaCl_2$ treatment group, not only electrolyte leakage of roots and leaves was decreased but O_2^- production rate and H_2O_2 contents in the leaves were also decreased. The activity of antioxidant enzymes was greater in NaCl + $CaCl_2$ treatment group than NaCl treatment group. $CaCl_2$ + inhibitor treatment decreased the activity of POD, APX and GR compared with their activity in $CaCl_2$ treatment under NaCl stress, but $CaCl_2$ + inhibitor treatment increased the H_2O_2 content in the leaves and the electrolyte leakage of the roots and leaves. Ca^{2+} showed influx transport in grapevine roots, but the net flow rate was significantly lower than control after one day of 200 mmol L^{-1} NaCl treatment. The net flow rate in $CaCl_2$ treatment recovered to the level of control, and in $CaCl_2$ + $LaCl_3$ treatment was lower than $CaCl_2$ treatment. In conclusion, exogenous $CaCl_2$ improved the salinity tolerance of grapevines by increasing the activities of antioxidant enzymes, and it also affected Ca^{2+} transport through both the Ca^{2+} channel and Ca^{2+} external flow system. © 2019 Friends Science Publishers

Keywords: Grapevine; Saline stress; Ca^{2+}; Calcium inhibitors; ROS

Introduction

Grapes (*Vitis vinifera* L.) are economically one of the most important fruit crops. Salinity is one of the most common environmental stresses that affect grapevine growth and development. Salinity stress can limit vine growth, photosynthesis, productivity, fruit quality and yield (Walker *et al*., 1981; Shani *et al*., 1993; Walker *et al*., 2002; Li *et al*., 2013a). The inhibition of grapevine growth and CO_2 assimilation is mainly due to changes in stomatal conductance, electron transport rate, leaf water potential, chlorophyll, fluorescence, osmotic potential and leaf ion concentrations. Salt stress can cause the formation of reactive oxygen species (ROS), membrane disorganization, metabolic toxicity and reduced nutrient acquisition, as well as the induction of several genes related to plant hormones (Cramer *et al*., 2007; Farooq *et al*., 2015; Mohammadkhani *et al*., 2018).

Calcium (Ca) not only is one of the macronutrients necessary for plants but also plays an important role in maintaining the structure and function of cell walls and cell membranes (Guimarães *et al*., 2011). Many studies have shown that exogenous Ca^{2+} can alleviate the damage of drought (Dai *et al*., 2012; Li *et al*., 2017), salt (Khan *et al*., 2010, 2012; Yang *et al*., 2016a, b), high temperature (Tan *et al*., 2011), low temperature (Dai *et al*., 2012), hypoxia stress (Gao *et al*., 2011), herbicide stress (Erinle *et al*., 2016) and heavy metal stress (Tian *et al*., 2011; Liu *et al*., 2014a) to plants, thus improve a plant's resistance. A certain concentration of exogenous Ca^{2+} can effectively mitigate the growth inhibition effect of saline stress on *Zea mays* L. (Hamada, 1994; Maeda and Nakazawa, 2008), *Triticum aestivum* (Liu *et al*., 2014b; Nemat Alla *et al*., 2014; Tian *et al*., 2015), *Atriplex halimus* (Nedjimi and

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Daoud, 2009), *Vigna unguiculata* L. (Murillo–Amador *et al*., 2006), *Glycine max* L. (Yin *et al*., 2014), and *Solanum lycopersicum* (Tuna *et al*., 2007; Manaa *et al*., 2014). Additionally, it can improve the activity of antioxidant enzymes in seedlings of okra (Qadira *et al*., 2017), naked oat (Xu *et al*., 2008), wheat (Nemat Alla *et al*., 2014; Tian *et al*., 2015), Jerusalem artichoke (Xue *et al*., 2008), *Cakile maritima* (Amor *et al*., 2010), *Catharanthus roseus* (Jaleel *et al*., 2007) and wild jujube (Yang *et al*., 2016b) under salt stress. It can also reduce membrane lipid peroxidation and thus enhance a plant's adaptation to saline stress as well as improve salt tolerance.

However, the difference in Ca salt types (Hamada, 1994; Renault and Affifi, 2009), Ca concentrations (Tuna *et al*., 2007; Nedjimi and Daoud, 2009; Liu *et al*., 2014b), and plant cultivars (Manaa *et al*., 2014) will lead to different mitigation effects of calcium among plants facing saline stress. Previous experiments have shown that 10 mmol L^{-1} $CaCl_2$ had the greatest mitigation effect on salt stress of grape seedlings (Tan *et al*., 2018). Based on this, the present research studied the effects of $CaCl_2$ and $CaCl_2$ + calcium inhibitors {plasma membrane calcium channel blocker $LaCl_3$, calmodulin antagonist (CPZ), Ca^{2+} releasing channel inhibitor heparin, and Ca^{2+}–ATPase inhibitor eosin (EB)} on the activity of antioxidant enzymes, accumulation of reactive oxygen, and Ca^{2+} transport rate in roots. The aims of this study were to not only investigate the effects of exogenous calcium in the salt tolerance of grapevines but also lay a foundation for further studies on mitigation and regulation mechanisms and application of calcium on grapevines under saline stress.

Materials and Methods

Experimental Materials and Treatments

The experiment was conducted in the glass greenhouse at the Institute of Pomology, Shanxi Academy of Agricultural Sciences, China from March 2015 to July 2016. The experimental materials were annual self–rooted seedlings of Tamina (*Vitis vinifera.* L.) that were planted in pots with a diameter of 25 cm and a height of 35 cm and filled with sand. When the plants grew 3–4 leaves, they were irrigated with Hoagland nutrient solution. When the aboveground parts grew to ~7–8 leaves, seedlings were treated with different concentrations of NaCl, $CaCl_2$ and calcium inhibitors: CK = control (Hoagland nutrient solution); T_1 = Na^+ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); T_2 = Na^+ + 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); T_3 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); T_4 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); T_5 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.4 mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); T_6 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.2 mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and T_7 = 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$). Each treatment was replicated three times, and each replicate included 10 seedlings. To avoid salt shock in the T_1–T_6 treatments, grapevines were first treated with 50 mmol L^{-1} NaCl in Hoagland nutrient solution for 1 day, and then the NaCl concentration increased by 50 mmol L^{-1} each day up to 200 mmol L^{-1} NaCl. On the day 200 mmol L^{-1} NaCl was reached, which was defined as day 0, the grapevines were treated with the above–described different solutions. The Ca^{2+} transport of grapevine roots was measured on day 1. On 10th day, the length of new shoots was measured, and the fresh plantlets were weighed (roots were first rinsed with distilled water). The middle–node (5~7) grapevine leaves were harvested and immediately frozen in liquid N_2 and then stored at –80°C. In October 2015, the activity of antioxidant enzymes, production rate of superoxide anions, and hydrogen peroxide content were measured. Moreover, shoot length and total biomass production was also recorded.

Electrolyte Leakage of Leaves and Roots

Electrolyte leakage was used as an indicator of the membrane permeability of leaves and roots and was measured in terms of the relative conductivity of the leaked solution from the leaves and roots. The leaves and roots (0.3 g) from the different treatment groups were washed twice with distilled water. Then, they were placed in a closed glass test tube containing 20 mL of deionized water and incubated at 25°C on a rotary shaker for 24 h. After determining the electrical conductivity of the solution (CE_0), the samples were autoclaved at 120°C for 20 min, and the final electrical conductivity (CE_t) was measured after equilibration at 25°C. The electrolyte leakage was defined following the method described in Lutts *et al*. (1996).

Production Rate of Superoxide Anions and Hydrogen Peroxide (H_2O_2) Contents

The production rate of superoxide anions was measured according to Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2^- at 530 nm. Hydrogen peroxide (H_2O_2) content was measured according to Brennan and Frenkel (1977) by monitoring the A415 signal of the titanium–peroxide complex.

Measurement of Enzyme Activity

Frozen leaves (1.5 g) were ground with 9 mL of precooled extracting solution [phosphate buffer with a pH of 7.8, containing 0.1 mmol L^{-1} ethylene diamine tetra acetic acid (EDTA) and 1% polyvinyl pyrrolidone (PVP)] in an ice

bath. Then, the mixture was centrifuged for 20 min at 12,000 × g at 4°C, and the supernatant was the extracted enzyme solution. The activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method described by Giannopolitis and Ries (1977). The catalase (CAT) activity was determined by following the consumption of H_2O_2 (extinction coefficient: 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min according to Aebi (1984). The ascorbate peroxidase (APX) activity was measured by monitoring the decrease in absorbance at 290 nm (extinction coefficient: 2.8 mM^{-1} cm^{-1}) according to Nakano and Asada (1981). The peroxidase (POD) activity was assayed following the methyl catechol method (Cakmak and Marschner, 1992). The glutathione peroxidase (GR) activity was measured following the oxidation of NADPH at 340 nm (extinction coefficient: 6.2 mM^{-1} cm^{-1}) according to the method described by Schaedle and Bassham (1977).

Measurement of Ca^{2+} Transport Rate in Grapevine Roots

The Ca^{2+} transport within grapevine roots was measured using noninvasive microtest technology (NMT), and the measurement system was the noninvasive microtest system BIO–001A from Younger U.S.A., L.L.C. (Amherst, M.A., 01002, U.S.A.) with reference to the method described by Sun *et al.* (2010). White absorbing roots 1 cm away from the root tip of the treated grape seedlings were selected and placed into a culture dish and then washed three times with deionized water. Then, the seedling roots were cleaned once with the test solution (0.1 mmol L^{-1} KCl, 0.1 mmol L^{-1} $CaCl_2$, 0.1 mmol L^{-1} $MgCl_2$, 0.5 mmol L^{-1} NaCl, 0.2 mmol L^{-1} Na_2SO_4, 0.3 mmol L^{-1} MES, pH 7.0). The sampled roots were then placed into testing solution to equilibrate for 30 min. After washing the roots with testing solution, the roots were fixed in the testing solution. The measurement position was 500 μm, which was used to measure the Ca^{2+} net flow. Each measurement position was measured for 2 min, and the test ended when the flow rate became stable after 10 min, which was repeated five times.

Statistical Analysis

Each of the reported data was the mean ± standard error (S.E.) of three replicates. Statistical analyses were performed by analysis of variance (ANOVA) using S.P.S.S. version 17.0 (S.P.S.S., Chicago, U.S.A.), and comparisons between the mean values were made by the least significant difference (L.S.D.) test at a 0.05 probability level.

Results

The Electrolyte Leakage of Leaves and Roots

When compared with the control treatment, NaCl treatment significantly increased the electrolyte leakage from cells of leaves and roots of Tamina by 45.9 and 69.3%, respectively (Fig. 1A, B); however, $CaCl_2$ addition in the saline solution obviously decreased the electrolyte leakage from NaCl–treated plants. $CaCl_2$ alone only increased significantly (13.6%) the electrolyte leakage of roots compared to that for the control plants. Under NaCl stress, compared with $CaCl_2$, $CaCl_2$ + $LaCl_3$, + CPZ or + EB had no significant effect on the electrolyte leakage of roots, while $CaCl_2$ + heparin obviously increased it by 7.6%; however, there was no significant differences among the four calcium inhibitor treatments. Under NaCl stress, compared with $CaCl_2$, $CaCl_2$ + calcium inhibitors obviously increased the electrolyte leakage of leaves.

The Production Rates of Superoxide Anions and H_2O_2 Contents in Grapevine Leaves

As shown in Fig. 2A and B, compared with the control condition, the production rate of superoxide anions and H_2O_2 contents in Tamina grapevine leaves under NaCl stress increased by 30.7 and 25.1% ($p < 0.05$), respectively. Addition of $CaCl_2$ significantly reduced the production rate of superoxide anions and H_2O_2 contents in grapevine leaves under NaCl stress by 31.6 and 22.6% ($p < 0.05$), respectively, compared with those under NaCl stress. Under NaCl stress, the production rates of superoxide anions in grapevine leaves in $CaCl_2$ + inhibitor treatment groups were all significantly lower than that of grapevine leaves in the $CaCl_2$ treatment group. The H_2O_2 contents of grapevine leaves treated with $CaCl_2$ + $LaCl_3$ or + heparin had no significant difference from that of plants under NaCl stress but were 20.5% ($p < 0.05$) and 14.5% ($p > 0.05$) higher, respectively, than grape seedlings treated with $CaCl_2$. The H_2O_2 contents of grapevine leaves in the $CaCl_2$ + CPZ and $CaCl_2$ + EB treatment groups were both significantly higher than those in the other treatments and were 23.2 and 14.3% ($p < 0.05$) higher, respectively, than those in the NaCl treatment group. The production rate of superoxide anions in grapevine leaves treated only with $CaCl_2$ was 16.1% ($p < 0.05$) lower than that of leaves in the CK group, and the H_2O_2 content showed no significant difference from the CK.

The Antioxidant Enzyme Activities of Leaves in Tamina

Compared with the CK, the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) of grapevine leaves under NaCl stress all declined significantly ($p < 0.05$, Fig. 3). $CaCl_2$ additional increased the activity of SOD, CAT, POD, APX and GR by 5.4% ($p < 0.05$), 9.1% ($p < 0.05$), 90.5% ($p < 0.05$), 122.8% ($p < 0.05$), and 43.7% ($p < 0.05$) compared with those of the NaCl treatment.

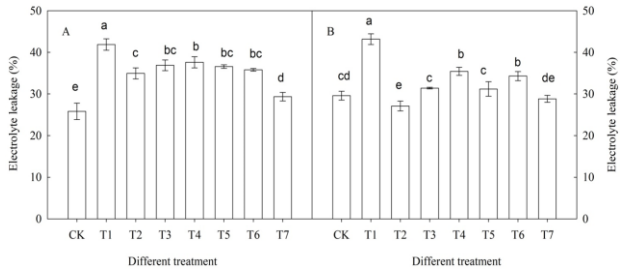

Fig. 1: Effects of $CaCl_2$ and calcium inhibitor treatments on the electrolyte leakage in the roots (**A**) and leaves (**B**) of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); $T_1 = Na^+$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); $T_2 = Na^+ + 10$ mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); $T_3 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 2$ mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); $T_4 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 2$ mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); $T_5 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 0.4$ mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); $T_6 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 0.2$ mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and $T_7 = 10$ mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$)

The data in the column followed by different small letters show significant differences between different treatments at the 0.05 level (by Fisher's LSD test)

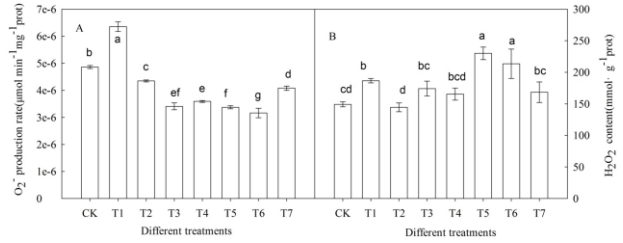

Fig. 2: Effects of $CaCl_2$ and calcium inhibitor treatments on the (**A**) O_2^- production rate and (**B**) H_2O_2 contents of Tamina grapevine leaves under NaCl stress

Here, CK = Control (Hoagland nutrient solution); $T_1 = Na^+$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); $T_2 = Na^+ + 10$ mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); $T_3 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 2$ mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); $T_4 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 2$ mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); $T_5 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 0.4$ mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); $T_6 = Na^+ + 10$ mmol L^{-1} $Ca^{2+} + 0.2$ mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and $T_7 = 10$ mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$)

The data in the column followed by different small letters show significant differences between different treatments at the 0.05 level (by Fisher's LSD test)

Under NaCl stress, the SOD activity of grapevine leaves treated with $CaCl_2$ + inhibitor EB or CPZ were 8.2% ($p < 0.05$) and 8.7% ($p < 0.05$) higher, respectively, than those treated with $CaCl_2$, the SOD activities of grapevine leaves treated with $CaCl_2$ + inhibitors were all significantly higher under saline stress, and the activities of the samples treated with only $CaCl_2$ were significantly lower than those of the control but were not significantly different from those of the NaCl treatment (Fig. 3A).

Under NaCl stress, the CAT activities of leaves treated with $CaCl_2$ + inhibitors were all significantly higher than those under NaCl stress and $CaCl_2$ treatments but were not significantly different from those of the control. The CAT activity of leaves treated only with $CaCl_2$ was significantly lower than that of the control but significantly higher than that under NaCl stress (Fig. 3B).

The POD activity of leaves in the $CaCl_2$ + $LaCl_3$ or EB treatment groups were both significantly lower than plants treated with $CaCl_2$ but had no significant difference with that under NaCl stress. The POD activities after treatment with $CaCl_2$ + heparin and $CaCl_2$ + CPZ were 13.4 and 3.5% ($p > 0.05$) lower, respectively, than in plants treated with $CaCl_2$. The difference between the POD activity of leaves treated with only $CaCl_2$ and those of the CK and NaCl + $CaCl_2$ groups was insignificant (Fig. 3C).

The APX activities of leaves under $CaCl_2$ + inhibitor treatments were all significantly lower than those treated with $CaCl_2$, and plants treated with $CaCl_2$ + $LaCl_3$ had a significantly higher APX activity than those under NaCl stress. The APX activity of plants under the heparin treatment did not differ significantly from those under NaCl stress, and the APX activities for the CPZ and EB treatments were significantly lower than those under NaCl stress. Only $CaCl_2$ treatment had no significant effects on the APX activity compared with that of the CK condition (Fig. 3D).

Under NaCl stress, the GR activities of the leaves from groups treated with $CaCl_2$ + inhibitors were all significantly lower than the GR activity of leaves in the $CaCl_2$ treatment group but did not differ significantly from the GR activity of leaves under NaCl stress. The GR activity of the $CaCl_2$ treatment group did not differ significantly from that of the CK and NaCl + $CaCl_2$ treatment groups, but it was significantly higher than that under NaCl stress (Fig. 3E).

The Growth of Tamina Grapevine

As shown in Fig. 4 (A, B), treatment for 10 days with 200 mmol L^{-1} NaCl not only inhibited the shoot growth but also reduced the total biomass of Tamina, which were 14.1 and 17.5% lower, respectively, than those of the control. The addition of $CaCl_2$ in NaCl solution increased the shoot length and total biomass of plants by 4.9% ($p > 0.05$) and 15.0% ($p < 0.05$), respectively, compared with those of the NaCl treatment group. $CaCl_2$ + calcium inhibitors had no significant effects on the shoot length compared with the NaCl and NaCl + $CaCl_2$ treatments (Fig. 4A); $CaCl_2$ + heparin or + EB obviously decreased the effects of $CaCl_2$ on the plant biomass, which were 13.5 and 11.5% lower, respectively than that in the NaCl + $CaCl_2$ treatment group (Fig. 4B). $CaCl_2$ alone had no significant effects on the grapevine growth.

The Ca^{2+} Flow Rate in Tamina Grapevine Roots

Under normal conditions, grapevine roots could absorb Ca^{2+}, while under NaCl stress, the Ca^{2+} absorption rate

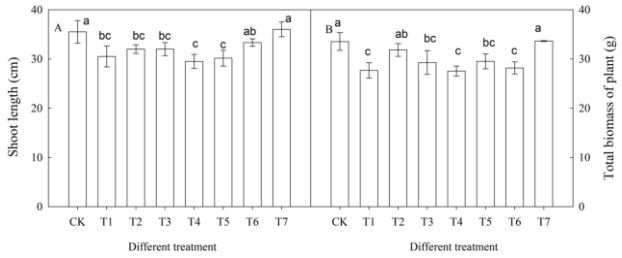

Fig. 4: Effects of $CaCl_2$ and calcium inhibitor treatments on the shoot length (**A**) and total biomass (**B**) of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T_1 = Na^+ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); T_2 = Na^+ + 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); T_3 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); T_4 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); T_5 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.4 mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); T_6 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.2 mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and T_7 = 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$)

The data in the column followed by different small letters show significant differences between different treatments at the 0.05 level (by Fisher's LSD test)

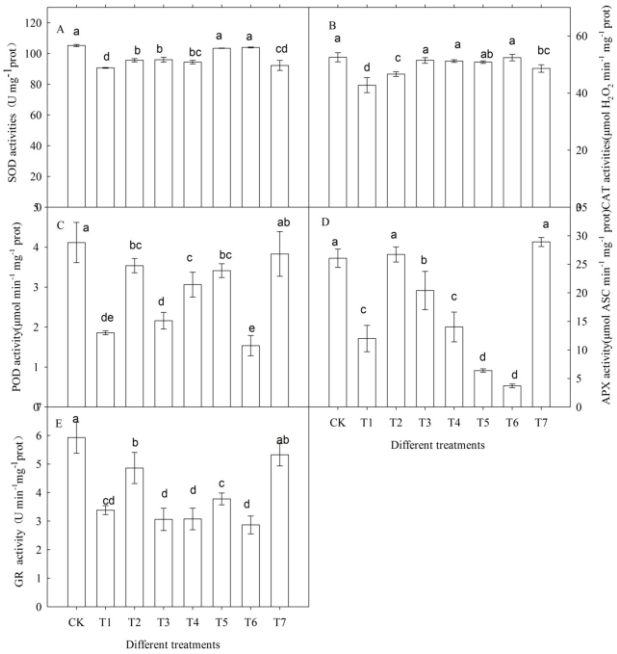

Fig. 3: Effects of $CaCl_2$ and calcium inhibitor treatments on the antioxidant enzyme activities of leaves from Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T_1 = Na^+ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); T_2 = Na^+ + 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); T_3 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); T_4 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); T_5 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.4 mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); T_6 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.2 mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and T_7 = 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$)

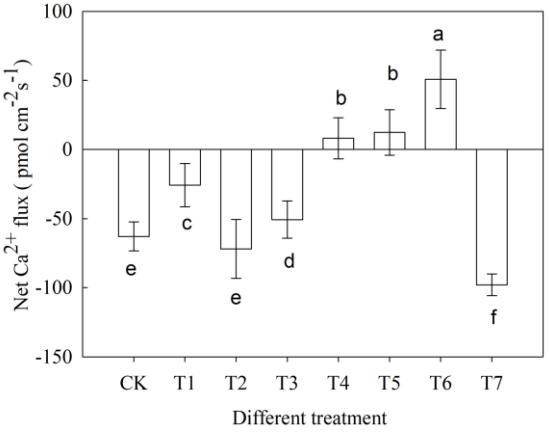

Fig. 5: Effects of $CaCl_2$ and calcium inhibitor treatments on the net Ca^{2+} flux of roots of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T_1 = Na^+ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl); T_2 = Na^+ + 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$); T_3 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} $LaCl_3$ treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} $LaCl_3$); T_4 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 2 mmol L^{-1} heparin treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 2 mmol L^{-1} heparin); T_5 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.4 mmol L^{-1} CPZ treatment (Hoagland nutrient solution +200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.4 mmol L^{-1} CPZ); T_6 = Na^+ + 10 mmol L^{-1} Ca^{2+} + 0.2 mmol L^{-1} EB treatment (Hoagland nutrient solution + 200 mmol L^{-1} NaCl + 10 mmol L^{-1} $CaCl_2$ + 0.2 mmol L^{-1} EB); and T_7 = 10 mmol L^{-1} Ca^{2+} treatment (Hoagland nutrient solution + 10 mmol L^{-1} $CaCl_2$)

The data in the column followed by different small letters show significant differences between different treatments at the 0.05 level (by Fisher's LSD test)

declined significantly ($p < 0.05$) to 41.1% of that of the CK treatment (Fig. 5). The addition of $CaCl_2$ significantly increased the Ca^{2+} absorption rate under NaCl stress, which was 178.4% higher than that under saline stress but did not differ significantly from that of the CK. Under NaCl stress, the Ca^{2+} absorption rate in grapevine roots treated with $CaCl_2$ + $LaCl_3$ was 96.3% higher than that under NaCl stress ($p < 0.05$), which was significantly lower than that of the CK and the $CaCl_2$ treatment groups ($p < 0.05$). When treated with $CaCl_2$ + heparin, + CPZ, or + EB, the Ca^{2+} transport in grapevine roots converted to an efflux, and the Ca^{2+} efflux rate of the EB treatment was significantly higher than those of the heparin and CPZ treatments. The Ca^{2+} absorption rate of the grapevine roots for the $CaCl_2$ treatment group was 55.8% ($p < 0.05$) higher than that for the CK group.

Discussion

Salt stress can lead to the production of reactive oxygen species (ROS), such as H_2O_2 (hydrogen peroxide) and $O_2^{\bullet-}$ (superoxide radical), and accumulation in plants (Tian *et al*., 2015; Hussain *et al*., 2018). In this experiment, 200 mmol·L^{-1} NaCl significantly increased the production rate of superoxide anions and H_2O_2 contents of grapevine leaves (Fig. 2A, B). The increased rate of ROS generation

and decreased scavenging of ROS contribute to overall oxidative stresses and damage, such as peroxidation of membrane lipids and loss of membrane permeability (Xue *et al*., 2008). In the present study, salt stress increased electrolyte leakage in leaves and roots (Fig. 1A, B). Plants have developed a series of both enzymatic and nonenzymatic detoxification systems to counteract ROS, thereby protecting cells from oxidative damage (Das and Roychoudhury, 2014; Hossain *et al*., 2017). The imbalance between ROS production and the capacity of the plant antioxidant system leads to drastic oxidative damage. Under 200 $mmol \cdot L^{-1}$ NaCl stress, the lower activities of antioxidant enzymes did not protect the cells from the higher H_2O_2 and $O_2^{\bullet -}$ levels (Fig. 2A, B). High levels of enzymatic and nonenzymatic antioxidants in plants have been reported to lead to greater resistance to oxidative damage (Chakraborty *et al*., 2016; Hussain *et al*., 2018). Previous studies have suggested that external Ca^{2+} application decreased the H_2O_2 contents and $O_2^{\bullet -}$ generation rate of the halophyte *Cakile maritima* (Amor *et al*., 2010) and wheat seedlings (Tian *et al*., 2015) under salt stress, which resulted in a decrease in electrolyte leakage. Our results showed that $CaCl_2$ decreased the electrolyte leakage of roots and leaves under salt stress. In the leaves, the lower production rate of superoxide anions and H_2O_2 contents of grapevine leaves treated with NaCl + $CaCl_2$ might be related to the relatively higher SOD, CAT, POD, APX, and GR activity compared with those of the NaCl treatment group. Studies have shown that $CaCl_2$ can increase the activity of antioxidant enzymes under NaCl stress (Xue *et al*., 2008; Amor *et al*., 2010; Tian *et al*., 2015). Additionally, after being treated with calcium inhibitors, the antioxidase activity of Baxi banana (Zhou *et al*., 2014), rice (Liu *et al*., 2002), and Jerusalem artichoke (Xue *et al*., 2008) under saline stress continued to decline and thus accelerated saline stress. In this study, the calcium inhibitors had different effects on the activity of antioxidant enzymes (Fig. 2). SOD (enzyme converting superoxide to H_2O_2) is usually considered the first line of defense against oxidative stress. The SOD activity of treatments with $CaCl_2$ or $CaCl_2$+ calcium inhibitors were higher than that of NaCl treatment (Fig. 2A), leading to a lower $O_2^{\bullet -}$ generation rate than that of NaCl treatment (Fig. 2A). H_2O_2 is eliminated by APX, CAT and different classes of PODs (Tanaka *et al*., 1991; Bowler *et al*., 1992; Parida *et al*., 2004). APX and GR are involved in the ascorbate–glutathione cycle, which eliminates H_2O_2 (Foyer and Halliwell, 1976). Thus, the lower activity of POD, APX and GR led to a higher content of H_2O_2 in the $CaCl_2$ + calcium inhibitor groups (Fig. 2B and 3), which resulted in the higher electrolyte leakage of leaves (Fig. 1B).

Under saline stress, the Ca^{2+} concentration of the cytoplasm increased (Chinnusamy *et al*., 2004), inducing plant cells to generate calcium signals; then, Ca^{2+} combined with CAM and other Ca^{2+}–binding proteins, activated a series of physiological and biochemical processes to regulate cellular metabolism and gene expression, and facilitated adaption to stress. Saline stress had a great effect on Ca^{2+} transport (Sanders *et al*., 1999). The Ca^{2+} flow rate measured with noninvasive microtest technology illustrated the final net ion flow rate. After continuous saline stress for 24 h, Ca^{2+} transport of tobacco roots showed a trend towards internal flow (Li *et al*., 2013b), and the results of the present study were consistent with this. After NaCl stress for 1 day, Ca^{2+} transport in grapevine roots presented an influx, but the net flow rate was lower than in the CK, and the net Ca^{2+} flow rate of the $CaCl_2$ treatment group recovered to the level of the CK group. With the addition of calcium inhibitors, the Ca^{2+} internal flow rate of the epidermal cells of tobacco roots declined or converted to an efflux (Li *et al*., 2013b). In this study, the Ca^{2+} internal flow rate of the epidermal cells of grapevine roots under treatment with $CaCl_2$ + $LaCl_3$ was lower than those of the $CaCl_2$ and CK treatment groups but was significantly higher than that under saline stress. The Ca^{2+} transport of epidermal cells of grapevine roots under treatment with $CaCl_2$ + CPZ, $CaCl_2$ + heparin, and $CaCl_2$ + EB all presented as external flow, which indicated that under saline stress, exogenous $CaCl_2$ had impacts both on the Ca^{2+} channel and Ca^{2+} external flow system. However, how $CaCl_2$ influences the Ca^{2+} channel and Ca^{2+} external flow system in grapevines still requires further study at the genetic and protein levels.

Conclusion

Exogenous $CaCl_2$ improved the salinity tolerance of grapevines by increasing the activity of antioxidant enzymes, and it also affected Ca^{2+} transport through both the Ca^{2+} channel and Ca^{2+} external flow system. Further studies are needed to determine how $CaCl_2$ influences the Ca^{2+} channel and Ca^{2+} external flow system in grapevines at the genetic and protein levels.

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