



Full Length Article

Effects of Exogenous CaCl₂ and Calcium Inhibitors on the Reactive Oxygen Species Metabolism and Ca²⁺ Transport of Tamina (*Vitis vinifera* L.) 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 CaCl₂ and inhibitors {LaCl₃, 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 O₂⁻ production rate and H₂O₂ 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 + CaCl₂ treatment group, not only electrolyte leakage of roots and leaves was decreased but O₂⁻ production rate and H₂O₂ contents in the leaves were also decreased. The activity of antioxidant enzymes was greater in NaCl + CaCl₂ treatment group than NaCl treatment group. CaCl₂ + inhibitor treatment decreased the activity of POD, APX and GR compared with their activity in CaCl₂ treatment under NaCl stress, but CaCl₂ + inhibitor treatment increased the H₂O₂ content in the leaves and the electrolyte leakage of the roots and leaves. Ca²⁺ 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 CaCl₂ treatment recovered to the level of control, and in CaCl₂ + LaCl₃ treatment was lower than CaCl₂ treatment. In conclusion, exogenous CaCl₂ improved the salinity tolerance of grapevines by increasing the activities of antioxidant enzymes, and it also affected Ca²⁺ transport through both the Ca²⁺ channel and Ca²⁺ external flow system. © 2019 Friends Science Publishers

Keywords: Grapevine; Saline stress; Ca²⁺; 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 CO₂ 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 Ca²⁺ 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 Ca²⁺ 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 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* (Ben Amor *et al.*, 2010), *Catharanthus roseus* (Cheruth *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 CaCl₂ 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 CaCl₂ and CaCl₂ + calcium inhibitors (plasma membrane calcium channel blocker LaCl₃, calmodulin antagonist (CPZ), Ca²⁺ releasing channel inhibitor heparin, and Ca²⁺-ATPase inhibitor eosin (EB)) on the activity of antioxidant enzymes, accumulation of reactive oxygen, and Ca²⁺ 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 *Vitis vinifera* L. Tamina 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, CaCl₂ and calcium inhibitors: CK = control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺

+ 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂). Each treatment was replicated three times, and each replicate included 10 seedlings. To avoid salt shock in the T₁–T₆ 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 Ca²⁺ 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 N₂ 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 (CE₀), the samples were autoclaved at 120°C for 20 min, and the final electrical conductivity (CE_i) 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 (H₂O₂) 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 O₂⁻ at 530 nm. The hydrogen peroxide (H₂O₂) 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 tetraacetic 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 H₂O₂ (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 Ca²⁺ Transport Rate in Grapevine Roots

The Ca²⁺ transport within grapevine roots was measured using noninvasive microtest technology (NMT), and the measurement system was the noninvasive microtest system BIO-001A from Younger USA, LLC (Amherst, MA, 01002, USA) 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 CaCl₂, 0.1 mmol L⁻¹ MgCl₂, 0.5 mmol L⁻¹ NaCl, 0.2 mmol L⁻¹ Na₂SO₄, 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 Ca²⁺ 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 (SE) of three replicates. Statistical analyses were performed by analysis of variance (ANOVA) using SPSS version 17.0 (SPSS, Chicago, USA), and comparisons between the mean values were made by the least significant difference (LSD) 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. 1); however, CaCl₂ addition in the saline solution obviously decreased the electrolyte leakage from NaCl-treated plants. CaCl₂ alone only increased significantly (13.6%) the electrolyte leakage of roots compared to that for the control plants. Under NaCl stress, compared with CaCl₂, CaCl₂ + LaCl₃, + CPZ or + EB had no significant effect on the electrolyte leakage of roots, while CaCl₂ + heparin obviously increased it by 7.6%; however, there was no significant differences among the four calcium inhibitor treatments. Under NaCl stress, compared with CaCl₂, CaCl₂ + calcium inhibitors obviously increased the electrolyte leakage of leaves.

The Production Rates of Superoxide Anions and H₂O₂ Contents in Grapevine Leaves

As shown in Fig. 2, compared with the control condition, the production rate of superoxide anions and H₂O₂ contents in Tamina grapevine leaves under NaCl stress increased by 30.7% and 25.1% (*p*<0.05), respectively. Addition of CaCl₂ significantly reduced the production rate of superoxide anions and H₂O₂ 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 CaCl₂ + inhibitor treatment groups were all significantly lower than that of grapevine leaves in the CaCl₂ treatment group. The H₂O₂ contents of grapevine leaves treated with CaCl₂ + LaCl₃ 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 CaCl₂. The H₂O₂ contents of grapevine leaves in the CaCl₂ + CPZ and CaCl₂ + 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 CaCl₂ was 16.1% (*p*<0.05) lower than that of leaves in the CK group, and the H₂O₂ 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). CaCl₂ 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.

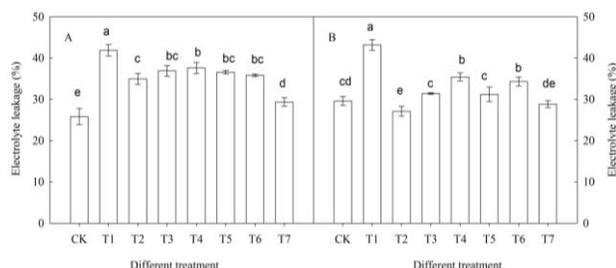


Fig. 1: Effects of CaCl₂ and calcium inhibitor treatments on the electrolyte leakage in the roots (A) and leaves (B) of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂)

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)

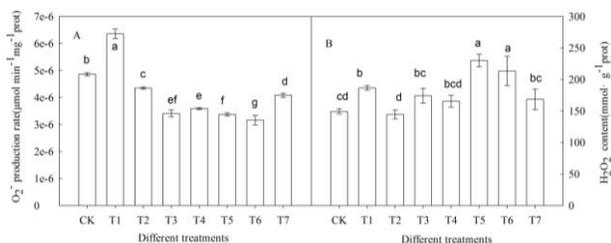


Fig. 2: Effects of CaCl₂ and calcium inhibitor treatments on the O₂⁻ production rate and H₂O₂ contents of Tamina grapevine leaves under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂)

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 CaCl₂ + inhibitor EB or CPZ were 8.2% (p<0.05) and 8.7% (p<0.05) higher, respectively, than those treated with CaCl₂, the SOD activities of grapevine leaves treated with CaCl₂ + inhibitors were all significantly higher under saline stress, and the activities of the samples treated with only CaCl₂ were significantly lower than those of the NaCl treatment (Fig. 3A).

Under NaCl stress, the CAT activities of leaves treated with CaCl₂ + inhibitors were all significantly higher than

those under NaCl stress and CaCl₂ treatments but were not significantly different from those of the control. The CAT activity of leaves treated only with CaCl₂ 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 CaCl₂ + LaCl₃ or EB treatment groups were both significantly lower than plants treated with CaCl₂ but had no significant difference with that under NaCl stress. The POD activities after treatment with CaCl₂ + heparin and CaCl₂ + CPZ were 13.4% and 3.5% (p>0.05) lower, respectively, than in plants treated with CaCl₂. The difference between the POD activity of leaves treated with only CaCl₂ and those of the CK and NaCl + CaCl₂ groups was insignificant (Fig. 3C).

The APX activities of leaves under CaCl₂ + inhibitor treatments were all significantly lower than those treated with CaCl₂, and plants treated with CaCl₂ + LaCl₃ 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 CaCl₂ 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 CaCl₂ + inhibitors were all significantly lower than the GR activity of leaves in the CaCl₂ treatment group but did not differ significantly from the GR activity of leaves under NaCl stress. The GR activity of the CaCl₂ treatment group did not differ significantly from that of the CK and NaCl + CaCl₂ treatment groups, but it was significantly higher than that under NaCl stress (Fig. 3E).

The Growth of Tamina Grapevine

As shown in Fig. 4, 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 CaCl₂ 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. CaCl₂ + calcium inhibitors had no significant effects on the shoot length compared with the NaCl and NaCl + CaCl₂ treatments; CaCl₂ + heparin or + EB obviously decreased the effects of CaCl₂ on the plant biomass, which were 13.5% and 11.5% lower, respectively than that in the NaCl + CaCl₂ treatment group. CaCl₂ alone had no significant effects on the grapevine growth.

The Ca²⁺ Flow Rate in Tamina Grapevine Roots

Under normal conditions, grapevine roots could absorb Ca²⁺, while under NaCl stress, the Ca²⁺ absorption rate declined significantly (p<0.05) to 41.1% of that of the CK treatment (Fig. 5). The addition of CaCl₂ significantly increased the

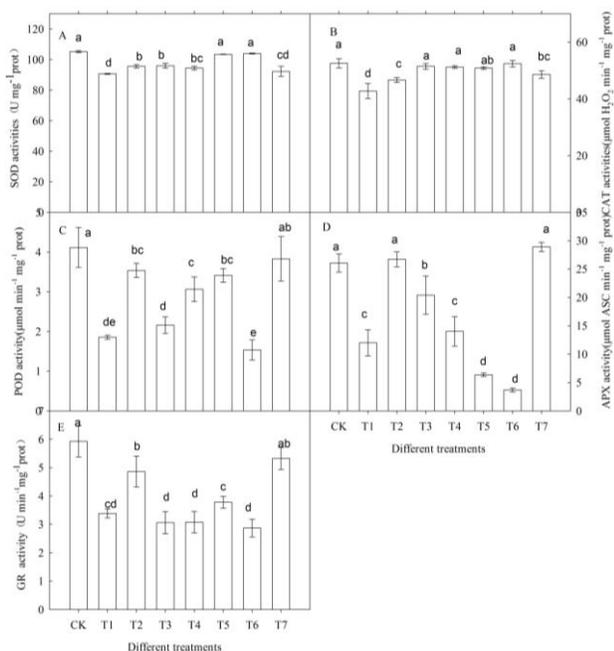


Fig. 3: Effects of CaCl₂ and calcium inhibitor treatments on the antioxidant enzyme activities of leaves from Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂)

Ca²⁺ 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 Ca²⁺ absorption rate in grapevine roots treated with CaCl₂ + LaCl₃ was 96.3% higher than that under NaCl stress (p<0.05), which was significantly lower than that of the CK and the CaCl₂ treatment groups (p<0.05). When treated with CaCl₂ + heparin, + CPZ, or + EB, the Ca²⁺ transport in grapevine roots converted to an efflux, and the Ca²⁺ efflux rate of the EB treatment was significantly higher than those of the heparin and CPZ treatments. The Ca²⁺ absorption rate of the grapevine roots for the CaCl₂ 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 H₂O₂ (hydrogen peroxide) and O₂⁻ (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

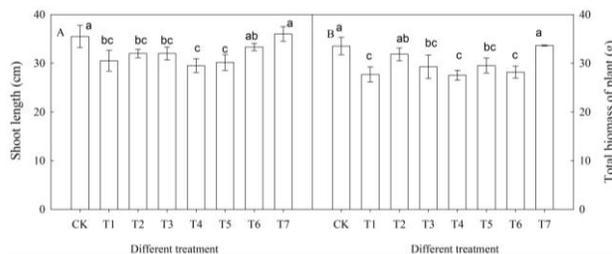


Fig. 4: Effects of CaCl₂ and calcium inhibitor treatments on the shoot length and total biomass of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂)

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)

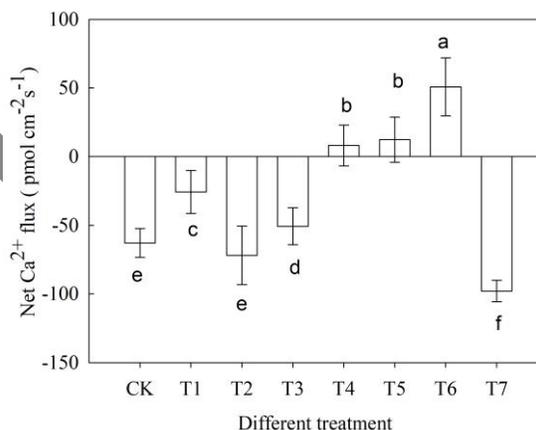


Fig. 5: Effects of CaCl₂ and calcium inhibitor treatments on the net Ca²⁺ flux of roots of Tamina grapevines under NaCl stress

Here, CK = Control (Hoagland nutrient solution); T₁ = Na⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl); T₂ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂); T₃ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ LaCl₃ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ LaCl₃); T₄ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 2 mmol L⁻¹ heparin treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 2 mmol L⁻¹ heparin); T₅ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.4 mmol L⁻¹ CPZ treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.4 mmol L⁻¹ CPZ); T₆ = Na⁺ + 10 mmol L⁻¹ Ca²⁺ + 0.2 mmol L⁻¹ EB treatment (Hoagland nutrient solution + 200 mmol L⁻¹ NaCl + 10 mmol L⁻¹ CaCl₂ + 0.2 mmol L⁻¹ EB); and T₇ = 10 mmol L⁻¹ Ca²⁺ treatment (Hoagland nutrient solution + 10 mmol L⁻¹ CaCl₂)

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)

rate of superoxide anions and H₂O₂ contents of grapevine leaves (Fig. 2). 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. 1). 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·L⁻¹ NaCl stress, the lower activities of antioxidant enzymes did not protect the cells from the higher H₂O₂ and O₂⁻ levels (Fig. 2). 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 Ca²⁺ application decreased the H₂O₂ contents and O₂⁻ generation rate of the halophyte *Cakile maritima* (Ben 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 CaCl₂ decreased the electrolyte leakage of roots and leaves under salt stress. In the leaves, the lower production rate of superoxide anions and H₂O₂ contents of grapevine leaves treated with NaCl + CaCl₂ might be related to the relatively higher SOD, CAT, POD, APX, and GR activities compared with those of the NaCl treatment group. Studies have shown that CaCl₂ can increase the activities of antioxidant enzymes under NaCl stress (Xue *et al.*, 2008; Ben 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 activities of antioxidant enzymes (Fig. 2). SOD (enzyme converting superoxide to H₂O₂) is usually considered the first line of defense against oxidative stress. The SOD activities of treatments with CaCl₂ or CaCl₂ + calcium inhibitors were higher than that of NaCl treatment (Fig. 2A), leading to a lower O₂⁻ generation rate than that of NaCl treatment (Fig. 2). H₂O₂ 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 H₂O₂ (Foyer and Halliwell, 1976). Thus, the lower activities of POD, APX and GR led to a higher content of H₂O₂ in the CaCl₂ + calcium inhibitor groups (Fig. 2 and 3), which resulted in the higher electrolyte leakage of leaves (Fig. 1B).

Under saline stress, the Ca²⁺ concentration of the cytoplasm increased (Chinnusamy *et al.*, 2004), inducing plant cells to generate calcium signals; then, Ca²⁺ combined with CAM and other Ca²⁺-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 Ca²⁺ transport (Sanders *et al.*, 1999). The Ca²⁺ flow rate measured with noninvasive microtest technology illustrated the final net ion flow rate. After continuous saline stress for 24 h, Ca²⁺

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, Ca²⁺ transport in grapevine roots presented an influx, but the net flow rate was lower than in the CK, and the net Ca²⁺ flow rate of the CaCl₂ treatment group recovered to the level of the CK group. With the addition of calcium inhibitors, the Ca²⁺ internal flow rate of the epidermal cells of tobacco roots declined or converted to an efflux (Li *et al.*, 2013b). In this study, the Ca²⁺ internal flow rate of the epidermal cells of grapevine roots under treatment with CaCl₂ + LaCl₃ was lower than those of the CaCl₂ and CK treatment groups but was significantly higher than that under saline stress. The Ca²⁺ transport of epidermal cells of grapevine roots under treatment with CaCl₂ + CPZ, CaCl₂ + heparin, and CaCl₂ + EB all presented as external flow, which indicated that under saline stress, exogenous CaCl₂ had impacts both on the Ca²⁺ channel and Ca²⁺ external flow system. However, how CaCl₂ influences the Ca²⁺ channel and Ca²⁺ external flow system in grapevines still requires further study at the genetic and protein levels.

Conclusion

Exogenous CaCl₂ improved the salinity tolerance of grapevines by increasing the activities of antioxidant enzymes, and it also affected Ca²⁺ transport through both the Ca²⁺ channel and Ca²⁺ external flow system. Further studies are needed to determine how CaCl₂ influences the Ca²⁺ channel and Ca²⁺ external flow system in grapevines at the genetic and protein levels.

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