Effects of Late Season Foliar Application of Calcium Chloride on Cold Hardiness in Grapevines (Vitis vinifera ‘Thompson Seedless’)

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As a major growth limitation, low temperature-induced injuries may adversely affect grape production in many areas. Ten-year-old grapevines ‘Thompson Seedless’ were sprayed with calcium chloride (CaCl₂) three times at 10-day intervals from 19th September to 8th October 2015 and again in 2016 in a commercial vineyard. Bud samples were collected in December 2015 and 2016, January 2015 and 2016 and February 2016 and 2017. The buds were exposed to freezing treatments: −12, −16, −20, −24, and −28°C for 3 hours, to assess their low temperature tolerance. Moreover, the relationships among freezing tolerance and changes in antioxidant enzyme activity, soluble carbohydrates, proline and total proteins were investigated. Irrespective of foliar spray treatments, the freezing tolerance of buds increased from December to January and decreased in February. Application of CaCl₂ at a 1% concentration resulted in increased bud freezing tolerance compared to the control plants. Application of 1% CaCl₂ considerably increased the concentrations of soluble carbohydrates and total proteins in buds, but had subtle and inconsistent effects on proline. Activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) were increased in response to foliar application of CaCl₂; however, inconsistent changes were found in the activities of catalase and peroxidase following CaCl₂ application. The results showed that application of 1% CaCl₂ increased freezing tolerance of grapevines predominantly via upregulating soluble carbohydrates and total proteins.

Key Words: electrolyte leakage, enzyme activity, proline, soluble carbohydrates.

Introduction

The grapevine (Vitis vinifera) is one of the most economically valuable fruit crops cultivated around the world. With a production level of 2.45 million tons of grapes, Iran is the ninth largest country grape-producing country in the world (FAO, 2016). However, in areas with harsh climatic conditions, low temperature can limit grapevine distribution and production. The frost hardiness of grapevines is related to genetic properties, climatic conditions, and management practices, including the type of training system, rootstock selection, and fertilization, control of cropping level and irrigation practices. Winter hardiness of deciduous trees is mainly dependent on their internal nutrient reserves (Zachariassen et al., 2004). Plant nutrition may improve freezing tolerance by manipulating the physiological characteristics, dormancy and nutritional status of plants (Apostolova et al., 1996; Karimi et al., 2016; Sarikhani et al., 2014; Stushnoff and Hamman, 2002). In autumn, calcium is transported through the phloem from leaves to the root system, and the remaining calcium is lost via abscised leaves. Reducing the amount of Ca stored in tissues after harvesting may affect winter hardiness (Domagala-Swiatkiewicz and Blaszczzyk, 2007). As a result, calcium application is a cultural practice in the late season to increase this element in plant tissues. Calcium is an important constituent of the cell wall. It has a vital role in cross-bridge formation with free carboxylic groups of galacturonic acid residues present in pectins that influence the cell wall strength. Calcium is a fundamental component for cell division, development, stability and repair, as well as the penetrability of cell walls (Taylor and Locascio, 2004). It affects cell wall integrity and is regarded as the last barrier before cell separation (Fry, 2004). White and Broadley (2003) showed that foliar calcium nutrition stabilizes the plant cell wall and inhibits cell wall degrading enzymes. Calcium plays a very important
role in the reduction of a plant’s vulnerability to environmental stresses. It has also been proposed that calcium functions as a secondary messenger in intracellular signal transduction during cold acclimation (Harandi et al., 2013).

The adaptation of grapes to low temperature stress involves adaptive metabolic pathways, including induction of antifreeze proteins, changes in membrane composition, the accumulation of osmoprotectants, and changes in the redox status that allow plants to function at low temperatures (Fennell, 2004).

To the best of our knowledge, however, little information is available about the effect of autumn foliar application of CaCl$_2$ on the cold hardiness of grapevine buds. Therefore, this study aimed to determine the effect of autumn application of CaCl$_2$ on the cold hardiness and enzymatic antioxidant system of grapevines cv. Thompson seedless.

**Materials and Methods**

**Plant materials**

This study was done on 10-year-old grapevines ‘Thompson Seedless’ in a commercial vineyard in Malayer, Iran (34.30° N, 48.82° E). All cultural practices were uniformly applied to grapevines throughout the study. In order to get more reliable results, the experiment was conducted in two years. The soil texture of the vineyard was clay—loam at soil layers of 0–40 cm and 40–90 cm (Table 1). Daily maximum and minimum temperatures for the period of November–April in 2015–2016 and 2016–2017 were recorded from the nearest weather station to the study site (Fig. 1).

<table>
<thead>
<tr>
<th>Experimental variant</th>
<th>Total calcium in leaves (%)</th>
<th>Total calcium in buds (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7b</td>
<td>3.2b</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>4.3a</td>
<td>4.7a</td>
</tr>
</tbody>
</table>

Mean values for each parameter (Total calcium) followed by the same lower-case letters in each column are not significantly different at $P \leq 0.05$ by Duncan’s multiple range test.

![Fig. 1. Daily maximum and minimum air temperatures in the experimental vineyard (2015–16 and 2016–17).](image)
Calcium application

Foliar application of CaCl₂ at three concentrations (0, 1, and 2% w/w) was done using manual sprayer three-times at 10-day intervals from 19th September to 8th October in 2015 and was repeated in 2016. The pH levels of the CaCl₂ solutions were adjusted to 6.8–7 using citric acid (CₓHₓOᵧ), and a few drops of tween 20 were added to solutions before spraying. The study was conducted using a randomized complete block design with four replications, and each experimental unit contained three adjacent grapevines for a total of 36 vines per experiment.

Application of 2% CaCl₂ caused some damage to the leaves; therefore, the results of this treatment were not referenced in this study.

Analysis of total calcium content of tissues

Calcium concentrations in leaves and buds were measured using the atomic absorption spectroscopy (AAS) method. Leaf and bud samples were collected on 21 October and their total calcium concentrations were measured. Samples were washed gently with distilled water in order to remove any CaCl₂ left on the leaves and buds.

The samples were prepared as described by Barbeau and Hilu (1993). Wet digestion was used in order to avoid mineral decomposition and destruction (volatilization) that may occur during ashing in a muffle furnace (Bergmann and Bergmann, 1985).

Freezing procedure

Cane samples were collected over two years, on 23rd December, 2015 and 2016, and the 30th January and 25th February 2016 and 2017. Canes were divided into two groups, and half of the samples were used to measure the cold-hardiness of buds in the laboratory and the other half were used to measure proline, soluble carbohydrates, antioxidant enzyme activity and total proteins. Low temperature treatments were done using a programmable freezing chamber (CRP-Z200; Kimia-Rahavard, Tehran, Iran). The treatment temperatures were −12, −16, −20, −24, and −28°C (Pool et al., 1990; Yamori et al., 2005). The cooling rate was 2°C per hour and samples were kept at final temperatures for 3 hours.

Subsequently, samples were transferred to room temperature in order to evaluate low temperature damage using an electrolyte leakage measurement.

Electrolyte leakage (EL)

After running frost tests, 40 mL of deionized water was added to tubes containing the bud samples (two buds per tube and two tubes per replication). Then, the electro conductivity (EC) of the shaken solutions for 24 h was measured (L₁) using a pH/Cond 720 EC-meter (WTW Ino Lab, Weilheim, Germany). The samples were then heat-killed in an autoclave (121°C and 105 kPa for 20 min), and the electro conductivity was measured again (L₂). Relative Electrolyte leakage (REL) was calculated for each sample using Eq. 1:

\[
(1) \text{REL} = \left( \frac{L_1}{L_2} \right) \times 100
\]

The lethal temperature (LT) at which 50% of the total electrolyte leakage occurred in cane or bud tissues (EL-LT₅₀) was calculated using the reported method of Andrews et al. (1984).

Soluble carbohydrates, proline and total protein

Soluble carbohydrate concentrations in buds were determined according to the method described by Yemm and Willis (1954). Free proline concentrations in cane and bud tissues (n = 6 per treatment) was measured according to Bates et al. (1973). Bud total nitrogen was determined using the micro Kjeldahl method (Bradstreet, 1954), and the total protein was then calculated as the conversion rate of proteins in grapevines (Cetin et al., 2011) using Eq. 2:

\[
(2) \text{Protein} = N \times 6.25
\]

Enzyme activity

Bud samples were homogenized in 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 × g for 30 min at 4°C, and the catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX) activities were measured using this supernatant. The activity of SOD was measured by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT). The activity of POD was assayed using a spectrophotometer at 470 nm for 1 min at 25°C. The activities of APX were determined by measurement of absorbance at 290 nm (Beauchamp and Fridovich, 1971; Chance and Maehly, 1955).

Statistical analysis

All statistical analyses were performed using SAS (SAS software version 9.1; SAS Institute, 2003). Duncan’s multiple range tests at the 5% level of significance (P ≤ 0.05) was used to compare the results.

Results

Total calcium

Application of 1% CaCl₂ resulted in higher Ca concentrations in leaves and buds compared to control plants; spraying CaCl₂ had a greater effect on increasing Ca concentration in the buds than the leaves as compared to non-sprayed grapevines (Table 1).

Cold hardness

Foliar application of CaCl₂ had a large influence on improving the cold hardness of buds in both years. Grapevines treated with 1% CaCl₂ had higher freezing tolerance of buds in December of 2015 and 2016 com-
pared to control plants. Regardless of calcium treatments, the freezing tolerance of buds increased in January; however, control plants showed a lower enhancement in bud freezing tolerance compared to CaCl$_2$ treated plants. Buds of grapevines treated with 1% CaCl$_2$ had higher freezing tolerance in January and February in both years. Deacclimation began at the end of February, so bud frost tolerance reduced somewhat in February (Table 2).

**Soluble carbohydrates**

The effect of CaCl$_2$ was significant on soluble carbohydrates in buds at all sampling stages. Buds of grapevines treated with 1% CaCl$_2$ had higher soluble carbohydrates compared to the control plants. Concentrations of soluble carbohydrates substantially decreased in February (Table 3).

**Proline contents**

Bud proline concentration was influenced by CaCl$_2$ nutrition in some sampling stages. However, inconsistent changes were found in bud proline concentrations following 1% CaCl$_2$ treatment (Table 3).

**Total protein**

The effect of CaCl$_2$ on bud total protein concentrations was significant at all sampling stages. Bud protein content increased from December to January and decreased in February. Application of 1% CaCl$_2$ resulted in elevated amounts of bud total proteins (Table 3).

### Enzyme activity

The effect of foliar application of CaCl$_2$ on the activity of CAT, SOD, APX, and POD enzymes in buds is shown in Table 4. The highest bud enzyme activities were found in January of both years. There were subtle and inconsistent changes in bud CAT and POD activities in grapevines treated with 1% CaCl$_2$ as compared to control plants. Grapevines treated with 1% CaCl$_2$ showed higher APX and SOD activities at all sampling stages compared to the control plants (Table 4).

### Relative water content (RWC)

Irrespective of CaCl$_2$ treatments, the RWC of buds decreased from December to January and increased in February. Grapevines treated with 1% CaCl$_2$ had higher RWC at all sampling stages compared to the controls; the greatest differences in bud RWC were found in January, the deep dormancy stage, in both years (Table 5).

### Discussion

Foliar application of CaCl$_2$ leads to an increased concentration of calcium in leaves and buds in the late season. As expected, concentrations of Ca in the leaves were higher than the buds, and the lower stomatal density in buds compared to leaves may explain these results (Nell and Barrett, 1986; White and Broadley, 2003). Late season application of CaCl$_2$ increased winter hardness of grapevines at all sampling stages. Surviving buds were significantly increased in both years following application of 1% CaCl$_2$. Calcium can control many aspects of cold hardness, including maintenance.
of membrane integrity and membrane transport functions (Miura and Furumoto, 2013; Percival and Barnes, 2008; Schaberg et al., 2011).

Freezing injury increases leakage of ions and organic solutes and water soaking of plant tissues. These symptoms suggest that the cell membrane is a site of freezing injury. Although potassium was found to be the major leaking ion, significant loss of cellular/membrane Ca$^{2+}$ during the early stages of freeze-thaw stress was also found (Han and Bischof, 2004; Reynolds, 2001). There is also some evidence that cellular calcium may play an important role in the recovery from freezing injury, which is associated with the activity of plasma membrane H$^+$-ATPase (Mahfoozi et al., 2006; Raese and Curry, 2010).

Increases in organic solutes such as proline and protein and/or an increase in nutrient minerals, mainly Ca$^{2+}$, may result in enhanced low temperature tolerance (Verslues et al., 2006). Sarikhani et al. (2014) found that there is a relationship between increasing freezing tolerance of grapevines and higher bud contents of protein, total proteins and soluble carbohydrates following potassium nutrition. In grape species, carbohydrate content is critical for vine health, especially in the winter when temperatures frequently fall below zero (Fennell, 2004; Karimi Alavijeh et al., 2015; Mohamed et al., 2010; Sarikhani et al., 2014). Several studies have shown that calcium plays an important role in carbohydrate metabolism in plants (Bowler and Chua, 1994; Fromm and Lautner, 2010). Soluble carbohydrates may enhance the freezing tolerance of plants by reducing the freezing point, serving as a cryoprotectant, osmotic buffer, and/or through stabilization of proteins and phospholipids (Wisniewski et al., 2003). According to Paquin and ST-Pierre (1980), an increased proline concentration is a protective measure against cold resistance. It is believed that proline can play a defensive role during low temperature-induced injuries by protecting macromolecules and cell membranes (Wisniewski et al., 2003). In this study, however, subtle and inconsistent changes in bud proline concentration were found following CaCl$_2$ application compared to untreated plants. Ghasemi Soloklui et al. (2012) showed that there was not a strong relationship between cold hardiness and proline content, as compared with soluble carbohydrates, in dormant stems of the pomegranate (Punica granatum L.). Application of calcium increased bud total proteins; the highest concentrations were found in January in both years (Table 6). In this study, there was a strong correlation between protein content and bud cold hardiness in both years (Table 6).

In this experiment, 1% CaCl$_2$ treatment enhanced SOD and APX activities in grapevine buds, but had a slight and inconsistent effect on CAT and POD activities. Low or negative correlations between CAT and POD activities and the freezing tolerance of buds following CaCl$_2$ treatment indicated that calcium did not enhance the freezing tolerance of buds primarily by im-

### Table 4. Evaluation of the effect of CaCl$_2$ sprays on the bud-specific activity of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX) in two years.

<table>
<thead>
<tr>
<th>Parameter/CaCl$_2$ spray concentration [% (w/v)]</th>
<th>Dec-2015</th>
<th>Jan-2016</th>
<th>Feb-2016</th>
<th>Dec-2016</th>
<th>Jan-2017</th>
<th>Feb-2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (U·min$^{-1}$·g$^{-1}$FW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.77$^a$</td>
<td>16.27$^b$</td>
<td>11.58$^b$</td>
<td>12.85$^a$</td>
<td>19.57$^a$</td>
<td>10.50$^a$</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>9.90$^b$</td>
<td>15.52$^a$</td>
<td>12.72$^a$</td>
<td>12.25$^a$</td>
<td>17.48$^b$</td>
<td>9.10$^a$</td>
</tr>
<tr>
<td>SOD (U·min$^{-1}$·g$^{-1}$FW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52.00$^b$</td>
<td>68.50$^a$</td>
<td>44.83$^b$</td>
<td>51.50$^b$</td>
<td>70.50$^b$</td>
<td>47.83$^b$</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>83.67$^a$</td>
<td>98.33$^a$</td>
<td>72.83$^a$</td>
<td>88.33$^a$</td>
<td>116.17$^a$</td>
<td>77.83$^a$</td>
</tr>
<tr>
<td>APX (U·min$^{-1}$·g$^{-1}$FW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.67$^b$</td>
<td>27.17$^b$</td>
<td>20.17$^b$</td>
<td>17.67$^b$</td>
<td>30.17$^b$</td>
<td>22.33$^b$</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>24.67$^a$</td>
<td>52.67$^a$</td>
<td>31.00$^a$</td>
<td>28.83$^a$</td>
<td>58.67$^a$</td>
<td>33.00$^a$</td>
</tr>
<tr>
<td>POD (U·min$^{-1}$·g$^{-1}$FW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.17$^b$</td>
<td>38.83$^a$</td>
<td>14.00$^a$</td>
<td>15.33$^a$</td>
<td>32.67$^b$</td>
<td>14.30$^a$</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>15.50$^b$</td>
<td>33.67$^b$</td>
<td>14.50$^b$</td>
<td>14.83$^a$</td>
<td>35.33$^b$</td>
<td>13.80$^b$</td>
</tr>
</tbody>
</table>

Mean values for each parameter followed by the same lower-case letter in each column are not significantly different at $P≤0.05$ by Duncan’s multiple range tests.

### Table 5. Evaluation of the effect of CaCl$_2$ treatments on bud RWC (%) of ‘Thompson seedless’ grapevines in two years.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>74$^a$</td>
<td>64$^a$</td>
<td>83$^a$</td>
<td>82$^a$</td>
<td>61$^a$</td>
<td>87$^a$</td>
</tr>
<tr>
<td>CaCl$_2$ 1%</td>
<td>61$^b$</td>
<td>42$^b$</td>
<td>67$^b$</td>
<td>63$^b$</td>
<td>38$^b$</td>
<td>66$^b$</td>
</tr>
</tbody>
</table>

Mean values followed by the same lower-case letter in each column are not significantly different at $P≤0.05$ by Duncan’s multiple range tests.
proving antioxidant enzyme activity (Table 6). As a mineral element, calcium can induce the signal transduction pathways for many antioxidant enzymes (Bose et al., 2011). CaCl$_2$ increases the activity of enzymes such as ascorbate peroxidase and superoxide dismutase that help scavenge reactive oxygen species (ROS) (Halman et al., 2008; Jiang and Huang, 2001; Jiang and Zhang, 2003).

Conclusions

In conclusion, the results showed a great impact of late season foliar application of CaCl$_2$ on the freezing tolerance of grapevines. However, Ca concentrations in buds, compared to leaves, were more important in terms of low temperature resistance of grapevines during winter. Calcium nutrition improved low temperature tolerance, especially in January, by increasing the concentration of osmoregulants and subsequently reducing the RWC of buds, as well as improving the plant’s antioxidant system. CaCl$_2$ treatment had a substantial impact on total proteins of buds and should be considered in future research. Generally, late season foliar application of 1% CaCl$_2$ increased freezing tolerance of grapevines primarily by upregulating soluble carbohydrates and total proteins.

**Literature Cited**


Jiang, Y. and B. Huang. 2001. Effects of calcium on antioxidant activities and water relations associated with heat tolerance

### Table 6.  Pearson correlation coefficients among EL-LT$_{50}$ values (in °C) and soluble carbohydrate, total protein, proline concentrations, antioxidant enzyme and RWC in the buds of ‘Thompson Seedless’ grapevines treated with CaCl$_2$ in two years.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble carbohydrate</td>
<td>0.60**</td>
<td>0.38NS</td>
<td>0.79**</td>
<td>0.66**</td>
<td>0.79**</td>
<td>0.77**</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0.57**</td>
<td>0.83**</td>
<td>0.79**</td>
<td>0.68**</td>
<td>0.88**</td>
<td>0.77**</td>
</tr>
<tr>
<td>Proline</td>
<td>0.39*</td>
<td>0.68**</td>
<td>−0.36NS</td>
<td>0.54**</td>
<td>−0.61**</td>
<td>−0.27NS</td>
</tr>
<tr>
<td>CAT</td>
<td>−0.26NS</td>
<td>−0.75**</td>
<td>0.65**</td>
<td>−0.45*</td>
<td>−0.80**</td>
<td>−0.32NS</td>
</tr>
<tr>
<td>SOD</td>
<td>0.62**</td>
<td>0.82**</td>
<td>0.78**</td>
<td>0.67**</td>
<td>0.88**</td>
<td>0.79**</td>
</tr>
<tr>
<td>APX</td>
<td>0.58**</td>
<td>0.82**</td>
<td>0.79**</td>
<td>0.67**</td>
<td>0.86**</td>
<td>0.68**</td>
</tr>
<tr>
<td>POX</td>
<td>0.10NS</td>
<td>−0.72**</td>
<td>0.14NS</td>
<td>−0.22NS</td>
<td>0.71**</td>
<td>−0.10NS</td>
</tr>
<tr>
<td>RWC</td>
<td>−0.67**</td>
<td>−0.83**</td>
<td>−0.75**</td>
<td>−0.61**</td>
<td>−0.87**</td>
<td>−0.77**</td>
</tr>
</tbody>
</table>

**NS** Significant at $P \leq 0.01$ or $P \leq 0.05$, respectively.