Calcium alleviates decreases in photosynthesis under salt stress by enhancing antioxidant metabolism and adjusting solute accumulation in *Calligonum mongolicum*

Danghui Xu¹*, Wenyin Wang¹, Tianpeng Gao², Xiangwen Fang¹, Xiaogang Gao¹, Jinhua Li¹, Haiyan Bu¹ and Jing Mu¹

¹State Key Laboratory of Grassland and Agro-ecosystems, School of Life Sciences, Lanzhou University, Lanzhou 730000, Gansu, China
²Centre of Urban Ecology and Environmental Biotechnology, Lanzhou City University, Lanzhou 730070, China

*Corresponding author: State Key Laboratory of Grassland and Agro-Ecosystems, School of Life Sciences, Lanzhou University, Lanzhou 730000, China. Email: dhxu@lzu.edu.cn

Calcium is known to affect photosynthesis under normal conditions and induces tolerance in plants to biotic and abiotic stresses through influencing physiological processes. In this study, physiological processes were investigated in the desert plant *Calligonum mongolicum* to examine how these processes were induced by calcium treatments to alleviate a decrease in photosynthesis under salt stress. Salinity decreased biomass and photosynthesis, regardless of the salt stress level, but increased superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activity and decreased malondialdehyde (MDA) content under 4 and 8 g/L NaCl and decreased all enzyme indices under 16 g/l NaCl stress. The plants with 5 and 10 mM added calcium had significantly greater biomass and photosynthesis than plants with the non-calcium application or calcium application with 20 mM at all salinity levels. Calcium-treated plants exhibited increases in photosynthesis and biomass along with higher levels of SOD, POD, CAT, APX, GR and total soluble sugar, and lower levels of MDA and proline. Alleviating the effects of salt stress on photosynthesis depended on the concentration of calcium used. Maximum alleviation of salt stress occurred with 5 and 10 mM calcium application. Our result indicated that calcium could be used in forestation and restoration of *C. mongolicum* in high saline soil.

Key words: Antioxidant enzymes, CaCl₂, photosynthetic activity, proline, salinity

Editor: Kevin Hultine

Received 7 June 2017; Revised 13 September 2017; Editorial Decision 14 September 2017; accepted 28 September 2017


Introduction

Salinity is one of the most important abiotic environmental factors, besides drought, extreme temperature, light and metal stress (Slama et al., 2015; Himabindu et al., 2016). High concentrations of salts, in particular sodium chloride (NaCl), because soil salinity is mainly due to the accumulation of NaCl, generally have profound detrimental impacts on major plant physiological processes (Yang et al., 2009). The physiological processes that are primarily affected by...
salt stress include ion toxicity, osmotic stress, nutrient deficiency and oxidative stress (Munns and Tester, 2008; Forieri et al., 2016).

Salt stress may negatively affect photosynthesis by causing stomatal closure and oxidative stress resulting in the formation of reactive oxygen species (ROS) (Nazat et al., 2011). Excessive amounts of ROS can enhance membrane lipid peroxidation and electrolyte leakage (Gunes et al., 2007) and damage chloroplast, inhibit photochemical reactions and decrease photosynthesis (Steduto et al., 2000). Malondialdehyde (MDA) contents are used as an indicator of lipid peroxidation. The increase of MDA content indicates the occurrence of serious lipid peroxidation, and results in a substantial accumulation of ROS that destroys membrane structures (Chen et al., 2017). As an adaptive response, plants activate several mechanisms to counteract the adverse effects of salt-induced ROS. These mechanisms include non-enzymatic antioxidants such as ascorbic acid, glutathione and carotenoids as well as antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and enzymes of the so-called ascorbate-glutathione cycle (ASC–GSH cycle); ascorbate peroxidase (APX) and glutathione reductase (GR). These mechanisms are able to catalyze or participate in the elimination of free oxygen radicals and H₂O₂ from cells (Noctor and Foyer, 1998; Lee et al., 2013). Plants containing high activities of antioxidant enzymes have shown considerable resistance to oxidative damage caused by ROS (Gapińska et al., 2008; Syeed et al., 2011). Saline stress is also known to affect many physiological activities related to the accumulation of ions and osmolytes such as proline and soluble sugar (Lee et al., 2013). The accumulation of these compounds plays a major role in the process of osmotic adjustment, limiting water loss and ion toxicity.

Calcium has been widely examined for its protective role in most abiotic stresses: drought, cold, heat, heavy metals and salt stress (Khan et al., 2010; Liu et al., 2014). Calcium is found to be crucial in altering the ion selectivity absorption and in enhancing salt tolerance in many species (Rengel, 1992; Zhu, 2003). Several studies suggest that calcium increased antioxidant enzyme activities, reduced lipid peroxidation and enhanced osmotic adjustment of cell membranes (Hernandez et al., 2003; Amor et al., 2010). It has also been shown that moderate concentration of calcium decreases oxidative damage generated by NaCl in Cakile maritima (Amor et al., 2010) and high calcium concentration improved salt tolerance in Nitraria tangutorum (Liu et al., 2014). These reports show that calcium has different effects in inducing stress tolerance that may depend upon the species or concentration of calcium applied. Numerous observations in both field and controlled experiments showed that plant yield increases in response to calcium application, although exact mechanistic background of calcium effects is not known. Therefore, it is critical to identify the physiological processes of plants differing in salt sensitivity, and examine how physiological processes are impacted by calcium application to alleviate the decrease in photosynthesis under salt stress.

Calligonum mongolicum is one of the most dominant plant species in the northern desert of China. It is widely used in resisting wind and stabilizing sand in desertification areas, and its branches are good forages for camels and sheep. But salinity is the most limiting factor to its growth and survival (Taia et al., 2007; Dashti et al., 2011). The normal range of salt tolerance for C. mongolicum is 5–8 g/l, and in field conditions, the concentration of NaCl above 20 g/l mM proved lethal (Li et al., 2011). Owing to obvious evidence of the adverse effects of NaCl stress on plant growth, it was hypothesized that calcium addition in this study can overcome the injurious effects of NaCl stress on the desert plant C. mongolicum, and can be used in vegetation restoration and reclamation under salt stress. Therefore, the main objective of this study was to examine whether or not calcium could alleviate the injurious effects of NaCl stress and regulate plant growth by adjusting the osmoprotectants (proline and soluble sugars) and antioxidant enzyme activities in stress tolerance.

Materials and Methods

Plant materials and treatments

The research was conducted in the Botanical Garden at Lanzhou University (N 35°56′, E 104°09′, 1745 m above sea level). Environmental conditions in the experiment field are typically semi-arid, the mean annual temperature is 10°C and rainfall is 320 mM. One-year-old C. mongolicum were obtained after germinating seeds directly from the field and were planted in plastic pots with diameter of 25 cm and height of 24 cm and filled with 3 kg sandy soil in early March, and put to a field trench with 22 cm depth and irrigated once a week. The soil in pots was dug from the area that the plant had colonized near Lanzhou city. The soil type was eolian sand, and soil bulk density, soil organic matter and pH values are 1.46 g/cm³, 1.07 g/l, 7.82, respectively.

After 3 months of accimation, the uniform plants were chosen, and then treated with five levels of NaCl (0, 4, 8, 12 and 16 g/l, equal to 0, 68.4, 136.8, 205.1 and 273.5 mM) with four levels of calcium (0, 5, 10 and 20 mM CaCl₂), respectively were arranged in a randomized, complete block design with four replicates, give a total of 20 containers in every treatment. These ranges of salts and calcium were chosen according to the field conditions and the most salt stress and/or calcium experiments (Amor et al., 2010; Li et al., 2011; Piwowarczyk et al., 2016). All the salts and calcium were weighted using a scale sensitive to 0.001 g, and dissolved in the tap water. Salt concentrations were increased stepwise with 4 g/l (68.4 mM) at 5 day intervals. The plants were well-watered (80–85% of maximum field capacity) with tap water (soil salinity concentration, conductivity and Ca²⁺ content in the tap water are 128 mg/l, 192 us/cm and 34 mg/l, respectively) and no fertilization treatment was applied. When the soil water content was decreased to 80% of field water capacity, enough tap water was applied to maintain a constant solute concentration in the pots (~400–500 ml tap water was...
added at 3 day intervals and a small amount of water was leached from the pots), and soil water content was investigated at 3 day intervals and soil salinity concentration was investigated at 15 day intervals. If soil salinity concentration was higher than the experiment design, more amount of water was leached from the pots to keep the solute concentration constant. To reduce the influences of probable environmental factors, pots of all parts were stochastically allocated to different positions every week.

**Biomass**

Plant samples from four pots for each treatment were harvested at the end of August. Plant roots were removed carefully from soil and then were washed cleanly in water. Fresh masses of all plant samples were immediately put in a drying oven for 48 h at 85°C, and weighed to calculate biomass.

**Gas exchange measurements**

Plants gas exchange of assimilating branches (we used branches instead of leaves because they are the main photosynthetic tissue for most desert plants) was measured from early July to the end of August, including net photosynthetic rate ($Pn$), transpiration rate ($E$) and stomatal conductance ($Gs$), with a Li-6400 Portable Photosynthetic System (Licor, NE, USA). Measurements were performed from 9:00 to 11:00 on clear days on July 6, 18, 27 and August 4, 13, 25 using photosynthetically active radiation (PAR) = 1800 $\mu$mol m$^{-2}$ s$^{-1}$ and flow rate = 350 $\mu$mol s$^{-1}$. Four measurements were taken per treatment and the results were presented as the average of all point mentioned above. After measurement, the parts of the assimilating branches were cut and the area were measured with a portable area meter (Li-3000C, Licor, NE, USA), in order to calculate $Pn$ and $E$ per unit leaf area.

**Enzyme assays**

Fresh assimilating branches (1 mg) were homogenized with 3 ml of 0.05 M sodium phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) polyvinylpyrrolidine. The homogenate was centrifuged at 13 000 $\times$ g for 20 min at 4°C. The supernatant was used immediately for enzyme assay.

The activity of SOD and POD was measured with the method of Tan et al. (2008). One unit of SOD activity was defined as the amount of enzyme per mg of protein sample causing 50% inhibition of the rate of nitro blue tetrazolium reduction at 560 nm. One unit of POD activity was defined as the changes in readings at 470 nm due to guaiacol oxidation. APX activity was followed by a decrease in the absorbance at 340 nm ($E = 6.2$ $\mu$M$^{-1}$ cm$^{-1}$) due to NADPH oxidation. GR activity was determined by the method of Foyer and Halliwell (1976), the activity was measured by following the decrease in absorbance at 340 nm ($E = 6.2$ $\mu$M$^{-1}$ cm$^{-1}$) due to NADPH oxidation. APX activity was followed by a decrease in the absorbance at 290 nm ($E = 2.8$ $\mu$M$^{-1}$ cm$^{-1}$).

CAT activity was determined at 25°C according to Aebi (1984) and it activity was estimated by decreased in absorbance of $H_2O_2$ at 240 nm. Content of MDA in chloroplasts was measured according to the method of Du and Bramlage (1992), and content was measured at 532 nm and corrected by subtracting the absorbance at 600 nm.

**Proline and total soluble sugars accumulation**

Free proline and total soluble sugars were extracted from 1 g of fresh leaves. The methanolic phase was used for the quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973). Soluble sugars were analyzed according to Irigoyen et al. (1992). The absorbance at 620 nm was determined in a spectrophotometer to calculate the content of soluble sugars.

**Statistical analysis**

All statistical analyses were performed using SPSS 13.0 for windows (SPSS Inc., Chicago, IL, USA). Before analysis, all data were tested for normality and all data met the normality distribution. Data were statistically analyzed using analysis of variance (ANOVA) with least significant differences (LSD test) at $P < 0.05$, and were presented as treatment mean ± SD of four measurements.

**Results**

**Plant biomass**

As shown in Fig. 1, biomass was significantly reduced by NaCl stress ($F = 70.46$, $P < 0.001$); however, this inhibition was significantly alleviated by calcium supplement. Under salt stress calcium significantly increased the biomass of C. mongolicum ($F = 62.06$, $P < 0.001$). Under non-salt stress condition calcium increased the maximum biomass at concentration of 10

**Figure 1:** Dry biomass of C. mongolicum grown with 0, 4, 8, 12 and 16 g/l NaCl and treated with 0, 5, 10 and 20 mM calcium respectively. Data are presented as mean ± SD ($n = 4$). Bars showing the same letter are not significantly different by LSD test at $P < 0.05$. 
and 20 mM. *Calligonum mongolicum* grown in the presence of calcium 5 mM showed a maximal growth potential at 4 g/l NaCl compared to other concentration of calcium addition under salt stress, and it increased 20.5% compared to 4 g/l NaCl stress with no calcium addition.

**Photosynthesis characteristic**

Of the note, 4 g/l salt has no effect on *Pn* (*F* = 0.606, *P* = 0.466), *Gs* (*F* = 1.994, *P* = 0.208) and *E* (*F* = 0.242, *P* = 0.640). *Pn*, *Gs* and *E* were significantly reduced by 31, 14 and 19% in 8 and 12 g/l and were reduced by 44, 33 and 39%, respectively in 16 g/l salt compared to the control treatment. Calcium application at 10 mM on plants proved effective in alleviating salt stress by increasing *Pn*, *Gs* and *E* 10, 9.9 and 29% under 4 g/l, 39, 30 and 8.4% under 8 g/l, 39, 22 and 11% under 12 g/l and 29, 43 and 47% under 16 g/l salt stress respectively compared to non-calcium application conditions. (Fig. 2).

**Antioxidant enzymes**

Salt stress increased SOD and POD by 88 and 169% under 8 g/l and increased APX and GR by 97 and 188% under 12 g/l, respectively compared to control (Fig. 3a–d). The effect of calcium application under non-saline conditions did not affect the activity of SOD (*F* = 3.333, *P* = 0.056), POD (*F* = 2.577, *P* = 0.102), APX (*F* = 0.918, *P* = 0.461) and GR (*F* = 3.358, *P* = 0.053). Plants grown with NaCl and treated with 5 and 10 mM calcium showed further increase in the activity of SOD, POD, APX and GR. Maximum activity of SOD and POD were recorded with 10 mM calcium plus 12 g/l NaCl, and the maximum activity of APX and GR was recorded with 10 mM calcium plus 8 g/l NaCl (Fig. 3b and c).

The responses of CAT and MDA content to the treatments are shown in Fig. 3. About 4 and 8 g/l NaCl increased the activity of CAT by 209 and 240% and 16 g/l NaCl decreased it by 49%, respectively compared to control. The effect of calcium application under non-saline conditions did not significantly affect the activity of CAT (*F* = 4.270, *P* = 0.209), but significantly increased under saline condition (*F* = 1447, *P* < 0.001). Calcium application at 10 mM resulted in maximal increase in the activity of CAT under 4, 8 and 12 g/l NaCl (Fig. 3e).

The content of MDA increased progressively with the increase in NaCl concentration (*F* = 639, *P* < 0.001) and application of calcium did not influence MDA content under non-saline conditions (*F* = 2.35, *P* = 0.124) (Fig. 3f). Application of 5 and 10 mM calcium resulted in the decrease of MDA content by 28 and 32% under 4 g/l, 38 and 40% under 8 g/l, 42 and 56% under 12 g/l and 32 and 46% under 16 g/l salt stress respectively compared to non-calcium application conditions (Fig. 3f).

**Proline and total soluble sugars accumulation**

No significant differences among the different calcium application were found for proline (*F* = 0.284, *P* = 0.836) and total soluble sugars accumulation (*F* = 1.295, *P* = 0.323) in assimilating branches of *C. mongolicum* under non-saline conditions (Fig. 4). Proline concentrations increased (*F* = 1289, *P* < 0.001) and total soluble sugars accumulation decreased (*F* = 830, *P* < 0.001) with the increase of salinity. Plants grown with NaCl treated with 5 and 10 mM calcium showed a decrease in proline content by 19, 23, 16 and 9.5% and increase in total soluble sugars by 15, 42, 44 and 74%, respectively, compared to non-calcium application under 4, 8, 12 and 16 g/l of NaCl stress.

**Discussion**

The reported research was undertaken to improve our understanding of physiological processes determining salt tolerance and the induction of such processes by calcium application for the alleviation of salt-induced decreases in photosynthesis. To counteract NaCl-induced oxidative stress, plants are endowed with an antioxidant defense system and potential osmotic regulation. The application of 5 and 10 mM calcium on NaCl-grown plants substantially decreased content of proline and MDA, and increased the activity of antioxidant enzymes.
enzymes such as SOD, POD, APX and GR, and osmotic adjustment substances such as total soluble sugars. This resulted in reduced negative effects of NaCl on biomass and photosynthesis.

The reduction in photosynthesis and biomass has been associated with stomatal closure (Steduto et al., 2000), reduction in osmotic adjustment solutes (Kohler et al., 2009) and the increased production of ROS in chloroplasts (Gunes et al., 2007) under salt stress. External calcium application increased salinity tolerance and improved the growth in *Cakile maritima* (Amor et al., 2010) and in *Nitraria tangutorum* (Liu et al., 2014). The increase in photosynthesis and biomass with application 5 and 10 mM calcium under salt stress with concurrent increase in stomatal conductance and intercellular CO2 concentration suggests stomatal and non-stomatal limitation to photosynthesis, and show that CO2 is utilized more efficiently with 5 and 10 mM calcium application under salt stress.

Salt stress increases ionic and osmotic effects leading to the generation of ROS and oxidative stress. These ROS can affect the integrity of cellular membranes, enzymes activities and the plant photosynthetic apparatus (Nazar et al., 2011;
Plant cells contain an array of antioxidant enzymes that scavenge or prevent the formation of the aggressive ROS, which protect cells from oxidative damage (Gunes et al., 2007). SOD, POD and CAT are usually considered as the key components of antioxidant defense of the plants (Rahman et al., 2016). In the present study, 4 and 8 g/l NaCl treatment (low and medium level of NaCl) increased SOD, POD, CAT, APX and GR activity and decreased those indexes under 16 g/l salt stress (severe NaCl stress). This indicated that *C. mongolicum* tolerance to low and medium salt is associated with enhanced activity of antioxidant enzymes (Wang et al., 2009). Our results also indicated that the application of calcium increases SOD and POD activity under salt stress. This implies that enhancement of SOD, scavenges superoxide radicals to protect from cellular oxidative damage (Hernandez et al., 2003; Amor et al., 2010). Efficient destruction of H$_2$O$_2$ in chloroplast requires the induction of APX via ascorbate-glutathione pathway. The application of calcium to salt-stressed plants maximally induces APX activity, exhibiting greater potential to detoxify H$_2$O$_2$. Those results partially agree with those described by other authors who have proposed that moderate calcium concentration induces increases of antioxidant enzyme activities and the ASC–GSH cycle enzyme activities (Amor et al., 2010).

It is already known that free radical-induced peroxidation of membrane lipids is a reflection of stress-induced damage at the cellular level (Jain et al., 2001). Therefore, the level of MDA, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. Our results indicate that the cell membrane was affected under salt stress. The constant content of MDA in combination with a high activity of SOD and POD suggests that under low and medium salt stress *C. mongolicum* plants are well protected from oxidative damage with calcium addition. It has been shown that Ca$^{2+}$ had the function of preventing cell membrane injury and leakage as well as stabilizing cell membrane structure under adverse environmental conditions (Guimarães et al., 2011). Our results showed that the treatment at 5 and 10 mM calcium leads to an enhancement of the antioxidant levels independent of salt. This suggests that *C. mongolicum* had a severe capacity for scavenging the ROS at 5 and 10 mM calcium levels.

Salt stress has been shown to affect carbohydrate partitioning, leading to the synthesis of new compounds in many plants, especially for the accumulating various solutes during salinity (Sharma et al., 1990). Their accumulation might be of importance for the adjustment of the cellular water potential under conditions of reduced water availability, and they can act as scavengers of ROS. In plants exposed to salinity, the total soluble sugar content in the assimilating branches was reduced significantly compared with plants not exposed to salinity. This could be related to limited carbohydrate availability, as a consequence of a decline in photosynthesis (Kohler et al., 2009). The application of 5 and 10 mM calcium on NaCl-grown plants substantially increased total soluble sugar in assimilating branches. Hyper accumulation of soluble sugar enabled the cells to improve the tolerance to salinity by increasing the osmotic pressure (Tian et al., 2015).

In contrast to soluble sugar, moderate and severe salinity increased proline accumulation in assimilating branches of *C. mongolicum*. It is reported that high level of proline protects plants against salt/osmotic stresses, not only by adjusting osmotic pressure, but also by stabilizing many functional units such as complex II electron transport, membranes and proteins and enzymes such as RUBISCO (Mäkelä et al., 2000). However, decreasing in proline concentrations were observed in plants inoculated with 5 and 10 mM calcium in response to moderate and severe salinity. This could indicate that moderate and severe salinity affected to a lesser extent the osmotic pressure, so they accumulated less proline (Hoque et al., 2007).

In conclusion, increased magnitudes of antioxidant activities and osmolytes concentration were more pronounced under 16 g/l NaCl than those under 4 g/l NaCl. In comparison with the non-calcium treatment, calcium applied to soil enhanced the biomass and photosynthesis characteristic of assimilating branches. Thus, the application of calcium, particularly at 5 and 10 mM, markedly increased SOD, POD, CAT, APX, GR and soluble sugar content, and decreased the content of MDA and proline in the plants. Moreover, we have demonstrated differences in response of antioxidant activities and osmotic adjustments to different concentration of calcium applications under salt stress for *C. mongolicum*. The presented results supported the view that calcium can contribute to protect *C. mongolicum* against NaCl stress by alleviating the oxidative stress. Our results further our understanding of the mechanisms of salinity tolerance and may assist with forestation conservation, management and plant restoration actions; for example, management to alleviate salt stress that may affect plant growth and survival by addition adequate calcium which could improve plant growth in saline condition, especially as the amount of land impacted by salinity increases globally.

**Acknowledgements**

We thank Jacob Weiner and two anonymous reviewers for their constructive comments and language revision on this manuscript.

**Funding**

This work was financially supported by the National Natural Science Foundation of China (NSFC) (31460162, 31600336 and 31370423). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.
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