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SHORT COMMUNICATION

Salicylic acid improves chilling tolerance by affecting antioxidant enzymes and osmoregulators in sacha inchi (*Plukenetia volubilis*)

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Abstract The effects of pretreatment of 0–5 mM salicylic acid (SA) on the sacha inchi (*Plukenetia volubilis*) seedlings during chilling stress were investigated. The stress induced damage to seedlings, and SA pretreatment decreased the level of damage. Lipid peroxidation, measured in terms of malondialdehyde content, increased significantly by stress, and SA pretreatment prevented the increase because SA decreased the production rate of superoxide anion (O_2) and H_2O_2 content. SA pretreatment increased the activities of antioxidant enzymes including superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase and decreased activity of dehydroascorbate reductase. In addition, SA pretreatment increased the content of proline and soluble sugar. Results suggested that the pretreatment of sacha inchi seedlings with SA may induce antioxidant enzymes which lead to increased chilling tolerance. The best protection appeared to be obtained from seedlings that were sprayed with SA at 0.5 and 1 mM. Induction of chilling tolerance in seedlings by exogenous application of SA may have a significant practical application in sacha inchi plantation.

Keywords Antioxidant system · Chilling · Sacha inchi · Salicylic acid

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Introduction

Environmental factors, such as light, rainfall, and temperature, affected the growth and production of plants. The suitable environmental condition would be beneficial to plant, but because of various reasons, plants often face kinds of stresses including drought, salt, low temperature, and heat. Low-temperature stress may be further categorized into chilling stress (0–15 °C) and freezing stress (<0 °C). For those plants that originate from tropical and subtropical area, chilling is a potential threaten for their growth (Thakur et al. 2010). Chilling stress affects the normal function of plants at various levels, viz., morphological, physiological, and cellular levels. The plants suffered from chilling stress may show various characters such as anthocyanin accumulation, stunted growth, wilting, reduced, and deformed leaves (Rymen et al. 2007). Severe chilling at reproductive stage ultimately affects the yield of crops manifested in the form of reduced fruit set (Kumar et al. 2011).

In recent years, with the development of industrial farming and the changes of people's lifestyle, the health of people has generally been declining, which led to a great demand for healthy foods including healthy natural oil. Therefore, more and more people have used vegetable oil for cooking instead of animal oil because of its higher unsaturated fatty acids (FAs). The seeds of sacha inchi (*Plukenetia volubilis* L., Euphorbiaceae) contain a large amount of polyunsaturated FAs comprising about 93 % of total FAs (Hamaker et al. 1992). In particular, the fatty acid composition of the seed oil differs markedly in containing large quantities of α-linolenic acid (Follegatti-Romero et al. 2009). The sacha inchi oil was consumed by Peruvian Indians, who have used them to prepare foods and beverages for hundreds of years (Guillen et al. 2003). In

addition, the seeds of sacha inchi contained amino acid, tocopherol, and sterol, showing good prospect in pharmacy, healthy care, and cosmetics (Simopoulos 1991; Balk et al. 2006). In order to use the sacha inchi, Xishuangbanna Tropical Botanical Garden introduced the crop in 2006.

Xishuangbanna, belong to Yunnan Province, is located in southwestern China. The government of Xishuangbanna will vigorously develop the industry of sacha inchi, but harsh winters (with minimum temperatures dropping below 10 °C) occurred in 1963–1965, 1969–1971, 1973–1974, 1980, 1999–2000, and 2013 in Xishuangbanna. For example, the temperature was as low as 3 °C in January in 1974 and 2 °C in 1999 (Yu et al. 2008), and some area was down to −5.4 °C in 1974 and −3.5 °C in 1999 (Huang et al. 2000). These low temperatures killed or damaged many tropical crops including rubber, coffee, tea, and litchi. Sacha inchi plant is native to Amazon rainforest and is a typical tropical crop. This means that the plants are sensitive to chilling temperatures; therefore, improving the tolerance to chilling is important for its plantation in Xishuangbanna.

Salicylic acid (SA) plays a regulatory role in plant physiology and metabolism and plays a great role in plant response to abiotic stress factors of various natures, i.e., temperature, salinity, drought, and heavy metal (Pál et al. 2013). SA can improve tolerance to these abiotic stress including by seed priming (Farooq et al. 2009; Carvalho et al. 2011; Sayyari et al. 2013; Pouramir-Dashtmian et al. 2014) or by foliage application (Kang and Saltveit 2002; Kang et al. 2003; Sayyari 2012; Mutlu et al. 2013b). Although sacha inchi plants have wide applications in the future, no paper has been reported to improve chilling tolerance by exogenous SA application. On the basis of the antioxidative role of SA, we hypothesize that SA pretreatment can induce the antioxidant system in plants during chilling stress. Thus, the purpose of this paper was to test the possibility that application of SA would protect the young plants of sacha inchi from damaging effects of chilling stress by changing the activity of certain antioxidant enzymes.

Materials and methods

The fruits of *P. volubilis* Linneo were collected in Xishuangbanna tropical botanical garden in September, 2012. After cleaning, the seeds were dried slightly and transported by car to Puer University. The seeds were sown in cylinder pots (15 cm diameter × 15 cm height) containing 2,000 cm^3 humus in November, 2012, and four seeds were sown in one pot. After most seeds germinated, one seedling was kept in each pot, and others were removed. The pods were placed in greenhouse with 71.1 ± 4.1 % average relative humidity (RH), and the seedlings were exposed to the natural photoperiod. The maximum, average, and minimum temperatures were 26.32 ± 1.71 °C, 21.65 ± 3.76 °C, and 17.98 ± 0.87 °C, respectively. The plants received 500 mL of water per pot every other day using a sprinkling can. The seedlings had 5–6 pairs of leaves after which the seeds were sown for 60 days, and SA pretreatment were done at this time. SA with concentrations of 0.5, 1, 2, and 5 mM was used for foliage spray, and the controls were sprayed by water. The first spray was done at 8:00 am, and the second one was at the same time next day. All plants were watered after the second spray. At 8:00 am of the third day, the plants sprayed by water were kept well watered as control, and the chilling stress was imposed by transporting the plants into plant growth chamber with 12/12 h photoperiod, 75 % RH, and 4 °C temperature for 2 days. After chilling stress finished, the fourth blades of each seedling were cut off and immediately stored at −20 °C for later analysis. Then, the seedlings were moved back in greenhouse, and all plants were watered. After 72 h, all plants were assessed to determine the extent of injury, and data were collected.

All seedlings were visually examined to determine the extent of chilling injury and classified by the following scale: none, no visible symptoms; slight, small necrotic areas on shoots but without growth restrictions (<5 % of leaf area necrotic); moderate, well-defined necrotic areas on shoots (<25 % of leaf area necrotic); severe, extensive necrotic areas and severe growth restrictions (>50 % of leaf area necrotic but plant still alive); and killed, entire plant necrotic and collapsed. By assigning values of 1, 2, 3, 4, and 5, respectively, to each group, the average injury for each treatment was calculated (Korkmaz 2002).

Lipid peroxidation was determined measuring the concentration of thiobarbituric acid reactive substances, estimated as malondialdehyde (MDA) equivalents (Luo et al. 2012). MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and was expressed as nmol g^{-1} dry weight.

The superoxide anion ($O_2^{\cdot-}$) production rate was determined spectrophotometrically by monitoring the nitrite formation from hydroxylamine based on the method of Li and Gong (2005). Sodium nitrite (10–50 mM) was used as a standard. The $O_2^{\cdot}$ production rate was expressed as nmol min^{-1} g^{-1} dry weight. H_2O_2 levels were estimated based on the method of Patterson et al. (1984). H_2O_2 content was expressed as μmol H_2O_2 mg^{-1} dry weight.

Extraction of antioxidant enzymes was carried out on ice unless stated otherwise. Frozen samples were homogenized with 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and 0.3 % Triton X-100 (v/v) and 5 % (w/v) PVP, with the addition of 1 mM AsA in the case of APX assay. The homogenate was centrifuged at 13,000g

for 15 min at 4 °C, and the supernatant was transferred to cryogenic vials and kept at −20 °C for enzyme activity assay. Total soluble protein content was determined according to the method of Bradford (1976) with bovine serum albumin as the standard. The activities of antioxidant enzymes were assayed according to Jiang and Zhang (2001) and Cia et al. (2012).

Superoxide dismutase (SOD) activity was assayed by the photochemical NBT method. The assay mixture contained 1.9 mL 50 mM phosphate buffer (pH 7.8) with 13 mM L-methionine, 75 μM NBT, and 16.7 μM riboflavin; and 100 μL enzyme extract. The photoreduction of NBT was measured at 560 nm. One unit of SOD is defined as that amount of enzyme causing half the maximum inhibition of NBT reduction.

Guaiacol peroxidase (GPOX) activity was measured with guaiacol as the substrate in a total volume of 2 mL over 2 min. The reaction mixture consisted of 1.99 mL 50 mM potassium phosphate buffer (pH 6.0) with 0.5 % (v/v) guaiacol and 1 % (v/v) H_2O_2, and 10 μL enzyme extract. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm. One unit of GPOX is defined as the 1 of increase of absorbance value per minute.

Catalase (CAT) activity was measured by monitoring the disappearance of H_2O_2 through measuring the decrease in absorbance at 240 nm over 2 min. The reaction mixture consisted of 1.9 mL 50 mM potassium phosphate buffer (pH 7.0) with 30 mM H_2O_2 and 100 μL enzyme extract. CAT activities were expressed as nmol H_2O_2 mg^{-1} protein min^{-1}.

Ascorbate peroxidase (APX) activity was determined by monitoring the rate of ascorbate oxidation at 290 nm over 2 min. The reaction mixture contained 1.9 mL 50 mM potassium phosphate buffer (pH 7.0) with 1 mM ascorbic acid (AsA), 0.1 mM EDTA and 4 mM H_2O_2, and 100 μL enzyme extract. APX activities were expressed as μmol AsA mg^{-1} protein min^{-1}.

Glutathione reductase (GR) activity was measured by monitoring the decrease of NADPH in absorbance at 340 nm over 2 min. The reaction mixture contained 1.8 mL 50 mM potassium phosphate buffer (pH 7.8) with 5 mM $MgCl_2$, 1 mM oxidized glutathione (GSSG) and 1 mM EDTA, 100 μL 2 mM NADPH, and 100 μL enzyme extract. The reaction was initiated by the addition NADPH and plant extract. GR activities were expressed as nmol NADPH mg^{-1} protein min^{-1}.

Dehydroascorbate reductase (DHAR) activity was measured by monitoring the increase of AsA at 265 nm over 2 min. The reaction mixture contained 1.8 mL 50 mM potassium phosphate buffer (pH 7.8) with 0.4 mM DHA, 3.5 mM reduced glutathione (GSH), and 1 mM EDTA; and 100 μL enzyme extract. DHAR activities were expressed as μmol AsA mg^{-1} protein min^{-1}.

Proline content was determined using the procedure described by Bates et al. (1973). Leaf samples were homogenized in 3 % (w/v) of sulphosalicylic acid, the homogenate was incubated in the boiling water for 15 min and centrifuged at 1,000×g for 5 min at 25 °C. 1 mL of supernatant was added to 1 mL acid ninhydrin solution (2.5 g ninhydrin, 60 mL acetic acid, and 40 mL 6 M phosphoric acid) and 1 mL glacial acetic acid, and incubated at 100 °C for 30 min and cooled to room temperature. The absorbance was measured at 520 nm, and the proline content was determined using a proline standard (0–100 μg mL^{-1}).

Total soluble sugars were measured by the anthrone method according to Yemm and Willis (1954). Leaf samples were homogenized in 70 % of ethanol, dropped into the boiling water for 10 min and centrifuged at 1,000×g for 5 min at 25 °C. 0.5 mL of supernatant was added to 3 mL acid ninhydrin solution (0.6 g anthrone, 300 mL dilute H_2SO_4), and incubated at 100 °C for 10 min and cooled to room temperature. The absorbance was measured at 620 nm, and the sugar content was determined using a sucrose standard (0–100 μg mL^{-1}).

Data were subjected to two-way analysis of variance (ANOVA). Multiple comparisons after ANOVA were performed using Duncan's test ($P < 0.05$). All analyses were performed with R software (Version R i386 3.0.1).

Results and discussion

Salicylic acid application was effective within the range of 0.5–5 mM in protecting sacha inchi seedlings against chilling stress. After exposure to chilling for 2 days, the seedlings not sprayed with SA exhibited typical chilling injury symptoms in moderate level (5–25 % leaf area necrotic), while SA-treated seedlings were slightly damaged (<5 % leaf area necrotic) or no visible symptoms by chilling stress. The leaves of non-SA-treated seedlings were moderately wilted and lost a portion of their foliage as indicated by higher injury rating values due to necrotic areas compared to SA-treated seedlings (Fig. 1). Seedlings treated with the highest concentration of SA (5 mM) had small necrotic areas on leaves, whereas those treated with lower concentrations of SA had almost no visible injury symptoms. The lowest injury rating was obtained from seedlings that were pretreated with 0.5 mM SA. Therefore, SA pretreatment led to the increased levels of chilling tolerance in sacha inchi seedlings. Similar results were also reported in other researches which showed that SA pretreatment induced cold tolerance of maize, rice, cucumber, banana, watermelon, and barley (Kang and Saltveit 2002; Kang et al. 2003; Sayyari 2012; Sayyari et al. 2013; Mutlu et al. 2013a, b). Moreover, although the injury degrees were not significantly different among concentrations of

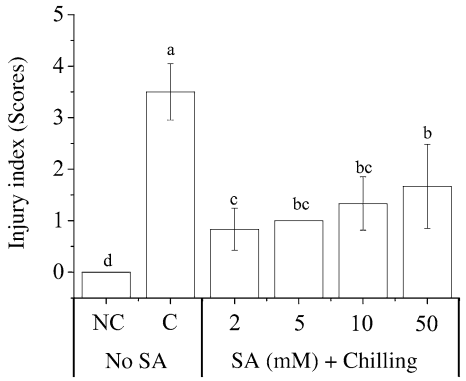

Fig. 1 Effect of SA pretreatment on injury index of sacha inchi seedlings subjected to chilling stress. NC and C are the control seedlings that were not pretreated by SA. NC, the control seedlings without chilling; C, the control seedlings with chilling. *Different letters* in figure showed that there was a significant difference at the level of 0.05. The notes of following figures are the same as this

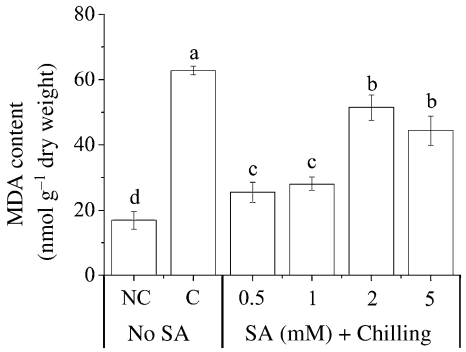

Fig. 2 Effect of SA pretreatment on MDA content of sacha inchi seedlings subjected to chilling stress

0.5, 1, and 2 mM, 0.5 mM, SA pretreatment led to the lowest injury index; therefore, 0.5 mM may be the most cost effective concentration in providing chilling tolerance.

SA treatment prevented lipid peroxidation during the drought period in *Ctenanthe setosa* (Kadioglu et al. 2011) and chilling period in cucumber seedlings (Sayyari 2012) and barley (Mutlu et al. 2013b). In our study, chilling without SA application significantly increased the level of lipid peroxidation, leading to higher MDA content (Fig. 2). SA with concentrations of 0.5–5 mM significantly decreased the increase of MDA ($P < 0.01$), and 0.5 and 1 mM decreased more than 2 and 5 mM ($P < 0.05$). This result indicates that SA pretreatment increased the activities of antioxidant enzymes, which caused a decrease in oxidative stress as shown by the decrease in MDA content.

Chilling treatment without SA application significantly increased production rate of $O_2^{\cdot-}$ and H_2O_2 content compared with control (Fig. 3). Application of SA significantly prevented this increase, and therefore efficiently decreased the level of lipid peroxidation. All concentrations (0.5–5 mM) of SA significantly decreased production rate of $O_2^{\cdot-}$ and H_2O_2 content compared with the 0 mM ($P < 0.01$). No significant difference was found among 0.5, 1, and 2 mM, and 5 mM led to higher level of $O_2^{\cdot-}$ and H_2O_2. These results indicate that lower injury induced by SA pretreatment with lower concentration was produced in sacha inchi leaves because of the lower level of ROS.

Plants that were exposed to certain environmental stresses tend to produce more ROS (Munne-Bosch and Penuelas 2003; Kadioglu et al. 2011; Saruhan et al. 2012). These stress factors lead to an increase in endogenous ROS levels in plants. In our work, chilling increased the production rate of $O_2^{\cdot-}$ (Fig. 3a) and induced the accumulation of H_2O_2 (Fig. 3b, $P < 0.01$), but these ROS were effectively reduced by SA pretreatment during chilling ($P < 0.01$). The decrease in certain antioxidant enzyme activities induced more MDA content under chilling, while the induction of antioxidant enzyme activities by SA decreased $O_2^{\cdot-}$ and H_2O_2 by chilling. This means that ROS generation caused by chilling was neutralized by the ROS scavenging in SA-treated seedlings. The case that the activities of some antioxidant enzymes in SA-treated seedlings were lower than in chilling seedling suggests that ROS may play a secondary role in the chilling signaling network by inducing defense pathways. As revealed from substantially lowered H_2O_2, $O_2^{\cdot-}$, and MDA contents, SA pretreatment may alleviate the effects of chilling stress.

All antioxidant enzyme activities significantly decreased under chilling stress (Fig. 4). SA pretreatment affected the activities of antioxidant enzymes during chilling, but each enzyme showed a different response to SA pretreatment. SOD activities were significantly increased under chilling stress after SA pretreatment, and 0.5 mM SA lead to the highest activities (Fig. 4a). There was a similar trend in GPOX (Fig. 4b) and CAT (Fig. 4c) activities. Differently, the pretreatment by 0.5 and 1 mM SA led to significantly higher activities than other concentrations ($P < 0.05$). For GR (Fig. 4e), pretreatment of 1 mM SA induced highest activity than other concentrations. Activities of APX (Fig. 4d) and DHAR (Fig. 4f) had no significant differences between SA pretreatment and non-pretreatment.

Other researchers also found that SA changes the activity of antioxidant enzymes such as CAT, APX, POD, and SOD during stress (Kang et al. 2003; Hayat et al. 2008; Saruhan et al. 2012; Mutlu et al. 2013b). Kang et al. (2003) found that SOD activity decreased after chilling stress and SA application further decreased the activity, meanwhile chilling stress decreased the activities of CAT and APX, but SA application prevented the decrease. Differently, in barley apoplast, chilling stress decreased the SOD activities in both cold-tolerant and cold-sensitive cultivars, and SA

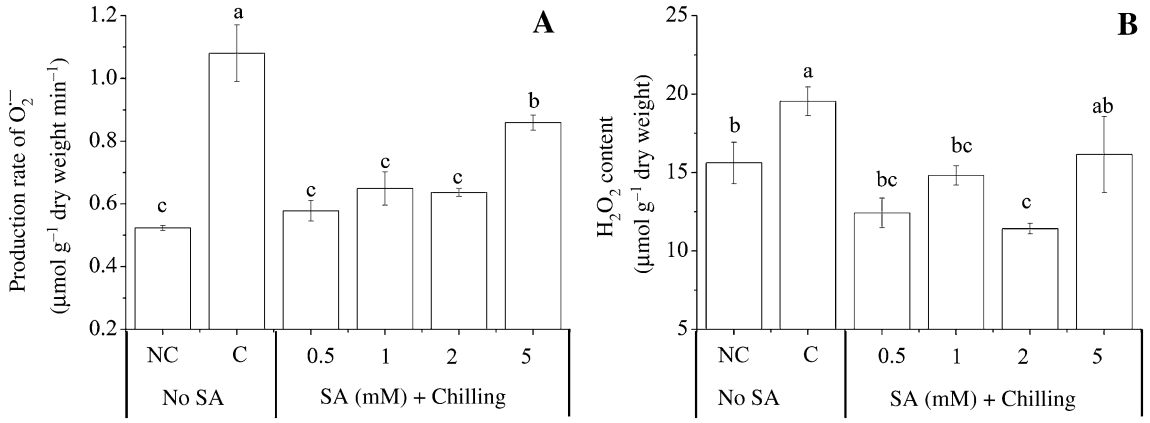

Fig. 3 Effect of SA pretreatment on production rate of $O_2^{\cdot-}$ and H_2O_2 content of sacha inchi seedlings subjected to chilling stress

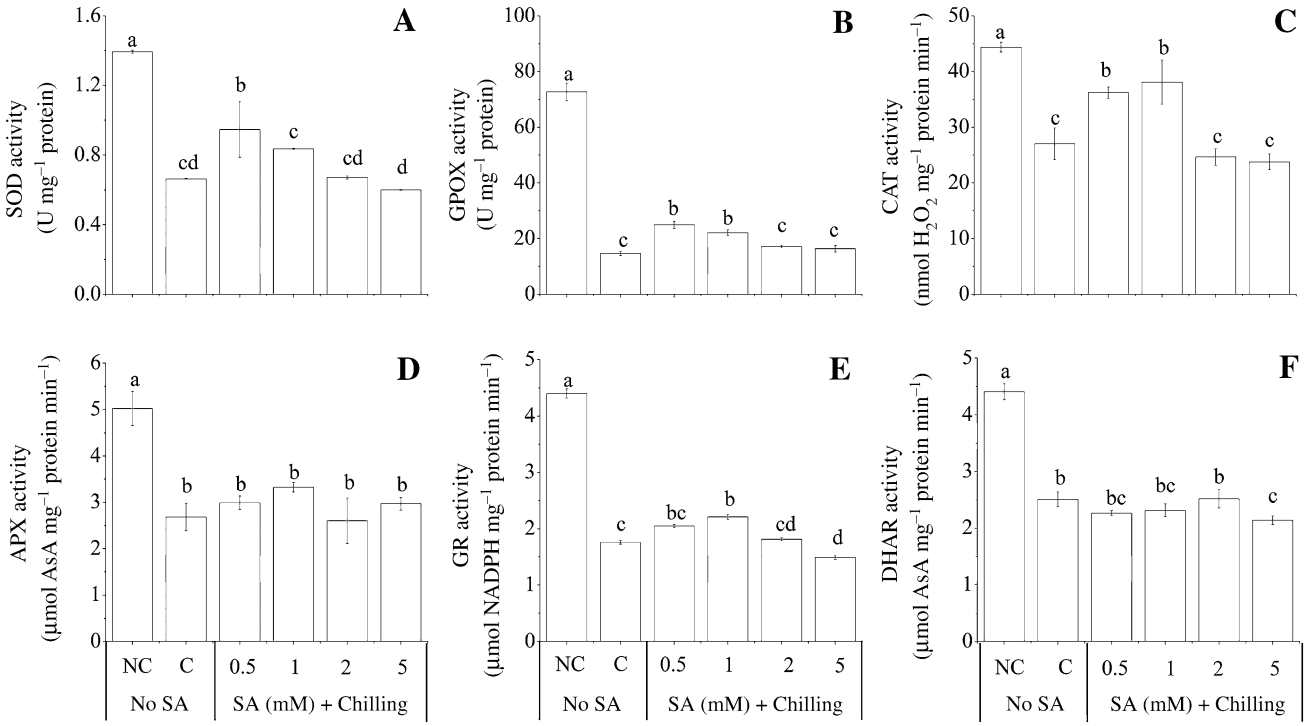

Fig. 4 Effect of SA pretreatment on SOD, GPOX, CAT, APX, DHAR, and GR activities of sacha inchi seedlings subjected to chilling stress

prevented the decrease, while CAT activities increased after chilling stress and SA enhanced the increase (Mutlu et al. 2013b). In maize, drought stress increased the activities of SOD, CAT, DHAR, MDHAR, APX, and GR in both drought tolerant and drought sensitive cultivars, and SA application enhanced the activities of all the enzymes (Saruhan et al. 2012). From above informations, we can see that each enzyme responds differently to stress, and SA application not always increases the enzyme activities. The reason that SA increases the tolerance to abiotic stress may be related to those enzymes whose activities enhanced by SA. Our results showed that activities of SOD, CAT, APX, GR, DHAR, and APX decreased after chilling, and SA application made the activities of SOD, CAT, GPOX, and GR be higher than that of chilling-stressed seedlings. Therefore, these enzymes may be related to the induction of antioxidant responses that protect the seedlings from oxidative damage.

In plants, the antioxidant enzymes such as APX, GR, and DHAR responsible for the AsA–GSH cycle may play great roles under stress (Sala and Lafuente 1999). APX is considered to be the primary H_2O_2 scavenging enzyme in plant, and GR plays a central role in maintaining GSH pool during stress (Contour-Ansel et al. 2006). GR and APX

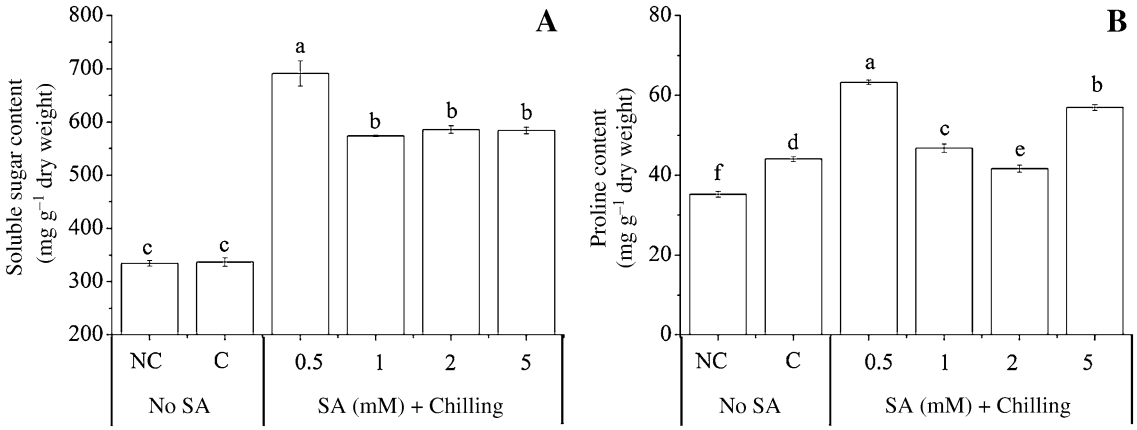

Fig. 5 Effect of SA pretreatment on proline and sugar content of sacha inchi seedlings subjected to chilling stress

activities were higher in sacha inchi seedlings that were pretreated by 0.5 and 1 mM SA. The results showed that AsA–GSH cycle enzymes may be involved to remove H_2O_2 produced in chilling stress. However, the DHAR activities in SA-treated seedlings were lower, and the DHAR cannot maintain the reduced ascorbate (AsA) pool. Since AsA can be produced by other ways, higher APX activities may play a role in scavenging H_2O_2.

Plants subjected to drought, chilling, or salinity stresses accumulate molecules such as sugars and proline in their tissues (Mohsenzadeh et al. 2006; Cao et al. 2011; Sayyari 2012). An accumulation of these molecules may serve as a means of osmotic adjustment, which improves plant's tolerance to stresses. Except for osmoprotective function, both proline and soluble sugar have other multiple functions (review in Couée et al. 2006; Ma et al. 2009; Szabados and Savouré 2010; Kavi Kishor and Sreenivasulu 2014). Proline is used for protein synthesis, has protective functions as antioxidant, contributes to the maintenance of the redox balance, can regulate development, and is a component of metabolic signaling networks controlling mitochondrial functions, stress relief, and development (Szabados and Savouré 2010; Kavi Kishor and Sreenivasulu 2014). Soluble sugars are involved in the responses to a number of stresses, and they act as nutrient and metabolite signaling molecules that activate specific or hormone-crosstalk transduction pathways, and thus resulting in important modifications of gene expression and proteomic patterns (Couée et al. 2006; Ma et al. 2009). In this study, proline content significantly increased after chilling stress (Fig. 5b), while soluble sugar content did not change significantly, but SA pretreatment significantly increased the content of the two molecules, and 0.5 mM induced the highest content ($P < 0.01$). This indicated that soluble sugar and proline had different strategies in protecting plant. Soluble sugar was not involved in cold-induced response, meanwhile proline played a role in it, but SA pretreatment made soluble sugar involved in. Although proline level increased after chilling stress and after SA pretreatment, its increase amount was smaller than soluble sugar; therefore, soluble sugar may play greater role in SA-induced tolerance to chilling.

In conclusion, foliage spray of SA on sacha inchi seedlings enhanced the capacity of antioxidants and the increased capacity of antioxidant enzymes and decreased the level of ROS. Additionally, soluble sugar and proline content were increased in SA-pretreated seedlings; they may play multiple functions against chilling stress. Therefore, foliage application of SA can help reduce the adverse effect of chilling and may have a key role in providing chilling tolerance in sacha inchi seedlings.

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