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Chilling Pretreatment Causes Some Changes in Respiration, Membrane Permeability and Some Other Factors in Soybean Seedlings

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Abstract: When plants with tropical and subtropical origins, like soybean, expose to low temperatures, suffer some injuries that some times are lethal for them. Cold temperature damage is a common problem for soybean in temperate regions. So it can be a good strategy that exposes these plants to low temperatures slightly above freezing temperature, to increase their chilling tolerance. Physiological responses to chilling, including antioxidative enzyme activity, respiration, membrane permeability were investigated in soybean to identify mechanisms of chilling tolerance. Plants were exposed to 15°C (cold-acclimated) or 25°C (nonacclimated) for 24 h, under 250 μmol m-2s-1 Photosynthetically Active Radiations (PAR). Then all plants were exposed to 4°C (chilling temperature) for 24 h and allowed to recover at 25°C for 24h. We analyzed the activity of Ascorbate Peroxidase (APX) and Guaiacol Peroxidase (GPX) in leaves. It revealed that the activity of APX and GPX induced in leaves. The respiration and membrane permeability of nonacclimated leaves were higher than the cold acclimated ones in chilling stress.

Key words: Acclimation, antioxidant, ascorbate peroxidase, chilling, guaiacol peroxidase, permeability, respiration

INTRODUCTION

Food crops of tropical and subtropical origins such as soybean (*Glycine max*), corn (*zea mays* L.) and tomato (*Lycopersicon esculentum* Mill) are cultivated in areas where temperatures fall below the optimum required for their normal growth and development. It is now known that exposure of chilling-sensitive plants, such as maize and tomato, to temperatures that are slightly above chilling, reduces chilling injury (Anderson *et al*., 1995; Gilmour *et al*., 1988; Prasad, 1996; Scebba *et al*., 1999; Venema *et al*., 2000). Various mechanisms have been suggested to account for chilling injury or tolerance in plants (Brasa, 2001).There is increasing evidence that chilling causes elevated levels of Active Oxygen Species (AOS), which contribute significantly to chilling damage (Prasad *et al*., 1994). AOS such as superoxide (O^{-}_{2}), hydrogen peroxide(H_2O_2), hydroxyl radicals(OH°) and singlet oxygen($^1O_2$), are present in plants at various levels at 25°C as a result of normal aerobic metabolism. Plants have evolved antioxidant systems to protect cellular membranes and organelles from damaging effects of AOS (Foyer *et al*., 1991). Antioxidant enzymes, such as Catalase (CAT) and various peroxidases such as Guaiacol Peroxidase (GPX) and Ascorbate Peroxidase (APX) can react with and neutralize, the activity of AOS (Foyer *et al*., 1991; Lee and Lee, 2000; Oidaira *et al*., 2000; Prasad, 1996; Scandalios, 1993). Beside these enzymes, antioxidant compounds such as ascorbate, glutation,β-carotene and α-tocopherol also play important roles in the removal of toxic oxygen compounds (Hodges *et al*., 1996; Wise and Naylor, 1987b). Cold acclimation increases tolerance to AOS in plants with an increase in antioxidant enzymes (Anderson *et al*., 1995; Scebba *et al*., 1999). In chilling sensitive plants, the ability to defend against oxidative damage is inhibited by the reduction expression of antioxidants such as ascorbate, glutation andα-tocopherol (Wise and Naylor, 1987a), CAT (Fadzillah *et al*., 1996) and SOD (Michalski and Kanjuga, 1982). Chilling tolerance improved when GSH peroxidase and CAT levels were enhanced (Upadhaya *et al*., 1989). Thus, it is important to determine the activity of various antioxidants during acclimation and chilling to assess their contribution to chilling tolerance. We aim to determine whether AOS- scavenging enzymes play a role in soybean tolerance to chilling stress.

MATERIALS AND METHODS

Seeds of soybean (Glycine Max cv. L17) were soaked in water for 6 h at 25°C and then were cultivated in Petri dishes on 2 layer of filter paper for 48 h at 25°C in an

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incubator. After that the soybean seedlings were transferred to pots containing washed sand (4 seedlings per pot) and were irrigated with half-strength Hoagland's nutrient solution. The plants were grown at 27/25°C (day/night) temperature, 70% relative humidity, with a 16h/8h day/night photoperiod under 250 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density. Seedlings at the three-leaf stages were placed at 15 C (cold-acclimated) or 25°C (nonacclimated) for 24 h. The acclimated and nonacclimated seedlings were then exposed to chilling at 4°C for 24 h and allowed to recover for 24 h at 25°C. Harvesting was done at the same time each day to avoid complications from diurnal fluctations in biochemical processes. Experiments were conducted from June to August in 2007 at biochemistry lab, Department of biology, Faculty of science, Urmia University, Iran. Means were separated by Tukey Multiple Range Test at p = 0.05. Values are the mean±SE of three replicates.

Enzyme extraction and assay: Samples were prepared for APX and GPX analyses by the method described by Chang and Kao (1998). About 0.5 g of frozen leaf and root materials from all treatments were harvested and homogenized with a mortar and pestle in 6 mL of extraction solution (0.05 M tris-Hcl buffer (pH 7.2), 3 mM $MgCl_2$ and 1 mM EDTA). Then the solution was centrifuged for 20 min at 5000 rpm. The supernatant was used for monitoring antioxidative enzymes mentioned above. APX activity was assayed by monitoring the ascorbic acid dependent reduction of H_2O_2 at 240 nm, as described by Asada (1992). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 0.5 mM ascorbate, 0.2 mL H_2O_2 (1%), 0.1 mL enzyme extraction. GPX activity was determined at 420 nm by the method of Chang and Kao (1998). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1 mL guaiacol (1%), 1 mL H_2O_2 (1%) and 2.5 mL enzyme extract.

Electrolyte conductivity: Electrolyte conductivity was measured according Huixia *et al.* (2004) with a slight modification. For each treatment, 6 leaves from three plants per event (2 leaves per plant, 6 laves per event) were measured for Electrolyte Conductivity (EC). Sampled leaves were rinsed with ddH_2O to remove possible ion contamination on the surface. Leaf discs (7 mm in diameter) were placed into a 20×150-mm glass test tube containing 13 mL distilled water. Tubes were placed at room temperature for 24 h. Conductivity of the solution was measured by using a conductivity meter (Thermo Orin 150 A+, Orion Research, Beverly, MA). The solutions were autoclaved at 100°C for 1 h to completely lyse the plant cell walls. The electrolyte conductivities of autoclaved solutions were recorded as the absolute conductivity. The percentage of EC was calculated by dividing the initial conductivity by the absolute conductivity.

Respiration: Respiration was measured according Jinn *et al.* (1995) with a slight modification by using an oxygen electrode and oxygen meter (WTW 730). Respiratory measurements were made in a 4 mL cuvette. Reaction mixtures contained 0.25 M sucrose, 0.01 M tris, 0.01 M K_2HPO_4, 0.005 M $MgCL_2$, 0.005 M EDTA, 0.5 mg mL^{-1} BSA and 3 mL of sample. Respiratory activity is expressed as mg O_2 to L reaction solution in 1 min.

RESULTS

Antioxidative enzymes in leaves: The baseline levels of antioxidative enzyme activities were generally the same between cold-acclimated and nonacclimated leaves. No differences were found in APX and GPX activities in cold-acclimated and nonacclimated leaves during chilling and recovery period. APX and GPX activities in cold-acclimated and nonacclimated leaves were similarly affected by chilling temperature (Fig. 1 and 2). However, recovery in cold-acclimated leaves for APX and GPX activities were better than in nonacclimated leaves. Significant changes in GPX activity was observed between cold-acclimated and nonacclimated leaves 24 h after acclimation (Fig. 1 and 3). GPX and APX activities in cold-acclimated leaves were higher than nonacclimated leaves.

Activities of APX and GPX in cold-acclimated and nonacclimated roots during acclimation and chilling were generally the same (Fig. 2 and 4). Roots of cold-acclimated plants showed higher activity of antioxidative enzymes

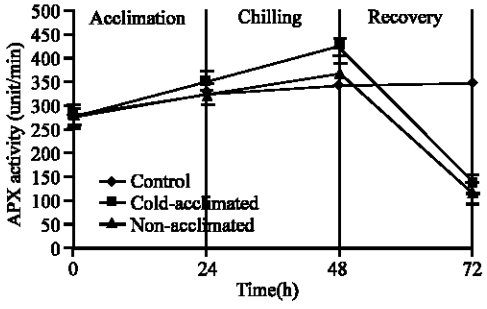

Fig. 1: Changes in leaf APX activity (unit/min) in cold-acclimated and non- acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements ±SE. p = 0.05

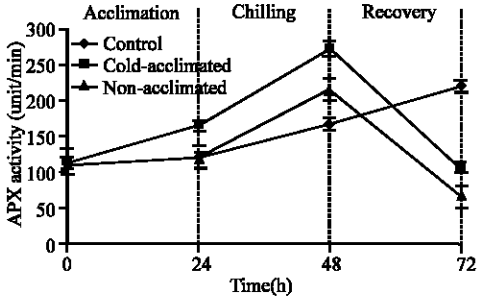

Fig. 2: Changes in root APX activity (unit/min) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements ±SE. p = 0.05

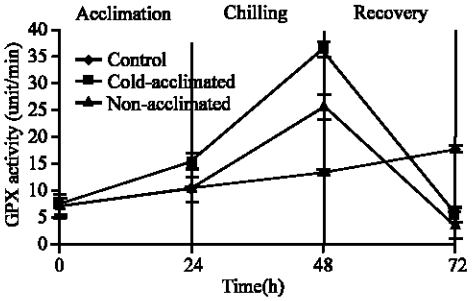

Fig. 3: Changes in leaf GPX activity (unit/min) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements ±SE. p=0.05

during the phase of the recovery period compared to roots of nonacclimated plants. APX activity in cold-acclimated roots was higher than in nonacclimated roots in recovery phase. GPX activity in cola-acclimated roots was significantly higher than nonacclimated roots (Fig. 4). In all plants APX and GPX activities in cold-acclimated roots was higher than nonacclimated and cold-acclimated roots had higher potential to recover than nonacclimated plants. However, APX activity were similar between cold-acclimated and nonacclimated roots during recovery.

Electrolyte conductivity: Cellular damage of treated seedlings due to cold-induced membrane lesions was estimated by measuring membrane damage or electrolyte leakage EC from the leaves of treated plants. The higher the EC, the sever the damage to the plant membrane and the less tolerant the plants were to low temperature challenging. In cold acclimated plants, the EC was lower than the nonacclimated ones and in recovery phase, the cold acclimated plants were recovered faster than the nonacclimated plants in comper with control plants.

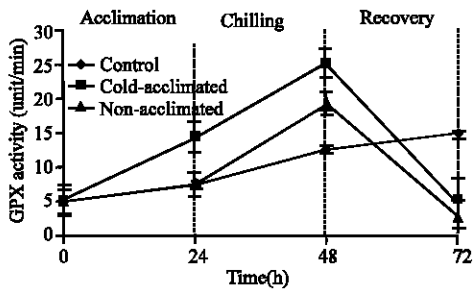

Fig. 4: Changes in root GPX activity (unit/min) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements ±SE

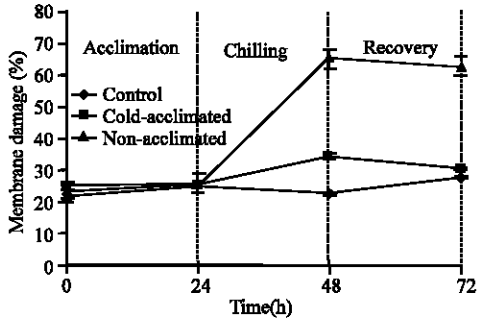

Fig. 5: Changes in Membrane damage in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements ±SE

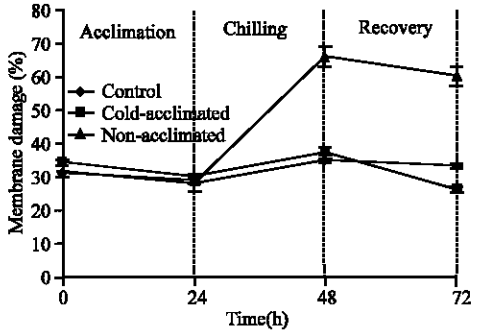

Fig. 6: Changes in Membrane damage in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements ± SE

(Fig. 5 and 6) This refers that cold pretreatment increased membrane tolerance to low temperature (4°C).

Respiration: The rate of respiration was increased as temperature was lowered. However, in 4°C, O_2

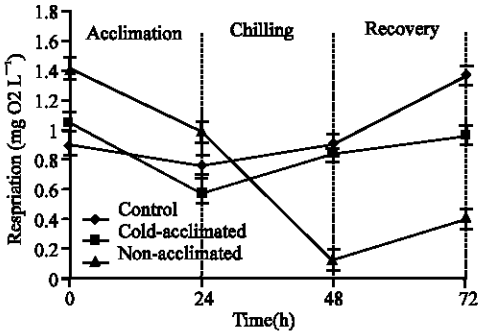

Fig. 7: Changes in respiration rate in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements ± SE

concentration was in lowest level (for nonacclimated plants) during assay. As it is obvious in Fig. 7, when plants were transferred to 25°C (recovery phase), O_2 concentration in buffer increased. According Fig. 7, cold acclimated plants recovered better than nonacclimated ones.

DISCUSSION

It has been reported that some chilling-sensitive plants acclimate if they are exposed to a low temperature slightly above the threshold of chilling temperature, in a process analogous in some respects to the acclimation that occurs in perennial plants in the autumn (Rikin *et al*., 1979). It has been shown that acclimation of maize seedlings to otherwise lethal chilling temperatures by a milder cold pretreatment was accompanied by catalase and peroxidase transcript accumulation (Prasad *et al*., 1994). To study the mechanisms of chilling injury or tolerance, most researchers utilize metabolic differences between chilling-sensitive and tolerant varieties as model systems (Pinhero *et al*., 1997; Saruyama and Tanida, 1995). However, this system is confounded by genetic differences between sensitive and tolerant varieties. Therefore, it is difficult to interpret the observed metabolic differences in relation to mechanisms of chilling tolerance when using different varieties of plants. Using a chilling-sensitive variety that can be cold-acclimated is advantageous for studying the mechanisms involved in chilling tolerance because it eliminates the complexity of genetic differences. In this study, only one variety was used to demonstrate whether chilling tolerance can be induced in soybean plants by cold acclimation and to examine whether an AOS-scavenging system is involved in tolerance to chilling stress. It has been reported that cold-acclimated rice plants shows higher tolerance to chilling stress than nonacclimated plants (Kuk *et al*., 2003). Also, a similar phenomenon was demonstrated in other cold-sensitive species, such as maize, tomato and *Arabidopsis thaliana* (L.) Heynh (Venema *et al*., 2000). Active oxygen species inactivate enzymes and damage important cellular component. The increased production of toxic oxygen derivatives is considered to be a universal or common feature of stress conditions. Plants have evolved a wide rage of mechanisms to contend with this problem. The antioxidant defense system of the plant comprises a variety of antioxidant molecules and enzymes (Arora *et al*., 2002). We have investigated the activity of APX and GPX in both leaves and roots of cold-acclimated and nonacclimated soybean plants. Ascorbate peroxidase activity has mainly been reported from chloroplast and cytosol (Chen and Asada 1989). However, some recent studies have also reported its occurrence in mitochondria as well. (Gomez *et al*., 1999). This enzyme involved in Asada-Halliwell pathway of hydrogen peroxide scavenging that also involves various antioxidant enzymes (Arora *et al*., 2002). Guaiacol peroxidase removes H_2O_2 from apoplast and vacuole and utilizes L-ascorbic acid as electron donor. In an abiotic environment it uses phenolic compounds such as guaiacol as redox intermediate (Chanda and Singh, 2000). In this study APX activity in leaves was found to be higher than roots. But significant changes were observed in roots. In recovery phase, cold-acclimated plants returned to normal condition more easily and rapidly than nonacclimated plants. Soybean plants exposed to chilling temperature exhibited all the aforementioned symptoms; however, cold-acclimated plants showed higher tolerance to chilling stress than nonacclimated plants. Cold-acclimated plants generally still showed similar level of injury as nonacclimated ones, but cold-acclimated plants showed the capability to recover from chilling injury. This was indicated by faster recovery of fresh weight of cold-acclimated plants compared with nonacclimated ones. The baseline levels of antioxidative enzyme activities were generally the same between cold-acclimated and nonacclimated leaves. No differences were found in APX and GPX activities in cold-acclimated and nonacclimated leaves during chilling and recovery period. APX and GPX activities in cold-acclimated and nonacclimated leaves were similarly affected by chilling temperature (Fig. 1 and 3). However, recovery in cold-acclimated leaves for APX and GPX activities were better than in nonacclimated leaves. Significant changes in GPX activity was observed between cold-acclimated and nonacclimated leaves 24 h after acclimation (Fig. 2 and 3). GPX and APX activities in cold-acclimated leaves were higher than nonacclimated leaves. The leaf disc leakage of solute into the incubation medium is thought to be

controlled by the plasma membrane. Increased leakage from injured seedlings might have resulted from damage to the plasma membrane and possibly to the tonoplast, which affects membrane structure, physical integrity and composition (Levitt, 1980). Chang *et al*. (2000) suggested that the three days of acclimation at 10°C maximized mungbean seedling tolerance to chilling at 4°C. Acclimation significantly decreased the leakage of solutes and cations from the leaves of seedlings chilled at 4°C. They showed that acclimation at 10°C for 2-3 days significantly decreased the conductivity and concentration of cations (K^+, Mg^{++} and Ca^{++}) in the leakage. The large amount of solute in leakage may be due to not only plasma membrane damage but also tonoplast. Duke *et al*. (1977) have shown that mitochondrial respiration of soybean embryonic axes decreased at 10°C. Raison and Lyons (1970) have indicated that initial state 3 respiration of plant mitochondria is slow and becomes progressively slower as temperature is lowered. According our results, respiration rate varies in response to changes of temperature; the more lowered the temperature, the more increased the respiration. In summary, pretreatment of seedlings at 15°C for 24 h reduced the severity of chilling injury at 4°C compared with those from nonacclimated plant tissues. Also at recovery phase, cold acclimated plants recovered faster than nonacclimated ones.

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