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Cold Pretreatment-Induced Changes in Antioxidant Enzyme Activities and Relative Water Content and Soluble Sugars in Shoots and Roots of Soybean Seedlings

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Abstract: Soybean (*Glycine max*) is a tropical crop, but is also grown in temperate regions in middle spring to late summer. This crop has an important role in human diet. Cold temperature damage is a common problem for this plant in temperate regions. Physiological responses to chilling, including antioxidative enzyme activity, Relative Water Content (RWC) and soluble sugar contents 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 24h and allowed to recover at 25°C for 24 h. We analyzed the activity of Ascorbate Peroxidase (APX), Catalase (CAT) and Guaiacol Peroxidase (GPX) and soluble sugar content and RWC in both shoots and roots of soybean seedlings. It revealed that the activity of APX and CAT and GPX induced in leaves and roots. Increased activity in roots is important for cold tolerance as compared to shoots. The amount of RWC decreased in both roots and shoots, but soluble sugar content increased, especially in shoots as compared to control plants. Chilling sensitive soybean plants can be made tolerant to cold by cold acclimation.

Key words: Acclimation, antioxidant, ascorbate peroxidase, catalase, chilling, guaiacol peroxidase, RWC, soluble sugar

INTRODUCTION

Food crops of tropical and subtropical origions 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 (Scebba *et al*., 1999; Venema *et al*., 2000). Various mechanisms have been suggested to account for chilling injury or tolerance in plants (Barsa, 2001). There is increasing evidence that chilling causes elevated levels of Active Oxygen Species (AOS), which contribute significantly to chilling damage (Wise and Naylor, 1987b). 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 (POX) 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; 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, 1987a). Cold acclimation increases tolerace to AOS in plants with an increase in antioxidant enzymes (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 and α-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 (Upadhayaya *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. Sugars appear essential in plant cold acclimation, as shown for example by the inability of an *Arabidopsis* sucrose synthase mutant to cold acclimation or the requirement for light in low-nonfreezing temperature-induced cold acclimation connected to sugar accumulation (Wanner and Junttila, 1999). Precise function of sugars is not known, but their high abundance in cold acclimated plants suggests a role in osmoregulation and less abundant

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sugars might also have a role in cryoprotection or as signaling molecules (Annikki and Palva, 2006). We aimed to determine whether AOS-scavenging enzymes and soluble sugar and relative water contents play a role in soybean tolerance to chilling stress.

MATERIALS AND METHODS

Seeds of soybean (Glycine max) were purchased from oilseeds center, Ardabil and were soaked in water for 6 h at 25°C, then were germinated in Petri dishes on two layers of filter paper for 48 h at 25°C in an incubator. Subsequently the soybean seedlings were transferred to pots containing washed sand (4seedlings per pot) and were watered with half-strength Hoagland 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 stage, 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 May to July in 2007 at biochemistry lab, Department of biology, Faculty of science, Urmia University, Iran. Statistical analyses were performed using SAS software. Measurements were mean of 3 replicats±SE.

Enzyme extraction and assay: Samples were prepared for CAT, APX andGPX analyses by the method described by Chang and Kao (1998). The amount of 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), 3mM $MgCl_2$, 1 mM EDTA). Then the solution was centrifuged for 20 min at 5000 rpm. The supernatant was used for monitoring antioxidative enzymes mentioned above. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm using the method of Chance and Maehly (1959). The reaction mixture contained 50mM potassium phosphate buffer (pH = 7. 4), 0.1 mL H_2O_2 (1%) and 2 mL enzyme extract. 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.

Relative water content: Chilling injury on leaves and roots was evaluated by changes in Relative Water Content (RWC). Relative water content was calculated using the formula has became below (Yong In Kuk *et al*., 2003). RWC= (1-dry weight of tissue/fresh weight of tissue)×100.

Soluble sugar content: Soluble sugars content was measured using phenol sulphoric method (Fales, 1979). The amount of 0.5 g of plant tissues (leaf or root) were sampled and homogenized with a mortar and pestle in 5 mL distilled water and then was filtered through Whatman NO. 1 filter paper. Two mL of filtered extract transferred into tube and 1 mL of phenol 5% (w v^{-1}), 3 mL of sulphoric acid 98% were added. After 1 h the absorbance was recorded at 485 nm using UV-visible spectrophotometer (WPA model S2100).

RESULTS AND 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). Therefor chilling injury can be alleviated if plants are exposed first to a low non-chilling temperature (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). Other correlations between cold acclimation and antioxidant defense have also been reported (Taqo *et al*., 1998). 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 differevces. 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 showes higher tolerance to chilling stress than nonacclimated plants (Yong *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). Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions. 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 defence system of the plant comprises a variety of antioxidant molecules and enzymes (Ajay *et al*., 2002). We have investigated the activity of APX, CAT 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 (Ajay *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 *et al*., 2000). Catalase is another defense enzyme that is present in peroxisoms (Scandalios, 1993). In this study APX activity in leaves was found to be higher than roots. But significant changes was observed in roots. In recovery phase, cold-acclimated plants returned to normal conditioin 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, GPX and CAT activities in cold-acclimated and nonacclimated leaves during chilling and recovery period. CAT, APX and GPX activities in cold-acclimated and nonacclimated leaves were similarly affected by chilling temperature (Fig. 1-3). However, recovery in cold-acclimated leaves for APX,GPX and CAT activities were

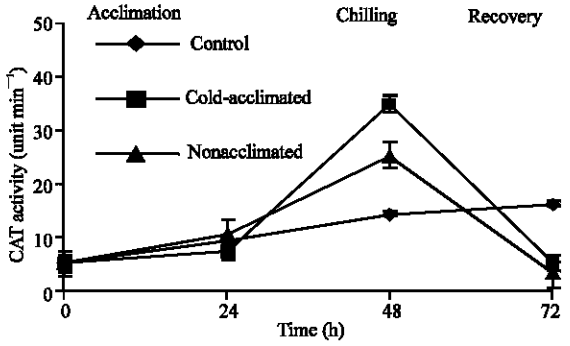

Fig. 1: Changes in leaf CAT activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

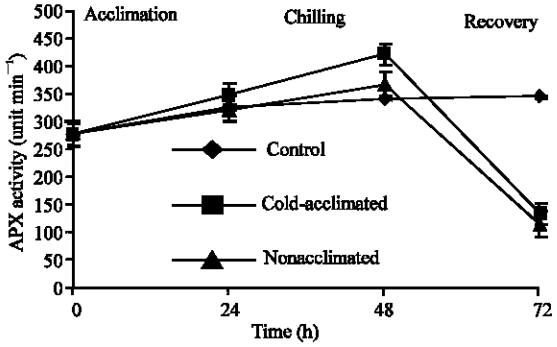

Fig. 2: Changes in leaf APX activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE. p = 0.05

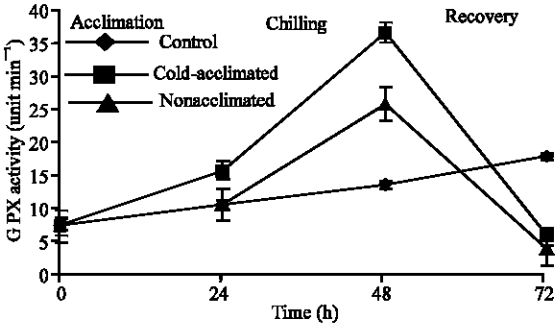

Fig. 3: Changes in leaf GPX activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

better than in nonacclimated leaves. Significant changes in GPX activity was observed between cold-acclimated and nonacclimated leaves 24 h after acclimation

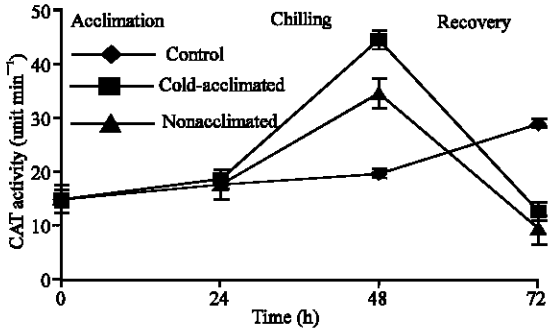

Fig. 4: Changes in root CAT activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

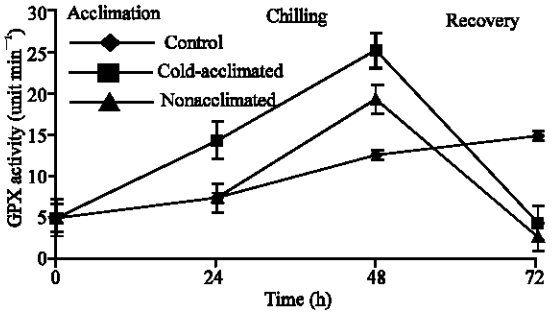

Fig. 6: Changes in root GPX activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements±SE

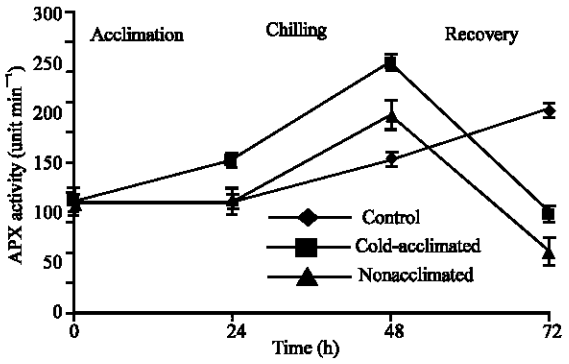

Fig. 5: Changes in root APX activity (unit min^{-1}) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

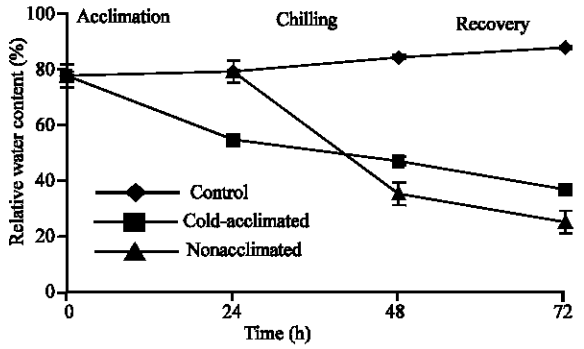

Fig. 7: Changes in root relative water content (%) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

(Fig. 2 and 3). GPX and APX activities in cold-acclimated leaves were higher than nonacclimated leaves.

Activities of CAT, APX and GPX in cold-acclimated and nonacclimated roots during acclimation and chilling were generally the same (Fig. 4-6). Roots of cold-acclimated plants showed higher activity of antioxidative enzymes 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-acclomated roots was significantly higher than nonacclimated roots (Fig. 6). In all plants APX, CAT and GPX activities in cold-acclimated roots was higher than nonacclimated and cold-acclimated roots had higher potential to recover than nonacclimated plants. However, CAT and APX activity were similar between cold-acclimated and nonacclimated roots during recovery. No significant differences were found in CAT activity between cold-acclimated and nonacclimated roots during acclimation (Fig. 4).

The minimal impact of chilling on Relative Water Content (RWC), high photosynthetic efficiency of cold-acclimated plants; and the full recovery of carbon fixation capability of cold-acclimated plants after chilling. Typical symptoms of chilling injury are wilting, yellowing of leaves,and inhibition of growth. Leaf fresh weights of cold-acclimated and nonacclimated soybean seedlings were reduced in chilling period (Fig. 7 and 8). Leaf and root fresh weight of nonacclimated plants rapidly declined when exposede to chilling temperature and did not recover from the chilling treatment properly. Leaf and root fresh weight of cold-acclimated plants also declined significantly 24 h after exposure to chilling temperature. Although the cold-acclimated plants did not completely recover from chilling injury compared with untreated control plants, the cold-acclimated plants showed a general increase in leaf and root weight compared with nonacclimated plants during the recovery period.

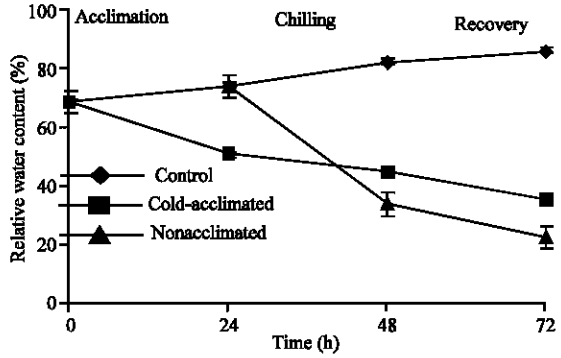

Fig. 8: Changes in leaf relative water content (%) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

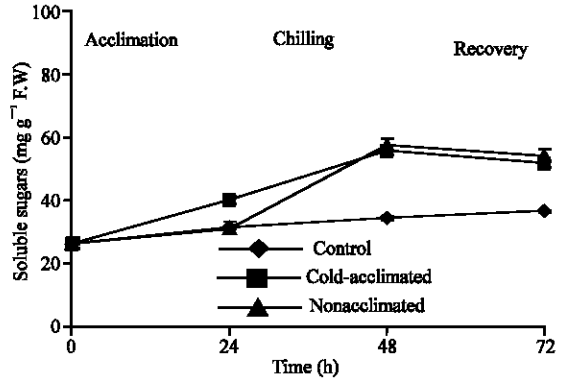

Fig. 9: Changes in root soluble sugars (mg g^{-1} F. W) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

Soluble sugars function as cryoprotectants and osmolites that protect cells from freezing damage (Xin and Browe, 2000; Huixia shou *et al*., 2004) showed that 24 h and 48 h cold acclimations significantly increased the soluble sugar levels in plants. Accumulation of sucrose and other simple sugars that occurs with cold acclimation also contributes to the stabilization of membrane as these molecules can protect membranes against freeze-damage because increase in sugar content lowers the freezing point of cell solution (Shilpi, 2005). Especially sucrose can increase the effectiveness of the heat stable proteins in preventing protein denaturation (Robertson *et al*., 1994). Soluble sugar levels were increased in roots. In cold-acclimated roots, soluble sugars content were started to increase in acclimation phase and downed in recovery phase. But in nonacclimated roots they increased in chilling phase suddenly (Fig. 9). In leaves changes were significant (Fig. 10). Soluble sugars content in

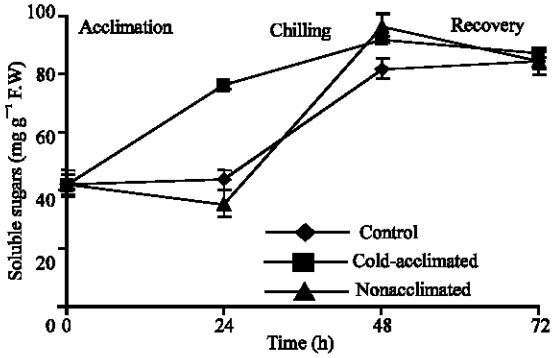

Fig. 10: Changes in leaf soluble sugars (mg g^{-1} F. W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurements±SE. p = 0.05

nonacclimated leaves, in acclimation phase, decreased, then increased suddenly in chilling phase and subsequently decreased in recovery phase.

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