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The Influence of Cold Acclimation on Proline, Malondialdehyde (MDA), Total Protein and Pigments Contents in Soybean (*Glycine max*) Seedlings

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Abstract: Low temperature damage is a common problem for early-planted soybean, because it is a tropical plant and is sensitive to low temperatures. In this research plant's response to cold acclimation and nonacclimation was investigated in soybean (*Glycine max*). Seedlings were exposed to 15°C (cold-acclimated) or 25°C (nonacclimated) for 24 h, under 250 μmol m^{-2} s^{-1} Photosyntetically Active Radiation (PAR). Then, all plants were exposed to chilling temperature at 4°C for 24 h and allowed to recover at 25°C for 24 h. Physiological responses to chilling, including, MDA, proline, chlorophyll a and b and carotenoids and total protein contents were measured in soybean to identify mechanisms of chilling tolerance. Relative water content showed that cold-acclimated plants were less affected by chilling compared to nonacclimated plants. Cold-acclimated plants also recovered faster from chilling injury than nonacclimated plants.

Key words: Acclimation, chilling, chlorophyll a and chlorophyll b, carotenoids, lipid peroxidation, proline, protein

INTRODUCTION

Each plant species has its unique set of temperature requirements, which are optimum for proper growth and development. Low temperature is one of the abiotic stresses that are principal cause of crop failure world wide, dipping average yields for most major crops (Bray *et al*., 2000). Many plants, especially those, which are native to warm habitat, exhibit symptoms of injury when exposed to low non-freezing temperatures. (Lynch, 1990). These plants include maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), tomato (*Lycopersicon esculentum*) and banana (*Musa* sp.) which are particularly sensitive to temperatures below 10-15°C and exhibit signs of injury (Lynch, 1990; Hopkins, 1999). The symptoms of stress induced injury in these plants appear from 48 to 72 h, later, however, this duration varies from plant to plant and also depend upon the sensitivity of individual plant to cold stress. (Shilpi *et al*., 2005). It is now known that exposure of chilling-sensitive plants, such as maize and tomato, to temperatures slightly above chilling reduces chilling injury (Anderson *et al*., 1995; Gilmour *et al*., 2000; Scebba *et al*., 1999; Prasad, 1996; Scebba *et al*., 1999; Venema *et al*., 2000). It has been reported that some chilling-sensitive plants acclimate if they are exposed to a low temperature slightly above the threshold chilling temperature, in a process analogous in some respect to the acclimation that occurs in perennial plants in the autumn (Daie and Campbell, 1981). Several factors involve in cold acclimation, such as plant hormones, especially Abscisic Acid (ABA), Ethylene (ET) and Gibberellic Acid (GA), proteins and carbohydrates (Annikki and Palva, 2006). Microtubules are key candidates for pronounced cold sensitivity of cell growth and depolymerize in response to low temperatures (Mizuta *et al*., 1995). During low temperature, ABA level raises. Elevated levels of ABA prevent microtubular destruction, which appears in response to chilling (Wang and Nick, 2001). Ethylene appears to be involved in cold acclimation in some plants, because of its increased levels during cold acclimation and ability of endogenous ethylene to induce number of antifreeze proteins (Yu *et al*., 2001). Gibberellic acid has been suggested to function as an ABA antagonist during cold acclimation (Annikki and Palva, 2006). Since soybean is sensitive to chilling temperatures, so, we investigated the effect of low temperature pretreatment on soybean's tolerance to chilling, which is one of the strategies to protect plants from chilling damage.

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 and then were germinated in Petri dishes on two layers of filter paper for 48 h at 25°C in an incubator. Subsequently the seedlings were transferred to pots containing washed sand (4 seedlings per pot) and were

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watered with half-strength Hoagland nutrient solution. The plants were grown at 27/25°C (day/night) temperature, 70% relative humidity, with a 16/8 h 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. Means were separated by Tukey Multiple Range Test at p = 0.05. Values are the mean±SE of three replicates.

Lipid peroxidation: Lipid peroxidation was estimated by the level of Malondialdehyde (MDA) production by a slight modification of the Thiobarbituric Acid (TBA) method described by Buege and Aust (1978). Absorbance at 532 nm was recorded and corrected for nonspecific absorbance at 600 nm. MDA concentrations were calculated by means of an extinction coefficient of 156 $mM^{-1}cm^{-1}$ and the following formula:

$$\text{MDA } (\mu\text{mol g}^{-1} \text{ fresh wt.}) = [(A_{532}-A_{600})/156]\times 10^{3}\times \text{dilution factor}$$

(Zhan Yuan and Bramlage, 1992).

Proline content: Free proline content was estimated using the acid ninhydrin method (Bates 1973). 150 mg of plant tissues (leaves and roots) was grounded in a mortar and pestle with 6 mL of 3% (w/v) sulfosalicilic acid aqueous solution and the homogenate was filtered through Whatman No. 1 filter paper, then 2 mL of the filtered extract was taken for the analysis to which 2 mL acid ninhydrin and 2 mL of glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1h and the reaction was finished in an ice bath. 4 ml of toluene was added to the reaction mixture and the organic phase was extracted, in which a toluene soluble reddish chromophore was obtained, which was read at 520 nm using toluene as blank by UV-visible spectrophotometer (WPA model S2100).

Total protein content: Total protein content was measured using Lowry method (Lowry *et al*., 1951).

Chlorophyll a and b and carotenoids content: Chlorophyll a (C_a) and b (C_b) and carotenoids (C_{x+c}) content were measured using Lichtentraler and Wellburn (1983) method. The absorbance of resulting supernstant was recorded at 470, 645 and 662 nm using UV-visible spectrophotometer (WPA model S2100). the amount of chlorophyll a and chlorophyll b and carotenoids were measured using the following formulas:

$$C_a = 11.75\times A_{662}-2.350\times A_{645}$$

$$C_b = 18.61\times A_{645}-3.960\times A_{662}$$

$$C_{x+c} = 1000\times A_{470}-2.270\times C_a-81.4\times C_b/227$$

RESULTS AND DISCUSSION

The increase in chilling tolerance that occurs with cold acclimation, is thought to involve the activation of multiple chilling tolerance mechanisms. Here we showed that changes in multiple metabolites such as total protein, proline, soluble sugars and MDA contents that are commonly observed to occur in plants during cold acclimation. There is evidence to indicate that each of these classes of biochemical alternations proline (Carpenter and Crowe, 1988; Nanjo *et al*., 1999) and MDA (Kacperska, 1989; Williams *et al*., 1988) protein (Annikki welling *et al*., 2002; Kee-Young Kim *et al*., 2004; Shilpi, 2005) contribute to an enhancement of chilling tolerance.

Proline accumulates in higher plants in response to various biotic and abiotic stresses such as water deficts and salinity and chilling stress (Stewart, 1981; Hanson and Hitz, 1982; Rhodes, 1987; Delauney and Verma, 1993; Samaras *et al*., 1995; Taylor, 1996; Rhodes *et al*., 1999; Sarah S. Gilmour *et al*., 2000) plays a major role in antioxidative stress as a hydroxyl radical scavenger (Matysik *et al*., 2002) regulation of NAD^+/NADH ratio (Alia and Saradhi 1993) and as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995). At low temperatures, proline accumulates in plants (Van Swaaij *et al*., 1985; Gilmour *et al*., 2000). It has been reported that proline content was increased in the leaves of potato hybrids when the plants were subjected to cold acclimation treatment (Van Swaaij *et al*., 1985). In our research proline accumulated when the plants were transferred to chilling temperature (4°C). In cold acclimated plants proline content was higher than nonacclimated plants and cold acclimated plants recovered faster than nonacclimated ones. So, because of proline's protective role in plants in stress conditions, we can say, cold acclimated plants could tolerate chilling temperature better than nonacclimated plants. In leaf samples, amount of praline

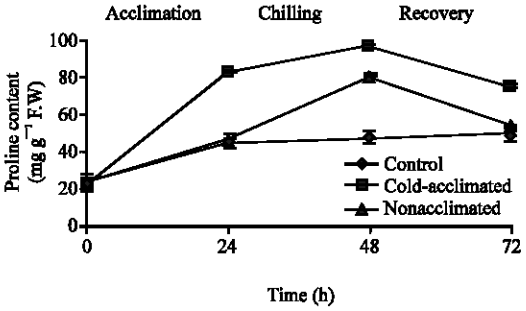

Fig. 1: Changes in root proline content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurments±SE. p = 0.05

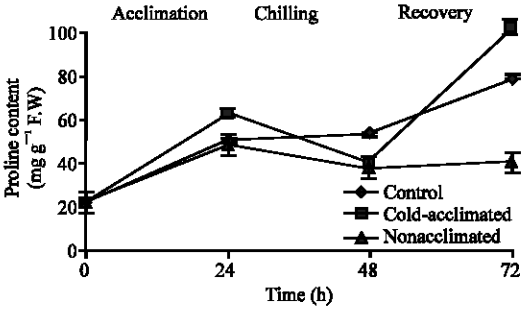

Fig. 2: Changes in leaf proline content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE. p = 0.05

increased in acclimation specially in cold acclimated plants, but it decreased in chilling phase and again increased in recovery phase (Fig. 1-2).

Cold acclimation proteins may play a physiological role similar to that of heat shock proteins (HSPs) (Key *et al*., 1981) in protecting organisms from injury at low temperatures (Chen *et al*., 1991). Genes encoding Early Light-Induced Protein (ELIP) were found to be the most highly upregulated in cold acclimated plants (Wei *et al*., 2005). HSPs in cells are vital for increasing thermotolerance (Yeh *et al*., 1997; Annikki *et al*., 2002). Heat shock proteins are associated with plasmalemma and are thought to be physiologically important in reducing cellular leakage of solutes in soybean seedlings (Lin *et al*., 1984). Like HSPs, cold acclimation proteins are associated with nuclei, mitochondria and ribosomes (Chen, unpublished data), which may explain why lower amount of amino acids and ions were found in the leakage of, chilled, cold acclimated seedlings than in that of chilled, nonacclimated seedlings. Chang *et al*. (2000)

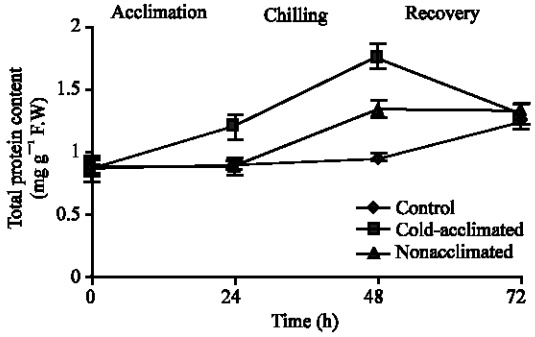

Fig. 3: Changes in root total protein content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean roots during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0.05

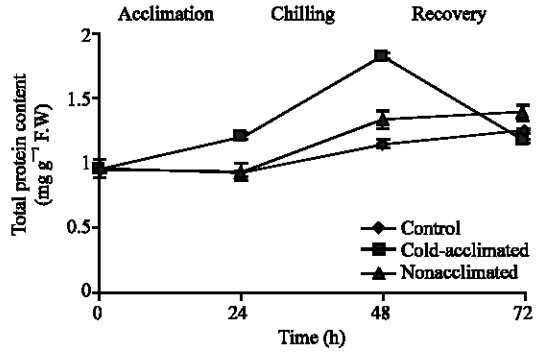

Fig. 4: Changes in leaf total protein content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0.05

showed that proteins in the cell sap of cold acclimated mungbean seedlings were about 60% higher than the control seedlings. In this research, total protein content was increased in cold-acclimated and nonacclimated plants. Cold-acclimated plants recovered faster than nonacclimated plants in recovery phase (Fig. 3-4).

The lipid membrane is composed of a mixture of phospholipids and glycolipids that have fatty acid chains attached to carbon 1 and 2 of the glycerol backbone by an ester linkage. The peroxidation reactions differ among these fatty acids depending on the number and position of the double bounds on the acyl chain. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products (Bradley and Minn, 1992) such as Malondialdehyde (MDA). In this research, MDA content of nonacclimated leaves was higher than cold acclimated ones in recovery phase. Cold acclimated plants recovered faster than nonacclimated leaves. It shows that

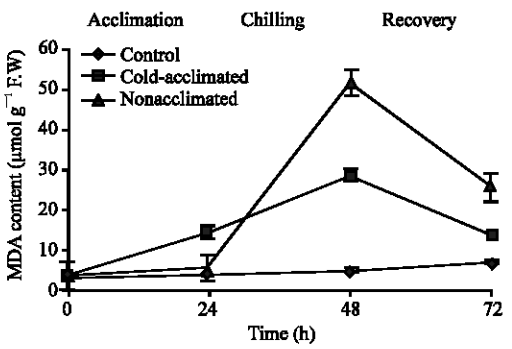

Fig. 5 Changes in leaf MDA content (µmol g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0.05

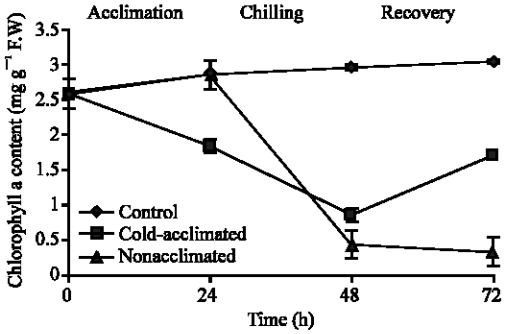

Fig. 6: Changes in leaf chlorophyll a content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0 05

pretreatment of cold temperature (15°C) can tolerate soybean plant against subsequent chilling temperature (4°C) (Fig. 5).

Carotenoids are C_{40} isoprenoids and tetraterpens that are located in the plastids of plant tissues. In chloroplasts, the carotenoids function act as accessory pigments in light harvesting, but a more important role is their ability to detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of the photosynthetic complexes by light. The xanthophylls, like zeaxanthin, are carotene derivatives. Low temperatures inhibit the formation of zeaxanthin (Bilger and Bjorkman, 1991) which normally quenches excitation energy in the antenna of photosystem II and dissipates it as heat (Demmig-Adams, 1990). Zeaxanthin production from violaxanthin is induced under normal conditions by high light and low thylakiod lumen pH, but this process is blocked at low temperatures. So

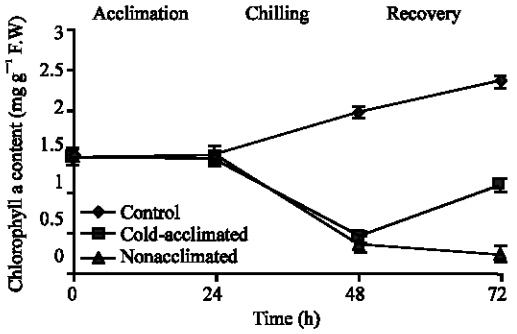

Fig. 7: Changes in leaf chlorophyll b content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0.05

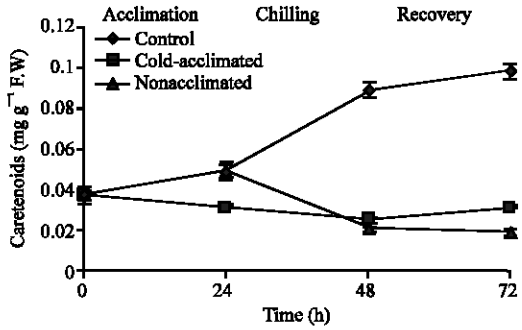

Fig. 8: Changes in leaf carotenoids content (mg g^{-1} F.W) in cold-acclimated and non-acclimated soybean leaves during acclimation, chilling and recovery. Mean of 3 measurments±SE p = 0.05

carotenoids and subsequently, chlorophyll a and b deplete after a lag time of 3 to 6 hours of exposure to low temperatures (Wise and Naylor, 1987). In present reaserch, the amount of chlorophyll a and b and carotenoids were decreased in chilling phase. In cold acclimated leaves this decrease was gradually compered with nonacclimated leaves. Cold acclimated leaves recovered better than nonacclimated leaves when they were transferred to 25°C (recovery phase) (Fig. 6-8).

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