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Antioxidants and Accumulation of α-tocopherol Induce Chilling Tolerance in *Medicago sativa*

SAMEERA O. BAFEEL AND MOHAMED M. IBRAHIM[1]†
Botany Department, Faculty of Science, King Abdel Aziz University, Jeddah, Saudi Arabia
†*Botany Department, Teacher's College, King Saud University,11491, P.O. 4341; Riyadh, Saudi Arabia*
[1]Corresponding author's e-mail: mibrahim@ksu.edu.sa; sameera12@yahoo.com

ABSTRACT

Chilling associated oxidative damage that enhanced the production of reactive oxygen species (ROS), slow down metabolism and modify membranes resulting in lipid peroxidation. A marked increase in the level of H_2O_2 was estimated in alfalfa leaves after dark chilling treatment and as a consequence, oxidation damage due to H_2O_2 accumulation could cause lipid peroxidation of membrane and result in a significant increase in malondialdehyde (MDA) content. After recovery period the MDA content decreased significantly due to the increase of phenolic compounds, which suppress lipid peroxidation. Also, the redox properties of α-tocopherol play an important role in adsorbing and neutralizing free radicals and provide some forms of antioxidant protection. Activity of superoxide dismutase (SOD) increased straight away the dark chilling stress, whereas catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities were slightly increased after chilling treatment. During the recovery period activities of CAT, APX and GR increased significantly, which restrict the recycling of active oxygen species associated with chilling stress. The results indicated that cold treatment may have initially caused injury, thereafter during the recovery period leaves coordinated and enhanced the capacity of the antioxidative system, thus diminishing the potential for active oxygen species.

Key Words: Antioxidant; Chilling; α-tocopherol; Alfalfa; Oxidative stress; Phenolic compounds

INTRODUCTION

Crop plants experience the lowest temperatures during the night and although chilling injury in the dark is not as severe as in the light, it is significant (Ting *et al*., 1991). Chilling stress adversely affects a wide range of processes, including seed germination, photosynthesis, flowering, plant growth and grain yield (Staggenborg & Vanderlip, 1996). The degree of chilling injury can vary with plant species, stage of crop development and with the conditions of irradiance and mineral nutrition during the stress (Verheul *et al*., 1996). The severity of damage due to chilling stress depends not only on conditions during chilling, but also on the environment before and after chilling, such as temperature, light conditions and water status of the plants prior to chilling and the light conditions after chilling (Smeets & Wehner, 1997; Szalai *et al*., 1996). Much of the injury to plants caused by chilling stress is associated with oxidative damage at cellular level (Bowler *et al*., 1992). Higher plants have active oxygen scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) and some non-enzymatic antioxidants, such as ascorbic acid, α-tocopherols, phenolic compounds and reduced glutathione (Bowler *et al*., 1992). In alfalfa plants Van Camp *et al*. (1996) demonstrated that Fe-SOD and Mn-SOD have different protective properties in response to chilling treatment when targeted to the chloroplast. Transgenic alfalfa (*Medicago sativa* L.) plants expressing a Mn-SOD had increased vigor after freezing stress and increased winter survival under field conditions (McKersie *et al*., 1996, 1997 & 1999). This may occur, because their biochemical properties differ or because their subcellular localization within the chloroplast is influenced by their different affinities for membranes.

Research into the mechanisms that contribute to cold tolerance in different plant species such as *Arabidopsis thaliana*, barley and wheat has led to the identification of a large number of low temperature responsive genes (Murata *et al*., 1992; Thomashow, 1999). The duration and intensity of any perturbation in redox homeostasis caused by increased reactive oxygen species (ROS) generation depends on the capacity of the major redox buffers of the plant cell, ascorbate, glutathione, carotenoids and α tocopherols. These antioxidants are present at very high concentrations in most plant organs, where they perform a variety of important biological functions (Noctor & Foyer, 1998). Ascorbate and glutathione, together with SOD and the enzymes of the ascorbate–glutathione cycle effectively

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remove superoxide and hydrogen peroxide generated as a result of photo-damage caused by oxidative stress (Foyer & Noctor, 2000). The synchronous action of antioxidant enzymes; SOD, APX, CAT GR, that operate both in the chloroplast and in the cytosol, are able to control the cellular concentrations of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), thereby preventing the formation of (ROS) (Noctor & Foyer, 1998). It has been also found that chilling stress increases APX and GR contents in *Arabidopsis* (O'Kane *et al*., 1996), whereas it inhibits the activation of catalase in rice (Fadzilla *et al*., 1996).

Lipid peroxidation increases in plants under environmental stress, including drought (Moran *et al*., 1994) and low temperatures (Prassad, 1996). The antioxidant activity of phenolic compounds, which are widely distributed in plants is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals or decomposing peroxides, suppressing lipid peroxidation (Rice-Evans *et al*., 1997; Javanmardi *et al*., 2003).

Tocopherols appear to be universal constituents of all higher plants (Threlfall, 1971; Kamal-Eldin & Appelqvist, 1996). In addition to their antioxidant function, tocopherols play a role in a range of different physiological phenomena including plant growth and development, senescence, preventing lipid peroxidation and to interact with the signal cascade that convey abiotic and biotic signals (Sattler *et al*., 2004). Tocopherols are able to scavenge lipid peroxides and oxygen radicals and to quench singlet oxygen (Horvath *et al*., 2006). The contents of ascorbic acid and α-tocopherol increased at low temperatures in chilling-tolerant cereal leaves (Streb *et al*., 1999), whereas in chilling-sensitive maize ascorbate and α-tocopherol levels decrease at low temperatures (Sattler *et al*., 2004).

Alfalfa, *Medicago sativa,* is an important crop in Egypt and Saudi Arabia (Alsuhaibani, 1996 ; Soliman Kamel and Shoukry, 2001), and is grown as a perennial and provides high quality forage and green manure. Currently there are 0.2-0.3 million ha of alfalfa planted in Egypt and acreage is rising each year (The Agricultural Research Center, Ministry of Agriculture, Egypt). However a little is known about the participation of α-tocopherols in the reaction of plants to chilling-evoked and information about antioxidant enzymes or compounds in *Medicago sativa* is not sufficient. Therefore, the aim of this research was to study the response of antioxidant enzymes and non-enzymatic antioxidant molecules of *Medicago sativa* during recovery period after exposure to dark chilling stress.

MATERIALS AND METHODS

Plant materials and treatments. Alfalfa seeds (*Medicago sativa* L. *c.v.* sewa, Egypt Agricultural Research Institute) were presoaked for five min. in 100% ethanol and washed with sterilized water. Subsequently, seeds were stirred for 30 min. in 4% sodium hypochlorite solution and then allowed to germinate on filter paper (Whatman No. 2) in a Petri dish containing distilled water for 3 days at 25°C and planted in a pot containing acid washed sand. The pots were placed in a growth chamber, 70% humidity, 25°C and light intensity of 420 μ mol m^{-2} s^{-1} with a photoperiod of 16 h at 25±2°C and a dark period of 8h at 20±2°C. After fifteen days from sowing, the pots were irrigated with half strength of Hoagland solution only up to twenty five day then the pots were divided into two sets. In the first set, the pots were transferred to a cold chamber and subjected to10°C under dark condition for 24 h, then transferred for recovery to illuminated growth chamber (420 μmol m^{-2} s^{-1}) at 25°C for 48 h under16/8 h day/night cycle. In the second set, pots containing plants without chilling treatment served as control. The third trifoliate leaves of *Medicago sativa* plants subjected to chilling or recovered were used biochemical analysis. The leaves were collected after 12 and 24 h of dark chilling and after 24 and 48 h of the recovery period.

Determination of lipid peroxidation. Malondialdehyde (MDA) was determined by spectrophotometer (Jenway 6305 UV/Vis, UK) as described by Dhindsa *et al*. (1981). The concentration of MDA was calculated using coefficient of absorbance of 155×10^6 cm^2 mol^{-1}.

Determination of total phenolics. Total soluble phenolics were determined as reported by Sgherri *et al*. (2003). Phenolics were extracted from fresh leaves (2 g) for 1 h with 50% methanol containing 1% HCl under continuous stirring at room temperature. After centrifugation at 12000 x *g* for 15 min, the supernatant was collected and the extraction was repeated three times on the pellet. Methanolic extracts were collected, dried and re-suspended in 80% methanol. A measure of total phenolics was obtained by recording A_{280}. The calculations were performed using the absorbance and by using a calibration curve for total phenolics.

Determination of tocopherols. Tocopherol content was assayed and calculated following the method of Taylor *et al*. (1976). After saponification of the sample with KOH in the presence of ascorbic acid non-saponifiable lipids were extracted with n-hexane. The organic layer was measured at 280 nm and 310 nm wavelengths.

Determination of H_2O_2. The H_2O_2 level was measured colorimetrically as described by Mukherjee and Choudhuri (1983). $H_2O_2$was extracted from homogenizing of leaf tissue (0.5 g) with 3 mL of phosphate buffer (50 mm, pH 6.5). The homogenate was centrifuged at 6,000 *g* for 25 min. Three ml of supernatant was mixed with 1 mL of 0.1% titanium sulfate in 20% H_2SO_4 (v/v). The mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1}cm^{-1}$.

Extraction of antioxidant enzymes. Leaves of control and treated seedlings (2-3 g) were homogenized in 100 mm potassium phosphate buffer (pH 7.8) containing 0.1 mm ethylenediamine-tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP) and 0.5% (v/v) Triton X-100

at 4°C. In case of APX activity however, leaves were homogenized in 100 mm sodium phosphate buffer (pH 7.0) containing 5 mm ascorbate and 1 mm EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18000 *g* for 20 min. at 4°C. The supernatant was used for determination of antioxidant enzyme activities. Protein content was measured according to the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) as a standard.

Assays for antioxidant enzymes activities. Determination of SOD activity was performed by the method of Beyer and Fridovich (1987). The reaction mixture containing 50 mm potassium phosphate buffer (pH 7.8), 9.9 mm methionine, 57 µM nitroblue tetrazolium (NBT) and the appropriate volume of plant extract and was initiated by light illumination. One unit of SOD is defined as the amount of enzyme, which causes a 50% decrease of the SOD-inhabitable NBT reduction.

Catalase activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient 39.4 mm cm^{-1}) at 240 nm following the method of Aebi (1974). The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and plant extract in a 3 mL volume. The reaction was initiated by adding 10 mm H_2O_2. One unit of catalase is defined as the amount of enzyme, which liberates half the peroxide oxygen from 10 mm H_2O_2 solution in 100 second at 25°C.

APX was assayed as described by Chen and Asada (1989) with slight modifications. A 2 mL reaction mixture contained 80 mm phosphate (pH 7.0), 0.5 mm ascorbate and 0.25 mm H_2O_2. The oxidation of ascorbate was recorded at 290 nm for 3 min. after adding 100 µL of leaf extract.

GR was assayed as described by Rao *et al*. (1996). A 2 mL reaction mixture contained 50 mm Tris–HCl (pH 7.5), 0.15 mm $NADPH_2$, 0.5 mm oxidized glutathione (GSSG) and 3 mm $MgCl_2$. The oxidation of $NADPH_2$ was recorded at 340 nm for 3 min after adding 200 µL of leaf extract.

Statistical analysis. The experiments were conducted in completely randomized design. Results are the mean of four measurements per treatment. The significance of difference between means values was determined by one-way analysis of variance. LSD was used to compare the means of treatments at $P \leq 0.05$.

RESULTS

A significant increase in H_2O_2 content was observed during the period of dark chilling stress, whereas with further progression of the recovery for previously dark chilled plants, however, H_2O_2 concentration continued to decrease, up to a level 65% lower than that of stressed plants after 48 h recovery (Fig. 2A). Oxidative damage to lipids estimated as malondialdehyde (MDA) content was significantly increased in response to dark chilling stress, while its content decreased significantly after completion of the recovery period, reaching 48% less than that of the 24 h dark chilled plants (Fig. 1B). Total phenolics was slightly increased after chilling stress in comparison to control; whereas during recovery period a highly significant increase was estimated after 24 h recovery treatment and reached 149% of the dark chilled plants. No significant difference was observed in total phenolics in control plants over the incubation period (Fig. 1A). Chilling stress increased significantly α-tocopherol concentration in stressed-leaves. On average, concentrations range from 5 to 100 µg α-tocopherol per g dry weight. As shown in Fig. 2B, total tocopherols concentrations found in the leaves at the start of chilling treatment are approximately 8.3 ± 0.6 µg tocopherol per g dry weight and increased significantly to 88.6 ± 3.8 µg tocopherol per g dry weigh by the end of chilling treatment. After one day of the recovery period total tocopherol concentrations dropped to 48.4 ±5.8 µg tocopherol per g dry weight and by the end of experimental period declined to 27.6± 0.6 µg tocopherol per g dry weight, which was still higher than the control values.

In comparison to the control, chilling stress induced a significant increase of the total SOD activity, whereas after 48 h of recovery the plants reached almost the same activity as control plants did (Fig. 3A). Activities of catalase and peroxidase were monitored at 12 and 24 h of chilling stress and after 24 and 48 h of the recovery period (Fig. 3 B & C). The foliar levels of catalase activity were decreased by chilling stress compared with the control. After recovery, of the previously dark chilled plants, the activity was significantly higher than the level at chilling stress. APX activity was enhanced in chilling stressed plants as compared with control plants. After 24 h of recovery treatment, the level of APX activity was significantly higher than that at chilling stress. The pattern of changes in APX activity was very similar to that of changes in H_2O_2 content (Fig. 1C). GR is an essential catalyzer in the conversion of H_2O_2 in order to maintain the redox state of ascorbate and glutathione. The foliar levels of GR activity were significantly increased by chilling stress as compared with the control. After the recovery until 48 h, the enzyme activity was significantly increased, but the level of enzyme activity was higher than the level at chilling stress in alfalfa leaves (Fig. 3D).

DISCUSSION

Reactive oxygen species induced by chilling stress, trigger a series of deleterious processes, such as lipid peroxidation and degradation of proteins and nucleic acids in the cell (Fridovich, 1978) and for chilling stress in plants, several secondary metabolites that include phenolics, accumulate to mediate these stresses (Christie *et al*., 1994). Therefore the rapidity with which these plants were able to recover from the chilling stress indicates some kind of antioxidant protection. Our results suggested that higher phenolic content, especially after recovery of the chilling stress, resulted in a greater antioxidant capacity (Fig. 1A). The relationship between antioxidant capacity and chilling tolerance of plants has been clarified and reported by Foyer

Fig. 1. Changes in phenolics (A) and MDA (B) contents in leaves of control and stressed plants of alfalfa leaves subjected to dark chilling (10°C) for 24 h and then transferred to 25°C at 420 µmol m^{-2} s^{-1} for another 48 h to allow recovery from dark chilling stressed. The controls for these measurements are non-chilled leaves. Each value represents the mea ± SE of five replicates. Significant differences (P ≤ 0.05) between treatments according to the LSD test are shown by an asterisk

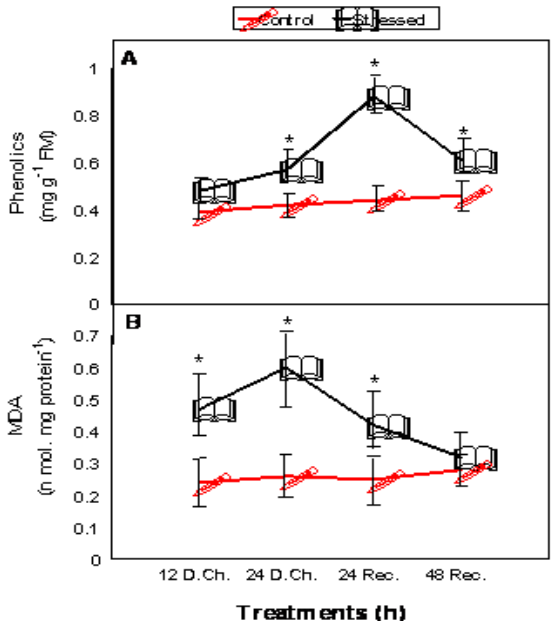
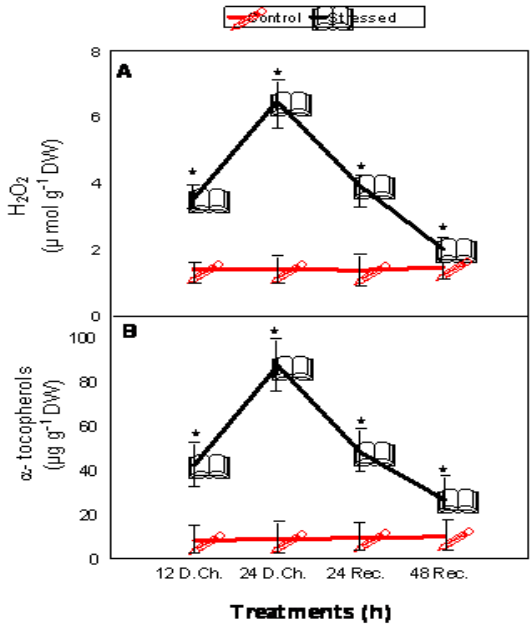


Fig. 2. Changes in H_2O_2 (A) and α-tocopherols (B) contents in leaves of control and stressed plants of alfalfa leaves subjected to dark chilling (10°C) for 24 h and then transferred to 25°C at 420 µmol m^{-2} s^{-1} for another 48 h allow recovery from dark chilling stress. The controls for these measurements are non chilled leaves. Each value represents the mean ± SE of five replicates. Significant differences (P ≤ 0.05) between treatments according to the LSD test are shown by an asterisk



et al. (2001).

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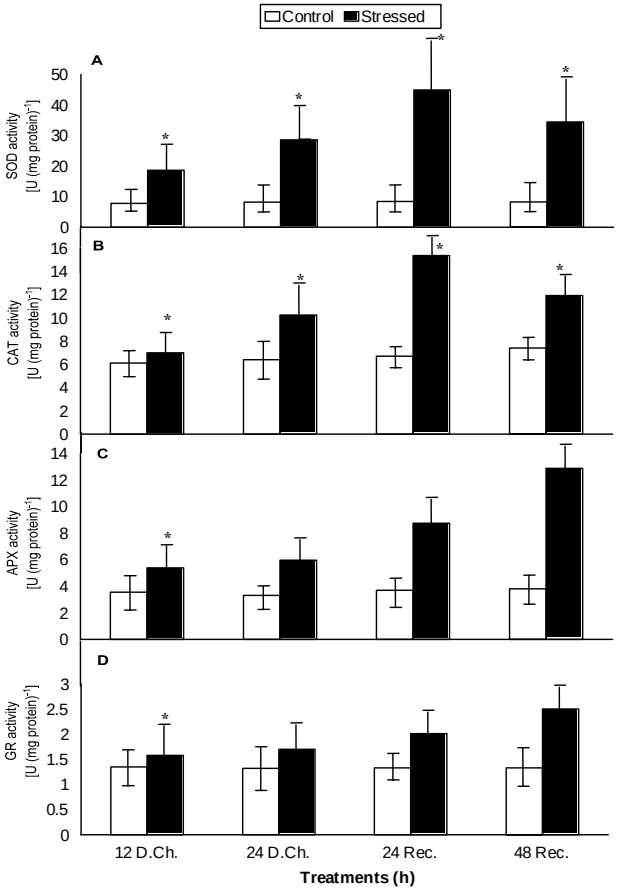
The result of the present investigation indicated that chilling treatment had a greater positive effect on tocopherol in leaves and was associated with the enhancement of lipid peroxidation (Fig. 1A). Therefore, tocopherol may be involved in the protection of the shoot tissues against oxidative stress resulting from chilling. It appears that the enhancement of tocopherol concentration in the alfalfa plants after chilling treatment (Fig. 2B) may be at least partly resulted from the degradation of chlorophyll (Ibrahim & Bafeel, 2007). Both tocopherol and chlorophyll contain a phytyl moiety as part of their molecules and chlorophyll-derived phytol has been suggested to be a substrate for the biosynthesis of tocopherols (Munné-Bosch & Alegre, 2002). Recently, phytol and phytyl-phosphate kinases have been shown to participate in this alternative way of tocopherol production (Gajewska & Sklodowska, 2007). Elevated levels of tocopherol have previously been reported in response to abiotic stresses (Horvath *et al*., 2006).

The activities of the antioxidant enzymes involved in scavenging of H_2O_2 such as CAT, APX and GR activities (Fig. 3B, C & D), as well as, phenolics and tocopherol contents, are regulated in alfalfa leaves by chilling treatment, and play a protective role during the recovery period following the exposure of plants to chilling stress, this indicated by the significant increase in phenolics and tocopherols concentrations during the recovery period (Fig. 1A & Fig. 2B). An additional explanation for our observations is that the enhanced scavenging ability for H_2O_2 in alfalfa plants during the recovery period inhibited the accumulation of reactive oxygen species and thus protected alfalfa plants from lipid peroxidation of membrane systems and oxidative damage under chilling stress (Fig. 2A), as was previously proposed by (Fadzilla *et al*., 1996; O'kane *et al*., 1996). This may have enabled the recovered plants to allocate more non-enzymatic antioxidants to the leaves compared with the control plants. Stress-induced increases in cytosolic APX transcript levels have been reported in pea (Mittler & Zilinskas, 1994). The increased APX activity in cold-conditioned jack pine could be a consequence of the increase in cytosolic GSH/GSSG ratio during conditioning. The changes of GR activity during cold conditioning were correlated with the changes in GSH/GSSG mole ratios. This

Fig. 3. Changes in SOD (A), CAT (B), APX (C) and DR (D) activities in dark adapted leaves of control and stressed plants of alfalfa leaves subjected to dark chilling (10°C) for 24 h and then transferred to 25°C and 420 µmol m^{-2} s^{-1} for another 48 h to allow recovery from dark chilling stress. The controls of these measurements are non-chilled leaves. Each value represents the mean ± SE of five replicates. Significant differences (P ≤ 0.05) between treatments according to the LSD test are shown by an asterisk



may suggest that cytosolic GR plays a role in the regulation of the oxidation-reduction status of glutathione. During the recovery period, these enzymes could protect the cells against the excessive reactive oxygen species production following exposure to chilling stress. The observed results that increased SOD activity in leaves of the recovered alfalfa plants was positively related to the improvement in winter survival of alfalfa. This assumption was supported by the original hypothesis of (Bryan *et al*., 2000) that perennial and winter annual plants experience oxidative stress during winter and that antioxidant defense systems contribute to winter hardiness.

In summary, the results in the present study suggest that high cellular levels of H_2O_2 accumulated during the dark chilling treatment can induce the activation of a defense mechanism against chilling stress or programmed cell death. The accumulation of H_2O_2 can be induced by the increase in SOD activity. Therefore, during the recovery treatment the accumulated H_2O_2, in turn, may activate a protective mechanisms that increase the activities of several antioxidant enzymes such as APX, CAT and GR .Also induce alterations in the relative concentration of several non-enzymatic antioxidant compounds such as phenolics and tocopherols.

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