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Photosynthetic Efficiency and Pigment Contents in alfalfa (*Medicago sativa*) Seedlings Subjected to Dark and Chilling Conditions

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ABSTRACT

The functional activities of the photosynthetic apparatus of alfalfa (*Medicago sativa* L.) was investigated after 24 h of dark chilling treatment at 10°C and after 48 h recovery period at 25ºC and 420 µmol m^{-2} S^{-1}. Dark chilling limits photosynthesis due to chilling-associated oxidative damage, which enhanced the production of active oxygen species (AOS). Dark chilling treatment resulted in a significant reduction in the CO_2 assimilation rate and decreased stomatal conductance. Stomatal limitation was largely responsible for the inhibition of CO_2 assimilation generated by affections in the PSII functioning and also in the Calvin cycle. Photosynthetic parameters and chlorophyll fluorescence analyses showed the same degree of inhibition during the dark chilling period. However, chlorophyll fluorescence analysis showed an increase in the process of energy dissipation in the dark chilled leaves. In addition, leaves showed a significant decrease in chlorophyll a:b ratio and higher carotenoids-to-total chlorophyll ratio providing photoprotection. Under recovery, leaves recovered photosynthetic activity thus diminishing the potential power for AOS generation.

Key Words: Chilling; Oxidative stress; Fluorescence; Stomatal conductance; Alfalfa

INTRODUCTION

Photosynthesis is known to be one of the most temperature-sensitive processes and it can be completely inhibited by low temperature before other symptoms of stress are detected (Berry & Björkman, 1980). Photosynthesis is severely affected for several days following a single cool night (Kingston-Smith *et al.*, 1997). Because photosystem II (PSII) is the most vulnerable component of the photosynthetic machinery, Fv/Fm is often used as an indicator of cellular stress and can indicates the overall health of photosynthetic machinery and its efficiency (Bazzaz, 1995; Maxwell & Johnson, 2000). Thus, chlorophyll fluorescence is sensitive and reliable method for detection and quantification of chilling induced changes in the photosynthetic apparatus (Van Heerden *et al.*, 2003).

The photosynthetic capacity declines in chilling-susceptible plants exposed to low temperatures, and this decline is related to a decrease in the quantum efficiency of PSII and the activities of photosystem I (PSI), the ATP synthase and the stromal enzymes of the carbon reduction cycle (Allen, 2001). There are varying degrees of photosynthetic susceptibility among alfalfa genotypes in response to light and temperature for growth (Barnes *et al.*, 1995). During the day, high air temperature of 27°C is optimum for seedling growth but optimum night temperature declines to 22°C as shoots develop (Frame *et al.*, l998). However, the flux through these side reactions is enhanced under the effect of chilling, increasing the risk of oxidative damage, that generate active oxygen species (AOS); namely singlet oxygen, superoxide, hydrogen peroxide and the hydroxyl radical (Foyer & Harbinson, 1999). AOS may cause a cascade of oxidative reactions of cellular components and they have been implicated in photoinhibition and cellular damage in chilling-susceptible plants exposed to low temperatures (Hull *et al*., 1997).

Inhibition of photosynthesis caused by chilling was particularly well documented in previous studies and reported a decrease in CO_2 assimilation rate and stomatal conductance in different plant species (Smirnoff, 1993; Van Heerden *et al*., 2003). The different sensitivity to chilling stress among plant is due in part to a differing antioxidant system. Carotenoids increased in response to abiotic stress, not only play a role as accessory light pigments but they also protect photosynthetic systems against active oxygen species (Asada *et al*., 1998; Loggini *et al*., 1999). Chilling-tolerant-varieties at low temperature showed a greater amount of carotenoids than chilling sensitive ones (Hodges *et al.*, 1997). The inactivation of photosynthesis by dark chilling treatment

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is also related to the damage of the membrane. According to (Blum & Ebercon, 1981), membrane instability is a primary symptom of chilling injury. In this sense, the conductivity test, based on the electrolyte leakage, has been proposed as a good indicator of chilling resistance (Shanahan *et al*., 1990; Ristic *et al*., 1996).

Alfalfa is grown in Egypt in arid and semiarid regions, and provides high quality forage and green manure (Kamel & Shoukry, 2001). Therefore, the purpose of our investigation was to provide a novel insight into the effect of dark chilling stress on photosynthetic activity and subsequent recovery in alfalfa.

MATERIALS AND METHODS

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 placed on a sterilized beaker with a 4% sodium hypochlorite solution and stirred for 30 min (Davis & Zhang, 1991). Seeds were grown in the greenhouse in plastic pots (15 cm diameter x 20 cm height). The pots were furnished with a hole at the bottom. Seeds were soaked in continuously aerated distilled water for 24 h in darkness. At the end of soaking period, twelve seeds were sown in each pot containing 1500 g sterilized sandy soil for twenty five days under 16/8 h day/night cycle. Light intensity was 420 µmol m^{-2} s^{-1} at the canopy of plant supplied by a mixture of fluorescent and incandescent lamps and at controlled temperature of 25/21˚C and 60/65 RH. After 25 d in pots, plants were transferred to 250 mL jars containing half strength Hoagland solution. For exposure to chilling, half the jars were transferred to a cold chamber and subjected to 10°C under dark for 24 h and then after completion of chilling treatment the same stressed plants were transferred to the illuminated growth chamber (420 µmol m^{-2} s^{-1}) at 25°C for 2 days under 16/8 h day/night cycle. The second set of jars containing plants are served as control without chilling stress. The third trifoliate leaves of alfalfa plants were subjected to chilling or recovered were used as the experimental materials. Trifoliate leaves of untreated plants were wrapped in aluminum foil to serve as a dark control. The leaves were collected after 12 h and 24 h of dark chilling stress and after 24 and 48 h of the recovery period.

Photosynthetic pigments were extracted from fresh samples ground at a low light intensity in 10 mL 80% acetone at 4°C. Absorbance of centrifuged extracts was measured with a spectrophotometer (Jenway 6305 UV/Vis, UK) at specified wavelengths required for computation of chlorophyll a, b and carotenoids from published formula (Lichtenthaler, 1987). Pellets remaining after centrifugations were dried at 60°C and weighed. Photosynthetic pigments contents were expressed as µg g^{-1} DM. Chlorophyll fluorescence were measured on the same leaves used for gas-exchange using a chlorophyll fluorometer (Model PAM 101, Watz, Effelrich, Germany). Stressed and control leaves were pre-darkened for 40 min before starting the measurements. The conceptual approach and detailed analytical derivation of the various parameters have been previously described by Maxwell and Johnson (2000). Photosynthetic rate [CO_2 assimilation rate (PN)] and stomatal conductance for water vapor (gs) were measured, one hour after the start of the recovery period following dark chilling, in fully expanded trifoliate leaves of ten control and dark chilled plants using a portable photosynthesis system (model LCA4, Analytical Developmental Co., Hoddesdon, UK), attached to an infra-red gas analyzer (Li-COR LI-6250) and a data logger, following the procedure of Foyer *et al*. (1994).

Chilling tolerance was estimated based on electrolyte leakage measured by conductivity meter (4010 data. Hani. Instruments, Padova, Italy) as described by Dicagno *et al*. (1999) with slight modification. Leaf disks (1 cm) were collected and washed with de-ionized water to remove surface electrolytes and incubated overnight at 25°C, with gentle shaken on a gyratory shaker. After measurements, the tubes were placed in boiling water for 15 min and then cooled to room temperature and shaken for 2 h, and conductivity determined again. The electrolyte leakage was calculated as the ratio of conductivity before boiling to that after boiling.

Statistical analysis. The experiment was conducted in completely randomized design. Gas exchange, fluorescence and electrolyte leakage results are the mean of ten independents measurements in each treatment. Pigments content values are the mean of four measurements per treatment. Significance of difference between means values was determined by one-way analysis of variance. Duncan's multiple range test was used to compare the means.

RESULTS

Chilling stress altered the chlorophyll and carotenoids contents in the treated plants (Fig. 1). A decrease in chlorophyll a:b ratio was shown in dark chilled plants, which was mainly caused by the decrease in chlorophyll a more than the decrease in chlorophyll b (data not shown). The carotenoids content increased significantly throughout the experimental periods, and thus carotenoids-to-chlorophyll ratio increased significantly in both stressed and recovered plants with respect to the control (Fig. 1B).

Dark chilling treatment also modified the chlorophyll fluorescence emission in stressed plants. Thus, initial fluorescence (F0) and (Fv) values were significantly reduced in treated plants at the end of dark chilling period in relation to the control treatment (Fig. 2A, B). Also maximum photochemical efficiency of PSII,

Fig. 1. Changes in total chlorophyll, chlorophyll a:b ratio and carotenoids-to-total chlorophyll ratio in leaves of control and stressed plants of alfalfa leaves subjected to dark chilling (10°C) for 24 h (12 D.Ch. 24 D.Ch.) and then transferred to 25°C at light intensity of 420 µmol m^{-2} S^{-1} for another 48 h (24 Rec. 48 Rec.) for recovery, while the controls were non chilled. Each value represents the mean ± SE of five replicates. Significant differences (P≤0.05) between treatments according to LSD test are shown by an asterisk

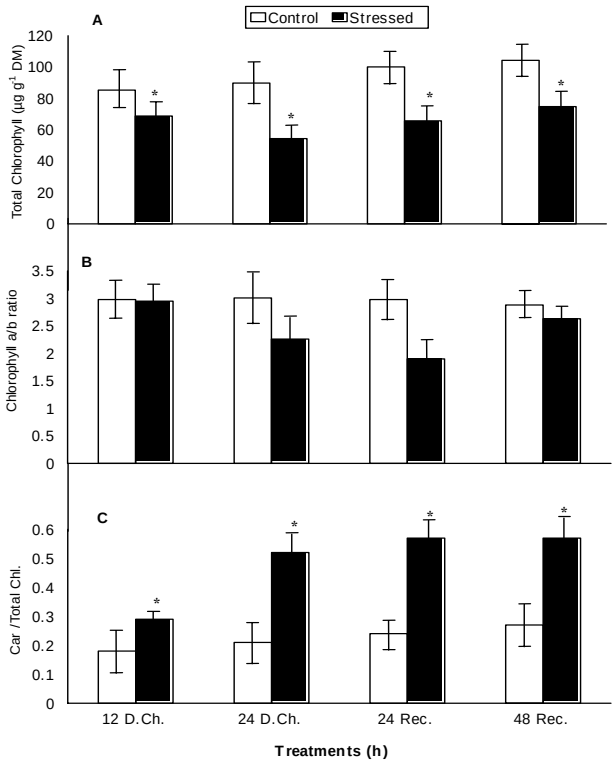
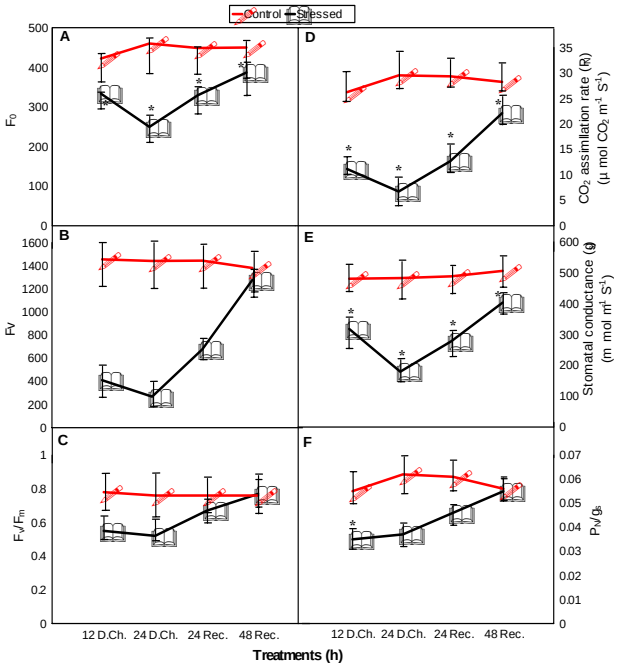


Fig. 2. Initial, FO and variable, Fv (A, B), the ratio of variable to maximum fluorescence of chlorophyll (C), net photosynthetic rate, P_N (D), stomatal conductance gs, (E), and water use efficiency, P_N/g (F) in dark adapted leaves of control and stressed plants of alfalfa leaves subjected to dark chilling (10°C) for 24 h (12 D.Ch. 24 D.Ch.) and then transferred to 25°C and light intensity of 420 µmol m^{-2} S^{-1} for another 48 h (24 Rec. 48 Rec.) to allow recovery from dark chilling stress, while the control were not chilled. 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



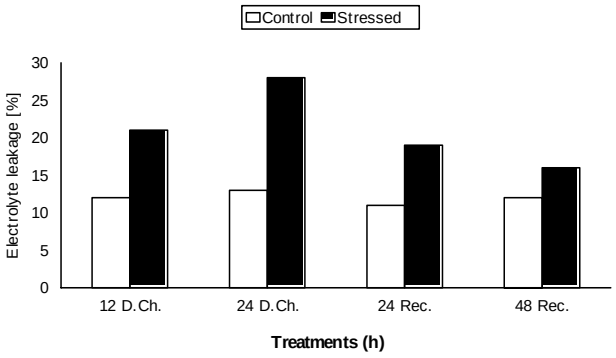
expressed as Fv/Fm (Fig. 2C) was reduced significantly in the stressed plants at the end of chilling treatment, reached to level 32% decrease in comparison with control. Whereas upon transfer of previously chilled plants to recovery condition, PSII function recovered completely after 48 h. Thereafter, Fv/Fm increased to values not significantly different to the control (Fig. 2C). The CO_2 assimilation rate (PN) measured immediately after the dark chilling period was significantly reduced (57 & 77%) in 12 and 24 h, respectively in chilled-treated-plants (Fig. 2D). Also under this condition, there was evidence of stomatal conductance (gs) influencing the inhibition of CO_2 assimilation in dark chilled treated alfalfa plants (Fig. 2E). After completion of recovery period for previously dark chilled plants, however, CO_2 assimilation rate and stomatal conductance ameliorated progressively and increased, ultimately to levels 78% and 80%, respectively relative to the control(Fig. 2D, E). Intrinsic water use efficiency (PN/gs) showed the same degree of changes in CO_2 assimilation rate and stomatal conductance during the dark chilling and recovery treatments (Fig. 2F).

The functioning of membrane was also altered by dark chilling stress. The efflux of electrolytes significantly increased in the dark chilled stressed plants, reached to approximately fourfold in comparison with control (Fig. 3). However, after the completion of recovery period for the previously chilled plants, a highly significant reduction in electrolyte leakage was observed with respect to stressed plants (Fig. 3).

DISCUSSION

The decrease of total chlorophyll and the increase of carotenoids-to-chlorophyll ratio in stressed alfalfa plants (Fig. 1A, B) suggest that these relationships could be used as indicator of tolerance and physiological status of the plant under these stress conditions. Our results showed a significant increase in the total carotenoids and higher carotenoids-to-chlorophyll ratio were observed

Fig. 3. Changes in electrolyte leakage (%) in leaves of control and stressed alfalfa leaves subjected to dark chilling (10°C) for 24 h (12 D.Ch. 24 D.Ch.) and then transferred to 25°C at light intensity of 420 µmol m^{-2} S^{-1} for another 48 h (24 Rec. 48 Rec.) for recovery. The controls for these measurements are no chilled leaves



throughout the experimental periods (Fig. 1B). Significant increase in the carotenoids content, suggest that this increase could be associated with the increased production of AOS during chilling treatment to support photoprotective mechanism. Therefore, leaves appeared to be more capable of avoiding and/or scavenge the production of AOS generated during chilling treatment. Our results are also in agreement with larger carotenoids pool in the plants grown under chilling stress (Young, 1991; Kingston-Smith *et al.*, 1997; Rahman *et al.*, 2004).

The results of this study confirm that PSII activity was drastically affected by dark chilling stress, while in recovered plants it was largely ameliorated. Usually, about half of the PSII complex must be inactivated before photosynthetic capacity becomes limited (Oquist & Malmber, 1989; How-Yeon *et al*., 1999). However, previous reports document that a slight inactivation of the PSI and PSII are closely correlated with changes in CO_2 assimilation rate (Feller *et al*., 1998; Law & Crafts-Brander, 1999). Dark chilling reduced the Fv/Fm ratio (Fig. 2C) indicating that a large portion of the PSII reaction centre was damaged. These damages are associated with structural modifications on PSII, especially in DI protein (Asada *et al*., 1998).

According to Allen *et al.* (2001), changes in the net rate of CO_2 assimilation reflect alteration in both stomatal conductance and/or mesophyll capacity for photosynthesis. In our case, the decrease in CO_2 assimilation observed in chilling-treated plants (Fig. 2D) was attributable to stomatal limitation, a significant decrease was observed in alfalfa stressed plants relative to control (Fig. 2E). Decrease in the stomatal conductance observed in treated plants indicated that the reduction in CO_2 assimilation during the chilling stress was limited by stomatal closure (Starck *et al.*, 2000).

An increase in electrolyte leakage was observed in stressed plants (Fig. 3), showing an increase in the permeability of membranes, as evident from diminished ability to retain solutes and water under dark chilling. However, in case of recovery of previously dark chilled plants, the permeability of the membrane was markedly improved (Fig. 3), indicating the maintenance of its functioning after recovery. The leakage of electrolyte has been often related to photosynthetic and mitochondrial activity reductions in plant cells (Ristic *et al*., 1996). Previous reports have documented that inefficient functioning of the membranes have been found in different species and under several stress condition (Chengkun *et al.*, 1996; Karim *et al*., 1999).

It can be concluded that dark chilling imposes metabolic limitation on photosynthesis and AOS are involved, to some degree, in the limiting photosynthetic capacity of alfalfa leaves. After recovery period the alfalfa plants showed physiological and biochemical changes that contribute to its superior dark chilling resistance and prevent the leaves from undergoing photooxidation damage and eventual death.

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