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Full Length Research Paper

The effect of light on ROS-scavenging systems and lipid peroxidation under cold conditions in saffron (*Crocus sativus* L.)

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Saffron is one of the most important crops cultivated in Iran. Because of low water demand and tolerance to winter cold as well as its high price and quality compared to that of other countries, Iranian saffron deserves closer attention. This experiment was conducted to study the effect of light under low temperature on the defense mechanisms of this plant. Corms of saffron were cultivated on the education and research farmlands of the University of Maragheh on October 1st 2007. Following the drop in weather temperature on December 23rd, 2007, some of the plants were covered in a way that sunlight could not reach them. After 6 h, the samples of saffron leaves were taken and the activity levels of superoxide dismutase (SOD, EC 1.15.1.1), catalases (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11) enzymes as well as the amount of lipid peroxidation (MDA content) in the samples were measured. The results indicated that the activity level of CAT, GR and APX in light and low temperature conditions were significantly higher than that of dark and low temperature conditions. Meanwhile, SOD activity was significantly lower in light and low temperature conditions compared to that of dark and low temperature conditions. Increased activity of CAT, GR and APX alleviated the damaging effect of light presence at a low temperature on cell membranes.

Key words: Antioxidant enzyme, lipid peroxidation, low temperature, saffron.

INTRODUCTION

Cultivated saffron (*Crocus sativus* L.) belongs to the Iridaceae family. It is sterile and propagates through its corms (Fernandez, 2004). Low water demand, tolerance to winter cold and of course expensiveness are important properties of saffron (Kafi, 2002). Iran and Spain are the major producers of saffron. Meanwhile, research findings indicate that crocin and picrocrocin contents of Iranian saffron is 5 to 10 times more than that of Spain and Indian ones (Caballero-Ortega et al., 2004). Furthermore, anti-cancer, anti-tumor and anti-depression properties of saffron have been approved by many studies (Abdullaev, 2004; Hosseinzadeh et al., 2004). Saffron can also reduce the amount of bilirubin, cholesterol and triglyceride in the blood (Duke, 1987).

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Generally, low temperatures, drought, salinity and other environmental stresses decrease the growth and development of plants. Moreover, low temperatures and other environmental stresses, often lead to the accumulation of reactive oxygen species (ROS). ROS can act as second messengers involved in the stress signal transduction pathway (Foyer and Noctor, 2005). But excessive ROS production under low temperatures can disturb plant cell metabolism (Kornyeyev et al., 2003; Yamazaki et al., 2003). ROS are partially reduced forms of atmospheric oxygen (O_2) which is produced in common processes such as photorespiration, photosynthesis and respiration (Jimenez et al., 1998; Kornyeyev et al., 2003; Taylor et al., 2003). To produce water in these processes, 4 electrons are required for perfect reduction of O_2. ROS typically results from the transfer of 1, 2 and 3 electrons respectively, to O_2 to form superoxide (O_2^-), hydrogen pero-

xide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (Mittler, 2002; Edreva, 2005). These species of oxygen are highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins and nucleic acids among others, causing lipid peroxidation, protein denaturing and DNA mutation, respectively (Scandalios, 1993; Van Breusegem et al., 2003; Quiles and López, 2004; Foyer and Noctor, 2005; Guo et al., 2006; Moller et al., 2007).

Under low temperature conditions, chloroplasts are the main source of ROS production. This production is due to the low temperature which decreases CO_2 fixation in the Calvin-Benson cycle (Yamazaki et al., 2003). Photon utilization for CO_2 fixation decreases as a result, leading to over-reduction of electron transport chain and hence over-production of NADPH, H^+. This may result in increased formation of ROS in the electron transport chain and subsequent damage to the photosynthetic system (Yamazaki et al., 2003). For example, $HO^{\cdot}$ interacts with and damages all molecular species present in chloroplasts. Singlet oxygen ($^1O_2$), a form of ROS, as well as O_2^{-} predominantly attack double bond containing compounds (unsaturated fatty acids, chlorophylls), thus damaging the chloroplast membrane system and the photosynthetic reaction centers. O_2 acts on aromatic amino acids such as tyrosine in D_1 protein and may cause destructive changes at the donor side of PSII. Calvin cycle enzymes, Fe^{2+} containing enzymes, D_1/D_2 proteins and Mn clusters in PSII have also been reported as sensitive targets to H_2O_2 (Niyogi, 1999).

Fortunately, plants have evolved various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration (Beak and Skinner, 2003). Enzymatic antioxidant system is one of the protective mechanisms including superoxide dismutase (SOD), which can be found in various cell compartments and catalyses the disproportionation of two $O_2^{\cdot-}$ radicals to H_2O_2 and O_2 (Scandalios, 1993; Khosravinejad et al., 2008). H_2O_2 is eliminated by various antioxidant enzymes such as catalases (CAT) (Kono and fridivich, 1983; Scandalios, 1993; Khosravinejad et al., 2008) and peroxidases (POX) (Jablonski and Anderson, 1982; De Gara et al., 2003; Khosravinejad et al., 2008), which convert H_2O_2 to water. Other enzymes that are very important in ROS scavenging system and function in ascorbate-glutathione and xanthophyll cycles are glutathione reductase (GR), monodehydro ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Yoshimura et al., 2000). ROS are inevitable by-products of normal cell metabolism (Martinez et al., 2001), but under optimal conditions, production and destruction of ROS are well regulated in cell metabolism (Edreva, 2005). When a plant is encountered with harsh conditions, ROS production will overcome scavenging systems and oxidative stress will burst. In these conditions, ROS attacks vital biomolecules and disturb the cell metabolism and ultimately the cell causes its own death (Sakihama et al., 2002).

As mentioned before, tolerance to low temperature is an important ability of saffron. Under these conditions the fixation of CO_2 decreases in the Calvin-Benson Cycle, which increases ROS production in the presence of light and can remarkably intensify damage to the plant. To the best of our knowledge there is no previous report about the effects of low temperature and light on saffron. This is the first study conducted to evaluate the response of this plant (saffron) to these two important environmental factors.

MATERIALS AND METHODS

Saffron corms were cultivated at 25 × 40 cm from each other on the education and research farmlands of the University of Maragheh on October 1, 2007. The soil type of the farmland was silty. The content of organic material was designated at 0.5% with a pH of 6.8. The plants flourished in 30 - 35 days. Following temperature drop on December 23, 2007, some of the plants were covered in a way to be protected from sunlight without any interference in the temperature rate. The plants underwent these conditions for 6 h after which leaf samples were taken and immersed in liquid nitrogen. The samples were preserved at -20°C until the measurement of the related parameters. Weather temperature and light intensity during samplings were 5°C and 900 $\mu molm^{-2}s^{-1}$ respectively.

Enzyme extraction

For SOD, CAT and GR extractions, leaf samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH= 7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay (Esfandiari et al., 2007b).

Enzyme activity assay

SOD activity was estimated by recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme (Sen Gupta et al., 1993). About 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.1 ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. 2 tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 µM) and placing the tubes below a light source of 2 15 W florescent lamps for 15 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal color. A non-irradiated complete reaction mixture, which did not develop color, served as blank. Absorbance was recorded at 560 nm and 1 unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

CAT activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH= 7), 0.5 ml of 75 mM H_2O_2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. The reaction started by adding H_2O_2 and a decrease in absorbance was recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

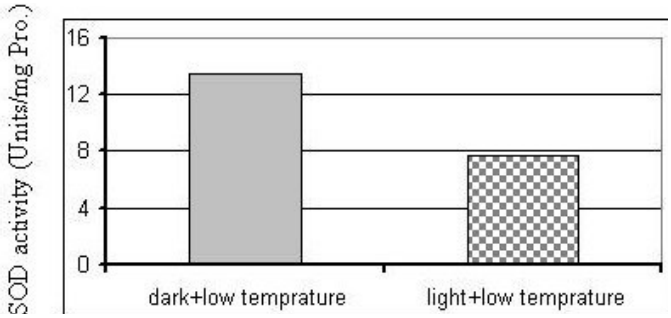

Figure 1. The effect of light and low temperature on SOD activity in saffron plant (LSD1% = 3.23).

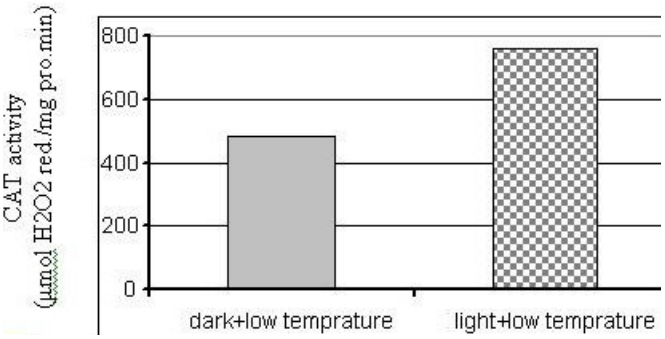

Figure 2. The effect of light and low temperature on CAT activity in saffron plant (LSD1% = 95.59).

APX activity was measured according to Yoshimura et al. (2000) by monitoring the rate of ascorbate oxidation at 290 nm (E= 2.8$mM^{-1}cm^{-1}$). The reaction mixture contained 25 mM phosphate buffer (pH= 7), 0.1 mM EDTA, 1 mMH_2O_2, 0.25 mM AsA and the enzyme sample. No change in absorption was found in the absence of AsA in the test medium.

GR activity was assayed by recording the increase in absorbance in the presence of oxidized glutathione (GSSG) and 5,5-dithiobis-2-nitrbenzoic acid (DTNB) (Sairam et al., 2002). The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer (pH= 7.5) containing 0.1 mM EDTA, 0.5 ml of 3 mM DTNB in 0.01 M potassium phosphate buffer (pH= 7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and distilled water to make up a final volume of 2.9 ml. The reaction was initiated by adding 0.1 ml of 2 mM GSSG. The increase in absorbance at 412 nm was recorded at 25°C over a period of 5 min on a spectrophotometer.

Protein content of samples was determined by the method of Bradford. Bovine serum albumin was used as a standard (Bradford, 1976).

Malondialdehyde (MDA) was measured by the colorimetric method (Stewart and Bewley, 1980). 0.5 g of leaf samples was homogenized in 5 ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in an ice bath. The samples were then centrifuged at 10000×g for 30 min. With the supernatant removed, absorption was read at 532 nm, and the amount of non-specific absorption at 600 nm was also read and subtracted from this value. The amount of MDA present was calculated from the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Enzyme activity and MDA content of samples were recorded in 7 replications. For identification of differences between various treatments, t-student's test was performed after Levene's test of equality of variances (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Study findings indicated a significant difference among antioxidant enzymes activity (CAT, APX and GR) in light and dark conditions and at low temperatures at 1% probability level. It was only SOD among the antioxidant enzymes, which exhibited lower activity in the presence of light than in darkness. Moreover, no significant difference was detected among the treatments, that is low temperature + dark and low temperature + light, in terms of lipid peroxidation ($p<1\%$) (Figure 1).

SOD catalyses the disproportionation of two $O_2^{\cdot-}$ radicals to H_2O_2 and O_2 (Scandalios, 1993; Sairam et al., 2002). Low SOD activity may lead to more $O_2^{\cdot-}$ accumulation and the occurrence of Haber-Weiss reaction, which in turn results in highly toxic levels of hydroxyl radical. On the other hand, $O_2^{\cdot-}$ radical can decrease the activity of CAT (Fridovich, 1989) and POX (Kono and Fridovich, 1983). Furthermore, the activity of some SOD isozymes is stopped due to sensitivity to high amounts of H_2O_2 (Martinez et al., 2001). All together, these events can intensify the oxidative stress in cells, though the study results here indicated the converse (Figures 2, 3 and 5). In fact, there was an increase in the activity of CAT (Figure 2) and APX (Figure 3) in the presence of light. The amount of MDA was identical for both treatments as well (Figure 5). Earlier studies reported that low SOD activity is concomitant with little damage to vital biomolecules (Vaidyanathan et al., 2003; Israr and Sahi, 2006; Esfandiari et al., 2007b). This might be the result of an increase in the amount of ascorbate and glutathione antioxidants, which can in turn, react directly with $O_2^{\cdot-}$ radical, turning them to H_2O (Vaidyanathan et al., 2003; Guo et al., 2006). Sen Gupta et al. (1993), Scandalios (1993) and Dionisio-Sese and Tobita (1998) reported that increased SOD activity is effective in improving plant's tolerance to environmental stresses. The activity of CAT and APX in saffron remarkably increased in the presence of light (Figures 2 and 3). These enzymes can completely reduce H_2O_2 to H_2O (Mittler et al., 2004; Edreva, 2005). As the activity of these enzymes increase, the tolerance of the plant would be enhanced against oxidative stress (Candan and Tarhan, 2003; Kornyeyev et al., 2003). APX is also involved in important cycles such as ascorbate-glutathione and Mehler. The ascorbate-glutathione cycle is involved in the full scavenging of H_2O_2, the utilization of reducing $NADPH,H^+$ units and the consequent supply of $NADP^+$ equivalents, as well as in the dissipation of excess excitation energy as heat. The Mehler cycle operates as a mechanism of ROS scavenging and dissipation of excess photons. In this way, these cycles minimize the overloading of the electron transport chain and contribute to normalization of the redox status in chloroplasts. By consuming $NADPH,H^+$, $NADP^+/NADPH,H^+$ ratio increases in the chloroplast and electron transport

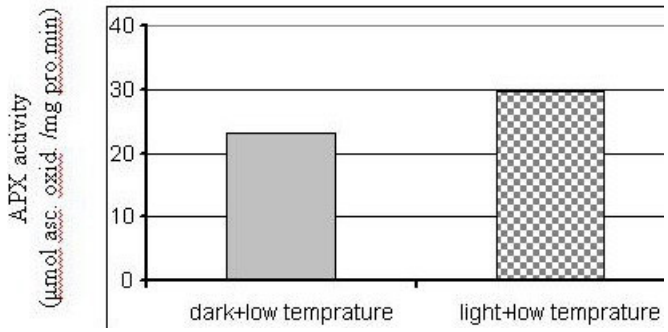

Figure 3. The effect of light and low temperature on APX activity in saffron plant (LSD1% = 4.98).

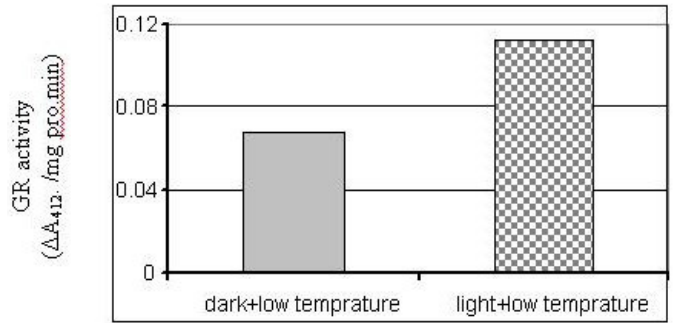

Figure 4. The effect of light and low temperature on GR activity in saffron plant (LSD1% = 0.012).

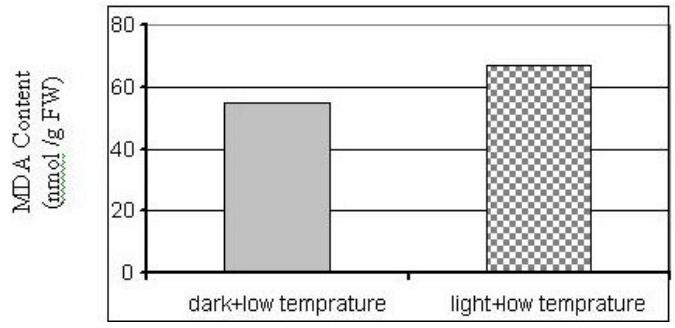

Figure 5. The effect of light and low temperature on MDA content in saffron plant (LSD1% =33.04)

occurs in common route that is, electron transport chain. As a result, the cycles significantly contribute in decreasing ROS production and damaging to membranes.

The activity of GR, an enzyme responsible for the conversion of oxidized glutathione (GSSG) into reduced glutathione (GHS) (Vega et al., 2003), increased in saffron upon exposure to sunlight (Figure 4). Higher GR activity regulates GSH/GSSG ratio and supplies GSH for GPX and DHAR, which convert H_2O_2 to H_2O and reduces oxidized ascorbate, respectively. GR acquires the reduction power from NADPH, H^+ and then dissipates this power which in turn increases $NADP^+$/NADPH,H^+ ratio. The effective involvement of GR can contribute to the efficiency of xanthophyll and ascorbate-glutathione cycles (Jiang et al., 2006; Shi et al., 2006). Higher activity of GR and APX removes the restrictions imposed on ROS scavenging and energy dissipation mechanisms systems such as xanthophyll, glutathione-ascorbate and Mehler cycles, passing the cells over more desirable conditions (Asada, 2006). The increased activity of these enzymes can help cells to maintain their redox potential, which can in turn decrease damage to membranes. Sairem et al. (2002) and Roa and Alschair (1991) reported an increase in the tolerance to environmental stresses in wheat and pea as GR activity increased. MDA is used as a biomarker to estimate damage to cell membranes (Esfandiari et al., 2007b; Xiao et al., 2008). The result of the present study showed that, presence of light under low temperatures had no significant effect on membranes damage. In other words, there was a negative relationship between the activities of CAT, APX and GR enzymes and the MDA content; these results are in accordance with the findings of Ping et al. (2006), Esfandiari et al. (2007a,b) and Esfandiari et al. (2008). The reason that saffron membranes underwent no damage when exposed to light under low temperature is explained by increased activities of GR, CAT and APX. Actually, these enzymes activities bring ROS production and scavenging systems activity into a balance, hence preventing oxidative stress in the cells. Finally, damage to membranes is decreased and the cell is rendered into more desirable conditions.

Ultimately we can claim that, high activities of antioxidant enzymes in the presence of light leads to successful scavenging of ROS thus alleviating damage to membranes. In addition, higher activities of these enzymes equilibrate the redox potential of cells in the presence of light and prevent further cell membranes damage.

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