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Comparative analysis of some biochemical responses of winter and spring wheat cultivars under low temperature

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Key words: Low temperature, *Triticum aestivum*, Superoxide dismutase, Peroxidase, Catalase.

Abstract

To compare changes of biochemical indices between spring (Kavir) and winter (Azar2) cultivars of wheat (*Triticum aestivum* L.) under low temperature, 14 days old wheat seedlings were exposed to cold. The seedlings were transferred into growth chamber for 9 days at 5/3 ˚C (day/night) as cold treatment, or at 20/18 ˚C as control. Proline content, total protein accumulation, activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymes, were assayed in the leaf extracts of control and cold treated plants. The results showed that cold led to an accumulation of proline and an increase in protein level, especially in winter cultivar. Rapid increases in proline and protein accumulations were observed during early stages of cold stress. SOD activity displayed no significant differences between the two cultivars during the first 3 days after cold stress, while in Azar 2, the level of SOD activity was gradually increased after 3 days of cold stress. The POD and CAT activity were higher in plants grown at cold stress than in the controls; however, their rate was different in winter and spring wheat cultivars. In general, Azar2 showed relatively higher POD and CAT activity compared to Kavir. Regarding antioxidant enzymes activities, cultivars respond differently under cold stress.

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Introduction

Low temperature is one of the most significant abiotic stresses for agricultural plants, affecting both plant development and yield (Kasuga *et al.*, 1999; Lang *et al.*, 2005). It is a major factor in determining the natural distribution of plants (Repo *et al.*, 2008). Cold tolerance is the result of a wide range of physical and biochemical processes that allow functioning at low temperatures, such as the induction of antifreeze proteins (Yeh *et al.*, 2000) and changes in the membrane composition (Huner *et al.*, 1987; Wang *et al.*, 2006).

During the cold treatment, the level of several metabolites, including free amino acids, undergoes a change. The cold-induced increases in the total amino acid concentration derived mainly from the accumulation of Pro, Glu and Gln in bluegrass (Dionne *et al.*, 2001). Proline is a proteinogenic amino acid with an exceptional conformational rigidity which accumulates in many plant species in response to environmental stress (Szabados and Savoure, 2010). Growth at low temperature may also cause an excessive excitation of the electron transport systems, possibly leading to an increase in the concentration of reactive oxygen species (ROS). If the plants are not able to control the intracellular ROS level, the membrane lipids, proteins and nucleic acids may suffer damage, leading to the death of the cells (Suzuki and Mittler, 2006; Krishnamurthy and Rathinasabapathi, 2013). Plants have well-developed defense systems against ROS, including both by limiting the production of or quenching the ROS. For this, plants detoxify ROS by up-regulating antioxidant enzymes, like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Raza *et al.*, 2007; Heidari and Golpayegani, 2012).

Several studies have been conducted on changes in the antioxidant activity in plants under stress conditions, including low temperature stress. However, most of these studies focused on cold-sensitive plants, such as rice (Huang and Guo, 2005), sorghum (Badiani *et al.*, 1997) or maize (Iannelli *et al.*, 1999). In this study, we compare the responses, changes trend and the effect of cold duration on some biochemical indices in winter and spring wheat cultivars.

Material and methods

Plant Materials and treatment

Seeds of two wheat cultivars, Azar2 (winter wheat) and Kavir (spring wheat), were planted and grown for 14 days in a growth chamber maintained at 20/18°C (day/night) under fluorescent white light (250 µmol m^{-2} s^{-1}), 12h photoperiod, 60% relative humidity. The seedlings were then exposed to chilling at 5/3°C (day/night). The samples (leaves) were taken after 3, 6 and 9 days of the cold (chilling) stress for analyses. Control plants were maintained in the constant temperature of 20/18°C (day/night).

Proline measurement

Free proline content was measured as described by Bates *et al.* (1973). 100 mg of frozen plant material was homogenized and extracted with 3% (w/v) sulphosalicylic acid. After filtration, ninhydrin reagent was added and dissolved with toluene and the absorbance was measured at 520 nm. The concentration of proline was calculated from a proline standard curve. The concentration of proline was expressed as µmol g^{-1} FW.

Protein content

Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) to standardize the method.

Enzyme assay

Superoxide dismutase (EC 1.15.1.1) activity assay was measured, based on the method of Beauchamp and Fridovich (1971). One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate at 560 nm in the presence of riboflavin in the light. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 75 µM NBT, 13 mM L-Methionine, 0.1 mM EDTA and 4 µM Riboflavin.

Catalase (EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease of H_2O_2 at 240 nm according to Aebi (1984). The reaction mixture consisted of 50mM potassium phosphate buffer, pH 7.0, 33mM H_2O_2 and enzyme fraction.

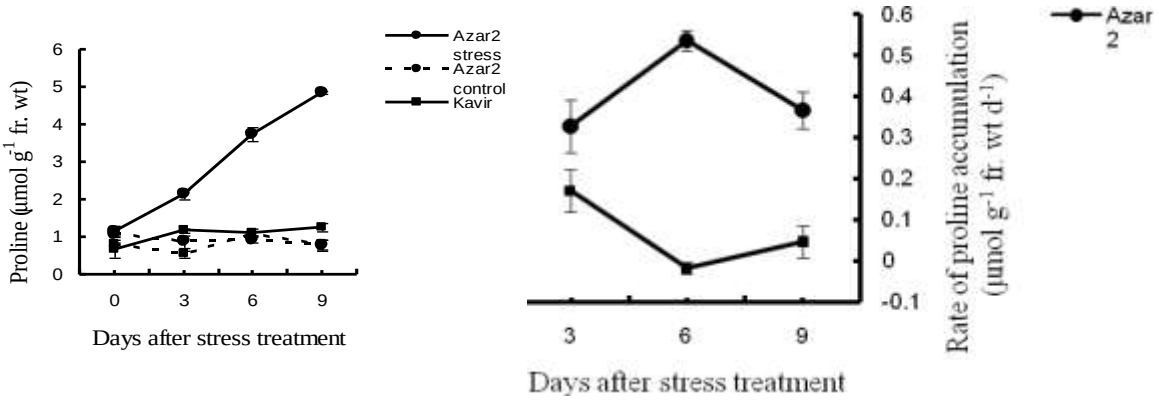
Peroxidase (POD) activity was assayed in a reaction mixture containing 20 mM guaiacol, 10 mM H_2O_2 and 50 mM potassium phosphate buffer (pH 7) and extract (Chance and Maehly, 1955). The reaction was started by adding H_2O_2 and guaiacol, and the activity determined by monitoring the increase in absorbance at 420 nm as a result of guaiacol oxidation.

Statistical analysis

For each measurement, three individual plants were used. The data for each variable was subjected to analysis of variance (ANOVA). Significant mean differences ($p<0.05$) were detected using LSD's pair-wise comparisons. Statistical analyses were performed using SAS (Version 9.1) software.

Results and discussion

The proline content of extended leaves was higher in cold stressed than controlled treatment in both cultivars (Table 1). Proline content in both cultivars (Azar2 and Kavir) were increased during cold stress, while, the rate of increase was higher in winter wheat cultivar (Azar2) compared to the spring wheat (Kavir) (Fig -1A). The proline content enhanced continuously in winter wheat cultivar, Azar2, and it was %243 higher than their control plant at 3 days after cold stress (Fig -1A). Under cold stress, Azar2 had a slight increase of proline level after 3 days of cold stress, but a sharp increase after 6 days. In addition, the mean rate of increase in proline level at 5/3°C for Azar2 and Kavir was 0.41 and 0.06 (µmol g^{-1} fr. wt d^{-1}), respectively (Fig 1B).



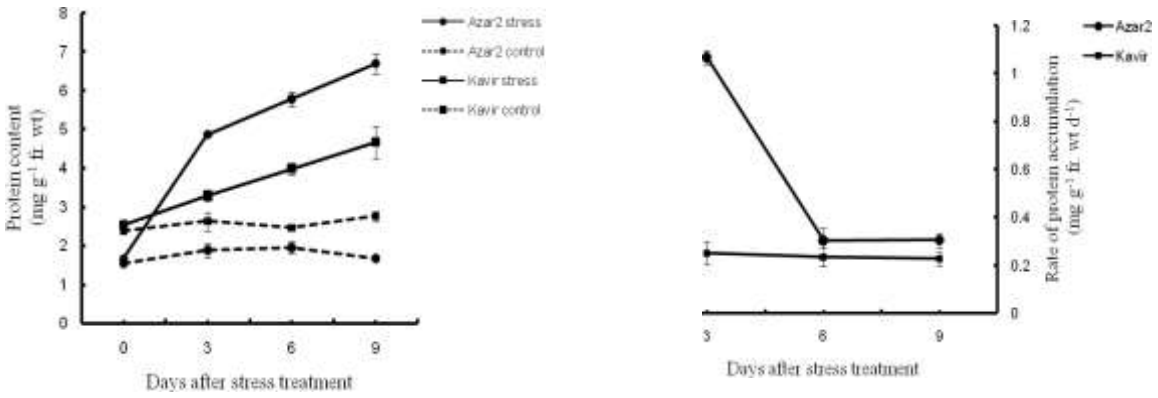
A B

Fig. 1. Effect of low temperature on proline content (A) in two cultivars of wheat (Azar2; Kavir). Rate of proline (B) accumulation at low temperatures.

The present results show that a fast induction of proline is an advantage because under low temperature condition, it might enables winter cultivar to prepare for the cold in comparison to the spring cultivar. Numerous papers have been published regarding correlations between proline accumulation and frost tolerance (Patton *et al.*, 2007). Exogenous application of proline to plants, before, during, or after stress exposure, has been shown to increase stress tolerance (Ashraf and Foolad, 2007). Several modes of action of proline have been proposed. As an amphiphilic molecule proline can bind to hydrophobic surfaces by its hydrophobic moiety thus converting them to hydrophilic ones. Such conversion enables the cell to preserve the structural integrity of cytoplasmic proteins under the dehydration conditions developed under drought, salinity and frost stress (Papageorgiou and Murata, 1995). Moreover, evidence for a function of proline as a molecular chaperone enable to protect

protein integrity, a signaling molecule in defense pathways (Szabados and Savoure, 2010) and a ROS scavenger (Matysik *et al.*, 2002; Hoque *et al.*, 2008) has been presented. Proline was shown to be a relatively good marker in breeding programs.

The protein content in both tested cultivars increased during cold stress, however, in comparison to their controls, protein level in Azar2 increased more than Kavir (Table 1). Protein accumulation in Azar2 and Kavir increased by 2.92 and 1.25-fold compared to their control after 3 days of cold stress, respectively (Fig -2A). In cold condition, the final protein level in Azar2 leaves was 6.63 (mg g^{-1} fr. wt) compared to 4.66 (mg g^{-1} fr. wt) in Kavir. The increase of protein content, which was probably attributable in part to the decrease of relative water content of cold stressed plants. Soluble protein changes may involve changes in water-binding proteins which would decrease free cellular water. This would establish a free energy gradient such that intracellular ice formation would be less likely during lower temperatures (Levitt, 1980). The analysis of protein content at low temperature condition, in both cultivars showed the similar pattern. However, rate of protein content was higher in Azar2 than Kavir after a 3-days stress (Fig -2B). The more protein content of winter cultivar than spring type may be due to it had a longer period of vernalization requirement than spring habit, which affected the expression of low temperature-induced proteins (Fowler *et al.*, 2001). Previous researches have demonstrated that antifreeze proteins are synthesized during low and freezing temperature (Yeh *et al.*, 2000; Galiba *et al.*, 2009). Also, Mohapatra *et al.* (1987) have reported that plants subjected to cold acclimation, change their both the amount and the type of polypeptides.



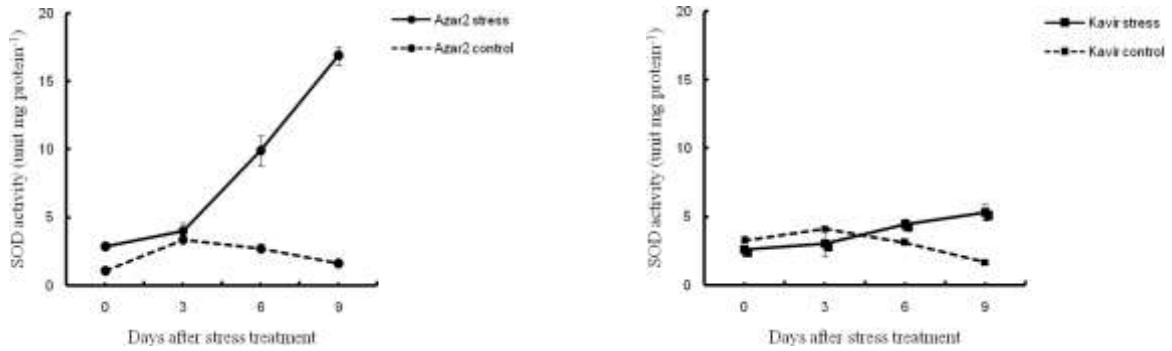
A B

Fig. 2. Effect of low temperature on protein content (A) in two cultivars of wheat (Azar2; Kavir). Rate of protein (B) accumulation at low temperatures.

Tolerance to low temperature stress in crop plants has been reported to be associated with an increase in antioxidant enzymes activity (Szalai *et al.*, 2009). In the present study, the activities of tow antioxidant enzymes SOD, CAT and POD were investigated in Azar2 and Kavir cultivars under low temperature condition.

SOD (superoxide dismutase) is a type of enzymes with metals, whose function is to get rid of oxygen free radicals or others and protect membrane systems (Urquiaga and Leighton, 2000; Apel and Hirt, 2004). In comparison to the control, SOD activity in both cultivars, was not significantly changed by cold treatment up to 3 days of exposure to cold stress, but interestingly, the level of SOD activity was gradually increased after 3 days of cold stress in Azar2 and the mean cultivars SOD activity did not change very much by the low temperature after exposure to 5/3°C (day/night) (Fig -3A,B). However, it is possible that the maintenance of constant SOD levels could be sufficient to ensure production against O^{2-} produced

during cold treatment due to the high SOD protein turnover (Scandalios, 1993). In contrast, SOD activity in Kavir was almost constant at low temperature stress. The maximum level of SOD activity was observed after 9 days of cold treatment in Azar2.

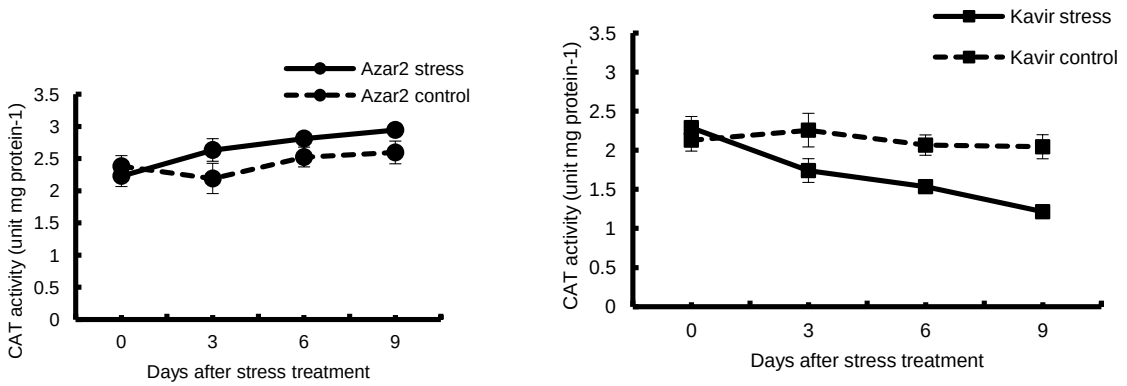


A B

Fig. 3. SOD activity of Azar2 (A) and Kavir (B) during low temperature and control conditions.

SOD activity, in the winter wheat cultivar, enhanced after 3days of cold stress that it seems to be attributable to an increased production of H_2O_2. In contrast, compared with a growth temperature of 20/18°C, cold stress did not cause any significant difference in the activity of SOD in the spring wheat cultivar. Therefore, these results suggested that the effect of cold stress on SOD activity depends on cold duration. At initial time of cold stress, there was not an increase in SOD activity, but it increased after continuation of chilling. Similar observations were demonstrated in other species, such as maize and rice (Iannelli *et al.*, 1999; Huang and Guo, 2005).

CAT (catalase) is enzyme that involved in scavenging H_2O_2 that is produced under normal and stressful conditions (Khedr *et al.*, 2003). The activity of CAT in leaves of both winter and spring wheat was similar to the control condition. In contrast, CAT activity in leaves for cold-stressed plants changed during the experimental period in both cultivars. Cold stress gradually increased leaf CAT activity of Azar2, while that in Kavir, it decreased continuously after cold condition (Fig -4A, B). At 3 days after cold stress, CAT activity in winter cultivar, Azar2, increased 19%, whereas in spring cultivar, Kavir, it decreased about 22% of their control plants.



A B

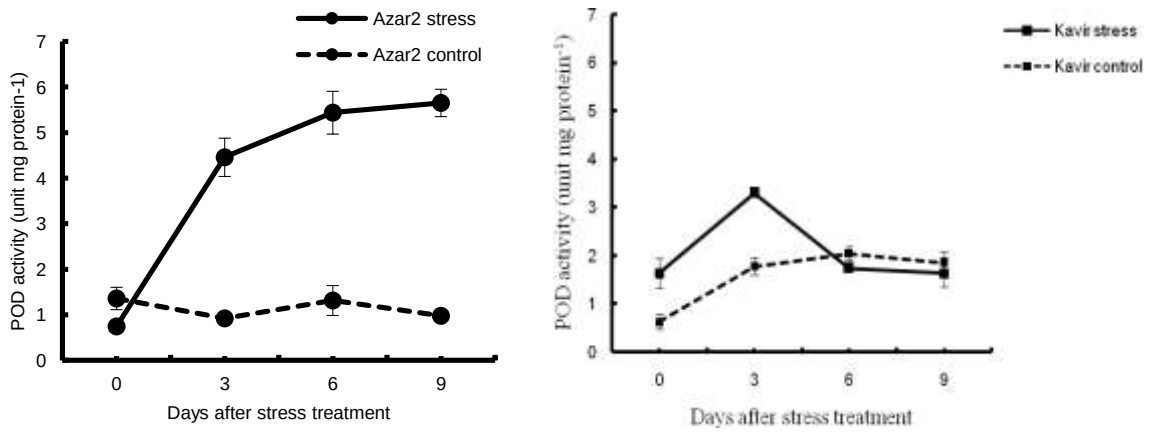
Fig. 4. CAT activity of Azar2 (A) and Kavir (B) during low temperature and control conditions.

According to the results, it could be argued that although CAT may contribute to the detoxification of ROS, its activity is not a direct key factor in the cold tolerance of wheat. In addition, the reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme (Abedi and Pakniyat, 2010).

POD (perioxidase) are enzymes that catalyze polyphenolic compounds into other products (Urquiaga and Leighton, 2000; Apel and Hirt, 2004). The activity of POD was monitored during cold stress. The level of POD activity was increased by cold stress as compared with the control (Table 1). However, POD activity was higher in winter wheat cultivar than in spring wheat cultivar. The winter wheat cultivar; Azar2, maintained higher POD activity under low temperature, while POD activity in Kavir decreased sharply after 3 days (Fig -5A, B). In other words, after 3 days of cold stress, POD activity in Azar 2 was not changed much by the low temperature treatment. In contrast, the activity in Kavir was decreased by cold after 3 days of stress. Otter and Polle (1994) have suggested that peroxidases are known to utilize phenolic compounds as a substrate, play a central role for the synthesis of secondary metabolites such as lignin. The enhanced scavenging ability for H_2O_2 in tolerant cultivars inhibited the accumulation of ROS and thus protects plants from lipid peroxidation of membrane systems and oxidative damages under cold stress. The results indicated that the tolerance for a specific cultivar of wheat to stresses, such as low temperature, depends upon its responses of antioxidative system. On the whole, the increase in POD might be considered as a key point for the decomposition of H_2O_2, especially under CAT inactivation.

Table 1. Average values of proline content, protein accumulation, SOD, CAT and POD activity in leaves of Azar2 and Kavir under control (20/18°C) and cold (5/3°C) conditions. Values are the means±SE (n=3) (pooled data for all times).

Cultivar	Proline (μmol g^{-1} fr. wt)		Protein (mg g^{-1} fr. wt)		SOD (unit mg protein^{-1})		CAT (unit mg protein^{-1})		POD (unit mg protein^{-1})	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Azar2	0.86±0.09	3.57±0.39	1.83±0.06	5.77±0.26	2.58±0.27	10.27±0.61	2.42±0.18	2.65±0.13	1.07±0.14	5.18±0.27
Kavir	0.80±0.11	1.18±0.04	2.62±0.15	3.97±0.25	2.96±0.36	4.26±0.46	2.12±0.16	1.69±0.11	1.89±0.1	2.22±0.28



A B

Fig. 5. POD activity of Azar2 (A) and Kavir (B) during low temperature and control conditions.

Conclusion

Low temperature significantly enhances the contents of protein and proline, and induces the activities of SOD, CAT (except in spring wheat) and POD in both winter and spring wheat cultivars. However, the winter cultivar responded more rapidly over the first time of cold treatment than the spring cultivar. The rates of proline and protein accumulation in the winter cultivar are more than those in the spring under cold stress. In addition, different responses to cold stress were observed in the two cultivars, from activities of antioxidant enzymes. The evidence from this study strongly suggests that changes induced by low temperature in the activities of antioxidant enzymes depend not only on the temperature, but also on the timing and duration of stress.

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