



Journal of
Plant Sciences

ISSN 1816-4951



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Effects of Cold Acclimation and Exogenous Phytohormone Abscisic Acid Treatment on Physiological Indicators of Winterness Wheat

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Abstract: We investigated the changes in the concentrations of protein, soluble sugar, proline, malondialdehyde and the antioxidative enzyme activities of superoxide dismutase, peroxidase, catalase of three wheat (*Triticum aestivum* L.) cultivars, DN1, Mironovskaya 808 (M808) and Chinese Spring (CS) under cold stress and exogenous abscisic acid treatment in the growth chamber. The results on physiological indices suggested that DN1 and M808 were higher than that of CS on cold resistance. We also found that abscisic acid had significant impacts on cold resistance, suggesting that low concentration of abscisic acid could improve cold acclimation of wheat under low temperature and the high concentration could inhibit cold acclimation. Therefore, physiological changes in wheat seedlings are useful to better understand chilling stress responses in plants which promise to improve cold resistance of crops at low temperatures.

Key words: Winterness wheat, cold acclimation, exogenous ABA, physiological indices

INTRODUCTION

Drought, salinity and extreme temperatures are among the major environmental stresses to crop productivity worldwide (Ashraf and Foolad, 2007). To improve crop tolerance to these abiotic stresses, researches have been focused on the physiological and molecular mechanisms of plant responses to the stresses. Cold is a major environmental stress to crop productivity and to the distribution of wild species. The cold stress includes low, above-zero temperatures ranging from 0 to 12 or 15°C. These temperatures can cause severe damage to plants of tropical and sub-tropical origin and chilling-sensitive plants such as *Zea mays*, *Glycine max*, *Lycopersicon* sp., *Cucumis sativus* or *Gossypium hirsutum*. In contrast, these temperatures induce important biochemical and physiological changes in chilling-tolerant plants such as winter cultivars of *Secale cereale*, *Triticum aestivum* or *Hordeum vulgare* which help them to survive sub-zero temperatures (Kosová *et al.*, 2007).

The phytohormone Abscisic Acid (ABA) is involved in various physiological processes of plants, such as adaptation to stressful environments, seed germination and seedling

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growth (Finkelstein *et al.*, 2002). Abscisic Acid (ABA) also can change carbohydrate levels and activities of the enzymes involved in carbohydrate biosynthesis (Blöchl *et al.*, 2005; Kobashi *et al.*, 1999; Taji *et al.*, 2002). Cold tolerance is also induced by exogenously added ABA. Abscisic Acid (ABA) has been suggested to have an important role in the enhancement of freezing tolerance in cold acclimated plants.

Both winter and spring cereal cultivars can acclimate to low temperature, but spring cultivars generally develop less chilling tolerance than do winter cultivars. Mironovskaya 808 (M808) was bred in the Mironovskaya Institute of Ukraine and is reported to be the hardiest winter cultivar among tetraploid and hexaploid wheat tested for freezing tolerance (Veisz and Sutka, 1990). The DN1 is the cultivar of winter wheat which can pass the winter securely in the alpine region of Heilongjiang Province and its greening rate is more than 80%. The DN1 with high cold resistance fill the gaps in the Northern of China and break the previous distribution between winter and spring wheat. The earlier studies showed the much higher freezing tolerance of M808 compared with that of the spring wheat Chinese Spring (CS) by a simple one-point assay (Ohno *et al.*, 2001; Kume *et al.*, 2005). The aim of this study was to investigate physiological traits in two different wheat seedlings with different resistance to cold stress and exogenous ABA treatment. In order to gain more knowledge about physiological metabolism under low temperatures and exogenous ABA treatment, the treated wheat seedlings were used to determine the contents of soluble sugar, proline, malondialdehyde (MDA) and to determine the activities of antioxidant with superoxide dismutase enzymes (SOD), peroxidase (POD) and catalase (CAT).

MATERIALS AND METHODS

Plant Materials and Stress Treatments

The experiment was carried out at the Key Laboratory of Agricultural Biotechnology of Liaoning Province, in Shenyang Agricultural University between 15th September to 30th November, 2009. The two accessions of winter wheat M808 and DN1 were used to analyze physiological indices. An accession of spring wheat CS was used as the control reference. Wheat seeds were planted in distilled water for 10 days in culture plates at 25°C. Half of the 10-day-old seedlings were exposed to chilling stress with a temperature of 4°C for 0, 2, 4, 8, 12, 24, 48, 72 or 96 h in a cold chamber. To mimic ABA treatments, ABA solutions of different concentrations (containing 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg L⁻¹) were directly added to the other 10-day-old seedlings for 3 days. Then the seedlings were exposed to a low temperature treatment 4°C for 3 days.

Determination of Soluble Protein Content

Soluble protein concentration was measured by the Bradford (1976) method with Bovine Serum Albumin (BSA) as the standard.

Determination of Soluble Sugar Content

Soluble sugar content was measured following the method described by Yemm and Willis (1954). Leaf materials were soaked in 25 mL distilled water. The solution was boiled (100°C) for 30 min to extract soluble sugar and centrifuged under 4000 rpm for 10 min. The extracts were decanted and the residue was re-extracted for twice more, with extracts being completed to 50 mL. In all, 0.1 mL extracts and 3 mL anthrone reagent (0.15 g anthrone+84 mL oil of vitriol+16 mL H₂O) were mixed and the absorbance of the mixture was recorded at 620 nm. The content of soluble sugar was calculated from a standard curve of glucose at 620 nm by colorimetry.

Determination of Proline Content

Determination of free proline content was done according to Bates *et al.* (1973). Leaf materials (0.5 g) were homogenized in 3% (w/v) sulfosalicylic acid and homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100°C for 1 h in a water bath. The reaction was then stopped by using an ice bath. The mixture was extracted with toluene and the absorbance of the fraction with toluene extracted from the liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as $\mu\text{g proline g}^{-1}\text{ FW}$.

SOD Activity Assay

SOD activity was assayed by monitoring the inhibition of photochemical reduction of Nitro Blue Tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The 3 mL reaction mixture contained 2.4 mL of 50 mM buffered phosphate solution (pH 7.8), 0.2 mL of 195 mM methionine, 0.1 mL of 3 μM EDTA, 0.2 mL of 1.125 mM NBT, 0.1 mL of 60 μM riboflavin and 40 μL enzyme extract. The reaction mixtures were illuminated for 20 min at a light intensity of 300 $\mu\text{mol/m}^2/\text{sec}$. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

POD Activity Assay

Guaiacol Peroxidase (POD) was determined by measuring the oxidation of guaiacol. The assay mixture contained 50 mM sodium phosphate (pH 6.0), 28 mL guaiacol and 19 mL 30% H_2O_2 . The absorbance was recorded five times at 470 nm at 30 sec intervals. Variation of absorbance per minute per gram fresh weight ($\Delta A_{470}/\text{g}/\text{min}/\text{FW}$) stands for enzymes activity.

CAT Activity Assay

Catalase (CAT) activity was determined according to Aebi (1984). The reaction mixture contained 2 mL 50 mM phosphate buffered solution (pH 7.0), 1 mL 10 mM H_2O_2 and 0.2 mL of enzyme extract. The reaction was initiated by the addition of enzyme extract and the activity was determined by monitoring the decrease in absorbance at 240 nm for 30 sec.

Determination of MDA Content

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to Heath and Pacher (1968). Fresh samples mixed with 5 mL phosphate buffer (pH 7.8) were crushed into homogenate in a mortar. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, using the supernate for MDA determination. A mixture of 1 mL extracts (MDA) + 2 mL 0.6% thiobarbituric acid (TBA) (0.6 g TBA + 1 M NaOH + 10% trichloroacetic acid complete to 100 mL) was produced, boiled for 15 min, cooled and centrifuged for 10 min (4000 rpm). The concentration of MDA was calculated from the absorbance at 600, 532 and 450 nm.

RESULTS

Protein Contents

The protein content in wheat seedlings declined slightly within the initial 12 h. The protein content was significantly higher than the control upon reaching the maximum at 72 h cold stress. After 72 h chilling, the protein content dropped (Fig. 1). The protein content in DN1 seedlings persistently increased until the ABA concentration was 0.6 mg L^{-1} . Then the content of protein declined. There was little change in the protein contents of M808 and CS (Fig. 2).

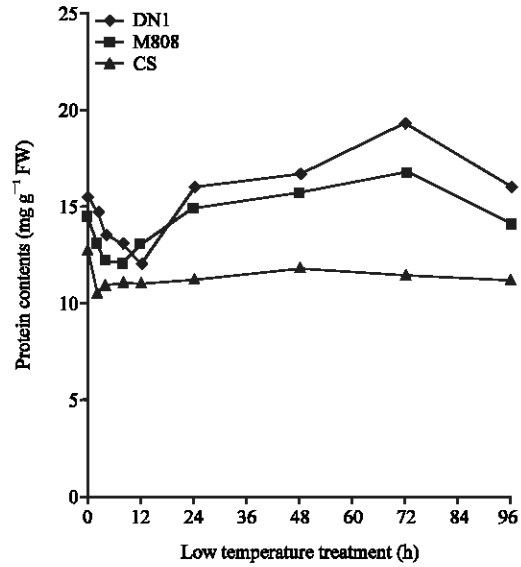


Fig. 1: Effect of low temperature on protein content in wheat seedlings

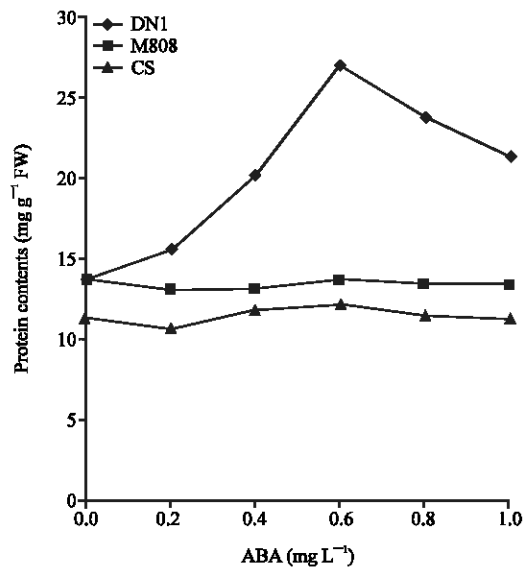


Fig. 2: Effect of exogenous ABA on protein content in wheat seedlings

Soluble Sugar Contents

Soluble sugar contents in the leaves of wheat seedlings showed a steep drop firstly under low temperature stress and then the protein contents increased subsequently (Fig. 3). The soluble sugar contents in DN1 and M808 seedlings increased markedly in exogenous ABA treatment, while the curve of CS was linear (Fig. 4).

Proline Contents

The contents of free proline increased quickly as the stress hours increased, the proline content of M808 and DN1 increased faster than CS (Fig. 5). The proline contents in three

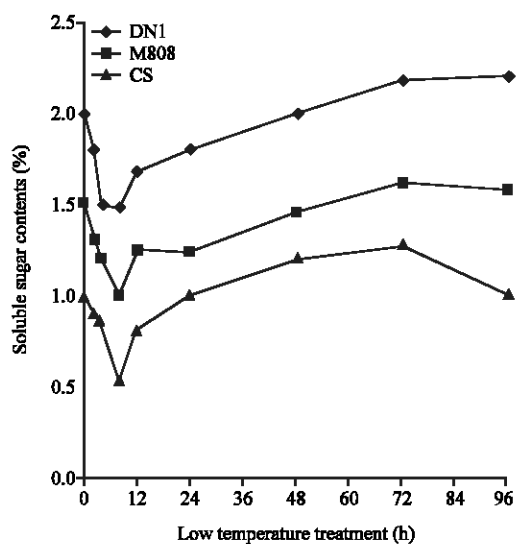


Fig. 3: Effect of low temperature on soluble sugar contents in wheat seedlings

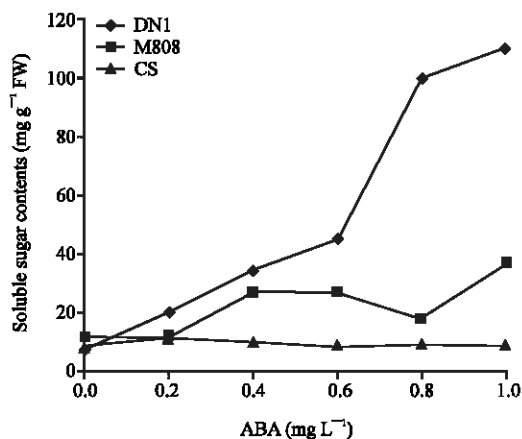


Fig. 4: Effect of exogenous ABA on soluble sugar contents in wheat seedlings

wheat seedlings persistently increased until the ABA concentration was 0.6 mg L⁻¹, then the contents of proline decreased as the concentration increase (Fig. 6).

SOD Activity

The activity curves of SOD in the leaves of wheat seedlings under low temperature stress were W shaped. The temperature on the same terms, the degree of DN1 and M808 changed greater than that of CS (Fig. 7). The SOD activity in DN1 seedlings persistently increased until the ABA concentration was 0.6 mg L⁻¹, then the activities declined. There was little change in SOD activity of M808 and CS (Fig. 8).

POD Activity

When compared to the control, POD activity decreased at the early stage of low temperature stress, while POD activity in DN1 and M808 increased, as the low temperature

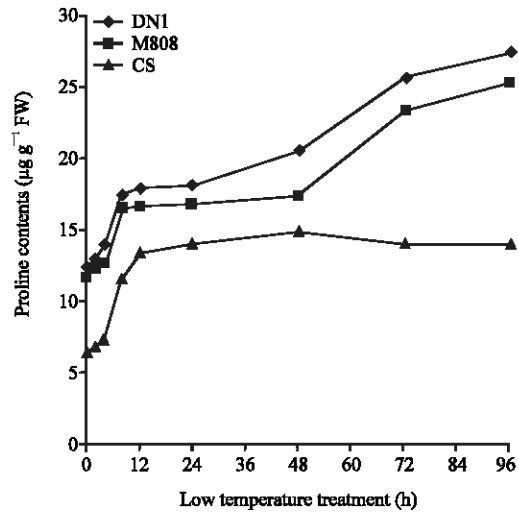


Fig. 5: Effect of low temperature on proline content in wheat seedlings

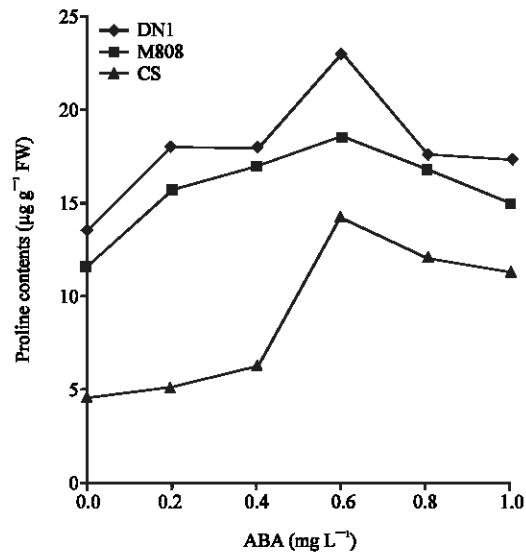


Fig. 6: Effect of exogenous ABA on proline content in wheat seedlings

hours changed (Fig. 9). The activity curves of POD in the leaves of wheat seedlings with exogenously added ABA were sigmoid curves (Fig. 10).

CAT Activity

The CAT activity in wheat seedlings declined at first under chilling stress and then increased slightly, finally the activity was down at the end of low temperature stress (Fig. 11). Under same temperature conditions the CAT activity of DN1 was higher than that of M808 and CS. The CAT activity in wheat seedlings in exogenous ABA treatment showed no apparent trend (Fig. 12).

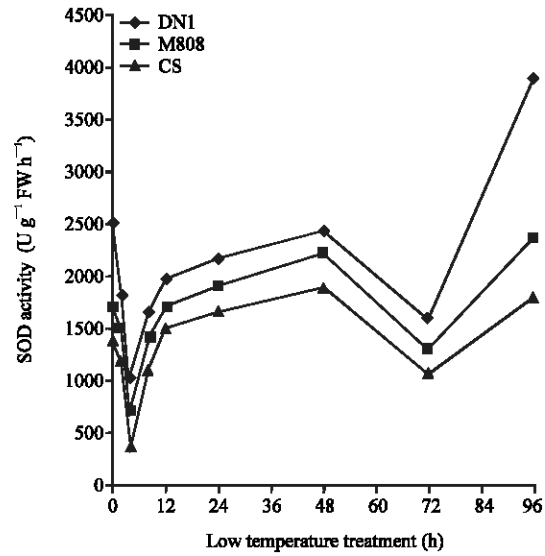


Fig. 7: Effect of low temperature on SOD activity in wheat seedlings

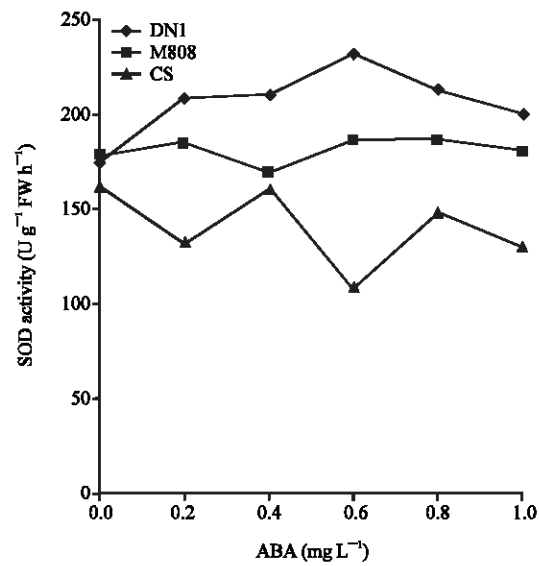


Fig. 8: Effect of exogenous ABA on SOD activity in wheat seedlings

MDA Content

The content of MDA in wheat seedlings significantly increased and then the change tended to stabilize. The MDA contents showed an increasing trend in general (Fig.13). The curves of MDA content were nearly linear, whereas with the concentration of ABA up to 0.6 mg L⁻¹, the content of MDA increased gradually. The increase degree of DN1 and M808 was smaller than that of CS (Fig. 14).

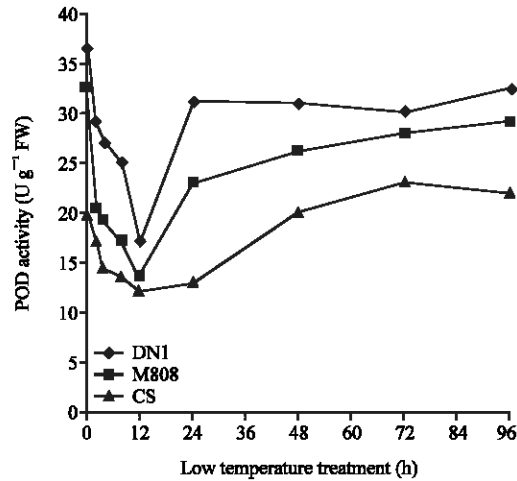


Fig. 9: Effect of low temperature on POD activity in wheat seedlings

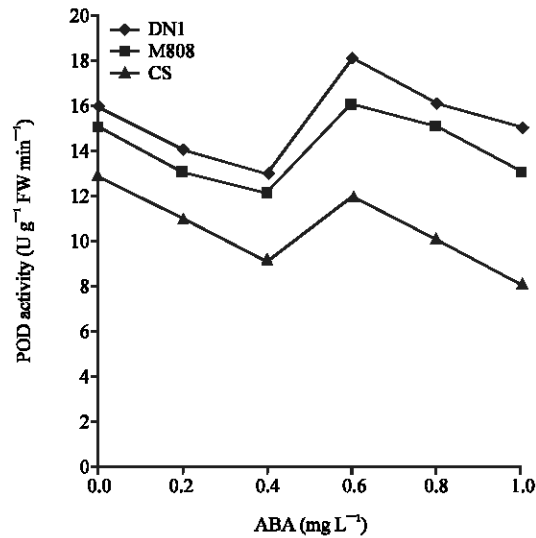


Fig. 10: Effect of exogenous ABA on POD activity in wheat seedlings

DISCUSSION

Some research shows that an important role is played by Abscisic Acid (ABA) during cold acclimation and ABA can also enhance freezing resistance (Pociecha *et al.*, 2008). A relationship between phytohormone ABA and chilling tolerance has been shown in rice cultivars (Lee *et al.*, 1995). According to our results, ABA can indicate cold tolerance in wheat can be induced by exogenously added ABA at a low temperature (4°C). Thus, ABA can substitute for the low temperature treatment in inducing cold hardiness in wheat. This result is in agreement with the previous view about the role of ABA in the stress acclimation process, namely: The ABA is one of the factors which regulated to stress adaptation

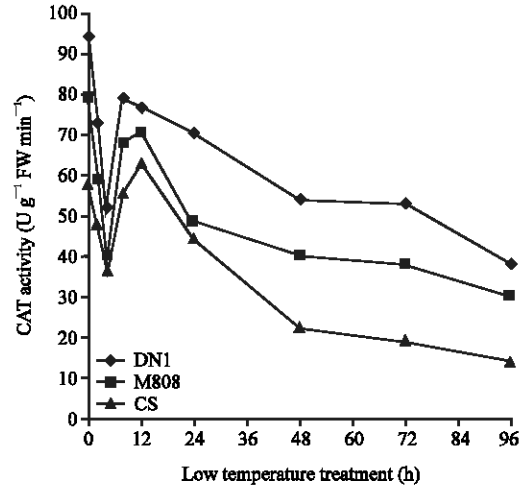


Fig. 11: Effect of low temperature on CAT activity in wheat seedlings

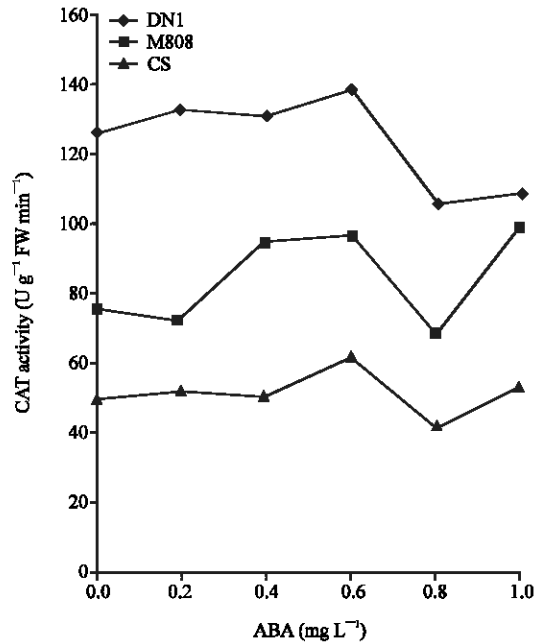


Fig. 12: Effect of exogenous ABA on CAT activity in wheat seedlings

(Shinozaki and Yamaguchi-Shinozaki, 1997). In present study, we also find the degree of cold tolerance obtained by ABA treatment was dependent on ABA concentration in the growth condition. Low ABA concentration can improve cold acclimation of wheat under low temperature and high concentration can inhibit the cold acclimation. When the ABA concentration was $0.6\ mg\ L^{-1}$, it resulted in maximal cold tolerance.

The effect of cold stress depends on the degree of severity and the time of exposure. Increased tolerance to abiotic stress in plants is necessary in order to increase productivity

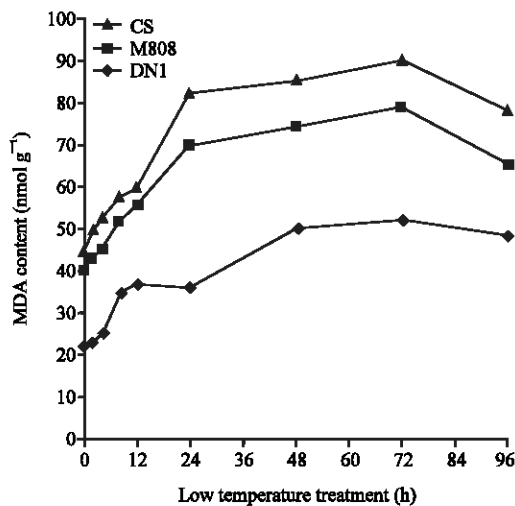


Fig. 13: Effect of low temperature on MDA content in wheat seedlings

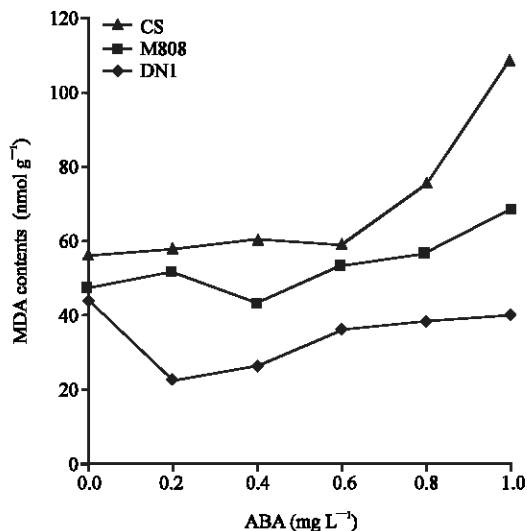


Fig. 14: Effect of exogenous ABA on MDA content in wheat seedlings

under cropping conditions with limited water supplies, high salinity and low temperature. Tolerant cultivars respond to abiotic stress with complex changes in their physiological and molecular status. In this study, the physiological responses to different stresses were studied to identify some of the key elements that may be responsible for abiotic stress tolerance in wheat. The concentrations of several stress-related molecules including protective enzymes, oxidative stress products and soluble sugar were analyzed.

Anderson *et al.* (1995) reported that the damage caused by chilling stress was, in part, due to membrane lipid peroxidation. The MDA is an indicator of lipid peroxidation and links to peroxidation of polyunsaturated fatty acids in the membranes thereby releasing free

radicals (Singh *et al.*, 2008). As low-temperature exposure time continued, the MDA contents of wheat seedlings dramatically increased. The results showed that the lipid membrane of the wheat seedlings was suffering from severe damage as the wheat seedlings were suddenly in low temperature treatment.

Enzymes of SOD, CAT and POD are important antioxidant systems and SOD catalyses the dismutation of O_2^- to H_2O_2 and O_2 , while CAT and POD scavenge H_2O_2 (Feng *et al.*, 2003). In present study, under low temperature stress, SOD, CAT and POD activities of wheat seedlings increased in varying degrees when stress time was increased. The researchers generally agree that SOD activity is related to the degree of cold tolerance of plants. As the activities of SOD increase, the cold resistance of plants is stronger, so our results suggest that the cold resistance of DN1 is higher than that of M808. Soluble sugar, soluble protein and free proline in plant cells are important osmotic substances. The contents of soluble sugar and free proline in cold-tolerant varieties were significantly higher than those of cold-sensitive varieties.

Comparing the physiological factors associated with cold-hardiness of different wheat, our results suggested that M808 and DN1 had remarkable changes on these physiological indices compared with the control CS and DN1 which tended to improve the cold-hardiness of wheat cultivars compared to M808. It is concluded that our studies will provide scientific guidance for exploring the cold resistance mechanism of wheat and will also provide reference for further screening of much more cold-resistant winter wheat varieties.

ACKNOWLEDGMENTS

We express our gratitude to the anonymous reviewers for helpful comments to improve the manuscript. This study was supported by the National Natural Science Foundation of China (Grant No. 30900894) and the Startup Foundation for Doctors of Liaoning province in China (Grant No. 20091069).

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