ANTIOXIDATIVE DEFENCE IN WINTER WHEAT PLANTS DURING EARLY COLD ACCLIMATION

P. Apostolova, I. Yaneva*

Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg. 21, 1113 Sofia

Summary. Changes in the activities of four enzymes involved in antioxidative plant defence against low temperature treatment were studied in winter wheat plants (*Triticum aestivum* L cv. Sadovo 1) grown on acid soil (pH 4.2, KCl) under controlled environmental conditions. To investigate the effect of molybdenum (Mo) deficiency on antioxidative defence of these plants, Mo was added to part of the plants at early second-leaf stage as $[NH_4]_6Mo_7O_{24}$ x 4 H_2O in 1 mg Mo/kg concentration. Enzyme activities of catalase (CAT, EC 1.11.1.6), ascorbic acid peroxidase (APX, EC 1.11.1.11), peroxidase (POX, EC 1.11.1.7), and glutathione reductase (GR, EC 1.8.1.7) were measured in leaves of both Mo-deficient and Mo-supplied plants during the early period of plant cold acclimation at 2°C. Results demonstrated higher level of APX activity and slightly increased activity of GR due to Mo-deficiency under normal growth temperatures. On its part CAT activity was found to be higher in Mo-supplied plants by contrast with POX activity. Mo-deficiency provoked changes only in APX and POX activities at low temperature.

Keywords: antioxidant enzymes, molybdenum deficiency, winter wheat, *low temperature.*

INTRODUCTION

Low temperatures (cold and frost) have profound negative impacts on global agricultural productivity. Many changes in physiological and biochemical parameters have been observed during the exposure of plants to low temperatures: modified

*Corresponding author, e-mail: yaneva@bio21.bas.bg

levels and activities of enzymes from various metabolic pathways, accumulation of carbohydrates, amino acids, soluble proteins, as well as appearance of new isoforms of proteins and altered lipid membrane composition (Thomashow, 1999). Low temperature-induced overproduction of reactive oxygen species has been shown to bring about serious cellular damage by rapidly reacting with DNA, lipids, and proteins (Sattler et al., 2000). Thus, the ability of cells to adjust their scavenging system capacity to elevated levels of active oxygen appears to be one of the basic elements in acquiring tolerance to environmental stress (Anderson et al., 1995). Both enzymatic and non-enzymatic systems are involved in protecting plants against oxygen toxicity (Pastori and Foyer, 2002). The enzymatic system consists of a set of enzymes like superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT), while the non-enzymatic system includes some antioxidants, such as ascorbate, carotenoids and proline (Prasad, 1996). In this study, data describing the effect of low temperature (cold) on the activities of antioxidant enzymes such as catalase (CAT) and peroxidase (POX), as well as on the activities of ascorbate peroxidase (APX) and gluthatione reductase (GR) in winter wheat grown on acid soil has been presented. In such soils (pH below 5.5), the availability of the micronutrient molybdenum (Mo) becomes limiting for normal plant growth (Georgieva et al., 1983). This enabled us to study also the effect of molybdenum deficiency on antioxidative defense after transferring the plants for cold acclimation at low temperature.

MATERIALS AND METHODS

Plant material

Winter wheat (*Triticum aestivum* L. cv Sadovo 1) seeds were planted on cinnamon forest soil with pH 4.2 (KCl) in plastic pots. Plants were grown at 210 μmol $m^{-2}s^{-1}$ light intensity, 16/8 h photoperiod, and 17/14°C light/dark temperature regime. Soil humidity was maintained at 70%. At the early second-leaf stage, half of the plants (11-d-old) were supplied with Mo added to soil as $[NH_4]_6Mo_7O_{24}$ x 4 H_2O at a concentration of 1 mg Mo per kg absolutely dry soil. Four days later, both Mo-supplied and Mo-deficient plants (15-d-old) were transferred to a temperature-controlled chamber for cold acclimation at 2 °C. Part of the plants remained at 17/14°C (controls). For the enzyme assays, leaves were harvested at the end of the light period, weighed, cut into small pieces, and immediately frozen in liquid nitrogen.

Extraction and assay of enzyme activities

Leave samples were ground with mortar and pestle in liquid nitrogen and homogenized in 100 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 0.5 % insoluble polyvynil pyrrilidone (the “tissue/buffer” ratio was 1:5, w/v).

The homogenate was centrifuged at 12 000 g for 20 min and the supernatant was used for analyzing the activities of catalase (CAT, EC 1.11.1.6) and peroxidase (POX, EC 1.11.1.7). CAT activity was assayed according to Aebi (1984). Oxidation of hydrogen peroxide was followed at 240 nm for 1 min at 25 ŕC. POX activity was estimated after Polle et al. (1994). Protein content in the supernatant was determined after Bradford (1976).

Leaf samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0), containing 5 mM EDTA, 2mM sodium ascorbate, 0.5 % insoluble polyvynil pyrrilidone (Nakano and Asada, 1981), and centrifuged at 12 000 g for 20 min. Ascorbic acid peroxidase activity (APX, EC 1.11.1.11) was analyzed in 3 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM sodium ascorbate, 6 mM hydrogen peroxide, and 0.2 ml supernatant. Oxidation of sodium ascorbate started by adding 9.7 M hydrogen peroxide and it was followed at 290 nm for 1 min at 22°C.

For glutathione reductase (GR, EC 1.8.1.7) analysis, samples were homogenized in 50 mM Tris-HCl (pH 7.5) buffer, containing 1 mM EDTA, 9.94 mM sodium ascorbate, 0.5% insoluble polyvynil pyrrilidone, and centrifuged at 12 000 g for 20 min. GR activity was analysed in 3 ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 3 mM $MgCl_2$, 1 mM oxidized glutathione, and 0.3 ml supernatant (Wang et al., 1991). The addition of 0.1 mM NADPH started the reaction and the decreasing absorption at 340 nm, resulting from NADPH oxidation, was followed for 1 min.

RESULTS

The activities of four enzymes involved in antioxidative plant defense against low temperature stress were investigated in Mo-deficient winter wheat during the early period of plant cold acclimation. The effect of Mo-availability on enzyme activity alterations imposed by low temperature was evaluated by additionally supplying winter wheat plants with Mo. Activities of CAT, APX, POX, and GR in 15-d-old winter wheat plants grown on acid soil at optimal temperature conditions, either additionally supplied or not supplied with Mo were determined. Enzyme activities were analyzed in leaves harvested just before transferring the plants to 2°C. Figure 1 presents values obtained for enzyme activities in Mo-deficient plants expressed as percentage of the respective activities in Mo-supplied plants. Our results demonstrated a significantly higher level of APX activity and slightly increased activity of GR due to Mo-deficiency (Fig. 1). CAT activity tended to decrease to some extent in Mo-deficient winter wheat, while POX activity seemed to be unaffected by Mo-deficiency (Fig. 1). Differences between Mo-treated and Mo-deficient plants with respect to APX and GR activities were observed also in 16-d and 17-d-old plants and

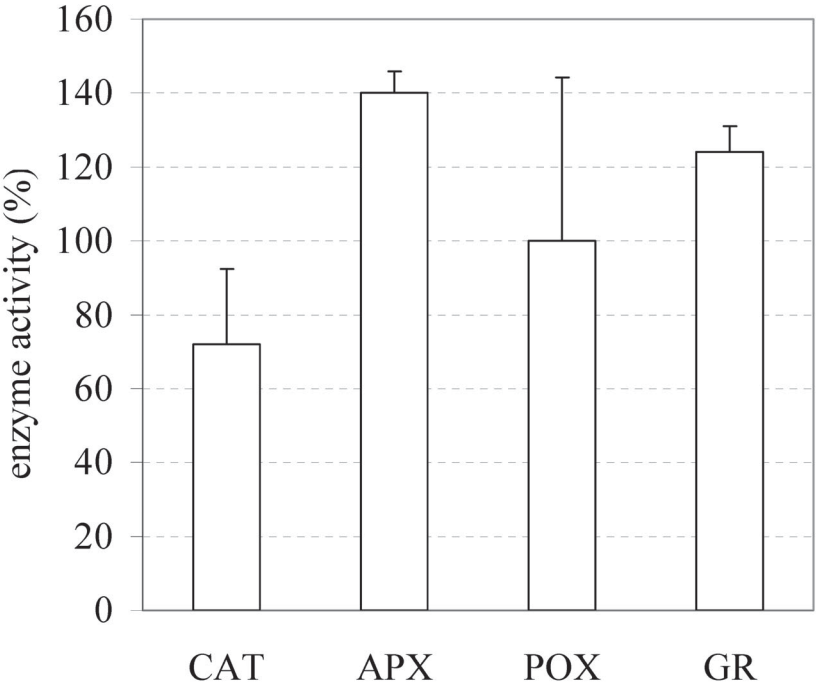

Figure 1. Effect of Mo-deficiency on the activities of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and gluthaione reductase (GR) in leaves of 15-d-old winter wheat. The enzyme activities in Mo-deficient plants are presented as percentage of the respective once in Mo-supplied plants. 100 % CAT = 8.07 mol oxidized hydrogen peroxide min^{-1} mg^{-1} protein; 100% APX = 16.06 mmol oxidized ascorbate min^{-1} mg^{-1} protein; 100 % POX = 67.83 mmol oxidized guaiacol min^{-1} mg^{-1} protein; 100 % GR = 1.95 mmol NADP min^{-1} mg^{-1} protein

tended to become even more significant (Table 1). Considerable differences regarding CAT and POX activities between both experimental groups were not registered (Table 1).

The effect of Mo-deficiency on plant antioxidative defense against low temperature was accessed by monitoring the activities of CAT, APX, POX, and GR in both Mo-deficient and Mo-supplied winter wheat after the first and second days of cold acclimation (2 °C). For evaluation the single effect of low temperature treatment on enzymes, activities in low temperature-treated Mo-deficient and Mo-supplied plants were compared to the values of the respective controls (grown at optimal conditions for the same period). APX activity in Mo-deficient winter wheat tended to decrease during the early period of cold acclimation (Fig. 2A, B), while POX activity showed no changes after one day at 2 °C (Fig. 2A) but increased significantly after the second day at 2°C (Fig. 2B). Activity levels of the other two enzymes, GR and CAT, remained relatively unaffected by cold acclimation in Mo-deficient plants (Fig. 2A,

Table 1. Enzyme activities (mmol oxidized donor min^{-1} mg^{-1} protein) in leaves of Mo-deficient (-Mo) and Mo-supplied (Mo) winter wheat grown at optimal growth conditions (see, Materials and Methods). The values ± SD are mean from 2 independent experiments.

Enzyme	16-d-old plants		17-d-old plants	
	-Mo	Mo	-Mo	Mo
catalase	7,27 ± 1,21	7,06 ± 0,67	7,67 ± 1,15	7,69 ± 1,32
ascorbate peroxidase	24,22 ± 3,48	17,80 ± 6,78	22,16 ± 2,32	10,74 ± 6,06
peroxidase	91,25 ± 34,13	103,16 ± 28,26	91,11 ± 24,73	103,48 ± 41,74
gluthaione reductase	1,86 ± 0,34	1,75 ± 0,28	2,03 ± 0,13	1,78 ± 0,27

B). Similarly, the activities of GR and CAT in Mo-supplied plants remained unchanged after the first day at 2 °C (Fig. 2A) but tended to decrease slightly after the second day of cold acclimation (Fig. 2B). APX activity exhibited lower levels in Mo-supplied plants after the first day at 2 °C (Fig. 2A) but it increased after the second day of cold acclimation reaching control level (Fig. 2B). In contrast to activity measured in Mo-deficient plants, POX was not significantly affected by the cold acclimation in Mo-supplied winter wheat (Fig. 2A, B).

DISCUSSION

Production of reactive oxygen species in plants is a general reaction in response to pathogen attack and photo oxidative processes induced by abiotic factors such as chilling, drought, salt, and ozone stress (Baker and Orlandi, 1995; Mittler 2002). The protective system against oxidative stress in plants involves several enzymes. In this paper, we report the activity changes of APX, CAT, POX, and GR involved in reactive species scavenging during the early period of plant cold acclimation of Mo-deficient winter wheat. In a previous study it was demonstrated that Mo-deficiency resulted in decreased freezing resistance of winter wheat (Yaneva et al., 1996). The present study is focused on Mo-deficiency impact on the efficiency of antioxidant defense system in winter wheat.

Recently, it has been reported that Mo treatment of 60-d-old winter wheat grown at normal temperatures caused an increase in POX and CAT activities by 30% and 53%, respectively (Xue-Cheng et al., 2006). The authors have also demonstrated that increased rates of these antioxidative enzymes in Mo-treated plants became even higher under low temperature stress. In our experiments, only CAT activity was found to be higher in Mo-supplied plants, while POX activity was not affected by Mo-deficiency (Fig. 1). Since plants we used were only 15-d-old, we assume that POX appeared to be less sensitive than CAT to Mo-deficiency in young winter wheat.

Both enzymes belong to the antioxidative system which is crucial for cold tolerance in many plants (Mittler 2002; Dunn et al., 2001). To evaluate the changes in

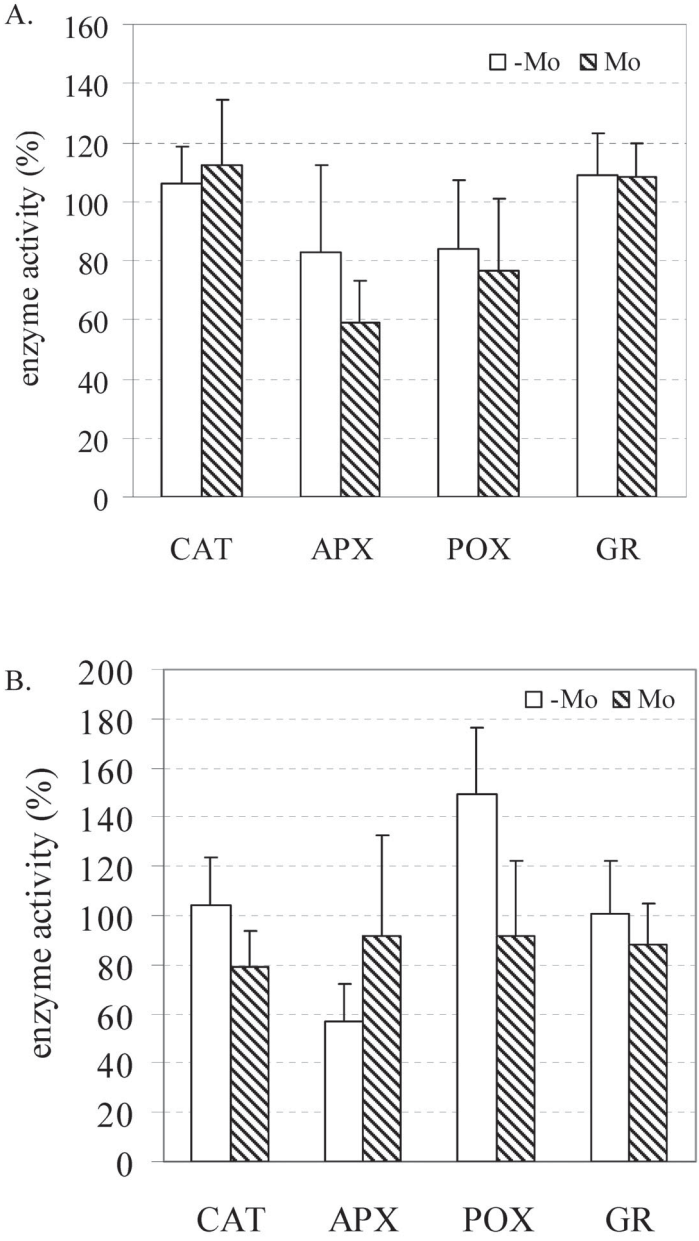

Figure 2. Effect of low temperature on the activities of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and gluthaione reductase (GR) in leaves of Mo-deficient (-Mo) and Mo-supplied (Mo) plants. A. Enzyme activities in winter wheat subjected to 2°C for 24 h. B. Enzyme activities in plants after 48 h at 2°C. All data are presented as percentage of the respective enzyme activities in plants maintained for the time of low temperature treatment at normal growth temperatures (the absolute values are presented in Table 1).

CAT and POX during early cold acclimation as dependent on Mo-availability in plants, we expressed measured activities in both Mo-deficient and Mo-supplied low-temperature treated plants as percentage of the respective activity values measured in plants that remained at normal growth temperatures. The results showed that after two days at low temperature, both enzymes exhibited higher activity in Mo-deficient winter wheat than in Mo-supplied plants (Fig 2B), suggesting higher production of ROS in Mo-deficient winter wheat and respectively higher sensitivity of these plants to low temperature exposure. It is likely that Mo-availability in winter wheat ensures higher capacity to scavenge active oxygen species still before transferring plants to low temperatures and thus may determine enhanced cold resistance of the plants.

In consistence with this suggestion, activities of the other two enzymes, APX and GR, were found to increase in Mo-deficient winter wheat irrespective of the low temperature treatment (Fig. 1). This indicates that reactive oxygen species generation rate is likely to be increased in Mo-deficient plants prior to low temperature transfer. Higher GR activity is probably necessary for maintaining a high GSH/GSSG ratio, which is supposed to ensure sufficient GSH to the H_2O_2-scavenging ascorbate-glutathione cycle. As an enzyme playing also an important role in eliminating H_2O_2 (for review, Noctor and Foyer, 1998), the increase in APX activity in Mo-deficient winter wheat might be accounted for by the increased need of preventing H_2O_2 accumulation in plant cells.

No significant changes in GR activity were observed in both Mo-supplied and Mo-deficient plants during the early cold acclimation at low temperatures. The level of GSH in plant cell has been shown to be dependent not only on GR activity but also on the activities of enzymes involved in glutathione synthesis (Kocsy et al., 1996). Hence, we assume that GSH levels in both Mo-supplied and Mo-deficient plants are likely to be high and as a result non-limiting by GR activity. Data on the other antioxidant enzyme, APX, showed that its activity tended to decrease steadily in Mo-deficient plants as compared to plants grown at optimal temperatures (Fig. 2A, B). Whereas in Mo-supplied plants, APX activity decreased after the first day at 2 °C, but increased again thereafter, reaching values measured in non-acclimated winter wheat (Fig. 2A, B). Despite of these differences, we conclude that Mo-deficiency do not limit substantially plant response to low temperature during the early period of cold acclimation.

***Acknowledgements*:** This study was supported by ”Progress in plant investigations for the improvement of sustainability of agriculture (PISA-INI14/01.09.2005)” Project (Bulgarian Ministry of Education and Sciences).

References

Aebi, H., 1984. Catalase in vitro, Meth. Enzyol., 105, 121-126.

Anderson, M.D., T.K. Prasad, C.R. Stewart, 1995. Changes in izozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings, Plant Physiol., 109, 1247-1257.

Baker, C.J., E.W. Orlandi, 1995. Active oxygen in plant pathogenesis, Annu. Rev. Phytopathol., 33, 299–321.

Bradford, M.M., 1976. A rapid and sensitive method for the quantititation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72, 248-254.

Dunn, M.A., G. O'Brien, A.P.C. Brown, S. Vural, M.A. Hughes, 2001. Resistance to abiotic freezing stress in cereals, Adv. Bot. Res., 34, 237-261.

Georgieva, D., G. Salcheva, C.H. Gramatikova, 1983. Effect of Mo on cold resistance and productivity of wheat varieties with different cold resistance. Proceedings of the II International Symposium on Mineral Nutrition in Plants, Varna 2, 379-382.

Kocsy, G., M. Brunner, A. Rüegsegger, P. Stamp, C. Brunold, 1996. Glutathione synthesis in maize genotypes with different sensitivity to chilling, Planta, 198, 365-370.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci., 7, 405-410.

Nakano, Y., K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts, Plant Cell Physiol., 22, 867-880.

Noctor, G., C.H. Foyer, 1998. Ascorbate and glutathione: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol., 49, 249–279.

Polle, A., T. Otter, F. Seifert, 1994. Apoplastic peroxidases and lignification in needles of norway spruce (*Picea abies* L.), Plant Physiol., 106, 53-60.

Pastori, G.M., C.H. Foyer, 2002. Common components, networks, and pathways of cross-tolerance to stress. The central role of "redox" and abscisic acid-mediated controls, Plant Physiol., 129, 460-468.

Prasad, T.K., 1996. Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities, Plant J., 10, 1017-1026.

Sattler, U., P. Calsou, S. Boiteux, B. Salles, 2000. Detection of oxidative base DNA damage by a new biochemical assay, Arch. Biochem. Biophys., 376, 26-33.

Tomashow, M.F., 1999. Plant Cold Acclimation: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol., 50, 571-599.

Wang, S.Y., H.J. Jiao, M. Faust, 1991. Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple, Physiol. Plant., 82, 231-236.

Xue-Cheng, S., H. Cheng-Xiao, T. Qi-Ling, 2006. Effects of molybdenum on antioxidative defense system and membrane lipid peroxidation in winter wheat under low temperature stress, J. Plant Physiol. Mol. Biol., 32, 175-182.

Yaneva, I., G. Mäck, R. Vunkova-Radeva, R. Tischner, 1996. Changes in nitrate reductase activity and the protective effect of molybdenum during cold stress in winter wheat grown on acid soil, J. Plant Physiol., 149, 211-216.