RESPONSE OF ANTIOXIDATIVE DEFENCE SYSTEM TO LOW TEMPERATURE STRESS IN TWO WHEAT CULTIVARS

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Summary. Wheat (*Triticum aestivum* L.) plants change their metabolic state in response to low non-freezing temperatures adjusting the cellular homeostasis for upcoming winter season. The induction of oxidative stress during cold hardening as well as in response to freezing stress and recovery was under investigation in this work. Here we present data about the performance of some enzymes involved in plant antioxidative network in two wheat varieties, winter and spring. Freezing induced plasma membrane disruption and considerable accumulation of different by-products like malondialdehyde and proline was observed in the non-hardy spring cultivar.

***Key words*:** antioxidative enzymes, cold hardening, freezing stress, wheat.

***Abbreviations**:* AOS – active oxygen species; APX – ascorbic acid peroxidase; CAT – catalase; CH – cold hardening; EL – electrolyte leakage; F – freezing; H_2O_2 – hydrogen peroxide; LT – low temperature; MDA – malondialdehyde; POX - guaiacol peroxidase; Pro – praline; R – recovery.

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INTRODUCTION

Plants encounter many various stresses during their life cycle. One of the biggest challenges for plant growth and productivity is to cope with the abrupt and often unpredictable temperature fluctuations. As the temperature falls below the subzero scale formation of ice in plant tissues is inevitable. Many overwintering plant species, including cereals, have developed adaptive strategies to sense the seasonal downshift in temperature adjusting their biology to the subsequent temperature regimen. The ability of plants to acquire tolerance to freezing temperatures is a consequence of numerous changes in plant cells during a period called hardening (Browse and Xin, 2001). This process is initiated by a stress response to low non-freezing temperatures and is connected with rapid changes at physiological, biochemical and molecular levels. The initial perturbations in plant metabolism are followed by alteration in plant development and morphology with a final result achievement of maximum freezing tolerance (Huner et al., 1998).

Under stressful conditions as a consequence of burst in the fine-tuned homeostasis plant cells accumulate in excess toxic by-products and among these are activated oxygen species (AOS). Potentially harmful to plant cells when in high levels, the AOS production during LT stress could have a role in stress perception and protection (Suzuki and Mittler, 2006). The tight control of AOS levels under optimal growth conditions and especially during stress events is attained by a network of antioxidative enzymes and small molecules, found in almost all cellular compartments (Mittler et al., 2004).

In this study, the effects of short-term hardening and freezing stress on the activities of the enzymes: ascorbic acid peroxidase (APX 1.11.1.11), guaiacol peroxidase (POX 1.11.1.7) and catalase (CAT 1.11.1.6), as well as on the levels of electrolyte leakage, free proline, malondialdehyde and hydrogen peroxide were examined in winter and spring wheat cultivars. Here, we put into focus the ability of winter wheat to harden for a short period and we made a comparison with the non-hardy spring cultivar to ascertain the differences in LT response of two varieties with different genetically determined capacity to cold harden.

MATERIALS AND METHODS

Plant material

Seeds of two wheat (*Triticum aestivum* L.) cultivars, the hardy winter wheat 'Sadovo-1' and the non-hardy spring wheat 'San Pastore' were grown for 12 days in loamy soil under controlled-environment conditions: PPFD - 210 μmol m^{-2} s^{-1}, photoperiod - 16 h and day/night temperature regime - 20/16 °C. Once the wheat plants had reached the stage of fully developed 2nd leaf (approx. 12 days after emerging) the seedlings were transferred to a temperature-controlled chamber with a constant temperature of 2 °C. The low temperature (LT) hardening was conducted for 7 days, in the light (16 h photoperiod and 210 μmol m^{-2} s^{-1} PPFD). Cold hardened plants were shifted to frost-chamber, where wheat plants were subjected to freezing stress. Freezing treatment was conducted for 24 h in dark with gradually decreasing temperature (1 °C per hour, final temperature –6 °C) and was followed by a short period of recovery (24 h) at 20/16 °C.

To evaluate the single effect of low temperatures all measurements of the LT treated plants were made in parallel with their respective control variants, where the growth conditions were maintained the same with the exception of the specific temperature treatments. In this respect, as controls for freezing stress (F control) served plants remained at 2 °C in the dark. They were subsequently transferred to optimal growth conditions and served as controls for recovery (R controls).

Determination of the membrane status

The plasma membrane intactness was estimated through the leakage of electrolytes, described by Sun et al. (2006). Fresh leaves (0.300 g) were placed in tubes, containing 30 ml bidistilled water and kept for 2 h in water bath at 30 °C for measuring the initial conductivity (EC1). The final electrolyte conductivity (EC2) was measured after boiling the plant samples for 15 min. The leakage percentage was calculated as (EC1/EC2) x100 %.

Lipid peroxidation was measured using the 2-thiobarbituric acid (TBA)

reaction (Heath and Packer, 1968). The plant homogenate (extracted with 0.1 % trichloroacetic acid) was mixed with potassuim phosphate buffer (pH 7.0) and the TBA-reagent (0.5 % thiobarbituric acid in 20 % trichloroacetic acid, w/v) in a ratio 1:1:2 (v/v) and the reaction was developed for 30 min in boiling water bath. The levels of TBA-conjugated substances was calculated using the extinction coefficient of 155 mM cm^{-1} from the data read at 532 nm after applying the correction read at 600 nm (for non-specific absorption).

Measurement of endogenous peroxides

Plant material (0.150 g) was homogenized with 7.5 % trichloroacetic acid and centrifuged for 20 min at 14 000 x *g*. The supernatant was mixed with potassuim phosphate buffer (pH 7.0) and 1M potassium iodide in a ratio 1:1:2 (v/v) (Jessup et al., 1994). The hydrogen peroxide content was measured at 390 nm and was determined using a standard curve.

Measurement of proline content

Determination of the free proline levels was done according to Bates et al. (1973). Proline was extracted in 3 % (w/v) aqueous sulfosalicylic acid. The reaction mixture contained supernatant, acetic acid and ninhydrin reagent in a ratio 1:1:1 (v/v) and was boiled for 1h in water bath. Proline quantity was estimated from the data received after reading the toluol fraction at 520 nm.

Enzyme activities

For the analyses of catalase (CAT 1.11.1.6) and guaiacol peroxidase (POX 1.11.1.7) 0.200 g plant tissue was homogenized in 100 mM potassium phosphate buffer (pH 7.0), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 % insoluble polyvynil pyrrilidone (the tissue/ buffer ratio of 1:5, w/v). After centrifugation (20 min at 12 000 x *g*) the activity of catalase was assayed according to Aebi (1984). The oxidation of hydrogen peroxide was followed at 240 nm for 30 sec. The guaiacol

peroxidase activity was estimated after Polle et al. (1994). To analyze the activity of ascorbic acid peroxidase (APX EC 1.11.1.11), plant tissue was homogenized in 50 mM potassium phosphate buffer (pH 7.0), containing 5 mM EDTA, 2 mM sodium ascorbate, 0.5 % insoluble polyvynil pyrrilidone (Nakano and Asada, 1981), and centrifuged at 16 000 x *g* for 5 min. The reaction medium contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM sodium ascorbate and 6 mM hydrogen peroxide. The oxidation of sodium ascorbate in the presence of APX started by adding of 9.7 M hydrogen peroxide and was followed at 290 nm for 30 sec.

Protein content in the supernatant was determined according to Bradford (1976).

RESULTS

Determination of the membrane status

The effect of low temperature on the plasma membrane intactness is shown in Fig. 1. Levels of electrolyte leakage in both wheat cultivars were not influenced by low positive temperatures (cold hardening) while treating the plants with subzero temperatures (freezing) provoked a strong increase in plasma membrane leakage estimated in leaves of winter (10-fold) and spring (15-fold) wheat plants. By contrast to tolerant wheat where the levels of membrane leakage 24 h after applying the stress have returned near control levels, the spring wheat still showed high levels of electrolyte leakage.

Data for the lipid peroxidation in response to low non-freezing temperature revealed quite similar results for the plasma membrane status of both winter and spring wheat. CH period induced a 3-fold increase in malondialdehyde content in leaves of winter and spring wheat plants (Table 1). However, while the levels of MDA in winter wheat plants remained relatively stable during subsequent treatments (freezing and recovery), a further 50 % increase was detected in spring cultivar after 24-h recovery.

Measurement of endogenous peroxides

The most pronounced effect on hydrogen peroxide levels had the period

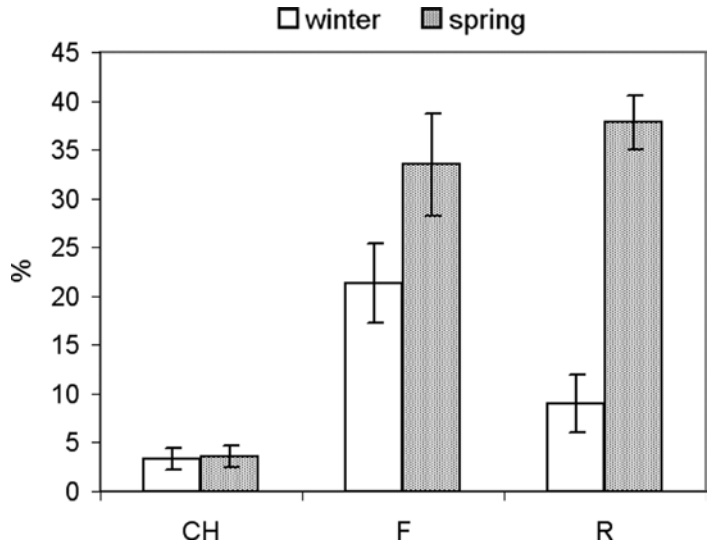

Fig. 1. Estimation of electrolyte leakage (EL) in two wheat cultivars – winter (open bars) and spring (dotted bars) type at the end of the short-term cold hardening (CH), freezing stress (F) and after 24 hours of recovery (R). The given EL results were estimated by the formula: EC1/EC2x100(%), as ratio of EL conductivity determined in wheat leaves after 3h at 25°C (EC1) and total EL conductivity after boiling the samples (EC2). The EC1 values for winter wheat are CH – 16.78±0.41 μS/g FW; F – 143.72±15.58 μS/g FW; R – 72.52±7.61 μS/g FW; for spring wheat are CH - 16.45±0.85 μS/g FW; F – 217.75±20.57 μS/g FW; R – 294.58±16.55 μS/g FW. Values ± SD are mean from 3 independent experiments.

of cold hardening giving a similar trend response in both winter and spring varieties. We estimated an increase (by 40 %) of endogenous H_2O_2 levels in leaves of winter wheat and the values for the spring cultivar were even higher – a 100 % increase was established. A slight hydrogen peroxide augmentation was observed during the following freezing and recovery treatments (Table 1).

Measurement of proline content

Exposure of plants to low temperatures provoked dynamic changes in the proline content (Table 1). Freezing stress did not affect further the content

of free proline found to be doubled in CH winter wheat (in respect to control plants). However, it was found that after 24-h recovery the proline content had increased almost 2-fold the levels estimated in CH plants. Moreover, as the proline levels in R control plants had begun to lower, it seemed that treated plants had increased almost 3-fold the proline content in response to the recovery procedure. The non-hardy spring wheat showed the same trend in proline accumulation and the estimated values were slightly higher than the winter wheat values.

Enzyme activities

Participation of three antioxidant enzymes, CAT, POX and APX in low temperature plant response was investigated in parallel between winter and spring wheat cultivars. As shown in Table 2, activity of catalase extracted from winter wheat was influenced only by low non-freezing temperatures. A stable activity state was observed after the initial decrease at the end of CH period. The spring cultivar, however, showed a non-stable CAT behavior with a gradual decrease during the whole experimental period accompanied by a decrease in control levels. Opposite to the CAT behavior were the changes in POX enzyme activity. We found insignificant changes in plant response to CH and F treatments for both wheat varieties. Transferring the frozen cultivars to optimal growth conditions, however, led to a dramatic increase in POX activity by 70 % and 50 % in winter and spring wheat, respectively (Table 2). It is interesting to notice that in spite of the higher estimated POX percentage for winter wheat, the spring R absolute values were higher than the winter R absolute values. Following the changes in APX enzyme activity (Table 2) during low temperature treatments we found dynamic fluctuations in both examined cultivars. Spring wheat APX was not significantly influenced by low non-freezing temperatures while the subsequent F stress caused an increase (by 60 %) in the activity of the peroxidase. A slight decrease in winter APX activity was found at the end of CH while F did not influence the enzyme activity. After returning to optimal conditions (24-h recovery) both examined cultivars showed an enhanced APX activity. The estimated APX activity in response to F stress was on the basis of the results obtained from control plants remained at

Table 1. Levels of the malondialdehyde (MDA, nmol g^{-1} FW), hydrogen peroxide (H_2O_2, nmol g^{-1} FW) and free proline (Pro, μmol g^{-1} FW) measured in leaf extracts from winter and spring type wheat plants at the end of short-term cold hardening, freezing stress and 24-h recovery. The control plants were not treated with the specific temperature, characteristic for each point. Values ± SD are mean from 3 independent experiments.

		Levels of MDA, H_2O_2, Pro					
		Winter wheat			Spring wheat		
		Hardening	Freezing	Recovery	Hardening	Freezing	Recovery
MDA	Treated plants	21.51±0.86	21.33±2.58	20.42±1.30	20.73±0,95	19.10±0.60	32.11±4.43
	Control plants	7.81±1.00	19.89±1.73	21.20±1.82	9.00±1.05	17.69±1.76	17.72±0.86
H_2O_2	Treated plants	3.75±0.53	3.93±0.43	3.90±0.45	3.48±0.55	3.93±0.84	3.85±0.48
	Control plants	2.71±0.59	3.44±0.35	3.70±0.66	1.73±0.14	3.69±0.63	3.25±0,05
Pro	Treated plants	149.75±64.24	178.73±50.25	304.73±74.62	194.71±56.26	205.66±70.73	375.20±33.76
	Control plants	72.03±8.67	129.80±18.93	114.35±17.26	73.58±10.74	164.96±23.82	108.13±29.24

2 °C in the dark. A rapid decrease in APX activity after switching off the light was noticed for both control plants.

DISCUSSION

Winter cereals grow and develop at geographical latitudes where freezing temperatures are common events. Freezing stress has a complex occurrence due to mechanical impairments through ice formation in plant tissues and physiologically by alterations in membrane properties, changes in enzymatic reactions and interactions among macromolecules (Palva et al., 2002). Resistance to low freezing temperatures therefore is a characteristic trait of tolerant plant species acquired after a period of cold hardening. Thus, fully hardened freezing-tolerant plants are able to survive temperature as low as –20 °C, while a temperature of –5 °C could be lethal for unhardened plants (Thomashow, 1998).

In cereals the plasma membrane is the most susceptible membrane structure to freezing damages, and the impaired integrity is connected with leakage of electrolytes and other solutes (Pearce, 2001). In our work, we found that low freezing temperatures had a profound negative effect on membrane stability in spring wheat plants. In contrast to hardy cultivar, the higher EL levels during freezing combined with the lack of ability to minimize the membrane leakage during recovery showed a well-pronounced LT sensitivity of the non-hardy spring cultivar.

The negative impact of freezing stress on spring wheat was further supported by the data for lipid peroxidation where continuous accumulation of the lipid breakdown products during recovery period was under consideration. The elevated MDA levels in response to CH was in agreement with previously reported results for wheat plants subjected to LT for 48 h and 72 h (Sun et al., 2006; Yordanova and Popova, 2007). Protection of cell membrane, thus, is critical for freezing survival and it is usually achieved by accumulation of compatible solutes in the cytoplasm and alterations in membrane lipid composition. Solutes accumulate during hardening and contribute to plant cell adjustment by reducing the extent of cellular dehydration and macromolecule denaturation (Steponkus, 1984; Pearce, 2001). As part of compatible substances network we investigated

Table 2. Enzyme activities of catalase (CAT), guaiacol peroxidase (POX) and ascorbic acid peroxidase (APX), extracted from leaves of winter and spring type wheat plants during low temperature treatment. Both varieties have undergone hardening period (7 days), freezing stress (24 h) and recovery procedure (24 h) while the respective control variants differ from the test ones in specific temperature treatment concerning every point. Values ± SD are mean from 3 independent experiments.

		Enzyme activity (μmol mg^{-1} protein min^{-1})					
		Winter wheat			Spring wheat		
		Hardening	Freezing	Recovery	Hardening	Freezing	Recovery
CAT	Treated plants	12.98± 1.50	11.92±0.91	11.77±0.85	11.30±1.06	9.06±0.96	8.18±0.83
	Control plants	15.82±1.93	11.41±1.13	11.05±1.65	13.62±1.90	10.83±1.15	11.56±1.19
POX	Treated plants	310.84±51.54	329.28±54.53	474.27±51.57	266.79±26.23	298.56±30.35	523.34±46.74
	Control plants	280.81±56.07	267.89±20.02	284.93±61.86	252.57±54.11	271.88±36.09	343.15±31.15
APX	Treated plants	34.07±6.33	28.16±3.14	35.87±6.99	34.14±7.17	37.24±4.57	50.95±6.51
	Control plants	39.03±7.59	27.13±8.89	28.75±6.43	37.37±6.46	23.52±2.56	35.74±7.25

the changes in proline content in response to LT stress. Doubled Pro levels at the end of CH were detected, while during F no changes were observed. A further increase in proline levels was found 24 h after transferring the plants to optimal growth conditions. The observed elevated levels of proline in response to salt stress (Reza et al., 2006) and H_2O_2-treatment (Upadhyaya et al., 2006) showed that Pro accumulation is not specific for freezing stress and could not be particularly related to hardiness acquisition. Moreover, Wanner and Junttila (1999) did not find a correlation between the continuous Pro accumulation and freezing tolerance in hardened Arabidopsis plants. According to the authors, although Pro accumulation is closely correlated to freezing tolerance, this could rather be a consequence than a cause for enhanced freezing tolerance (Wanner and Junttila, 1999).

Another effect of LT stress on plant homeostasis is the imbalance of metabolite pathways and enhanced production of AOS. As a consequence of their dual role, acting both as toxic compounds and as signal mediators, plants must exhibit the possibility to lower the excess of AOS and to modulate precisely the low levels for signalling purposes (Mittler, 2002). In our experiment, levels of endogenous peroxides were strongly increased in the spring cultivar in response to CH, and to a lesser extent in the winter wheat. Further F and R treatments enhanced insignificantly the H_2O_2 levels.

Scavenging the harmful AOS and particularly decomposition of hydrogen peroxide is assessed by plant antioxidant network, part of which are the enzymes CAT, APX and POX. Low non-freezing temperature resulted in decreased CAT and APX activities for both wheat varieties. Our results are in contrast to results observed by Janda et al. (2007). These authors found a strong induction of both CAT and APX in winter wheat after 12 days of cold hardening. The short CH period in our case could be of importance for the studied enzymes, despite of their different affinities to H_2O_2, as well as by their differences in redox status sensitivity (Mittler, 2002).

The following F and R treatments imposed an enduring decrease in spring CAT activity, indicating lesser capacity of this wheat variety to cope with the LT stress. In spite of this result was the observed stable CAT activity in winter wheat plants. Interpretation of the results for APX activity in response to freezing stress and recovery, however, suggested an

interesting dependence on the activities found in control plants. As already mentioned above, the APX in winter wheat seemed to be strongly induced upon recovery as a result of diminished APX activity in the respective control plants, while in the spring cultivar the elevated APX levels were not supplemented by lower control levels.

The results for POX showed a similar performance in both examined cultivars. Being not influenced by CH and F, this enzyme increased strongly its activity upon transferring the plants to normal growth conditions.

In summary, the short-term cold hardening of wheat plants did not cause significant differences in the responses of both examined cultivars. However, freezing stress appeared to be a challenge to a greater extent for the spring cultivar, particularly in relation to plasma membrane intactness. An obvious stress response, more intense in spring wheat could be detected based on the results obtained for enzyme activities and levels of MDA and hydrogen peroxide. Acquisition of cold hardiness is a complex process and involves many physiological changes in plant cells, part of which are directly related to hardiness, but others result from the non-specific response to stress events.

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