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Impact of cold-induced antioxidant activity on frost resistance in androgenic *Festulolium* genotypes

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Summary

The aim of the study was: (i) to state if selected in the field conditions androgenic *Festulolium* genotypes are diverse in frost tolerance, and (ii) to investigate if changes in anitoxidant activity could be recognized as a physiological marker of this type tolerance. Antioxidative system induced by prehardening (12°C) and hardening (2°C) temperatures was investigated in 6 androgenic genotypes generated from a *Festuca pratensis* × *Lolium multiflorum* (2n = 4x = 28) amphidiploid hybrid (four genotypes derived from the F_1 hybrids, and two genotypes derived from the *Festulolium* cultivar 'Rakopan'). The electrolyte leakage, frost resistance expressed as the values of temperature causes 50% damages (LT_{50}), the activities of superoxide dismutase (SOD), catalase (CAT), non-specific peroxidase (PX) and ascorbate peroxidase (APX) were measured. The results obtained indicated weak diversity of frost tolerance among studied androgenic genotypes. Only the one genotype was chosen as the most resistant to frost, while the other genotypes demonstrated no significant differences in values of LT_{50} coefficient recorded after hardening. Prehardening temperature of 12°C caused an increase in cell membrane permeability in all genotypes studied. After hardening ion leakage from cells declined up to the control level. Generally, cold activated SOD, PX and APX in leaves of the genotypes studied, and inhibited strongly CAT activity. The most frost tolerant genotype was characterized by high PX activity after hardening process.

Abbreviations: APX – ascorbate peroxidase, CAT – catalase, EDTA – ethylenediaminetatraacetic acid, LT_{50} – letal temperature causing 50% membrane cell damages, NBT – nitroblue tetrazolium, PPFD – photosynthetic photon flux density [µmol m^{-2} s^{-1}], PX – non-specific peroxidase, RH – relative humidity, ROS – reactive oxygen species, SE – standard error, SOD – superoxide dismutase

Introduction

Grasses are a great important plant group to sustainable agriculture. Especially at present, according to European Union (EU) guidelines relating fodder quality the culture of forage grasses comes into prominence. There is a challenge of a creation of new cultivars characterized by high quality of biochemical composition and resistance to environmental stresses (Humphreys et al., 1998). Species within the *Lolium-Festuca* complex belong to the most important forage grasses. They offer many valuable and complementary traits, including the high productivity and forage quality of *Lolium*, and the persistency and resistance to environmental stresses of *Festuca* (Thomas and Humphreys, 1991). So, *Festuca* species can be used as sources of genes for winter hardiness, drought tolerance as well as disease resistance. *Lolium* and *Festuca* species hybridise and their chromosomes pair and recombine in hybrids, thus, it is possible to transfer stress resistance genes from *Festuca* into *Lolium* by backcross breeding procedures (Zwierzykowski et al., 1999; Kosmala et al., 2006). Whilst conventional plant breeding programmes provide many opportunities for combining useful traits, pre- and post zygotic selections limit access to all potentially useful gene combinations. In addition, many useful gene combinations in breeding programmes may remain 'hidden' due to the non-expression of recessive alleles, epistasis, or pleiotropy (Thomas et al., 2003).

Androgenesis (*in vitro* anther culture of microspores) is a frequent component of plant breeding methodology for several crop species to a rapid achievement uniform and homogeneous genotypes. In grasses within the *Lolium-Festuca* complex androgenesis was found to be an effective way to select new gene combinations which have rarely or never been recovered by conventional backcross breeding programmes (Humphreys et al., 1998; Leśniewska et al., 2001). In some experiments androgenesis enhanced gene expression for complex traits, including freezing-tolerance (Rapacz et al., 2005), in excess of that found in the parent genotypes.

Overwinter grasses should be characterized by good winter hardiness including tolerance to frost and other factors occurring during winter. Frost can damage cells, tissues, whole organs, and finally lead to plant death. Tolerance to low temperature depends on tolerance to dehydratation of the cell and damages of cell membranes. Accumulation of low molecular compounds decreasing osmotic potential and its freezing temperature is the most common mechanism initiated during plant hardening at cold (Crow et al., 1984). Hardening to frost is preceded by prehardening. Prehardening proceeds at day temperature of about 12°C. Night temperature and light intensity during this period do not influence so significantly plant acclimation to cold. Prehardening effects the frost tolerance *via* stimulation of photosynthesis at hardening temperature and increasing energy amount for biochemical processes by simultaneous inhibition of elongation growth. During plant hardening proceeding usually at 1-5°C the accumulation of soluble carbohydrates, a decrease in gibberellin content and an increase in growth inhibitor level were indicated (Rapacz and Janowiak, 1998). After cold-hardening in cell membranes many structural changes are observed. Mainly there should be mentioned changes in proportion between saturated and non-saturated fatty acids, an increase of synthesis of phospholipids and a decrease in sterol content. These changes influence a decrease in membrane permeability, which protects cells against unfavourable electrolyte leakage (Yoshida and Uemura, 1990; Skoczowski, 1999). High correlation between frost tolerance and membrane permeability in hardened plants of barley, meadow fescue and winter rape was found (Płażek and Żur, 2003).

During cold conditions also activity of antioxidant enzymes is changed, as an effect of generation of reactive oxygen species (ROS). ROS traditionally considered as toxic products play also a positive role, i.e. they control programmed cell death, participate in defence response to abiotic stress and pathogens and are mediators in signal transduction pathways. So, a balance between ROS accumulation and antioxidant activity is important for plant reaction to stresses (Alscher et al., 1997).

ROS are generated as a result of disturbances on different metabolic pathways. Chloroplasts and mitochondria are the most important sources generating superoxide radical ($O_2^{\cdot -}$). At low temperature high-energy state electron is not utilised by photosynthetic CO_2 fixation but is converted to reactive O_2 form. Increase of ROS concentration due to cold could be a result of over-reduction of the electron transport chain in mitochondria. ROS cause oxidative damages to protein,

nucleic acid and lipid peroxidation, and membrane deterioration (Bestwick, 1998; Pastori, 2000).

Plants are equipped with complex antioxidant systems composed by low molecular mass antioxidants and enzymes protecting against oxidative damages. Essential for ROS detoxification are such ROS-scavenging enzymes as superoxide dismutase (SOD), which converts $O_2^{\cdot -}$ to H_2O_2, catalase (CAT), ascorbic peroxidase (APX) and other peroxidases (PX; mainly wall-bound ones), which decompose H_2O_2 to H_2O and O_2. APX is probably responsible for the fine modulation of ROS because of its low concentration. Similarly, low concentration of hydrogen peroxide takes part in signal transduction involved in resistance mechanisms (Mitler, 2002; Vranová et al., 2002).

The aim of the study was to investigate the relationship between changes in antioxidant enzyme activities during prehardening and hardening and frost tolerance of studied plants. Obtained results could serve as physiological markers for breeding of new cultivars characterized by higher winter hardiness.

Material and methods

The study was performed on six androgenic genotypes generated from the amphidiploid *Festuca pratensis* × *Lolium multiflorum* (2n = 4x = 28) hybrid (named *Festulolium*) (Zwierzykowski et al., 2001). Four genotypes, nos. 715, 716, 729 and 768, derived from F_1 hybrid plants, and two genotypes, nos. 561 and 621, derived from the *Festulolium* cultivar 'Rakopan'. The genotypes were chosen in the previous study conducted in the field conditions in Polish Breeding Station in Lopuszna on the basis of visual scale estimated physiological state and regrowth after two winter seasons 2002/2003 and 2003/2004 (Rapacz et al., 2005). Selected plants were cloned and grown in glasshouse condition at 18°C (day/night) and day light for 5 weeks (September), then were prehardened for 2 weeks at 12°C (day/night) and 10h-photoperiod at photosynthetic photon flux density (PPFD) of 250 µmol m^{-2} s^{-1} and 80% RH and next hardened for 3 weeks at constant temperature of 2°C at 8h-photoperiod at the same light intensity. The plants were fertilized weekly with Hoagland's liquid medium. Analyses were performed at 18°C (control), after prehardening and hardening.

Electrolyte leakage

Five leaf disks cut to a size of 5 mm in diameter were placed in a vial containing 13 cm^3 ultrapure water. They were shaken (1.7 s^{-1}) at 20°C. Conductivity was measured using CI317 conductometer (Elmetron, Poland) after 2 h, and the vials were stored at -50°C overnight. After thawing, the vials were shaken for 2 h and the conductivity was measured again. The measurements performed on frozen and thawed tissue represent the conductivity of the total ion content in the tissue. Membrane permeability is expressed as a percentage of total electrolyte leakage.

The LT_{50} coefficient

Frost resistance of leaves was determined by lethal temperature of 50% of the leaf samples using electrolyte leakage test. Leaf sections of about 15 mm long were cut from the middle part of the leaf. The sections were immediately placed on ice (to avoid excessive undercooling by ensuring ice nucleation) in plastic vials and frozen for two hours at -3, -6, -9, -12 and -15°C. The applied rate of freezing was approximately 12 $K \cdot h^{-1}$. Samples were thawed for approximately 24 h at 2°C on a shaker. Electrical conductance of the solution were measured using a CI317 conductometer (Elmetron, Poland). The index of injury was calculated according to Flint et al. (1967) and LT_{50} was calculated based on the linear regression fitted within the linear range of the relationship between freezing temperatures and the percentage of killed plants (at least 3 temperatures). Determinations were performed in 10 replications for each freezing temperature.

Assay of superoxide dismutase (SOD) (E.C.1.15.1.1) activity

Leaf samples were homogenized at 4°C with 50 mM phosphate buffer pH 7.5 and centrifuged at 16 000 x *g*. The resulting supernatant was used as crude extracts. The reaction mixture (final volume of 3 cm^3) contains 50 mM phosphate sodium buffer (pH 7.8) with 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2 µM riboflavine and 50 µl of supernatant. Reaction was started by lightening with a fluorescence lamp (for 10 min.) and stopped by its switching off. Spectrophotometric measurement was done at 560 nm. Unlighted reaction mixture does not change colour and is treated as a control. Extract volume relating to 50% inhibition of reaction is considered as an enzyme-activity unit (Beauchamp and Fridovich 1971).

Assay of catalase (EC 1.11.1.6) activity

Catalase activity was estimated according to Aebi (1984). Samples of leaves were homogenised at 4°C with 50 mM phosphate buffer (pH 7.5) and 1 mM EDTA and centrifuged at 16 000 x *g*. CAT activity was assayed in reaction mixture (3 cm^3 final volume) composed of 50 mM phosphate buffer pH 7.5, to which 30% (w/v) H_2O_2 was added to reach an absorbance value in the range of 0.520-0.550 (λ = 240 nm). The reaction was started after adding to the reaction mixture 200 µl of crude extracts. CAT activity was measured as the decrease in absorbance at 240 nm (using spectrophotometer LKB Ultrospec II) as a consequence of H_2O_2 consumption. The decrease in absorbance of 0.0145 responded with 1 µmol H_2O_2 decomposed by CAT. Activity of the enzyme was expressed as µmol H_2O_2 decomposed per minute per mg of protein. The determination of CAT activity was done in 5 replicates (in five leaves from different plants of each object).

Assay of total peroxidase (PX) activity

Peroxidase activity was measured spectrophotometrically according to the method described by Bergmeyer (1965). Leaf samples were ground to a fine powder with liquid nitrogen and extracted with 50 mM phosphate buffer (pH 7.0) and 1 mM EDTA. The extracts were centrifuged (16 000 x *g*) at 4°C for 10 min and the resulting supernatants were used as the crude extracts. Two cm^3 of 50 mM phosphate buffer (pH 7.0) were mixed with 12 µl of 0.5% p-phenylenediamine and with 12 µl of crude extract. The oxidation of p-phenylenediamine was initiated by addition of 12 µl of buffered H_2O_2 (0.15 cm^3 of H_2O_2 mixed with 50 cm^3 of extract buffer) to the prepared mixture. The absorbance was measured at λ = 460 nm (using a spectrophotometer LKB Ultrospec II). The peroxidase activity was expressed as difference of absorbances of sample recorded at the beginning of measurement and after 1 min. Assays were done in 5 replications (in five leaves from different plants of each object).

Assay of ascorbate peroxidise (APX) (EC 1.11.1.11.) activity

Ascorbate peroxidase activity was estimated spectrophotometrically according to Nakano and Asada (1981). Sample of leaves were homogenised and extracted with 50 mM phosphate buffer (pH 7.0) and 1 mM EDTA then centrifuged at 16 000 x *g*. One cm^3 of reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.25 mM ascorbic acid, 0.5 mM H_2O_2 and 10 µl of crude extract. The absorbance was measured at λ = 290 nm (using Spectrophotometer LKB Ultrospec II). The peroxidase activity was expressed as µmol of ascorbic acid $\cdot$ $min^{-1} \cdot mg^{-1}$ protein. Assays were done in 5 replications (in five leaves from different plants of each object).

Protein determination

Protein content was determined according to the method described by Bradford (1976) using the Bio-Rad protein assay (Munich, Germany) with bovine serum albumin as a calibration standard.

Statistical analysis

The obtained results were statistically analysed independently by analyses of variance (ANOVA/MANOVA) with Statistica Version 6.1. software. The values demonstrated in graphs are the means ± SE (standard error).

Results

Cell membrane permeability and frost tolerance (LT_{50} coefficient)

Prehardening at 12°C significantly influenced cell membrane permeability in all studied genotypes (Fig. 1). In all cases prehardening caused significant increase in ion leakage from leaf cells. The highest membrane permeability after prehardening was observed in leaf cells of genotype 768. Other genotypes demonstrated at this time the same level of ion leakage.

After hardening membrane permeability was lower than initially found in genotypes 715, 716 and 729. In other genotypes electrolyte leakage declined to the level noted before prehardening. Frost tolerance evaluated by mean of LT_{50} test after prehardening increased in genotypes 561, 621, 715 and 729, while in genotypes 716 and 768 temperature of 12°C did not effect on LT_{50} coefficient values (Fig. 2). In these two genotypes only hardening at 2°C increased frost tolerance. In the case of genotypes 561, 621 and 715 after hardening the LT_{50} values were higher comparing to values after prehardening but lower than in the control plants (before prehardening). In the case of genotype 729 LT_{50} values after prehardening at 12°C did not differ from the values after hardening at 2°C. On the basis of LT_{50} test genotype 561 could be recognized as the most frost resistant.

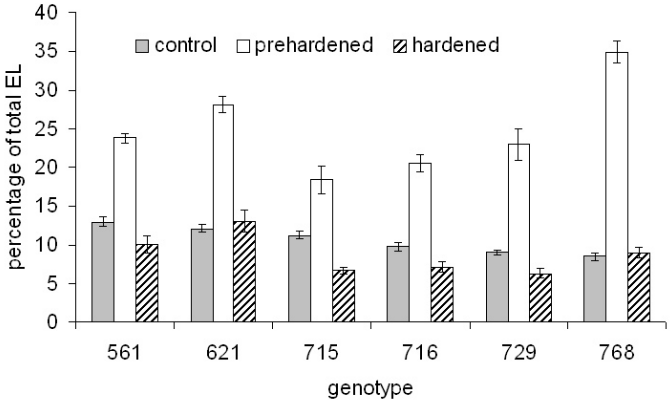

Fig. 1: The electrolyte leakage from leaves of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 10 replicates ± SE.

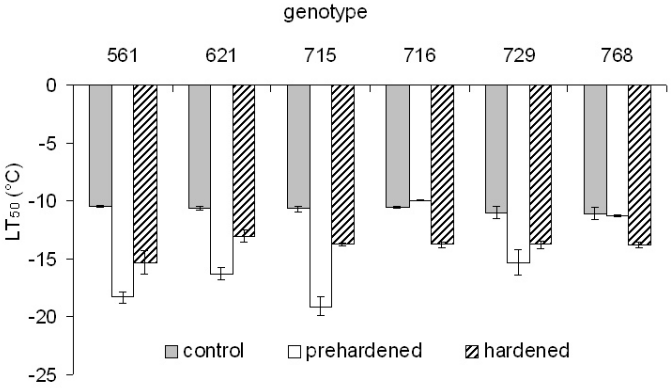

Fig. 2: The frost resistance expressed as the values of LT_{50} (temperature causing 50% of damages) of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 10 replicates ± SE.

SOD activity

Prehardening at 12°C activated superoxide dismutase in leaves of only two genotypes: nos. 561 and 768 (Fig. 3). Hardening at 2°C increased activity of that enzyme in genotypes 561, 621 and 716. In the case of genotypes nos. 715 and 729 prehardening and hardening temperature did not affect activity of SOD. The correlation between LT_{50} coefficient and SOD activity was found in the case of only two genotypes: 561 ($r = 0.72$; $P < 0.05$) and 716 ($r = 0.97$; $P < 0.05$).

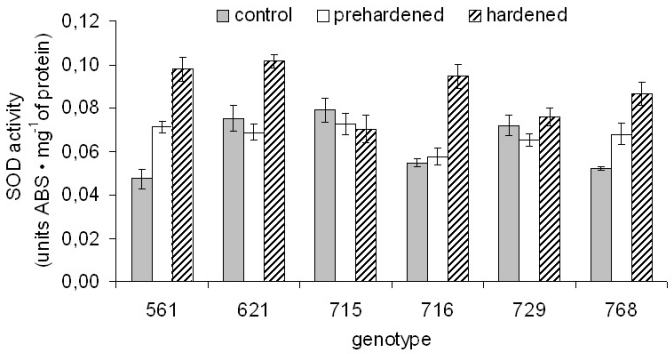

Fig. 3: The activity of superoxide dismutase (SOD) in leaves of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 5 replicates ± SE. (ABS-absorbance).

Statistical analysis showed that the CAT activity in leaves of all investigated genotypes rapidly decreased after prehardening in comparison with the control, however activity of that enzyme after hardening increased, remaining still significantly lower than the control values (Fig. 4). The highest CAT activity was noted in the case of control plants genotypes nos. 621 and 768. Control plants of other

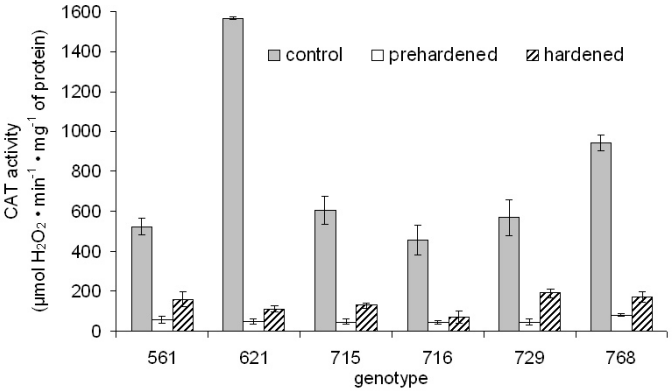

Fig. 4: The activity of catalase (CAT) in leaves of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 5 replicates ± SE.

genotypes demonstrated CAT activity on the same level. Negative correlation between LT_{50} coefficient and CAT activity was found in the case of two genotypes: no. 561 ($r = -0.96$; $P < 0.05$) and no. 715 ($r = -0.88$; $P < 0.05$).

PX activity

Total peroxidase activity in leaves was significantly higher after 2 weeks of prehardening at 12°C in all studied genotypes regardless of their frost tolerance (Fig. 5). After hardening for 3 weeks at 2°C an increase in activity of that enzymes in comparison with the prehardened plants was observed only in two genotypes: 561 and 715, however in the case of this first one PX activity was significantly higher.

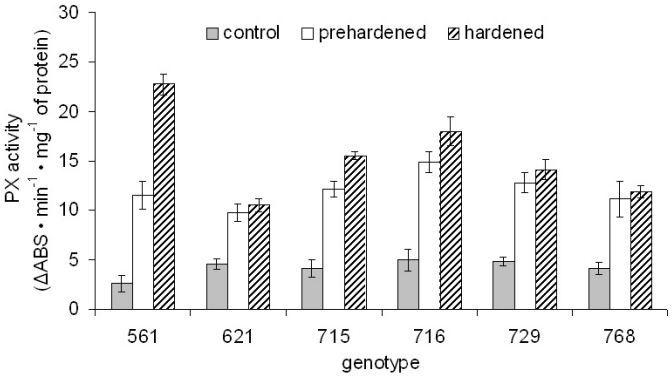

Fig. 5: The activity of peroxidase (PX) in leaves of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 5 replicates ± SE. (Δ ABS – difference in absorbance per min per g of protein).

APX activity

All studied genotypes demonstrated the same level of APX activity in control plants (Fig. 6). The temperature of prehardening (12°C) resulted in rapid APX activity in all investigated genotypes in comparison with the control. After two weeks of hardening at 2°C also in all cases, significant decrease of that enzyme activity was observed. However, leaves of hardened plants of genotypes nos. 561, 715, 729 and 768 showed still considerably higher APX activity than the control samples. The correlation between LT_{50} coefficient was found in the case of two genotypes: 561 ($r = 0.95$; $P < 0.05$) and 715 ($r = 0.97$; $P < 0.05$).

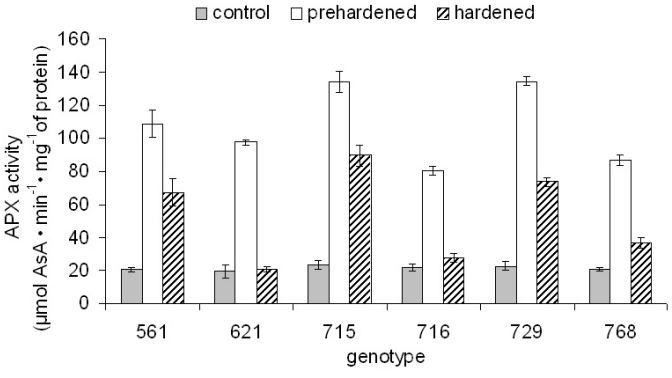

Fig. 6: The activity of ascorbate peroxidase (APX) in leaves of control (18°C), prehardened (12°C) and hardened (2°C) plants of androgenic *Festulolium*. Mean values of 5 replicates ± SE. (AsA – ascorbate acid).

Discussion

The hardening temperature stimulates several metabolic processes that could play a important role in the plant acclimation to cold and frost. Physiological background of cold acclimation involves apart from a decrease in osmotic potential mainly rebuilding of membrane structure (Yoshida and Uemura, 1990; Uemura and Steponkus, 1994). According to these authors characteristic response of plant cells to cold stress is a change in cell membrane permeability. The destabilization of membranes causes strong cell dehydratation and synthesis of abscisic acid (ABA) or ROS generation. Bravo et al. (1998) showed correlation between ABA concentration and frost tolerance of barley. Carruthers and Melchior (1986) demonstrated that „fluidity" of cell membranes influences activity of membrane enzymes and receptor proteins fixed in plasmalemma. Gaudet et al. (1999) supposed that the resistance mechanism for winter factors includes common physiological processes induced during cold acclimation. Processes that enhance frost tolerance may also increase resistance to snow mould pathogens, which are very significant factors deciding about winter hardiness of overwintering crops.

In all *Festulolium* genotypes studied after prehardening at 12°C cell membrane permeability significantly increased and then after hardening at 2°C it returned to the control values. It is worth to add that prehardening caused the greatest ion efflux from cells, while the following temperature of 2°C did not cause membrane injuries. This result indicates the very positive role of prehardening in 12°C for acclimation to frost. The most favourable membrane rebuilding preventing ion leakage from cells during hardening was noted in plants of genotypes nos. 715, 716 and 729, so in those genotypes, which were not recognized as the most frost resistant. Płażek and Żur (2003) showed that cell membrane permeability in barley, meadow fescue and winter rape hardened at 5°C for 6 weeks was specific for each plant species and may change relating to time of hardening. Generally, initially ion leakage from cells of these species increased, and after 6 weeks decreased significantly.

In four androgenic *Festulolium* genotypes studied prehardening increased their frost tolerance expressed as LT_{50} coefficient. After following hardening the LT_{50} values increased, but they were still lower than before prehardening. In two studied genotypes nos. 716 and 768 temperature of 12°C did not cause changes in frost tolerance. Only at 2°C these genotypes demonstrated an increase of frost tolerance. This result could indicate a positive effect of 12°C-treatment for processes influencing resistance to frost, for example rebuilding of cell membranes and synthesis of specific proteins protecting plasmalemma and cytoplasm before ice-caused injuries, which appear during frost temperatures. Similar results were obtained by Rapacz (1998), which showed the positive effect of prehardening at 15°C to frost acclimation of winter rape.

Hardening at 2°C may stimulate ROS accumulation and activation or inhibition of some antioxidant enzymes. According to some authors oxidative stress usually is connected with decrease in activity of antioxidants (Scebba et al., 1999; Shim et al., 2003). At prehardening temperature of 12°C Scebba et al. (1999) found a decrease in SOD activity, an increase in guaiacol peroxidase and no changes in CAT activity, however the same authors noted an increase in activity of all antioxidants after freezing at -4°C. Shim et al. (2003) reported that in rice seedlings subjected to 8°C, CAT activity was reduced by low temperature and this decrease was much sever in the low-temperature-sensitive cultivar comparing to the tolerant one. Oidaira et al. (2000) observed in rice seedlings exposed to low temperature a transient increase in APX activity and slow increase in SOD activity. In contrast, the CAT activity was not significantly affected. Inhibition of CAT at cold condition was observed also in maize seedling (Leipner et al., 1999; Pastori et al., 2000), and in meadow fescue, barley and winter rape (Płażek and Żur, 2003). Results obtained by Zhao and Blumwald (1998) suggested that enzymes activated during cold acclimation,

involving in the ascorbic-glutathione cycle play a protective role against ROS generated in jack pine seedlings after exposure to freezing temperatures. These authors found no changes in APX activity in roots of seedlings after 1 and 2 week-growth at 5°C but it increases after 4 week-cold. Kuk et al. (2003) noted significant activation of CAT and APX in cold treated rice leaves, while SOD, CAT, APX and glutathione reductase in roots. They concluded that increased activity of antioxidants in roots was more important for cold tolerance than the increase of their activity in shoots. Results obtained by Seppänen and Fagerstedt (2000) suggested that although SOD activity in potato hybrids may contribute to their freezing tolerance and acclimation capacity, cold-induced acclimation capacity was not related to level of that enzyme activity. According to Jouve et al. (2000) the temporary rapid increase of SOD after 2 days at 10°C in poplar could be due to chilling.

In four androgenic *Festulolium* genotypes studied after cold hardening activity of SOD increased, while in two genotypes it remained unchanged. Simultaneously, hardening caused the decrease in CAT activity in all investigated genotypes. APX in the hardened plants was higher than in the control ones of four genotypes and in the others it achieved the control values. Obtained data partly agreed with results of Kubo et al. (1999). According to these authors chilling temperature in *Arabidopsis thaliana* increased activity of APX, whereas it decreased CAT activity and did not influence SOD activity. According to Asada (1992) APX is the major scavenger of hydrogen peroxide in plant cells, and its activity increases in response to various environmental stressors. Ascorbate-glutathione cycle is present in almost all cellular compartments (chloroplasts, cytosol, mitochondria, peroxisome and apoplast) suggesting its crucial role in controlling the ROS level. According to another author (Mitller, 2002) APX and CAT belong to different classes of scavengers. APX is probably responsible for the fine modulation (activity of μM range), while CAT (activity of mM range) could be responsible for scavenging excess of ROS during stress. It is worth to add, that CAT showed its activity mainly in peroxisomes and is closely associated with photosynthesis intensity. At 2°C this process is strong inhibited.

In the presented work hardening caused an increase in PX activity of all genotypes. This phenomenon could be explained by involving of apoplastic peroxidases in lignification process in cell walls. Obtained results indicate that activity of SOD converting superoxide to hydrogen peroxide was associated with activity of total peroxidases, for which H_2O_2 is a substrate. Initially SOD activity was also followed by APX activity, however after hardening at 2°C its activity significantly decreased. This result could be explained by the domination of PX activity in cell walls in lignification processes over APX one.

Concluding, the investigation indicate that studied androgenic genotypes were not diverse in frost resistance. Moreover, although the studied plant material was derived from *Festuca pratensis* × *Lolium multiflorum* amphidiploid hybrid (four genotypes derived from the F_1 hybrids, and two genotypes from the *Festulolium* cultivar 'Rakopan'), the origin of these plants did not influence studied parameters. Only one genotype obtained from 'Rakopan' was distinguished significantly by frost tolerance. It may be explained by different factors that initiate the response mechanisms to cold stress. On the basis of obtained results it could be supposed that only an increase in total peroxidase activity may be assumed as a physiological marker of frost tolerance in grasses within *Lolium-Festuca* complex.

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