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Cell membrane permeability and antioxidant activities in the rootstocks of *Miscanthus x giganteus* as an effect of cold and frost treatment

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Summary

The aim of the study was to estimate the ability of *Miscanthus x giganteus* to acquire frost tolerance. Field grown rootstocks were transferred into pots and cultivated in a glasshouse at 20°C. After 5 weeks plants were pre-hardened at 12°C for a further 2 weeks and then hardened at 5°C for another 3 weeks. After this time, plants were frozen at -8°C or -15°C for 1, 3 or 5 days, after which their regrowth at 20°C was investigated. The membrane permeability (electrolyte leakage), activity of the catalase (CAT), non-specific peroxidase (PX), and protein content in stolons were measured, before and after pre-hardening, as well as after hardening and freezing.

Both pre-hardening and hardening decreased membrane permeability of the rootstock cells, and this effect was observed further, after 5-week of regrowth at 20°C. Freezing at both temperatures increased ion leakage gradually over the period of treatment. On the basis of total ion content, damage to the cell membranes of frozen stolons after recovery was stated. Prehardening increased CAT activity, while hardening did not alter it. However, after 5-week de-hardening, CAT activity decreased significantly. Freezing at -8°C for 5 days increased significantly the activity of this enzyme. At -15°C CAT activity was lower than in the control after only one day of freezing. PX activity decreased both in the rootstocks of cold (12°C and 5°C) and frost treated plants. Protein content increased significantly in the stolons of both pre-hardened and hardened plants, although not immediately after cold treatment, but only after a 5-week re-growth period in a glasshouse at 20°C. This phenomenon was observed also in the stolons of plants frozen at -15°C for 5 days. From frozen rootstocks no new stems in regrowth conditions were obtained. The results obtained indicated, that although frozen stolons cannot produce new shoots, they do demonstrate some metabolic vitality. So, it could be supposed that the frost susceptibility of studied plants resulted from the strong sensitivity of shoot apical meristems to the cold. Further studies will analyse the survival of Miscanthus in milder frost temperatures.

Abbrevations: CAT – catalase, EDTA – ethylenediaminetetraacetic acid, FW – fresh weight, PPFD – photosynthetic photon flux density [µmol m^{-2} s^{-1}], PX – non-specific peroxidase, ROS – reactive oxygen species,

Introduction

Miscanthus x *giganteus* is a sterile triploid (hybrid of the diploid *Miscanthus sinensis* and tetraploid *M. sacchariflorus*), which reproduces only vegetatively from rootstocks. Recently, Miscanthus has received the interest of producers because of the possibility of its use as a biofuel or cellulose source. This is a perennial plant of a C_4-photosynthesis type, characterized by high CO_2 absorption, high yield potential and low water consumption. It does not demand high mineral nutrition and can be cultivated on poor soils. Moreover, because of many tangled stolons it prevents soil erosion. These properties are important for contemporary, sustainable agriculture designed to keep wastelands in a good condition (LEWANDOWSKI, 2006).

However, *Miscanthus* plants are sensitive to frost, especially during the first winter. The loss of many plants results in the necessity of spring re-planting, so increasing production costs. The consequence of vegetative reproduction is a small range of genetic variability within this species, so it is very difficult to select plants with higher frost resistance.

The mechanisms responsible for freezing tolerance are not very well understood, and have a very wide background. Herbaceous plants from temperate regions survive temperatures ranging from -5°C to -30°C, while plants from tropical regions generally have little or no capacity to survive even the slightest frost. Many important crops, such as rice, maize, soybean, cotton and tomato are chill sensitive and incapable of cold acclimation. Moreover, they cannot tolerate ice formation in their tissues. Nevertheless, the temperature threshold for chilling damage is lowered even in chill-sensitive crops by prior exposures to sub-optimal low temperatures (CHINNUSAMY et al., 2007).

Freezing tolerance is induced during cold hardening at non-freezing temperatures. Cold acclimation involves the stabilization of plasma membranes against freeze-induced damages, synthesis of proteins preventing molecules from precipitation and direct physical damages caused by the accumulation of intercellular ice. Cold acclimation includes an increase in fatty acid desaturation in membrane phospholipids and changes the levels and types of membrane sterols and cerebrosides (THOMASHOW, 1998). MURATA and LOS (1997) reported that changes in membrane fluidity under cold treatment regulate the activity of receptor-mediated phospholipase C and protein kinase C, key enzymes, in signal transduction during thermoacclimation.

Low temperature evokes the accumulation of proteins, especially those of small molecular weight with antifreeze properties (THOMASHOW, 1998; KARIMZADEH et al., 2000; CHINNUSAMY et al., 2007). These proteins protect membranes and other cell structures from mechanical injuries, regulate cell metabolism, and control their osmotic potential (ANTIKAINEN and GRIFFITH, 1997; YU and GRIFFITH, 1999; THOMASHOW, 1998; CHINNUSAMY et al., 2007).

Oxidative stress is the secondary effect of numerous abiotic and biotic stresses. Exposure to low temperatures causes changes in the activity of various antioxidant enzymes, which is connected with the higher production of reactive oxygen species (ROS). In maize, many studies have linked chilling tolerance to antioxidant capacity (PASTORI et al., 2000). Hydrogen peroxide is recognized as a signal molecule initiating the defence responses of plants to many stressors. This compound mediates the cross-talk between signalling pathways, and is a key signalling molecule contributing to the phenomenon of cross-tolerance. Hence, the mediating role of H_2O_2 takes into account any programme aimed at improving crop tolerance to environmental stresses (NEILL et al., 2002). Among genes up-regulated by hydrogen peroxide are those, which code the proteins required for peroxisome biogenesis, senescence-related proteins, antioxidant enzymes, protein kinases and phosphatases, and DNA damage repair proteins (DESIKAN et al., 2000). Hydrogen peroxide also regulates stomata closure in response to abscisic acid (ALLAN and FLUHR, 1997). The accumulation of hydrogen peroxide under cold treatment in many plant species was observed (SCEBBA et al., 1998; SEPPÄNEN and FAGERSTEDT, 2000; PASTORI et al., 2000). The plant defence antioxidant mechanism includes enzymatic and non-enzymatic scavengers of ROS, so their amount in cells may be estimated on the basis of antioxidant activities. The main antioxi-

dant enzymes that scavenge hydrogen peroxide are catalases and peroxidases. They are activated by different H_2O_2 concentrations (MITTLER, 2000), and catalase (CAT) as chilling-sensitive, while peroxidase (PX) as chilling-tolerant are recognized (SCEBBA et al., 1999). The aim of the study was to estimate, the processes taking place in the rootstocks of *Miscanthus x giganteus* undergoing pre-hardening or hardening temperatures, and if the changes induced by low temperature are stable during regrowth in a warm glasshouse. The frost tolerance was estimated on the basis of changes in membrane permeability, as well as the activities of enzymes scavenging hydrogen peroxide in the rootstocks of plants undergoing pre-hardening, hardening and frost treatment.

Material and methods

Plant growth conditions

In August, the rhizomes of Micanthus were transferred from the field to pots and grown in a glasshouse in natural light at 20°C. The pots contained a mixture of soil : peat : sand (2 : 2 : 1 v/v/v) at pH 5.8. Six-week-old plants were pre-hardened for 2 weeks in a growth chamber (12°C, 10 h-photoperiod, 250 µmol m^{-2} s^{-1} of PPFD) and than hardened for a further 3 weeks (5°C, 8 h-photoperiod, light intensity as above). After this time the plants were frozen at -8ºC or -15ºC for 1, 3, or 5 days in the dark. Some pre-hardened, hardened and frozen plants were taken for analyses, while others were transferred to a glasshouse (conditions as described above) for 5 weeks after which their regrowth ability was examined. Pre-hardened plants transferred to 20°C for 5 weeks were marked as RP (regrowth after prehardening), hardened ones as RH (regrowth after hardening, also known as dehardened plants), and frozen plants for 1, 3 or 5 days, as R1, R3 and R5 respectively, for each frost temperature. Control plants were grown in a glasshouse and were divided into three groups: C1, C2 and C3 – plants growing in the glasshouse for 5, 7 and 10 weeks respectively. Their age responded to age of prehardened (C2) and hardened as well as frozen plants (C3). C1 was the initial control. The estimation of membrane permeability and total ion content per g of FW was performed on stolons from prehardened, hardened and frozen plants, as well as after 5-week regrowth at 20°C, while in the case of other measurements, frozen plants after the regrowth period were not analyzed.

Analyses

Ion leakage

The conductivity was expressed as the ion leakage *via* cell membranes. Stolon disks (approximately 0.3 g of FW) were placed into vials containing 13 cm^3 of ultrapure water, and were shaken (100 rpm) at 20°C. After 2 h, the electrical conductivity (E_1) was measured using a conductometer (CI 317, Elmetron, Poland). Then, the vials with the samples were boiled for 5 min and shaken again. The conductivity was re-measured and this value was taken to represent the total ion content (E_2) in the tissue. Membrane permeability was expressed as a percentage of total electrolyte leakage ($E_1 x 100/E_2$). Moreover, total ion content per g of FW in the stolons was calculated. The measurements were done in 50 replicates (10 discs from any of 5 stolons for each treatment).

Catalase (EC 1.11.1.6) (CAT) activity

Frozen samples of stolons (each weighing 0.2 g of FW) were ground to a fine powder with liquid nitrogen and extracted with 50 mM of phosphate buffer (pH 7.5). The extracts were centrifuged at 4°C for 10 min at 14 000 × *g* and the supernatants were used as the crude extracts.

CAT activity was assayed in a reaction mixture (3 cm^3 final volume) composed of 50 mM of 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 A at 240 nm. The reaction was started by adding 200 µl of crude extracts. CAT activity was measured by monitoring the decrease in absorbance at 240 nm (using spectrophotometer LKB Ultrospec II, Umea, Sweden) as a consequence of H_2O_2 consumption. The decrease in absorbance of 0.0145 A corresponded to 1 µmol H_2O_2 decomposed by CAT (AEBI, 1984). The activity of the enzyme was expressed as µmol H_2O_2 decomposed per minute per g of FW of stolons. Catalase assays were done in 15 replicates (3 samples were collected from any of 5 stolons for each studied object).

Total peroxidase (PX) activity

Peroxidase activity was measured according to the method described by BERGERMEYER (1965). Stolon discs were ground to a fine powder with liquid nitrogen and extracted with 50 mM of phosphate buffer (pH 7.0) and 1 mM EDTA (SIGMA-ALDRICH). The extract was centrifuged (14 000 rpm) at 4°C for 10 min and the supernatant was used as the crude extract. Two cm^3 of 50 mM phosphate buffer (pH 7.0) was mixed with 12 µl of 0.5% *p*-phenylenediamine and 12 µl of crude extract. The oxidation of *p*-phenylenediamine was initiated by the addition of 12 µl of buffered H_2O_2 (0.15 cm^3 of 30% H_2O_2 (w/v) mixed with 50 cm^3 of extract buffer) to a prepared mixture. The absorbance was measured at 460 nm. The total peroxidase activity was expressed as an increase in absorbance of the sample after 1 min and expressed as activity per 1 mg of FW. The determination of PX activity was completed in 15 replicates.

Protein determination

Protein concentration was determined according to BRADFORD (1976) using the Bio-Rad (Munich, Germany) protein assay reagent. Bovine serum albumin (Sigma-Aldrich) was used as a calibration standard. The measurements were taken in 15 replicates.

Statistical analysis

All the effects of various temperatures on studied parameters in stolons were tested with the *F*-test (analysis of variance ANOVA, Statistica 5.0).

Results

Statistical analysis showed the significant effect of applied temperatures on all studied parameters. Although the pre-hardening temperature did not influence the membrane permeability to a significant degree, some tendency to improve (the decrease) cell membranes permeability under a 12°C-treatment could be seen (Fig. 1). After 5-week-growth after pre-hardening at 20°C, a considerable decrease in ion leakage from the stolon cells (RP) was observed. A temperature of 5°C caused a significant decrease in ion leakage immediately after hardening. Less membrane permeability remained on the same level after 5-week-growth (RH) in glasshouse conditions. In the case of freezing at -8ºC and -15ºC, the membrane damages increased throughout the duration of the treatment. Electrolyte leakage from cells of stolon frozen at -8°C and -15°C for 1 day (R1 and R2) in regrowth conditions, did not change from those tested just after freezing, while the membrane permeability of rootstocks frozen at both frost temperatures for 3 and 5 days (R3 and R5) decreased at the control temperature.

Total ion content in the stolons of pre-hardened plants did not differ significantly from the control (C2), although an increase in this para-

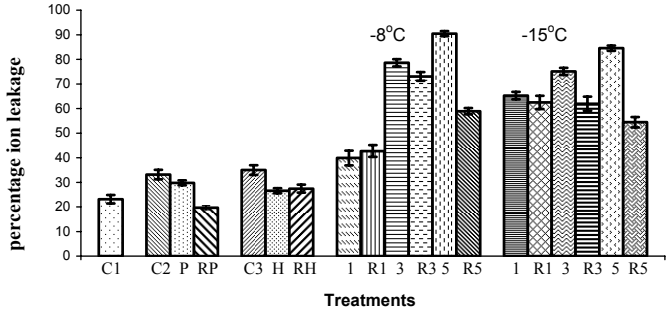

Fig. 1: Electrolyte leakage from the rootstocks of prehardened (P) at 12°C, hardened (H) at 5°C and frozen at -8°C and -15°C for 1, 3 or 5 days Miscanthus plants (-8/1, -8/3, -8/5; -15/1, -15/3 -15/5). C1 – initial control; C2 – control for prehardened plants; C3 – control for hardened and frozen plants. RP – pre-hardened plants regrowing at 20°C for 5 weeks; RH – hardened plants re-growing at 20°C for 5 weeks; R1, R2 and R3 – frozen plants at both frost temperatures for 1, 3 or 5 days respectively and regrowing at 20°C. Mean of 50 replicates ± SE.

meter could be seen especially after the recovery period (Fig. 2). Hardening caused a considerable increase in ion content in tissue compared with C3 plants and after 5-weeks of de-hardening this process was even stronger. Frozen stolons demonstrated no significant difference in ion content compared to the rootstocks of hardened plants. However, some influence of prolonged time and a stronger frost on a parameter decrease was observed. After 5-week regrowth at 20°C independent of the time and temperature of frost treatment, an almost total ion efflux from the stolons was observed.

CAT activity during prehardening at 12°C increased significantly and after 5-week-recovery at 20°C, it did not change (Fig. 3). The rootstocks of hardened plants demonstrated few changes in CAT activity, while

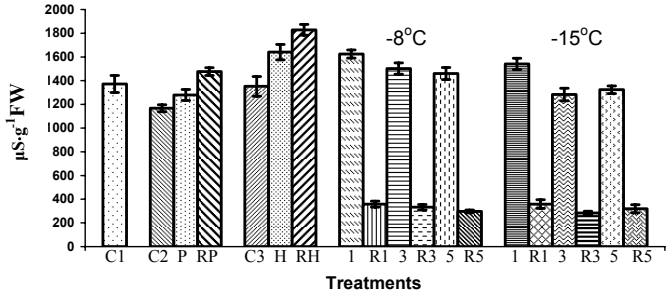

Fig. 2: Ion content [µS g^{-1} FW] in the rootstocks of prehardened, hardened and frozen Miscanthus plants. Treatment labels the same as on Fig. 1. Mean of 50 replicates ± SE.

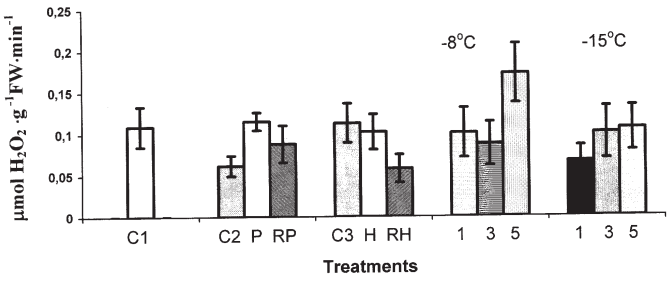

Fig. 3: Catalase activity in the rootstocks of prehardened, hardened and frozen Miscanthus plants. Treatment labels the same as on Fig. 1. Mean of 15 replicates ± SE.

during de-hardening (RH) at 20°C they showed a decrease. Generally, both applied frost temperatures did not cause significant changes in CAT activity, and only freezing at -8°C for 5 days increased the activity of this enzyme.

PX activity decreased along with plant age (C1, C2, C3) (Fig. 4). Prehardening resulted in a decrease in the activity of this enzyme, and the decrease was also observed later in the stolons of plants transferred back to a glasshouse at 20°C (RP). Neither hardening nor -5°C exerted changes in PX activity. Plant freezing at -15°C for 5 days increased PX activity in comparison with frozen plants at -8°C and at -15°C for 1 and 3 days. However, the level of PX activity in the stolons of frozen plants at -15°C for 5 days, returned to the level of the C3 plant rootstocks.

Protein content per FW unit strongly increased in both pre-hardened and hardened plants, not immediately after cold treatment, but only after 5-week regrowth in a glasshouse at 20°C (Fig. 5). This phenomenon was observed also in the stolons of plants frozen at -15°C for 5 days. Analysis after freezing showed that many stolons demonstrated no visible damage. However, in the case of both frost treatments (temperature x time) no new shoots from rootstocks kept in regrowth conditions were obtained. This result suggests that temperatures applied independently of time treatment were lethal for Miscanthus apical meristems.

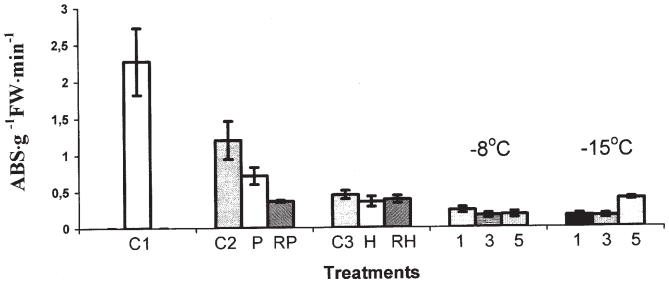

Fig. 4: Non-specific peroxidase activity in the rootstocks of prehardened, hardened and frozen Miscanthus plants. Treatment labels the same as on Fig. 1. Mean of 15 replicates ± SE.

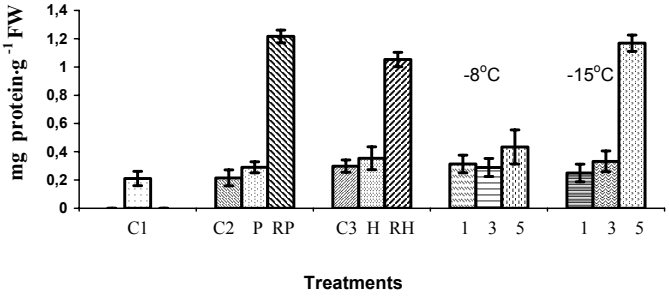

Fig. 5: Protein content in the rootstocks of prehardened, hardened and frozen Miscanthus plants. Treatment labels the same as on Fig. 1. Mean of 15 replicates ± SE.

Discussion

The main problem for plants originating from other climatic zones acclimatizing to Polish environmental conditions, is their weak resistance to the cold and freezing temperatures in winter.

For thermophilic plants even temperatures of 10°C during spring or autumn can evoke cold damage. In the case of Miscanthus, the first winter in the field determines its further cultivation.

Changes in the structure of cell membranes are often observed as the

effects of cold temperatures (Yoshida and Umera, 1990; Murata and Los, 1997; Thomashow, 1998). These changes relate especially to the proportion between the amounts of saturated and non-saturated fatty acids, the polar phospholipids content, and H^+-ATPase activity, which is closely connected with the selective characteristic of membranes. According to Carruthers and Melchior (1986), selective characteristics of cell membranes significantly influence the activity of several membrane-enzymes. This fact points to the positive role of cold in the activation of membrane proteins, functioning as receptors in the signal transduction initiated during the defence responses to any stress.

In the presented experiment, ion leakage from cells was measured in the rootstocks of Miscanthus. After the first cold days in autumn the leaves of this species dry very quickly, and as such the metabolic changes effected by prehardening, hardening and frost were studied in the stolons, because their physiological condition before and during winter determines whether Miscanthus survives the winter. Quamme and Brownlee (1997) used ion leakage from apple-tree root pieces for estimation of resistance to winter injury of rootstocks of various cultivars. The laboratory tests of frost resistance agreed with reports of field survival.

The prehardening of Miscanthus plants did not change significantly the membrane permeability of stolon cells. However later, during de-prehardening, the positive effect of this treatment on the selectivity of membranes was observed. Hardening caused a considerable decrease in ion leakage from cells and this effect remained during de-hardening. Cold-improving membrane permeability could be concluded on the basis of total ion content in the tissue. In the earlier study, Płażek and Żur [2003] stated that in winter rape, spring barley and meadow fescue, cold hardening at 5°C decreased the membrane permeability of leaf cells, while de-hardening increased electrolyte leakage to the control level. In the frozen stolons, ion leakage increased gradually throughout the time treatment, possibly resulting from frost damaged tissue. Also, in this case after 5-weeks re-growth at 20°C, the electrolyte leakage decreased similarly to the effect observed by de-prehardened and de-hardened rootstocks. This result could be explained by partial ion efflux from damaged tissue to the soil, although many stolons seemed to be uninjured. This is why no other measurements in stolons re-grown after frost-treatment were performed. Arora and Palta (1991) stated in freeze-thawed onion bulbs a higher ion leakage rate, which decreased during recovery. The authors explained this was the result of frost reduction of plasma membrane ATPase activity, which then returned to the control level.

In wheat under water stress, Pereyra et al. (2006) observed no changes in membrane structure and permeability in root cells contrary to those of the leaves. Kang and Saltveit (2002) stated that chilling maize, cucumber and rice seedlings at 15°C, affected the increase in electrolyte leakage from leaves but did not influence this parameter in radicals. The change pattern of CAT and PX activity in Miscanthus rootstocks under cold treatment, differ from the effects noted in leaves by other authors (Scebba et al., 1999; Back and Skinner, 2003; Ella et al., 2003). These results could indicate that roots differ in reaction to the stresses from leaves.

Oxidative stress has been proposed as one of the reasons for cold-temperature damage (Scebba et al., 1999). Reactive oxygen species are supposed to be responsible for frost-induced injuries because they are produced at higher concentrations during freezing stress. The higher tolerance to oxidative stress evoked by freezing is induced by cold-acclimation temperatures (Scebba et. al., 1998). ROS generation is different in various plant parts. Scebba et al. (1999) showed, that the roots of cold-treated wheat demonstrated higher an activation of SOD (superoxide dismutase) and CAT, while in cold-hardened leaves a decrease in activity of both enzymes compared to the control was observed. The main source of ROS in leaves are electron transport *via* thylacoids and mitochondrial membranes, and the reactions taking place in peroxisomes during photooxidation, while in rhizomes ROS are produced mainly during the electron transportation chain in mitochondria. Hydrogen peroxide could moreover be generated in apoplast in both organs – leaves and rhizomes. In Miscanthus stolons, prehardening temperature of 12°C increased CAT activity, while a hardening temperature of 5°C did not change it. The cold effect (the decrease in CAT activity) was observed much later in de-hardened plants, although interpretating this result is not easy. A significant increase in activity of this enzyme in the stolons of frozen plants was observed at -8°C, but only after 5 day-treatment. The activity of antioxidants in frozen rootstocks during recovery was not measured, because no plants from these rootstocks were obtained, although it could indicate that both applied freezing temperatures were lethal for this plant species. CAT is recognized as a cold-sensitive enzyme, and usually its lower activation level in cold is observed (Leipner et al., 1999; Płażek and Żur, 2003), although during recovery its activity increases to the control level (Ella et al., 2003; Płażek and Żur, 2003]. Thus, the decrease in CAT activity in stolons, 5 weeks after hardening, may be difficult to explain. It's very possible, that despite no obvious visible damage, in the cold aftermath some metabolic processes in Miscanthus rootstocks were altered. Although no correlation between cell membrane permeability and CAT activity was found, it could be supposed that in stolons of prehardened and hardened plants, this enzyme protected cell structures against the cold.

PX was drasticly inhibited in the Miscanthus stolons during prehardening, while lower temperatures during hardening and freezing did not influence PX activity, except at -15°C lasting for 5 days, when PX activity increased to the control level. Peroxidase is recognized as cold-tolerant, so the obtained result relating to the CAT increase and PX decrease in frost indicates, that rhizomes react differently from leaves and the interpretation of these results could not be compared to processes occurring in the leaves. Moreover, the increase in CAT activation could be a response to the dramatic decline in PX activation, and it was caused by an increase in hydrogen peroxide accumulation. The different response of Miscanthus stolons at 12°C and the much lower temperatures could suggest, that this plant species may also be classified as cold-sensitive equally with maize, sorghum and cotton, for which even 15°C is a stress-temperature.

Low temperature evokes the accumulation of various proteins, especially those of small molecular weight (Thomashow, 1998; Karimzadeh et al., 2000; Chinnusamy et al., 2007). These proteins with antifreeze properties protect membranes and other cell structures from mechanical injuries, regulate metabolic processes, and control the osmotic potential of cells (Antikainen and Griffith, 1997; Yu and Griffith 1999; Thomashow, 1998; Chinnusamy et al., 2007).

Cold hardening resulted in protein accumulation especially in winter cereals (Antikainen and Grffith 1997; Yu and Griffith, 1999). These proteins demonstrated mainly antifreeze properties. Karimzadeh et al. (2000) confirmed higher protein accumulation in the roots of hardened wheat compared to the plants growing at 20°C. According to some authors (see Karimzadeh et al., 2000), the cold treatment in various plant species had no effect on protein synthesis, for example in *Lolium temulentum*. In contrast, the tropical cereal *Sorghum bicolor* grown initially at 35°C, responded markedly to low temperatures. In the studied Miscanthus rootstocks, a drastic increase in protein accumulation after prehardening at 12°C and hardening at 5°C was observed, but only after 5-week regrowth at 20°C. Freezing of plants at -8°C for 5 days caused some increase in the protein content in stolons, although this change was not statistically significant. In contrary, the influence of -15°C for 5 days, increased (by 3.5 times) the accumulation of proteins in Miscanthus stolons, than in the case after freezing for 1 or 3 days.

From the frozen stolons no new stems in re-growth conditions were obtained. This result could be explained by the strong sensitivity of the meristematic tissues of Miscanthus to the cold. According to field observations, after the first autumn chilling, the leaves of this plant

species remain green, while the apical part of stems inside the leaf sheaths become brown and atrophy. The results obtained indicated, that although frozen stolons can not produce new shoots they do demonstrate some metabolic vitality. Further studies will analyze the survival possibility of Miscanthus in milder frosts than applied in the presented experiment.

Conclusions

1. Prehardening at 12°C and hardening at 5°C decrease cell membrane permeability, while freezing temperatures increase ion leakage from the cells of Miscanthus rootstocks. Less membrane permeability remains during further plant cultivation, which could be recognized as some symptoms of the cold acclimation of this plant species.
2. A temperature of 12°C significantly increases catalase activity, and decreases non-specific peroxidase activity in Miscanthus stolons.
3. Prehardening, hardening and frost temperatures do not influence protein accumulation in Miscanthus stolons. However, a significant increase in protein content does occur in the aftermath of chilling.
4. The frost sensitivity of Miscanthus is determined by a high susceptibility of apical meristems to frost.

References

Aebi, H., 1984: Catalase in vitro. Methods of Enzymol. 105, 121-126.

Allan, A.C., Fluhr, R., 1997: Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell. 9, 1559-1572.

Antikainen, M., Griffith, M., 1997: Antifreeze protein accumulation in freezing-tolerant cereals. Physiol. Plant. 99, 423-432.

Arora, R., Palta, J.P., 1991: A loss in the plasma membrane ATPase activity and its recovery coincides with incipient freeze-thaw injury and postthaw recovery in onion bulb scale tissue. Plant Physiol. 95, 846-852.

Baek, K., Skinner, D.Z., 2003: Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. Plant Sci. 165, 1221-1227.

Bergermeyer, H.U., 1965: Methods of enzymatic analysis. Verlag Chemie GMBH Weinheim/Bergstr., Academic Press, New York and London.

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

Chinnusamy, V., Zhu, J., Zhu, J.K., 2007: Cold stress regulation of gene expression in plants. Trends Plant Sci. 12, 444-451.

Carruthers, A., Melchior, D.L., 1986: How bilayer lipids affects membrane protein activity. Trends Biochem. Sci. 11, 331-335.

Desikan, R., Neil, S.J., Hancock, J.T., 2000: Hydrogen peroxide-induced gene expression in *Arabidopsis thaliana*. Free Radical Biology and. Medicine. 28, 773-778.

Ella, E.S., Kawano, N., Ito, O., 2003: Importance of active oxygen-scavenging system in the recovery of rice seedlings after submergence. Plant Sci. 165, 85-93.

Kang, H., Saltveit, M.E., 2002: Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid. Physiol. Plant. 115, 571-576.

Karimzadeh, G., Francis, D., Davies, M.S., 2000: Low temperature-induced accumulation of proteins is sustained both in root meristems and in callus in winter wheat but not in spring wheat. Ann. Bot. 85, 769-777.

Leipner, J., Fracheboud, Y., Stamp, P., 1999: Effect of growing season on the photosynthetic apparatus and leaf antioxidative defenses in two maize genotypes of different chilling tolerance. Environ. Exp. Bot. 42, 129-139.

Lewandowski, I., 2006: Miscanthus – a multifunctional biomass crop for the future. In: Jeżowski, S., Wojciechowicz, M.K., Zenkteler, E. (eds.), Alternative plants for sustainable agriculture, vol. 5, 83-90. PAGEN, Centre of Excellence in Plant Agrobiology and Molecular Genetics. Institute of Plant Genetics, Polish Academy of Sciences, Poznań.

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

Murata, N., Los, D.A., 1997: Membrane fluidity and temperature perception. Plant Physiol. 115, 875-879.

Neil, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T., 2002: Hydrogen peroxide and nitric oxide as signalling molecules in plants. J. Exp. Bot. 53, 1237-1247.

Pastori, G., Foyer, C.H., Mullineaux, P., 2000: Low temperature-induced changes in the distribution of H_2O_2 and antioxidants between the bundle sheath and mesophyll cells of maize leaves. J. Exp. Bot. 51, 107-113.

Pereyra, M.A., Zalazar, C.A., Barassi, C.A., 2006: Root phospholipids in *Azospirillum*-inoculated wheat seedlings exposed to water stress. Plant Physiol. Biochem. 44, 873-879.

Płażek, A., Żur, I., 2003: Cold-induced plant resistance to necrotrophic pathogens and antioxidant enzyme activities and cell membrane permeability. Plant Sci. 164, 1019-1028

Quamme, H.A., Brownlee, R.T., 1997: Cold hardiness evaluation of apple rootstocks. VI International Symposium on Integrated Canopy, Rootstock, Environmental Physiology in Orchard Systems. ISHS Acta Hort. 451,

Scebba, F., Sebastiani, L., Vitagliano, C., 1998: Changes in activity of antioxidative enzymes in wheat (*Triticum aestivum*) seedlings under cold acclimation, Physiol. Plant. 104, 747-752.

Scebba, F., Sebastiani, L., Vitagliano, C., 1999: Protective enzymes against activated oxygen species in wheat (*Triticum aestivum* L.) seedlings: Responses to cold acclimation. J. Plant. Physiol. 155, 762-768.

Seppänen, M.M., Fagerstedt, K., 2000: The role of superoxide dismutase activity in response to cold acclimation in potato. Physiol. Plant. 108, 279-285.

Tomashow, M.F., 1998: Role of cold-responsive genes in plant freezing tolerance. Plant Physiol. 118, 1-7.

Yoshida, S., Uemura, M., 1990: Responses of the plasma membrane to cold acclimation and freezing stress. In: Larsson, C., Mřller, I.M. (eds.), The plasma membrane: structure, function and molecular biology, 293-319. Springer-Verlag, Berlin, Heidelberg,

Yu, X., Griffith, M., 1999: Antifreeze proteins in winter rye leaves form oligomeric complexes. Plant Physiol. 119, 1361-1369.

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