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Convener

P. M. Pukacki

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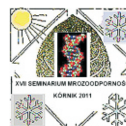

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Editorial
This book of proceeding contains contributions submitted by authors. The results and opinions presented, as well as the texts of the contributions are under full responsibility of the authors.

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Preface

This book is the collection of papers on plant cold hardiness that were presented at the 17th Cold Hardiness Seminar in Poland (17th CHSinP), hosted by the Institute of Dendrology Polish Academy of Sciences, Kórnik, in May 17–18, 2011. This was organized by the Lab of Physiology of Abiotic Stress, Institute of Dendrology. There were 21 participants representing 6 universities and institutes: Florence (Italy), Kraków, Kórnik, Poznań, Skierniewice and Warsaw (Poland). The presentations at the 17th CHSinP centred on various aspects of molecular, physiological mechanisms of plant freezing stress and fundamental research of plant cryopreservation.

Accordingly the participants are grouped under three themes:

- Physiological and molecular aspects of low temperature adaptation
- Desiccation, cryoprotection and genetic stability
- Occurrence and prevention of frost injury in field conditions

We would like to thank the authors for their cooperation in preparing their manuscripts for publication. The conference and book would not have been without support of: the Committee of Horticultural Sciences, Polish Academy of Sciences and Institute of Dendrology PAS, Kórnik.

Finally, we are grateful to Professor Jacek Oleksyn, Director of Institute Dendrology PAS in Kórnik, for the kindness and helpfulness during conference.

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Physiological and molecular aspects of low temperature adaptation

Tissue specific DHN24-dehydrin from *Solanum* species has cryoprotective ability towards freezing sensitive enzymes

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Abstract

We have analyzed tissue, cellular localization and potential function of a dehydrin from *Solanum sogarandinum*, designated DHN24. Our initial studies revealed that accumulation of the DHN24 protein in response to low temperature was only observed in the *Solanaceae species* that have ability to acclimate to cold and was organ dependent. Further studies revealed that accumulation of the DHN24 protein was also observed under water deficit in the Solanaceae species with ability to adaptation to water deficit. Tissue immunolocalization studies of DHN24 protein in organs of *S. sogarandinum* revealed that the DHN24 protein only localizes to sieve-tube cells and companion cells of the phloem tissues in roots, stems and leaves. At the subcellular level DHN24 is present in the cytosol and in nucleus. The DHN24 is phosporylated in the plant tissues at poly-serine track by a serine/threonine protein kinase. The CD analysis indicated that the recombinant AtHIRD11 was disordered in the water solutions. DHN24 displays cryoprotectve ability *in vitro* towards freezing sensitive enzymes (LDH). Potential function of the DHN24 *in planta* is still unknown.

In Polish territory plants start growth usually early in April, and in May, annular plants are at the seedlings stage and horticultural ones are under blooming. Short frosts that come usually in May caused major yield losses in agricultural crops and horticultural plants productivity.

Cultivated potato varieties originate from *Solanum tuberosum* species exhibit low freezing tolerance and low ability to cold acclimate. On the contrary, many of wild uncultivated *Solanum* species are freezing tolerant and have ability to cold acclimation. Note that most of potato cultivars also exhibit low ability to adaptation to water deficit. Increase in freezing tolerance and ability to cold acclimation of cultivated potatoes would allow to start growing earlier in spring and speed up the harvest in summer. Then the main stage of the plant growth would precede the massive development of potato pests and pathogens in summer and help to maintain a stable yield. On the other hand, increase of potato tolerance to water deficit would help to maintain the yield stability during summer drought.

In our approach to improve freezing capacity and acclimation ability to cold, first we focused on isolation of genes the expression of which is associated with freezing tolerance and acclimate to cold in *Solanum* species. The isolated genes would help to select genotypes of better performance against the stressful conditions. On the other hand, analysis of a potential function of the protein products of the cold acclimating genes enable better understanding the mechanisms of acclimate to cold in *Solanum* species.We have screened more than 100 genes

from a cold acclimating *Solanum sogarandinum* the expression of which increased in response to low temperature treatment. They encode proteins involved in different functions in the plant cell (Rorat et al. 1997; Rorat et al. 1998, Rorat 2000). However, analysis of their expression in response to cold in *Solanum* species differing in ability to cold acclimation revealed that expression only some of them was associated with acclimate to cold. Three of the isolated genes, designated *Dhn10*, *Dhn15* and *Dhn24* encoded dehydrin proteins,

```
Dhn15                                                                Dehydrin subclass
                   Y
1      MAQYGNQDQMRKTDEYGNHVQETGAYQGTGTGGMMGTGGMGGGYGTQGTG
                                       S                 K
51     TGMGMGTGGTQGMAHHHEGQQQLRRSDSSSSSEDDGEGGRRKKGMKEKIM
                                            K
101    EMPGGHSQQEGEYGAQHGQTTGYGTTEEKKGMMDKIKDKIPGMH          YSKn
                                                             (typical dehydrin
                                  S                          type)
Dhn10
                                                   K
1      MAGIIHKIEEKLHIGGGHKEEEHKKEEHKGEGHKEGFVEKIKDKIHGEES
                                         S
51     GEHHKDGKEKKKKKDKKEKKEKKHDGHDSSSSSDSD
                                                             KnS

Dhn24
1      MADQYEQNKPSVEETVGANVEATDRGLFDFIGKKEEEKPSHAHEEEAISS
                                         S
51     EFCEKVKVSEEEEHKEKEKKEEKKLHRSSSSSSSSSDEEEEIGEDGQIIK
             K                                          K
101    KKKKKGLKEKIKEKISGDHKEEVKTEDTSVPVEKYEETEEKKGFLEKIKE    SKn
                                                K
151    KLPGGGHKKTEEVAAPPPPPPAAVDHEAEGKEKKGFLDKIKEKLPGYHSK
201    TEEEKEKEKD
```

Fig. 1. Amino acid sequence of the DHN15, DHN10 and DHN24 dehydrins from *Solanum sogarandinum*. The cDNA clones coding for the DHN proteins are deposited in the GeneBank database under the accessions AA076679, AF542504 and AY292655 for the *Dhn15*, *Dhn10* and *Dhn24* clones, respectively. Specific conservative features, i.e. segment Y, poly serine track (S) and lysine rich segment (K) were underlined.

distinctive features such as a consensus motif T/VDEYGNP (the Y-segment), a track of Ser residues (the S-segment) and lysine-rich 15-amino acid domain, EKKGIMDKIKEKLPG, named the K-segment. Our initial data of expression analysis of the *Dhn* genes at the transcriptional level showed that actually only the expression of the *Dhn24* gene, encoding a DHN24 dehydrin (a SKn-type) was associated with acclimate of *Solanum* species to low temperature.Expression of the *Dhn10* gene was primarily regulated by factors related to an organ type and leaf developmental stage during vegetative growth (Rorat et al. 2004) but expression of the *Dhn15* gene was shown to be more associated with adaptation to water deficit than to cold and was regulated by ABA-dependent signal pathway.To gain more

insight into the involvement of DHN24 dehydrin in plant acclimate to cold, we investigated *Dhn*24 gene expression, at the protein level in different organs of *Solanum* species differing in ability to acclimate to cold subjected to low temperature treatment. hese data allowed the separation of two groups in terms of ability to acclimate to cold, a one group able to acclimate to cold, constituted by *S. sogarandinum* line 2 and *S. tuberosum*, cv. Aster, and a second one, composed of a *S. sogarandinum* line 1 that lost ability to cold acclimation, and of *S. tuberosum*, cv. Irga, displaying low ability to cold acclimation (Rorat et al. 2006). was analysed in relation to cold acclimation in organs of the two groups of *Solanum* species differing in the ability to acclimate to cold. Antibody was prepared against the DHN24 protein produced in bacteria and used for analysis of DHN24 abundance in plant tissues by Western blot. In two week-old *S. sogarandinum* plants grown at normal growth conditions DHN24 dehydrin was observed in all organs, however much more of the protein was observed in roots and stem, less in apical part of shoot but the protein was barely detected in leaves. The DHN24 was also detected in tubers (Rorat et al. 2006). These data revealed that expression of the *Dhn24* gene was under control of organ dependent factors.

Abundance of the DHN24 protein was analysed in organs of *S. sogarandinum* and *S. tuberosum* plants subjected to low temperature treatment. With the exception of leaves, substantial increase in the protein levels was observed in the organs of the cold acclimating plants. No increase in the accumulation of DHN24 was observed in organs of line and species that do not have ability acclimate to the stressful conditions (Fig. 1), (Rorat et al. 2006).

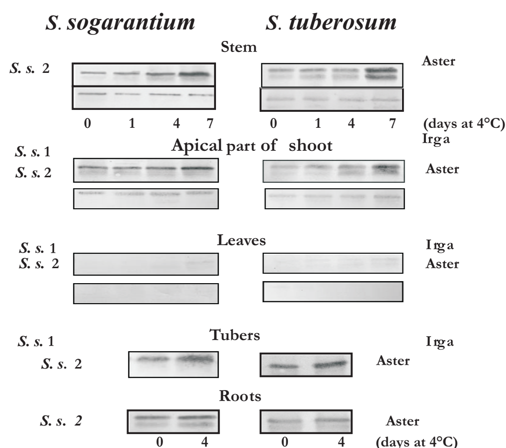

Fig. 2. Western blot analysis of DHN24 protein abundance in organs of *S. sogarandinum* and *S. tuberosum* plants subjected to low temperature treatment. *S.s.*1 - *S. sogarandinum*, line 1, *S.s.*2 - *S. sogarandinum*, line 2.

The data in the Fig. 2 revealed that accumulation of the DHN24 protein in response to low temperature was only observed in *Solanum* species that have ability to acclimate to cold and was organ dependent (Rorat et al. 2006). *Lycopersicon esculentum*, *Lycopersicon chilense, Nicotiana tabacum* and *Capsicum annum* the proteins homologous to DHN24 in response to cold was only observed in *L. chilense* and *C. annum*. These species display ability to adaptation to water deficit.
Abundance of DHN24 protein in Solanacea species was also analysed in response to water deficit. Opposite to the accumulation of DHN24 in response to low temperature treatment, gradual decrease in the DHN24 levels was observed in the organs of cold acclimating *Solanum* species subjected to water deficit (Fig. 3 A), (Rorat et al. 2006).

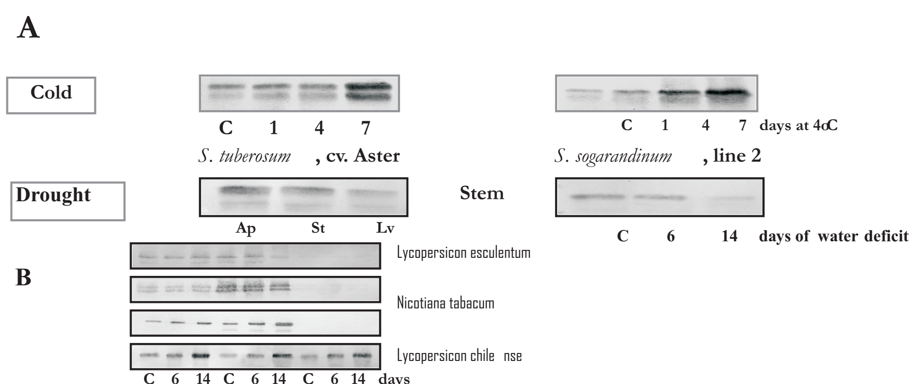

Fig. 3. Western blot analysis of DHN24 abundance in three week-old Solanaceae species subjected to water deficit for 13 days. Ap - apical part of shoots; St - stems; Lv - leaves. C - control well watered plants, 6 - plants not watered for 6 days; 14 - plants not watered for 14 days.

However, when the DHN24 abundance was analysed under water deficit in other Solanaceae species differing in capacity to adaptation to water deficit, increase in the DHN24 amount was observed in *L. chilense* and *C. annum.* These species have ability to adaptation do water deficit (Fig. 3 B). Expression analysis of the Dhn24 revealed that accumulation of its expression product, the DHN24 dehydrin during cold treatment is associated with acclimate of Solanaceae species to cold and under water deficit with adaptation to drought conditions. Tissue and subcellular localization of DHN24 protein.
In the first step to determine a potential role of the DHN24 in acclimation to low temperature tissue and subcellular localization of DHN24 was analysed.

A tissue immunolocalization method was applied to localize the DHN24 protein in the *S. sogarandinum* tissues. The DHN24 was detected in the tissues with using the anti-DHN24 antibody as the primary one and the secondary antibody conjugated with green fluorescence dye. In the transverse sections of stem, roots and leaves the green fluorescence dye that reflects presence of the DHN24 in the tissues was only observed in the phloem tissues. Note green fluorescence was observe in much more phloem cells in the sections prepared from the organs of cold acclimated plants.In order to distinguish the phloem cells the DHN24 is localized in which, the longitudinal section of the stem was first incubated with the primary and secondary antibodies to detect the green fluorescence and subsequently with aniline blue to detect callose occurring in pores of the transverse walls of the sieve tubes and thereafter with DAPI to detect the nucleus in the cells. The data shown that green fluorescence was observed in the two types of the cells, a long large cells, called sieve elements (sieve tubes) and in the smaller ones adjacent to the sieve tubes, called companion cells. The callose was only observed in the transverse walls of the sieve tubes but nuclei were only found in the companion cells. Altogether these data revealed that the DHN24 protein localizes to the two type cells of the phloem tissue.

To localize the DHN24 inside the cell, organelle (nuclei, mitochondria and chloroplsts) and subcellular compartments (cytosol) were isolated from apical part of *S. sogarandinum* three-week-old shoots and the DHN24 was detected in the proteins prepared from them by Western blot. As shown in the Fig. 4 the DHN24 was localized in the cytoplasm and nuclei, however, much more protein was present in the cytoplasm.

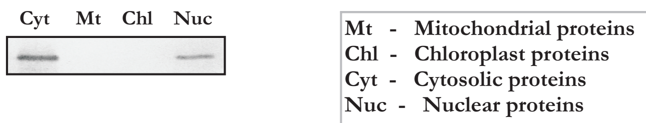

Fig. 4. Subcellular localization of the DHN24 in isolated of organelle and cell compartments of *S. sogarandinum*.

Tissue immunolocalization shown that in *S. sogarandinum* plants grown both at room temperature and under cold, DHN24 protein only localises to the phloem tissues in all organs, and in the phloem tissue DHN24 is present in the sieve-tubes and companion cells. At the subcellular level, at room temperature the DHN24 localises to the cytoplasm, and under cold conditions the protein is present both in the cytoplasm and nucleus. In order to confirm organ and tissue specific expression of the *Dhn24* gene and its association with acclimate to cold and adaptation to water deficit we have isolated the gene encoding DHN24 protein from the *S. sogarandinum* nuclear DNA and analysis of its promoter region revealed many *cis*-dominat

elements that are responsible for the regulation of expression of the *Dhn24* gene in response to dehydration conditions caused either by low temperature or water deficit, and also lead to identification of the elements responsible for the tissue and meristem specific expression. These data thoroughly justifies the experimental ones that expression of the *Dhn24* in associated with adaptation of Solanaceae species to cold and drought conditions and is organ and tissue specific (Rorat et al. 2006).

DHN24 dehydrin is phosphorylated in plant tissue

Molecular mass of the DHN24 protein isolated from the plant tissues was shown to be higher than that produced in bacterial cells measured by SDS-PAGE. This would suggest that the DHN24 might be modified in the plant cell. When the protein extract from the plant tissue was subjected to the alkaline phosphatase digestion the molecular mass of the DHN24 decreased, and opposite phosporylation of the DHN24 produced in bacteria by a serine/threonine protein kinase (Casein kinase II) increased its molecular mass (Fig. 5). These data strongly suggested that the DHN24 is phosphorylated in the plant tissues.

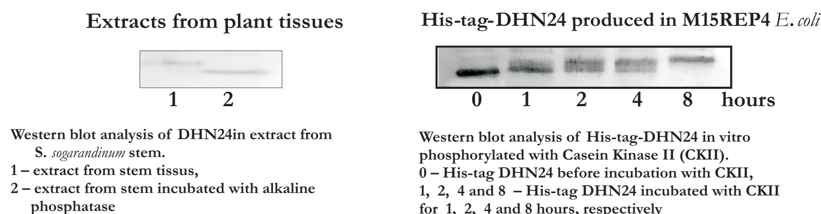

Fig. 5. Analysis of DHN24 phosphorylation *in vitro.*

To get direct proofs that the DHN24 is phosphorylated in the plant tissues we prepared mutant forms of the *Dhn24* coding sequence using a direct-site-mutagenesis method. Amino acid sequence analysis of the DHN24 protein using NetPhos 2.0 i NetPhosK 1.0 (Technical University of Denmark) programs revealed that there are three potential sites in the amino acid sequence of the DHN24 that may contribute to the protein phosphorylation. The KLHR that is a binding site for the protein kinases A and C (CPKA and PKC protein kinases), poli serine segment that may be a potential phosphorylation site and DEEE motif that is a serine/threonine protein kinase binding site.Two mutant variants of the *Dhn24* coding sequence were prepared, a one devoid of the poly S segment (DHN-DLSER), and a second

one lack of a serine/threonine kinase binding site DEEE (DHN-DELDE). The mutated *Dhn24* genes and the native one (DHN24) were transformed into *Arabidopsis* via *Agrobacterium* transformation and the proteins from each of the transgenic *Arabidopsis* lines were analysed in respect of the DHN24 protein by Western blot. The data revealed that both mutant forms of the DHN24 displayed higher migration ability in the SDS-PAGE than the control-unchanged DHN24 transgen. These data confirmed that from the in vitro experiments that the DHN24 is phosporylated *in planta* in the poly serine track by the serine/threonine kinase such as Casein kinase II. So, the DHN24 must function in the phloem tissue as a phosphorylated protein.

Potential function of DHN24

What is its a potential function of the DHN24 in the phloem tissue under the stressful conditions caused either by freezing or water deficit? It may be supposed that DHN24 participates in protection of transport in the transporting organs under the stress conditions, (a) in direct way by interaction with the components of the transporting system to maintain their functional status under freezing or (b) indirectly participating in formation of an appropriate environment inside the cells that enable the transporting system to function under drought conditions. (c) It can not be excluded that the phosphorylated DHN24 may bind Ca^{+2} and functions as a calcium buffer under the stressful conditions. All these possibilities are considered now.

In our first approach to determine a role of the DHN24 in cold tolerance, transgenic cucumber plants were prepared that expresed the *Dhn24* gene under control of the Ca35S promoter region. In comparison with the control non-transgenic cucumber plants, the Ca-35-DHN24 transgenics showed a significantly reduced chilling injury value (severity of chilling injury, SCI) compared to the non-transgenic line. These results let us conclude that the DHN24 dehydrin originated from a cold-acclimated species *S. sogarandinum* is linked to low temperature tolerance in cold-sensitive species such as cucumber and can be used to improve chilling/freezing tolerance through a transgenic approach (Yin et al. 2006).

Then a function of the DHN24 was analysed in *in vitro*, and cryoprotective ability of the DHN24 towards freezing sensitive enzymes such as cytoplasmic Lactate Dehydrogenase (LDH) was measured. Activity of LDH is completely diminished after the 8-9 freeze/thaw cycles but in the presence of the DHN24 in the incubation mixture at the concentrations indicated at the Fig. 7, enzymatic activity of the LDH was restored and in the presence of 100 μg/ml of DHN24 in incubation mix (2.5/1 molar ration of DHN/LDH) the activity of the LDH

was thoroughly preserved. This cryoprotective ability of the DHN24 was even higher than that of albumin (Fig. 6).

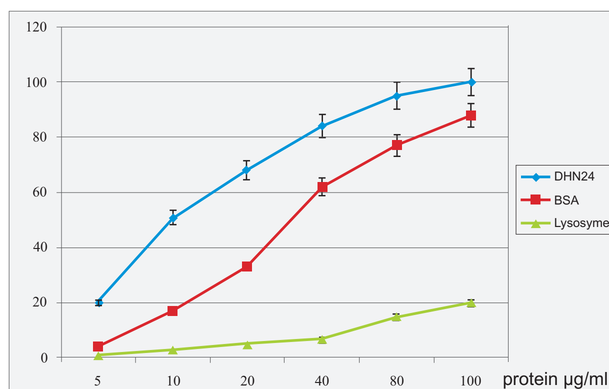

Fig. 6. LDH activity (40 µg/ml) was measured in the presence either of DHN24, BSA or Lysozyme at the concentrations indicated above after nine freeze/thaw cycles when the activity of the DHN24 was diminished completely. Relative LDH activity in the presence of the DHN24, BSA or Lysozyme was referred to the control (unfrozen) LDH activity that was taken as 100.

The above data clearly indicate that DHN24 displays cryoprotective ability *in vitro* towards freezing sensitive enzymes such as freezing-sensitive cytoplasmic LDH.

How the DHN24 may interact with other proteins. Circular dichroism (CD) analysis of the DHN24 in the water solution showed that there is only a one minimum in the elipticity shape at 198 nmn suggesting that there are rather not any internal secondary structures in the DHN24 protein in water solutions and the DHN24 displays a disordered structure (random coil), (Fig. 7). However, in the presence of SDS, that is a factor that stimulates formation of the internal secondary structures some decrease in the wave shape at 220 nm was observed that suggests partially formation of α-helix structures in the DHN24 in the presence of SDS(Fig. 7).

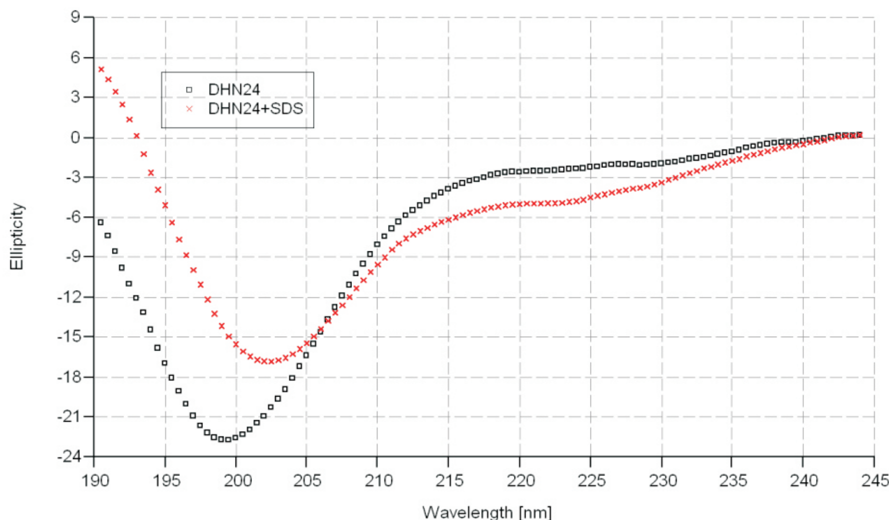

Fig. 7. Circular dichroism (CD) spectrum for the DHN24 in the presence of SDS.
The scan was performed from 190 to 245 nm. Concentration of the DHN24 in each of the samples was 200 µg/ml. Dots show the means from two-three measurements. The spectrum was done using the Jasco 810 spectropolarimeter.

The CD analysis data indicate that in the plant cell the DHN24 may function as a disordered protein. Though we were unable to define a way the DHN24 functions in the plant cell, very likely the DHN24 interacts with other proteins through its unfolded structure by electrostatic forces.

Conclusions

1.Accumulation of the SK3–type DHN24 dehydrin in Solanaceae species is organ dependent and associated with cold acclimation and adaptation to water deficit.
2. DHN24 only localizes to the sieve-tubes and companion cells of the phloem tissue.
3. Inside the cells the DHN24 localizes to the cytoplasm and nucleus.
4. DHN24 is phosphorylated in the poly-serine segment in the plant cells by a serine/threonine type protein kinase.
5. The CD analysis indicated that in vitro the DHN24 does not form a well-defined structures inside the protein, even if in the presence of SDS.
6. DHN24 exhibits ability to protect in vitro activity of freezing-sensitive enzymes against freezing. phosphorylation of the DHN24 does not influence its cryoprotective ability in vitro.

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Biotechnological tools for the *ex situ* conservation of woody plant germplasm

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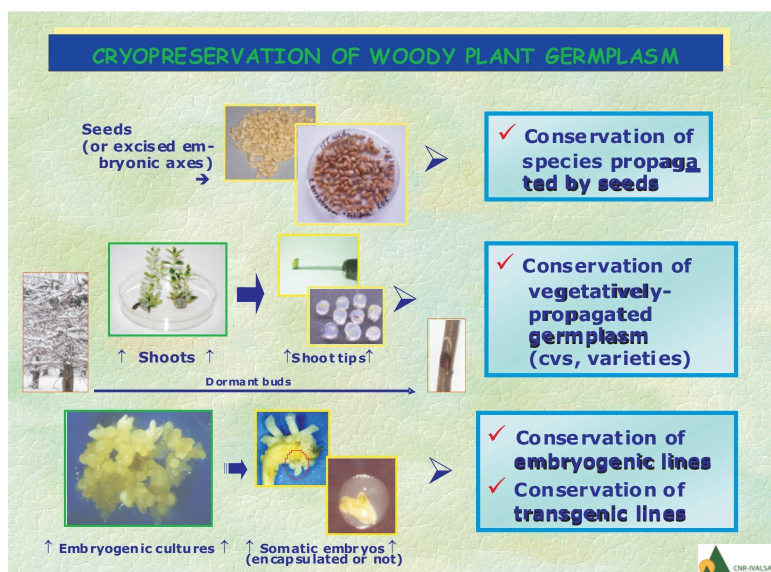
The storage of shoot cultures in slow growth and the cryopreservation of organs and tissues are today important biotechnological tools for the medium and long-term *ex situ* conservation of woody plant germplasm. First approach is pursued in commercial micropropagation laboratories by means of the maintenance of stock cultures at low (above-freezing) temperature and in darkness, thus reducing the growth of microshoots up to a minimum allowed by each species and extending significantly the interval between subcultures. Here, experience with woody plants has shown that the storage period in such condition can vary consistently among species, from few months up to two years (Lambardi and De Carlo, 2003). Some factors can interact with temperature and dark conditions, influencing at different levels the maximum time of conservation and the potential of shoot regrowth after cold storage, such as the initial quality of shoot cultures, the characteristics of containers in terms of gas permeability, the composition of the storage medium and its osmotic potential. As for long-term conservation, first attempt to cryopreserve woody plant material dates back to early '60, when Akira Sakai showed that one-year-old twigs of several *Populus* species were not injured when cooled to –196°C, if they were first held at low temperatures for 6-24 hours (Sakai, 1960). Thirty years later, Sakai and co-workers developed the Plant Vitrification Solution n°2 (PVS2) which showed to be very effective for the induction of vitrification in nucellar cells of *Citrus sinensis* during ultra-rapid freezing in liquid nitrogen (Sakai et al., 1990). Since then, the number of PVS2-based protocols, developed for one-step freezing cryopreservation of woody plant shoot tips, increased yearly, while at the same time new and effective encapsulation- and droplet-based methods were also proposed. Today, cryopreservation opens important prospects to the safeguard of woody plant genetic resources, allowing the conservation in liquid nitrogen of organs and tissues from *in vitro* culture, such as shoot tips, entire seeds or embryonic axes, embryogenic callus and somatic embryos (Panis and Lambardi, 2006). In addition, a method recently developed for apple, the cryopreservation of dormant buds, used after thawing for propagation by chip-budding, has opened the door to a further simplification and cost reduction of germplasm conservation by the cryogenic technique (Lambardi et al., 2011). The use of such different approaches for the cryopreservation of fruit and forest species is critically analyzed.

Acknowledgements

Carla Benelli, Anna De Carlo and Aylin E. Ozudogru, Ph.D., are gratefully acknowledged for the valuable work, carried out in collaboration with the Author, in the development and exploitation of the cryogenic technology at the CNR-IVALSA of Florence.



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Recalcitrant seeds; the occurrence, characteristics and research directions

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Abstract

Seeds of plant species vary in sensitivity to desiccation. Orthodox seeds can be dried to low moisture content without loss of viability, and their longevity increases logarithmically with decrease in water content and storage temperatures. Seeds of the *recalcitrant* category are characterized by the lack of a dehydration stage during seed ripening, so when shed from the parent plant, they have a high moisture content and active metabolism. They are sensitive to dehydration during development and after maturation (i.e., when they are shed from the parent plant). Such seeds quickly lose their germination capacity if they are dried below a certain, relatively high threshold value of moisture level or if they are stored in a hydrated state. Seeds which are more tolerant to desiccation than recalcitrant and less than orthodox belong to the intermediate (suborthodox) category. Oxidative stress is an important factor in the loss of viability of recalcitrant seeds during drying and storage. During seed dehydration, reactive oxygen species (ROS) formation drastically increases as a result of unbalanced metabolism and the impairment of respiratory electron transport. ROS react with cellular macromolecules, such as proteins, lipids and nucleic acids, causing damage and the disruption of cell function. This damage results in the peroxidation of membrane lipids, followed by membrane disintegration and cell death. Recalcitrant seeds vary between species in terms of their sensitivity to drying. Antioxidant systems are essential to scavenging excess of ROS during dehydration of seed tissues. The major low molecular weight antioxidants ascorbic acid (ASA) and glutathione (GSH) are water soluble and can scavenge ROS directly or as electron donors in cooperation with enzymes participating in the ascorbate-glutathione (A-G) cycle: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). ROS are also scavenged by another antioxidative enzyme like superoxide dismutase (SOD), catalase (CAT), peroxidases. Very important low molecular antioxidant α -tocopherol protect membranes. For example of seeds of temperate climate trees will be presented the results of studies on the changes occurring in the antioxidant system during desiccation of seeds of silver maple (*Acer saccharinum*), during development and desiccation of seeds of closely related species of the genus *Acer*: *A. platanoides* (orthodox) and *A. pseudoplatanus* (recalcitrant), and results on increased tolerance to desiccation of recalcitrant seeds of *Quercus robur* and *A. saccharinum* by changing the desiccation rate conditions and treatment of selenium.

Seeds of plant species vary in sensitivity to desiccation. Orthodox seeds can be dried to low moisture content without loss of viability, and their longevity increases logarithmically with decrease of water content and storage temperatures. Seeds of the *recalcitrant* category are characterized by the lack of a dehydration stage during seed ripening, so when shed from the parent plant, they have a high moisture content and active

metabolism. They are sensitive to dehydration during development and after maturation (i.e., when they are shed from the parent plant). Such seeds quickly lose their germination capacity if they are dried below a certain, relatively high threshold value of moisture level or if they are stored in a hydrated state (Roberts, 1973). Seeds which are more tolerant to desiccation than recalcitrant and less than orthodox belong to the intermediate (suborthodox) category (Ellis et al., 1990). So far, the reactions to dehydration of seeds are known only in about 2.5% of all plant species listed in *Compendium*. The great majority from them (c. 90%) are orthodox, c. 7% recalcitrant, and 2% intermediate (Dickie and Pritchard, 2002).

To the recalcitrant category belong seeds of economically important species growing mainly in tropical and humid zone. However, some species growing in the temperate zone also produce a recalcitrant seeds. Most species with recalcitrant seeds occur in the families: *Fagaceae, Sapindaceae, Arecaceae, Moraceae, Rutaceae, Myrtaceae, Clusiaceae, Sapotaceae, Annonaceae* (Dickie and Pritchard, 2002), where species growing in temperate climate occur in the families *Fagaceae* and *Sapindaceae*. The *Fagaceae* include 5 two genera *Quercus* and *Castanea* which 80.2 % species are desiccation-sensitive. In *Sapindaceae, Aceraceae* and *Hippocastanaceae* include 31 % species recalcitrant. To the genus *Acer* belong species which produce typically orthodox seeds eg. Norway maple (*Acer platanoides*), as well as species with recalcitrant seeds eg. sycamore (*A. pseudoplatanus*) or silver maple (*A. saccharinum*). Recalcitrant seeds differ from orthodox in terms of content of some factors, or activities of some processes which contribute to achieve at a certain stage of development, tolerance to desiccation. Despite increasing interest in recalcitrant seeds, the cause of their desiccation sensitivity is still insufficiently understood.

The differences between recalcitrant and orthodox seeds.

Recalcitrant seeds are often characterized by large size. During the maturation recalcitrant seeds do not accumulate insoluble reserve materials, or accumulate them in small amounts. Respiration rates of mature recalcitrant seeds are relatively high, indicating that these seeds are metabolically active and suggesting that metabolic activity may be associated with desiccation sensitivity. The cells of mature seeds often posses many vacuoles and maintain high water content. They do not tolerate dehydration below the level of free water. Most of these seeds are non dormant, and if so, the dormancy is often imposed anatomically.

Orthodox seeds are generally smaller than recalcitrant. During maturation, they accumulate a lot of reserve materials, soluble and insoluble, often in vacuoles, displacing water from them. Orthodox seeds tolerate dehydration and can lose a considerable proportion of the structure-associated water. During the maturation they slow down metabolism, decline respiratory rate with substantial de-differentiation of mitochondria and other organelle and decline in respiratory substrates. Approximately 80% of orthodox seeds is characterized by a deep dormancy.

Research directions on recalcitrant seeds

1. Improvement methods for storage
2. Research on the causes of the loss of viability during desiccation
3. Attempts to increase the tolerance to desiccation and study changes in the embryonic axes and cotyledons under their influence.

Recalcitrant seeds are adapted to living conditions and the species that produce them, in its natural environment, well to develop. However, because of their economic values for human there is a need to develop methods for prolonging their viability during storage. It is difficult, because in addition to their sensitivity to drying, they are also chilling sensitive and after shedding they quickly started to germinate. Hence, the cryostorage in liquid nitrogen (LN) at -196°C, presently appears to offer the only option for long-term storage of these seeds (Berjak and Pammenter, 2008). Researchers' efforts are directed to developing methods for cryopreservation of seeds or their organs of particular species and improvement of protocols in that area.

The study of mechanisms of sensitivity to dehydration of recalcitrant seeds back to the eighties of last century. In those years it was found that the cause of recalcitrant seeds lose viability during drying are destructive changes in the membranes, leading to the loss of their semipermeability, manifested as increased electrolyte leakage during imbibition (Becwar et al., 1982). Pukacka, (1989) showed that during desiccation of *A. saccharinum* seeds the decrease of their viability was accompanied by increased electrolyte leakage, and decrease of total and particular phospholipid contents as well as decrease of biosynthesis of these membrane components. The later experiments showed that the cause of membrane deterioration is the action of free radicals, which are produced in cells in excess during the water loss. Free radicals (later known as reactive oxygen species, ROS) can induce the peroxidation of unsaturated fatty acids and phospholipid deesterification resulting in a loss of membrane structure integrity (McDonald,1999, Pukacka, 1998, Hendry et al., 1992). The rapid accumulation of ROS in recalcitrant seeds during desiccation was stated for seeds of

such temperate species as *Quercus robur, Castanea sativa* and *Aesculus hippocastanum* (Hendry et al., 1992, Finch-Savage et al., 1994) and in tropical species *Shorea robusta* (Chaitanya and Naithani, 1994). The dehydration of *Q. robur* seeds was accompanied by decreasing activity of antioxidant enzymes as superoxide dismutase (SOD) and ascorbate peroxidase (APX) and low molecular antioxidant α-tocopherol. Pukacka and Ratajczak (2006) investigated the production of ROS by desiccated *A. saccharinum* seeds and antioxidative response of enzymes and metabolites of ascorbate –glutathione (A-G) cycle. The desiccated seeds gradually lost their germination capacity and this was strongly correlated with an increase in electrolyte leakage from seeds (Fig.1).

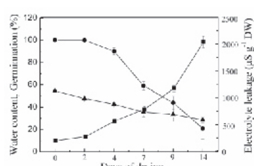


Fig. 1. Changes in water content (▲), germination percentage (●)and electrolyte leakage (■) during drying of *A. saccharinum* seeds.

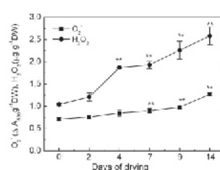


Fig. 2. The ROS production in *A. saccharinum* seeds during desiccation. (Pukacka and Ratajczak, 2006 – changed)

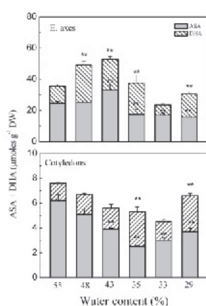
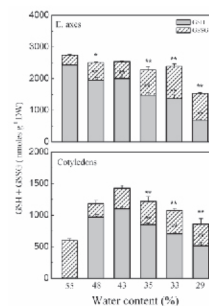


Fig. 3. Changes in ASA and DHA contents in embryonic axes and cotyledons of A. saccharinum seeds during desiccation. (Pukacka and Ratajczak, 2006)

Fig. 4. Changes in GSG and GSSG contents in embryonic axes and cotyledons of A. saccharinum seeds during desiccation. (Pukacka and Ratajczak, 2006)



Simultaneously the increase of ROS (superoxide radical – $O_2^{-\cdot}$ and hydrogen peroxide – H_2O_2) production was observed (Fig.2). The high negative correlation between ROS production and seed germination capacity during desiccation was stated. The results indicated that remarkable decrease in the concentrations and redox status of low molecular antioxidants, ascorbate and glutathione occurred in embryonic axes and cotyledons, toward a more oxidative state. After seed shedding, concentrations of ascorbic acid (ASA) and the reduced form of glutathione (GSH) were higher in embryonic axes than in cotyledons and their redox status was high in both embryo parts. At the first stages of desiccation, up to a level of 43% of water content (WC), ASA content in embryonic axes and GSH content in cotyledons increased (Figs 3and 4). Below this level of WC, the antioxidant contents as

well as their redox status rapidly decreased. The high positive correlation between decline of ascorbate and glutathione redox status and seed viability suggested that this decline may be a reason for the loss of metabolic balance which resulted in intensification of oxidative reaction, ROS accumulation, lipid peroxidation and finally cell death. The enzymes of the ascorbate–glutathione pathway: ascorbate peroxidase (APX) (EC 1.11.1.11), monodehydroascorbate reductase (MR) (EC 1.6.5.4), dehydroascorbate reductase (DHAR) (EC 1.8.5.1) and glutathione reductase (GR) (EC 1.6.4.2) increased their activity during desiccation, but mainly in embryonic axes. The changes were probably required for counteracting the production of ROS during desiccation, but this system was not efficient enough to oppose metabolic imbalance.

The comparison of desiccation sensitive seeds with tolerant orthodox seeds can clarify our understanding of desiccation tolerance mechanisms and the causes of recalcitrance. Such research on closely related seeds of genus *Acer*, differing in desiccation tolerance: Norway maple (*A. platanoides* L., orthodox) and sycamore (*A. pseudoplatanus* L., recalcitrant) were conducted by Pukacka and Ratajczak (2007). The results showed remarkable differences in the concentration and redox balance of ascorbate and glutathione between these two kinds of seeds during development, and a significant dependence between glutathione content and acquisition of desiccation tolerance in Norway maple seeds (Fig. 5). In seeds of this species the glutathione pool clearly increased from the time of acquisition of desiccation tolerance (18 WAF) and was high until the end of seed maturation. In mature seeds the glutathione pool was significantly higher in *A. platanoides* – orthodox seeds than in *A. pseudoplatanus* – recalcitrant seeds.

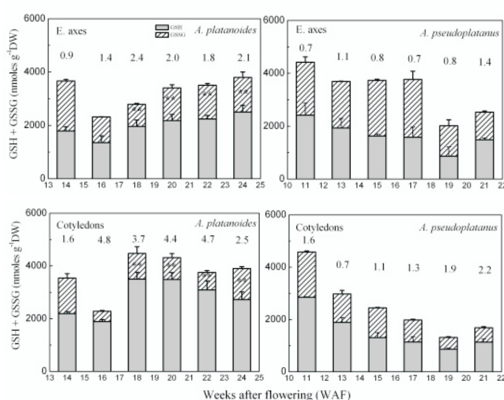


Fig. 5. Changes in the content of reduced (GSH) and oxidized (GSSG) forms of glutathione and GSH/GSSG ratio (redox status) in embryonic axes and cotyledons of *A. platanoides* and *A. pseudoplatanus* seeds during development. (Pukacka and Ratajczak, 2007)

There were relatively small differences between the species in the activities of enzymes of the A-G cycle. When sycamore seeds were desiccated to a moisture content of less than 26%, there was a marked decrease in seed viability and an increase in the production of reactive oxygen species (Figs. 6 and 7). During desiccation, Norway maple seeds had a more active defence system, which was reflected in a higher glutathione content, a higher glutathione redox status (Fig. 8), a higher ascorbate redox status, and higher activities of enzymes of A-G cycle, APX, MR, DHAR, GR and GPX in embryonic axes.

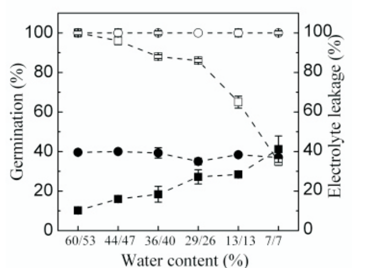


Fig. 6. The germination capacity (open symbols) and electrolyte leakage (closed symbols) in seeds of *A .platanoides* (○,●) and *A. pseudoplatanus* (□,■) during desiccation. (Pukacka and Ratajczak,2007)

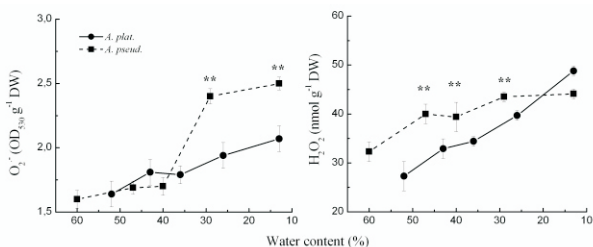


Fig. 7. Levels of ROS in seeds of *A. platanoides* (●) and *A .pseudoplatanus* (■) during desiccation after rippening. (Pukacka and Ratajczak,2007)

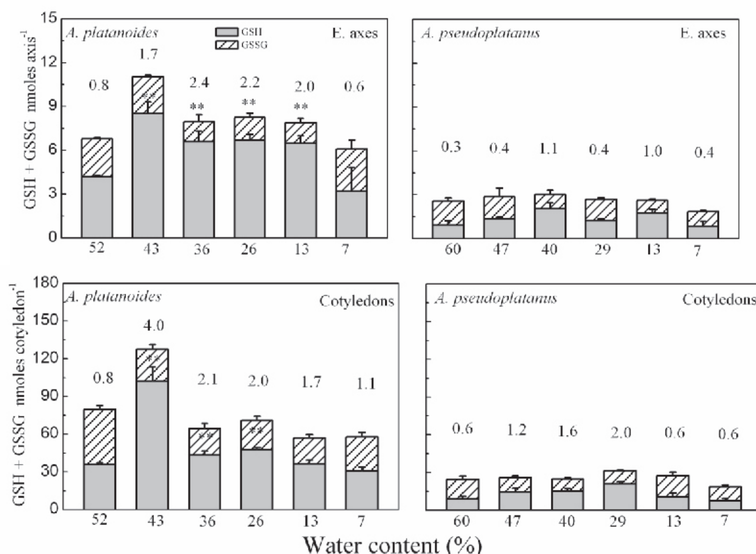
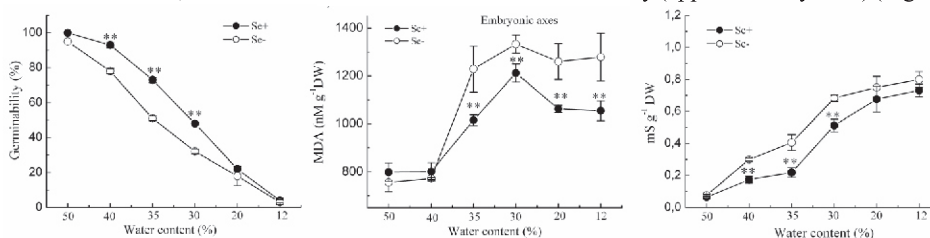


Fig. 8. Changes in the content of reduced (GSH) and oxidized (GSSG) forms of glutathione and GSH: GSSG ratio (redox status) in embryonic axes and cotyledons of *A. platanoides* and *A. pseudoplatanus* seeds during desiccation. (Pukacka and Ratajczak , 2007)

The results showed that the difference in the desiccation tolerance among the seed of *A. platanoides* and *A. pseudoplatanus* to a large extent could be result from the differences in the glutathione metabolism.

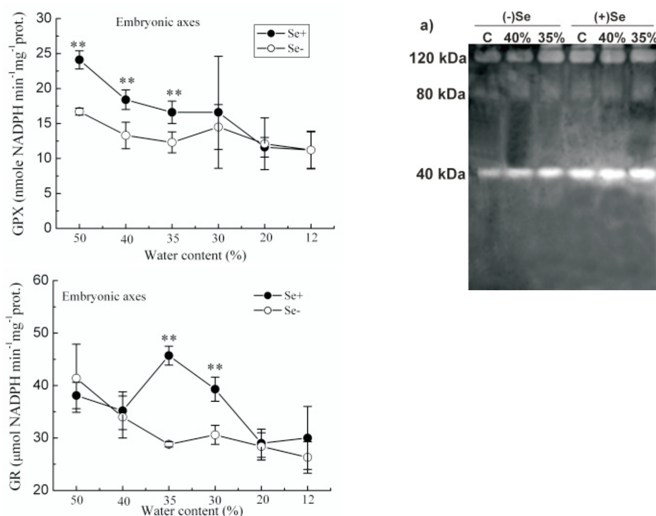
Recently, there have been reports of research to increase the tolerance to drying of recalcitrant seeds under the influence of various factors, among them were the factors enhancing the antioxidant activity in the cells. Beardmore and White (2005) showed that isolated embryonic axes of recalcitrant *A. saccharinum* seeds, treated with abscisic acid (ABA) (20 μM) and tetcyclasis (substance enhances ABA concentration) and then desiccated to 10% WC, reached the germination capacity 95 %. They endured 24 h cryopreservation in LN, thawing, and after that germinated in 55%. Pretreatment of the axes with ABA and tetcyclasis increased the ABA content in the axes and stimulated the synthesis of storage and dehydrin-like proteins, believed to have a role in the desiccation tolerance of orthodox seeds.

Pukacka et al. (2011) found the increase of viability of seeds of *A. saccharinum* during desiccation, after pretreatment by sodium selenite. Freshly harvested silver maple (*A. saccharinum* L.) seeds were soaked in either sodium selenite (10 mg/L) or water for 6 h. After washing and air drying, seeds were desiccated at 20°C at a RH of 45- 50% to comparable water levels from 50 to 12%. Germination capacity was significantly higher in seeds treated with selenium and desiccated [from 50 to 40, 35 and 30% of water content (WC)] than in water-soaked seeds. At 20% WC, the seeds from both treatments had low viability (approximately 20%) (Fig.9).



Figs. 9-11. The germination capacity, MDA contents and electrolyte leakage of silver maple (*A. saccharinum*) seeds previously soaked for 6h in a 10mg/L sodium selenite (● Se^+) and in water (○ Se^-).(Pukacka et al. 2011)

The electrolyte leakage and the MDA content were significantly lower in the embryonic axes of seeds soaked in selenite than in seeds soaked in water (Figs 10 and 11). It was also found that the activity of glutathione peroxidase (GPX) of embryonic axes from selenium-treated seeds that were not desiccated, or from seeds that were desiccated to 40 and 35% WC was significantly higher than that of non-treated axes (Fig.12). No difference in GPX activity was detected in cotyledons. This was confirmed by activity staining of GPX after native PAGE of proteins extracted from embryonic axes (Fig. 13). An increase in glutathione reductase (GR) activity was also observed in embryonic axes of seeds treated with selenium and dried to 35 and 30% WC compared to non-treated samples (Fig. 14).



Figs. 12-14**.** The activity of glutathione peroxidase (GPX), native PAGE of glutathione peroxidase and activity of glutathione reductase (GR) in embryonic axes of Se^+ and Se^- *A. saccharinum* seeds during desiccation. (Pukacka et al., 2011)

Presented results showed that selenium caused the increase of GPX activity in embryonic axes of *A. saccharinum* seeds just after soaking. It had a beneficial effect on membrane protection against ROS produced during desiccation. Maximal increase of GR activity occurred later in the desiccation process (at 35 % WC) and was probably the result of a lowered GSH concentration due to GPX action. GR also contributed in the slowing down the loss of viability of seeds during desiccation, by preferential protection of embryonic axes. One can suppose that the combined action of GPX and GR was critical in mitigating the effect of oxidative stress on membranes in Se+ seeds.

Bai et al., (2011) showed that nitric oxide (NO) enhances tolerance to desiccation of reacalcitrant seeds of *Antiarix toxicaria,* the tropical tree growing in Asia, Africa and Australia. Pretreatment of seeds with NO increases the activity of antioxidant ascorbate-glutathione pathway enzymes and metabolites and in this way diminishes ROS production, particularly H_2O_2. Desiccation increases the protein carbonylation levels and reduces protein S-nitrosylation of the A-G antioxidant enzymes. These effects can be reversed with NO treatment. Thus, NO via inhibitory effect on protein carbonylation and reduction of protein S-nitrosylation of A-G antioxidant system decreases desiccation-induced H_2O_2 accumulation and enhances desiccation tolerance of seeds. The Authors suggests, that NO

reinforces recalcitrant seeds desiccation tolerance by regulating antioxidant enzymes activities to stabilize H_2O_2 accumulation at an appropriate concentration.

Recently, were published a few papers on the impact of desiccation rate on the viability and activity of antioxidant system of recalcitrant seeds. The effect of desiccation rate on the viability of recalcitrant seeds was previously investigated in the seeds of such species as *Avicenia marina* (Farrant et al., 1985), *Landolphia kirkii* (Pammenter et al., 1991), *Quercus rubra* (Prithard, 1991), *Ekbergia capensis* (Pammenter et al., 1998), *Theobroma cacao* and *Ginkgo biloba* (Liang and Sun, 2000, 2002). None of these studies are affected by dehydration-induced oxidative stress. However, ultrastructural observations of cells of embryonic axes of *E. capensis* seeds dried to the same water content (WC) using the different drying rates showed different patterns of damage (Pammenter et al., 1998). Many other studies have noted that the cells of rapidly desiccated recalcitrant seeds are less vulnerable to the consequences of unbalanced metabolism and ROS-associated damages (Walters et al., 2001, Berjak and Pammenter, 2008). Varghese et al., (2011) demonstrated that isolated embryonic axes (EAs) of recalcitrant seeds of tropical plant *Trichilla dregeana* survive dehydration to a much lower level of water during rapid drying (0-5 h) as comparing to slow drying (0-60 days) all seeds. Rapid drying allowed these EAs to survive dehydration to much lower WC (at ca. 24% WC - 73% germination) compared with slow drying, where 90% EAs lost viability at ca. 44% WC. In EAs slowly dried the level of hydroxyl radical ($OH^{\cdot}$) and lipid peroxidation (measured as TBRS level) were significantly higher compared with those dried rapidly to comparable WCs. During slow drying enzymatic antioxidant levels were not sustained and declined significantly with prolonged drying. In contrast, sustained activity of enzymatic antioxidants was detected in rapidly dried EAs even at relatively low WCs. Also the greater decline in GSH/GSSG ratio was stated in EAs slowly dried compared with rapidly dried EAs. It is apparent from presented study that greater retention of viability to lower WCs in rapidly dried recalcitrant seeds *T. dregeana* may at least be partly explained by the retention of functional antioxidant status.

The activity of antioxidant system was also investigated during slow and rapid drying of seeds of species of temperate zone, pedunculate oak (*Q. robur)* (Pukacka et al., 2011). The seeds were subjected to slow (S) and rapid (R) desiccation at desiccation rates of 0.16 and 0.39 % H_2O per hour respectively. Till ca 40% WC the germination capacity of seeds in the S and R variants was high (ca 100%). Between 40 and 28% WC, germination capacity declined to 20% and 50% in S and R variants, respectively. The decrease in seed

viability was accompanied by a significant increase of electrolyte leakage from embryonic axes (28% for S and 15% for R variants) (Fig. 15)

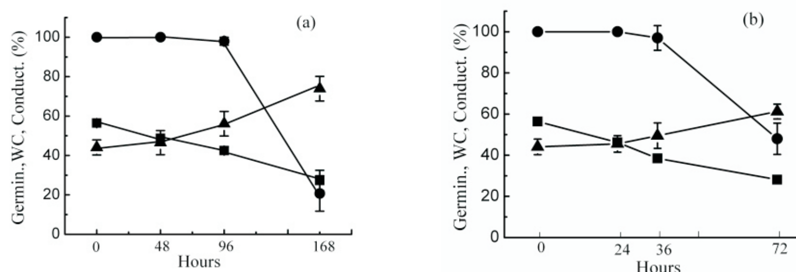
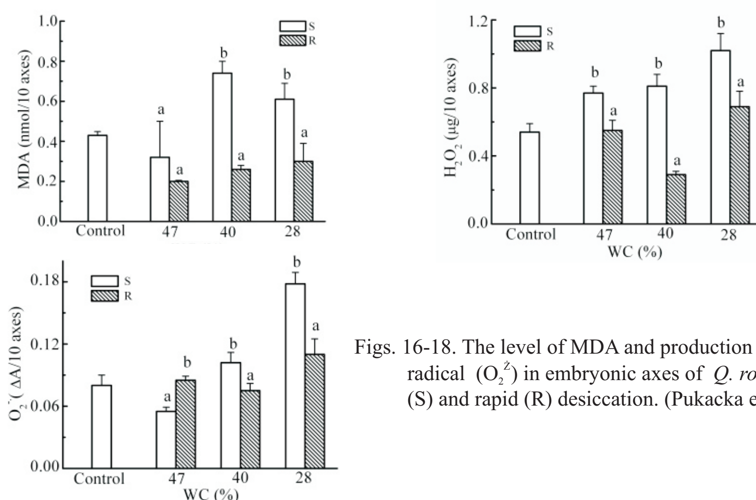


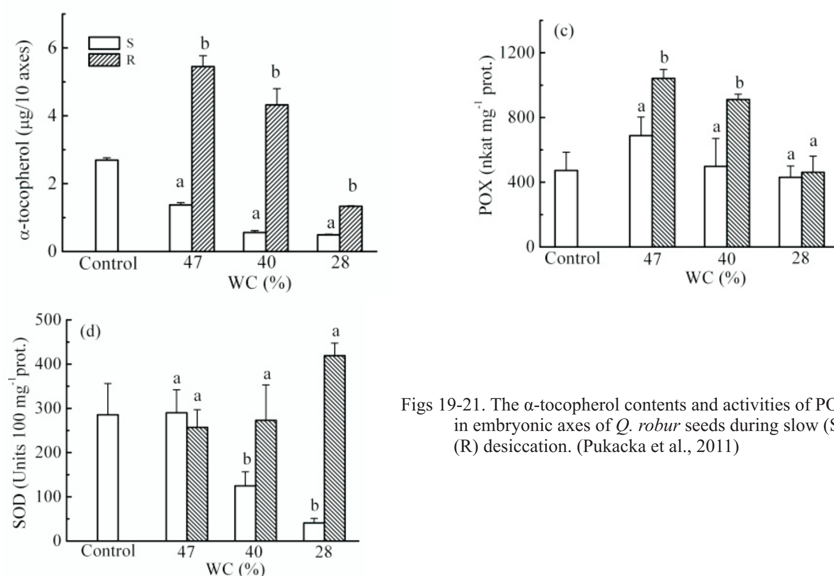
Fig. 15. The effect of slow (a) and rapid (b) dehydration of *Q. robur* seeds on germination capacity (*circle*), water content (*square*) and electrolyte leakage (*triangle*) of embryonic axes. (Pukacka et al., 2011)

In the embryonic axes of seeds subjected to slow desiccation, malondialdehyde (MDA) (Fig.16) and free fatty acid (FFA) contents not shown were significantly higher than those in R variants, indicating greater membrane damage due to lipid peroxidation. The production of ROS (H_2O_2 and $O_2^{\cdot -}$) was significantly higher in S than in R variants Figs 17 and 18). The low molecular weight antioxidants α-tocopherol, ascorbic acid (ASA) and phenolic compounds indicated different reactions in response to desiccation stress. ASA levels decreased during desiccation to a similar degree in both the S and R variants. A significant decrease of total phenols was observed in R variant, which coincided with a significant increase of guaiacol peroxidase (POX) activity (Fig. 20). α- tocopherol content was significantly higher in the embryonic axes of seeds subjected to rapid drying (Fig. 19).



Figs. 16-18. The level of MDA and production of H_2O_2 and superoxide radical ($O_2^{\cdot -}$) in embryonic axes of *Q. robur* seeds during slow (S) and rapid (R) desiccation. (Pukacka et al., 2011)

The activities of the enzymatic scavengers APX and GR data not shown had similar runs and were slightly higher in R variant. The activities of POX and SOD (Figs 20 and 21) were significantly higher in the embryonic axes of seeds subjected to rapid drying.



Figs 19-21. The α-tocopherol contents and activities of POX and SOD in embryonic axes of *Q. robur* seeds during slow (S) and rapid (R) desiccation. (Pukacka et al., 2011)

These results show that rapid dehydration of *Q. robur* seeds leads to the greater mobilization of antioxidant system in embryonic axes, particularly increased levels of α-tocopherol and POX and SOD activities, in the first stages of water loss. This mobilization has a greater impact on maintenance of higher viability of seeds after drying to lower level of WC. Generally, the presented studies confirm that, to some degree of dehydration of recalcitrant seeds cells it is possible to increase their tolerance to desiccation by modulating the activity of antioxidant system, either by a faster decrease in osmotic potential, or by external factors like Se ions or nitric oxide NO. However, it is noteworthy that desiccation tolerance of seeds is a complex and multifaceted property involving a multiple of genes whose expression ultimately leads to mechanisms of both cellular protection to sustain limited damage during drying itself and cellular repair, to reverse any desiccation induced changes when the appropriate hydrated conditions are re-established. Finding the direct cause of seed recalcitrance is difficult and so far, there are several desiccation-sensitive mutants (in *Arabidopsis* and several crop species) of which the wild-types are orthodox, desiccation tolerant seeds. However, there are no reports of seed desiccation tolerant mutants in species with recalcitrant seeds.

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Molecular aspects of tolerance to low temperature stress in fruit plants

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Abstract

Low temperatures during winter dormancy and spring frost at the beginning of vegetative phase belong to the most important abiotic factors limiting the production of fruits in many areas of the holarctic zone. Depending on the intensity of the stress factor it may lead to a reduction in yield (effect of secondary metabolic changes) or its total loss (due to damage to the flower buds), as well as irreversible damage, and consequently to the death of plants. The scale of damages is dependent on the genetic potential of the species / cultivars and the level of plant acclimatization. The regulation of low temperature stress has been widely studied in herbaceous model-plants using transcriptomics, proteomics, and transformation technologies. However, only recently, have these same approaches been used to study stress tolerance in fruit plants. Evidence suggests that although there is high level of conservation in mechanism of stress tolerance, but the perennial habit can resulted in additional pathways. An overview of recent results of study on stress response in selected fruit plants and abilities to develop comprehensive understanding of their tolerance to low temperature stress will be presented.

Accumulation of the *SsBBX24* protein containing zing finger domains is regulated in Solanum species by circadian clock and stress dependent

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A *SsBBX24* gene corresponding to cold-induced mRNA was screened from *S. sogarandinum* cDNA library by differential screening. The *SsBBX24* gene displayed high homology with genes encoding plants B-box zinc finger proteins. The *SsBBX24* gene contains an open reading frame of 702 bp encoding a protein of 234 amino acids with a calculated molecular weight of 25.84 kDa. Analysis of *SsBBX24* protein sequence showed that the protein contains two tandem B-box domains (38 amino acids of each) located at the N-terminus.

The database search revealed that SsBBX24 sequence from *Solanum sogarandinum* shares the highest homology with STO (salt tolerance) and STH (salt tolerance homolog) proteins from *Arabidopsis thaliana* (70 and 69 % identity, respectively). Based on this data SsBBX24 protein has been classified to the DBB (Double B-box) protein family. The characteristic features of the Double B-box protein family is that they possess two tandem of the B-box domains located at the N-terminal and a lack the CCT domain (CO/COL) at the C- terminus.

We have thoroughly analyzed the relationship between the SsBBX24 protein level and resistance of the *Solanum* species to a low temperature, water deficit and salinity. Abundance of the SsBBX24 protein was analysed in response to low temperature, water deficit and salinity in the organs of four *Solanum* lines and species differing in their ability to cold acclimate and adaptation to the water deficit. We reported that under the control conditions the *SsBBX24* protein abundance strongly varies as a function of organ type. A substantial SsBBX24 protein abundance was observed in the well-expanded leaves, young leaves and stems of two week-old *S. sogarandinum* and *S. tuberosum* cultivars, Irga and Ursus. The protein amount was much lower in the old leaves and the rhizomes. We also investigated the SsBBX24 abundance in 2-month-old *S. tuberosum* plants at the flowering stage. In the extracts from open flowers the highest protein level was noticed in the stamens and pistils whereas in the petals and in the sepals the SsBBX24 was barely detected. These data showed that the SsBBX24 protein abundance is differentially regulated as a function of organ type and developmental stage.

Under the stressful conditions a noticeable changes in the SsBBX24 protein abundance was only found in the young leaves of the phytotron-grown plants after three days of NaCl treatment (watering with a nutritive solution containing 0.2 M NaCl). No changes in the SsBBX24

protein levels was noticed in all organs of phytotron-grown plants subjected to low temperature and the water deficit. Altogether, these data indicate that expression of the *SsBBX24* gene is associated neither with acclimation of *Solanum* species to low temperature nor with the plants adaptation to the water deficit.

Further studies showed that the abundance of the SsBBX24 protein undergoes significant changes during the circadian clock. Under the control non-stressful conditions at the 14-h dark/light cycles, the highest SsBBX24 level was found during the light phase while the lowest protein level was observed during the dark period. In contrast, under the stressful conditions the level of the SsBBX24 protein was not decreased in the dark phase at the 14-h dark/light cycle. These results indicate that the stressful conditions dramatically modify the circadian clock regulation of the *SsBBX24* gene expression.

In the cell the SsBBX24 protein is localized both in the cytosol and in the nucleus, however, under light much more protein is present in the nucleus.

Desiccation, cryoprotection and genetic stability

Proteomic changes in *Gentiana cruciata* (L.) cell suspension during adaptation to osmotic stress in cryopreservation protocol

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Keywords: encapsulation-dehydration, sucrose treatment, two dimensional electrophoresis

Abstract

The tolerance to dehydration and freezing, which is induced at the stages of pretreatment, is a requirement for maintaining high viability of plant material after cryostorage. Changes related to adaptation to stressful conditions reach the molecular level of the material studied. Proteomic analysis allows observing physiological response of cell manifested in the post-translational level. The aim of presented studies was preliminary analysis of proteomics changes during dehydration in cryopreservation protocol for encapsulated *G. cruciata* embryogenic cell suspension culture. We would like to define how the sequence of pretreatment induces changes in protein expression patterns. There are only few reports about proteomics analysis in cryopreservation. Proteins were isolated according to Wang's procedure and the protein content was determined by Bradford assay. The protein samples were separated in two dimension technique and stained with Coomassie. In obtained electrophoretical profiles different types of proteins were found: some of them presented up-stream growth of activity, other proteins were connected with decreasing. In preliminary mass spectrometry identification 8 of 12 different spots have been found. Our preliminary results confirmed the previously posted limited information concerning changes in protein profiles in the analyzed process. Proteomic analysis of multi-stage osmotic dehydration allows to observe changes induced by the prolonged and gradually intensified dehydration stress. These types of observations have not yet been described in the literature.

Introduction

Contemporary plant biotechnology enables to conserve valuable genetic resources and conduct fundamental research which aim to understand the processes on every level of plant organisation. Micropropagation, regeneration, transformation or conservation require storing of plant material. Cryopreservation is a technique which is used frequently for long-term preservation of the material. It allows to store various type of the material. However, only seeds and dormant buds are physiologically adapted to freezing. Safe storage of plant tissue, cells requires working out appropriate procedures. It includes the range of activities which

aim to protect the material from dehydration stress, freezing, storing in liquid nitrogen, and thawing. Tolerance to freezing and dehydration, which is induced at the stages of pretreatment, is a condition for maintaining high viability in plant material. Changes related to adaptation to stressful conditions reach the molecular structure of the material. Understanding the molecular basis of response to the cryopreservation protocol permits optimizing the methodology and assuring safety procedures as well as plant material long-term storage at ultra low temperatures (Suzuki et al. 2006).

Gentians species in vitro cultures has been studied widely. Gentians cell suspensions have been investigated with respect to mass propagation, somatic embryogenesis (Mikuła et al. 2005; Chen et al. 2009) and second metabolite production (Hayta et al. 2011). The broad programme of genetic manipulation (Nakatsuka et al. 2008), transformation and somatic hybridization by *Gentiana* taxa was executed (Rybczyński et al. 2008). For researchers it is important to preserve cell suspensions without changing competency. It is needed to develop a technique which would allow to avoid loosing cell line, genetic changes through somaclonal variation or DNA damages. The best technique that can help to preserve tissue competency is cryopreservation.

Three cryopreservation methods: controlled rate cooling, vitrification and encapsulation, are commonly used for plant material. The investigations confirms that cryopreservation by encapsulation is the most reliable procedure for preserving the viability and embryogenic competence and recovery of gentian cell suspension culture (Mikuła et.al. 2005). It turned out that the stages of pretreatment are crucial for maintaining high viability in cryopreserved plant material. In order to induce freezing tolerance, as much as possible water must be removed from plant cells before freezing. Media in appropriate sequence with different sucrose concentration were used for gentians in pretreatment in order to dehydrate cells and to induce freezing tolerance.

The successful cryopreservation of embryogenic suspension cultures is often assessed by tissue survival, recovery, ability to produce somatic embryos or the regeneration of complete plants (Mikuła et al. 2008). Genetic stability and absence of molecular changes were confirmed in plant material which is exposed to cryopreservation protocol and storage in ultra low temperature (Mikuła et al. 2011). The next level of analysis focused on detection of proteomic changes which probably occur during the induction of freezing tolerance in successive stages cryopreservation protocol.

For successful cryopreservation it is necessary to induce high levels of dehydration tolerance in plant cells. Understanding induction mechanisms of such dehydration tolerance is necessary for optimizing preculture conditions and improving survival in cryopreservation (Suzuki et al. 2006). Only a few papers have concerned description of physiological changes occurred in the aftermath of preculture treatment and how it affects the survival following cryopreservation protocol (Hitimi et al. 1999; Vandenbussche et al. 1999; Suzuki et al. 2006).

The effect of the cryopreservation procedure for Getiana cruciata, with the application of growing sucrose concentration to 1.0 mol, is controlled with the help of morphogenic potential expressed by the number of regenerated somatic embryo and growing plantlets in post thawing culture. The genetic uniformity of post thawing regenerants predisposes the system for studies on proteomics on posttranslational level of stress treated cell (Mikuła et al. 2008, 2011).

Recently, proteomics has become an essential methodology for large scale analysis of protein in several aspects of plant biology (Padney and Mann, 2000). Two-dimensional gel electrophoresis is one of the most widely used techniques for resolving complex protein extracts (Sheoran at al. 2009). The resulting gel profiles allow to observe changes for every single spot - the protein (Lopez, 2007). Abiotic stress cause specific reactions as well as non-specific damage and adaptive responses at the cellular level and the whole organism (Beck et al. 2007). All these changes are based on the biochemical changes that involve not only stress proteins (eg. dehydryns) but also the enzymatic, structural or receptor ones (Riccardi et al. 1998). Computer analysis of gels with full proteome profile of the material made with 2D PAGE technique, subjected to various stressful conditions, allows to detect statistical changes in proteins (Riccardi et al. 1998).

The aim of presented studies was preliminary analysis of proteomics changes during dehydration in cryopreservation protocol for encapsulated *G. cruciata* embryogenic cell suspension culture. We would like to define how the sequence of pretreatment induces changes in protein expression patterns. Proteomic analysis of multi-stage osmotic dehydration allows to observe changes induced by the prolonged and gradually intensified dehydration stress. These types of observations have not yet been described in the literature.

Methods

Cell suspension maintaining and cryopreservation

Gentiana cruciata embryogenic cell suspension is maintained as described before (Mikuła et al. 2005). Embryogenic cell suspension of *Gentiana cruciata* was encapsulated in

calcium alginate. The capsules were incubated in medium containing 0.3M, 0.5M, 0.75M sucrose, for 48hrs in each concentration and finally transferred to 1M sucrose for 24h. After this pretreatment capsules were harvested and surface-dried by air in laminar-flow chamber for 5hrs at room temperature. Capsules after air drying were transferred to cryotubes and directly cooled in liquid nitrogen.

Protein analysis

Proteomic patterns were done on each cryopreservation protocol stage with sucrose concentration changing. Proteins were isolated according to Wang's procedure (Wang et al. 2006).

The protein content was determined by Bradford (1978) assay. The protein samples were focused using 3-10 nonlinear IPG strips for the 1st dimension, in second dimension separated on 12.5% acrylamide gel and stained with Colloidal Coomassie Brilliant Blue 250.

The image comparative and statistical analysis (ANOVA) were carried out with the Image Master 2D Platinum 7.0 software.

Mass Spectrometry (MS) analysis was performed. Spots were identified by MS on the basis of peptide mass matching following digestion with trypsin, and their peptide sequences which were obtained in a fragmentation process

Results

Alginate encapsulated embryogenic cell suspension has never been used for isolation of proteins before. Wang's method of isolation has allowed to obtain protein profiles with a very large number of spots without smearing and streaking (Figure 1).

The average number of protein spots observed in gels run from 839 to 918 in studied groups. Among them about 90 percent were matched across all tested variants (Table 1).

Tab. 1. Comparative analysis of 2D-PAGE proteomic images from every stages of application of sucrose in cryopreservation protocol.

	Encapsulated cell suspension (control)	After sucrose concentration treatment			
		0,3M	0,5M	0,75 M	1,0M
No. of spots in gel	851	918	869	839	876
% of matches	94,00	91,67	95,67	95,67	88,67

The results of statistical analysis showed that, under drought stress, the expression of various proteins changed qualitatively as well as quantitatively. Out of differential protein spots, 10 spots occurring during the process were observed. A large group of proteins under stress has changed its level of expression. In the case 28 spots expression increased. Twice as many spots revealed a statistically significant decrease in expression. In additon, it was observed a group of 14 proteins with different profiles of changes specific to each protein (Table 2).

Tab. 2. Quantitive and qualitative changes in 2D – PAGE protein profiles during the application of sucrose in cryopreservation protocol.

The number of proteins with altered expression (at significance level 0.05)				
total	new	up-regulated	down-regulated	changing expressio n
104	10	28	52	14

The proteins which were differentially expressed were taken forward for MS identification. In preliminary MS identification 8 of 12 different spots have been found. Remaining spots probably contained not enough protein to be faithfully detected. It should be also underline that protein database for *Gentiana cruciata* is only residual so we were forced to used very broad databases. However, some proteins still might be unidentified because of lack of appropriate database. The full names of the proteins, their theoretical mass weight (MW) values and pI, data from the mass spectrometry analysis, such as their score and the protein amino acid sequence coverage by the matching peptides are presented in Table 3.

Tab. 3. Mass Spectrometry identification results of up-regulated selected proteins during investigated dehydration process.

No. of spot	Protein name	Plant species from database	MW (kDa)	pI	Sequence coverage (%)	No. of unique peptides	Score
1	Pyruvate decarboxylase isoenzyme 1	*Zea mays*	65,39	6,62	3,61	1	70,27
2	1-Cys peroxiredoxin A	*Oryza sativa* subsp. indica	24,06	5,97	10,45	3	159,90
3	Glucose and ribitol dehydrogenase homolog	*Arabidopsis thaliana*	31,37	6,12	7,99	2	100,29
4	Glucose and ribitol dehydrogenase homolog	*Daucus carota*	31,48	6,28	7,22	1	81,76
5	Alcohol dehydrogenase	*Malus domestica*	41,38	6,52	11,32	3	180,11
6	10kDa late embryogenesis abundant protein	*Helianthus annus*	10,03	5,18	14,13	2	65,51
7	Aldo/keto reductase family protein	*Arabidopsis thaliana*	37,83	5,89	4,06	1	101,52
8	Chalcone synthase	*Arabidopsis thaliana*	43,00	6,1	12,91	6	223,56

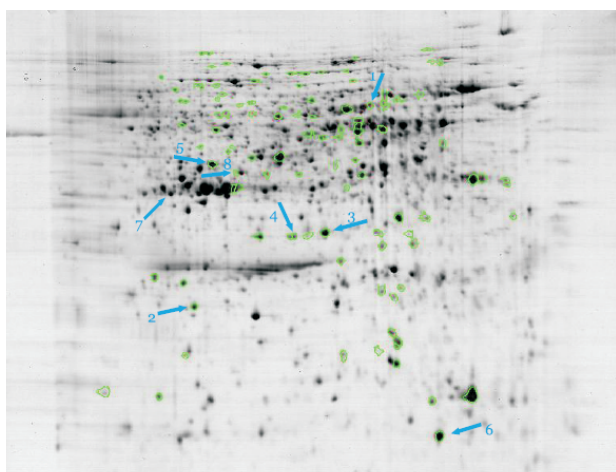

Fig. 1. Example of 2D PAGE gel in proteomic analysis of embryogenic cell suspension of *Gentiana cruciata* in dehydration process (green circles - differential proteins, blue arrows – identified proteins from Table 3).

Discussion

There are only few reports about proteomics analysis related to cryopreservation. In addition these studies are performed basically on model or crop plants. Among the objects of the research we can list the tomato apical shoots. They are osmotically dehydrated in preculture with 0.4 M sucrose and after that treated with osmoprotectants before freezing in liquid nitrogen. Effect of the applied treatment was illustrated in 2D PAGE and subjected to protein identification manifested changes (Criel et al. 2006). Laboratory of Tropical Crop Improvement (K.U. Leuven, Belgium) based their research on the multiple shoot meristem cultures of the banana as a model material for proteomic analysis. They examined tissue reaction in acquisition to osmotic stress (Carpentier et al. 2007). In subsequent reports some various stages of proteomic research were presented including optimization methods for isolation of proteins (Carpentier et al. 2005), the development of proteomic analysis of cryopreservation protocol (Carpentier et al. 2006) methodology for 2-DE analysis (Carpentier et al. 2007). The research led to the complete proteome analysis of banana meristems protected by cryopreservation technique. Identification of proteins altered during treatment of high concentration of sucrose led to a full description of stress tolerant pathways. Acquisition of tolerance to osmotic dehydration and freezing-induced incubation in constant concentrations of sucrose (0.4 M) at the level of protein metabolism have also been studied on the basis of somatic embryos of carrot. Detailed analysis allowed to conclude that the proteins have been observed in the mass range of 10 to 30 kDa and pH of about 6.8 were a key fraction for the acquisition of resistance to dehydration of somatic embryos of carrot (Thierry et al. 1999). There are no references to analysis based on protocols in which osmotic dehydration treatment is caused by elevated levels of sucrose especially for the material in the form of totipotential cells from the basis of the cell suspension.

Until now, our research demonstrated that osmotic dehydration plays a key role in the acquisition of freezing tolerance and lifting embryogenic capacity of cell suspension culture of *G. cruciata* (Mikuła et al. 2011) and *G. kurro* (Mikuła et al. unpublished). Therefore, we started presented above proteomic studies of multistage osmotic dehydration with the use of 0.3 M to 1.0 M sucrose concentration in cryostorage protocol preculture medium. The presented project analyzed the process of cryopreservation of gentiana cell suspension which produces a much higher level of dehydration by material incubation, eventually reaching the highest optimum level of osmotic stress at 1M sucrose concentration in preculture medium. These types of observations have not yet been described in the literature.

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Effects of desiccation and cryopreservation on viability *in vitro* of embryonic axes of *Fagus* and *Acer* seeds

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Abstract

Seeds have been categorized into three groups according to their tolerance to desiccation and storage physiology: *orthodox, recalcitrant* and *intermediate*.

Our studies concern the native genera of trees, with seeds orthodox - Norway maple (*Acer platanoides*), recalcitrant - sycamore (*Acer pseudoplatanus*) and intermediate beech (*Fagus sylvatica*). The aim of our studies was to determine possible type of damages of isolated embryonic axes (EA) during the cryopreservation phases. The ice crystallization and melting was determined by DTA (Differential Thermal Analysis). In EA of *A. platanoides* and *F. sylvatica* the ice crystallization don't occur below 26.8% water contents (WC).While in *A. pseudoplatanus* the limiting WC for the ice crystallization is below 36.2 %. The WC for melting is similar for both species (ca. 45%). The increase of electrolyte leakage above 50% as a result of dehydration was observed only in EA of *A. pseudoplatanus* below 20% WC. According to the analysis of leachate conductivity the safe WC for freezing in -40°C and -196°C is below 25% WC in *A. platanoides*, while the lowest electrolyte leakage (ca. 60%) in freezing EA of *A. pseudoplatanus* is 20-15% WC. Dehydration and freezing was accompanied with gradual increase in superoxide (O_2^-) and hydrogen peroxide (H_2O_2) accumulation in both species. The increase of H_2O_2 in desiccated axes was 28.4% more in *A. platanoides* than in *A. pseudoplatanus.* While in *A. pseudoplatanus* the increase of O_2^- was 48.8% higher. The accumulation of AOS as a result of dehydration and freezing was associated with decrease in activity of guaicol peroxidase (POX). While the superoxide dismutase (SOD) activity increase during dehydration in *A. pseudoplatanus* and it reached at the end of dehydration a value 13.1% higher in comparison with control. In turn during dehydration in EA of *A. platanoides* and freezing in both species activity of SOD decrease.

The EA of *A. platanoides* and *F. sylvatica* tolerated the dehydration to 10% WC, while *A. pseudoplatanus* to 15%. Successful cryopreservation in LN was obtained for EA of *A. platanoides* and *F. sylvatica* desiccated to 15-10% WC and for EA of *A. pseudoplatanus* desiccated to 20-15% WC.

Introduction

Long-term storage under low relative humidity conditions and temperatures -17°C or -20°C is the conventional practice in most seed banks, but is appropriate only for desiccation-tolerant *orthodox* seeds. Which water contents is the range 4-8%, and none of the cellular water will freeze (Vertucci 1989), thus facilitating storage at the liquid nitrogen (LN).

However , the seeds of many woody plants species are desiccation tolerant. These seeds termed as *recalcitrant*, tolerating little or no water loss.

The extreme cold of liquid nitrogen profoundly limits chemical physical reactions. Indeed, there are increasing numbers of reports that demonstrate viability of germplasm after 10-20 years of storage (Walter at al. 2011). Cryopreservation is the storage of living cells and tissues at ultra-low temperature in liquid nitrogen (-196°C), using a minimum of space and maintenance, since during the storage cell division, metabolic and biochemical processes stop ((Walters 2004, Reed *et al.,* 2005 Sakai 1995). Seeds of different plant species vary in the degree of tolerance to desiccation and freezing stress. Mature seeds of *orthodox* category survive immersion in liquid nitrogen and storage without losing their viability. Seeds *suborthodox* and *recalcitrant* type characterized by distinct boundaries of resistance to water loss and low temperature. The objective of the present research was to determine possible causes of damage of embryonic axes of the typical s*uborthodox* seeds of beech (*Fagus sylvatica*), *orthodox* seeds (*Acer platanoides*) and *recalcitrant* seeds (*Acer pseudoplatanus*) during the step-by-step of cryopreservation process. Their tissues can be damaged during: (1) dehydration, (2) pre-cooling at -40°C, (3) freezing in LN (-196°C) and thawing cycle. The desiccation and low temperature stresses are accompanied by the formation in plant cells reactive oxygen species (ROS). ROS can damage cellular structures (Apel and Hirt 2004, France et al. in 2007), including DNA, proteins and lipids, causing membrane disruption. Detoxification of harmful ROS is carried out by a highly efficient antioxidant system, consisting of both non-enzymatic: ascorbic acid (AsA), glutation (GSH) and enzymatic antioxidants: superoxide dismutase (SOD), guaiacol peroxidase (POX) and catalase, CAT) present in plant cells. On the other hand several reports indicate that at least within the species, the relative water content in meristemic region is an important factor of survival. This factor limits the availability of freezable water and therefore reduce the likelihood of intracellular ice formation. The present study was undertaken to determine the extent survival of embryonic axes of beech, Norway maple and sycamore seeds, following desiccation and freezing stress in LN, without the addition of cryoprotectants and the potential ability to regenerate plant.

Materials and Methods

Seeds were harvested directly under trees of species representing 3 seed categories: *orthodox* (*A. platanoides* L.), *suborthodox (F. sylvatica* L.), and *recalcitrant*: *(A. pseudoplatanus* L.) growing at forest divisions in Poland. In the laboratory, the seeds weredried to a water content of 11% (*orthodox* and *suborthodox* seeds), and 45% (*recalcitrant* seeds) and

stored at -3°C in sealed jars. Cleaned seeds were then surface-sterilized and then the embryonic axes (EA) of seeds were isolated and next desiccated on nylon gauze inserted across a PVC pipe of 95 mm in diameter and 220 mm in length, which acted as a support for the axes. A computer C.P.U. cooling fan (12V, 1W), diameter 90 mm was mounted in the middle of this PE pipe, with air flow directed towards the gauze (Wesely-Smith 2001).

Cryopreservation

After dehydration embryonic axes were placed in 0.5 x 5 cm Al-tubes (and frozen in LN (-196°C) using a two-step method. First EA were frozen to -40°C (cooling rate 15°C/h) and after 30 min immersion, in LN for 24 h. Following LN exposure , the Al-tubes were rewarming rapidly, transferred to sterile distilled water in bath at 10°C for 30 s (warming rate: 6.2°C/s) (Pukacki et al. 2007).

Recovery of excised embryonic axes

The regrowth of excised embryonic axes was determined by *in vitro* germination concentrations of each of two growth hormones (NAA at 0.02 and 0.1 mg l^{-1}, BAP 0.5 and 1.0 mg l^{1}) were combined in a factorial design and we added to a full-strength WPM medium, (Lloyd and McCown 1981) 0.7% agar and 0.6% glucose in order to find a suitable medium for *in vitro* culture of embryonic axes. The pH of the medium was adjusted to 5.2 before sterilization. Embryonic axes of were excised and cotyledon segments were trimmed down as much as was possible before culture. Axils of elongating stems were surface-sterilized using the 3-step protocol: (1) mercuric chloride 0.1% for 1 min; (2) Domestos 10% (sodium chlorate < 5%) for 10 min; and (3) 75% ethyl alcohol for 5 min. For regrowth, embryonic axes were placed on WPM medium in Perti dishes (8 ml medium) and placed in a growth chamber Mytron WB 750 KHFL Germany. Growth chamber conditions were at 23°C, with a 16 h photoperiod at PAR 35-45 mol $m^{-2}.s^{-1}$ photon flux. After 7 weeks, surviving explants were transferred on the same medium in culture tubes (150 x 25 mm). Survival and plant recovery was were evaluated 1-week intervals after cryopreservation. Regeneration was recorded as the percentage of total number of samples.

Thermal analysis (DTA)

The DTA was used to determine the crystallization of apoplastic water. The technique determines the difference in temperature between live tissues and a reference dry sample, as both are cooled at the controlled rate.15°C/h The EA were cooled in an aluminium block placed in a computer-controlled freezer (Pukacki and McKersie 1990). The course of the analysis was controlled and registered with the help of an IBM computer equipped with an Analog/Digital Board (Type WB-AAI-B8) and software developed by Omega Engineering, USA.

Assessment of degradation of cell membranes

The level of membrane lipid peroxidation was assessed by measuring MDA content according to Heath and Packer (1968). The concentration of MDA was calculated from the difference in absorbance at 535 nm and 600 nm and excitation coefficient of 155 $nM^{-1}cm^{-1}$ (Heath and Packer 1968).

Superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2)

For the assays of O_2^-, 5 embryonic axes in tree replicates were soaked in phosphate buffer (pH 7.8) with 0.05% nitroblue tetrazolium (NBT) and 10 mM NaN_3, and incubated at room temperature for 90 min in the dark. The extract was heated at 65ºC for 15 min (Doke 1983). The total concentration of O_2^- was expressed as the ratio of measured absorption of the extract (read at 580 nm) to the dry weight of the EA.

To assay total H_2O_2, 5 axes and tree replicates were ground in liquid nitrogen. The powders obtained were homogenized with 5% TCA. The homogenate was centrifuged at 12 000 *g* for 15 min. The total amount of H_2O_2 in the supernatant was analysed by the ferrithiocyanate method (Thurman et al. 1972). The total concentration of H_2O_2 was expressed as the ratio of measured absorption of the extract (read at 480 nm) to dry weight of EA.

Enzyme extraction and assays (SOD and PO)

The 20 EA and tree replicates were powdered in liquid nitrogen and then the powder was transferred to a cold extraction buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.0) and polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15 000 *g* for 20 min. The supernatant was used as the crude enzyme for analysis of superoxide dismutase (SOD) and guaiacol peroxidase (PO)

Total SOD activity (EC. 1.15.1.1.) was determined by measuring its ability to inhibit the photochemical reduction of NBT according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 1.3 mM riboflavin, 0.1 mM EDTA, 1mM dithioeritritol, 63 mM NBT, and 50 l of enzyme extract. Tubes were placed under a fluorescent lamp and absorbance was recorded at 560 nm. A nonirradiated reaction mixture served as the control. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction. SOD activity was expressed as U g^{-1} DW of EA.

PO (EC.1.11.1.7.) activity was measured in the reaction of oxidation of guaiacol at 460 nm (e = 26.6 mM^{-1} cm^{-1}) according to Chance and Maehly (1955). The reaction mixture contained of 100 mM phosphate buffer pH 7.0, 1% guaiacol, 0.2 M H_2O_2, and 50- 100 l of enzyme extract; the activity was expressed as kat g^{-1} DW.

The protein content of crude enzyme extract was estimated according to Bradford (1976) using bovine serum albumin as a standard. All spectrophotometric analyses were performed in a UV-VIS 2401 PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

Results and Discussion

Desiccated embryonic axes of *A. platanoides, A. pseudoplatanus,* and *F. sylvatica* were subjected to differential thermal analysis (DTA) to determine the temperature of water crystallization and ice melting. In *F. sylvatica,* DTA detected water crystallization after desiccation to 26%, (Fig. 1), while in *A. pseudoplatanus,* more can be frozen (36%). This indicates that in embryonic axes of *A. pseudoplatanus*, water is bound more strongly in the cells. In *A. platanoides,* water crystallization in embryonic axes is observed at a similar level as for whole seeds, while in *A. pseudoplatanus* the water content limit of crystallization is higher in axes (Pukacka and Pukacki 1997).

The first symptoms of damage in tissues, caused by stress factors, can be observed in cytoplasmic membranes. Seed tolerance to desiccation can be assessed by measuring cell membrane permeability to ions. As a result of dehydration, in embryonic axes of the orthodox seeds of *A. platanoides* and the *intermediate* seeds of *F. sylvatica,* no lethal changes were observed in cell membrane permeability. Similar results have been reported for *Phaseolus vulgaris* (Chandel et al. 1995). However, membrane permeability to ions increased in desiccated embryonic axes of several species whose seeds are classified as

recalcitrant (Chandel et al. 1995, Ntuli et al. 2011, Song et al. 2004, Xin et al. 2010). In embryonic axes of *A. pseudoplatanus,* the increase in membrane permeability above the lethal level after desiccation to less than 20%, suggests that seeds of this species are sensitive to dehydration stress (Fig. 3a).

As a result of the next stages of cryopreservation (freezing at -40°C and -196°C), further damage may take place in membranes, leading to a loss of their semi-permeability and finally to cell death (Figs 3,4). An increase in cell membrane permeability was observed in the studied species if tissues with a high water content were frozen as a result of ice crystallization (Fig. 1). The decrease in water content of embryonic axes was associated with an increase in osmotic pressure. This resulted in a decrease in freezing temperature and in cytoplasm vitrification, which prevented ice crystallization in cells (Wolfe and Bryant 2001). The optimum range of water content of embryonic axes, without further tissue damage during freezing, was 10-25% in *A. platanoides* and 12-21% in *F. sylvatica*. In the recalcitrant *A. pseudoplatanus,* the range was the highest and reached 15-25%. At such levels of water content for individual species, cell membrane permeability after freezing was similar to the level immediately after desiccation. Similar results after freezing were obtained for embryonic axes of another species with recalcitrant seeds – *Aesculus hippocastanum* (Wesley-Smith et al. 2001).

A high permeability of cell membranes to ions in plant cells is associated with accumulation of the products of membrane fatty acid peroxidation (Leprince et al. 1995). In this study, we measured the level of thiobarbituric acid (TBA)-reactive products of peroxidation, mostly malondialdehyde (MDA) (date not presented). In embryonic axes of *A. platanoides,* because of the low concentration of the polyunsaturated linolenic acid in its membranes, assessment of the content of TBA-reactive products of peroxidation, is not a reliable method for peroxidation assessment (Pukacka 1999). By contrast, in *A. pseudoplatanus* as a result of desiccation and freezing at -40°C and -196°C, TBA-reactive products of peroxidation were accumulated. MDA content in *A. pseudoplatanus* increased with increasing dehydration stress, and reached the highest values after desiccation to less than 25%. After freezing at -40°C and -196°C, malondialdehyde accumulation was higher at higher levels of water content, but it decreased at the water content range of 10-25%, which was also associated with a decrease in membrane permeability to ions.

Desiccation and freezing of plant tissues is linked with ROS production. ROS cause oxidative damage, including fatty acid peroxidation in cell membranes. ROS accumulation,

e.g. of the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) under the influence of dehydration, has been reported for embryonic axes of seeds classified as intermediate, e.g. *Azadirachta indica* (Varghese and Naithani 2008), or *recalcitrant: Antiaris toxicaria* (Xin et al. 2010) and *Acer saccharinum* (Pukacka and Ratajczak 2006). By contrast, there is little published data for *orthodox* seeds (Bailly et al. 2001). In embryonic axes of the studied *Acer* species and *F. sylvatica* – an increasing desiccation stress was linked with a growing production of superoxide radical and hydrogen peroxide (Fig. 5). As a result of dehydration to a water content of 10%, about 25% more hydrogen peroxide was recorded in *A. platanoides*. In *A. pseudoplatanus,* superoxide radical production was 49% higher than in *A. platanoides*. In contrast, ROS level drastically increasing with desiccation stress to 300% in *F. sylvatica* (Fig. 6). After freezing at -40°C and -196°C, ROS level increasing in *F. sylvatica* and *A. platanoides* was stable, whereas in *A. pseudoplatanus* an increase in water content was connected with a decline in ROS production. It was the lowest for the water content range of 10-25% if also the level of membrane damage caused by freezing was the lowest.

For proper cell metabolism, an effective antioxidant system is necessary, involved in ROS degradation. The enzymatic antioxidant system includes e.g. superoxide dismutase (SOD) and guaiacol peroxidase (PO). SOD catalyses the disproportionation of superoxide radical to hydrogen peroxide, which is next transformed by PO into water. In control samples, a 43% higher SOD activity was detected in embryonic axes of *A. platanoides,* whereas PO activity was 11% higher in *A. pseudoplatanus*. The highest SOD activity in both species was detected at a water content of 40%. Its activity was about 40% higher than in the control in both species (Fig.6). As a result of further desiccation, in *A. platanoides* SOD activity declined, and at a water content of 10% it was 35% higher than in the control. By contrast, in *A. pseudoplatanus*, the lowest SOD activity was observed in the control. After freezing at -40°C and -196°C, SOD activity declined in both *Acer* species, and the decline was substantial at a water content of 40%. At other water content thresholds, a larger decrease was observed for axes of *A. pseudoplatanus* than for *A. platanoides* (Fig.6). Desiccation and the successive stages of cryopreservation (freezing at -40°C and -196°C), caused a slight decrease in PO activity in both *Acer* species (Fig. 6). The greatest changes were recorded after freezing at -40°C and -196°C, from embryonic axes with a water content of 40% in *A. platanoides* and *A. pseudoplatanus.* Despite their different tolerance to desiccation, they have a similar level of activity of antioxidant enzymes under the influence of stress factors. As a result of the ineffective

activity of antioxidants in the studied species, ROS production increased. A lower SOD and PO activity was detected also for embryonic axes of *Quercus robur* (Hendry et al. 1992), *Theobroma cacao* (Li and Sun 1999), and *Trichilia dregeana* (Song et al. 2004). Seeds of *A. platanoides* tolerate dehydration to 3% (Hong and Ellis 1992), while in embryonic axes, if water content declined to 10%, survival rate was 50% lower than in the control. Embryonic axes of *A. pseudoplatanus* were much more tolerant to desiccation than whole seeds, as the latter did not tolerate desiccation to a water content lower than 45% (Dickie et al. 1991). Survival rate of embryonic axes was maintained at a high level even after drying to 15%, and a higher decrease was observed only at a water content of 10% (Fig. 4). Seeds of a species from the *intermediate* category – *F. sylvatica* – very much like in *A. platanoides,* tolerate water loss to a moisture content of about 3%. By contrast, for isolated embryonic axes of *F. sylvatica,* survival rate declined after desiccation to 8% (Fig. 2).

Cryopreservation at -196°C of embryonic axes of *A. platanoides, A. pseudoplatanus,* and *F. sylvatica* proved to be possible after a 3-step procedure: desiccation, initial freezing at -40°C, and storage at -196°C. Dehydration made it possible to remove the water that could be otherwise transformed into ice, leading to cell damage and death. Desiccation is the most common method used for cryopreservation of embryonic axes isolated from seeds. It allows safe freezing at -196°C of axes of *Camellia sinensis* (Kuranuki and Yoshida 1996*), Aesculus, Castanea, Quercus* (Pence 1990), *Poncirus trifoliata* (Radhamani and Chandel 1992), and *Azadirachta indica* (Varghese and Naithani 2008). For *A. platanoides,* a species whose seeds tolerate desiccation, an optimum water content for freezing at -40°C and next at -196°C, was 10-15%. By contrast, for the species with recalcitrant seeds, the best results of cryopreservation were achieved for a higher water content (15-20%). For embryonic axes of *F. sylvatica,* survival rate was the highest after cryopreservation if before freezing they were dried to a water content of 10-18%.

Conclusions

1. Free water crystallization in embryonic axes is not observed if water content is lower than 26% in *A. platanoides* and *F. sylvatica*, compared to 36% in *A. pseudoplatanus***.**
2. In embryonic axes of *A. pseudoplatanus,* as a result of desiccation to 20%, membrane permeability to ions exceeded the lethal level. However, in *A. platanoides* and *F. sylvatica*, drying to 10% did not cause any critical damage to cell membranes.

3. Membrane permeability measurement showed that the safe range of water content for freezing at -40℃ and -196℃ was 25-10% in *A. platanoides*, 25-15% in *A. pseudoplatanus*, and 21-10% in *F. sylvatica.*
4. After desiccation to 10% in *F. sylvatica*, the increase in superoxide radical ($O_2^{\cdot-}$), was 400% higher than in control. Whereas axes of *A. platanoides* the increase in production was only 20% higher.
5. Desiccation and freezing at -40℃ and -196℃ resulted in a decline in PO activity in both species of *Acer*.
6. SOD activity in embryonic axes grows as a result of dehydration in *A. pseudoplatanus*, but it declines in *A. platanoides.* Freezing at -40℃ and -196℃ lowered SOD activity in both species.
7. Desiccation of embryo axes to 10% caused a significant loss of regeneration potential in *A. pseudoplatanus,* whereas axes of *A. platanoides* and *F. sylvatica* regenerated well under the desiccation stress.
8. The safe range of seed water content for survival and development of embryonic axes subjected to initial freezing at -40℃ and next at -196℃ is 10-15% for the *orthodox* species *A. platanoides*, 10-18% for *F. sylvatica*, and 15-20% for the *recalcitrant A. pseudoplatanus*.

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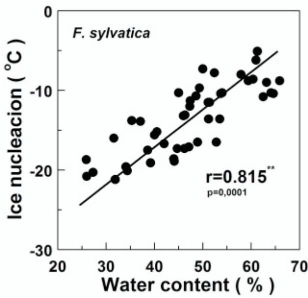


Fig. 1.

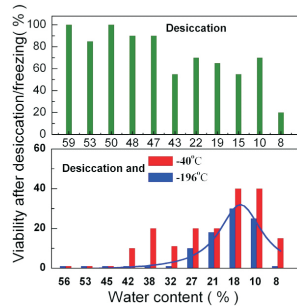
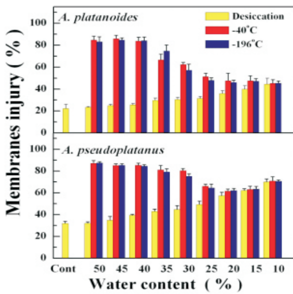


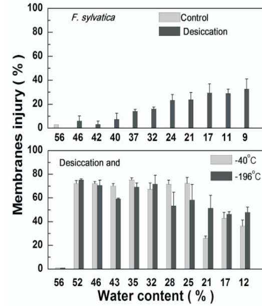
Fig. 2.

Fig. 1. Effect of desiccation stress (water content) in *Fagus sylvatica* embryonic axes on temperature of ice nucleation measured by DTA method.

Fig. 2. Effects of desiccation and freezing in -40℃ and -196℃ on viability of embryonic axes of *Fagus sylvatica* seeds.

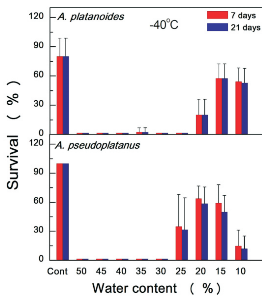


(a)

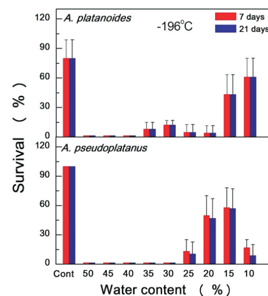


(b)

Fig.3. Effects of desiccation and freezing in -40℃ and -196℃ on injury of cell membranes of *Acer* species **(a)** and *F. sylvatica* of embryonic axes (**b**).



(a)



(b)

Fig. 4. Survival, regrowth of embryonic axes of *A. platanoides* and *A. pseudoplatanus* after dehydration and freezing in -40°C (a) and -196°C (b) on WPM. Results are expressed as means SM of 10 embryonic axes with 3 replicates.

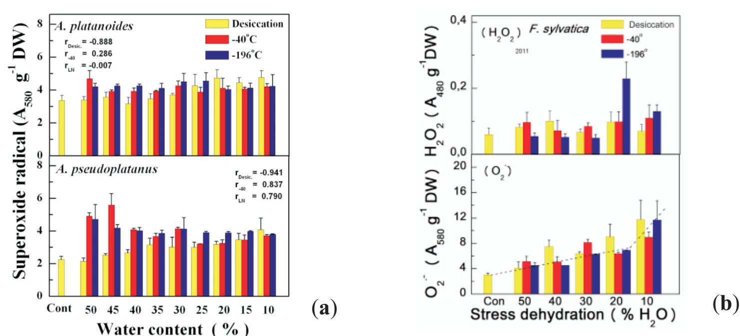


Fig.5. Effects of dehydration and freezing on content of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) in embryonic axes of *A. platanoides, A. pseudoplatanus* (**a**) and *F. sylvativa* (**b**) seeds

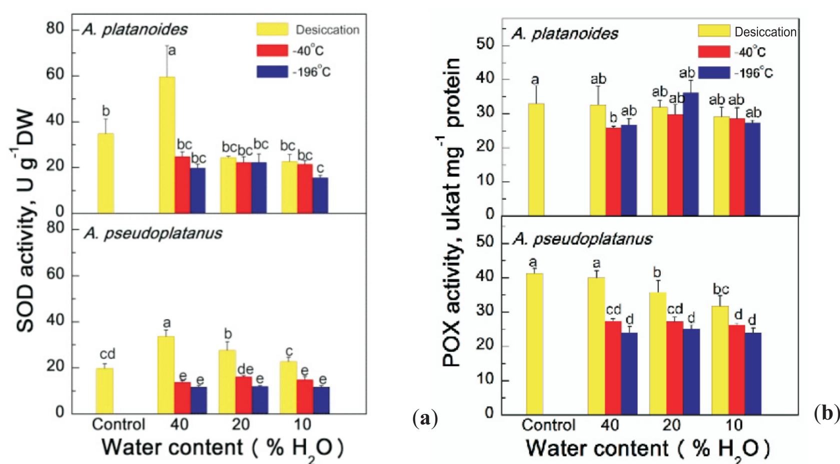


Fig. 6. Changes in activity of superoxide dismutase (SOD)-(**a**) and guaiacol peroxidase (PO)-(**b**), during cryopreservation of embryonic axes *A. platanoides* and *A. pseudoplatanus* seed. Results are expressed as means ± SD of 20 embryonic axes with 3 replicates. The same letters mean no significant difference on $p<0.05$ according to Newman-Keuls multiple range test.

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Cryopreservation by the vitrification method and genetic stability of embryogenic cultures of Norway spruce (*Picea abies*)

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Introduction

Currently, in the cryopreservation of cells, tissues or plant organs, procedures based on the phenomenon of vitrification are frequently used. Vitrification is a direct transition of water from a liquid phase to an amorphous phase, while avoiding the formation of ice crystals. Engelmann (2004) lists eight such procedures. One of them, called the pre-growth-dehydration technique, relies on short-term treatment of the explants during growth with cryoprotectants, then dehydration in a chamber with laminar air flow or over silica gel, and quick freezing in liquid nitrogen.

The aim of this study was to investigate if the pre-growth-dehydration technique can be used in cryopreservation of Norway spruce embryogenic tissue, and if the tissue stored with this technique and the somatic embryos derived from it are genetically stable.

Material and Methods

Plant material

Embryogenic tissue (ET) of Norway spruce *Picea abies* was obtained from mature zygotic embryos (explants) taken from seeds of Swedish origin, which were achieved from seed storage in Forest Technology Centre in Jarocin.

Preculture of ET

Picea abies ET was subjected to 7-day preculture on half-strength LM medium (Litvay et al. 1985) with sucrose (0.25 M for 1 day; 0.5 M for 1 day, 0.75 M for 2 days; 1 M for 3 days) and abscisic acid (ABA, 10µM). The tissue precultured with sucrose and ABA (variant SA) was compared to fresh ET (control).

Air-drying and freezing of ET

After the preculture, ET was air-dried over silica gel for 2 h at 25 °C, to a water content of approximately 20%, and then directly frozen in liquid nitrogen for 24 h (variant SA-LN).

Fast thawing of ET

After removing the ET samples from liquid nitrogen, the material was quickly thawed in water at 42 ºC and then placed on ½ LM medium for 63 days. During the growth of ET on the proliferation medium, weight gain measurements were made every 3 weeks.

ET ability to form somatic embryos

ET was placed on ½ LM medium without growth regulators but with activated charcoal (10 g/l) for a week. Next, ET was transferred to the medium with 20 μM ABA and 1 μM IBA, in order to stimulate regeneration of somatic embryos. After a 5-week treatment with these growth regulators, we counted somatic embryos at the cotyledonary stage (CSE) obtained from 1 gram of ET in the control and in variants SA and SA-LN.

Genetic stability testing

The genetic stability of cryopreserved ET and of SE derived from this tissue was analysed at 5 microsatellite loci: SpAGC1, SpAGC2, SpAGG3, SpAC1H8, SpAC2F7. We compared mature zygotic embryos (ZE), fresh ET (control), precultured ET (SA) and somatic embryos obtained from the thawed tissue (SE-SA).

Results

After ET preculture with increasing concentrations of sucrose (0.25-1.00 M) and ABA (10 μM), the water content of the tissue was 67%. Before freezing in liquid nitrogen, tissue water content was lowered to the optimum level (19%) by air-drying over silica gel for 2 h.

About 21 days after rapid thawing from liquid nitrogen, the weight of ET on proliferation medium ½ LM started to increase (Fig. 1). The thawed tissue showed the highest ability to proliferate after 63 days, although the rate of weight gain of cryopreserved ET was 5-fold lower than in the control (Fig. 1). This difference resulted from the stress of preculture and freezing of the tissue in liquid nitrogen.

The number of cotyledonary somatic embryos (CSE) per 1 gram of ET was 4-fold higher after preculture (203 CSE) than in the control (55 CSE). A similar trend was observed in tissue that was thawed from liquid nitrogen after preculture (222 CSE, Fig. 2).

The analysis of 5 microsatellite loci showed that the samples had repeatable amplification and the same genotype (Table 1). The obtained results indicate that the applied method of cryopreservation had no effect on the studied DNA regions.

Discussion

Before freezing in liquid nitrogen, treatment of plant material with high concentrations of soluble sugars, especially sucrose, contributes significantly to an increase in its tolerance to drying (Pence 1998). Thus it facilitates the necessary procedure of lowering of water content before freezing the cells, tissues or plant fragments in liquid nitrogen. This procedure eliminates the possibility of frost damage during storage of plant material under conditions of ultra-low temperature (-196ºC).

Our earlier research on the freezing of Serbian spruce with the method of pre-growth-dehydration showed that the optimal level of water content, which enabled survival and growth of tissues after thawing from liquid nitrogen, was approximately 20% (Hazubska-Przybył et al. 2010). A similar result was obtained for Norway spruce in this study (19%) after preculture on the medium with increasing concentrations of sucrose (0.25-1.00 M) and ABA (10µM) and air-drying over silica gel for 2 h.

ABA is added to the media at the preculture stage, often with sucrose (Mikuła 2008), as a factor involved in enhancing freezing tolerance of tissues of many plant species (Jitsuyama et al. 2002). In our study, ABA and sucrose added to the medium had a stimulating effect on ET weight growth after thawing from liquid nitrogen, which was already evident on day 42 of ET proliferation on ½ LM medium (Table 1). ET treated with ABA produced more somatic embryos, both after preculture alone (SA) and after preculture and freezing (SA-LN), as compared with the control (Table 2).

Plant material obtained by somatic embryogenesis and stored in liquid nitrogen should be genetically stable. However, some changes have been observed, although to a limited extent, in genetic material of somatic plants (Isabel et al. 1996), especially plants subjected to

cryopreservation (DeVerno et al. 1999). According to Burg and colleagues (1999), in trees cultured in vitro, mutational changes occur very frequently in the sequences of microsatellites (SSRs). Therefore, there is a need to monitor the genetic diversity within these sequences, also in plant material stored in liquid nitrogen.

On the basis of our analysis of the genetic stability of the examined microsatellite regions, there were no changes in precultured ET and in somatic embryos obtained from cryopreserved tissue. These results suggest that the plant material preserved its genetic stability after cryopreservation using the pre-growth-dehydration technique.

Conclusions

The tested cryopreservation procedure, based on the vitrification phenomenon, has proved to be effective in storage of ET of Norway spruce, which retains the ability to grow and to produce somatic embryos after thawing from liquid nitrogen. The application of ABA and sucrose in the preculture positively affects the growth of ET after cryopreservation. The tissue was characterized by good growth and a higher capacity to produce cotyledonary somatic embryos, as compared with the control. Treatment before freezing of ET in liquid nitrogen had no effect on the studied microsatellite regions in the genome of the tissue, suggesting genetic stability of plant material stored in liquid nitrogen using the pre-growth-dehydration technique.

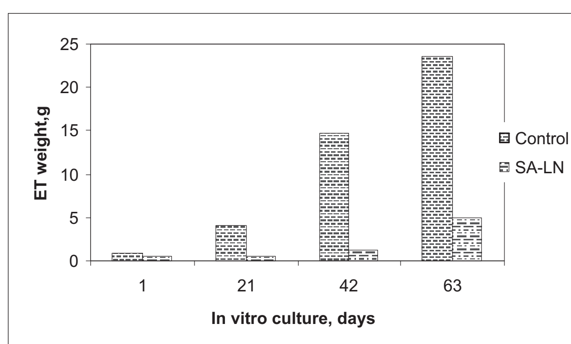

Fig.1. Embryogenic tissue (ET) weight on the proliferation medium (½ LM) during the 63-day culture after thawing from liquid nitrogen. Control = ET not precultured and not frozen; SA-LN = ET precultured with sucrose (0.25-1.00 M) and ABA (10 μM) and next frozen.

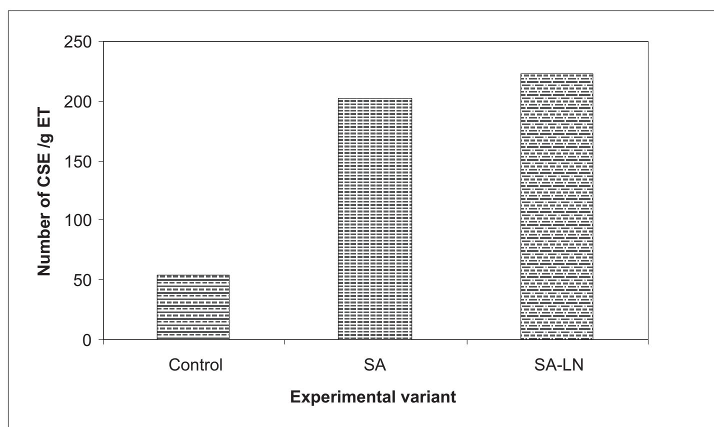

Fig.2. Somatic embryos at the cotyledonary stage, obtained from 1 gram of embryogenic tissue (ET) in a 5-week culture on ½ LM medium supplemented with ABA (20 μM) and IBA (1 μM). Control = ET not precultured and not frozen; SA = ET precultured with sucrose (0.25-1.00 M) and ABA (10 μM); SA-LN = ET precultured and next frozen.

Tab. 1. Genotype for the 5 microsatellite regions in the DNA of precultured ET and somatic embryos obtained from cryopreserved tissue; bp = base pair.

Locus	**SpAGC1**		**SpAGC2**		**SpAGG3**		**SpAC1H8**		**SpAC2F7**	
Allel	a	b	a	b	a	b	a	b	a	b
pb	99	103	86	86	128	132	92	102	98	110

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The cryo-conservation of apical meristems of dormant buds of Siberian and Asiatic woody plants

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Abstract

The Kórnik Arboretum including 3500 species and cultivars of woody plants, is third largest in Europe. The in situ conservation of Siberian woody plants is often dangerous, in conditions of the climate of Poland. In this study we investigated for cryopreservation of important 18 species of the commonly Asiatic and Siberian origin. Our results guaranteed successful recovery at average 80% of freezing desiccated dormant bud after preserved long-term in LN (-196°C).

Introduction

Low temperatures during winter dormancy and spring frost at the beginning of vegetative phase belong to the most important abiotic factors limiting the growth interesting Siberian woody plants species introduced in Europe. The scale of frost and cryo-tolerance is dependent on the genetic potential of the species and the level of plant acclimatization (Reed 1999, Volk et al. 2008). The important factors for cold tolerance of dormant buds seem to be acclimation, moisture content, cooling rates and thawing procedures (Jenderek et al. 2011).

Results

The dormant buds in the terminal end of the annual shoot from 18 species Asiatic and Siberian regions were collected in February 2010 from the Kórnik Arboretum. They were cut into 40 -50 mm section. The freezing-dehydration of buds was conducted at -10°C in open jars cooling chambers for 3 weeks. During this period buds were desiccated to 15% - 29% moisture content (fresh weight basis), depend on species.

Cryopreservation.

The desiccated buds were sealed in aluminum cryovials (5.0 X 0.5 cm) and frozen slowly (prefreezing) to -40°C at 0.2°C/min, after 30 min followed by immersion in liquid nitrogen (-196°C) for 48 h. Re-warming was performed by placing the Al-cryovials in water bath at 10°C for 2 min (Pukacki et al. 2007). After thawing, the apical meristems

were extracted and sterilized with ethanol, sodium hyperchlorite solution, 10% Domestos. The apical meristems were transferred to solid WPM medium (McCown and Llyd 1981) for recovery. Survival was determined as the percentage of explants resuming growth after thawing. These results show that survival and regrowth of cryostored buds depend on species. Regrowth percentages (average 82%), ranged between 45 and 100%. On the other hand, when dormant buds were freezing-dehydrated at -10°C for 3 weeks, apical meristems excised from buds, after culture *in vitro* in WPM medium displayed recovery ranged between 10 to 100% (average 69%), (Fig.1,2).

Determination of cold hardiness of species by freeze-shoots in laboratory freezing chamber

This was performed according to Pukacki and Pukacka (1987). The shoots of species wrapped in aluminium foil were placed in a computer-controlled freezer, the temperature was decreased by 3°C h^{-1} at 5°C intervals, for 24 h. After freezing and thawing, the 1.5 cm section of shoots were put into deionised water (0.1 g ml^{-1}). The percentage of ion leakage from the samples, determined after 24 h incubation in room temperature and than by measuring its electrical conductivity. The cold hardiness of shoots was defined as the lethal temperature (LT_{50}) and was calculated to the temperature giving 50% ion leakage (Fig. 3). Most species showed high survival rates (Fig. 4).

Cryopreservation of cold hardy woody plants of dormant apical meristems by dehydration-freezing procedure has potential for using in long-term conservation programs.

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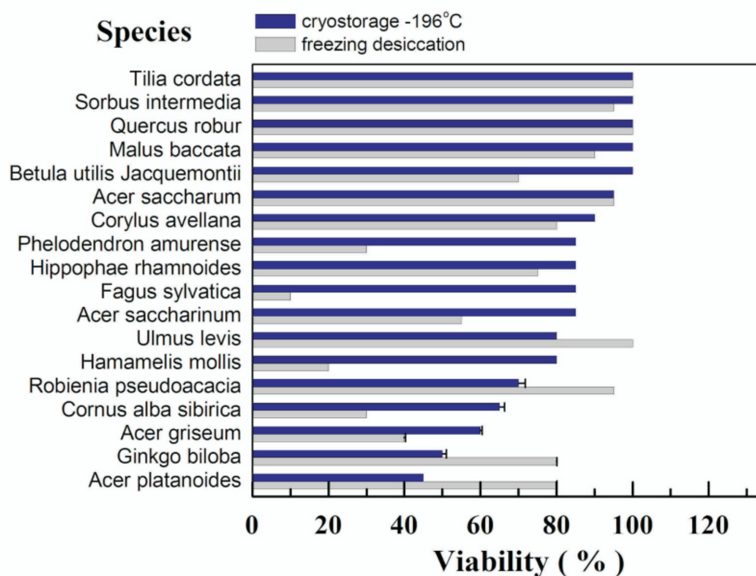
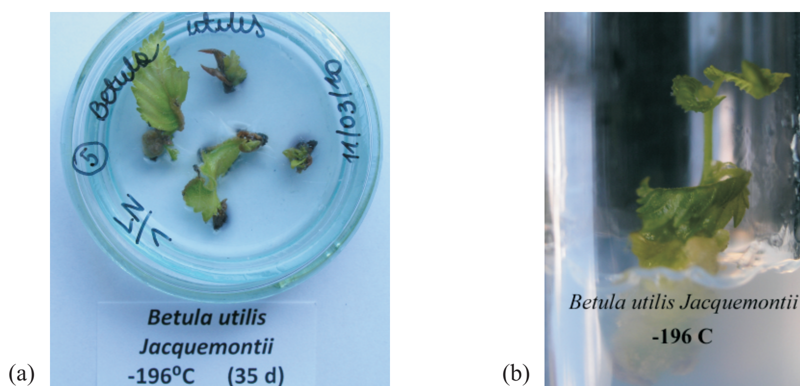


Fig.1. Percentage viability, regrowth post-thaw cryoconserved and freezing desiccated of Asiatic and Siberian origin of woody plant species



(a) (b)

Fig.2. Recovery growth of cryopreserved the meristems of buds *Betula utilis Jacquemontii* after 35d (a) and 6 months (b), of culture on WPM; at PAR 25-35 mol $m^{-2}s^{-1}$, 16 h photoperiod.

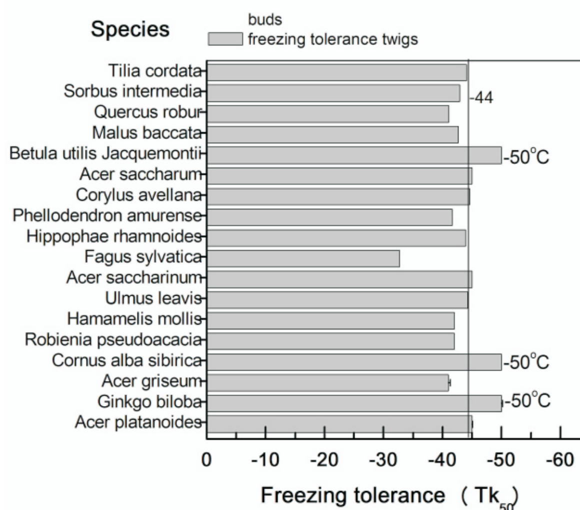


Fig.3. Winter freezing tolerance (February) estimated from electrolyte leakage (Tk_{50}) of cold acclimated 18 species. Twigs collected from the Kórnik Arboretum at the end February 2011.

Fig.4. Laboratory test of winter freezing tolerance (February) of, one-year shoots of *Cornus alba sibirica*, following freezing at -30°C, -35°C, -40°C and -45°C for 24 hr and recovery at 22°C for 28 days.

Cryopreservation of wild rose achenes from a natural habitat in Krakow

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Roses are a leading group of ornamental plants in horticultural market. Wild rose species, apart from their decorative value, are an important substrate in pharmaceutical and cosmetic industry, and are a source of food and shelter for numerous wild animals. Wild rose populations found in Poland in natural habitats have been decreasing systematically due to destructive human activity (1). Relevant approach to protection of endangered species involves the creation of gene banks, and the most recent method, cryopreservation (2).

Four species of wild roses: *Rosa agrestis, Rosa canina*, *Rosa dumalis*, *Rosa rubiginosa,* found in south district of Krakow had been observed. Achenes were separated from mature rose hypanthia collected at the end of October. Then they were destined for stratification in humid peat at 25°C for 10 weeks, followed by 14 weeks at 3C. During the stratification, successive portions of seeds (50 pieces) were desiccated at 25C under laminar flow of sterile air and afterwards were placed in cryotubes submerged in liquid nitrogen. Achenes were frozen in 1st, 7th, 11th, 17th and 23rd week of stratification. They were thawed for 3 minutes in a water bath at 37C. Subsequently, the achenes were sawn in rows in trays filled with a standard substrate Basis Substrate 1 (Kronen Klasmann). Achenes which went through the full stratification process but were not placed in LN, were the control.

Germination was observed 7 months after sowing. Percent of germinated seeds depended on stratification level and species. The best germination percentage was noted for the control (from 30% for *R. agrestis* to 80% for *R. dumalis*). Germination percentage of the achenes after cryopreservation was higher when they finished stratification process and then were cryopreserved (from 27% for *R. agrestis* to 78.1% for *R. dumalis*). Germination of achenes was very low when they were cryopreserved during the stratification process (Fig. 1).

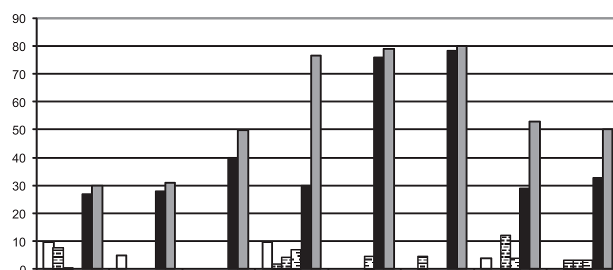

Fig. 1. The effect of cryopreservation term during stratification (1- 23 week) on seeds germination (%)

Acknowledgements

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Occurrence and prevention of frost injury in field conditions

Resistance to the low temperature stress of the Polish strawberry cultivars frozen in controlled conditions

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Abstract

Resistance to the low temperature stress of frigo plants kept at the cold store of four strawberry cultivars ('Elkat', 'Selvik', 'Filut' and 'Vikat') bred at the Research Institute of Pomology and Floriculture (now Research Institute of Horticulture) was evaluated in the years 2009-2010. The standard cultivars were: 'Elsanta' (the Netherlands) and 'Ventana' (USA). Plants were frozen artificially using a freezing chamber (BINDER GmbH, Germany). Frigo plants of investigated cultivars were kept in a cold store in the temperature -1,7 to -2,0°C. Before freezing they were planted into small pots (dimension 9x9x10 cm; capacity 0,81 l) filled with moist mixture of peat substrate and sand (4:1). The freezing was performed on 23-27 March 2009 at different temperatures (-5°C, -10°C, -15°C and -20°C) and on 25-29 January 2010 also at different temperatures (-5°C, -8°C, -10°C, -12°C and -15°C). Plants were frozen for 3 hours, the temperature was reduced with the speed of 1°C per hour. Immediately after freezing plants in pots were again placed in the cold store and at the beginning of April they were transferred to the uncovered frame. The control plants were treated in the similar ways except for artificial freezing. Each experimental combination consisted of 24 plants (4 replications with 6 plants). Plant survival and plant growth vigor after freezing were performed six weeks after removing plants from the cold store. Each plant was estimated individually using the ranking scale 0-5, where 0 - means dead plants and 5 – the most strong vigor within each cultivar.

In 2009, very pronounced differences in cold hardiness were observed when the plants were frozen at the temperature of -10°C. This temperature caused dying of 100% plants of cultivars 'Elsanta' and 'Ventana', 29,2% plants of 'Selvik', 25% plants of 'Elkat', 20,8% plants of 'Vikat' and 4,2% plants of 'Filut'. The average growth vigor of frozen plants was moderate for 'Selvik' and 'Vikat' (the average value was 2.5 in the ranking scale 0-5), 'Elkat' and 'Filut' (3.0) comparing with the unfrozen plants (5,0). After freezing of plants in the temperature -15°C, most of the plants died, only a few plants of cultivar 'Filut' remained alive (4.2%).

In 2010, the temperature of -10°C proved to be the most harmful to the plants of 'Elsanta' (70.8% dead plants), while plants of the other cultivars were more hardy – dead plants from 20.8% for 'Vikat' to 33.3% for 'Elkat'. The average growth vigor of frozen plants was weak or moderate (from 1.5 for 'Elkat' to 2.2 for 'Selvik'). The temperature -12°C killed the most plants of all cultivars (from 80 to 100% dead plants depending on the cultivar).

The studies showed that the Polish strawberry cultivars ('Elkat', 'Selvik', 'Filut' and 'Vikat') are more resistant to the low winter temperature stress, than the cultivars originating from the warmer climate, like 'Ventana' bred in California or 'Elsanta' bred in the Netherlands.

Frost injuries of apricot (*Prunus armeniaca* L.) and peach (*Prunus persica* L.) flower buds during winters of 2008/2009 and 2009/2010 and their affect on yielding

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Abstract

Apricot (*Prunus armeniaca* L.) and peach (*Prunus persica* L.) are of continental climate origin and posses short dormancy period. After breaking dormancy, during longer periods of winter thaws, trees lose their frost hardiness. Under Polish climatic conditions, the winters are not stable and are characterized by high fluctuation of temperatures, which often leads to frost injuries of flower buds and even shuts of apricot and peach trees. Large decreases in temperature occurred during winters of 2008/2009 and 2009/2010.

In spring of 2009 and 2010, the rate of flower buds survival of chosen apricot and peach varieties and breeding clones was examined at the Dąbrowice Research Orchard near Skierniewice. Damaged flower buds in apricots were rated on April 6-7th in 2009 and 2010 and in peaches on April 9-10th 2009 and April 13-14th 2010. Depending on the number of trees in collection of varieties/clones, the evaluation of flower bud injuries was performed on 2 or 4 trees of each apricot and peach genotype. The rating was done at the phase of swelling healthy buds but before fall - off damaged ones. The flower buds were divided into two categories: developing buds (undamaged) and not developing buds (damaged). When 4 trees of given genotype were to our disposal, the percentage of damaged buds was evaluated on 8 branches, 50 flowers on each. In case of only 2 trees, evaluation was made on 4 branches, 100 buds on each. Each genotype was evaluated on a sample of 400 buds, in four replications, 100 flowers per plot. The buds were counted on the branches located on the opposite sides of the trees, at 1.5-2.0 m above soil surface.

In the winter of 2008/2009 the lowest temperature (-23 °C at a height of 2 m) was noted on January 6th. In spring of 2009 damage of apricot flower buds, varied from 50.5 to 98.0% (average 76.1%) depending on the variety. During the winter of 2009/2010 the maximum drop in temperature (-28 °C at a height of 2 m) was noted on January 26th. During that winter temperatures were quite stable, however the temperatures were lower, damage to apricot buds was lower than after the winter 2008/2009 and varied from 41.0 to 85.3% (average 64.4%) depending on variety. Apricot produced higher yield than in 2009.

After the winter 2008/2009 peach flower buds have been damaged to varying degrees (depending on variety) from 30.5 to 84.0% (average 65.8%) in 2009 and in 63.3 – 95.3% (average 79.1 %) in 2010. Despite of more serious damage in 2010 than 2009 good weather conditions during flowering time in 2010 contributed to good fruit set and higher yields of peach trees than in 2009.

Results of this study indicate that the most frost resistant varieties of apricot and peach, in cold but stable winters can achieve and maintain a high frost resistance level which allows the good winter survival when the temperatures drop to - 28°C. Those varieties retain enough of undamaged flower buds to set good number of fruits and produce moderate or even high yield. In such a case there is no need to thin fruit sets, leading to high quality yield. Results of observations 2010 indicated that during cold winters but without large fluctuations in temperature at the time of deep dormancy, flower buds of apricot trees may gain higher frost resistance than flower buds of peach.

Resistance to the low temperature stress of the polish apple rootstocks after artificial freezing

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Abstract

Resistance to the low temperature stress of 9 Polish vegetative apple rootstocks (P 2, P 14, P 16, P 22, P 59, P 60, P 66, P 67 and P 68) bred at the Research Institute of Pomology and Floriculture (now Research Institute of Horticulture) was evaluated in the years 2009-2010. As standard rootstocks were used M.7, M.9, M.26, M.106 and Antonovka seedling. The rootstocks were frozen artificially using a freezing chamber (BINDER GmbH, Germany). Until the time of freezing all rootstocks were kept in a cold store in the temperature 0°C. The freezing was performed on 3-5 April 2009 and on 15-17 February 2010 at different temperatures: -8°C, -10°C and -12°C (10 rootstocks in each temperature, freezing time – 3 hours, the temperature was reduced with the speed of 2°C/hour). During freezing the rootstocks were placed in sealed plastic bags to protect them before drying. After freezing the rootstocks were again placed in the same cold store, where they stayed until planting in the field – 14 April in the year 2009 and 9 April in the year 2010. Controls were the same rootstocks which were not frozen. After planting in the field, all rootstocks were cut 5 cm above the soil level. In 2009 and 2010 the following measurements and observations were performed, individually for each genotype:

- diameter of rootstocks measured at the height of 5 cm from the ground level (mm) – at different terms – 28.04.2009 and 23.04.2010 and the end of plants' vegetation – 28.10.2009 and 18.10.2010
- height of rootstock's shoots (cm) – 29.10.2009 and 18.10.2010
- length of all newly grown shoots (cm) – 29.10.2009 and 18.10.2010
- weight of rootstock (g) – after the end of the vegetation period – 05.11.2009 and 25.10.2010

After two years of studies the most resistant to the low temperature stress were the following rootstocks: P 60, P 68 and P 67

Poster Presentations

(Abstracts)

POSTER No 1

Cryoprotective activity of thermal hysteresis proteins of boreal evergreen plants

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Thermal hysteresis proteins (THPs), also known as anti-freeze proteins (AFPs inhibit the growth of ice by binding to the surface of ice crystals, and thus preventing the addition of water molecules, to cause a local depression of the freezing point. Recent findings demonstrate that apoplastic THPs from Norway spruce needles show a cryoprotective and also anti-ice nucleation activity (Jarząbek et al. 2009). Here, we have focused on the most active THPs from nine frost-hardy conifers of North American and Eurasian boreal forest: *Abies alba A. grandis, Picea abies, P. glauca, P. pungens, Pinus nigra*, *P. sylvestris, Pseudotsuga menziensii, and Tsuga canadensis,* and additionally from one alpine (2100 m a.s.l.) evergreen shrub *Loiseleuria procumbens*. The objective of this study was to determine whether THPs from these species influence survival at subzero temperatures by modifying the freezing process and/or by acting as cryoprotectants. Apoplastic extracts were obtained by vacuum infiltration of leaves with 5 mM ascorbic acid, and the extracts were concentrated by using a 10 kDa cutoff Ultrafree centrifugal filter device (Millipore, USA). Apoplastic proteins were separated by SDS-PAGE. Their cryoprotective activity was determined with the use of the freeze/thaw inactivation, by four cycles in liquid nitrogen (-196°C) and room temperature. The assay of lactate dehydrogenase (LDH) was performed as described by Wisniewski et al. (1999). Thermal hysteresis activity was determined by using the droplet freezing assay (Vali 1971). Our results show that evergreen plants produce THPs that are secreted into the apoplast. When we examined the possible role of conifer THPs in cryoprotection, we found that LDH activity was higher after the freeze-thaw cycle in the presence of THPs, as compared with bovine serum albumin (BSA) or buffer alone. The presence of THPs (250 $\mu g\ ml^{-1}$) preserved 90% and 100% of LDH activity after the freeze/thaw cycles. In comparison with BSA, the cryoprotective activity of THPs proteins from the *P. pungens and L. procumbens* was respectively 2-fold and 4-fold higher in terms of LDH activity. The accumulation of proteins in extracellular spaces in winter as a result of seasonal cold acclimation indicates that these proteins may also play a significant role in freezing tolerance of needle cells in conifer.

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POSTER No 2
Cryopreservation of embryonic axes of *Fagus sylvatica* L. seeds using dehydration procedures

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Cryopreservation in liquid nitrogen (LN, -196°C) allows safe and long-term conservation of many plant species (Uchendu et al., 2010). The objective of the presented research was to determine the possible damages of embryonic axes of the typical *suborthodox* seeds of beech (*Fagus sylvatica*) during the step-by-step cryopreservation process. Their tissues can be damaged during dehydration and/or the LN-thawing cycle. Ice nucleation during freezing was determined by differential thermal analysis. The non-freezable water was estimated when the tissues were dehydrated below a water content (WC) of 24%. Damage to membranes during desiccation and cryopreservation was assessed based on electrolyte leakage and analysis of lipid peroxidation, as the concentration of its end product – malondialdehyde (MDA). Accumulation of MDA slightly increased when desiccation progressed, and a further increase was observed after freezing in LN. Dehydration alone slightly impaired plasma membrane integrity, while the increase in membrane breakdown was observed after pre-freezing to -40°C and then after cryo-freezing if WC was above 23%. No substantial changes were observed in levels of particular fatty acids within the phospholipid fraction. Desiccation and cryopreservation caused an increased production of reactive oxygen species: superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2). The strong rise in $O_2^{\cdot-}$ production appeared during cryopreservation stress. Ultrastructural examination of tissues dehydrated to a WC of 20.7% and next frozen, showed mild injury of the cell wall, cell membrane and nuclear envelope, but also mitochondrial swelling and coalescence of lipid bodies. Optimal survival of tissues after cryogenic stress was achieved when they were desiccated to a WC of 14.0% and 16.2%. Comparison between the effects of desiccation over silica gel and fast desiccation by air-flow on the cryopreservation, showed that axes survival was dependent on water content, not on desiccation method. Embryonic axes subjected to variable levels of desiccation stress showed a minor decrease in viability and vigour, but after extreme desiccation, an increase in electrolyte leakage appeared, indicating a impaired membrane integrity. Embryonic axes of *F. sylvatica* survived freezing at -196°C when their WC was between 22.3 and 11.4%. Antifreeze protein fraction <30kDa (compared to buffer alone) had a protective effect on the cold-labile enzyme lactate dehydrogenase (LDH) activity after freezing to -196°C.

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POSTER No 3

Cryoprotective activity of thermal hysteresis proteins of embryonic axes of orthodox and recalcitrant seeds.

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Introduction: Thermal hysteresis proteins (THPs), also know as antifreeze proteins (AFPs), lower the nonequilibrium freezing point, of water below the melting point by a noncolligative mechanism. During cold acclimation, the accumulation of THPs is correlated with increased freezing tolerance of plants.

Methods: Protein extracts from embryo axes (EA) of *Acer platanoides, A. pseudoplatanus, Fagus sylvatica and Quercus robur* seeds were obtained by grinding in phosphate buffer followed by centrifugation at 10000g to recover the proteins. Protein extracts were filtered with a molecular weight cut-off of 3-kDa and 30-kDa (Ultrafree-CL Centrifugal Filter, Millipore). Three protein fractions were distinguished: P-total (7–97 kDa), THP >30 kDa), and THP <30 kDa). Then proteins were submitted to purification and concentration with Ultrafree-CL Centrifugal

Results:

The possible role of *A. platanoides, A. pseudoplatanus, F. sylvatica* and *Q. robur* embryonic axes protein in cryoprotection was investigated. It was found that cold-labile enzyme lactate dehydrogenase (LDH) activity was higher after freezing in the presence of embryonic axes proteins. After several freeze/thaw cycles at -196ºC/20ºC of LDH, in the presence of EA *protein,* it was shown their cryoprotective activity, which was comparable to that of the AFPs of spruces needles (Jarząbek et al. 2009). Especially, high cryoprotective effect indicated *protein fraction below 30 kDa*. It was about 3-18 times more effective than glucose, sucrose and bovine serum **albumin (**BSA). Our further research on the protein of fraction <30 kDa should broaden our knowledge of the range and nature of the cryoprotective function of this fraction.

Conclusions:

Our results demonstrate that THPs from *Acer, Fagus and Quercus* embryo axes have specific cryoprotective activity and modify the freezing process in tissues. Especially, high cryoprotective effect indicated protein fraction below 30 kDa form EA of *A. platanoides*. THPs of embryo axes also inhibit the propagation of ice *in vitro,* so that no freezing occurs until the tissue reaches its equlibrium freezing points. The THPs from *Acer platanoides* EA froze 3-fold slowly than BSA or water.

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Jarząbek M., Pukacki P.M., Nuc K. 2009. Cold-regulated proteins with potent antifreeze and cryoprotective activities in spruces (*Picea* spp.). Cryobiology, 58:268-274.

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POSTER No 4

Successful application of a vitrification method for cryopreservation of embryogenic tissues of Serbian spruce (*Picea omorika*)

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Clumps of embryogenic tissue (ET) of Serbian spruce (*Picea omorika* (Pančić) Purk. were successfully cryopreserved using a vitrification-based procedure, namely pregrowth-dehydration (Engelmann 2004). ET clumps were precultured for 7 days at 25ºC in the dark on standard Litvay medium (LM, Litvay et al. 1985) with half-strength inorganic salts, and the following concentrations of sucrose: 0.25 M for 24 h, 0.5 M for 24 h, 0.75 M for 2 days, and 1.0 M for 3 days. Next the clumps were air-dried for 2 h over silica gel, down to a 20% water content (based on fresh weight), then placed in cryovials, and immersed in liquid nitrogen (LN). After 24 h, ET clumps were thawed within the vials at 42ºC, and rehydrated in Phytagel-solidified LM medium with decreasing concentrations of sucrose. After about 3 weeks of in vitro culture, we noticed that surviving clumps were similar in their morphology to non-frozen clumps. We obtained high rates of ET survival (99%) after 28 days of in vitro culture by using this kind of vitrification. The frequency of bacterial and fungal contamination was higher if ET was frozen in LN-containing vials than in LN-free vials. The novel protocol of ET cryopreservation can be applied for long-term storage of the endangered *Picea omorika* germplasm *ex situ*.

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Seminarium towarzyszył okolicznościowy wernisaż malarstwa przyrodniczego emerytowanego profesora Instytutu Dendrologii PAN Henryka Chylareckiego

"Marzą mi się „miasta-ogrody"
w miejsce kamiennych pustyń, w których
znacznie większe dobory drzew
i krzewów ozdobnych stworzą warunki
dla zdrowego wychowania
młodych generacji."

Kórnik, Maj 2011

Tło: Zachód słońca w Biernatkach
Strona tytułowa: Sekwoje, bambusy i klony japońskie w Arboretum Przelewickim
Fotografie: P.M.Pukacki; Opracowanie graficzne: Wiktoria Pukacka

Obszarem zainteresowania Profesora była zmienność morfologiczna drzew i krzewów (głównie cisów, świerków, modrzewi), a przede wszystkim studia nad potencjałem produkcyjnym, rozwojem i ekologią drzew obcych, które wyróżniają się dużą zdolnością przystosowawczą i przydatnością gospodarczą. Dla nich opracował własną, oryginalną metodę oceny adaptacji do nowego środowiska, która sprawdziła się na 220 leśnych powierzchniach doświadczalnych w calej Polsce.

Duże znaczenie społeczne mają badania ekologiczne odnośnie aklimatyzacji drzew i krzewów ozdobnych w aglomeracjach miejskich i one posłużyły do opracowania regionalizacji doboru drzew ozdobnych w Polsce. Niemniej ważne były badania nad mrozoodpornością gatunków drzew obcych, ozdobnych w obrębie 22 rodzajów np.: Magnolia, Rhododendron, Prunus, Viburnum.

W czasie 35 lat pracy w Instytucie Dendrologii prof. Chylarecki zgromadził bogate zbiory przyrodnicze (2400 pozycji kat.), które dały początek Muzeum Dendrologicznemu.