EUROPEAN COOPERATION IN THE FIELD OF

SCIENTIFIC AND TECHNICAL RESEARCH

COST 871 ACTION:

CRYOPRESERVATION OF EUROPEAN CROP SPECIES

OVIEDO, SPAIN

13 – 14 APRIL 2007

FIRST MEETING OF WORKING GROUP 1

“Fundamental Aspects of Cryopreservation/Cryoprotection and Genetic Stability”

Organized by:

Instituto Universitario de Biotecnología de Asturias (IUBA).

Departamento de Biología de Organismos y Sistemas.

Universidad de Oviedo.

Oviedo, Spain.

Dr. M. Ángeles Revilla Bahillo (local organizer)

Dr. Rubén Álvarez Fernández (layout)

COST 871

PROGRAMME OF THE MEETING

Oviedo April 13-14, 2007

FRIDAY 13TH

09.00-09.30	Registration of the participants
09.30-09.45	Welcome of the Vice-Rector for Research
09.45-10.00	Welcome of the Chair of the COST Action 871, Dr. Bart Panis

Theme 1.1. Fundamental aspects of cryopreservation and cryoprotection

10.00-10.45 Keynote lecture: "The effect of a sucrose induced acclimation on sugar, polyamine, sterol and fatty acid composition and its role in cryopreservation of banana meristems"
G.-Y. Zhu, J.M.C. Geuns, S. Dussert, R. Swennen and B. Panis

10.45-11.05 "Effect of the osmotic agent chosen for the preculture step during the *Pelargonium* apices cryopreservation process"
A. Grapin and N. Dorion

11.05-11.25 "The effect of osmotic pretreatments on the post-thaw regrowth and the proteome of banana meristems"
S.C. Carpentier, R. Swennen and B. Panis

11.25-11.55	Coffee break

11.55-12.15 "Effects of cryoadditives and suspension density in the cryopreservation of *Pinus pinaster* embryogenic cultures"
L. Marum, M.M. Oliveira and C.Miguel

12.15-12.35 "Cryoprotective and antifreeze activity of apoplastic proteins in winter hardiness of conifers"
P.M. Pukacki, M. Jarząbek and P. Nuc

12.35-12.55 “Dedifferentiated plant cell lines for the evaluation of physiological parameters of cryotolerance”
E. Heine-Dobbernack, A. El Banna, H. Kiesecker and H.M. Schumacher

12.55-13.15 “The effect of phase transition on recovery rate of *Hypericum perforatum* L. shoot tips after cryopreservation”
M Skyba and E Cellarova

13.15-15.00	Lunch

15.00-15.20 “The use of differential calorimetric analysis of plant shoot tips in cryopreservation”
A. Bilavcik, J. Zamecnik and M. Faltus

15.20-15.40 “Contaminated liquid nitrogen storage vessels as vectors for plant pathogens
B.W.W. Grout and G.J. Morris

15.40-16.00 “Fundamental aspects of cell suspensions cryopreservation of *Gentiana* spp.”
A. Mikuła and J.J.Rybczyński

16.00 -16.20 “Cryopreservation of strawberry”
A. Nukari, M. Uosukainen and A. Flyktman

16.20-16.50	Coffee break

16.50-17.10 “Cryopreservation of Scots pine”
H. Häggman, J. Krajňáková ,L.Ryynänen, T.Aronen, S. Jokipii and J. Vuosku

17.10- 17.30 “Cryopreservation of embryogenic cultures of Norway spruce”
M Vágner, J Spacková, K Eliasová, L Fischerová and Z Vondráková

17.30- 17.50 Tissue culture as a tool for ex situ conservation of wild pear
E Caboni, MG Tonelli, MA Palombi and C Damiano

18.00	Sightseeing visit of Oviedo
21.00	Dinner at the Hotel “La Gruta”

SATURDAY 14TH

Theme 1.2. Genetic stability and authenticity

09.00-09.45 "Cryopreservation and genetic stability"
A.M. Vázquez

09.45-10.05 "Transgene stability in cryopreserved cork oak somatic embryos"
R. Álvarez, M.A. Revilla and R.J. Ordás

10.05-10.25 "Genetic fidelity of *Pinus pinaster* somatic embryogenic cultures following cryopreservation"
L. Marum, M. Rocheta, M.M. Oliveira and C. Miguel

10.25-10.45 "Assessment of developmental competence, genetic stability and biochemical potential of cryopreserved *Hypericum perforatum* L. shoot tips"
E. Cellarova, M. Urbanova and J. Kosuth

10.45-11.15	Coffee break

11.15-11.35 "Assessment of genetic stability of cryopreserved and cold stored hop samples"
E.L. Peredo, B.M Reed, R. García-Arroyo and M.A. Revilla

11.35-11.55 "Approaches to detect somaclonal variation and epigenetic changes in cryopreserved chrysanthemum apices"
C. Martín and M.E. González-Benito

11.55-12.15 "Molecular validation of a micropropagation-cryopreservation procedure for red chicory (*Cichorium intybus* L.) selected lines"
A. De Carlo, A. Previati, C. Benelli, F. Da Re, M. Giannini and M. Lambardi

12.15-12.35 "Genetic fidelity of cryopreserved embryogenic cultures from mature *Quercus robur* trees"
S. Valladares, M.T. Martínez, C. Sánchez, M.C San-José, N. Vidal and A.M. Vieitez

12.35-13.30 General discussion and final considerations

13.30	Lunch
16.00	Sightseeing visit of Gijón

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Caboni, Emilia	Tissue culture as a tool for ex situ conservation of wild pear	e.caboni@propag.org	38
Carpentier, Sebastien	The effect of osmotic pretreatments on the post-thaw regrowth and the proteome of Banana meristems	sebastien.carpentier@biw.kuleuven.be	16
Cellarova, Eva	Assessment of developmental competence, genetic stability and biochemical potential of cryopreserved *Hypericum perforatum* L. shoot tips	eva.cellarova@upjs.sk	50
De Carlo, Anna	Molecular validation of a micropropagation-cryopreservation procedure for red chicory (*Cichorium intybus* L.) selected lines	decarlo@ivalsa.cnr.it	56
González-Benito, Elena	Approaches to detect somaclonal variation and epigenetic changes in cryopreserved chrysanthemum apices	me.gonzalezbenito@upm.es	54
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Häggman, Hely	Cryopreservation of Scots pine	hely.haggman@oulu.fi	32
Marum, Liliana	Genetic fidelity of *Pinus pinaster* somatic embryogenic cultures following cryopreservation	marum@itqb.unl.pt	48
Nukari, Anna	Cryopreservation of strawberry	anna.nukari@mtt.fi	36
Oliveira, Margarida	Effects of cryoadditives and suspension density in the cryopreservation of *Pinus pinaster* embryogenic cultures	mmolive@itqb.unl.pt	18
Panis, Bart	The effect of a sucrose induced acclimation on sugar, polyamine, sterol and fatty acid composition and its role in cryopreservation of banana meristems	bart.panis@biw.kuleuven.be	12

Peredo, Elena	Assessment of genetic stability of cryopreserved and cold storaged hop samples	elperedo.uo@uniovi.es	52
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Rybczyński, Jan	Fundamental aspects of cell suspension cryopreservation of *Gentiana* spp.	jjryb@ob.neostrada.pl	30
Schumacher, Heinz	Dedifferentiated plant cell lines for the evaluation of physiological parameters of cryotolerance	mas@dsmz.de	20
Skyba, Matus	The effect of phase transition on recovery rate of *Hypericum perforatum* L. shoot tips after cryopreservation	skybos@extel.sk	24
Vágner, Martin	Cryopreservation of embryogenic cultures of Norway spruce	vagner@ueb.cas.cz	34
Valladares, Silvia	Genetic fidelity of cryopreserved embryogenic cultures from mature *Quercus robur* trees	silviav@iiag.cesga.es	58
Vázquez, Ana	Cryopreservation and genetic stability	anavaz@bio.ucm.es	42

Fundamental aspects of cryopreservation and cryoprotection

Chairperson: Pawel Pukacki

The effect of a sucrose induced acclimation on sugar, polyamine, sterol and fatty acid composition and its role in cryopreservation of banana meristems

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Cryopreservation is a valuable alternative to the in vitro collection for conserving biodiversity in banana. For each species and tissue type, a cryopreservation protocol needs to be developed / adapted to the natural freezing resistance of the species, explant size and type, and water content. Until now, all cryopreservation protocols are developed through 'trial and error'. The main aim of the EU FP5 CRYMCEPT project was (i) to understand the physico-biochemical changes associated with tolerance towards cryopreservation (ii) based on this knowledge, to develop new / adapted cryopreservation protocols.

We have previously shown that a two-week acclimation phase on 0.4 M sucrose medium is essential for the survival of cryopreserved meristems of banana (1). Measured differences with respect to intracellular sugar contents, however, could not explain the differences in survival after cryopreservation observed between different cultivars. Therefore we also investigated sucrose-induced changes in proteins, membrane components and polyamines (2). Membrane component analysis revealed that sucrose pretreatment induced in banana meristems an increase in stigmasterol / sitosterol ratio, total fatty acid content of neutral lipid fraction and of the glyco- and sphingolipid fraction. Sucrose pretreatment also induced changes in individual fatty acid content for both phospholipids and free fatty acids. Consequently, the double bound index (DBI), an indication of the unsaturation degree of the fatty acids, also changed due to the sucrose pretreatment.

By linking the membrane component and polyamine data to the cryopreservation, we were able to conclude that although the sucrose pretreatment is required for a high survival after cryopreservation (effects on water contents of the cells and its possible protecting effects on proteins and membranes), it also induces stress in the banana meristems. Most important parameters which could be closely associated to the post thaw shoot regeneration rates are a minimal change of stigmasterol / sitosterol ratio, a minimal change of double bound index in phospholipids and free fatty acid, an increase in linoleic acid (C18:2) content induced by sucrose pretreatment. For polyamines and aromatic amines, increase in polyamines (particularly free putrescine) and a decrease in aromatic amine (particularly dopamine) were linked to the cryopreservation ability.

These results suggest that the application of specific sterols, fatty acids (preferably unsaturated ones) or polyamines into the sucrose preculture medium might enhance cryopreservation efficiency. Therefore, different sterols, fatty acids combined with

glycerol, polyamine or aromatic amine were applied to the sucrose preculture medium. Indeed, following the simple freezing method, shoot regeneration rates increased when the proliferating meristems were precultured on the sucrose medium supplemented with sterols, putrescine or fatty acids combined with glycerol. This increase was only observed for the cultivars belonging to ABB and AAA genomic group, while for the cultivars belonging to AAA highland group, no large effect was observed. In order further understand possible mechanism of the cryopreservation process, the sterol-polyamine-sucrose pretreated meristems were reanalysed. This proved that cryoprotection process is very complex: besides changes of sterols, fatty acids, and polyamines, there are still other parameters or limiting factors involved in the cryopreservation. Moreover it was shown that there is a strong interaction between sterols and polyamines/aromatic amines in relation to cryopreservation.

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Effect of the Osmotic Agent Chosen for the Preculture Step during the *Pelargonium* apices cryopreservation process

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Apex cryopreservation studies were undertaken to guarantee the safe, long-term conservation of a *Pelargonium* collection. Plant regrowth was obtained with a modified encapsulation-dehydration process (1, 2). The first step consists of a preculture in a sucrose rich medium in order to confer dehydration tolerance to the apex. Choosing *P.* x *peltatum* 'Balcon Lilas' as a model, the main objective of this study was to determine if sucrose is specific in conferring dehydration tolerance to apices. The following hypothesis was tested: if this step consists only in a mild osmotic desiccation due to the high sucrose concentration of the preculture medium, preparing apices to the later dehydration on silica gel, other osmotic agents, at the same osmolarity, could be as efficient as sucrose. In the preculture medium sucrose was replaced by glucose, glucose with 3 % sucrose, or sorbitol at similar osmolarities (1200 - 1250 mosm). The concentrations determined were: sucrose: 256.5 g L^{-1} (0.75 M), sorbitol: 174 g L^{-1} (0.95 M), glucose: 168 g L^{-1} (0.93 M) and 148 g L^{-1} (0.82 M) (in a mixture with 3 % sucrose (0.09 M). At the end of the 40 h preculture step with the media at the same osmolarity, bead water content was approximately 1.5 times higher with glucose and sorbitol than with sucrose. The water content of 0.25 g/g DW, optimal for tolerance to dehydration, was reached after 3 h of dehydration in case of sucrose preculture. Four-hour dehydration was necessary to obtain the optimal water content with the other osmotica. None of the osmotic agents tested produced a level of tolerance to desiccation as high as that of sucrose. A high concentration of glucose was toxic and did not induce a dehydration tolerance. We compared these results with previous reports: only osmoticum molarity was indicated and sucrose was not the only one to provide dehydration tolerance (3, 4, 5)

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The effect of osmotic pretreatments on the post-thaw regrowth and the proteome of Banana meristems

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The Laboratory of Tropical Crop Improvement (K.U.Leuven, Belgium) hosts, under the authority of Bioversity International, the global *in vitro* collection of banana varieties (*Musa* and *Ensete* spp.). Bananas and plantains, with an annual production of about 100 million tons, are one of the oldest domesticated crops and the fourth most important food commodity in the developing countries of the (sub)tropics. Through human selection and prevailing agro-ecological conditions, numerous varieties arose with different degrees of tolerance towards (a)biotic stresses. All edible varieties do not produce seeds and propagate vegetatively. In banana, shoot meristems are the most convenient structures to be used for cryopreservation (1). For successful storage at -196 °C, the meristems need to survive first a severe dehydration process prior to freezing. In general, tolerance towards this dehydration is achieved by an osmotic stress acclimation during a pretreatment phase (2). Yet, more than half of the accessions stored at the Bioversity International collection consists of varieties that have a nonexistent or low cryopreservation survival rate (3). In view of the importance of conserving the world's plant biodiversity, it is thus crucial to get a deeper insight into the mechanisms behind acclimation and tolerance towards desiccation and cryopreservation.

The study of the physiology behind acclimation and the understanding of gene function and gene expression profiling can be executed using different approaches. Its success depends to a great extent on the genomic progress reached for the organism under investigation. A proteomic approach is currently for banana the most suitable in view of its poor genomic characterisation. A large scale proteomic study contributed to a preliminary understanding of the mechanisms behind acclimation mediated by the sucrose pre-culture (4-5). For the first time, a large scale study of a meristematic proteome was reported. The results suggest that the maintenance of an osmoprotective intracellular sucrose concentration, the enhanced expression of particular genes of the energy-conserving glycolysis and the conservation of the cell wall integrity are essential to maintain homeostasis, to acclimate and to survive. By comparing a dehydration tolerant variety (ABB) with a dehydration sensitive variety (AAA), it was possible to distinguish several genotype specific proteins (isoforms) and to associate the dehydration tolerant variety with proteins involved in energy metabolism (*e.g.* phosphoglycerate kinase, phosphoglucomutase, UGPase) and proteins that are associated with stress adaptation (*e.g.* OSR40-like protein, ASR).

To get a deeper insight into the different aspects of the sucrose mediated acclimation process (sucrose as osmoticum, sucrose as compatible solute, sucrose as signalling molecule and sucrose as metabolite) a dynamic experiment was set up using control conditions and sucrose, sorbitol and mannitol as osmotic active molecules. Protein samples were taken at 0, 1, 2, 4, 8, 14 and 22 days. As expected, control conditions have

no beneficial effect towards cryopreservation since there is no acclimation. The mannitol and sorbitol treatment also proved to have no significant beneficial effect towards cryopreservation probably because they only cause an osmotic shock. In contrast, the sucrose treatment lowers the mortality rate considerably. Although the sucrose pre-treatment and the concomitant high intracellular sucrose concentration are required for a low mortality rate after cryopreservation, it also proved to induce negative stress effects. The right timing of the pretreatment is therefore crucial. Preliminary results clearly show that sucrose pre-culture is correlated to high relative abundance of phosphoglycerate kinase, UGPase and OSR40 and a low relative abundance of SuSy, lectin and chitinase and abscisic stress ripening protein-like protein (ASR). We hypothesise that a low mortality rate after cyopreservation can be achieved by a low relative abundance of SuSy and high relative abundance of phosphoglycerate kinase, OSR 40, UGPase, ASR and storage proteins such as lectin and chitinase. A first screening of several other cultivars belonging to different genetic backgrounds link several proteins and specific isoforms to the tolerant B genome, which opens perspectives to apply proteomics for taxonomical characterization and classification. Upon further discovery and validation, a tool will become available to screen for better pre-culture treatments (time and treatment) and to maximize survival rate after cryopreservation.

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Effects of cryoadditives and suspension density in the cryopreservation of *Pinus pinaster* embryogenic cultures

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Introduction

The cryopreservation of embryogenic cultures has become an important tool in reforestation and breeding programs, especially when conifers are concerned. With few exceptions, so far the initiation of somatic embryogenesis from conifers is only possible from juvenile material with unknown genetic properties. To ensure the juvenility of selected genotypes until field testing, the cryopreservation is a crucial step. Maritime pine (*Pinus pinaster* Ait.) is the main forest species in Portugal and an *in vitro* system consisting of somatic embryogenesis has already been established for this species. A successful method to cryopreserve embryogenic lines creates new possibilities for clonal forestry. Pre-treatments with cryoadditives can help to reduce damage caused by both solution and intracellular ice injury. Others factors for a successful cryopreservation should be considered, such as the cell suspension density used before application of pre-treatments and thawing/pos-thawing procedures.

The main objective of this work was to optimize a protocol for cryopreservation of somatic embryogenic cultures of *P. pinaster*.

Materials and Methods

Embryogenic cell lines of *Pinus pinaster* were established from immature zygotic embryos (1). The standard cryopreservation and the regrowth methodology used were based on methods described by Nørgaard *et al*. (2), with some modifications (Devillard, personal communication).

Varying densities of the embryogenic suspension were prepared before application of the pre-treatment, respectively 50, 150 (control) and 250 mg ml^{-1}.

In addition to sorbitol used in the standard cryopreservation procedure, three other cryoprotective carbohydrates, at the same final concentration of 0.4 M, were tested in the 1st and 2nd days of pre-treatments: sucrose, maltose and glucose. On the 3rd day of the pretreatment, a solution of 10 % (w/v) polyethylene glycol (PEG 4000), 10 % (w/v) sucrose and 10 % (v/v) DMSO (PSD) was used as cryoprotectant instead of DMSO alone.

During the regrowth procedure the effect of the cryoprotective carbohydrate was tested in decreasing concentrations. The thawed suspension was either directly sub-cultured to proliferation medium, or to medium containing decreasing concentrations of sorbitol (0.4 M, 0.2 M and 0.0 M sorbitol). The influence of post-thawing of the washing suspension was also evaluated. After thawing, the suspension was sedimented and the replacement of 1 ml of cryoprotectant solution by the same amount of liquid medium without cryoprotecting agents was tested. Plating of the suspension culture for regrowth

was also tested using nylon supports with 1000, 300 and 50µm pore size in comparison with the standard filter paper discs (55 mm Watman2).

Results and Conclusions

The results obtained in the present study show that the pre-treatments tested before the immersion in liquid nitrogen had a strong influence on the culture survival, which was not observed when testing post-thawing treatments.

The regrowth rates (assessed as variations in fresh weight) after thawing were higher when the initial density of the suspension culture was 250 mg ml^{-1} (as compared to 50 and 150 mg ml^{-1}). A statistically significant difference ($p \leq 0.05$) was found only between the densities of 150 mg ml^{-1} and 250 mg ml^{-1}. The regrowth rate of the embryogenic lines pretreated with maltose was significantly higher than that achieved using the other carbohydrates (as assessed 8 weeks post-thawing). The use of DMSO in a mixture of PEG and sucrose (PSD solution) instead of DMSO alone (at the same final concentration) had a clear beneficial effect in culture survival post-cryopreservation. The mean regrowth rate for samples treated with PSD solution was significantly higher than that observed in control samples ($p \leq 0.001$) at the end of analyses period (8 weeks). However, an exponential growth was already observed after the 2nd subculture period (2x2 weeks). There were no significant differences between regrowth rates of somatic cultures subjected or not to post-thaw washing. The filter paper discs used as a support for regrowth were better for growth and survival than the nylon supports. The effect of cryoprotective carbohydrate in decreasing concentrations had no influence on the regrowth when compared to the control.

The establishment of an improved protocol for cryopreservation of *P. pinaster* embryogenic cell lines was based on the optimization of the density of embryogenic suspensions and on the type of treatment applied to samples before freezing. The selection of the cryoprotective carbohydrate and the addition of a solution combining DMSO with PEG and sucrose (PSD solution) instead of using DMSO alone, allowed a significant increase in the efficiency of cryopreservation, as evaluated 8 weeks post-thawing, from survival and regrowth rates of the cultures. Also a suspension density of 250 mg ml^{-1} proved appropriate for both survival and regrowth rate.

The results achieved with the optimized method represented a significant improvement when compared to the previously used cryopreservation procedure (66-78 % against the present 96 % efficency). We consider this method as an efficient procedure for the successful storage of a large number of genotypes of *Pinus pinaster*.

References

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Dedifferentiated plant cell lines for the evaluation of physiological parameters of cryotolerance

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At DSMZ a collection of more than 700 undifferentiated cell lines is maintained from about 600 different plant species. These cell lines are used for basic and applied research, like elucidation of biosynthetic pathways, defining the influence of metabolic compounds and xenobiotics on plant metabolism or isolation and investigation of known and novel secondary plant compounds. Cryopreservation is a need for stable long term storage of such materials.

Even after application of different cryopreservation approaches controlled rate cooling turned out to be the method best suited for this kind of material. To facilitate the adaptation of the basic method of Withers and King to the needs of specific cell lines a Mini Test system has been worked out. The Mini Test system can be used to determine cell activity after each step of the controlled rate freezing method. It allows to quickly find out which combination of variables concerning all steps of controlled rate freezing favours cell survival best.

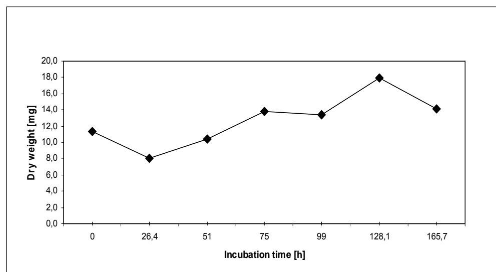

Fig. 1 Cell growth achieved in 24 multiwell plates

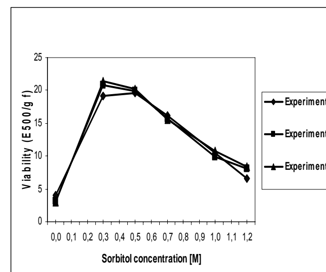

Fig.2 Reprodcucibility obtained in 24 multiwell plates

The system has been also used to investigate physiological parameters associated with osmotic stress like the formation of prolin, glutathion and the induction of proteins. To use cell lines as model systems for cryopreservation research we made the tests with three different potato cultivars performing differently in droplet cryopreservation.

Intact plants of these cultivars have been used in the CRYMCEPT project for the investigation of changes of the protein pattern. The proteins finally found in both cases were stress proteins. Whether they were induced because of the stress or whether there induction really contributes to an increase in survival after cryopreservation remains unclear.

At DSMZ we started to investigate this question by means of molecular biology. New transformation vectors have been constructed which in the future shall allow the quantification of the over-expression of a certain `target` genes in a physiologically active form by the quantification of a reporter gene. The vectors are based on the use of viral IRES elements for the co-expression of two genes.

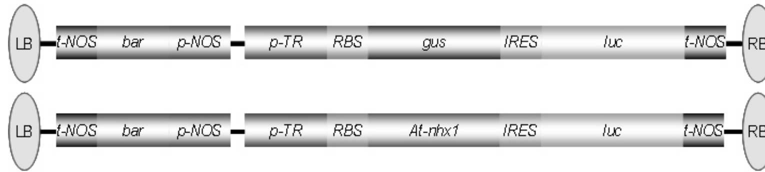

Fig.3. Vectors for over-expression of `target` proteins

In addition to the investigation of physiological mechanisms we would like to investigate genetic and epigenetic stability of dedifferentiated cell line. Possible methods are HPLC metabolite fingerprints 2-D protein patterns, DNA methylation status, RAPD technology and flow cytometry. Whether cell lines can be model systems in this case also remains to be worked out.

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Cryoprotective and antifreeze activity of apoplastic proteins in winter hardiness of conifers

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Antifreeze proteins (AFPs), also known as thermal hysteresis proteins (THPs), inhibit ice crystal growth by a non-colligative mechanism, lowering the freezing point of water below the melting point, thereby producing a so-called thermal hysteresis (2) (Table 1). AFPs are well known in crop plants, but only a few species recently have been identified in woody plants (3). Apoplastic extracts were obtained by vacuum infiltrating of needles with 5 mM ascorbic acid, followed by centrifugation to recover the infiltrate. Proteins extracted from, *Picea abies*, *P. glauca, P. pungens, and Abies alba* needles were separated on 12.5 % (w/v) of SDS-polyacrylamide gels. As shown by SDS-PAGE, antifreeze proteins are accumulated in apoplast coniferous species and contain from 3 to 7 polypeptides. These proteins have a molecular mass from 10 to 80 kDa. The 27 kDa polypeptydes from *P. abies* and *P. pungens* trees are homologous to PR proteins and also provide protection against pathogens. Cryoprotective activity of two fraction: AP-1 and AP-2 of AFP was determined using the freeze/thaw inactivation assay of lactate dehydrogenase, LDH (EC 1.1.1.27, from rabbit muscle, Sigma) according to Crowe (1) The samples with LDH were frozen in –196 ºC for 30 s and then thawed. The freeze-thaw process was conducted four times. Antifreeze activity was determined as the ice nucleation activity, using a droplets Vali technique (4). When we examined the possible role of conifers proteins in cryoprotection, we found that lactate dehydrogenase (LDH) activity was higher after freeze-thaw cycle in the presence of AFPs compared with BSA or buffer. The presence of 250 µg ml^{-1} of AFP preserved 90 % and 100 % of LDH after freeze/thaw cycles (Fig.1).

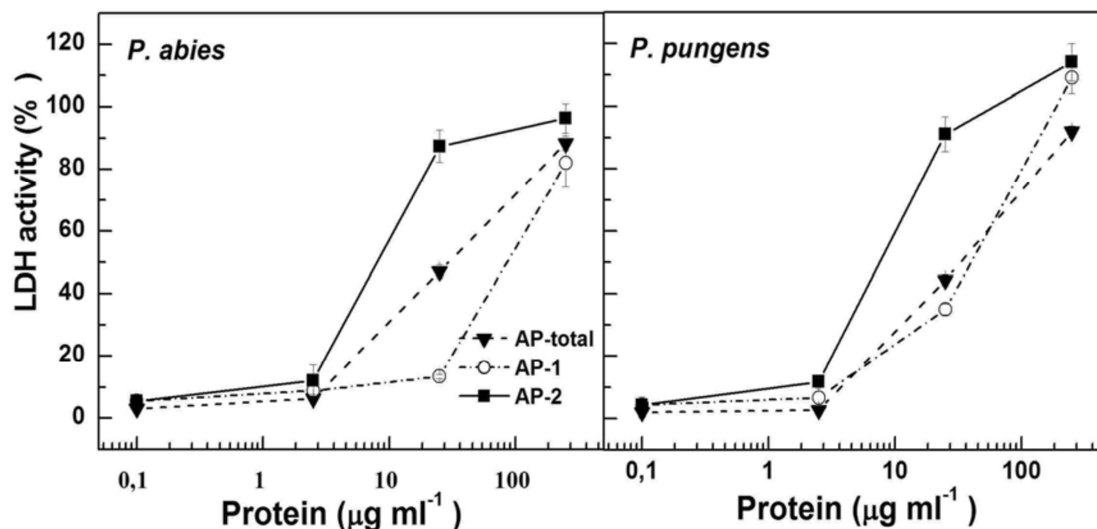

Figure 1. Cryoprotection of LDH. The curve show the percentage LDH activity remaining after freeze/thaw (from 20 ºC to –196 ºC) cycle in the presence of difference fraction apoplastic proteins, AP-total, AP-1 (20-80 kDa) and AP-2 (<20 kDa) of *P. abies* and *P. pungens*. Mean values ± SD (n=6).

These results shown that conifers produces cryoprotective proteins that are secreted into apoplast. The accumulation of proteins in extracellular spaces in winter as a result of seasonal cold acclimation, indicates that these proteins may play a significant role also freezing tolerance of needle cells of coniferous species. The physiological significancence of in-vitro cryoprotective activity of AFPs is not clear yet.

Table 1. Thermal hysteresis activity (TH =Tf – Tm) apoplastic proteins from *Picea abies* and *P. pungens* needles and freezing tolerance (LT_{50}). TL_{50}, (visual method, temperature causing a 50 % of injury) of one-year-old Norway spruce needles. Mean ± SD, n = 6

Species	Tm	Tf	TH	LT_{50} (°C)	
	Temperature (°C)			Winter	Summer
P. abies	–0.39 ± 0.16	–2.58 ± 0.98	2.19 ± 0.83	–35	–7
P. pungens	–0.30 ± 0.06	–2.32 ± 0.54	2.02 ± 0.40	–45	–7

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The effect of phase transition on recovery rate of *Hypericum perforatum* L. shoot tips after cryopreservation

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The aim of this study was to investigate biophysical processes with special attention to phase transition of cryoprotective agents during freezing and their possible impact on recovery rate of *Hypericum perforatum* L. shoot tips. Freezing studies were conducted using PLANER series 300-500 freezer and thermal sensor KTY 81-120 measuring temperature (Figure 2) directly in samples cooled under controlled rates between 0.4 and 2.0 °C min^{-1}. In this study, we have discovered a heating phenomenon occuring during phase transition that is cooling rate dependent (Fig. 1). The higher the cooling rates are, the more heat releases, which cause the system to warm up. Recovery rate (Fig. 3) is also cooling rate dependent. Lower cooling rates (under 1 °C min^{-1}.) resulted in high recovery rate (36 % maximum recovery with cooling rate 0.4 °C min^{-1}), whereas high cooling rates between 1 and 2 °C min^{-1} resulted in recovery rate between 0 and 3.33 %. We have also created a freezing model simulating processes occurring in cryoprotective solutions during the cooling, that has been proven by a computer simulation and comparison with experimental data. We assume that released heat affects recovery rate, although we do not know the exact mechanism of its influence on this recovery (Table 1).

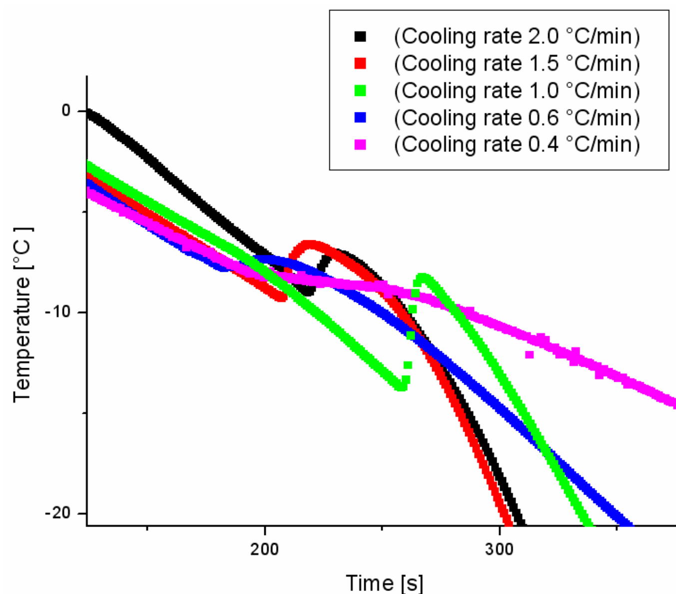

Figure 1. Phase transitions using different cooling rates

Table 1 Recovery rate of cryopreserved *Hypericum perforatum* L. shoot tips.

Cooling rate	Precultivation agent	RcSt /CpSt	Recovery rate [%]
2.0 °C min^{-1}	0.076 μM Abscisic acid	0/120	0.000
	0.3 M Mannitol	0/110	0.000
1.5 °C min^{-1}	0.076 μM Abscisic acid	7/100	7.000
	0.3 M Mannitol	3/110	2.727
1.0 °C min^{-1}	0.076 μM Abscisic acid	12/210	5.714
	0.3 M Mannitol	11/190	5.789
0.8 °C min^{-1}	0.076 μM Abscisic acid	10/90	11.111
	0.3 M Mannitol	1/120	0.833
0.6 °C min^{-1}	0.076 μM Abscisic acid	12/110	10.909
	0.3 M Mannitol	9/100	9.000
0.4 °C min^{-1}	0.076 μM Abscisic acid	3/100	3.000
	0.3 M Mannitol	22/110	20.000

CpSt- Number of cryopreserved shoot tips
RcSt- Number of recovered shoot tips

Figure 2: Equipment used to measure temperature directly in cooled samples.

Figure 3: Differentiation of recovered *H. perforatum* L. shoots 8 weeks after cryostorage.

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The use of differential scanning calorimetric analysis of plant shoot tips in cryopreservation

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Introduction

Differential scanning calorimetry (DSC) belongs to thermal methods that can be used for measurement and determination of phase and glass transitions for cryopreservation. In principle, the DSC measures the temperatures and heat flows associated with transitions in plant material as a function of time and temperature. It gives information about endothermic or exothermic changes or changes in heat capacity. The obtained data can be used for determination of glass transition, temperature of ice nucleation, melting, boiling, crystallization time and kinetic reaction —the most important characteristics useful for cryopreservation—. As a critical point of plant survival at ultra-low temperatures is considered danger of nucleation and subsequent intracellular ice crystallization leading to frost damage during cooling and rewarming of the samples (1). The results from the field of molecular biophysics suggest that the main factor influencing the success of cryopreservation protocol is keeping of the glassy state in plant samples and avoiding ice nucleation (2,3). That is why majority of new progressive techniques uses and develops glassy state in biological material dedicated for cryopreservation.

Materials and methods

DSC method is based on regulated temperature decrease/increase of the sample and reference and measurement of temperature and heat flow corresponding to the sample. There are two different types of the differential scanning calorimeters. The power compensation DSC type measures directly heat release/uptake from the sample and the heat flow type measures differences of temperature between reference and sample and recalculates the differential heat flux. The most common cooling/heating rate of the sample is 10 °C min^{-1}.

In our experiments we used shoot tips of *in vitro* cultures of apple and pear, and dormant apple buds cv. ´Spartan´. Samples with different water content were obtained by air dehydration of in alginate encapsulated shoot tips in flow box or by dehydration at –4 °C of *in vitro* cultures or dormant apple buds, respectively. For DSC measurement dissected shoot tips and dormant apple buds were placed in aluminium sample pans and measured by Differential scanning calorimeter TA2920. Samples were cooled down to –120 °C (rate 10 °C min^{-1}). The data were collected during heating to 20 °C (rate 10 °C min^{-1}). The purge gas was either nitrogen or helium.

Results

The evaluation of measured plant sample is made preferably on the heating curve because the sample may supercool during cooling. After ice nucleation of the supercooled sample high amount of heat releases which influences cooling speed of the

sample —it becomes slower and is not linear. In dehydrated shoot tips, the ice crystals were detected till the water content decreased to 0.24 g water/ g dry matter (gW/gDM). Glass transitions were detected at samples dehydrated more than 0.7 gW/gDM. The glass transition of shoot tips ranged from –88 °C (0.72 gW/gDM) (Fig.1) to –45 °C (0.24 gW/gDM). The area of endotherms in dormant buds decreased with decreasing water content of measured apple buds. No exotherms were detected in the frozen dormant buds below the water content 0.2 gW/gDM.

Conclusion

Differential scanning calorimetry allows determining the basic thermal characteristics of plant samples used for cryopreservation, *e.g.* ice crystallization, the amount of ice crystals and the glass transition. On basis of measurement of thermodynamic characteristics of plant material the most appropriate cryopreservation protocols can be proposed.

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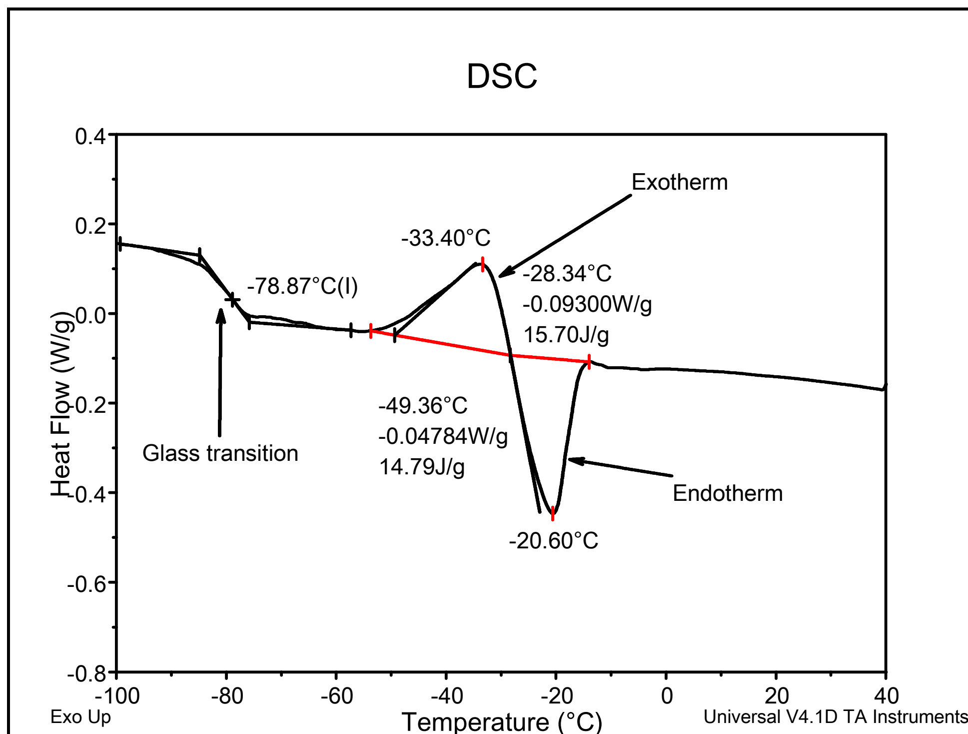

Figure 1. Thermal characteristic of apple *in vitro* culture shoot tips encapsulated in alginate beads were measured by differential scanning calorimetry (DSC). The characteristic peaks corresponding to release (exotherm) or uptake (endotherm) of heat were measured during crystallization or melting. Glass transition had typical S–shape of the curve. The heat capacity of the sample changes in the process of the glass transition.

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Contaminated liquid nitrogen storage vessels as vectors for plant pathogens

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Plant propagules are cryopreserved for long-term viability and genetic stability, using sample containers stored in liquid nitrogen or its associated vapour phase. Potential pathogens may enter storage vessels from leaking sample containers, contaminated container surfaces and the storage facility atmosphere, accumulating in a viable condition over time (1). In animal cell systems concerns over sample contamination from the storage vessel have been widely raised, with recent reports indicating the concensus that care over sealing and opening containers can contain the risks to the samples themselves (2, 3). However, the associated issue of contaminated storage vessels as pathogen vectors in their own right has not been satisfactorily dealt with, especially where national and international transport of material is concerned. Plant cryobiologists have to address the same issues and, to date, the debate is well advanced. This study investigates the potential for the movement of plant pathogens in contaminated storage containers, which may pose a threat, albeit of unknown dimension, to agriculture and natural ecosystems

Bulk liquid nitrogen was dosed either with $2x10^6$ sclerotia/ L^{-1} of *Sclerotinia* sp., a plant pathogen not present as an airborne contaminant in the investigating laboratory, and used to cool both dry shippers and a programmable LN freezer. The atmosphere of the shippers and freezer were monitored subsequently for the pathogen. After a single cooling run the chamber of the programmable freezer was heavily contaminated with the fungal pathogen, with viable spores recovered from the free atmosphere, walls of the freezing chamber and sample vessel surfaces. Glass slides coated with adhesive film were left in the shipper for 6 hours, removed and transferred to PDA plates. Subsequent growth of *Sclerotinia* confirmed that the vapour phase of the dry shippers was contaminated.

Contamination was also monitored using a sucrose hemiheptahydrate assay system (4) where 25 L of liquid nitrogen was contaminated with 0.01 g L^{-1} of crystalline sucrose hemiheptahydrate and used to cool a dry shipper to working temperature. After 24 h Petri plates of 70 % aqueous sucrose were placed in the dry shipper and the lids removed for 1 h before transfer to a –20 ºC deep freeze. After 7 days numerous crystals of sucrose hemiheptahydrate formed on the assay plates further demonstrating contamination in the dry shipper

The conclusion is that viable pathogens can be transported in LN storage containers, in tandem with sealed samples that may be pathogen-free. Contaminants are present in the vapour phase and on surfaces of storage containers and are likely to be persistent. Consideration has to be given to the use of LN-free cooling systems and the explicit regulation of procedures to minimize surface and aerial contaminants. Liquid nitrogen storage vessels and dry shippers may also be a risk to operators.

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Fundamental aspects of cell suspension cryopreservation of *Gentiana* spp.

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Embryogenic suspension cultures consist in unlimited morphogenetically active cells which could play important role in studies of numerous chemical and physical stresses connected with low temperature treatment. There is a number of attempts to reduce the stress effect on cell survival after thawing, among them sugar concentration increasing in extant preculture. The influence of increased concentrations of sucrose, 0.4 M sorbitol, DMSO and vitrification solution (PVS2) on the ultrastructure of non-frozen and frozen suspensions of *Gentiana tibetica* King ex Hook. F. tissue cells was investigated (1). Embryogenic aggregates were composed of three groups of cells of different size with various types of plastids. The ultrastructural changes resulting from increasing the sucrose concentration in the medium from 3 % to 6 % for 4-weeks and from treatment with 0.4 M sorbitol for 48 h were similar. Observations showed replacement of large vacuoles by numerous small ones, condensation of cytoplasm, accumulation of starch, and fragmentation of endoplasmic reticulum. Treatment with PVS2 led to degradation of starch, coalescence of amyloplasts and to shrinking of nucleoli from third group of cells originating from 6 % sucrose medium. The mitochondria initially presented various shapes, and after PVS2 treatment showed only spherical shapes with sparse cristae. After programmed freezing of tissue protected by sorbitol and DMSO, lethal damage was observed: membrane and nuclei degradation, and cell destruction. Reversible changes after freezing were observed in tissue pretreated with vitrification solution: dilation of cell membranes, mitochondria with electron-lucent vessels, aggregation of numerous vesicles, and degradation of starch in amyloplasts. In cells cooled by a vitrification method, cell organelles appeared normal as early as 5 h after thawing, and anomalies were not observed after 48 h of post-thawing culture.

We examined three techniques of freezing for 10-year-old embryogenic cell suspensions of *G. tibetica*: (I) controlled-rate cooling with various cryoprotectants (0.1-0.5 M DMSO, 0.5-1.0 M sucrose, 0.5-1.0 M glycerol, 0.25-1 M proline) or preculture with 0.4 M sorbitol and cryoprotectants (0.065-0.1 M DMSO, 0.2-0.8 M proline), (II) vitrification (PVS2) and (III) encapsulation (2). After controlled-rate cooling the majority of cells were lethally damaged, with only 3 % viability (TTC test) observed. Vitrification and encapsulation approaches were more effective, assuring high levels of post-thaw viability ca. 85 % and 70 %, respectively. The encapsulation procedure gave faster recovery of the culture suspension than did vitrification, and ensured culture homogeneity and embryogenic competence. With the application of this method the efficacy of cryopreservation for cell suspensions of *Gentiana tibetica* (11-year-old) and *G. cruciata* (2-year-old) was described in relation to (a) the viability of aggregates cells (b) the recovery of suspension cultures (c) the preserve of embryogenic competence and (d) the monitoring of genetic stability in DNA content of the cell suspensions and regenerants. The tissue of *G. tibetica* originated from medium with 3 % (w/v) or 6 % (w/v) sucrose survived cryopreservation with ca. 50 % or 70 % viability (TTC test) and

G. cruciata (from medium with 6 % sucrose) – 85 % viability (Fig. 1). Relative water content amounted about 28 % ensured mentioned above viabilities. Aggregate size and the time of tissue sampling after last subculture did not influence on the *G. tibetica* viability. Higher level of survival, additional 15 %, was achieved when the smaller than 500 µm of *G. cruciata* aggregates in the five-day of culture were taken. Regrowth of cryopreserved cell aggregates was studied by culturing them on: agar, liquid and agar/liquid culture. The combined type of culture significantly effected on the biomass increase and stimulated recovery of suspension culture (Fig. 2). Both of the recovered suspensions produced somatic embryos and had the same appearance as the unfrozen controls. Somatic embryos were developed, germinated and produced normal green plants. Flow cytometry analyses showed that the cryopreservation not induced alteration in nuclear DNA content of proembryogenic cells and regenerated from them plantlets. PEM and regenerants of both species had ca. 9 pg DNA.

In conclusion: cryopreservation by encapsulation is reliable procedures for conserving of the viability, recovery and embryogenic competency of gentian cell suspensions and can be routinely applied for the cryostorage.

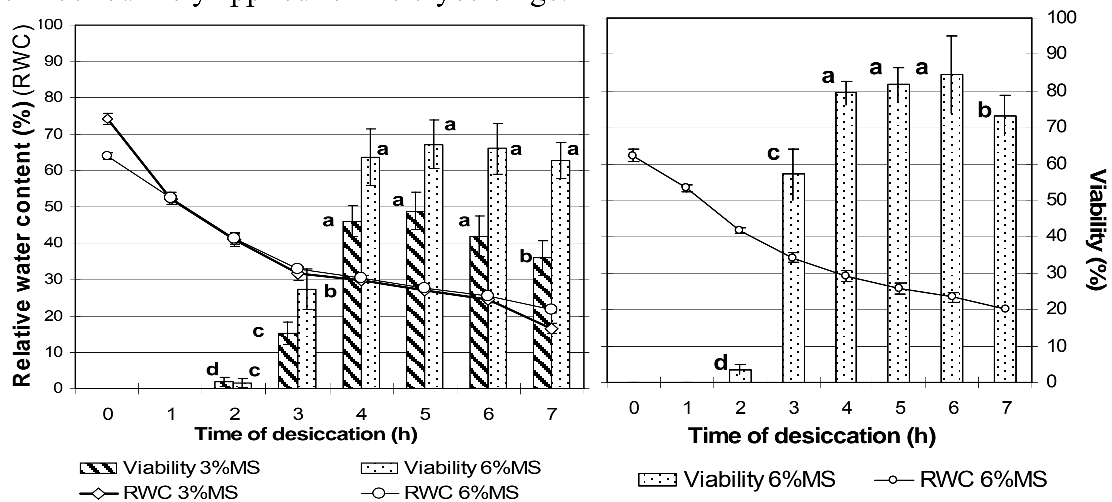

Figure 1. The correlation of the relative water content changes of capsulated cell aggregates of *G. tibetica* and *G. cruciata* and their viability. For *G. tibetica* suspension culture additionally this correlation depending on concentration sucrose (3 % or 6 %) in the liquid medium was assessed. Bars represent SD. Values followed by the same letter within different derive of *G. tibetica* PEMs indicate not significant differences at p < 0.05.

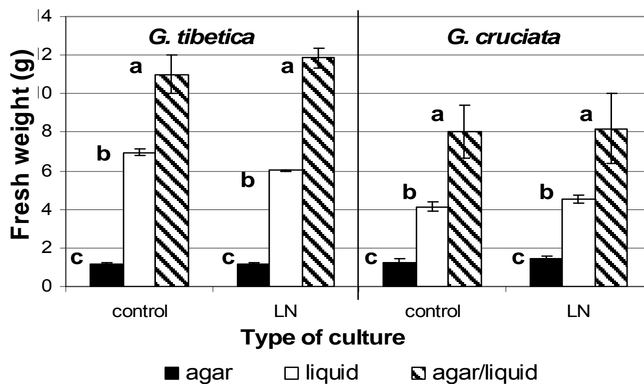

Figure 2. The effect of culture type: agar, liquid and agar/liquid on the biomass increase after 4 weeks post-thawing culture. Control – encapsulated and desiccated cells; LN – thawing cells (p < 0.05).

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Cryopreservation of Scots pine

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In pine (*Pinus)* species the recent progress in somatic embryogenesis, the production of genetically modified plants and the efforts towards plantation forestry have emphasized the need for germplasm conservation with functional cryopreservation protocols (1, 2, 3, 4). Scots pine (*Pinus sylvestris* L.) is one of the keystone species of forest ecosystems on large areas in the Eurasian boreal forest zone. In Finland, almost half of the growing stock consists of Scots pine and its proportion is slowly increasing. However, it has been proposed that the species will suffer due to the climate change which underlines the need for germplasm conservation.

As in some other conifers, also in Scots pine reliable long-term maintenance of embryogenic cultures requires that the cultures will be stored by cryopreservation. We have developed a cryopreservation protocol applicable for Scots pine using classical method that involves the pre-treatment of the material and a slow cooling down to a defined prefreezing temperature, followed by a rapid immersion in liquid nitrogen (Häggman et al. 1998). This protocol maintained the morphological appearance of the cultures and RAPD assays suggested that the cryostorage treatment used preserved their genetic fidelity. In the more recent work, we have paid more attention on growth phase appropriate on recovery and regrowth of the cryopreserved material. We found that the best cryotolerance and regrowth was achieved when the sampling was done 2 to 4 weeks after subculture. Cold-hardening and preculture phases were not necessary and the time needed for the whole process could be shortened remarkably. The cryoprotective substances, sucrose and sorbitol, worked as well when regrowth ability of was investigated but sucrose seemed to result more embryos than sorbitol.

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Cryopreservation of embryogenic cultures of Norway spruce

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The use of somatic embryogenesis for cloning of superior trees generates a need for long term storage of embryogenic cultures. Cultures should be kept genetically unchanged for a long time until the clones are evaluated in field test. Cryopreservation seems to be the best method for that, the main benefits are summarized bellow: 1) low costs - resulting from a cut in labor and savings in laboratory consumables, 2) the arrest of metabolic functions in ultralow temperature which markedly decreases the possibility of genetic changes of the clone. Cryopreservation is also a unique method of customary preservation of the large numbers of cell lines in the laboratory: the great variability of cell lines is a common feature of embryogenic cultures and it is a good reason for using a large number of cell lines in research.

The first successful cryopreservation of embryogenic cultures of Norway spruce is documented by Gupta *et al.* (2). Enhanced embryogenic capacity as a consequence of cryopreservation was reported by Bercetche *et al.* (1). Different cell lines show different cryotolerance; however, no conclusions were reached about the relationship of cryotolerance and the distinct morphological or biochemical characteristic of the cell line (3).

The embryogenic cultures of Norway spruce (different cell lines were purchased as gifts, or induced in our lab in the past) were maintained on agar-solidified GD medium (2) containing 5 μM 2,4-D, 2 μM benzylaminopurine and 2 μM kinetin. Cultures were grown in darkness at 24 °C (4, 5).

The cryopreservation protocol of Bozhkov was used and slightly adapted (Bozhkov, pers. comm., 2002). Embryogenic cultures grown on solidified medium were passed to roller flasks (3 g of fresh weight per 30 ml of liquid medium) and cultivated for 1 week. Cultures were then treated with sorbitol (0.16 ml of 4 M sorbitol was added to the flask ten times within 30 min). The resulting concentration of sorbitol was 0.2 M. The cultures were put back into the roller overnight. The following day, the procedure was repeated (this time using a 0.4 M of sorbitol). On the third day the cultures in flasks were placed on ice and treated with DMSO (0.175 ml DMSO ten times during 30 min, final concentration DMSO is 5 %). After a further 15 minutes the cultures were filtered through the sieve. Ice-cooled cryotubes were filled with cell suspension and closed.

We used two different cooling systems. Cryotubes were either put in a simple cryobox (Mr. Frosty, Nalgene) filled with isopropanol and cooled in a laboratory freezer, or cooled in a programmed cryomachine (Glacier, Sy-Lab). The temperature in the first system was monitored directly by a thermometer inserted in one cryotube. After cooling, cryotubes were transferred into liquid nitrogen.

After cryostorage, the cryotubes were put into sterile water (45 °C) for aprox. 1 min. At the melting point of their contents, cryotubes were placed into sterile water (+ 4 °C) for a couple of minutes. After the surface sterilization of the vials in 70 % (v/v) ethanol, the

cultures were layered on filter paper placed on a proliferation medium in a petri dish. The filter paper with the culture was further transferred onto a fresh medium after 1h, and once more after another 1 d. When tissue growth started, the filter paper was removed. Further subculture was the same as usual.

Cryopreservation using both cooling systems resulted in a regrowth of embryogenic cultures. After thawing, the growth of the embryogenic cultures is visible in 1-2 w. The cells of meristematic heads (the only part of the culture which remained alive) started to divide. Suspensor cells, damaged by cryostorage, recovered (Fig. 1). The rate of growth of cultures is changed during the period of lag-phase. This period is different for different cell lines. There is no doubt that cryopreservation represents a strong selection pressure which could eventually change the embryogenic capacity of the culture.

Fig 1.: Regrowth of Norway spruce embryogenic culture 14 d after cryopreservation.

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Cryopreservation of strawberry

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Introduction

In Finland MTT has been named as the main location of the preservation of vegetatively propagated agricultural and horticultural plants. Finland has as a part of the COST 863 activity taken the responsibility of the preservation of 12 strawberry cultivars ‘Frigg’, ‘Glima’, ‘Heino’, ‘Hella’, ‘Hiku’, ‘Mari’, ‘Pervagata’, ‘Rubina’, ‘Surprise des Halles’, ‘Xenion’, ‘Ydun’ and ‘Zefyr’. In addition 7 other cultivars have been chosen for long term preservation. For this purpose the project at MTT Plant Production Research aims to develop and utilise cryopreservation techniques *e.g.* for strawberry. At the moment MTT is still the only unit in the Nordic countries exploiting cryopreservation for horticultural plants.

At the moment along with field collections preservation methods like *in vitro* maintenance in slow growth conditions are being utilised but cryopreservation is needed as a new backup system. It is planned to become the primary backup method for long-term preservation of strawberry collections at MTT.

Traditional slow freezing has been tested for strawberry (1, 2). Modern methods like encapsulation-dehydration (3, 4) or vitrification (5) based methods like encapsulation-vitrification (6) are more efficient. After encapsulation-vitrification combined with cold acclimation good survival and regrowth have been obtained with *in vitro*-grown meristems of strawberry (7, 8). The latest, work-efficient method is droplet-vitrification (9), in which the encapsulation step is not needed. This method has not earlier been studied with strawberry.

Materials and methods

The experimental part was started at MTT in February 2006. The modified droplet-vitrification cryopreservation method was optimized for strawberry using seven different cultivars: ‘Cavendish’, ‘Jonsok’, ‘Kent’, ‘Korona’, ‘Lina’, ‘Polka’ and ‘Senga Sengana’. The apical shoot tip and axillary bud meristems were excised from *in vitro* propagated cultures. As pre-treatment media with increasing sucrose concentrations were used. The effect of 0.1 % Supercool X-1000 (21st century Medicine) added to the plant vitrification solution (PVS2) was tested. Survival and regeneration rates were observed.

Results

Screening of different cultivars indicated that survival and regeneration can be attained after cryopreservation but there are differences between cultivars. A useful droplet-vitrification method was developed for ‘Polka’. For the other cultivars some final adjustments are still needed. Shoot tips or buds smaller than 1 mm were not as competent for cryopreservation as larger ones.

Discussion

The positive results obtained indicate that the droplet-vitrification cryopreservation method is applicable for the preservation of meristems from *in vitro*-grown gene resources of strawberry. It is easy to be optimized for routine laboratory work but requires in vitro propagation facilities. The implementation of cryopreservation in the certified plant production of strawberry will enables the long-term preservation of more extensive strawberry collections than the traditional preservation methods. The experiences from long-term cryopreservation are yet limited and unexpected problems might appear. This aspect must be further investigated.

Aknowledgements:

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Tissue culture as a tool for *ex situ* conservation of wild pear

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In situ germplasm preservation plays an important role in the maintenance of biodiversity and avoidance of genetic erosion, but the preservation of woody plants in the field gene banks requires huge land areas and it is very expensive. More importantly, the materials are threatened by attacks of pests, diseases and other natural hazards (1, 2, 3). *Ex situ* conservation techniques, including the use of slow growth storage and cryopreservation for *in vitro* cloning have also been developed. Cryopreservation has been considered as an alternative/complementary choice for a long-term conservation of germplasm of plants including woody species (4).Thus, the plant materials can be stored for an indefinite period of time (1). Moreover, the materials are stored in a small volume, which largely avoids contamination and requires only very limited maintenance work.

However, the availability of efficient and reliable cryogenic protocols, which yield a high percentage of recovery, is basic requirement.

In recent years, several new techniques have been developed for cryopreservation of shoot tips of tropical and temperate plant species (1, 3). Encapsulation dehydration, originally described by Fabre and Dereuddre (1990)(5) for cryopreservation of *Solanum* shoot tips, is one of these techniques. This approach has been also applied to cryopreservation of shoot tips of woody plants such as *Malus* (6, 7), *Pyrus* (8, 9), and *Vitis vinifera* (10, 11).

In the present paper we report a protocol for cryopreservation of *in vitro* grown shoot tips of wild pear, interesting for forestation and for the productions of timber wood, highly valuated on the market, using encapsulation-dehydration techniques.

Shoot tips (2-4mm, in size) were excised from proliferating shoot (micropropagated plantlets) culture and were suspended in LP inorganic medium supplemented with Na alginate and sucrose. The mixture, with the shoot tips, was dispensed with a sterile micropipette into a $CaCl_2$ solution to form beads containing one or two shoot tip. Then, the beads were precultured on liquid medium LP enriched with sugar (sucrose or glucose) at two concentrations 0.75 and 1.0 M, for 1, 2, 3 or 4 days.

Dehydration was performed in small jar with silica gel for 4, 6, 8, 15 or 20 hours, at room temperature. Then, beads were transferred into a cryotube and directly immersed in liquid nitrogen (LN).

Encapsulated shoot tips after thawing were cultured on Petri dishes containing multiplication medium maintained in the dark at 24 °C for 7 days and then transferred to the light.

The highest survival after thawing was 54 % obtained with 1 day in 0.75 M sucrose and 20 hours in silica gel.

Proliferating and rooting ability of recovered material was evaluated. Multiplication rates were the same as the cryopreserved material, already after one subculture. Rooting

ability was lower in cryopreserved clones after 2 subculture, but it was fully restored after 4 subculture.

Genetic stability of conserved material is now under evaluation through DNA analysis. The preliminary estimation of genetic stability on stored material through RAPDs has not shown any somaclonal variation induced by the cryopreservation process.

Some biochemical analysis are now in progress to characterize the cryopreserved material.

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Genetic stability and authenticity

Chairperson: M. Ángeles Revilla

Cryopreservation and genetic instability

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Cryopreservation has proved to be a useful tool for germplams conservation. The principal objective of a germoplasm collection is to maintain intact a specific gene combination, so if after a period of storage, the genotype is not the same as the original material that was introduced, the validity of the system must be questioned. Therefore the maintenance of viability cannot be the sole criterion of successful cryopreservation, as genetic stability must also be maintained.

The process includes several steps, tissue culture being implied in some of them. In all the stages in which this technique is involved, the so-called somaclonal variation could be induced. Other treatments, commonly used during cryopreservation such as cold-hardening or cryoprotectant treatments, could also increase the level of genetic instability.

Two types of variation could be promoted: genetic and epigenetic. In the first case inheritable changes are produced. Epigenetic variation is theoretically non-heritable, however heritable changes in DNA methylation have been increasily referred to in recent years, thus the methylation persisted through meiosis, is transmitted to the descendant and on occasions is maintained during generations. We already know that some phenotypic variations that have been described as recessive mutations, are due to permanent methylation (1,2) and the paramutation phenomena are also explained by epigenetic changes. As modifications other than the alteration of the DNA sequence could also be inheritable, do we need to change our concept of mutation? (3).

Among the plants produced through tissue culture several types of genotypic variations have been reported: chromosome changes, point mutations, mobile element activation and persistent methylation are among the most cited. Different mechanisms seem to be involved in these changes and probably several of them, if not all, are operating to promote these alterations. Only in some cases the proposed mechanisms have been experimentally confirmed, however new data assert the real implication of some of them. (4). In several cases the involvement of epigenetic changes in the promotion of genetic modifications such as chromosome structural changes or point mutations have been stated (5). It is well known that hypomethylation causes the activation of mobile elements and the excision-insertion phenomena could account for many of the genotypic alterations found. Mobile elements are activated by other stresses, some of them also related with tissue culture such as wounding. Cold is also involved in some cases of activation of mobile elements so, the transposition frequency of Tam3 in *Antirrhinum majus*, is tightly controlled by temperature: higher temperature resulted in hypermethylation, whereas lower temperature resulted in reduced methylation and the transposon became active (6).

Reference has been made (*see* the Harding revision, 7) that in some species, cultivars and/or cryopreservation methodologies the genetic instability is an unimportant phenomenon and the possible variation, if any, does not suppose any trouble on the possible application of this technique. However in other cases it is necessary to be sure that the plant recovered after storage does not present any change. Several methodologies could be applied to assess genetic stability.

Phenotypic variation, including morphological and biochemical analyses, has been commonly reported. However, the analysis of the progeny from the regenerated plant must be carried out as plants which look normal segregate abnormal plants in the R1 and R2 generations depending on the autogamous or allogamous nature of the species in study. The virtue of this approach resides in the fact that all the genome is studied simultaneously, at least the genes which are expressed in the phenotype, because all the theoretical mutations must appear and segregate. However this kind of study is very tedious and time consuming, and could be impossible for some of the cryopreserved species.

Genotype changes could be observed in the recovered plant when cytological or molecular studies are performed. Chromosome number and structure as well as DNA content have been studied in many cases and are a good reference of genome stability at a gross level. The molecular marker methods to assess the stability of the recovered plants are at present widely used. Some of the applied techniques study specific sequences (RFLPs, SSRs, ribosomal DNA probes...) but others study a random part of the genome (RAPDs, ISSRs, IRAPs, REMAPs, AFLPs...). Methylations are equally studied, for instance, by means of a combination of the use of restriction endonucleases, which are sensitive or not to methylation, with some of the other techniques.

Changes in the pattern of DNA methylation have been reported in the cryopreserved recovered plants. In most cases we will assume that this modification will be non permanent and so lacking in importance. However, long term activation of the Karma retroelement during several generations after tissue culture associated to hypomethylation has been observed (8), so the epigenetic modifications must be also carefully controlled.

We must be aware that even when in theory the probability of change is randomly distributed along the genome, this is not always the case as hypervariable regions exist (9, 10, 11), and that some epigenetic changes often occur at a higher frequency. We must also take into account that in most of the cases the observation of a variable band does not indicate the kind of mutation promoting the change, even after sequencing the band, because usually we cannot discern which type of mutation occurs.

Not all the techniques detect the same level of variability and in some cases only some of them are able to discern the changes. The reproducibility of the band patterns observed with the different methodologies has been also questioned (12).

Harding (7) states that there is an increasing requirement to determine whether plants derived from cryopreservation are ‘true to type’ and to measure the extent of this near ‘normal phenotype’ in cryopreserved plants and estimate the degree of closeness to the ‘true’ parental genotype. The molecular marker techniques are an easy approach to measure the variability but, normally only a minimal part of the genome is tested.

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Transgene stability in cryopreserved cork oak somatic embryos

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The cork oak (*Quercus suber* L.) is one of the most characteristic species of the Mediterranean ecosystem. Besides its great ecological value, it produces cork, a natural renewable product of economic interest. Owing to the long generation time lag caused by seed germination and flowering this species has been left unaltered in relevance to genetic improvement or molecular breeding.

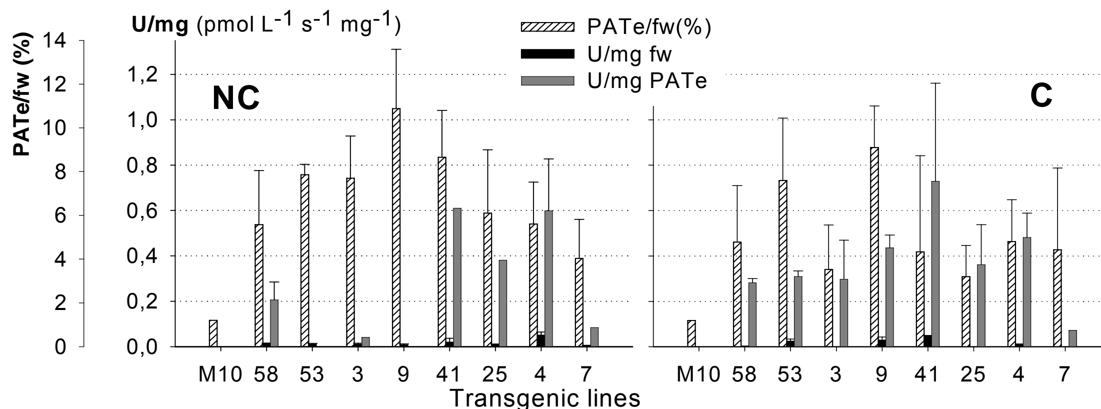
Herbicide resistance is a desirable trait not present within breeding population of *Quercus suber* L. A genetic transformation protocol of mature cork oak has been reported (1), which opened the possibility to insert this trait into desired genotypes (Alvarez et al. 2007, *in preparation*). Cork oak somatic embryos were genetically transformed with AGL1 pBINUbiBAR. The plasmid pBINUbiBAR contains the genes *nptII* and *bar*, which respectively confer resistance to the antibiotic kanamycin and the herbicide phosphinothricin. Hoping to be able to perform field analysis in a future, the transgenic embryogenic lines were cryopreserved and its genetic stability was tested.

Thirteen transgenic embryogenic lines were cryopreserved by vitrification following the protocol by Valladares et al. (2). Transgene stability of cryopreserved and non-cryopreserved embryos was assessed with molecular, resistance and enzimatic assays. Molecular assays were performed by polymerase chain reaction —PCR— and southern blot; resistance was tested both for kanamycin, culturing the explants with the antibiotic, and for phosphinothricin, culturing them with the herbicide and using the chlorophenol red assay —CR—); finally, the explants were tested for phosphinothricin acetyl transferase —PAT— enzimatic activity.

Our data on cryopreservation efficiency concurred with those by Valladares et al. (2), who reported a survival rate of about 90 %. Explants were subjected to a thermal analysis. Somatic embryos isolated or clustered (2-11 mg) were sealed in aluminium crucibles and frozen (-70 ºC)/thawed (20 ºC) in a N_2 atmosphere with a cooling/warming rates of ±10 ºC min^{-1}, and the effect of three variables on the freezing, thawing and transition temperatures was studied using differential scanning calorimetry (DSC). The variables were: explant type (isolated embryo/clustered embryos), preculture on a medium with 0.3 M sucrose for 3 days, and pretreatment at 0 ºC in PVS2 for 30 and 60 min.

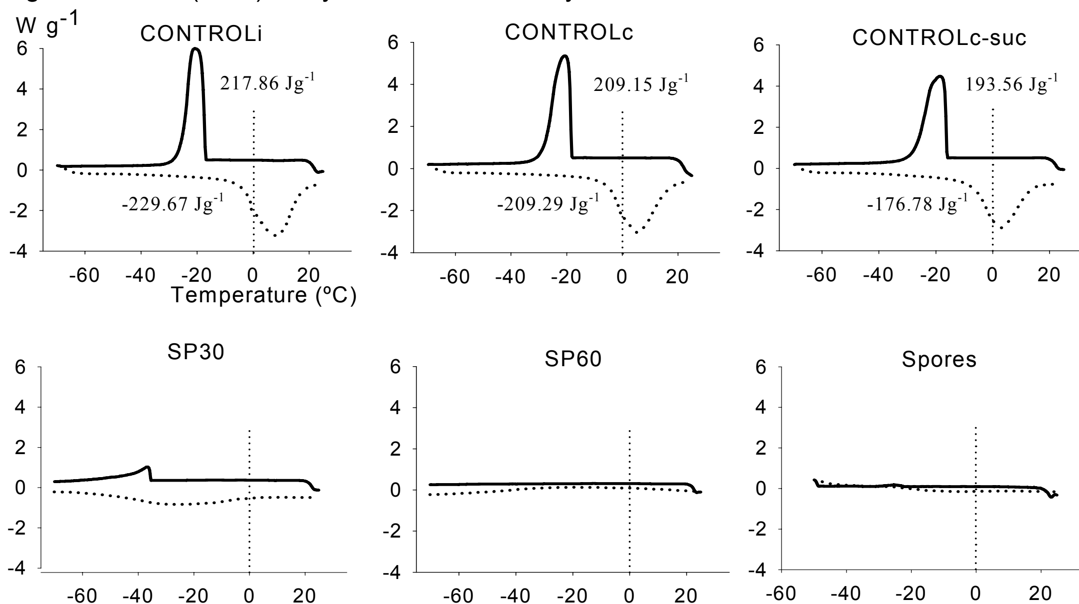
Presence and expression of both *nptII* and *bar* transgenes were assessed in all the embryogenic lines studied. The enzymatic assay showed that differences in expression exist between the transgenic lines (Fig. 1) The DSC (Fig. 2) showed that a pretreatment with sucrose reduced the cellular water nucleation, and confirmed that this treatment applied along with 30 to 60 min in PVS2 effectively avoids the nucleation.

Fig. 1. PAT expression levels between cryopreserved and non-cryopreserved embryogenic lines.



The crude extract from non-cryopreserved (NC) and cryopreserved (C) explants from a control (M10) and eight transgenic lines (rest) was enriched for the PAT enzyme by a differential $(NH_4)H_2SO_4$ precipitation (30–60 %). *U/mg fw*, units per mg fresh weight; *U/mg PATe*, units per mg protein in the enriched extract; *PATe/fw*, percentaje of enriched extract relative to the initial fresh weight. Both *U/mg fw* and *U/mg PATe* were normalized to the activity detected in the control.

Fig. 2. Thermal (DSC) analysis of somatic embryos



CONTROLi/c, isolated/clustered explants without pretreatments; CONTROLc-suc, clustered explant pretreated for 3 d with 0.3 M sucrose; SP30, SP60, clustered explants pretreated for 3 d with 0.3 M sucrose and for 30 or 60 min at 0 ºC in PVS2; Spores, sample of orthodox spores from the fern *Polypodium cambricum*.

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Genetic fidelity of *Pinus pinaster* somatic embryogenic cultures following cryopreservation

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Introduction

For clonal propagation of long-living trees using *in vitro* systems it is important to assess the maintenance of genetic fidelity after cryopreservation. The cryopreservation methodology has already been established for embryogenic cultures of maritime pine (*Pinus pinaster*), and dimethyl sulfoxide (DMSO) has been used in pretreatments in this process. However this cryoprotector has been described in the literature as a potential mutagenic compound which should thus be monitored. The sensibility, reproducibility, co-dominance and strong discriminatory power of SSR markers (simple sequence repeats) make them a useful tool for detecting somaclonal variation. SSR (microsatellite) size modifications represent manifestations of genomic instability and they have been described as ideal markers for assessing mutability in somatic embryogenic tissues.

The main objective of our work was to monitor genetic stability/instability of cryopreserved embryogenic cultures of *P. pinaster* using SSR markers.

Materials and Methods

Embryogenic cell lines of *Pinus pinaster* were established from immature zygotic embryos (1). The embryogenic cultures were cryopreserved following the protocol described by Marum *et al*. (2). Twenty four different samples were selected for analyses.

For the DNA extraction, cryopreserved and non-cryopreserved cultures with 4 months after initiation have been used. Non-cryopreserved embryogenic cultures were also used to monitor genetic stability after various periods of tissue proliferation (6, 14, 22 months). The isolated DNA was amplified using seven pairs of specific nuclear microsatellite primers (FRPP91; FRPP94; ITPH4516; PtTX3107; NZPR114, NZPR544, RPtEST11), according to previous publications (3, 4, 5). The fragment analysis was performed using a Capillary sequencer Beckman Coulter equipment (CEQ™ 8000) and the fragment length of the PCR-products was evaluated by CEQ™ 8000 Genetic Analysis System Software.

Results and Conclusions

From the twenty-four embryogenic samples analyzed with seven nuclear SSRs, a total of 39 alleles has been obtained. The number of alleles ranged from 2 to 11 per locus (Table 1). The FRPp91 locus was the most polymorphic one with eleven different alleles.

For the tissues revealing variations in SSR sizes, the fragment analyses were repeated at least three times and consistent results were obtained.

The analysis of tested loci suggests that the genetic integrity of cryopreserved embryogenic cell lines of maritime pine was not affected by freezing and storage.

However, in the embryogenic lines under prolonged *in vitro* culture conditions a 0.6 % of frequency variation was detected in three different SSR loci (FRPp91, ITPh4516 and PtTX3107).

Cryopreservation is usually seen as a process to avoid and prevent possible somaclonal variation induced by prolonged *in vitro* culture. Although it is not yet possible to establish a precise correlation between SSR variation occurring during culture and somaclonal variation, our results suggest that embryogenic tissues of *P. pinaster* should be cryopreserved as soon as possible after establishment, to reduce/prevent genetic instability. Some peculiarities of the cryopreservation technology such as the blocked cell metabolism and absence of subculturing, allow storage and maintenance of lines for long periods while reducing the risk of genetic and epigenetic alterations (6).

Table 1. Repeat motif, allele size range, number of tested samples (n), number of alleles from nuclear SSRs of *P. pinaster*.

SSR *Loci*	Repeat motif	Allele size range (bp)	n	Number of alleles
FRPp91	$(CT)_{20}$	160-192	24	11
FRPp94	$(CT)_{22}$	134-150	24	06
ITPh4516	$(CT)_{27}$	124-166	24	09
PtTX3107	$(CAT)_{14}$	148-164	24	02
NZPR114	CA_{15}... $CA_{13}TA_{22}$	179-187	24	04
NZPR544	$CA_5AC_{12}TA_5$	246-252	24	03
RPtEST11	$(ATC)_7$	204-214	24	04

The loss of maturation capacity of *P. pinaster* embyogenic cultures, when the proliferation period is extended over 12 months in culture (data not shown) may be related to increased somaclonal variation. Some authors have shown increased genetic variation associated to prolonged *in vitro* culture. The loss of totipotency could be explained by either loss or mutations in genes responsible for regeneration or by changes in ploidy level (7).

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Assessment of developmental competence, genetic stability and biochemical potential of cryopreserved *Hypericum perforatum* L. shoot tips

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Hypericum perforatum L. is a medicinal plant considered as an important source of secondary metabolites with a wide range of pharmacological attributes. The most important, biologically active constituents are hypericins and phloroglucinol derivatives that show antidepressive, anticancer, antiviral and antibiotic properties (1, 2, 3). The content of these metabolites, which is influenced by genetic, physiological, metabolic an environmental conditions is very variable (4). Therefore, a search for genotypes with high and relatively stable content of these compounds, and conditions for their preservation without genetic and metabolic alterations is of great interest.

This work is aimed at determination of the effect of two pre-treatment steps prior to cryopreservation (pre-culture and cryoprotection) and cryostorage in LN on survival rate, mitotic index, cytogenetic and DNA stability revealed by SSR primers and naphthodianthrone content.

Shoot tips from *in vitro* cultured diploid *Hypericum perforatum* L. genotypes were subjected to assessments of developmental competence, genetic stability and biosynthetic ability to identify critical points during the cryopreservation. Cryopreservation of shoot apices was performed by application of pre-cryogenic steps and cooling using slow freezing method. Survival rate (Fig. 1), chromosome number stability, mitotic activity, alteration in VNTR sequences and hypericin content were evaluated after pre-culture and two subsequent cryogenic steps (cryoprotection and cooling) in plants recovered from cryopreserved meristems. Pre-culture and cryoprotection treatments did not reveal any significant differences in the characteristics studied. Genetic stability was assessed by chromosome counts and analysis of variability in the VNTR sequences (Fig. 2). No changes in chromosome number and mitotic activity (Table 1) were detected in comparison with the untreated control but minor alterations were revealed in non-coding sequences. The content of hypericin after the recovery of cryopreserved meristems remained comparable with the unfrozen control (Table 2).

Table 1. Mitotic index of cryopreserved *Hypericum perforatum* L. meristematic cells

Genotype	Mitotic index (% ± S.E.)	
	Cryopreserved meristems	Control meristems*
6/7/1	97.99 ± 1.59	98.44 ± 0.16
2/7/9	98.74 ± 0.19	98.74 ± 0.35
5/7/8	97.83 ± 0.97	98.01 ± 0.56
7/7/5	97.01 ± 0.00	99.05 ± 1.55
2/7/7	98.44 ± 0.39	99.16 ± 0.16

* Mitotic index in shoot-tip meristems of the same plant

Table 2. Comparison of hypericin content in control and cryopreserved *H. perforatum* genotypes

Genotype	Mean content of hypericin [µg/g]	
	Non-cryopreserved control [x ± SD]	Plants regenerated from cryopreserved meristems [x ± SD]
2/7/7	365.380 ± 71.375	528.000 ± 81.529
2/7/9	577.820 ± 119.733	603.135 ± 125.448
5/7/8	355.505 ± 167.680	304.863 ± 47.906
6/7/1	769.085 ± 191.145	665.831 ± 119.643

Figure 1. Differentiation of *H. perforatum* shoots from meristems 12 weeks after cryostorage

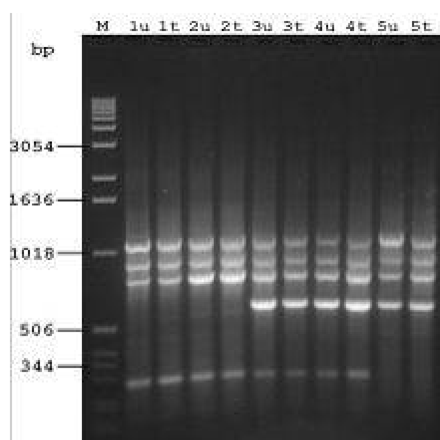

Figure 2. VNTR amplification patterns of diploid *H. perforatum* plants regenerated after pre-culture amplified with primer OGRB01. The numbers refer to compared couples of untreated controls (u) and derived treated plants (t). M: molecular weight marker

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Assessment of genetic stability of cryopreserved and cold storaged hop samples

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The genetic stability of 51 accessions of wild hop plants and hop cultivars that were cryopreserved or kept in cold storage was analysed by RAPDs and AFLPs. All the plant material was supplied by the USDA, National Clonal Germoplasm Repository (Corvallis, OR, USA).

Plant material

Cold storage (1): 2 to 3 cm plantlets grown in MS medium were transferred to two 5-chambered semipermeable tissue-culture bags with 10 ml NCGR-HUM medium without growth regulators per chamber. Sealed cultures were grown for a week in the growth room then for a week under cold acclimation (CA) conditions (–1 ºC 16 h dark, 22 ºC 8 h light).

Cryopreservation (1): Shoot tips of 1 or 2 weeks CA plantlets were dissected and precultured on NCGR-HUM medium with agar, Gelrite and DMSO for 48 h under the same conditions as the parents shoots. The tips were then transferred to plastic cryovials with 0.25 ml of MS liquid medium and the cryoproctentant PGD was added up to fill the cryovials. After the samples were equilibrated on ice, they were frozen to –40 ºC at 0.1 ºC min^{-1} with nucleation at –9 ºC and immersed in liquid nitrogen. The vials were thawed in 45 ºC for a minute and then in 23 ºC water for 2 min. The cryoprotectant was replaced with liquid MS medium. The shoot tips were grown in NCGR-HUM medium.

Molecular techniques

The RAPDs reactions were carried out according to Pillay and Kenny (1996) with slight modifications. Fifteen µl of each sample were loaded in a 2 % agarose gel and run at 70 V for 4 h. Six independent reactions of one sample were compared to assess the reliability of the band pattern. Twelve annealing temperatures ranging from 36 ºC to 58 ºC were tested for each RAPD primer; two repeats of the sample were tested in each annealing temperature.

The AFLP analysis was performed according to Vos *et al*. (1995) with the modifications described by Cervera *et al*. (1998). DNA was digested with *Mse*I and *Eco*RI. Aliquots (6 µl) of final sample were loaded on 6 % polyacrilamide gels, electrophorased for 2 h. A standard silver staining protocol was used to reveal the bands.

RESULTS

Plantlets remained in cold storage for an average of 14.1 ± 3.5 months. The range for individual accessions was 6 to 26 months. There were significant differences in length of storage for cultivars (14.6 ± 3.4) vs. wild accessions (12.6 ± 3.2). The slow-cooling procedure was successful for all the accession tested. All the cultivars and wild accessions tested with 2-week CA recovered at acceptable rates for storage, over 40 % in all cases. Recovering after 1-week CA ranged from 7 % to 65 % (1).

RAPD analysis

Fifty one hop accessions were analysed. Samples of 11 of them had been cryopreserved and/or kept in cold storage while 40 were only kept under cold storage conditions. In each accession one control plant and two or four independent samples that had been cryopreserved or kept under cold storage were analysed (total = 169). Eleven RAPD primers were selected with a total of 125 loci detected. Over 20 000 bands were scored. There were differences in the band patterns in each accession but no differences were found between the controls and the samples from the same accession no matter which storage protocol was used.

AFLP analysis

The genetic stability of five accessions kept under cold storage conditions and 5 cryopreserved was tested in the AFLP assay. The average number of detected bands per sample and primer combination was 70.5 ± 1.7. Nearly 5 000 bands were scored. No differences in the band patterns of the controls and samples were detected in any of the accessions.

Due to the known influence of the genotype in the risk of genetic instability we decided to analyse as many cultivar and wild hop accessions as possible. We did not find any behaviour difference in any of the 51 of them so we can assure that both kind of samples, wild and cultivars, are genetically stable according to our results as no altered bands were detected but further methylation analyses are needed in order to assess if the recovered plants are true-to-type.

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Approaches to detect somaclonal variation and epigenetic changes in cryopreserved chrysanthemum apices

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Cryopreservation techniques allow long-term preservation of vegetatively propagated plants using *in vitro*-grown explants (*e.g.* shoots apices), from which the whole plants can be obtained. However, there is concern about the possible occurrence of somaclonal variation induced either by the *in vitro* procedures, used before or after cryostorage, or by the cryoprotection procedures. Some studies have already revealed that *in vitro* conservation techniques are associated with changes in the DNA methylation state (1), and those changes have been related to somaclonal variation. There are evidences that epigenetic changes, as differences in the methylation pattern of DNA, play a role in the occurrence of somaclonal variation through, for example, activation of transposable elements and silencing of genes, however the exact mechanism of this process remains unknown (2).

In a previous work on genetic stability of cryopreserved chrysanthemum apices (3), we detected a somaclonal variant using RAPDs markers. In order to evaluate the genetic differences appeared in this variant, we have determined and compared the DNA sequences of the variant and the stable control. For this study, bands from both samples (somaclonal variant and stable control) were excised from the gel, eluted, cloned and sequenced. In a first step, the comparison of the sequences obtained from the variant and the stable control allowed confirming the differences found through RAPD analyses. Besides, the sequences were queried against the Genbank databases by BlastX at the NCBI website to identify possible homologies.

Since somaclonal variation occurrence seems to be related to DNA methylation changes, as it has been stated before, we studied possible changes in the methylation pattern of our variant sample. We chose the technique based in the pre-treatment of DNA with sodium bisulphite, a process that converts unmethylated cytosine residues to uracil (4). Subsequently, PCR analysis of the bisulphite-modified DNA was carried out. For amplification, arbitrary primers (RAPDs) were employed, similarly to the process described by Joyce *et al*. (5) to study developmental variation in micropropagated potatoes.

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Molecular validation of a micropropagation-cryopreservation procedure for red chicory (*Cichorium intybus* L.) selected lines

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Red chicory (*Cichorium intybus* L.) is one of the most economically important vegetables of the Veneto region, North-East of Italy. Here, over 9 000 ha are annually cultivated with red chicory, *i.e.*, almost 85 % of the Italian, and 60 % of the total World surface occupied by this vegetable. Introduced in Italy in the XV Century, in recent years it has been interested by an intense work of selection and breeding, which has produced in time several improved typologies (such as 'Rosso di Treviso', 'Rosso di Verona', 'Rosso di Chioggia', 'Variegato di Castelfranco'), highly appreciated for their agronomic and organoleptic characteristics (4). In order to assure a high-quality production, a great effort is made by the "Veneto Agricoltura" State Institution of Italy to provide red chicory farmers of seeds of superior characteristics. Here, high-performance lines for the main typologies of red chicory are selected and, after in-field evaluation, are introduced *in vitro* and reproduced by micropropagation. The *in vitro*-cultured stock lines are used as a source of plants, which are yearly acclimatized in greenhouses and then transferred in the soil for controlled cross-breeding. Seeds are then collected and made available to farmers for the obtaining of high-quality red-chicory crops. The core of this process (*i.e.*, the continuous micropropagation of stock shoot lines) requires particular *in vitro* conditions (21 °C and a 12 h photoperiod) and a regular subculturing every 21 days (2), which can be in the long time the cause of undesirable somaclonal variation. Hence, it is of fundamental importance to achieve to an effective system of long-term preservation of selected lines, thus reducing the costs of stock culture maintenance, and the risks of contamination and genetic alteration of lines.

A study for the optimization of micropropagation and the development of cryopreservation procedures for four red chicory typologies ('Rosso di Chioggia', 'Rosso di Treviso precoce', 'Rosso di Treviso tardivo' and 'Variegato di Castelfranco') was initiated in 2002, in the frame of a collaboration between the "Veneto Agricoltura" and the CNR/IVALSA on preservation of autochthonous germplasm of Veneto. Among the main aims of the study was the assessment of the maintenance of plant genetic fidelity during the whole process of *in vitro* propagation and conservation, through a series of molecular analyses carried out after both repeated subculturing and cryopreservation. As for cryopreservation, a procedure of "vitrification/one-step freezing" was developed with shoot tips from selected lines of the 4 red chicory typologies (2). In short, shoot tips (1-2 mm, in size) were excised from proliferating shoot cultures, cold hardened (2 days) on hormone-free MS medium, and loaded for

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30 min in CP (2 M glycerol and 0.4 M sucrose) at 25 °C inside 2-ml cryovials. The shoot tips were treated with PVS2 at 0 °C for 60 or 90 min, and then plunged directly into liquid nitrogen (LN). After thawing (one minute in a 40 °C waterbath), washing (20 min in 1.2 M sucrose-containing liquid MS medium), and plating onto a BA-containing proliferation medium, all the lines showed to survive satisfactorily to the storage in LN. Maximum explant survival ranged between 65 % ('Treviso precoce') to 77 % ('Treviso tardivo'). In comparison with the "vitrification" procedure, the application of an "encapsulation-vitrification" technique showed to be less effective in terms of survival to cryopreservation. After a period of about 3 months of *in vitro* regrowth, rooted shoots were transplanted in pots and *in vivo* acclimatized with 100 % survival.

To assess the maintenance of the genetic fidelity of the selected lines, RAPD profiles were generated using 10 arbitrary 10-mers as primers, four of which were then selected for the reproducibility, the legibility and the stability of the RAPD pattern: 1253 (5), 1247 (3), M3 and A5 (1). All the 4 selected primers produced strong and reproducible bands, their number varying from 5 to 12 for each primer. Each primer generated a set of amplification products of a size ranging between 350 bp and 3000 bp. Molecular assays were performed with two lines ('Chioggia CHP/101' and 'Treviso precoce TVP/124'), in order to validate both the micropropagation procedure (after 5 and 10 repeated subcultures), and the optimized cryopreservation technique (through analyses carried out before and after the storage in LN). Up to now, no change in the molecular pattern has been evidenced by the comparison of shoots from micropropagation and cryopreservation, but still under in vitro culture. Both molecular and morphological observations are in progress to compare acclimatized plants with the original mother plants.

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Genetic fidelity of cryopreserved embryogenic cultures from mature *Quercus robur* trees

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Oaks have recalcitrant seeds for storage, and this along with difficulties in vegetative propagation (1) hinders the conservation of high-value genotypes. The induction of somatic embryogenesis and regeneration of clonal plants from embryogenic lines originated from leaves of selected mature oak trees has been reported (2). Cryopreservation of these embryogenic cultures appears to offer the best prospects for conservation of these highly valuable genotypes. The aim of the present study was to define a cryopreservation protocol for embryogenic lines derived from mature selected oak trees based on the vitrification procedure. Genetic stability of cryopreseved material is an essential requisite for conservation and clonal forestry, hence a further objective was to obtain an assessment of the clonal fidelity of cryostored oak somatic embryos (SE) and regenerated plantlets by employing molecular markers as RAPDs.

Six embryogenic lines (CR-0, CR-7, B13, B17, B18 and Sainza) initiated from six specimens of *Quercus robur* (all over 100 years old) growing in North-western Spain were used for cryopreservation experiments. Somatic embryos were induced on expanding leaf explants excised from epicormic shoots forced from branch segments taken from these trees. The embryogenic lines used for this study had been maintained for more than two years by secondary embryogenesis. Samples consisting of 4-6 mg clumps of globular- or heart-shaped embryos were dissected from each of the six stock embryogenic lines and were precultured for 3 days on 0.3 M sucrose-supplemented medium, and then treated with PVS2 vitrification solution (3) at 0 ºC for different periods, before plunging in liquid nitrogen (LN). After one week and one year of cryostorage, these materials were retrieved from LN and used to evaluate embryo survival and embryo recovery capacities, as well as to assess the genetic fidelity of cryopreserved germplasm.

All six oak embryogenic lines showed high levels of tolerance to preculture in sucrose medium and PVS2 application (treated controls). Cryopreserved samples turned brown within 2-3 days after thawing. Direct regrowth of the pre-existing embryos did not occur; rather the new globular-stage embryos started to proliferate from surviving cells of original embryos after two weeks' culture in recovery medium. Recovery levels of only 5-31 % were recorded for clumps from the material that had been precultured on sucrose medium but which had not received PVS2 treatment. These values were significantly increased in cryopreserved embryos with PVS2 applied for 60 or 90 min, although no significant differences were found between these two periods. All tested lines were successfully cryopreserved using the vitrification method, with the resulting embryo recovery rates ranging between 57 to 91 %. Although there were differences in regrowth between different genotypes, the method can be considered feasible for the different genetic backgrounds employed in this study.

RAPD analyses were conducted on all six embryogenic lines to evaluate the genetic stability of somatic embryos cryostored for one week and for one year in comparison to the non-cryostored stock cultures (controls). Additionally, treated control embryos (PVS2 application and non-cryopreserved) were also included in the analyses to test the possible mutagenic effect of cryoprotectant DMSO of the vitrification solution. Forty SE from each embryogenic line (ten per treatment) were used. The genetic fidelity of plantlets generated from cryopreserved SE from lines CR-0, CR-7, B17 and Sainza was also evaluated by including ten plantlets from each line in the analyses. The reliability of 40 arbitrary 10-base primers was initially tested using Kits A and S (Operon Technologies). The primers OPA-6, -11, -12, -16, -19 and OPS-2, -6, -10 and -15 were discarded in all lines, and primer OPS-4 was also discarded in lines B18 and CR-0, as they failed to generate clear and reproducible RAPD profiles. The size of the amplification products was approximately in the range of 300-2 500 bp. The number of scorable bands per primer ranged from 2 (OPS-7 and OPS-9) to 18 (OPA-3 and OPS-14) with a mean of 8.8. Each primer used produced different RAPD patterns in all six genotypes, thereby allowing them to be distinguished. The 30 primers used with B18 and CR-0 produced a total of 293 and 262 possible bands, respectively, and the 31 primers used with B13, B17 and Sainza a total of 303, 273 and 231 possible bands, respectively; in each of these five lines, the banding pattern for each primer was highly uniform for cryopreserved materials and controls. In line CR-7, the amplification products were monomorphic for all samples, except in only one embryo from the material that had been cryopreserved for one year. In this single SE, the OPA-15, OPS-8 and OPS-12 revealed 1, 2 and 4 polymorphic fragments, respectively. However RAPD fragment patterns of all regenerated plantlets were identical to those of controls for all primers tested. In conclusion, the vitrification-based procedure used in this study appears to be suitable for cryopreservation and true-to-type clonal propagation of mature oak genotypes.

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