

Cryopreservation of crop species in Europe

CRYOPLANET – COST Action 871
20th-23rd of February 2008, Oulu, Finland

Jaana Laamanen, Marjatta Uosukainen,
Hely Häggman, Anna Nukari and Saija Rantala (eds.)



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Preface

CryoPlanet, i.e. COST Action 87 "Cryopreservation of Crop Species in Europe", is the instrument to bring together European plant cryopreservation specialists. The flexibility of the COST Actions allows the coordination of nationally funded research on a European level. The purpose of the action is to create a network that brings together European scientists with an expertise and/or interest in plant cryopreservation. The main aim is to develop and implement efficient cryogenic procedures for the preservation of crops that are vegetatively propagated and/or produce non-orthodox seeds. Emphasis is placed on applying cryopreservation to European plant germplasm collections as a complementary technique. So far, cryopreservation procedures have been developed for the *in vitro* tissues and non-orthodox seeds of about 200 plant species. This Action will run until December 2010.

The scientific programme of Cryoplanet was developed with the input of researchers from 17 different COST countries. The researchers in this Action represent a unique assembly of European scientists, with large experience in plant cryopreservation, tissue culture, stress physiology and/or genebank management. Two Working Groups are distinguished within the Action: WG1 on fundamental aspects of cryopreservation/cryoprotection and genetic stability, and WG2 on technology implementation, transfer, application and validation in plant genebanks, culture collections and research groups. These two Working Groups have strong links and interactions between them.

The most important mode of action in Cryoplanet is to organise Working Group meetings on a yearly basis separately or combined with the other WG. In 2007 the first WG meetings were organized separately; WG1 in Oviedo, Spain and WG2 in Florence, Italy. In 2008 one combined meeting takes place in Oulu, Finland. This meeting is organized by the University of Oulu and MTT Agrifood Research Finland. At this 2nd meeting of Working Groups 1 and 2 scientists from 19 European Countries and USA give 39 presentations dealing widely with various aspects of plant cryopreservation. Combined Working Group meetings enhance the integration of activities, addressing primarily the interfaces between the different fields.

Dr. Bart Panis (Belgium) is the Chair and Prof. Paul Lynch (UK) is the Vice-Chair of the Action. The coordinators of WG1 are Prof. Pavel Pukacki (Poland) and Dr. M. Angeles Revilla Bahillo (Spain) and the coordinators of WG2 are Dr. Florent Engelmann (France) and Dr. Joachim Keller (Germany). They as well as the University of Oulu, Department of Biology and MTT, Plant Production have given extensive support to the organizing team.

On behalf of the organizing team:

Marjatta Uosukainen
Senior Scientist
MTT, Plant Production
Nursery Group

Hely Häggman
Professor
University of Oulu
Department of Biology

Organizers and sponsors



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COST Action 871:

Cryopreservation of Crop Species in Europe



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The University of Oulu is an international scientific community known for high-quality research and education. The University promotes well-being and education in Northern Finland.



MTT Agrifood Research Finland is the leading Finnish research institute in the agriculture and food sector. MTT is an expert body operating under the Finnish Ministry of Agriculture and Forestry.

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Operational cryopreservation of multi-genera plant genetic resources collections at the National Center for Genetic Resources Preservation

D. D. Ellis & M. M. Jenderek

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Abstract

The U.S. National Center for Genetic Resources Preservation (NCGRP) is focused on research associated with the long-term preservation of plant and animal genetic resources in conjunction with the long-term storage of these resources. The Center securely stores over 730,000 inventories of plant genetic resources, the majority consisting of the base collection for the National Plant Germplasm System (>480,000 inventories). These collections are predominately seed which is dried to between 6% - 10% moisture content and stored at -18°C. However, ~8% of this base collection is stored in the vapour phase of liquid nitrogen. The criteria of which accession to store in liquid nitrogen and which to store conventionally (-18°C) is largely made on a genus-by-genus basis and usually cryopreservation is not considered for large sized see and seed with high oil content. Germination of every seed accession which is considered for liquid nitrogen storage is tested both with and without a 24 hour liquid nitrogen exposure to ensure that a particular accession can tolerate liquid nitrogen exposure.

In addition to the seed accessions stored at the NPGRP, we also have a large program in the operational storage of vegetatively-propagated accessions in the National Plant Germplasm System. At present, many of these accessions are maintained in a single field site and only ~10% of the 30,000 vegetatively-propagated accessions in the National Plant Germplasm System are backed up in cryopreservation. Due to the very high labour requirements for cryopreserving clonal germplasm collections (20-40 accessions/person/year), the NCGRP has operationally developed a priority system based on existing technology to determine which crops to process for long-term storage. Practically, our efforts are concentrated on crops with developed cryopreservation methods and where support of the crop curator is available. Examples of this collaboration include:

1. Apple (and sour cherry), where a robust dormant bud cryopreservation technology exists. Over 2,500 apple accessions are in cryostorage at NCGRP yet all phases of this work are dependent on the curator doing all grafting to test post-cryo viability as well as carrying out the monitor testing over time.
2. Garlic, where shoot tips are cryopreserved from field grown bulbs. The quantity of bulbs and number of accessions cryopreserved per year is very much dependent on the curator's ability to do the field work.
3. Accessions that require tissue culture plantlets as a source of the shoot tips (mint, strawberry, currants, blackberries, hops, sweet potato, pear). The NCGRP currently does not have the resources to do the isolation and establishment of the shoot cultures and therefore the curator must be willing to do this work in order to have the crop cryopreserved.

Operationally, our criteria for having an accession successfully back-up in cryostorage are a minimum of 40% viability in the cryopreserved shoot tips and a minimum 60 viable shoot tips cryostored. For most crops, we store 10 shoot tips/cryo vial and with the criteria for success mentioned above, we have a 99% chance of having at least one viable shoot tip per vial. Fortunately, for the crops we work with, vial-to-vial variability is very low.

Research in our operational setting focuses on applying existing techniques to our accessions and laboratory. Often, we need to make minor modifications in techniques so that the techniques are applicable in our system. This research phase may take 1-2 years before we consider a technique for a crop ready to start on a large-scale germplasm preservation project. Banana is a crop in this category in our laboratory where very good existing techniques exist yet it takes time to fully implement them into our operational system.

Another area of research in our applied preservation setting is extension of the apple dormant bud cryopreservation system to other woody crops. A major determinant for this research is the relative ease of preserving entire collections if dormant buds can be cryopreserved. This operational research approach was only initiated two years ago but we already have initial success with butternut and preliminary data indicates that material from our warmer-winter sites where vegetatively-propagated material is grown (west coast of the U.S.) can be cryopreserved as well as from colder growing regions. The focus crops for this research include a *Prunus* program (apricot, almond, peach, sweet cherry and plum), walnut, pear, hazelnut, blueberry, pomegranate and pistachio.

Key words: almond, apple, apricot, blueberry, garlic, hazelnut, hop, mint, peach, pear, pistachio, plum, pomegranate, Prunus, sweet cherry, walnut, strawberry, currents, blackberries, hops, sweet potato

Concepts in Cryobionomics: a Case Study of *Ribes* Genotype Responses to Cryopreservation in Relation to Thermal Analysis, Oxidative Stress, Nucleic Acid Methylation & Transcriptional Activity

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Abstract

Introduction

Cryobionomics is a conceptual framework linking disparate aspects of cryo-injury to genetic instability through a fundamental knowledge of an organism prior to its re-introduction into the environment [1]. Cryo-injury as a differential genotype response remains one of *the* most significant restrictions to applying cryopreservation to clonally propagated plants. *Ribes*, is generally amenable to cryobanking and genotypes display diverse responses to cryo-preservation [2-5]. These cannot be attributed to biophysical factors alone as demonstrated by DSC [5,7]. *Ribes* has been used effectively [2,3] to identify critical point factors in technology transfer, so it is unlikely that genotype variability can be assigned to technical parameters. Multi-disciplinary approaches [7-11] are required to elucidate causal factors of genotype variability. An ongoing case study [2-11] of *Ribes* exploring the molecular-physiological basis of genotype response to cryopreservation is described. Temperate woody perennials are ideal subjects, as their amenability to cryopreservation is moderated by acclimation, a programmed life-cycle adaptation. A model is presented to elucidate the roles of, and connectivity between epigenetic and oxidative processes in genotypic responses of clonal crop plants to cryopreservation.

Materials and methods

Four *Ribes* genotypes were selected, based on their known differential responses [4,11] to cryopreservation: *Ribes ciliatum* (sensitive), *Ribes nigrum* cv Ben Tron and *Ribes sanguineum* cv King Edward (intermediate tolerance) and *R. nigrum* cv Ben More (tolerant). *In vitro* shoots were subcultured on MS-*Ribes* medium [6]. Shoot meristems were acclimated [11] and cryopreserved by encapsulation-dehydration [4,6,11]. Gas chromatography headspace volatile analysis [11], antioxidant and pigment assays [9,10,11] were performed on *in vitro* shoots. Nucleic acids were extracted from nodal/shoot tissue and digestion reactions optimised [8]. Sample nucleoside concentrations were determined by HPLC analysis using external standards according to peak area as described [8].

Results and discussion

Objectives of CryoPlanet COST Action 871 require improved fundamental knowledge to widen the applicability of cryostorage to a large pool of germplasm. Profiles of oxidative stress markers and ethylene revealed genotypic differences in responses of acclimated *Ribes* germplasm to cryopreservation using encapsulation-dehydration. Two behaviours were identified: (i) a programmed oxidative burst (enhanced $\bullet\text{OH}$) resulting in elevated antioxidants and tolerance to cryopreservation; (ii) an oxidative stress reaction manifested by symptoms of photo-oxidation and delayed, incipient apoptosis. Parallel studies of DNA methylation revealed that epigenetic changes were differentially induced in more tolerant genotypes, whereas de-methylation occurred in the sensitive genotype in response to

acclimation and cryopreservation. Transcriptional activity was inversely correlated to levels of DNA methylation in both tolerant and sensitive genotypes during acclimation but this effect was less obvious following cryopreservation. Post-transcriptional activity correlated with tolerance to acclimation and cryopreservation. A network model is presented (Fig 1) integrating these findings with the objective of determining the role of stress and epigenetic activity in differential genotype responses.

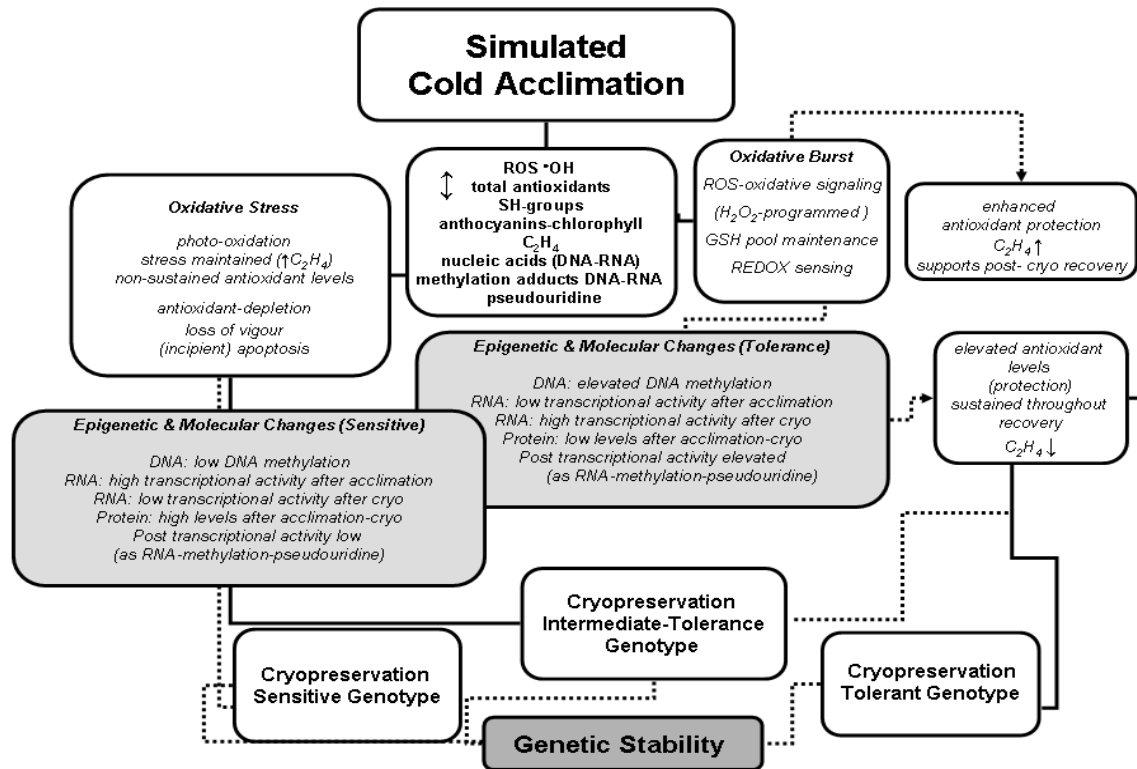


Figure 1. A network model exploring epigenetic and oxidative processes in differential genotypic responses of acclimated woody perennials to cryopreservation

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Key words: cold tolerance, cryo-injury, cryobanking, cryopreservation, encapsulation-dehydration, Ribes ciliatum, Ribes nigrum, Ribes sanguineum, temperate woody perennials

Detection of changes in the DNA-methylation patterns of cryopreserved apices of chrysanthemum

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Abstract

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 (e.g. 2), 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 remain unknown (3).

Genetic stability of cryopreserved chrysanthemum apices using RAPDs markers was evaluated in a previous work (4), founding in this study a somaclon between the regenerated material. From those results, techniques to detect possible changes in the DNA-methylation patterns of the cryopreserved apices have been developed in our laboratory.

The detection of changes in the DNA-methylation pattern in somaclonal variation studies using the technique CRED-RA (coupled restriction enzyme digestion and random amplification; 1) is evaluated in this work. The technique is based in the capability of the restriction enzymes MspI y HpaII to cleave the same sequence (5' CCGG 3'), but showing differential sensitivity to the presence of methyl residues. HpaII does not cut the sequence when any of the two residues of C are methylated, while MspI cuts the sequence when the internal C is methylated (5' CmCGG 3'). Subsequent PCR amplification of the digested DNA may reveal differences in the methylation pattern.

Ten samples from the cryopreserved chrysanthemum apices were studied (6 of them from the encapsulation-dehydration technique, and the other 4 from the vitrification technique), together with their corresponding pre-cryopreservation controls. The technique reveals its capability to show differences in the DNA-methylation pattern when used in the study of genetic stability of cryopreserved material. Differences between cryopreserved samples and their controls were found, mainly in samples deriving from the encapsulation-dehydration technique.

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Key words: in vitro-grown explants, somaclonal variation, genetic stability, encapsulation-dehydration, vitrification

Epigenetic stability of cryopreserved and cold-stored hops

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Abstract

Three hop accessions representative of commercially cultivated hops were selected for the analysis of epigenetic stability; females of different origins, including a cultivar developed in New Zealand (Calicross) from American cultivars, a landrace derived European cultivar (Tardif de Bourgogne), and a breeding accession (USDA 21055) obtained from crosses of English and American cultivars and wild American plants. Each accession included a total of five samples: one control, two cryopreserved samples and two samples kept under cold storage. The genetic stability of the accessions was previously assessed by RAPDs and AFLPs (1).

Cold acclimation, cryopreservation and cold storage method are described by Reed et al., 2003 (2). The MSAP analysis was performed according to Cervera et al., 1998 (3). Samples were electrophoresed in an automatic sequencer ABI PRISM® 3100 Genetic Analyzer and band patterns were analysed with the program Genemapper and corroborated by visual inspection of the electropherograms.

Table 1. Percentage of polymorphic loci and distribution of the epigenetic variation in hop accessions.

	USDA 21055	Calicross	Tardif
Polimorphic loci	38.28	36.70	47.40
Percentage of variation explained by both treatments			
Total	68.37	72.46	50.00
Demethylation	44.90	34.78	26.83
Methylation	7.14	17.39	15.85
Other	16.33	20.29	7.32
only cryopreservation			
Total	13.27	7.25	20.73
Demethylation	9.18	4.35	3.66
Methylation	1.02	1.45	12.20
Other	3.06	1.45	4.88
only cold storage			
Total	11.22	7.25	18.29
Demethylation	4.08	4.35	10.98
Methylation	3.06	0.00	7.32
Other	4.08	2.90	0.00
not explained	7.14	13.04	10.97

Over 36% of the detected MSAP loci presented some sort of modification after cold storage or cryopreservation protocols. It is noticeable that over 87% of the total variation

could be related to each or both protocols due to their presence in all the plants recovered from one or both procedures. Surprisingly the major part of the variation was shared by the cryopreserved and cold stored samples (50 to 72%) with demethylation the most frequent change comprising 27 to 45% of the total detected variation. On the other hand, different amounts of variation related to each specific treatment were found for every hop accession. Variation ranged from less than 8% in Calicross to around 20% in Tardif de Bourgogne. Nonetheless, in any of the cultivars the variation explained by the storing method was higher than the amount of variation shared by both treatments. This shared pattern could be explained by epigenetic changes related to the cold acclimation step present in both treatments. This consisted of a week or two of growth with temperature/photoperiod set of -1°C 16h dark/ 22°C 8h light. Cold acclimation is a complex process, achieved by short daylength and low temperatures, which results in the reprogramming of metabolism and gene expression. Cold stress regulates the plant transcriptome through transcriptional, post-transcriptional, and post-translational mechanisms which appear within hours of cold exposure. As there are exclusive methylation changes in the cold-stored plants or in the cryopreserved ones of each accession, we can assume that those treatments had at least some effects on the genome. The amount of variation detected is similar for cold-stored (2.6 to 8.6%) or cryopreserved (2.6 to 9.8%) hops. Methylation changes were reported in cryopreserved apple and strawberries (4,5) and citrus callus under slow growth (6). Cold acclimation was not used prior the storage protocols for any of these studies.

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Key words: demethylation, cold acclimation, gene expression, transcriptional, post-transcriptional, post-translational mechanisms, methylation changes

Epigenetic studies in embryogenic cultures of *Pinus pinaster*

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Abstract

Introduction

The main objective was the analysis of total genomic DNA methylation in somatic embryogenesis of *Pinus pinaster*. Epigenetic aspects of somaclonal variation would therefore involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change (2).

DNA methylation represents one of the key processes that play an important role in transcriptional control of gene expression (5). Methylation in animal genomic DNA occurs predominantly at the cytosine residues of sequence such as dinucleotide cytosine-guanine (CpG). In plants, beside in CpG sequences, DNA methylation is detected in the trinucleotide cytosine (CpNpG) (1). Variation in DNA methylation is suggested as an important factor in tissue culture-induced mutagenesis, which can also lead to alterations in chromatin structure and changes in gene expression.

In this study was done an evaluation of global methylation along the somatic embryogenesis process, as a evaluation of the effect of the cryopreservation method in this propagation process.

Methodology

Embryogenic cell lines of *Pinus pinaster* were established from immature zygotic embryos (3) and the cryopreservation procedure was performed as described in Marum et al (4).

A method for the quantification of global DNA methylation was performed by HPCE (High Performance Capillary Electrophoresis) for the detection of the relative percentage of 5-methyl-cytosine in DNA samples of *P. pinaster*. The pine samples included genomic DNA from needles of emblings and seedlings, germinated embryos and mature somatic embryos in different stage of development from tissue cryo and non-cryopreserved.

Results and discussion

The procedure using DNeasy Plant Mini Kit (QIAGEN) with minor modifications for DNA extraction resulted in a high purity DNA and in a complete RNA (figure 1).

In a total of 84 samples analysed, only 3 of them were not RNA-free. The quantification of global methylation was performed with success, in the majority of the samples. In figure 1, the electrophogram indicates a successful separation of the nucleosides.

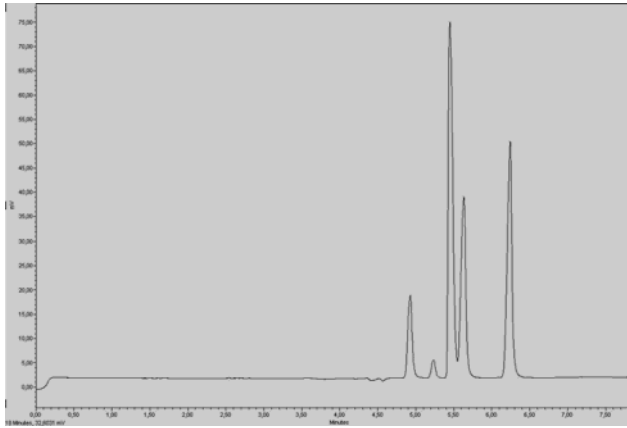


Figure 1. Electropherogram obtained by HPCE after enzymatic hydrolysis of RNA-free genomic DNA, from needles of seedlings germinated *in vitro*.

The starting material for induction of somatic tissue had 18.7% of mdC. After the maturation step, the somatic embryos show an increase in the DNA methylation, with 29% of mdC. The levels of global methylation decrease until the emblings is completely developed. The conditions *in vitro* do not seem to interfere with the process of development because the levels of methylation of emblings *in vitro* and in the field are similar (approximately 17, 4% of mdC).

According to these results the somatic embryogenesis process does not result in any changes in global methylation in emblings tissue. The 5-methyl 2'-deoxycytidine obtained in emblings was similar to the values obtain for seedlings, with the same time in the field (17% mdC).

Acknowledgements

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Key words: total genomic DNA methylation, somaclonal variation, gene silencing, gene activation, trinucleotide cytosine, tissue culture-induced mutagenesis, global DNA methylation

Plant freezing tolerance – from phenotypes to molecules

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Abstract

Considerable effort has been directed towards understanding how plants adapt to low temperature. In common with many plants, the model plant *Arabidopsis thaliana* is able to increase its freezing tolerance when exposed to low, nonfreezing temperatures. Additional improvements in freezing tolerance can be achieved by exposing cold acclimated plants to mild freezing temperatures (sub-zero acclimation). The freezing tolerance of *Arabidopsis* in all three states (nonacclimated, cold acclimated, sub-zero acclimated) is strongly influenced by the geographical origin of the investigated genotype (ecotype). In general, freezing tolerance increases with increasing latitude (from 16 to 66° Northern latitude) and with decreasing habitat temperature during the growth season. Additional genotypic and phenotypic variability can be created by crossing different ecotypes.

Plant freezing tolerance is a multigenic trait. Recently, gene expression studies with microarrays and metabolite profiling experiments using gas chromatography-mass spectrometry have revealed thousands of changes in gene expression and hundreds of changes in metabolite levels in response to cold acclimation and sub-zero acclimation. These changes show significant differences in different *Arabidopsis* ecotypes, opening the possibility of characterizing the functional significance of such changes through correlation with the freezing tolerance phenotype. Through such analyses we are able to identify candidate molecules with a high probability of being functionally important for plant freezing tolerance.

We are interested in two types of molecular changes: those responsible for low temperature signal transduction and regulation of gene expression (mainly transcription factors that regulate the expression of many other genes) and molecules that directly protect cellular structures during freezing and/or severe dehydration. To better understand the regulation of gene expression we are currently investigating the interplay of low temperature and circadian clock regulation of gene expression during cold acclimation and the regulation and function of transcription factors during both cold acclimation and sub-zero acclimation. To identify molecules that may directly affect cellular stability, we use metabolite profiling by mass spectrometry based techniques. This allows us to search for correlations between the cellular content of many compounds and the freezing tolerance of the tissues. The function of compounds of specific interest (e.g. oligosaccharides, LEA proteins) is investigated in detail using biophysical approaches such as fluorescence spectroscopy and infrared spectroscopy to determine their exact mechanisms of action.

Key words: Arabidopsis thaliana, genotypic, phenotypic variability, microarrays, metabolite profiling, low temperature signal transduction, regulation of gene expression, dehydration

Transgenic approach for basic research in cryopreservation - problems and chances

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Abstract

In many fields of basic physiological research molecular methods are applied to create model systems. For the investigation of basic mechanisms of cryopreservation such methods have rarely been used so far.

A possible approach for cryopreservation research could be the over-expression of a protein providing a specific physiological trait in cells or plants and the further analysis of their behaviour in different cryopreservation procedures. Although this approach offers a lot of chances certain problems arise especially when cell cultures are used. One of the problems is the monitoring of protein expression and the correlation with the desired physiological trait. To allow a simple and easy detection of gene expression transformation vectors have been constructed at DSMZ linking a target gene by a viral IRES element to a reporter gene. Such vector achieves a co-expression of both genes forming independent proteins instead of creating a fusion protein. Therefore the target gene can exert its physiological function and this function can be monitored directly by measuring the marker gene expression.

Although co-expression of reporter and target gene is achieved the choice for a suitable marker gene in such vector systems is a problem. The luciferase gene provides the highest sensitivity but it also needs ATP as co-substrate. Green fluorescent protein on the other hand is easily detected and even quantified in each specific cell but is less sensitive and may accumulate in the cells. Cell cultures normally show a certain degree of genetic and physiological variation among the single cells. Our previous results show that cell selection processes during the regrowth period of a cryopreservation experiment can not be excluded and may aggravate the interpretation of results.

Also the comparison of transgenic and wild type cell lines may be difficult. Apart from the desired physiological effect of the transgene other parameters may be different between transgenic and wild type cells. At DSMZ, for cryopreservation experiments with potato cells, we therefore started to characterize physiological aspects of osmotic tolerance of cell lines derived from different potato cultivars before starting transformation work. The data acquired with different un-transformed cultures should broaden the basis for a comparison with transformed cells. At present transgenic potato cell lines have been obtained from the cultivar Desire which probably show increased osmotic and salt tolerance. Transformed Desiree plants presently regenerated will allow a comparison of results obtained with plants and cell lines.

Although problems have to be solved transformed cells lines may offer the chance to investigate the importance of certain physiological traits in specific steps of cryopreservation procedures or the role of certain proteins for providing cryotolerance. Furthermore the natural variation occurring in cell lines may be useful to investigate selection processes due to cryopreservation.

Key words: over-expression of a protein, reporter, target gene, potato, osmotic tolerance, salt tolerance, cryotolerance

Study of hydrophobic proteins and protein complexes involved in cryopreservation of banana (*Musa spp.*) meristems

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Abstract

Cryopreservation (or conservation at ultra-low temperatures (-196 °C)) of meristems is the best method to preserve the banana (*Musa spp.*) diversity safely. A prerequisite for successful application of this technique is the avoidance of irreversible cell membrane damage caused by the formation of intracellular ice crystals. Ice crystallization can only be prevented through a reduction of the cellular water content (= dehydration) to the strict minimum. Acclimation is often essential to survive such a low water content (1). Membrane proteins likely play an important role in the acquisition of dehydration tolerance. As such, a study of the change in the membrane proteome of banana meristems of a dehydration tolerant and sensitive variety will expand the current knowledge of the physiology underlying cryo- and dehydration tolerance. This information will be used to improve the efficiency of current cryopreservation protocols.

One approach to study the meristem proteome is by its separation through two-dimensional electrophoresis (2DE) (2). However, highly hydrophobic membrane proteins largely escape from “classical” 2DE analysis because of their low abundance and their limited solubility in neutral detergent/urea lysis buffers (3).

Low abundance can be solved by enriching fractionation steps. Physical, as well as chemical methods have been described. For banana meristems, fractionation was executed by differential centrifugation in order to obtain a microsomal fraction. However, further chemical enrichment of this fraction was needed. Seigneurin-Berny *et al.* (1999) developed a simple technique to extract highly hydrophobic proteins from chloroplast membranes (4). The method is based on the differential solubilization of membrane proteins in chloroform/methanol mixtures. We optimized this extraction method for banana meristems by determining the ideal proportion of chloroform/methanol to be used as well as by testing alternative precipitation methods and different acrylamide concentrations. Subsequently, the optimised method was applied to search for differential proteins of a dehydration tolerant and sensitive banana variety.

An alternative technique to study hydrophobic proteins, which also gives information about the organization of protein complexes and/or protein-protein interactions, is Blue native PAGE (BN-PAGE). This technique, originally developed by Schägger and von Jagow (5) allows separation of protein complexes and hydrophobic proteins in the mass range of 10 kDa to 1 MDa.

This technique comprises (i) the use of mild, neutral detergents for solubilisation and (ii) the application of Coomassie Brilliant Blue G 250 to give a negative charge to proteins and protein complexes. This will allow separation according to molecular mass. The technique

was optimized and applied to study protein complexes present in the microsomal fraction of banana meristems. Preliminary results are presented.

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Key words: diversity, ice crystallization, dehydration, dehydration tolerance, membrane proteome, meristem proteome

Comparison of some physiological markers prior to and post vitrification in *Hypericum perforatum* L.

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Abstract

The aim of this work is to present the differences between survival rate of *Hypericum perforatum* L. shoot tips cryoprotected with PVS2 or PVS3 and to compare some physiological patterns prior to and post vitrification procedure.

H. perforatum shoot tips pretreated either with 0.076 μ M abscisic acid (ABA) for 10 days or 0.3M sucrose for 16 hours were cryoprotected with two different cryoprotective solutions, PVS2 (10% v/v glycerol, 20% w/v sucrose, 10% v/v DMSO) or PVS3 (50% w/v sucrose, 50% v/v glycerol). Survival rate was determined 7 weeks after thawing. As Table 1 shows we have observed 1.47 to 8.6 times higher survival rates (except for the genotypes 40/7/3 and 42/7/3) using PVS3 after ABA pretreatment, whereas in case of sucrose pretreatment survival rate of most genotypes exposed to the same cryoprotection procedure decreased (except for 29/7/5 and 34/7/1, respectively). Recovered plants were subjected to assessment of some physiological markers. Conductivity, H₂O₂ and MDA content were determined in recovered samples and their control plants (up to 100 mg FW). Our preliminary results indicate that at least one of the parameters studied exceeded level of control values (prior to cryopreservation) after recovery of cryopreserved samples (Figure 2) with an exception of one sample. Possible effect of these findings will be presented and discussed.

Table 1. Survival rates of *H. perforatum* L. samples exposed to different cryoprotective solutions PVS2 and PVS3 after pretreatment with 0.3M sucrose or 0.076 μ M ABA.

Genotype	0.3 M sucrose recovery rate [%]		0.076 μ M ABA recovery rate [%]	
	PVS2	PVS3	PVS 2	PVS 3
5/7/2	3.13	0.00	4.00	34.40
5/7/4	23.30	16.00	12.00	29.40
24/7/5	12.50	3.60	13.30	20.00
29/7/5	0.00	17.86	4.00	21.20
34/7/1	6.60	28.57	4.00	30.00
36/7/2	6.25	0.00	6.00	13.90
40/7/2	3.13	0.00	10.00	14.70
40/7/3	6.25	0.00	12.00	7.89
42/7/3	3.13	0.00	0.00	0.00
42/7/5	12.50	0.00	0.00	12.82

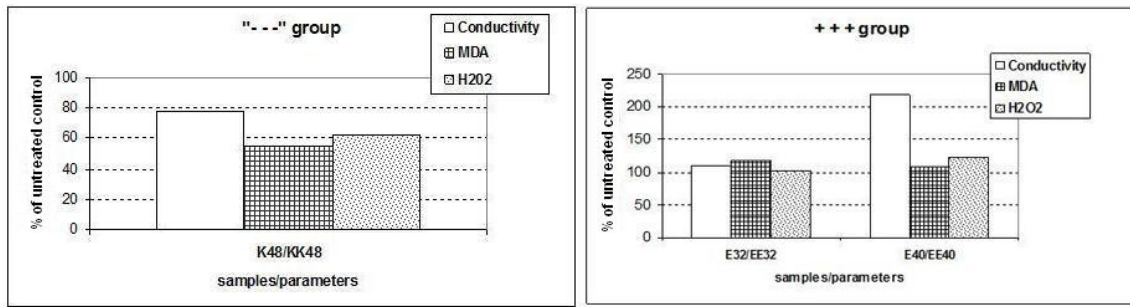


Figure 2. Recovered samples not exceeding (left) and exceeding (right) all three control values

Key words: abscisic acid (ABA), cryoprotective solutions, sucrose pretreatment

Use of secondary somatic embryos improves genetic fidelity of cocoa (*Theobroma cacao* L.) following cryopreservation

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Abstract

Because of the recalcitrant nature of cocoa seed and the vulnerability of field collections it is a priority to establish a replicated cryopreserved base collection of existing cocoa germplasm. Thus approximately 600 accessions of cocoa are being cryopreserved at Reading University through the encapsulation-dehydration of floral-derived somatic embryos (SEs) (1). This vitrification-based procedure involves the rapid cooling of the prolific secondary SEs obtained from cultured cotyledonary explants of primary SEs.

Analysis of embryogenic development using environmental scanning electron microscopy has revealed that, while primary SEs arise from intermediate callus, secondary SEs are generally initiated directly from epidermal cells. Due to concern about somaclonal variation arising as a result of the protracted callus phase involved in the generation of these propagules, their genetic fidelity has been tested and primary SEs have been found to exhibit a significant mutation frequency (2). In this study nuclear microsatellite-based screening has been applied to each of the cocoa linkage groups in SEs sampled from sequential stages of the cryopreservation procedure (ie following culture, sucrose pretreatment, dehydration over silica and thawing after storage in liquid nitrogen) and compared with profiles for the donor tree. For all 48 regenerants tested in duplicate none exhibited aberrant profiles with respect to the donor tree for any of the 12 microsatellites screened. We conclude that, within the limits of this test population, no gross chromosomal changes occurred during cryopreservation and that, until an efficient means of apical shoot culture is established for cocoa, secondary SEs constitute the best target tissue for cryopreservation of germplasm.

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Key words: cotoledonary explants, encapsulation-dehydration, somaclonal variation

Cryopreservation of olive embryogenic cultures

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Abstract

Introduction

Embryogenic cells have a high value for Biotechnology as they are the material of choice for applications such as genetic transformation, in vitro mutagenesis and in vitro selection. However, such cultures can be lost through contamination or possible genetic changes can take place due to somaclonal variation. Moreover, the embryogenic capacity decreases during long-term maintenance on nutrient medium. In consequence, it is recommended to apply a safe, long-term conservation method assuring maintenance in optima conditions. Cryopreservation is considered as the only available method for such safe long-term storage of plant material.

Materials and methods

Embryogenic cultures of olive (*Olea europaea* L.) were initiated from radicle segments of mature zygotic embryos of cultivar 'Picual' (1) and maintained by repetitive embryogenesis on OMe medium according to Pérez-Barranco et al. (2).

To test the influence of the protective solutions utilized in the vitrification-based procedures on survival and proliferation of olive embryogenic cultures, we treated them with loading solution (LS) and PVS2 solution during different time periods without LN exposure. Olive embryogenic cultures can be very heterogeneous, with cell lines proliferating as calli and others containing mainly somatic embryos at different developmental stages. To test the influence of plant material, both extremes (callus vs. somatic embryos) were tested in these experiments: non-organized embryogenic tissues, selected from the cell line P4, (P4.1) and somatic embryos (1-3 mm), selected from the cell line P1, (P1).

In the first cryopreservation experiment with exposure to LN, three cryopreservation protocols were compared using P4.1: 1) the vitrification-based protocol of Thinh et al. (3), 2) an ultra fast method with droplet vitrification on aluminium foil strips (4) and 3) a slow freezing method (1°C/min) (5). In the first and the second procedures, dehydration with PVS2 solution at 0°C was carried out for 30 and 60 min.

In the second experiment, we tested the effect of a long-term preculture treatment. For this, we used P4.1 and embryogenic material selected from cultures of the P4 cell line maintained during 7-8 weeks in proliferation medium supplemented with 0.4 M sucrose (P4 suc). Here, only the droplet vitrification method was used.

Results and discussion

Prolonged treatment of embryogenic cultures with LS had an adverse influence on the regrowth rate (assessed as the increase in fresh weight) of both, organized and non-organized embryogenic tissues.

Dehydration by using the vitrification solution PVS2 resulted in a significant reduction in the proliferation rate and a decline of cultures appearance. Six weeks after dehydration

treatment, cultures proliferation rate was significantly affected by the time of incubation in the PVS2 solution as well as by its interaction with culture type (P4.1 or P1).

Olive embryogenic cultures responded differently to the three cryopreservation methods tested. After controlled-rate cooling only 10% viability (determined as percentage of cultures resuming proliferation) was observed. Vitrification approaches were more effective assuring high levels of post-thaw viability (77.78-100%). The highest viability percentages and regrowth rates were obtained with the ultra fast method with droplet vitrification on aluminium foil strips. Embryogenic cultures responded better to the droplet method when previously dehydrated with PVS2 during 60 min. At these conditions, 100% of cultures showed embryogenic proliferation at the end of the first reculture.

Long-term preculture on medium containing high sucrose concentration showed a significant influence on the initial cultures response. In sucrose precultured material new proliferation was already observed 7 days after thawing while for embryogenic tissues taken directly from proliferation medium, survival was not observed until 18 days after thawing. However, preculture treatment did not significantly improve survival percentage or regrowth rate after cryopreservation. Twelve weeks after initiation, the main effect of the preculture treatment can be attributed to protection to the vitrification solutions.

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Key words: droplet vitrification, long-term preculture, non-organized embryogenic tissue, organized embryogenic tissue, post-thaw viability, slow freezing, vitrification-based protocol

Temperature Modulated Differential Scanning Calorimetry - a tool for evaluation of plant glass transition at low temperatures

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Abstract

There are two main cryopreservation groups of methods for plant meristematic tissue. The first group of cryopreservation methods is based on water freezing inside the tissue (mostly outside cells) after slow cooling rates. The second group of the methods is based on vitrification. Low water content and high cooling rates leads to the glassy state in plant tissue. The differential scanning calorimeter (DSC) is an appropriate instrument for both types of the methods to give exact information for controlling and/or improving the cryoprotocol. Water together in liquid, solid and vapour forms play a crucial role for survival of shoot tips after cryopreservation.

The DSC measurement can help us to measure the amount of frozen water within cryopreservation protocol. In addition to these characteristics, the DSC determination of ice nucleation temperature is of importance too. For the first method supposing that plants are able to tolerate frozen water outside the cells, the information about ice nucleation temperature is crucial. If the ice nucleation temperature occurs at too low temperatures the burst freezing after deep supercooling inside the cells causes the lethal injury and death. The DSC is important also for the second method – vitrification because of the glass transition temperature. It should be close to higher temperatures, close to zero, to decrease the probability of water crystallization during samples exposition to low temperatures. The measurement of ice nucleation is important for vitrification cryoprotocols as well. Vitrification is a method avoiding the ice nucleation, mainly by high rate of cooling and warming. In conclusion, there are four important thermal characteristics which can be obtained by DSC measurements: ice nucleation temperature, melting temperature, amount of frozen water and glass transition temperature.

According to our experience the glass transition temperatures of plant samples were very close to their thawing temperatures. In some cases these two thermal events are so close that it is too difficult or in some cases completely impossible to differentiate them. At these cases the conventional DSC technique is unable to separate the thermal events. Recently, the differential scanning calorimetry with modulated temperature gave good tool for measurement of such samples.

Since 1992 a new DSC method based on sinusoidal temperature modulation was introduced by Reading and co-workers. By temperature modulated differential scanning calorimetry (TMDSC) it is possible to obtain more information about sample in comparison with conventional DSC using linear change of temperature. TMDSC heat flow signal is composed of two parts: a) reversing heat flow - heat capacity component, heating rate dependent responsible for glass transition and some melting and b) nonreversing heat flow - kinetic component, time dependent responsible for crystallization, some melting and enthalpy relaxation. Conventional DSC can only measure the sum of these two components. Heat capacity (C_p) is generally calculated from the difference in heat flow between blank run and sample run under identical conditions including cooling/heating rate. In TMDSC, C_p is determined by dividing the modulated heat flow amplitude by the modulated heat rate amplitude.

The modulation type is specific for each instrument. Sinusoidal and jig-saw type of temperature modulation controlling the sample temperature is mostly used and is typical for each instrument of each producer. A new method – stochastic modulated temperature was recently published /2/.

Quasi-isothermal temperature modulated DSC (QITMDSC) method is based on analysis of sample response to modulated temperature around the constant temperature. After constant output of heat capacity of the sample the temperature abruptly changes to new modulated temperature. Discrete Fourier transformation is used mostly for data evaluation of TMDSC and QITMDSC methods. The evaluation is a complex of difficult mathematical system of equations but modern software of the instrument is able to evaluate the measured data.

TMDSC has several significant practical advantages. For example, in glass transitions studies the limit of detection and resolution increases without loss of sensitivity, that makes the correct assignment more certain and quantification of amorphous phases is more accurate /1/.

It is advantage to know the temperature range of glass transition before application of temperature modulated DSC. On the base of preliminary measurement by convenient DSC it is possible to decide which parameters of thermal analysis methods are appropriate for measuring TMDSC or QITMDSC. Three parameters can be chosen independently for each method with modulated temperature - modulated amplitude, modulated period and rate of cooling or heating. Typical modulated amplitudes are between 0.1 – 1 °C with modulated period 60 - 100s (0.017 – 0.1Hz). Typical cooling/warming rate used is 10 °C/min. Larger amplitude leads to higher sensitivity, smaller amplitude leads to higher resolution. So far, it has not been possible to change the type of modulated signals in an instrument. This option is influenced by setting of particular instrument by individual producer. Usually, the measurement by temperature modulated DSC technique is performed and the data for analysis are collected during sample warming because stochastic event of ice nucleation is avoided. A proper choice of amplitude and modulation allows keeping plant sample at permanent thawing or at both thawing and freezing events during modulation of the sample. For melting/crystallization studies “heat-only” amplitude should be used. There is an exact calculation of the maximum temperature amplitude (T_{amp}) for “heat-only” modulation: $T_{amp} = Hr P/2\pi$, where: Hr is average heating rate, P is period of modulation.

Examples of application

Because the plant samples are very complex it is impossible to distinguish overlapping melting and glass transition event by convenient DSC in some cases. For example, temperatures of both thermal events were close to each other in apple dormant buds and *Allium* shoot tips. A broader temperature range of delta enthalpy change was typical in these species.

Acknowledgement

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Key words: meristematic tissue, shoot tips, cryopreservation, ice nucleation temperature, water crystallization, melting temperature, reversing heat flow, nonreversing heat flow,

Cryoprotection of *Cyathea australis* spores and gametophytes

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Abstract

Introduction

Ferns present unique plant experimental material because of haploid generation presents different level of morphogenic potential with single cell spore and in majority one cell layer composed of mitotically and photosynthetically active cell. Woody one originate from tropics, the glob areas never caused by glacial era and drying stress. Mention above reasons make the woody ferns excellent plant experimental material for cryopreservation. The aim of studies was to develop crypreservation system for one of most popular species among woody ferns *Cyathea australis* (R.Br.) Domin, which vegetative propagation by gametophyte culture was earlier described elsewhere (1,2).

Material and methods

Experiments have been carried out with young stages of gametophytogenesis of *Cyathea australis* using spores and gametophytes. Spores were collected from immature (closed) and mature (open) sporangia. Gametophytes derived from directly germinated spores in prothallium or their adventitious culture. All media and preculture solution based on hormone free 0.5MS medium (3).

Cryo-methods used:

Spores were directly immersed in LN, gametophytes or their fragments were cryopreserved with application of encapsulation methods. Following treatment combinations were hold: 1) 1-2-week long preculture with 0.25 M sucrose with or without ABA, 2) osmotic desiccation – 3 or 6 day long pretreatment, 3) air desiccation - air dried up with sterile air (5 hrs), 4) direct immersion of vials with material into LN. The viability of plant material was assessed after 10-day-long spore culture or 4-week-long gametophyte culture (express in the % of viability). Cell viability was assessed with the application of natural response in the presence of filter BV440-440 (chlorophyll auto-fluorescence). Five grades scale of gametophyte viability response was employed: from non-disturbed to completely non-viable cell.

Results

Cyopreservation of spores

Spore freezing without any protection resulted in surviving of low temperature stress and mature spores were able to carried on over 75% surviving non depending on treatment. The stress of desiccation and low temperature help to germinate freshly collected non-mature spores. Their ability to germination increased from 1.9% to 40.5% after cryopreservation. However, LN resulted in reduction of spore germination from 57.1% to 3,2% in case of 11day long air desiccation prior cyropreservation.

Table 1. Spore germination ability (%) treated with 24-hrs freezing in LN after 11-day-long air desiccation at room temperature.

Spores	Freshly collected		After desiccation	
	-LN	+LN	-LN	+LN
mature	75.4 ± 12.1	79.5 ± 11.3	0.0	0.0
immature	1.9 ± 0.6	40.5 ± 6.3	57.1 ± 6.3	3.2 ± 4.2

Cryopreservation of gametophyte

One-week long preculture in liquid medium in comparison to two-week agar medium appeared insignificant on the increase of capsulated gametophyte surviving after freezing, however, double extension of time negatively resulted in explant viability (Fig. 1). Medium supplementing with ABA effected in 4-7 fold of explant surviving. The reduction of 6-day long pretreatment down to 3 days did not result in decreasing of gametophyte surviving after freezing. There was not found various response of explant directly developed from spores and adventitious gametophytes. In all cases 80% of explants survived. It is necessary to stress that in all explants the number of cells capable to survive the low temperature treatment was very high and was estimated about 25%. In many explants only a few cells were able to survive, however, in our system of gametophyte multiplication, that was sufficient to regenerate adventitious gametophytes.

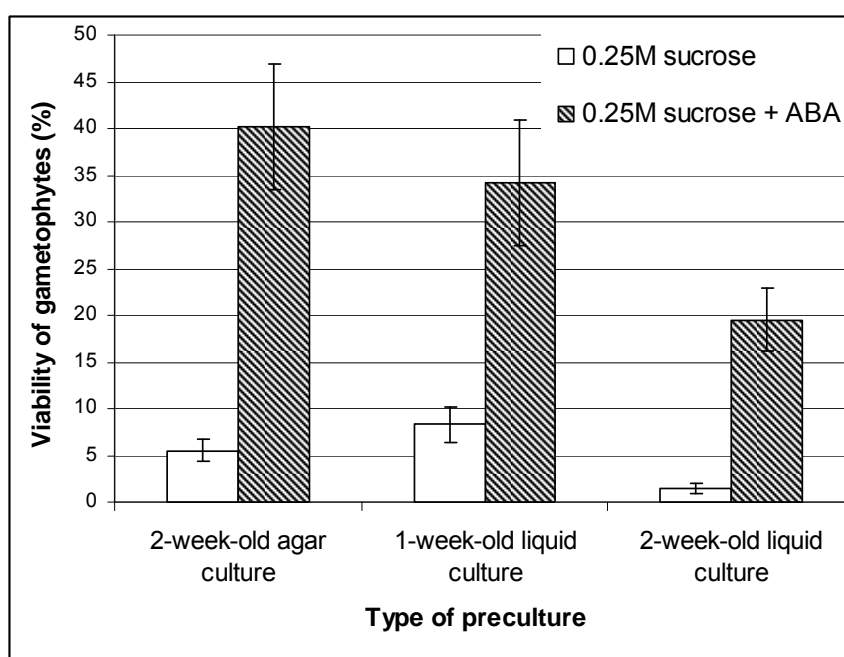


Figure 1. Effect of type preculture (agar or liquid) and abscisic acid (ABA) on the gametophyte viability (2-week-culture with ABA: 1st week only 0,25M sucrose, 2nd week 0.25M sucrose + 0.1M ABA).

Conclusions

- 1) Spores are able to survive deep freezing without any cryoprotection reaching 40-75%.
- 2) The gametophyte surviving was very varied presenting level 40-80%, however, relatively low level of particular cell surviving requires additional experiments and it should be improved.

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Key words: woody ferns, encapsulation methods, sucrose, ABA, osmotic desiccation, air desiccation, direct immersion into LN, cell viability

The current status of conservation of plant genetic resources in IBISS and related cryopreservation activities

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Abstract

The presentation has two parts: to describe the current status of genetic resources stored in plant tissue culture at Institute for Biological Research “Sinisa Stankovic” (IBISS), Belgrade, Serbia and to present some results on fundamental aspects of cryopreservation research.

The IBISS represents the largest and the most complete research institution in the field of biological sciences in Serbia. The researchers at the IBISS are primarily focused on fundamental studies in biology and are included in projects supported by Ministry of Science of Republic of Serbia. The IBISS is organized on the basis of research teams working within specific departments. The profile of researches performed at Department of Plant Physiology is characterized by basic lines of developmental plant physiology (photomorphogenesis, *in vitro* morphogenesis and cytodifferentiation) as well as of experimental plant biology (genetic engineering, secondary metabolite production, fungal growth and development). The IBISS has a specialized unit for experiments on *in vitro* plant cultures (160 m²) and developed protocols for micropropagation of more than 50 plant species. The summary of endemic, endangered and pharmaceutically valuable plant species kept as *in vitro* cultures in IBISS will be presented. Cryopreservation is a valuable alternative to the *in vitro* collection for conserving different types of the plant material which is planned to develop in future.

Second part of presentation is some fundamental aspects of cryoprotection. This study investigated the effects of one month acclimation on the accumulation of soluble sugars (sucrose, fructose and glucose) and polyols in *Fritallaria meleagris* bulbs formed in culture *in vitro*. Analysis of carbohydrate content revealed that sucrose was the dominant soluble carbohydrate in bulblets of *F. meleagris*. There was no high increase of sucrose accumulation in bulblets after low temperature storage. Monosacharides (glucose and fructose) are present at significantly lower content than sucrose at standard conditions. The significantly increased accumulation of glucose and fructose in bulblets was observed after low temperature treatment but the magnitude of change was much less for glucose than for fructose. The analyses of polyol content in bulblets of *F. meleagris* revealed more than twice higher accumulation of sugar alcohols after low temperature treatment (Table 1). Also the SOD activity during and after acclimation will be presented.

Table 1. The carbohydrate content in bulbs after one month acclimation at 4°C.

Treatments	Water (%)	Carbohydrate (mmol/100 g dry weight)			
		Sucrose	Glucose	Fructose	Polyols
25°C	77.64	30.42 ± 1.47	3.54 ± 0.39	2.18 ± 0.18	0.64 ± 0.17
4°C	90.67	32.01 ± 2.11	4.78 ± 0.77	7.73 ± 1.17	1.46 ± 0.16

Each value represents the mean ± standard error.

Key words: in vitro collection, acclimation, Fritallaria meleagris, carbohydrate content, polyol content

Characterization of cryoprotective activity of thermal hysteresis protein in evergreen plants

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Abstract

Thermal hysteresis proteins (THPs) inhibit the growth of ice by binding to the surface of ice crystals, preventing the addition of water molecules to cause a local depression of the freezing point. Recent findings demonstrate that apoplastic THPs from Norway spruce show a cryoprotective and also anti-ice nucleation activity (Jarzabek et al. 2008). Here, we have focused on the most active THPs from five frost-hardy conifers of North American and Eurasian boreal forest: *Abies grandis* (Douglas ex D.Don)Lindl., *Picea pungens* Engelm., *Pinus nigra* J.F.Arnold, *Pinus sylvestris* (L.), and *Tsuga canadensis* (L.) Carriere, and additionally from one alpine (2100 m a.s.l.) evergreen shrub *Loiseleuria procumbens* (L.). The objective of this study was to determine whether these thermal hysteresis proteins of frost-hardy plants (THPs) influence survival at subzero temperatures by modifying the freezing process and/or by acting as cryoprotectants. Apoplastic extracts were obtained by vacuum infiltration of leaves with 5 mM ascorbic acid, and the extracts were concentrated by using a 10 kDa cutoff Ultrafree centrifugal filter device (Millipore). Proteins were separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Leammler 1970). Cryoprotective activity of apoplastic proteins was determined with the use of the freeze/thaw inactivation, by four cycles in liquid nitrogen (-196°C) and room temperature while the assay of lactate dehydrogenase (LDH) was performed as described by Wisniewski et al. (1999). Antifreeze activity was determined by using the droplet freezing assay (Vali 1971).

Table 1. Properties of apoplastic cryoprotectant proteins from evergreen woody plants

Species	FP ^a	MP ^b	TH ^c	LDH ^d	ST ^e
	[°C]			THPs, BSA [%]	[°C]
<i>Abies grandis</i>	-4.33	-1.00	3.33±1.2	41.0 ±2.5	-10.6 ±1.4
<i>Picea pungens</i>	-2.30	-0.30	2.00±0.4	44.3 ±1.2	-8.9 ±1.0
<i>Pinus nigra</i>	-2.28	-0.16	2.12±0.6	31.6 ±0.5	-9.0 ±1.5
<i>Pinus sylvestris</i>	-1.45	-0.16	1.39±1.7	12.0 ±0.5	-10.5 ±1.0
<i>Tsuga canadensis</i>	-4.87	-0.68	4.19±1.9	35.9 ±4.2	-11.2± 0.04
<i>Loiseleuria procumbens</i>	-3.13	-0.40	2.73±1.0	90.1 ±3.5	-7.6± 0.4
BSA (control)	-0.13	0.22	0.35	23.1 ±2.5	-

^aFP, freezing points (the temperature of ice crystal growth)

^bMP, melting point

^cTH, thermal hysteresis, is the difference between the freezing and melting point of samples, means and ±SD

^dLDH, lactate dehydrogenase activity compared with the activity of unfrozen control, in vitro for concentration of 25 µg ml⁻¹ of THPs or bovine serum albumin (BSA), means and ±SD.

^eST, supercooling temperature water in needles (leaves). ST were analyzed by DTA method.

The results showed that the frost-hardy conifer species during winter, and the alpine plant also in spring, accumulated THPs in the apoplast of leaves. Five major polypeptides were separated from these extracts by SDS-PAGE. When the possible role of THPs of alpine shrubs and conifers in cryoprotection was examined, it was discovered that LDH activity was higher after freezing in liquid nitrogen in the presence of THPs, as compared with the unfrozen control or with bovine serum albumin (BSA) (Table 1). In comparison with apoplastic THPs of *P. sylvestris*, the cryoprotective activity of AFP proteins from the alpine shrub *L. procumbens* (LpTHPs) was 7-fold higher in terms of LDH activity. Similarly in comparison with BSA, LpTHPs at 25 µg concentration was about 4 times more active. The present study provides a procedure for purifying THPs and provided that THPs from frost-hardy plants has both cryoprotective and antifreeze activity (Table 1). Further research is necessary to establish if the observed *in vitro* cryoprotective activity of these apoplastic THPs is important for cryoprotection *in vivo*.

Acknowledgement

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Key words: anti-ice nucleation activity, Abies grandis, Picea pungens, Pinus nigra, Pinus sylvestris, Tsuga canadensis, Loiseleuria procumbens, antifreeze activity, droplet freezing assay

Dehydration of grapevine dormant buds in relation to cryopreservation

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Abstract

Introduction

In most cases, cryopreservation of dormant buds by slow freezing procedure needs to remove excessive water content from dormant buds before the freezing part of the protocol takes place (1,2). For grapevine cultivars belonging to plants that do not survive very low temperatures the dehydration of buds is an important factor for their cryopreservation. The aim was to evaluate dehydration tolerance of dormant buds of selected grapevine cultivars before the first step of cryopreservation protocol with regard to their thermal characteristics measured by Differential scanning calorimetry (DSC).

Materials and methods

One-year old shoots of grapevine; ‘Auxerrois’, ‘Muller-Thurgau’, ‘Portugal blue’ and ‘Rhein Riesling’ were collected from vineyards in Research Station for Viticulture, Karlstejn, CRI during January and February 2007. Uni-nodal cuttings were cut from shoots and subjected to 6-week dehydration at -4 °C in freezing box. Regrowth tests and thermal evaluations by the differential scanning calorimetry (DSC) were provided during the dehydration period. Control buds for regrowth tests were placed into water and establishment of growth was evaluated. At the level of lethal dehydration (LD50) only 50 % of buds were able to grow. The calorimetry measurement was done in Differential scanning calorimeter TA2920. Samples were cooled down to -100 °C and then warmed up to 20 °C. The cooling/heating rate was 10 °C/min. The purge gas was helium. Water content of buds was determined gravimetrically. Dry matter was expressed after drying at 85 °C to constant weight (minimally for 2 days).

Results

After 6 weeks of dehydration, the lowest water content of dormant buds was measured in ‘Rhein Riesling’ and ‘Muller-Thurgau’, 0.27 g H₂O/g dry matter and 0.28 g H₂O/g dry matter, resp. In contrary, ‘Portugal blue’ and ‘Auxerrois’ dehydrated in the same conditions to 0.33 g H₂O/g dry matter and 0.35 g H₂O/g dry matter, resp. Dormant buds dehydrated more than internode segments. The amount of crystallized water positively correlated with the water content in the buds, see fig. 1. The lowest percentage of crystallized water in the buds of the same level of dehydration had ‘Rhein Riesling’ and ‘Portugal blue’ contrary to ‘Muller-Thurgau’ and ‘Auxerrois’. In dormant buds dehydrated to the level of 0.29 g H₂O/g dry matter glass transition was detected. In such dehydrated dormant buds only small or none exotherms were detected. Regrowth analysis revealed different sensitivity of evaluated cultivars to dehydration. The highest dehydration tolerated ‘Rhein Riesling’ (LD50 at 0.37 g H₂O/g dry matter) and the lowest dehydration tolerated ‘Portugal blue’ (LD50 at 0.50 g H₂O/g dry matter).

Conclusion

Evaluation of dehydration phase of the first step of cryopreservation protocol for dormant grapevine buds was performed. The course of dehydration of dormant grapevine buds of four cultivars was evaluated according to their thermal measurement by differential scanning calorimeter. Dormant buds were dehydrated from 0.65 g H₂O/g dry matter down

to 0.27 g H₂O/g dry matter and the percentage of crystallinity ranged from 58% to 3%. At dehydration below 0.29±0.018 g H₂O/g dry matter the glass transition in buds was monitored. Further study will be focused on the survival of dormant buds after cryopreservation procedure.

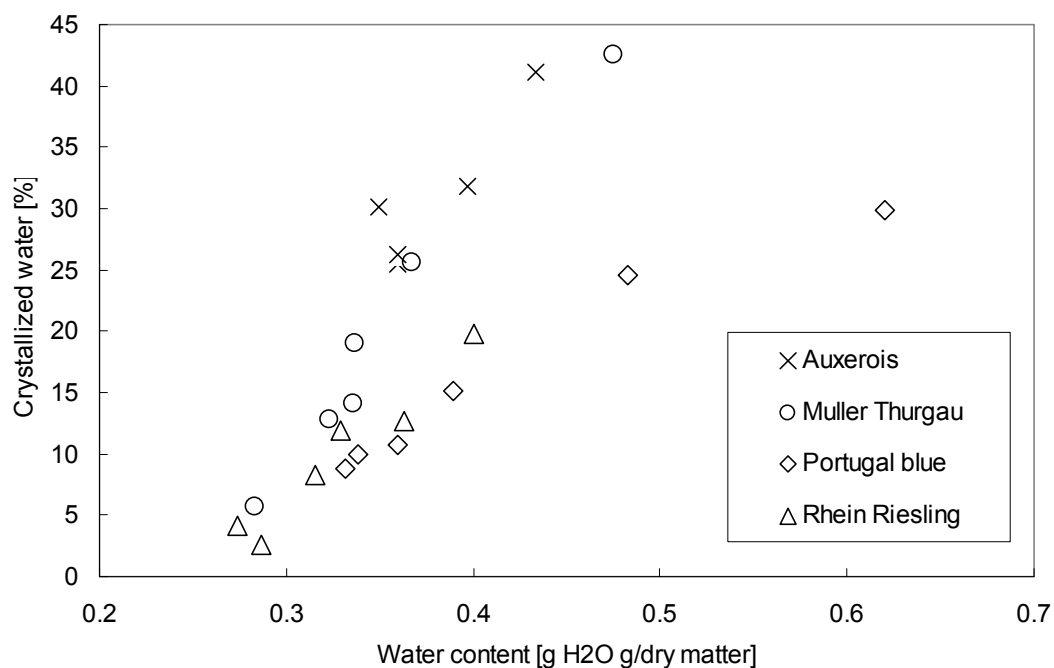


Figure 1. The amount of crystallized water of in dormant grapevine buds of cultivar 'Auxerois', 'Muller-Thurgau', 'Portugal blue' and 'Rhein Riesling' was measured by differential scanning calorimetry (DSC). The amount of crystallized water in the bud was counted from the area of the endothermic peak appearing during heating of the sample. The samples were cooled down to -100 °C and after equilibration of the signal they were heated up to 20 °C. Cooling/heating rate was 10 °C/min.

Acknowledgement

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Key words: crystallized water, glass transition, slow freezing procedure

Development of cryopreservation strategies for a recalcitrant seed species using differential scanning calorimetry

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Abstract

Introduction

The development of cryo-conservation for sensitive germplasm requires the consideration of both cryogenic (cryoprotectant and low temperature) and non-cryogenic (pre- and post-storage culture) factors (see references 1-4). Thermal analysis using differential scanning calorimetry (DSC) is applied to help optimise vitrification-based cryoprotection strategies with the aim being to integrate fundamental and applied approaches to assist the development of storage protocols for *Parkia speciosa* species that produce recalcitrant seeds.

Materials and methods

- shoot tips obtained from *in vitro* seedlings were precultured on MS medium supplemented with trehalose at (2.5, 5 & 10%) for 3 days, encapsulated in alginate beads and cryoprotected using PVS2 for various periods before storing in liquid nitrogen for 24 hours as described in Figure 1.
- DSC was used to investigate the water phase transitions in the shoot tips during cooling and warming cycles.

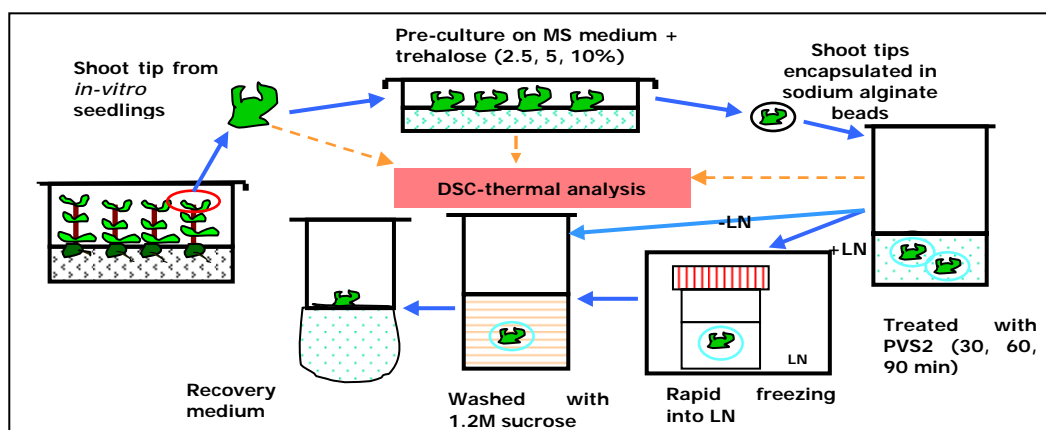


Figure 1. Schematic diagram for the encapsulation-vitrification cryopreservation strategies

Results

- Control shoot tips showed clear evidence of ice nucleation during cooling and ice melting events during subsequent rewarming.
- Concentration of trehalose does not have a significant effect on the water thermal behaviour.
- PVS2 duration did show a significant effect of dehydration where free water was completely removed after 60 min treatment.
- Though 90 min PVS2 treatment showed stable thermal profiles, no survival was recorded for this treatment.
- 60 min PVS2 treatment was found optimum for these shoot tips with survival above 70% after freezing.

Conclusions

DSC elucidated the critical points at which vitrification occurred in *P. speciosa* shoot tips exposed to trehalose and PVS2. A 60 min PVS2 treatment supporting ca. 70% survival was found optimal for stable glass formation during cooling and rewarming.

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Key words: Parkia speciosa, differential scanning calorimetry, vitrification, trehalose

Progresses in cryopreservation of *Pyrus* spp and evaluation of genetic stability of the recovered shoots

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Abstract

Encapsulation-dehydration and vitrification methods were applied to shoot tips (2-4 mm in length) of *Pyrus communis* (Cv William, Decana and Rootstock FAR 40) and *Pyrus pyraister* to established a suitable protocol of cryopreservation for application in *Pyrus* germplasm collection of the CRA - Fruit Tree Research Center in Rome.

The protocol, previously established for *Pyrus pyraister* with recovery of 60% after liquid nitrogen immersion and consisting in dehydration of Na-alginate beads for 2 days in 0.75M sucrose and desiccation to 20% moisture content (fresh weight basis), is being tested on *Pyrus communis* genotypes optimising the procedures of pre-treatment, of dehydration (Glucose or sucrose at various concentration and with time of application ranging from 1 to 5 days) and of desiccation in silica (8 to 20 hours of application) according to the genotype. The highest recovery (26%) has been so far obtained in William with a dehydration in sucrose 1M for 3 days and a silica desiccation of 10 hours. Other factors, such as cold and/or high sugar concentration pre-treatments, are now under evaluation to optimise the protocol. The cryopreservation of the pear genotypes by the vitrification method was also considered. PVS composition and time of application of the vitrification solution were tested and preliminary results will be discussed.

Genetic stability of the shoots of *Pyrus pyraister*, recovered after cryopreservation by encapsulation-dehydration, was also evaluated. Fifteen single bud derived lines were used for genetic analyses by RAPDs and SSRs. In RAPD analysis, among a total of 24 ten-mer primers used to amplify all the genotypes, 15 showed reproducible and well resolved bands. These primers produced a total of 66 fragments ranging from about 500 to 2500 base pairs size. SSR marker amplification was performed using 19 pair-primers which produced 57 reproducible fragments. Microsatellite fragments ranged from 60 to 600 base pairs. Both markers did not reveal any polymorphism between cryopreserved shoots and the original genotype. These results cannot represent the final proof that no somaclonal variation occurs cryopreserving wild pear by encapsulation-dehydration method, however, RAPDs and SSRs were shown to be more efficient than other markers in determine somaclonal variation in tissue culture and their combination gives more robust results than when they are used singularly, allowing us to be more confident in the stability of the cryopreserved material and in the possibility of a wider application of the encapsulation-dehydration method for long term conservation of *Pyrus* genotypes of the germplasm collection.

Key words: dehydration, desiccation, vitrification, encapsulation-dehydration

Protein and carbohydrate analyses of abiotic stress underlying cryopreservation in potato

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Abstract

Cryopreservation complements classical conservation methods, which are carried out in the field or *in vitro*. It involves the storage of biological material in liquid nitrogen (-196°C). At this temperature all chemical and physical processes are stopped, allowing a safe storage over an unlimited period of time. The aim of our work is to understand the effects of pretreatments, by increasing the osmotic pressure of the medium, on the cryopreservation ability of potato shoot tips and more specifically to try to evaluate the effects of such pretreatments on the metabolism of potato. Indeed, drought acclimation is known to improve recovery after cryopreservation in potato and other species

In vitro Désirée potato shoots were precultured for 21 days on regular MS and MS complemented with 0.055M, 0.11M, and 0.22M sorbitol. Directly after pre-treatment, leaf and shoot tip samples were taken and stored at -80°C for proteomic and carbohydrate analyses. In addition, cryopreservation was carried on the precultured shoot-tips.

For the cryopreservation, potato shoot tips were cut from pre-treated plants and incubated in a high osmotic Loading Solution. Afterwards, shoot tips were placed on an aluminum foil strip in droplets of Plant Vitrification Solution 2 and plunged into liquid nitrogen (Agrawal, 2004). Thawing was done in a highly osmotic Recovery Solution at room temperature, to prevent osmotic shock. After cryopreservation, shoot tips were transferred into the dark for 1 week (Panis, 2005). During the initial days of post-culture, shoot tips were maintained on MS media containing 0.3M sucrose. Afterwards, regular MS media were used. After 30 days, recovery was calculated as the percentage of shoot-tips forming new shoot.

Proteins from shoot tip and leaf samples were extracted, using a TCA/Acetone ex-traction method. After quantification, 40 mg protein was labelled using three different fluorescent dyes (Cy2, Cy3 and Cy5). In this way, 2 samples and an internal standard - containing a mix of all the samples - was loaded on the same IEF strip (pI 4-7). Isoelectric focusing was carried out using the GE Healthcare Ettan IPGphor. The second dimension was run in a GE Healthcare Ettan Dalt Six electrophoresis system. Gels were scanned using the Typhoon 9400 scanner and subsequently analysed with the GE Healthcare DeCyder program and EDA module. As such, differences in protein expression could be quantified and differentially expressed spots picked by the GE Healthcare Ettan Spot Handling Workstation (Renaut, 2006). In a following step, spots were digested, using Trypsin and spotted on a MALDI plate. Protein identification was done, using the Applied Biosystems MALDI 4800 TOF/TOF analyzer.

For the analysis of carbohydrates and polyols, roughly 100 mg leaf samples was used for carbohydrate and polyol extractions. The following carbohydrates and polyols were measured: sucrose, glucose, fructose, galactose, stachyose, arabinose, melibiose, maltose,

trehalose, inositol, mannitol, galactinol and sorbitol. Carbohydrates were analyzed on a Dionex HPLC ICS2500-Bio LC, using a Carbopac PA-20 column. HPAEC-PAD analyses for polyols was conducted on a Dionex DX-500 chromatograph, using a Dionex Car-bopac MA1 column.

Recovery rates without sorbitol were around 50% but increased with increasing concentration of sorbitol pre-treatment up to a recovery rate up to 80%.

For 2D-DIGE proteomics, preliminary analysis of the gels showed differences in protein patterns, when plants were precultured on different sorbitol media. Fifteen up- or down-regulated proteins were isolated and identified. Interestingly, these preliminary results indicate strong alteration of the primary metabolism and more precisely in carbon fixation. These results are sustained by the carbohydrate and polyol analyses. Indeed, sucrose, glucose, fructose, mannitol, arabinose, galactinol, meli-biose and stachyose increased with increasing concentrations of exogenously supplied sorbitol up to 0.11M sorbitol. At 0.22M sorbitol their concentrations decreased. Trehalose and sorbitol concentrations increased exponentially with an increasing molarity of sorbitol pretreatment. Maltose was only observed when plantlets were treated with 0.22M sorbitol.

When plants were treated with up to 0.11M sorbitol during 21 days, carbohydrate and polyol concentrations increased. When 0.22M sorbitol was applied as pre-treatment, most sugar concentrations decreased. Since high intracellular osmolyte concentrations are needed to allow successful recovery after cryopreservation, the results from carbohydrate and polyol analysis (Bhandal, 1985) may explain the higher recovery rate after cryopreservation observed after sorbitol treatment. Extra cryopreservation experiments are needed to confirm these results. Beside the sugar analysis, these changes affecting the primary metabolism have been observed at the protein level by using differential in gel electrophoresis. Further experiments including also chilling pretreatments will lead to a better understanding of the physiological status of the tissue and will hopefully explain the reason of the better results of cryopreservation.

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Key words: shoot tips, drought acclimation, sorbitol, recovery, sucrose, glucose, fructose, galactose, stachyose, arabinose, melibiose, maltose, trehalose, inositol, mannitol, galactinol, sorbitol.

Cryopreservation of forest trees – potentials and applications in Metla

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Abstract

Introduction

Development of cryopreservation methods for forest trees has been going on in Finnish Forest Research Institute, Metla, since the middle of 1990's. Research on cryopreservation techniques was initiated, firstly because there was need for alternative or back-up method for *in vivo* germplasm conservation under fast changing environment. The second call was from biotechnological research; to save tissue cultured material safely without contamination under minimum space and maintenance requirements, together with the necessity to maintain regeneration ability of *in vitro* grown SE-cultures of conifers. In Metla, cryopreservation methods have been developed for the economically important forest tree species growing in Finland, e.g. for silver birch (*Betula pendula*, Roth), hybrid aspen (*Populus tremula* L. x *Populus tremuloides* Michx.) and Scots pine (*Pinus sylvestris* L.). For Norway spruce (*Picea abies* (L.) Karst.) there are applicable methods developed elsewhere.

The easiest protocol to cryopreserve birch and aspen is to cryopreserve dormant vegetative buds. No cryoprotectants are needed, but a freezer for slow cooling of buds is necessary. The recovery (58 - 92%) of buds after cryostorage is equal to initiation of micropropagation from non-cryopreserved buds, and is dependent on the genotype of the tree, too (3). An important application of this protocol is cryopreservation of dormant buds of trees growing in the greenhouse at 5°C in the autumn. Special targets of this method are transgenic lines of silver birch and hybrid aspen, which are not allowed to be transferred outdoors (2,8).

Both slow cooling and vitrification are usable methods for cryopreservation of *in vitro* apical shoot tips and axillary buds of silver birch, but cryopreservation of *in vitro* hybrid aspen is successful with vitrification only. Substitution of ammonium in the medium by 10 mM KNO₃ already during cold hardening of *in vitro* shoots for 4 weeks at 5°C under SD (8/16, light/dark conditions), and using the same inorganic substitution in different media for three days after thawing resulted to highest recovery of silver birch by using either slow cooling or vitrification method (2,7).

In slow cooling method of silver birch, the cold hardening was followed by precultivation of dissected apical shoots or axillary buds on the same medium with added 5% DMSO for 72 h. After precultivation the material was transferred into cryotubes on ice and PGD cryoprotectant (10% polyethylene glycol w/v, 10% glucose w/v, and 10% dimethylsulfoxide v/v in water) was added dropwise for 30 min, and the tubes were kept on ice for additional 30 min before freezing (7). In vitrification, after cold hardening, the excised axillary buds were precultured on WPM containing 0.7 M sucrose for 24 h under the same conditions, followed by treating with loading solution containing 2 M glycerol and 0.4 M sucrose for 20 min at room temperature. Finally the material was dehydrated with PVS2 cryoprotectant (30% glycerol w/v, 15% ethylene glycol w/v, and 15% DMSO v/v in WPM) followed by direct immersion in liquid nitrogen. The average recovery of silver birch after vitrification (71.2%) was significantly better than that after slow cooling

protocol (51.8%) (4). Vitrification of aspen was performed as that of silver birch except cold hardening without any ammonium substitution for 3 weeks. Recovery of vitrified hybrid aspen genotypes varied from 2.5% to 75%, being significantly lower than the controls (2).

The main goal for cryopreservation of embryogenic cultures of Scots pine, derived from immature seeds, was to avoid the loss of embryogenic potential during long-term subculture. The best regrowth was achieved by using the method quite similar to slow cooling of deciduous trees: the embryogenic cultures were pretreated on the medium supplemented with 0.2 M sucrose for 24 h and with 0.4 M sucrose for an additional 24 h following incubation in PGD solution, performed as above. From the cryopreserved cell lines representing three mother trees, 78% remained viable after cryopreservation. After 7 weeks' cultivation the growth rates (Wo/Wi) of the lines varied significantly, from 3 to 24. Most of the cells retaining their regrowth ability were embryonal head cells. When the proliferation rate of the frozen cultures had started, their morphological appearance was the same as the non-frozen cultures (1). SE cultures derived from mature trees of Scots pine can also be cryopreserved successfully by using the same protocol (unpublished data).

Genetic fidelity of the cryopreserved materials

For all the applications, genetic fidelity of cryopreserved materials is essential. In our study, where growth rate and morphology of regenerated silver birch plants growing in the greenhouse were evaluated after slow cooling of *in vivo* and *in vitro* material no differences were found between the differentially cryopreserved plants and controls. In addition, the regenerated plants showed no genetic changes compared to the original donor trees, when the plants were evaluated using RAPD assays together with chromosome analysis (5). The protocol used to cryopreserve dormant buds of transgenic silver birch lead to high regeneration percentages combined with transgene stability and functioning (8). According to the morphology and the RAPD profiles of regenerated vitrified plants of silver birch and hybrid aspen in the greenhouse as well as SE cultures of Scots pine, the genetic fidelity of the material seems to have remained unchanged (1,2,4).

Future prospects

So far cryopreservation has been used mainly for research purposes, with the exception of conservation of some specific birch forms (6). The future applications planned include the following: I) Selected birch genotypes cryopreserved for breeding; i.e. crossing of the regenerated plants is possible within a few years due to accelerated flowering in greenhouse (9). II) More extensive cryostorage of specific tree forms of different tree species for gene conservation and landscaping purposes. III) Cryopreservation of coniferous SE-cultures during the field testing of clonal material, the selected lines being used in production of forest regeneration material.

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Key words: axillary buds, Betula pendula, hybrid aspen, Norway spruce, Picea abies, Pinus sylvestris, Populus tremula x Populus tremuloides, preculture, silver birch, Scots pine, vitrification

Examples of Integration of Cryopreservation in different Plant Biotechnology Programmes

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Abstract

Cryopreservation appears as an alternative to the preservation of biodiversity due to the risks to lose a part of the genetic resources by climatic disaster, human activities, pests and diseases. Genetic resources are a prerequisite for the establishment of classical and modern plant breeding programmes. By integrating cryopreservation in different biotechnology programmes, the breeders contribute to the preservation of genetic resources and ensure the chance to maintain the plant improvement process. The first step is the creation of a core collection representative of the genetic diversity and including wild and improved varieties. Considering recalcitrant species like coffee and cacao, the development of cryopreservation permitted to secure the core collection. But another advantage is the possibility to maintain embryogenic potential of strains established for elite clones for further propagation. On another hand, to maintain by in vitro culture chicory selected parental clones for seed production or tissues culture stock established for various research purposes is time consuming and exposed to the various risks like contaminations, loss of strain or somaclonal variation. Cryopreservation has been used as a solution to preserve various plant materials for new potential research programme and to reduce efforts to subculture collection. Cryopreservation is now routinely used at the R&D Centre on various species comprising a large number of coffee, cacao and chicory varieties as well as on a collection of tissue cultures established for pharmaceutical, cosmetic purpose of more 100 plant species.

Key words: biodiversity, genetic resources, plant breeding, core collection, coffee, cacao, chicory

Conserving genes and genotypes of trees

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Abstract

Conservation of genes and genotypes in trees is important e.g. for maintaining genetic biodiversity, for tree breeding, and for preserving valuable material for research. Viability of tree populations in future is dependent on genetic variation enabling to adapt to changing environment. The difficult task in conservation genetics is to identify most suitable populations and genotypes for conservation. Recent progress e.g., in methods and knowledge on the genetic basis of adaptive variation will help to improve conservation plans. In tree breeding and in basic research, preservation of valuable genotypes is needed. Use of different methods for preserving genetic variation and genotypes situ and ex situ (e.g. cryopreservation) will be discussed.

Key words: biodiversity, tree breeding

Steps Towards the Validation of *Allium* and Strawberry Cryopreservation

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Abstract

To enable the routine utilisation and wider application of plant germplasm cryo-conservation techniques by other laboratories, there is a need to identify critical factors that effect germplasm survival and implement ‘best practice’ procedures for their validation. This is of fundamental importance for a protocol to be effective and reliable in the context of genebanking and transfer of germplasm, however the validation of cryopreservation protocols between different laboratories is not yet commonplace.

Cryopreservation protocols for clonally propagated crop species have been developed, at the University of Derby for the use of *Allium sativum* (garlic) stem disc explants and *in vitro* shoot tips of *Fragaria x ananassa* Duch. (strawberry) and demonstrated to be applicable to a number of genotypes. However, to indicate their potential for more widespread use these protocols require to be validated to facilitate their application to germplasm conservation.

To assist the validation process, the encapsulation/dehydration technique was optimised using cultivars supplied by IPK Genebank to cryopreserve garlic stem disc explants during a technology exchange programme between IPK Genebank and the University of Derby. A detailed description of the protocol was sent to the IPK Genebank and using stored garlic cloves that were physiologically ‘standardised’ validation experiments took place simultaneously between both laboratories.

Following the validation process, several parameters were identified as critical factors during the technological exchange, including: (i) differing technical experience in relation to garlic explant tissue excision; (ii) encapsulation of the stem-discs and (iii) differences in facilities, particularly relating to *in vitro* culture rooms. Nonetheless, this exercise proved to be of paramount importance to validate this encapsulation/dehydration protocol for the cryopreservation of garlic stem-disc explants between the IPK Genebank and University of Derby laboratories.

Moreover, a further cryopreservation validation exercise is currently being conducted using a Plant Vitrification Solution 2 (PVS2) based protocol for the vitrification of strawberry *in vitro* shoot tips cultures, in a technology exchange programme between the University of Derby and the Institute of Fruit Breeding, Dresden-Pillnitz. This cryopreservation exchange programme has been initiated through a Short-Term Scientific Mission, as part of COST Action 871 by Dr.Monika Höfer with the University of Derby.

This Mission's outcome, so far has enabled Dr. Höfer to gain specific and essential 'hands on' experience and technical advice regarding the application of the PVS2 protocol. Perhaps, more significantly, this opportunity to visit colleagues at the University of Derby has enabled both collaborators to compare and contrast differing technical experiences, particularly in terms of explant characteristics crucial to the survival of strawberry *in vitro* shoot tips cultures and successful application of the PVS2 protocol. Additionally, the COST Mission provided the occasion to identify potentially critical factors including variation in size of explant and in the use of micropropagation media assisting in the transfer of this protocol to Dr. Höfer institution. An assessment of the significance of these factors will be undertaken as the basis for future validation experiments which will be conducted between both Institutions.

The value of this exchange programme will be instrumental in the future design of experiments to use 'newly' initiated *in vitro* cultures from the Institute of Fruit Breeding, Dresden-Pillnitz. Studies will incorporate strawberry varieties which have been shown at the University of Derby to be responsive to the PVS2 protocol and ones which are significant types representative of the strawberry germplasm collection from the Institute of Fruit Breeding, Dresden-Pillnitz.

Based on these two case studies illustrating the cryopreservation of clonally propagated crop species for *A. sativum* stem disc explants and *in vitro* shoot tips of *Fragaria x ananassa* the issues associated with effective technology transfer and the validation of cryopreservation protocols will be discussed.

Key words: Allium sativum, garlic, in vitro shoot tips, Fragaria x ananassa, encapsulation/dehydration, garlic stem-disc explants, vitrification

Establishment of cryobank of potato and hop apices in the Czech Republic

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Abstract

Introduction

To eliminate risk of genotype losses in collections of potato and hop germplasm in the Czech Republic we decided to establish cryo collections of these crops as a safe duplicate. The objective of the research project was to develop cryopreservation methods and to store selected potato cultivars and hop clones in liquid nitrogen at temperature of -196 °C.

Materials and methods

Fifty potato cultivars, especially of the Czech origin, and fifty important hop clones were selected and multiplied at the Potato Research Institute and the Hop Research Institute, respectively. Virus free *in vitro* plants were transferred to the Laboratory of Plant Physiology and Cryobiology at the Crop Research Institute. The method based on plant pretreatment and shoot tip desiccation was developed and used for the crops cryopreservation. The plants recovered from cryopreserved shoot tips were return to the crop institutions for genotype identity evaluation.

Results and discussion

Simple preculture-desiccation methods were developed for potato and hop cryopreservation. The method for potato (1) was based on preculture of nodal cuttings on modified MS medium (2) with adding of 2 M sucrose and following air desiccation of shoot tips on aluminium foils for about 1.75 h as long as the moisture content achieved 0.4 g water per g DW. Aluminium foils with shoot tips were then frozen by quenching into liquid nitrogen. Thawing was performed by plunging the aluminium foil directly into sterile water at room temperature. Recovery was performed on modified MS medium with growth regulators (0.5 mg l⁻¹ IAA, 0.5mg l⁻¹ kinetin, 0.2 mg l⁻¹ GA₃). Preculture-desiccation method for hop (1) was based on nodal cuttings preculture with 0.7M sucrose at 4°C. Shoot tip desiccation, freezing and thawing was done the same way as in potato cryoprotocol. Recovery was performed on modified MS medium with growth regulators (0.01 mg l⁻¹ IBA + 0.1 mg l⁻¹ BAP + 0.02 mg l⁻¹ GA₃).

Several modifications and improvements of the methods were done during germplasms cryopreservation. In case of hop, originally ten times more concentrated growth regulators were used for both, shoot tip loading and plant recovery. However, high phytohormone concentration resulted in callus production and limited shoot growth. In our study (3) it was found that ten times lower concentration of growth regulators increased hop plant regeneration from shoot tips.

Average recovery after cryopreservation of fifty potato cultivars was 24.8% and average hop recovery was 30.5%. Plant recovery improved due to cryoprotocol and media modifications and the average recovery in year 2007 of potato and hop was 29.1% and

35.5%, respectively (Fig. 1). The highest frequency of plant recovery was near to the average recovery in both crops.

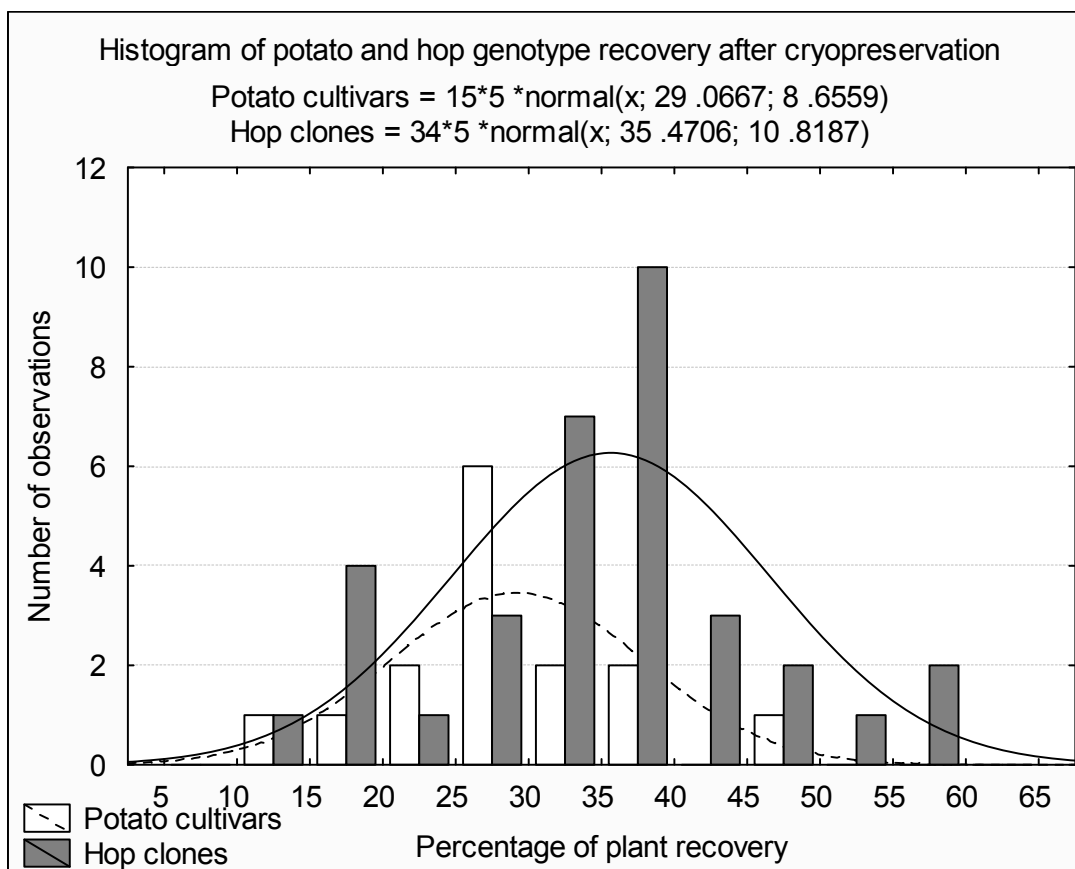


Figure 1. Histogram of hop and potato genotype recovery obtained in year 2007 by preculture-desiccation method. Each value represents result of at least 20 plants.

Conclusions

Fifty potato cultivars and fifty hop clones were cryopreserved by developed preculture-desiccation methods. Last modification of the methods improved plant recovery to 29% in potato and 35% in hop. Genotype identity of recovered plant was analyzed by biochemical methods and morphological trait comparison. No changes in genotypes stability were found in both crops. Next research will be focused on improvement of plant recovery and decreasing of variability in plant recovery of potatoes.

Acknowledgment

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Key words: pretreatment, shoot tip desiccation, preculture-desiccation, phytohormone concentration

Application of cryopreservation to the long-term storage of poplar and aspen (*Populus* spp.) germplasm

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Abstract

Introduction

Recent intensive research work explored the *Populus* genome, facing also the necessity of reliable methodologies for the preservation of its large genetic variability, both natural and clonal. In this context, the cryogenic technology should be regarded as an important strategy, offering unrivalled opportunities for cost-effective long-term germplasm preservation (4). In the past, attempts of cryopreserving poplar explants (e.g., buds, seeds and callus samples) used both slow cooling (controlled freezing) and one-step freezing procedures with promising results (2). Nevertheless, experimental protocols optimized with one species, or even specific genotypes, may give unsatisfactory results when different species/genotypes are tested (3). The present study was aimed at exploring on a large scale the possibility to cryopreserve the *Populus* biodiversity. First trials evaluated (i) the possibility of adapting the protocol of PVS2 vitrification, previously optimized for the cryopreservation of *P. alba* (1), to valuable local clones of the species, as well as to a hybrid aspen (*Populus tremula* x *Populus tremuloides*) clone, (ii) the efficiency of alternative one-step freezing cryotechniques (encapsulation-vitrification, droplet-freezing method); (iii) the response to cryopreservation of shoot tips, excised from shoot cultures showing marked signs of etiolation, blanching and decay after 6 months of storage at 4°C in the dark, and (iv) the natural tolerance to ultra-rapid freezing of seeds from *P. tremuloides* (“quacking aspen”) and *P. trichocarpa* (“black cottonwood”).

Materials and methods

Shoot cultures from three Italian clones of white poplar (*P. alba* L.) of high interest in terms of germplasm preservation were used, i.e., the cv Villafranca, and two clones (‘CSM’ and ‘MLF’), established from ancient monumental trees located in the Tuscany region. Shoots from a hybrid aspen clone (*P. tremula* L. x *P. tremuloides* Mincx.) were also included in this study. The shoot cultures of both ‘Villafranca’ and aspen clones were previously cold hardened for 2 weeks at 4 °C, under a 8/16 h photoperiod. The shoot cultures of clones ‘CMS’ and ‘MLF’ had been stored in the dark at 4°C from over 6 months and they were used directly for shoot-tip excision. Shoot tips, consisting of the apical meristem and 4-5 leaflets, were excised under a laminar-flow hood from both apical and axillary buds (cv Villafranca), or only from apical buds (clones ‘CSM’ and ‘MLF’, aspen). In all the experiments, the shoot tips were precultured for 48 h on hormone-free MS medium at 4°C and under a 8/16 h photoperiod. The basic procedure of cryopreservation by vitrification developed by Lambardi *et al.* (1) was followed, using a 60-min exposure to PVS2 with aspen, ‘CSM’ and ‘MLF’ clones. Differently, two exposure times (30 and 60 min) were tested with ‘Villafranca’.

As for encapsulation-vitrification, shoot tips from the ‘CSM’ and ‘MLF’ clones were first encapsulated in 3% alginate, loaded with cryoprotectant (CP) for 1 h at 25°C, treated with PVS2 for either 60 or 120 min at 0°C and then plunged into liquid nitrogen (LN). In the droplet-freezing method, 10 µl of PVS2 solution were dropwise poured on small aluminum strips (0.5x2 cm), placed over frozen tiles. One shoot tip was then included inside each

drop and maintained for 30 or 60 min. Afterwards, the strips were put inside the cryovials (containing LN), which were then plugged and immersed in LN. Thawing was done in a water bath at 40 °C or at air temperature (droplet-freezing method). Afterwards, the shoot tips (naked or encapsulated) were washed and plated as previously reported (1).

In a preliminary experiment seeds of *P. tremuloides* (Colorado provenance) and *P. trichocarpa* (Montana provenance) were used to value their natural potential to remain germinable after the direct immersion in LN without any dehydration pre-treatment. After thawing for 1 min at 37°C, the seeds were soaked for 6 hours in water at room temperature and plated on wetted filter paper.

Results

With the vitrification procedure, the shoot tips of both ‘CSM’ and ‘MLF’ clones showed good survival percentages (44% and 54%, respectively), although they came from etiolated and blanched shoot cultures. As for the cv Villafranca, best survival (64%) was recorded with shoot tips from axillary buds treated with the PVS2 solution for 60 min, in comparison with shoot tips from apical buds (17%). However, the overall survival percentage (77%) was achieved when the 60-min PVS2 incubation time was used to the hybrid aspen clone. No survival was achieved with the ‘encapsulation-vitrification’ technique, although the non-frozen encapsulated shoot tips tolerated even a 120-min PVS2 treatment (67% of regrowth). The droplet-freezing method determined an improvement of shoot-tip survival, in comparison with the PVS2 vitrification procedure. This effect was more evident with the clone ‘CSM’, which showed a regrowth of 74% in shoot tips treated with PVS2 for 60 min. To our knowledge, this is the first report for successful application of the droplet-freezing method for the cryostorage of forest species germplasm. All seeds of *P. tremuloides* maintained 100% germinability after direct immersion in LN and plating on moist filter paper. Only 20% of *P. trichocarpa* seeds germinated, which was lower than the initial germinability of black cottonwood seeds (45%). With this species, experiments are in progress to achieve maximum germinability after cryopreservation by testing different time of dehydration before the direct immersion in LN.

Conclusions

In this study, different kind of techniques for the cryostorage of *Populus* spp. and an aspen hybrid were successfully applied, using both shoot tips and seeds as experimental material. Of particular interest is the possibility of a direct use of shoot cultures from slow growth storage, even when the material shows evident physiological deviations. Moreover, results from the application of the droplet-freezing method are promising and raise the chances of further usage of this technique in woody plants.

Acknowledgements

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Key words: vitrification, clone, encapsulation-vitrification, droplet-freezing method, shoot tips, seeds

Influence of cryoprotectors on the viability of cryopreserved carob tree immature pollen

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Abstract

In this work the viability of carob tree (*Ceratonia siliqua* L.) cryopreserved immature pollen was evaluated after 5 and 8 months of storage. Anthers isolated from male and hermaphrodite flowers at developmental stages I and II with microspores at the late uninucleate to early binucleate stage, were cryopreserved by using ultra-rapid cooling method. Different types of cryoprotectants at different concentrations were used, namely carbohydrates (sorbitol: 0.5 M, 1 M, and 2 M; mannitol: 0.5 M and 1 M; sucrose: 0.5 M, 1 M and 2 M; glucose: 0.5 M, 1 M and 2 M; and other chemical substances (dimethylsulfoxide - DMSO: 3.5, 5, 7 and 10%, v/v; glycerol: 5, 10 and 15%, v/v; glycerol + DMSO: 2.5% + 2.5%, 1% + 5% and 5% + 5%, v/v; proline: 10%, v/v; ethyleneglycol: 2.5%, v/v and poliethyleneglycol: 10%, v/v. Anthers were pretreated by the addition of 10 ml of the cryoprotective solution to test tubes containing the anthers, divided in 4 applications of 2.5 ml, at 5 min intervals. The test tubes were maintained 24 h in a roller, at 25 rpm at RT. The anthers were then dehydrated by addition of 1 ml of the cryoprotective solution to each tube, divided in 4 applications of 0.25 ml, at 5 min intervals. After 20 min, the tubes were plunged to LN for 1 h, and stored at -80°C . In other assay, the anthers were cryopreserved without the addition of the cryoprotective solution. After storage, the samples were rewarmed in a 40°C water bath for 15 min, the cryoprotectant solution was drained off and the viability of pollen was evaluated by using the fluoresceine diacetate (FDA) test. Regardless of the type of cryoprotectant used both the application of the cryoprotectants and their presence during the period of storage generally promoted pollen viability compared with the control. The anthers excised from flowers at developmental stage I were more suitable for cryopreservation. When carbohydrates were used as cryoprotectants, pollen viability was not significantly affected by the developmental stage of the flowers from which the anthers were taken, and the most effective treatment consisted of a pretreatment with 0.5 M sucrose and a storage period of 5 months (~60% of viability). When DMSO was used as cyoprotectant pollen viability was affected not only by flower developmental stage but also by the sexual type of the donor plant, with the best results observed with anthers taken from hermaphrodite flowers at stage I. When using other chemical substances, it was observed that the use of solutions containing two different cryoprotectants were more effective than cryoprotectants alone. The best results were obtained with glycerol (2.5%) + DMSO (2.5%) (88% of viability). When using carbohydrates as cryoprotectants, the maximum percentage of viability obtained was lower than that observed with the application of other chemical substances. Although there is a need to increase the viability levels and to evaluate the response of cryopreserved microspores to induction of androgenesis, these results show that the cryopreservation of immature pollen of carob tree is possible, by using a relatively simple technique and maintaining high viability.

Key words: Ceratonia siliqua, sorbitol, mannitol, sucrose, glucose, dimethylsulfoxide, glycerol, proline, ethyleneglycol, poliethyleneglycol

Monitoring clonal stability with retrotransposon-based markers

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Abstract

Retrotransposons elements constitute a major component of dispersed repetitive DNA in all eukaryotic genomes. Retrotransposons are largely quiescent, but activated by stresses, such as wounding, pathogen attack and cell culture (7). The activation of retrotransposons has been shown to be accompanied by for example cytosine demethylation (3). Several retrotransposon-based marker systems have been developed that reveal insertional polymorphisms generated by the transposition of the retrotransposons. These have been found useful for linkage, evolution and genetics studies (4). Retrotransposon marker systems generally rely on PCR to generate fingerprints. The marker systems generate fingerprints, or multilocus profiles, for the members of given families of retrotransposons. The inter-retrotransposon amplified polymorphism (IRAP) technique (1) generates PCR products from retrotransposons inserted near enough to each other to allow efficient amplification. The IRAP amplification primers are commonly designed to match segments of LTRs conserved within element families. REMAP, retrotransposon-microsatellite amplified polymorphism (1), uses one LTR primer, together with a simple sequence repeat primer with one additional base at the 3' end of simple sequence repeat (SSR) primer (for example: 5'(CA)₉G-3'). The SSAP –method combines AFLP analysis with retrotransposons (2,6), i.e. measures the distance between a retrotransposon insert and a restriction site.

Whereas retrotransposon insertions are only heritable in sexually propagated plants if the insertion event occurs in a cell that gives rise to a gamete, insertions in somatic tissues can be passed along in vegetatively propagated crops. Other genetic rearrangements in somatic nuclei that affect the position of priming sites for markers would likewise be detectable. Using retrotransposon markers in IRAP analysis, we have observed occasional clonal changes in several different species, for example blueberry and apple. However, the most promising method of utilizing retrotransposon based markers for detecting intra-clonal variation is the SSAP –analysis (5).

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Key words: apple, blueberry, intra-clonal variation, IRAP, REMAP, SSAP

Cryopreservation of encapsulated *Ribes* meristems and variation between accessions within a cultivar

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Abstract

In vitro cultures of crop plants are used to complement field collections and reduce the potential impact of biotic and abiotic hazards as well as . Maintaining *in vitro* collections can reduce this risk and has been used to producing disease-free plants. However, previous studies have shown that, in some instances, somaclonal variation has occurred to produce aberrant regenerated plants (1,3). Such a risk is dramatically reduced by cryopreservation of excised meristems from *in vitro* culture, that can be used for subsequent plant proliferation. Latterly, the encapsulation/dehydration technique, borrowing the basics of synthetic seed technology, has significantly improved the success of this strategy for cryopreservation (2).

In this study, using *in vitro*-grown *Ribes nigrum* (blackcurrant) meristems, the extent of injury caused by the individual, preparative steps of the encapsulation/cryopreservation procedure have been documented. For the three accessions investigated the excision, desiccation and encapsulation steps had a relatively low impact on survival and the terminal, freeze/thaw step was the most injurious. The water content of the bead immediately before freezing is critical to success and the drying temperature used to determine this has to be carefully chosen to avoid underestimation.

To be of optimal value for the conservation of genetic diversity the encapsulation cryopreservation technique has to be robust and readily transferable between laboratories. In this study two accessions of the same cultivar were investigated, one with a prehistory of 9 years culture *in vitro* whilst the other had only been cultured for 1 year prior to study. Clear differences in biochemical properties were seen between the accessions, together with a markedly different response to cryopreservation. Frozen-thawed plants of the two accessions continue to show significant differences, including disease resistance, after three field seasons. The issues raised by this study concerning the significance of the *in vitro* history of material prior to selection for cryopreservation will be discussed

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Key words: meristems, in vitro culture, encapsulation/dehydration, Ribes nigrum, blackcurrant

Cryopreservation of *Pelargonium* species by droplet-vitrification

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Abstract

In order to guarantee safe, long-term conservation of the National Institute of Horticulture (I.N.H.) *Pelargonium* collection, meristem cryopreservation studies have been undertaken since 1999. An encapsulation-dehydration process has been first developed. More recently, studies were undertaken in order to adapt the droplet-vitrification procedure (1) to this genus.

In order to optimize the process, its main steps were studied choosing *P. x peltatum* 'Balcon Lilas' as a model, using plants grown in a greenhouse. The best results were obtained dehydrating apices in two steps, 20 min in LS and 15 min in PVS2 and then immersing them directly in Liquid Nitrogen (LN). After thawing and unloading in the recovery solution at room temperature during 15 min, apices were regenerated onto a semi-solid medium (2). This simple protocol without any pretreatment, was tested on 28 different *Pelargonium* accessions representative of the diversity of our collection. For each accession, at least 3 repetitions were performed on different days, reaching a minimum of 24 cryopreserved apices per genotype. An average of 65% survival rate was obtained ranging from 14.8% for *P. fragans* to 90% for *P. capitatum* and *P. x hortorum* Neurot. Plants were regenerated for each genotype, except *P. x peltatum* Papa Crousse. The genotype dependent tolerance at each step of the process is discussed.

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Key words: Pelargonium x peltatum, dehydrating, Pelargonium fragans, Pelargonium capitatum, Pelargonium x hortorum

Cryopreservation of hairy root cultures from *Maesa lanceolata*

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Abstract

Introduction

Maesa lanceolata is a medicinal shrub growing in the tropics of Africa and Asia. It produces a number of different saponins for which was shown that they exert antiviral, hemolytic, molluscicidal and anti-angiogenic activities (1, 2).

To evaluate the production of saponins in *Maesa lanceolata* grown under *in vitro* conditions, hairy roots were established. *In vitro* culture of hairy roots is, however, labor intensive and expensive, and there is a risk of contamination and genetic changes. Because of this we are currently developing a protocol for cryopreservation of root tips.

Materials and methods

Maesa lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). Surface sterilized seeds were germinated on basal medium supplemented with 0.8% (w/v) agar. The basal medium consisted of MS full strength basal salts with MS vitamins and 3% (w/v) sucrose. Cultures were maintained at $26\pm 1^\circ\text{C}$ with a 16h photoperiod.

Maesa lanceolata hairy roots were induced using *Agrobacterium rhizogenes* (strain LBA) transformation on leaf discs. GFP was used as a visible marker for the screening of transgenic material. Hairy roots were placed on SH medium supplemented with 0.8% agar and were subcultured every 4 weeks. The medium consisted of SH full strength basal salts with SH vitamins and 3% (w/v) sucrose. Cultures were maintained at $25\pm 1^\circ\text{C}$ in the dark.

In order to evaluate the toxicity of the vitrification solution (PVS2) prior to freezing, root tips (2-3 mm) excised from 2-week old root cultures were isolated. These roots were precultured for 1 day at 25°C in the dark on SH solid medium containing 0.3M sucrose. The following day samples were incubated at 0°C with PVS2 solution, during 0, 5, 10, 15 or 20 min. Half of the samples were first treated with loading solution during 10 min before they were subjected to the PVS2 treatment. Afterwards the hairy roots were placed on SH solid medium with 3% sucrose to regenerate.

For encapsulation-dehydration root tips ($\pm 0.5\text{cm}$) from 2-week old root cultures were isolated. They were immediately encapsulated in calcium-alginate beads, using a 3% (w/v) alginate solution and a 0.1M CaCl_2 solution. Encapsulated hairy roots were pretreated for 3 days in liquid basal SH medium with increasing sucrose gradient (0.5 – 0.75 – 1M sucrose). After 3 days the beads were dehydrated by the air of the laminar flow until approximately 38% of the initial weight was achieved. Sufficiently dehydrated beads were placed in cryotubes and brought immediately into liquid nitrogen. After a freezing period of 3 days, the encapsulated hairy roots were thawed quickly and placed on solid SH medium with 3% (w/v) sucrose to regenerate.

Results to date

Because PVS2 solution contains the cytotoxic compound DMSO, we assessed hairy root growth after different times of PVS2 treatment. 150 roots were incubated with PVS2 solution during 0, 5, 10, 15 and 20 minutes, and subsequently washed. Another 150 roots

were treated with a loading solution during 10 min and then incubated with PVS2 during 0, 5, 10, 15 and 20 minutes, and also washed. Regrowth was tested by incubating roots on normal SH solid medium. The results are shown in table 1. Treatment with loading solution prior PVS2 incubation had a positive effect on viability of hairy roots. Vitrification experiments with freezing in liquid nitrogen are currently being set up. Roots will be treated for 10 minutes with a loading solution and afterwards for 5 minutes with PVS2 solution.

Table 1. % of hairy roots that grow back after treatment with PVS2 solution for different time points, with or without a 10 min pretreatment with loading solution.

Duration of treatment	% regrowth of HR	
	with loading solution	without loading solution
0 min (controle)	90	100
5 min	66,67	16,67
10 min	3,33	10
15 min	20	0
20 min	10	6,67

An encapsulation-dehydration protocol was set up to avoid the cytotoxic effects of the vitrification solution. Encapsulated hairy roots were dehydrated for different periods and subsequently frozen in liquid nitrogen. After thawing, beads were placed on solid SH medium and the number of roots protruding from the beads were counted. As shown in table 2, regenerating roots are observed at all time points of freezing. Regenerated roots are currently being analyzed to determine the saponin content.

Table 2. Effect of dehydration time on weight loss of the beads and survival of the encapsulated hairy roots with and without freezing.

Time point of freezing	% of the initial weight	% of regrowth of HR	
		dehydrated (-LN)	dehydrated (+LN)
0 hours	100	100	0
2,5 hours	39,95	60	33,3
3 hours	39	80	22,5
3,5 hours	38,58	80	55
4 hours	37,07	40	20

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Key words: encapsulation-dehydration, vitrification

Evaluation of encapsulation and droplet vitrification methods in gene preservation work

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Abstract

At MTT Nursery Group we were looking for a suitable cryopreservation procedure that could be applicable for a wide range of species and genotypes. Different techniques for the cryopreservation of raspberry were evaluated at the University of Helsinki. Encapsulation was combined with vitrification or used together with dehydration methods (3).

At MTT we started the work by combining the vitrification with encapsulation according to the studies of Dr. Wang, but encapsulation was not compulsory for raspberry. Instead of encapsulation-vitrification we started to use our own modified droplet vitrification method for conservation of raspberries and also other vegetatively propagated plants like strawberry.

In the long term gene preservation of vegetatively propagated plants and especially as we are using *in vitro* grown meristems, it is optimal to preserve healthy plant material free of viruses and phytoplasmas (5, 6). Therefore, application of cryotherapy is a practical tool for producing healthy propagation material, that later on can be transferred into long-term preservation using cryopreservation methods (2).

In cryopreservation the focus is on conserving plant material in high amounts using such techniques that the survival after thawing is optimal. In cryotherapy however, the focus is not on high survival of plant cells. Instead, it is essential that the viability is conserved, but only the meristem cells are viable after cryopreservation and a minimum amount of the surrounding tissues survive (3). Cryotherapy is more efficient than the traditional virus eradication methods like meristem culture or thermotherapy that were introduced to Finnish raspberry and potato production already in the 70s. Combining cryotherapy with preceding thermotherapy was the solution also in the case of the raspberry bushy dwarf virus, that had earlier been practically impossible to eradicate from the diseased tissues with the traditional methods.

Cryopreservation of somatic embryos is potentially a method to cryopreserve increased amounts of accessions. In our project we have tested the methods to produce somatic embryos with several potato and two raspberry cultivars and cryopreservation of the somatic embryos using the encapsulation technique will be studied as the next step.

As the conclusion these methods are compatible with each others and we can use them in the Finnish gene preservation work (1). The basic cryopreservation methods are quite practical and effective, but the adaptability of one technique can be more suited for a particular purpose than the adaptability of the other techniques. In cryotherapy and cryopreservation of somatic embryos, encapsulation is especially useful. In large scale preservation the droplet vitrification method is more applicable as a working method.

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Key words: cryotherapy, potato, raspberry, somatic embryos

***In vitro* methods used in preservation of fruit germplasm in Serbia**

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Abstract

In the Fruit Research Institute which is situated in Čačak (Serbia), there is a significant collection (1,136 cultivars) of different fruit species. Our research, among other things, involves clonal selection of autohtonous plum and sour cherry cultivars (*Prunus domestica* L. and *Prunus cerasus* L.) and some apples (*Malus domestica* Borkh.) with good storage capacity. Our breeders are interested in many local varieties with some good characteristics, i.e. resistance to pest and diseases, long shelf life etc, and also in prevention of their extinction.

From these reasons many years ago we included some *in vitro* methods to establish *in vitro* germplasm collection, as a possibility of storing germplasm for medium-term conservation, for germplasm exchange, as well as for the possibility of its rapid propagation when necessary.

We used cold storage technique in the simple conditions, i.e. at +5°C in the total darkness. Before transferring the cultures into cold chamber we subcultured the plants for 7, 14 and 21 days to find the best duration time of precondition subculture. After cold storage we kept these cultures for 3 days in growth room for adaptation and after that the parameters of multiplication were measured, and also after 1 month. In that way plum cv Požegača was successfully stored up to 10 months with very good multiplication index and regrowth (1).

Cherry rootstocks Gisela 5 and Tabel Edabriz were also successfully stored for 3 months in the same conditions with 100% survival rate and good regrowth, i.e. the multiplication index increased after that by 1.8 and 4.8, respectively. The measured quantity of N taken from medium during cold storage was lower than in standard conditions (2).

As *in vitro* storage of the germplasm of small fruit could be easily achieved, the loss of the material being only accidental, 3-month maintenance of raspberry cv Meeker in our lab under cold temperature conditions has proved to be beneficial, accompanied by high viability rate (exceeding 67%). Similar results were also obtained for other raspberry cultivars (3). Thus, multiplication index and shoot length of raspberry cv Meeker were increased by 1.2 and 1.5 times respectively in the first subculture upon cold storage. The nitrogen uptake of plants in cold storage was also very low, i.e. 32.9% of N was absorbed as compared to the initial nitrogen quantity in the medium. Low uptake of N from the medium shows that this element does not have to be added to the medium during storage under chilling conditions, i.e. slow growth is accompanied by slow uptake of this constituent from the medium (4).

However, our results showed that the shoots of these species could be stored under very simple conditions with normal growth and enhanced multiplication after conservation, thus providing the conditions for the development of standard protocol for maintenance in *in vitro* germplasm.

Last year we started with encapsulation of shoot tips of blackberry cv Čačanska bestrna and raspberry cv Meeker in calcium alginate, i.e. preservation in alginate beads at 5°C in the darkness. Sodium alginate was dissolved in water with 4% glucose, or 3% sucrose, and in MS medium (5) without growth regulators with 4% glucose or 3% sucrose (6). Regrowth ability of the stored explants and *in vitro* multiplication in three successive subcultures were evaluated. In cv Čačanska bestrna 18.8% survived encapsulation after 1 month and gave regrowth, while in cv Meeker 6.3% survived encapsulation after 1 month and gave regrowth, and 18.8% after 2 months. We did not obtain viable explants after three months of encapsulation in both genotypes. Regrowth was only determined in both genotypes in alginate beads with MS and 3% sucrose. Multiplication index decreased from the first to the third subculture in blackberry. However, it slightly increased in raspberry towards the third subculture.

These results are a small step towards developing a new storage protocol technique for fruits in general and its introduction in our country, leading to cryopreservation technique.

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Key words: plum, sour cherry, Prunus domestica, Prunus cerasus, Malus domestica, raspberry, nitrogen uptake, encapsulation, blackberry

Application of cryopreservation of *in vitro* shoots for setting-up a cryo-genebank of *Betula*

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Abstract

Introduction

When micropropagated selected forest trees are planted out in field trials the question occurs how to conserve the *in vitro* cultures safely and economically in a juvenile easy-to-propagate state for the duration of the field testing. Depending on the species periods of eight to more than twenty years have to be bridged. One option, cold storage under slow growth conditions e. g. at 5 ± 1 °C, is less labour intensive than maintenance of *in vitro* cultures under the temperature regime for rapid propagation.

Shoot cultures of birch could be cold stored in our lab for up to five years. However, with increasing numbers of stored clones in the collection even the necessary reculture at 24 ± 1 °C every two to four years binds too much working capacity. This problem can be solved by cryopreservation of the shoot cultures. As soon as results of the field trials are available superior clones can be taken from the cryo-genebank at short notice and used for commercial large-scale propagation.

Field trials were established with 94 selected clones of *Betula pendula*, *Betula pubescens* and hybrids of *Betula pendula* x *B. platyphylla* var. *japonica* in recent years. So far, *in vitro* shoot cultures of these clones were conserved parallel to the field testing in cold storage. Last year we began to set-up a cryo-genebank with this material.

Material and methods

Details of our micropropagation protocol for *Betula* are described by Meier-Dinkel (1). *In vitro* shoot tips of *Betula pendula*, *Betula pubescens* and hybrids of *Betula pendula* x *B. platyphylla* var. *japonica* were cryopreserved applying a PVS2-vitrification protocol (Meier-Dinkel, 2). 70 shoot tips per clone were frozen in liquid nitrogen (LN) in order to establish a cryo-genebank for the long-term storage of selected birch genotypes. 10 explants per clone were rewarmed after a few days in storage LN in order to determine survival and shoot regeneration.

Results

Data on survival and shoot regeneration after cryopreservation of 35 clones are presented and discussed. Depending on the clone, shoot formation was up to 80 %.

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Key words: clones, *Betula pendula*, *Betula pubescens*, *Betula pendula* x *B. platyphylla* var. *japonica*, PVS2-vitrification protocol

Cryopreservation of embryogenic tissues of hybrid firs: the effect of sorbitol on the tissue regrowth and post-thaw recovery

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Abstract

Introduction

Somatic embryogenesis has been initiated in many conifer species belonging to different genera. In several species cryopreservation for the long-term storage was successfully applied. Within the genus *Abies* cryopreservation has been tested for *Abies nordmanniana* (3) and *Abies cephalonica* (1). In both species the slow freezing method resulted in successful post-thaw recovery. Embryogenic cultures of hybrid firs have been repeatedly initiated from immature and mature zygotic embryos and the tissues are being maintained on solid culture media (4, 5). In order to replace the time and labour intensive storage on solid media, cryopreservation of selected cell lines of *Abies* hybrids has been tested.

Material and methods

Plant material: Embryogenic tissues of hybrid firs *Abies alba* x *A. cephalonica* (cell lines AC1, AC4, AC78) as well as *Abies alba* x *A. numidica* (cell line AN72) were initiated from immature (enclosed in megagametophytes) and mature zygotic embryos (dissected from seeds) and cultured on DCR medium (2) supplemented with BA 1 mg/L.

Cryopreservation procedure: Embryogenic tissues were cultured on proliferation medium containing sorbitol (0.4 or 0.8 M) and maintained on this medium for 24, 48 and 72 hrs. Then, the tissues were resuspended in liquid proliferation medium with corresponding concentrations of sorbitol. DMSO was added stepwise to reach a final concentration 5%. This mixture was pipetted into 2 ml cryovials and the cryovials were placed into Mr. Frosty container (Nalgene TM) and stored in deep freezer (-80°C) until the temperature in cryovials reached -40°C. Then the cryovials were plunged into liquid nitrogen for 30 min. Thawing occurred in water bath at 40 °C. After thawing the content of cryovials was poured in stacked filter paper disc and the liquid was adsorbed. Post-thaw recovery took place on DCR proliferation medium.

Growth characteristics: Regrowth of tissues after thawing was evaluated and compared with the pretreated non-frozen tissues. Post-thaw growth was evaluated two months after thawing and cryopreserved and non cryopreserved non-pretreated tissues were compared.

Microscopic observations: From small pieces of cryopreserved embryogenic tissues squash preparations were prepared and observed under a light microscope (Axioplan 2, Carl Zeiss, Jena, Germany).

Results and discussion

Pretreated but non-frozen tissues (designated as control 1, C1) resumed growth soon after thawing (4 to 5 days) and plating. The tissues grew very rapidly and the frequency of regrowth in most of the cases (for all cell lines and treatments, except AC4 pretreated with 0.4M sorbitol for 48 hrs) reached 100%, suggesting limited negative influence of chemical pretreatment on the growth of tissues. There was a short delay in the growth of cryopreserved tissues in comparison to C1. All the tested cryopreserved cell lines resumed

growth 7 to 10 days after plating. The regenerated tissues showed typical features of conifer embryogenic tissues such as rapid growth and mucilaginous consistence. The recovery frequencies (in terms of percentage of cultures that showed regrowth) differed depending on cell line and treatments. For cell line AC4 we observed 100% regrowth after a 24, 48 as well as 72 hrs pretreatment on 0.4 M sorbitol. For cell line AC1 the short treatment (24 hours on 0.4 M sorbitol) resulted in very poor regrowth (20%). For cell lines AC78 and AC72, however, a longer pretreatment (72 hrs) was beneficial.

Although the results can be considered as preliminary, and no statistical evaluation has been done yet, the results indicate that the duration of pretreatment is more important than the sorbitol concentration itself.

Post-thaw growth rates have also been evaluated as the fresh as well as dry mass increase 2 weeks after inoculation to new medium. The growth of cryopreserved tissues was comparable to the growth of non-pretreated and non-frozen tissues (designated as control 2, C2). In cell line AN72 no statistically significant differences in fresh as well as dry mass increase could be found between cryopreserved and C2 tissues after two weeks of growth. Significant differences (P 0.05) in dry mass weight were observed for cell line AC4 at the beginning of growth (day 0) pretreated with sorbitol for 48 or 72 hrs.

The conifer embryogenic tissues contain somatic embryos – bipolar structures consisting of embryonal part with meristematic cells and suspensor with long vacuolated cells. Microscopic observations revealed no differences in the somatic embryo micromorphology of cryopreserved tissues of hybrid firs.

The maturation capacity of cryopreserved cell lines is being tested at present. For the maturation experiments, tissues regenerated from the best treatments have been selected. The maturation medium contains abscisic acid (ABA, 10 mg/L) and PEG-4000 (10%) and the maturation capacity of cryopreserved (selected according treatments) and C2 tissues is compared.

The slow freezing method of cryopreservation was applied to *Abies nordmanniana* (3) and *Abies cephalonica* (1). In *Abies nordmanniana* after an initial lag phase the tissues resumed growth and complete embryos were formed. Regrowth capacity, however, was strongly genotype dependent (3). The authors also link the regeneration ability with cryotolerance. For *Abies cephalonica* several pretreatments were used (PEG, DMSO, glucose). In the DMSO treated but non-frozen samples the RAPD profiles showed intraclonal variation, indicating the cryoprotectants may cause somaclonal variation (1).

Our preliminary results indicate the slow freezing method is convenient for the embryogenic tissues of hybrid firs. Tissue regeneration after cryopreservation occurred in all the tested cell lines.

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Key words: conifer species, Abies alba x Abies cephalonica, Abies alba x Abies numidica, slow freezing method, Abies nordmanniana, Abies cephalonica, cryoprotectants, somaclonal variation

Different protocols - different situations - different genotypes from the research laboratory to application in genebanks

Some subjects of discussion

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Abstract

Genebanks are application fields of preservation and maintenance methods, also of those for cryopreservation, in a broader scale. When moving some scientific method from the state of fundamental research in a university's laboratory into broader application, some aspects have to be considered. The main problems are:

1. In/stability of a given method. We need to be sure that the method developed under experimental conditions using a model genotype is applicable also under every-days laboratory conditions which may differ with the season, with the personnel, sometimes with fluctuations in surrounding technical parameters. They should be applicable with other genotypes than the models as well and they should be not too sensitive in circumstances when material is not grown or received under optimum conditions. Some factors which influence the results are the physiological conditions of the material and hidden endophytes.
2. Logistics using a method in a larger scale. Whereas the attention of a fundamental researcher can be drawn on every single step of the procedure, and it is no need to limit his time for one given part of the protocol, in routine work it is necessary to pass all the steps sufficiently quick to get an efficient amount of explants into cryopreservation. Therefore, the simplest and shortest protocol will always have priority when decision between several options is needed. Furthermore, the output of the whole procedure is depending on the number of acting persons. Should we have more persons available, specialisation could take place. This is especially important in regard of the explant preparation procedures. Participation of several persons will, on the other hand, increase the standardisation needs.
3. Genebanks are the most capable actors to compare aspects of cryopreservation in a range of taxa within a given systematic group. This potential is by far not fully utilised. It would be interesting to compare species in a given group which differ in certain parameters e. g. water content (e. g. there are genera where there is a full range from water plants to succulents, or use of resurrection plants), cold- or salt tolerance. Results obtained in routine screening in genebanks could, thus, give another feed-back link to fundamental research.
4. Finally, the existence of a well-elaborated method is only one prerequisite for success in genebanks. Collaboration between several laboratories will increase the general effect of these methods by mutual validation and chances to establish safety duplication systems.

Key words: stability of a method, standardisation, safety duplication systems

Cryopreservation plant genetic diversity for sustainable agriculture

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Abstract

Plant genetic diversity is highly essential in genetic improvement of crops for sustainable agriculture, and its gradual loss is as a consequence to rapid human population growth, industrialization, deforestation, and natural calamities. This great loss is occurring especially in the developing countries, and will have an adverse impact on sustainable crop production and environment in the future. The conservation, distribution and proper utilization of plant genetic diversity/resources become necessary for the development and improvement of plant cultivars for sustainable crop production; can be achieved by establishing gene/germplasm banks both at the national and international levels. The Gene bank should encourage researchers in the developing countries to survey and monitor the genetic diversity of natural populations and landraces on farmers' fields. Cryopreservation or cryo-storage is an excellent system for long-term conservation of elite genetic material of forest trees and horticultural crops. For this, *in vitro* cultures are suitable, e.g. somatic embryos/ cell suspension, callus, and should be able to regenerate plants without somaclonal variation. For the first time, cryo-storage of date palm somatic embryos was done in Tunisia, FAO/IAEA project, and plant regeneration is yet to be accomplished. Weyerhaeuser Inc., USA has cryo-stored embryogenic cultures of conifers, and maintains a large germplasm pool of elite genetic material. The performance of cryo-stored material is being evaluated and so far don't show any morphological variation. In Belgium, more than 130 banana accessions are cryo-stored. In Asia, National Bureau of Plant Genetic Resources (NBPGR, India) is the biggest germplasm bank, and conserves mainly local germplasm seed and vegetative propagated crops and introduces new crops as well. In Malaysia, they have recently started a germplasm conservation program with international support. The importance cryo-storage in germplasm conservation, distribution, and utilization will be discussed in the context of the developing countries.

Key words: utilization of plant genetic diversity, elite genetic material, forest trees, horticultural crops

Cryopreservation at CNR-IVALSA in Florence: reflections upon ten years of good results and some “failures” with woody plants

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Abstract

The first experiments on cryopreservation at CNR-IVALSA in Florence date back to 1998. They were a response to the necessity to explore new and innovative systems for the *ex situ* conservation of woody plant germplasm, complementary to the traditional approach of in-field collection (clonal orchards). In Italy, the IVALSA Institute has one of the largest germplasm collections of fruit species (over 1800 accessions of peach, olive, plum, pear, persimmon, cherry, apple and quince) spread over the 60 ha of its experimental farm. Year by year, the maintenance costs of the collections are becoming heavier to bear. Furthermore, in the middle of the '90s the Sharka disease (Plum Pox Virus) spread through the large plum collection, obliging the burning of a clonal orchard of over 150 accessions of *Prunus domestica* and *Prunus salicina*. Hence, cryopreservation technology was regarded as a possible complementary strategy for the future, reducing to a minimum the risks of germplasm loss.

Taking advantage of the considerable expertise in plant tissue culture, a small cryo-laboratory was soon established to begin with. The first experiments were with poplar (*Populus* spp.) and olive (*Olea europaea*), two species of great importance in Italy in terms of germplasm preservation. Since then, various cryopreservation techniques, numerous species (mainly, but not exclusively, woody plants) and various kinds of explants have been tested, mostly with the practical aim of developing effective and reproducible protocols for long-term conservation.

Among the cryoprotocols which were satisfactorily developed (thanks also to National and International collaborations), the following are particularly worthy of mention: the cryopreservation of shoot tips from white poplar (*Populus alba*), plum (*Prunus domestica*) and a non-woody species (*Cichorium intybus*), of embryogenic lines from olive and horsechestnut (*Aesculus hippocastanum*), and of seeds from *Pistacia* and *Citrus* spp.

However, it has not been ten years of only successes, not at all! On the contrary, enthusiasm over some very good results has often been followed by deep frustration deriving from disappointing experiments. One example: after ten years of trials, testing all the different cryotechniques today available (included slow cooling), the cryopreservation of olive shoot tips is still unreliable, a long way from being proposable as a complementary approach to the conservation in field. Other “failures” have been the shoot-tip cryopreservation of a grape rootstock (‘Kober 5BB’) and of redwood (*Sequoiadendron giganteum*), as well as the cryopreservation of an embryogenic line of ash (*Fraxinus excelsior*).

What have we learnt from our ten-year experience in cryopreservation? What are the possible reasons for species adapting extraordinarily well to -196°C (e.g., white poplar,

red chicory), and others being highly “realcitrant” (e.g., olive)? Do we know all the potentialities, as well as all the limits, of the technology?

First reflection: before working with plant cryopreservation, I had many years of experience in the field of somatic embryogenesis of woody plants. In my opinion, the two techniques share a “practical” similarity when approaching the study of a new species, i.e., for both, if after the first preliminary experiments you get “nil” (i.e., no explant producing an embryogenic callus, as well as no explant surviving to -196°C), there is little hope that it will be possible to develop a successful protocol, whatever you change in the methodology. If you get at least one positive (i.e., a fully-developed plantlet), it is worthy to go on! That means much more than only a glimmer of hope!

Second reflection: working with woody plants, species having structural “difficulties” in the initial stages of micropropagation (e.g., big problems with the *in vitro* establishment, low proliferation rates, very slow shoot elongation) will be, with most probability, also “difficult” in the development of an effective cryopreservation protocol. One example: olive is very difficult and slow during the introduction and the establishment *in vitro*. So, is the difficulty in developing an effective shoot-tip cryopreservation protocol strictly due to its low tolerance to ultra-rapid freezing? Or, rather, is it due to the accentuation of a “basic” problem when a sort of second establishment *in vitro* is required (i.e., the shoot-tip development after thawing and plating)?

Third reflection: a big mistake was made in the ‘80s in the field of micropropagation, i.e., private users were given to believe that every species could be, soon or later, successfully micropropagated, it just required experiments and tests of different medium/growth regulator combinations. In Italy, that idea led to the proliferation of commercial laboratories in the early ‘90s, which soon found themselves fighting to produce and offer the same relatively-few species which could be actually produced *in vitro*. The majority of them went broke in a few years! Of course, cryopreservation is a totally different story (first, it is a non-profit activity). However, we must be very careful to avoid the same mistake when “promoting” cryopreservation.

Last but not least: *a warm welcome to projects like “Crymcept” and “CryoPlanet”!!* Certainly, we are all convinced that plant cryopreservation still needs much experimentation and, more important, a continuous confrontation between scientists with different experiences, made with different species and in different scientific and national contexts.

Key words: fruit species, Olea europaea, shoot tips, white poplar, Populus alba, plum, Prunus domestica, Cichorium intybus, olive, horsechestnut, Aesculus hippocastanum, seeds, Pistacia, Citrus, grape rootstock, redwood, Sequoiadendron giganteum, Fraxinus excelsior

Ex situ conservation of plant genetic resources in Russia: history, current status and perspectives

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Abstract

The N.I. Vavilov Institute of Plant Industry (VIR) is the only research institution in Russia whose activities and responsibilities include the collecting of plant material from its source populations, maintenance and conservation of plant genetic resources. The VIR and its history are widely known.

In 1920th outstanding Russian plant scientist Nikolay Vavilov was elected as Director of VIR. Nikolai Vavilov first called attention to the importance of collecting, storing and utilization of exotic germplasm for increasing agricultural production and providing humankind with more food. To this time the origin of conservation of plant genetic resources can be traced. Since 1920th Nikolai Vavilov and his colleagues visited 65 countries where they systematically collected plant germplasm of crop species and their wild relatives to accumulate and store plant genetic resources *ex situ* at VIR. Over the 100 years of its history, VIR has organized 1,550 expeditions (both international and domestic) to all continents in all of the climatic zones. The VIR's collecting missions and collection's evaluation are based on the Vavilov's concepts: 1. The theory of centres of origin of cultivated plants. 2. The law of homologous series in variation. 3. Vavilov's 'differential method' and approach to study ecogeographical diversity.

At present VIR holds one of the biggest and oldest plant germplasm collections worldwide. Its collection represents plant diversity of crop species and their wild relatives encompassing 323,000 accessions of 64 botanical families, of 376 genera, of 2,169 species including bred varieties, landraces, mutants and wild populations. Plant germplasm collections of VIR are maintained at the experimental stations in different regions of Russia. The germplasm collections are catalogued and characterized. The web-based database (www.vir.nw.ru) provides passport data for VIR collection.

The main objectives of VIR are to collect, maintain, document, characterize, evaluate, conserve, regenerate plant germplasm and distribute of seeds and clonal stocks. Much of the material is phenotyped. Inter- and intraspecific diversity of plants is evaluated for resistance to biotic and abiotic stresses and for quality traits.

Current stocks of VIR gene bank include seed base collections, field-grown plants of vegetatively propagated species, *in vitro* base collections, cryopreserved material, DNA extracts.

VIR provides long-term conservation of seed collections which are stored in sealed glass bottles or in foil packets and kept at -10°C and +4°C in a chambers. Plant seed accessions are divided into: basic (25,000 accessions) and active (more than 200,000 accessions) collections. Long-term storage of seeds at low temperature is the most convenient, traditional method for plant germplasm conservation. However, this method is not applicable for vegetatively propagated species. The collections of vegetatively propagated species consist of small berries, fruit crops and grapevine, including about 23,000

accessions, and a potato collection including about 9,000 accessions. Most germplasm of the vegetatively propagated crops at VIR is maintained in field collections. However, these collections are endangered by diseases, pests and abiotic stress. To avoid the possible loss of these germplasm collections, new strategies, technologies and information are developed.

Currently about 700 stocks of clonally propagated germplasm are stored under *in vitro* conditions, among them about 300 accessions of berries and fruit crops (raspberries, blackberries, strawberries, cherries, plums, currant, blue honeysuckle) and about 300 stocks of potato. Program of preservation of clonally propagated crops under *in vitro* conditions includes: testing and eliminating of viral and bacterial diseases, establishing *in vitro* culture, micropropagation, virus indexing, medium-term *in vitro* preservation and genotyping. DNA (RAPD and SSR) and isoenzyme markers have been applied to establish a database for cultivar genotyping of potatoes, raspberries and blackberries.

Cryogenic facilities have been recently installed at VIR and they are available for developing cryopreservation. At present there are pollen cryocollections (samples of 198 accessions of wild species of some fruit and small berry crops) and cryopreserved winter buds of about 80 accessions of apple). Cryopreservation of *in vitro* meristem of potato has been recently applied.

Long-term conservation of biodiversity of vegetatively propagated plants needs further developing of the theoretical and methodical approaches of *in vitro* and cryopreservation. This requires greater scientific co-operation in developing and standardizing techniques and methods.

Key words: varieties, landraces, mutants, wild populations, seeds, clonal stocks, in vitro base collections, cryopreserved material, DNA extracts, berries, fruit crops, potato

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