Cryopreservation of crop species in Europe

COST Action 871

Proceeding of the final meeting

AGROCAMPUS OUEST INHP, Angers - FRANCE
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COST- the acronym for European Cooperation in Science and Technology- is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds.

The funds provided by COST - less than 1% of the total value of the projects - support the COST cooperation networks (COST Actions) through which, with EUR 30 million per year, more than 30 000 European scientists are involved in research having a total value which exceeds EUR 2 billion per year. This is the financial worth of the European added value which COST achieves.

A "bottom up approach" (the initiative of launching a COST Action comes from the European scientists themselves), "à la carte participation" (only countries interested in the Action participate), "equality of access" (participation is open also to the scientific communities of countries not belonging to the European Union) and "flexible structure" (easy implementation and light management of the research initiatives) are the main characteristics of COST.

As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a "bridge" towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of "Networks of Excellence" in many key scientific domains such as: Biomedicine and Molecular Biosciences; Food and Agriculture; Forests, their Products and Services; Materials, Physical and Nanosciences; Chemistry and Molecular Sciences and Technologies; Earth System Science and Environmental Management; Information and Communication Technologies; Transport and Urban Development; Individuals, Societies, Cultures and Health. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

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Organizers and sponsors

This is the final meeting proceeding of COST Action 871 CryoPlanet "Cryopreservation of crop species in Europe".

It has been jointly organized by AGROCAMPUS OUEST Angers and IRD (Institut de recherche pour le développement, Montpellier).

The companies and institutes below are duly acknowledged for their strong financial and organisational support to this meeting.

Part I
Introduction

This book represents contributions, oral as well as posters, of the final meeting of COST Action 871, CRYOPLANET (Cryopreservation of crop species in Europe) held in Angers. Local organizers of the meeting were Dr. Agnès Grapin (AGROCAMPUS OUEST – Angers) and Dr. Florent Engelmann (Institut de Recherche pour le Développement).

COST Action 871 started in December 2006 with a Kick-off meeting at the COST office in Brussels and officially ended in December 2010. Twenty-one COST Action Countries (see figure 1) and 3 non-COST institutes (New Zealand Institute for Crop & Food Research (New Zealand), Vavilov Institute of Plant Industry (Russian Federation), Faculté des Sciences de Sfax (Tunisia)) participated actively in this initiative.

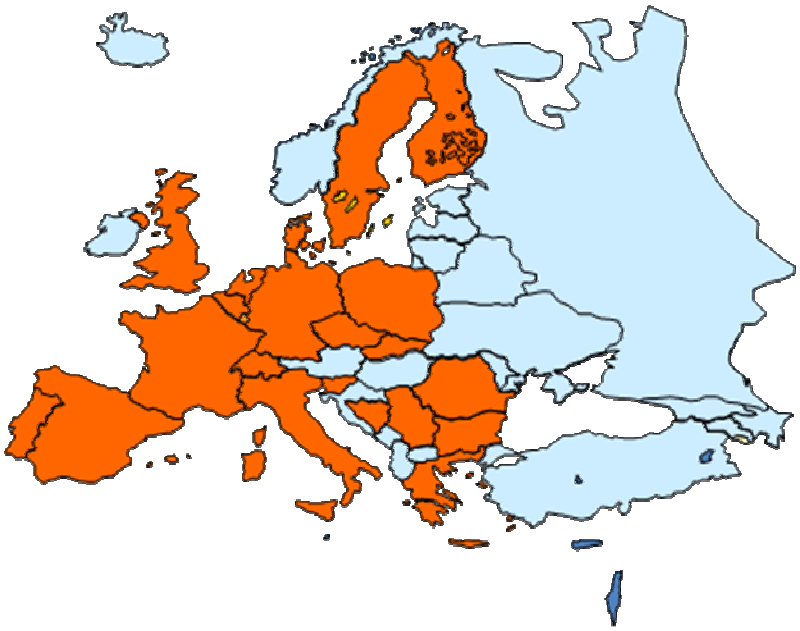

Figure 1. Participating countries at COST Action 871.

The Action was created because plant cryobiologists realized that plant cryopreservation was hardly applied in Europe. This was mainly due to the fact that (i) efficient and robust cryopreservation protocols applicable to many plant species and diverse germplasm types were not available, (ii) plant researchers were unacquainted to recent developments in cryogenic storage methods and (iii) there was a lack of coordinated research in Europe on plant cryopreservation. The main objective of this action was therefore “to improve and apply technologically advanced techniques for plant genetic resources conservation of crops that are grown/ and or conserved in Europe with main emphasis on long-term conservation through cryopreservation”. In order to achieve this, 2 working groups (WGs) were established; (i) a working group on fundamental aspects of cryopreservation/cryoprotection and genetic stability and (ii) a working group on technology, application and validation of plant cryopreservation.

Major activities of the Action included the organization of workshops, STSMs (short term scientific missions) and work group meetings.

The 5 training schools/workshops held in the framework CRYOPLANET, concentrated on two major topics, i.e. (i) the use of differential scanning calorimetry (DSC) as a tool to understand cryopreservation damage and (ii) cryopreservation through the dormant-bud technique as an alternative to the classical cryopreservation of in vitro material and were organized in Prague (on DSC), Florence and Copenhagen (both on dormant buds).

In total, 24 STSMs with focus on early carrier scientists were executed with a duration of one week to three months and proved to be one of the strongest tools of COST. These STSMs were beneficial for those young researchers as well as the host labs, confirmed by the numerous publications in peer reviewed journals that resulted from such collaborations.

Three WG 1 meetings (Oviedo (Spain), Wakehurst (UK) and Poznan (Poland), three WG 2 meetings (Florence (Italy), Leuven (Belgium) and Gatersleben (Germany)) and 2 common WG1/WG2 meetings (Oulo (Finland) and Angers (France)) were organized during the 4 years of this COST action. All participants from COST countries as well as invited speakers acknowledged the high quality of presentations at the different Workgroup meetings and emphasized the uniqueness of this initiative. They especially appreciated that a significant amount of researchers could be mobilized for this often "scientifically underestimated" topic.

Members of the action were also very active with respect to publication of their results (see Figure 2). The number of publications related to plant cryopreservation from members of the COST Action members increased considerably with the start of the action (end 2006). What is more important, the number articles in peer reviewed journals almost doubled.

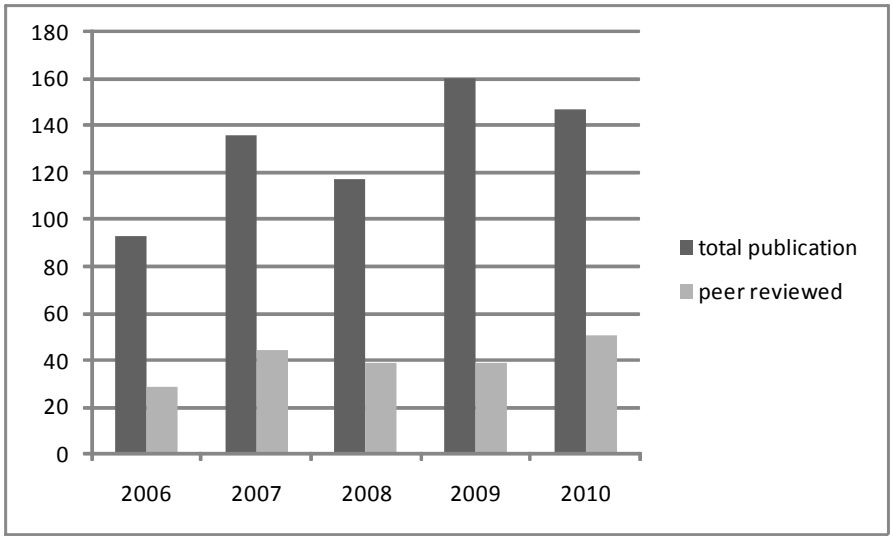

Figure 2. Publications by COST Action 871 members

This special issue is divided in 3 parts; one devoted to WG1 topics "fundamental aspects of cryopreservation/cryoprotection and genetic stability"; one devoted to WG2 topics "technology, application and validation of plant cryopreservation" and a last one with country reports with most important activities on plant cryopreservation that took place during the running of this action.

I would like to conclude this foreword by thanking all the participants of COST Action 871 for their active participation to all events that were organized; These four years showed that good quality science can take place in a very friendly and open atmosphere. Especially, the Action's Executive Committee (Paul Lynch, Joachim Keller, Florent Engelmann, Pawel Pukacki, Marjatta Uosukainen and Angeles Revilla Bahillo) are gratefully acknowledged for their advice and tremendous help throughout these 4 years. All the contributors of this issue are thanked for their excellent papers and Agnès Grapin for the perfect organization of the final meeting. Finally I would like to acknowledge the COST office for the opportunity that they have given us to put European plant cryopreservation on the world map.

Bart Panis, Chair of COST Action 871

In Europe, France is a country with a long history in plant cryopreservation research. The two French representatives in the Management Committee of COST Action 871 "Cryopreservation of crop species in Europe" were researchers from AGROCAMPUS OUEST and IRD. It was thus logical that AGROCAMPUS OUEST and IRD volunteered to jointly organize the final meeting of this COST Action in France.

This meeting, which brought together 68 participants from 24 COST and non-COST countries, took place in AGROCAMPUS OUEST INPH Angers, from 8 to 11 February 2011.

As conveners of this final meeting, we would like to thank all the participants for coming to Angers and for making the scientific presentations and exchanges so interesting and lively, and all members of the scientific and local organizing committees for their very efficient and friendly support. We also would like to thank our numerous sponsors for their strong financial and organizational support and COST for supporting the cost of publishing this document.

This book comprises a total of 54 articles, involving both oral and poster presentations related to the two Working Groups of the Action (WG1: fundamental aspects of cryopreservation/cryoprotection and genetic stability; WG2 technology, application and validation of plant cryopreservation) as well as country reports, presenting the research activities performed during the course of the Action and plans for the future.

We hope that this publication will provide a comprehensive overview of the status of cryopreservation research in Europe in 2011, and that it will be a useful document for the plant genetic resource community at large, both in and outside Europe, including researchers, engineers, students, genebank managers and decision-makers.

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Part II
Working Group 1: Fundamental Aspects of Cryopreservation / Cryoprotection and Genetic Stability

Design of transgenic cell cultures as model systems for cryopreservation research

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1. Introduction

In recent years plant cell and tissue cryopreservation improved a lot from the development of new techniques. It has been shown that plant cells during these procedures are exposed to different stresses. Addition of anti-stress compounds like antioxidants improved survival. Further improvement of survival during different cryopreservation procedures can be expected from an improved induction of plants own anti-stress mechanisms.

Undifferentiated plant cell lines have been widely used as model systems to investigate plant stress on the cellular level like the formation of phytoalexines (Schumacher *et al*. 1987) or phytochelatines (Grill *et al*. 1985). Also anti-stress mechanisms relevant to cryopreservation like salt tolerance (Blumwald and Poole 1985) and osmotic tolerance (Droillard *et al*. 2004) have been investigated using plant cell lines.

A disadvantage for the use of undifferentiated plant cell lines in fundamental research is their heterogeneous population of cells with different traits. Differences among single cells of a culture may arise from chromosomal aberrations (Bayliss *et al*.1980), point mutations, transposon activation (Alves *et al*. 2005) or even epigenetic changes (Kaeppler *et al*. 2000). Transgenic cell cultures may be further affected by post-transcriptional gene silencing (Kanno *et al*. 2000) or residual wild type cells outgrowing the transgenic ones. The heterogeneity of transgene expression in transformed cell cultures has recently been documented by Nocarova and Fischer (2009). They suggested cloning to yield more homogenous cultures or the use of *gfp*-tagged proteins for monitoring target gene expression. If the physiological function of a protein should be investigated by over-expression, protein fusions may influence protein function by steric effects. An alternative approach is the co-expression of physically independent proteins. Co-expression can be arranged by linking genes with spacers, 2A elements or viral IRES elements (Halpin 2005).

In the present approach a viral IRES element has been used to construct dicistronic vectors co-expressing a target and a reporter gene. From these vectors a unique mRNA is transcribed. During translation the IRES element, acting as stop codon, leads to cap-independent recruitment of ribosomes. Thus two physically independent proteins are formed. The reporter gene *luc* is used as second cistron and target genes providing traits relevant for cryopreservation as first cistrons. The constructed vectors were tested for expression monitoring in cell cultures.

2. Materials and Methods

2.1. Vector construction

The construction of the transformation vector containing the At*nhx1* gene as first cistron was carried out as described in Ali *et al.* (2010) and the construction of the transformation vector containing the *pr-10a* gene as first cistron was carried out as described in El Banna *et al.* (2010) (Fig. 1).

Fig. 1. Dicistronic transformation vectors containing a target gene (At*nhx1* or *pr-10a*) as first cistron and a reporter gene (*luc*) as second citron linked by the *TMV*cp148 IRES element.

2.2. Transformation of tobacco plants

Agrobacterium mediated transformation of tobacco plants and regeneration of transgenic plants was done by the leaf disc method as described in Ali *et al.* (2010). Confirmation of transformation was done by PCR and Southern blot methods as described in Ali *et al.* (2010).

2.3. Initiation of cell cultures

Initiation of callus cultures from leaf and stem material of wild type tobacco of the cultivar Samsung and transformed tobacco was done as described in Ali *et al.* (2010). Callus initiation took place on solid 4X medium (www.dsmz.de) containing 5mg/l phosphinothricin.

2.4. Transformation of potato cell cultures

Potato cell cultures cv. Desiree (DSMZ No. PC-1182) from the DSMZ collection were transformed by *Agrobacterium* mediated transformation. Co-cultivation was done as described in El Banna *et al.* (2010). Selection of transformed cells was carried out on solid 4X medium (www.dsmz.de) supplemented with 5mg/l phosphinothricin. Subsequent selection based on the transgene expression level was carried out under an Imager (Fuji LAS 3000) as described in El Banna *et al.* (2010).

2.5. Growth tests for salt- and osmotic tolerance

Growth tests in liquid medium were performed in 100 ml Erlenmeyer flasks, inoculated with 1 g of cells harvested from the logarithmic growth phase. Growth was carried out in control 4X medium and 4X medium supplemented with 320mM NaCl or 500mM sorbitol at 23°C on a gyratory shaker at 100 rpm. Every 2 days, 5 flasks were harvested independently for each treatment and dry weight was detected.

2.6. Glutathione and Proline tests

Proline and Glutathione tests were carried out as described in El Banna *et al.* (2010)

3. Results

3.1. Transgene monitoring in Atnhx1 expressing transgenic cell lines

To test expression monitoring a target gene with known function was used for the construction of the transformation vectors. The At*nhx1* gene chosen is known to provide increased salt tolerance (Apse *et al.* 1999). In a dicistronic vector the At*nhx1* sequence was linked to a firefly luciferase gene by the *TMV*cp148 bp IRES sequence (Fig.1). Both genes

were expressed under the control of a mannopine synthase promoter, providing high expression in cell cultures. Tobacco plants of the cultivar Samsung were transformed by *Agrobacterium* mediated transformation. Callus and suspension cultures from the wild type as well as from the T_0 generation of transgenic plants were initiated. These suspension cultures were used to proof a coincidence of *luc* activity and the expected At*nhx1* mediated salt tolerance in comparative growth studies.
Exposed to salt concentrations from 50 to 200 mM NaCl, the decrease of fresh weight accumulation was less pronounced for the transgenic cell line. At concentrations of 50 mM and 100mM NaCl transgenic and wild type cultures showed a significant difference (P = <0,001) in fresh weight accumulation indicating increased salt tolerance of the transgenic cell line (Fig. 2A). In parallel the luminescence of the transgenic cells was measured in another set of growth tests (Fig. 2B). Increased salt tolerance at 50 mM and 100 mM salt challenge coincided with an increase of luciferase activity in the transgenic cell line.

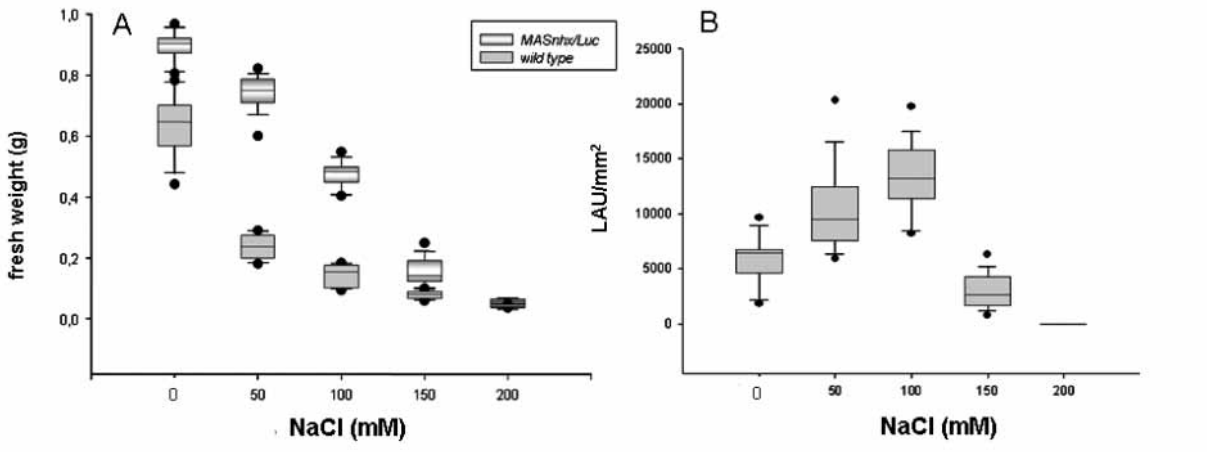

Fig. 2. Growth rates of transgenic versus wild type cells on solid medium: (A) increase of fresh weight on media supplemented with different salt concentrations over a growth period of 3 weeks starting from the same inoculum, (B) luciferase activity (LAU- Linear arbitrary units) quantified by a Fuji LAS 3000 Imager and Aida® quantification software.

3.2. Establishment of pr-10a over-expressing cell cultures.
To identify proteins relevant for salt and osmotic tolerance, a potato cell culture cultivar Desiree from the DSMZ collection was challenged with osmotic stress provided by sorbitol, sucrose and NaCl under equiosmolar conditions. The proteome was analyzed with 2D gels. Six spots, formed de-novo in high amounts after salt and osmotic stress, were selected for identification. The most prominent spot was identified as PR-10a protein (STH-2 (PR-10a, gi!169551)). This protein was chosen for over-expression experiments.
Based on the sequence information for the PR-10a protein from the NCBI database (accession no. gi!169551), the *pr-10a* gene was isolated from the potato cultures by PCR methods and used as first cistron in the dicistronic transformation vectors (El Banna *et al.* 2010). The same potato cell culture was also used for *Agrobacterium* mediated transformation. Transformed cells were regrown on solid medium with supplementation of phophinothricin as selective agent. A second selection step using microcalli was carried out based on the expression level of the transgene, measuring luciferase activity in a semi-quantitative assay (Fig. 3). Since the test is none-destructive highly expressing cell clusters could be manually picked. From selected calli suspension cultures were established.

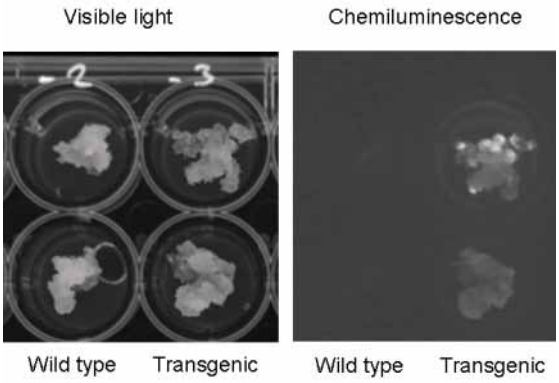

Fig. 3. Semi-quantitative luciferase assay. Luciferase activity was detected by spaying luciferin on top of calli. In a Fuji LAS 3000 Imager chemiluminescence was visualized and quantified by Aida® quantification software. Highly luminescent sections of callus were picked and sub-cultured.

3.3. Characterization of the pr-10a over-expressing cell line

To investigate the influence of *pr-10a* over-expression, growth in liquid culture under osmotic and salt challenge was tested. Dry weight accumulation was recorded over a period of 18 days for the wild type and the transgenic cell line under equiosmolal conditions using 4X medium supplemented with 0.5 M sorbitol or 0.32 M NaCl and 4X medium as control. Dry weight accumulation was the same under control conditions for both cell lines. Under osmotic challenge, the transgenic cultures showed an almost unaffected onset of the growth curve, whereas the wild type cell line started growth only after a prolonged lag phase. In addition, the transgenic culture reached a higher dry weight level after 18 days of growth under osmotic challenge. A more severe effect was observed under salt stress. In this case the wild type culture showed almost no growth (Fig.4.)

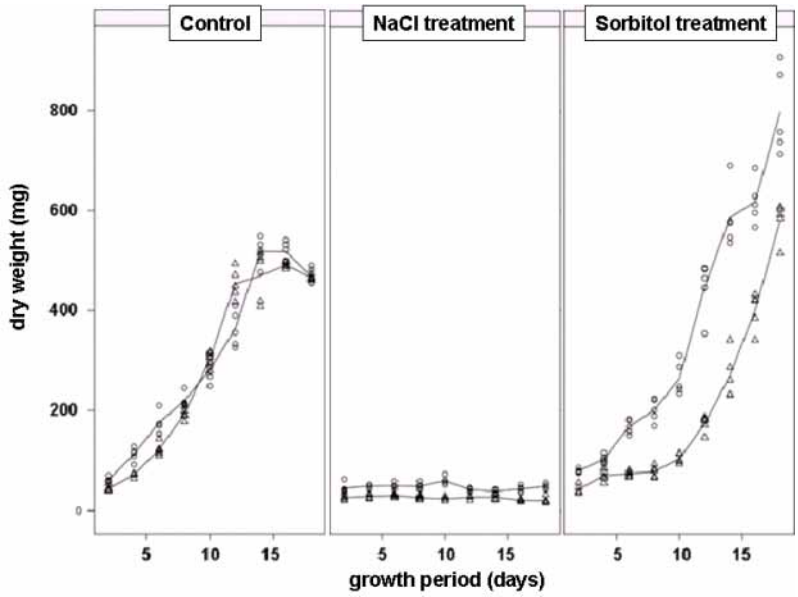

Fig. 4. Growth of the wild type (circles) and transgenic cell line (triangles) under osmotic and salt stress. Dry weight accumulation in liquid culture was measured under control conditions, sorbitol (0.5 M) and NaCl (0.32 M) stress conditions. Dry weight was measured for 5 replicates harvested every second day for wild type and transgenic culture.

Apart from comparing growth behaviour under salt and osmotic stress proline and glutathione content was measured under normal and stress conditions as indicators of osmotic and salt stress. Under control conditions the concentration of free proline and the percentage of oxidized glutathione from total glutathione were the same in both cell lines. Under osmotic and salt stress the transgenic cell culture contained more free proline (Fig. 5A) and a higher percentage of oxidized glutathione (Fig. 5B).

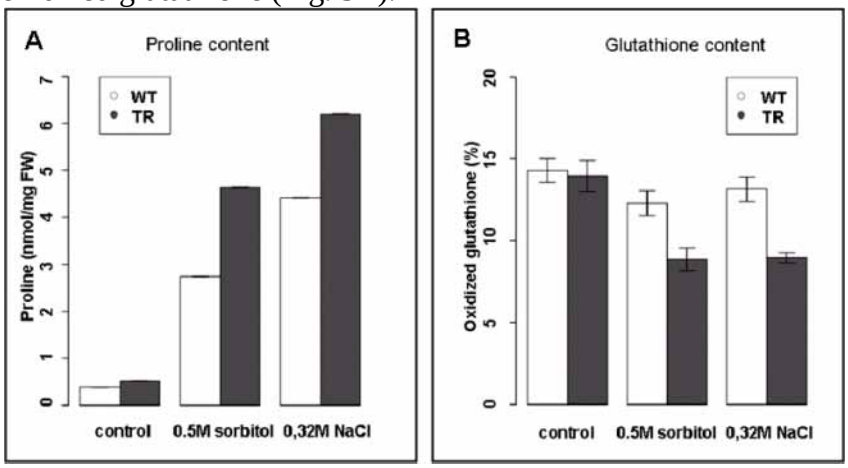

Fig. 5. Effect of PR-10a over-expression on proline and glutathione metabolism. (A) Proline content of wild type (WT) and transgenic potato cell cultures (TR) under control conditions and under osmotic (0.5M sorbitol) or salt (0.32M NaCl) challenge after three days of growth. (B) Percentage of oxidized from total glutathione in transgenic (TR) and non-transgenic (WT) cell cultures grown for three days under control or different stress conditions (0.32M NaCl, 0.5M sorbitol).

4. Discussion

In the present study dicistronic transformation vectors were developed combining a target gene as first cistron and a reporter gene as second cistron under the control of the same promoter. Since both genes are expressed in a fixed ratio (Dorokov *et al.* 2002) the expression of the target gene can be monitored by simple measurement of the reporter gene. Both gene products are physically independent proteins therefore no influence on the physiological function of the target gene should take place. With a gene of known function it could be demonstrated that expression monitoring in cell cultures is possible. The same approach could also be applied to a target gene with unknown function. This facilitates the use of undifferentiated cell cultures to investigate unknown gene functions by over-expression experiments. The use of such vectors in cell culture systems allows easy monitoring of transgene expression. Transgenic cell cultures which are exposed to epigenetic variation (Kaeppler *et al.* 2006) or post-transcriptional silencing (Kanno *et al.* 2000) can be standardized on a specific expression level. The approach circumvents the time consuming clone selection procedure or the use of GFP-tagged proteins suggested by Nocarova and Fischer (2009).

Furthermore the strategy allows using a transgenic culture without supplementing the medium with selective agents to guarantee the transgenic character. Since target and reporter gene in the presented approach are under the control of the same promoter gene silencing affects both genes in the same way and does not influence monitoring results. Nevertheless it has to be pointed out that monitoring visualizes only the transcription level of the target gene. Post-translational effects like different turnover of proteins have to be taken into account.

It was demonstrated that the present approach can be used to investigate traits like salt and osmotic tolerance which are relevant for cryopreservation.

5. Acknowledgements

The research of Z Ali at DSMZ has been financed by DAAD and the PhD work of A El Banna at DSMZ was supported by a grant of the Egyptian government.

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Transgenic potato cell cultures – Application as a model system to investigate the relation of cryopreservation and osmotic tolerance

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1. Introduction

Presently the DSMZ maintains a collection of more than 700 undifferentiated plant cell lines from more than 600 species routinely by continuous sub-culturing. Cryopreservation would be the only applicable long term preservation method to avoid loss of cultures by laboratory failure, contamination or genetic drift.

The most important problem for plant cell cryopreservation is the high water content of plant cells and the sub-cellular presence of water in the vacuoles. Therefore dehydration of cells is necessary for freezing success, exposing the cells to considerable osmotic stress and possibly salt stress. A better understanding of the molecular mechanisms of salt and osmotic tolerance could help to improve cryopreservation.

Cell cultures provide an excellent model system to investigate stress mechanisms on the cellular level, like the function of Na^+/H^+ pumps (Queirós *et al*. 2009), aquaporines (Kobae *et al.* 2006) and the formation of compatible solutes (Hoque *et al*. 2007). In the present study we therefore looked for cell cultures with different levels of osmotic tolerance as model systems to investigate the basic relation between osmotic tolerance and cryopreservation success. Proline accumulation, which is a known reaction on osmotic challenge (Khedr *et al*. 2003) and the percentage of oxidized glutathione from total glutathione, which indicates the degree of oxidative stress (Tausz *et al*. 2004) were determined as indicators for osmotic tolerance. The relation of osmotic tolerance and cryopreservation success was investigated by two different approaches. In a first approach plant cell lines initiated from 3 different potato cultivars Unicopa, Desiree and Ijsselster were used. They were selected due to the different behaviour of their apical meristems in ultra-rapid cryopreservation. In a second approach a wild type cell line of potato cv. Desiree and a transgenic cell line of this cultivar showing osmotic tolerance by over-expression of the PR-10a protein were applied.

2. Materials and Methods

2.1. Plant material

The following cell lines were used: *Solanum tuberosum* cv. Desiree wild type (DSMZ No. PC-1182) and transgenic (El Banna *et al.* 2010), cv. Unicopa (DSMZ No. PC-1189) and cv. Ijsselster (DSMZ No. PC-1188).The cell cultures were routinely subcultured in 300ml Erlenmeyer flasks containing 100ml suspension using 4X medium (www.dsmz.de). The Erlenmeyer flasks were incubated on a gyratory shaker (TR-250, Infors AG, Basel, Switzerland) with 50mm orbit (100 rpm) at 23°C.

2.2. Proline determination
Proline content was measured as described in El Banna *et al*. (2010).

2.3. Glutathione determination
Quantification of oxidized and total glutathione was performed as described in El Banna *et al*. (2010).

2.4. Semi-quantitative detection of luciferase activity
Luciferase activity was monitored according to El-Banna *et al*. (2010).

2.5. Cryopreservation
For cryopreservation experiments the controlled rate freezing method was performed in a minitest system previously worked out at DSMZ (Heine-Dobbernack *et al*. 2008).
An inoculum of 30% packed cell volume was used. Cells were subjected to different sorbitol concentrations for 2d, then incubated in 5% DMSO as cryoprotectant for 1h (first approach) or 1.5h (second approach) and finally cooled down with a programmed cooling rate of -0.25°C/min to -40°C. After a holding time of 15min at -40°C the cryovials were immediately transferred into liquid nitrogen. Regrowth behaviour after freezing was determined by quickly thawing the cryovials at 40°C in a water bath for 2min 45sec. As soon as the last visible ice had melted the content of the cryovials was poured on 4X agar covered with 3 sterile filter paper discs. After 2h the uppermost filter was transferred to fresh 4X agar and the agar plates were incubated for 2 to 4 weeks. Recovery growth was measured by dry weight determination.

2.6. Dry weight determination
For determination of dry weight, cells grown on a filter paper disc (see 2.5) were quantitatively transferred to a pre-washed (0.9% NaCl + 0.05% HCHO), dried (60°C for 3d) and pre-weighed filter. Cells on the filter were dried to constant weight at 60°C for 3d. Afterwards cells and filter were weighed again.

3. Results

3.1. Comparative studies with Solanum tuberosum cultivars Unicopa, Desiree and Ijsselster
Plant cell lines were initiated from the 3 different potato cultivars. In ultra-rapid cryopreservation experiments the meristems of Unicopa had shown highest plant regeneration rates (app. 75%), cv. Desiree had shown medium results (app. 20%). Least plant regeneration rates had been achieved with cv. Ijsselster (app. 10%).
3.1.1. Osmotic stress behaviour of Unicopa, Desiree and Ijsselster cell lines
Osmotic and salt tolerance of the 3 plant cell lines Unicopa, Desiree and Ijsselster were characterized based on proline accumulation and relative content of oxidized glutathione.
After 3 days of osmotic (0.5 M sorbitol, 0.42M sucrose) or salt challenge (0.32 M NaCl) compared to control (4X medium) in 24 well titer plates, the cells were harvested and proline content and glutathione content were determined.
Cell cultures of the cultivar Unicopa showed the highest proline accumulation under all stress conditions. In contrast to the two other cultivars there was an increase compared to control (Fig. 1A).
The percentage of oxidized glutathione (GSSG) from the total glutathione pool (GSH (reduced) + GSSG (oxidized)) was lowest in Unicopa cell cultures, showing that this cultivar had the highest protection against oxidative stress, whereas in cultures of Ijsselster the

percentage of oxidized from total glutathione was highest after three days of osmotic or salt challenge (Fig. 1B). The parameters measured indicate that cell cultures of the cultivar Unicopa have the highest and cultures of Ijsselster the lowest degree of osmotic tolerance.

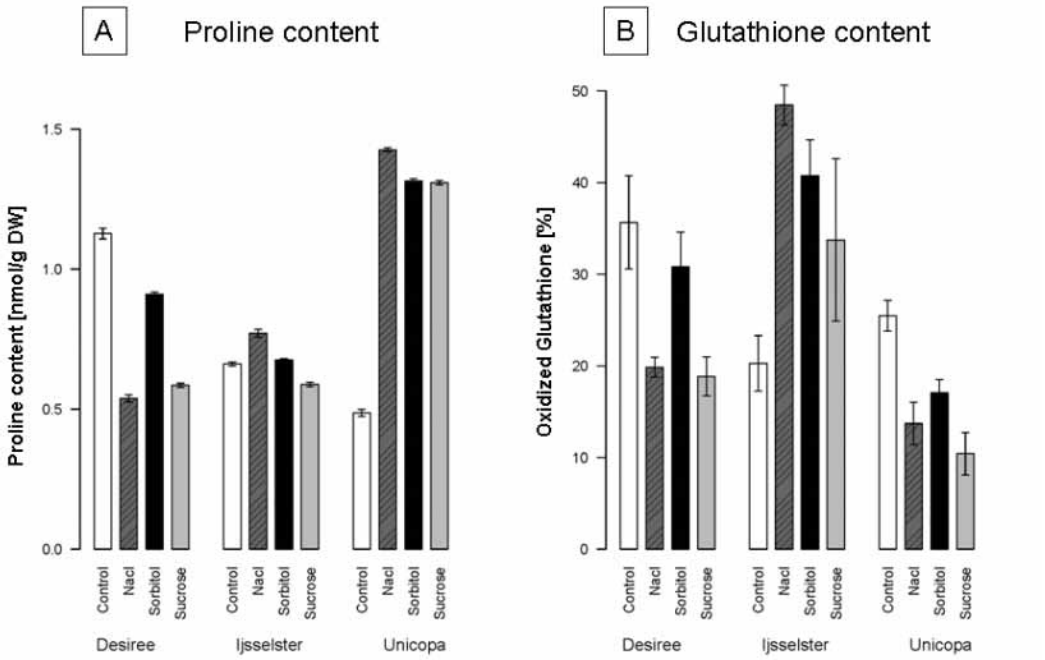

Fig. 1. Osmotic stress behaviour of *Solanum tuberosum* cv. Unicopa, Desiree and Ijsselster. Proline content (A) and percentage of oxidized from total glutathione (B) after 3 days of osmotic (0.5 M sorbitol, 0.42M sucrose) or salt challenge (0.32 M NaCl) compared to control (4X medium). DW: dry weight. Data shown represent the mean of 3 replicates. Error bars indicate standard deviation.

3.1.2. Cryopreservation behaviour of Unicopa, Desiree and Ijsselster cell lines

Comparing the cryopreservation behaviour of the three cell lines of *Solanum tuberosum* cultivars a different ranking was obtained than for osmotic tolerance. Identical cryopreservation conditions were applied to the three cell lines and cryopreservation success was measured as dry weight accumulation of cells re-grown after freezing (Fig. 2).

Ijsselster regrew faster than the two other cultivars. Unicopa showed the slowest regrowth.

In addition Ijsselster regrew over the whole range of preculture conditions, even without sorbitol in the preculture medium. In case of the two other cell lines a distinct optimum of regrowth at 0.5 to 0.7M for Unicopa and 0.7M for Desiree was observed. For these two cell lines a minimum sorbitol concentration of 0.3M was necessary to enable at least some regrowth. The absolute values of dry weight are not directly comparable because the cells of Ijsselster had to be harvested already after 12 days whereas in case of Desiree and Unicopa first regrowth was observed after 2 to3 weeks and therefore cells were harvested after 29 days.

Hence in studies with the three cell lines of Unicopa, Desiree and Ijsselster cryopreservation success could not be correlated with osmotic tolerance, measured by proline content and content of oxidized glutathione.

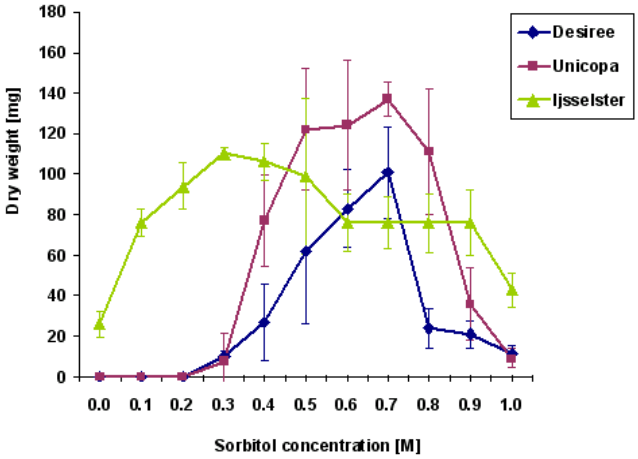

Fig. 2. Cryopreservation behaviour of *Solanum tuberosum* cv. Unicopa, Desiree and Ijsselster. Cryopreservation conditions: 30% pcv, 2d in sorbitol, 5% DMSO 1h, -0.25°C/min. Cryopreservation success was measured as dry weight accumulation of cells re-grown after freezing. Data shown represent the mean of 8 replicates. Error bars indicate standard deviation.

3.1.3. Cell size of Unicopa, Desiree and Ijsselster cell lines

The size and degree of vacuolization may also have an influence on survival of cells during freezing. Therefore the cell size of the different cultures was compared. The cells of the Ijsselster cell line were generally smaller than those of the two other cultivars (Fig. 3). Thus it seems possible, that effects like cell size and morphology may superimpose the effect of anti-stress mechanisms concerning cryopreservation success.

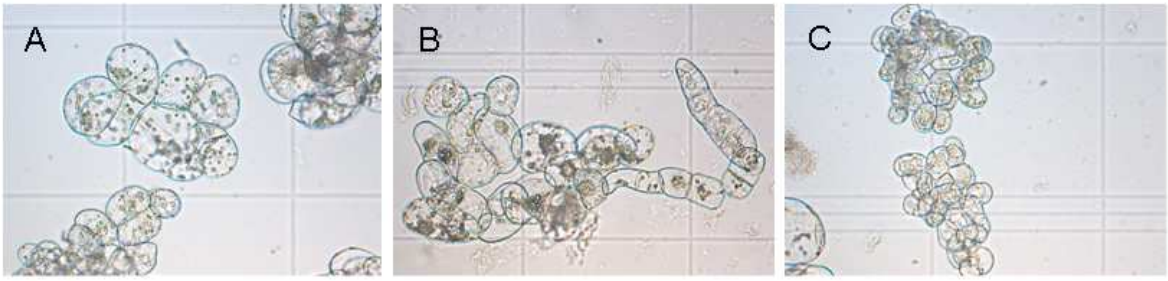

Fig. 3. Cell size of *Solanum tuberosum* cv. (A) Unicopa, (B) Desiree and (C) Ijsselster. For estimation of cell size by microscopy (objective: 10fold) a Fuchs Rosenthal chamber (depth 0.2 mm, 0.0625 mm^2) was used.

3.2. Investigations with wild type and an osmotic tolerant transgenic cell line of Solanum tuberosum cv. Desiree

To overcome the problem of comparing the cryopreservation behaviour of cells differing in more than the factor under investigation a transgenic approach was used. A cell line of *Solanum tuberosum* cv. Desiree over-expressing the *pr-10a* gene showed increased salt and osmotic tolerance concerning growth**,** proline content and glutathione content compared to the wild type (El Banna *et al*. 2010). Using this transgenic cell line and the corresponding wild type, allowed the comparison of cells of the same size and morphology but differing in osmotic tolerance.

Before starting a cryopreservation experiment the expression of the target gene under investigation was controlled by semi-quantitative luminescence measurement of the luciferase activity in the transformed cell line. Cryopreservation could therefore be performed without any selective agent in the medium of the transgenic cell culture.

Cryopreservation behaviour of wild type and transgenic cell line

To investigate whether over-expression of *pr-10a* also resulted in a higher level of cryotolerance wild type and transgenic cell cultures of *Solanum tuberosum* cv. Desiree were cryopreserved under equal experimental conditions optimal for the Desiree cells. Cryopreservation success was measured as dry weight accumulation of cells re-grown for 29 days after freezing.

When applying different sorbitol concentrations, cryopreservation success of wild type and *pr-10a* over-expressing cell cultures turned out to differ considerably (Fig 4A). For the transgenic culture equal amounts of surviving cells were determined over the whole range of sorbitol concentrations. It showed a broad range of cryopreservation success similar to the cultivar Ijsselster, whereas the wild type, as in previous experiments, showed a distinct optimum of surviving cells at 0.5 to 0.8M sorbitol. However within this optimal range no significant difference between cryotolerance of wild type and transgenic cell cultures could be observed.

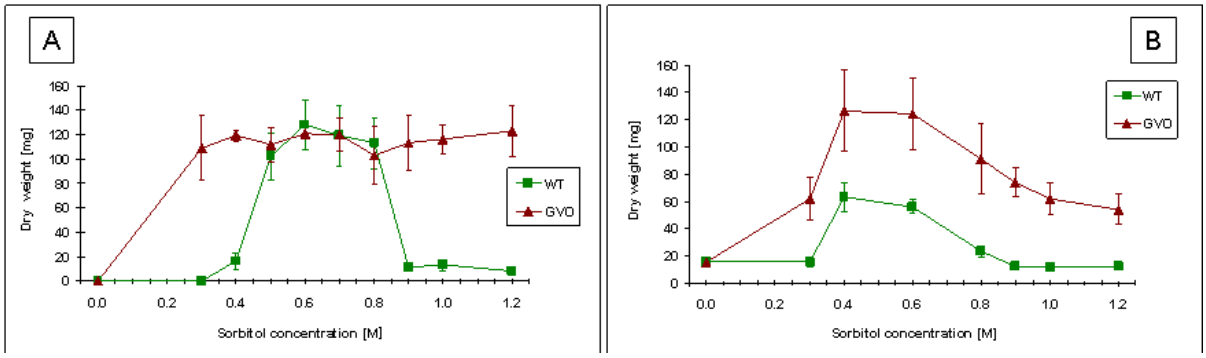

Fig. 4. Cryopreservation behaviour of wild type (WT) and transgenic (GVO) cell line. Cryopreservation success, measured as dry weight accumulation of cells re-grown after freezing, was compared under (A) standard conditions (after 29 days) and (B) improved conditions (after 15 days) of regrowth. Data shown represent the mean of 6 (B) to 8 (A) replicates. Error bars indicate standard deviation.

Measurement of survival after cryopreservation was improved by harvesting cells already after 15 days. Cultures recovered from freezing were exposed to cryopreservation again as above, thawed and re-grown. Under these conditions the transgenic cell line showed a better performance over the whole range of sorbitol concentrations (Fig. 4B).

4. Discussion

Osmotic tolerance helps cells to overcome dehydration stress caused by water loss, necessary for successful cryopreservation (Thierry *et al*. 1999). In studies with the three cultivars Unicopa, Desiree and Ijsselster cryopreservation success could not be correlated with proline content and content of oxidized glutathione, stress parameters often used to characterize osmotic tolerance (Khedr *et al*. 2003, Tausz *et al*. 2004).

However the compared cells differed in cell size and possibly also in other morphological and physiological characters. Therefore it seems possible that effects like cell size, may superimpose the effect of anti-stress mechanisms concerning cryopreservation success.

To establish a model system of cells differing only in the degree of osmotic tolerance a transgenic approach was used. A transgenic cell culture over-expressing the *pr-10a* gene showed increased osmotic tolerance compared to the wild type (El Banna *et al*. 2010). This

approach proved to be better suited as a model system to investigate the relation of cryopreservation and osmotic tolerance.
Applying a regrowth period of 29 days cryopreservation behaviour of the transgenic cell line and the wild type cell line differed, but a simple correlation between cryopreservation success and osmotic tolerance could not be detected. Under optimal conditions for the wild type cell line no difference between transgenic and wild type cells was observed. The samples of both cell lines showing best survival may already have reached the stationary phase and therefore differences were no longer detectable. Therefore survival was determined by harvesting cells after 15d. Under these conditions the transgenic cell line showed better performance over the whole range of sorbitol concentrations, indicating a correlation between cryopreservation success and osmotic tolerance. Based on these results a more detailed analysis of the relation of PR-10a formation, osmotic tolerance and cryopreservation success is under investigation.
Dobranszki *et al*. (2003) showed that osmotic tolerance of callus cultures initiated from different potato cultivars resembled that of the corresponding *in vitro* potato shoots. It can therefore be expected that results obtained from cell culture research are also relevant for the cryopreservation of intact plants.

5. Acknowledgements

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Safekeeping parameter for evaluation of plant thermal transitions important for cryopreservation by vitrification methods

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1. Introduction

A variety of theories have been proposed to understand why some systems easily vitrify while others do not. There are many publications about glass-forming ability (GFA) and glass stability (GS). GFA is the easiness to vitrify a liquid during cooling, while GS is the glass resistance against devitrification during warming (Nascimento *et al.* 2005). Glass forming ability and stability are important not only for bulk metallic glasses, alloys and oxide glasses but also for cryoprotectants and cryopreservation. In some cases, the ice nucleation and subsequent intracellular ice formation is important especially using the vitrification method by the cryopreservation procedures.

The GFA is related to the ratio of T_g/T_l [Eq.1, Tab.1], to the so-called reduced temperature (T_{rg}) where T_g and T_l are the glass transition temperature and liquidus temperature, respectively. The glass-forming tendency should increase with the reduced glass temperature T_{rg},. Liquids with glass temperatures as high as 2/3 T_l**,** if seed-free; thus they easily could be supercooling to the glass state. Liquids with glass temperature close to $T_l/2$ could, be chilled to the glass state only in relatively small volumes and at high cooling rates (Turnbull 1969). These liquids are good glass formers.

There are several parameters regarding GFA and GS during cooling and warming. For instance, (Turnbull 1969) [Eq.1, Tab.1] proposed a parameter based on the assumption that the nucleation frequency in a supercooling melt is inversely proportional. Melting point is characterized by temperature of the liquidus (T_l), instead of equilibrium melting point (T_m), Turnbull adopted the classical nucleation theory and used parameter as a criterion for GFA as the avoidance of a single nucleation event. Weinberg (1994) was the first who used another parameter [Eq. 7, Tab.1]. Actually Weinberg used the peak maximum of crystallization and not the onset of crystallization. In this paper we assumed the temperature of maximum crystallization and onset temperature is in high correlation and thus there are interchangeable. Hrubý (1972) proposed his own parameter (K_H) to probe glass stability against crystallization on heating [Eq. 8, Tab. 1]. According to Hrubý, the higher the value of K_H for a certain glass, the higher its stability against crystallization is on heating and, presumably, the higher the glass ability to vitrify is on cooling. Recently, Lu and Liu (2003) proposed a new criterion [Eq. 5, Tab. 1], which was tested for 49 metallic glasses, 23 oxide glasses and 25 cryoprotective aqueous solutions. There are many other parameters which the authors expressed the relationship of the tree parameters (T_l, T_x and T_g) with the aim to express the GFA and GS (see Tab. 1 for details). Many other parameters, which are not shown, are modifications of these mentioned in the Tab.1.

Vitrification methods based on the glassy state in the plant cell are currently most widely used in cryopreservation. The plant cells and tissues are able to keep regeneration ability at ultralow temperatures (e.g. liquid nitrogen) in glassy state for long-term.

The aim is to get together all these thermal transitions characteristics (T_l, T_x and T_g) in relation to expressed potentially danger temperature zone at which the plant can be lethally damaged by ice crystals. The safekeeping parameter was established to express the potentially

danger temperature zone at which the plant can be lethally damaged by ice crystals growing during cooling.

2. Methods for thermodynamic characteristics measurement

The modern methods are used for measurement of the thermodynamic characteristics of plant material. Differential scanning calorimetry with the modification like temperature modulated and quasi-isothermal temperature modulated are mostly used. By these techniques three main characteristic of plant tissue important for cryopreservation can be obtained: equilibrium freezing point (T_m) or temperature of liquidus (T_l), onset of crystallization temperature (T_x) and glass transition temperature (T_g). The T_g is usually determined by thermal analysis measurement as the temperature at which heat capacity is halfway between liquid and glass values.

3. Results

3.1. Equilibrium temperature of freezing point depression/liquidus temperature (T_m/T_l)

The only danger for cells and tissues is intracellular ice formation which is usually lethal for the plant tissues. The ice crystals may not to be formed between 0 °C to the equilibrium freezing point (T_m) during cooling. The equilibrium freezing point depression is strongly dependent on the osmotic potential of the substances. With higher concentration of osmotic substances either by dehydration or by uptake of the osmotic active cryoprotectants consequently the T_m is depressed to the lower temperatures. The temperature at which ice melts (0 °C) is well-defined in contrary to the temperature at which water freezes. Melting of a solution is determined as equilibrium temperature of freezing by the concentration and types of substances dissolved in the solution.

The higher the melt viscosity at the melting temperature, the lower is its crystallizability glass forming tendency. It was confirmed and extended prior indications that permeating cryoprotective additives decrease the ice nucleation temperature of cells, and have determined some possible mechanisms for the decrease. A simple equation describes the relationship between osmotic potential (Ψ_s [osmol]) of a solution in equilibrium with ice at subzero temperature (T [°C]) $\Psi_s = T/1.86$. Nucleation could be a limiting factor for storage of vitrified tissue at temperatures near T_g within the same narrow temperature range. Increasing solute concentration seems to depress homogenous and heterogeneous ice nucleation temperature more than corresponding melting point depression often about twice as much as the melting point ($T_x \sim T_m/2$).

3.2. Ice crystallization temperature (T_x)

When cells or whole tissues remain in the liquid state at the temperatures below the equilibrium freezing point ($\sim T_m$) they are in the metastable supercooling state. The nucleus presence is essential for initiation of the ice nucleation, which is an aggregation of water molecules with an ice-like structure, sufficiently large to grow. With decreased temperature decreases the size of the water molecule clustering required for ice nucleation at onset of ice crystallization temperature (T_x) and subsequently increase ice growing rate. The crystallization rate of a supercooling liquid is then specified by the rate of crystal nucleation and by the speed, with which the crystal-liquid interface advances (Hrubý 1972). The ice nucleation temperature decreases also by dehydration. The positive correlation of ice nucleation and the osmotic potential was founded.

3.3. Glass transition temperature (Tg)

The glass transition temperature (T_g) is the temperature at which amorphous (ice free) matter is formed inside the cells. Since observations indicate that T_x and T_g are close and differ only by a few degrees (usually T_x is higher than T_g). For long-term temperature storage at which vitrification occurs is the glass transition temperature (T_g). The glass transition is usually defined as the half high or inflection point of the change of heat flow or the changes of heat capacity of the sample. For pure substances, T_g is typically found near 2/3 T_l on the absolute temperature scale (Debenedetti and Stillinger, 2001).

It is important to note, not only for cryobiologists, that T_l of solution can be reduced by dehydration and moved closer to T_g because of solutes increasing concentration. So the change of T_l is on the other side of the glass transition temperature (Fig. 1). T_g depends on cooling and warming rate. A variety of theories have been proposed to understand why some systems easily vitrify while others do not. Ultimately, a high GFA is associated with slow crystallization rate.

3.4. Origin of new safekeeping parameter.

The safekeeping parameter (Sk) gets together all these three temperature characteristics of thermal transitions in to the relation: $Sk=(T_x - T_l)/(T_g - T_l)$ [Eq. 13, Tab.1]. The safe keeping parameter tell us how breadth is the dangerous temperature range at which ice nucleation and ice crystals can occurred. The safekeeping parameter during cooling close to 0 means the largest dangerous temperature range for ice nucleation and ice growth. The minimal temperature range for ice formation is when the parameter is close to 1 ($T_x \sim T_g$). During warming of the samples from cryogenic temperatures the meaning of safekeeping parameter is on the opposite meaning. The safekeeping parameter close to 1 during warming means the larger dangerous range of temperature for ice nucleation and cold crystallization. The safekeeping parameter was compared in the relationship with the GAS and GS.

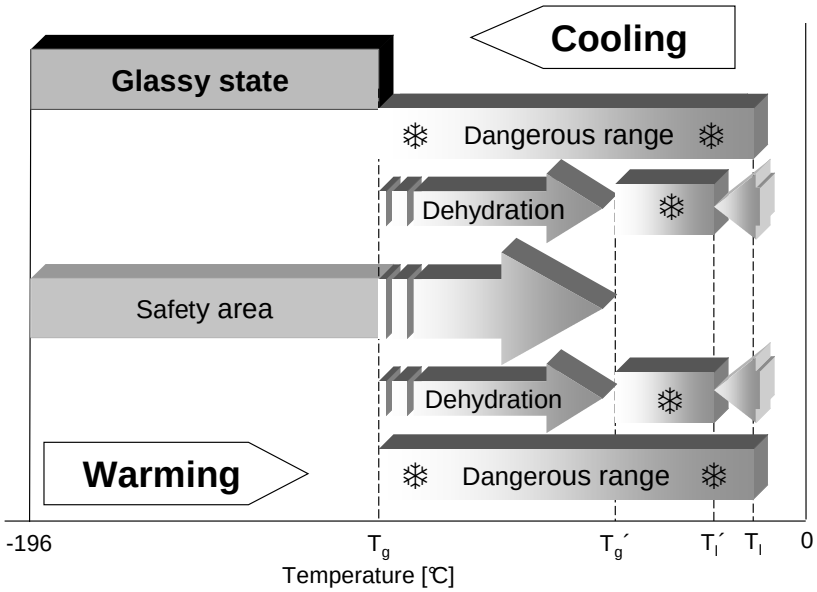

Fig. 1. This scheme showed the shortening of dangerous temperature range by dehydration at which the ice crystals appear. The dehydration increases the glass transition temperature (from T_g to T_g') and simultaneously decrease the melting temperature (from T_l to T_l'). The biological objects in glassy state at temperatures below the glass transition temperature are considered as safekeeping plant storage. The dehydration has a shortening effect on both dangerous temperature ranges during cooling and also during warming. In a dangerous range the ice crystallization temperature (T_x) can caused intracellular crystallization resulting cell dead.

Application of safe keeping parameter it might be of help during improving the cryoprotocol. The safekeeping parameter was tested on the state diagram of sucrose as main cryoprotectant and on the plant thermal parameters measured by differential scanning calorimeter. Some examples of safekeeping parameter for 30% w/w sucrose solution during cooling at 2 °Cmin^{-1} Sk = 0.43 and during warming (2 °C min^{-1}) it was Sk=0.44 (data from Sikora *et al.* 2007). For *Allium* shoot tips at cooling/warming rate 10 °C min^{-1} and at various dehydration Sk varied from 0.39 to 0.70.

Tab1. Parameters expressing the glass-formation ability (GFA) and glass stability (GS)

Equation	Eq. No.		GFA/GS
$T_{rg}=T_g/T_l$	[1]	Turnbull (1969)	GFA/GS
$\Delta T_x=T_x - T_g$	[2]	Inoue *et al.* (2001)	GFA
$\Delta T_{rg}=(T_x - T_g)/(T_l - T_g)$	[3]	unknown	
$\delta=T_x/(T_l - T_g)$	[4]	Chen *et al.* (2006)	GFA
$\gamma=T_x/(T_g + T_l)$	[5]	Lu and Liu (2003)	GFA
$\gamma_m=(2T_x - T_g)/T_l$	[6]	Du *et al.* (2007)	GS
$K_W=(T_x - T_g)/T_l$	[7]	Weinberg (1994)	GS
$K_H=(T_x - T_g)/(T_l - T_x)$	[8]	Hrubý (1972)	GS
$\alpha=T_x/T_l$	[9]	Mondal and Murty (2005)	GFA
$\beta=T_x T_g/(T_l - T_x)^2$	[10]	Yuan *et al.* (2008)	GFA/GS
$\zeta=T_g/T_l + \Delta T_x/T_x$	[11]	Du and Huang (2008)	GFA
$\omega=(T_g/(T_x - 2T_g))/(T_g + T_l)$	[12]	Long *et al.* (2009)	GFA/GS
$Sk=(T_x - T_l)/(T_g - T_l)$	[13]	this paper	GFA/GS$^+$

Note: T_x - onset of crystallization temperature; T_g - glass transition temperature; T_l - liquidus temperature;$^+$ based on correlation with other GFA/GS parameters.

The result of both possible ways is a reduction of equilibrium freezing point depression by this also increasing the safekeeping parameter during cooling. This parameter Sk is important not only to evaluate new cryoprotectants but also to predict GFA/GS in plants alone and/or after plant treated by cryoprotectant. Application of the cryoprotectant concentration with GFA increase the temperature of glass transition not only by dehydration described above but also by GFA of the cryoprotectant itself.

The safekeeping parameter is a good measure of all plant treatments leading to improving the regeneration of plants after storage them at ultralow temperatures. By the safekeeping parameter, we can measure how effective the individual steps of cryopreservation procedure are when we compare them with the regeneration rate of plant material. The same procedure can be used for testing how the specific treatment improve the same e.g. during warming the smaller the safekeeping parameter the better improvement of the cryoprotocol. The safekeeping parameter is temperature rate dependent so the same rate of cooling and warming during measurement as during cryopreservation is recommended if we would like to compare the Sk parameter of different samples.

After normalization of all parameters (Fig 2a) as maximal values is 1 and minimal values is 0. The relationship of Sk parameter after transformation is equal to the other parameters ($T_{rg,}$, ΔT_x, ΔT_{rg}, δ, γ, γ_m, K_W, α, Sk); Sk close to the value 1 is more sensitive to the parameters at temperature of T_x closer to T_g in the following order (ζ, β, K_H) less sensitive to the parameters (ω) (Fig 2b)

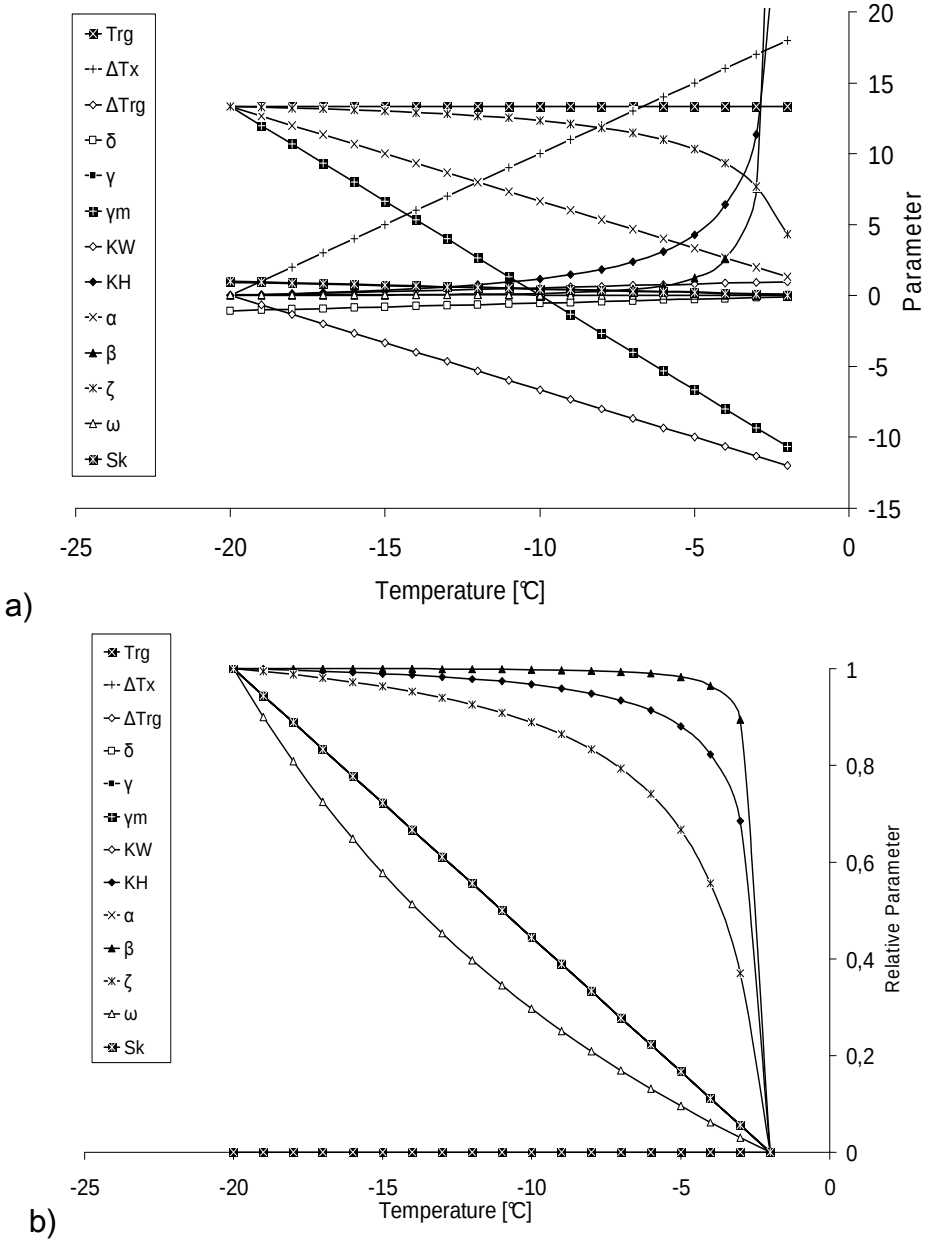

Fig 2 a) The value of different glass-formation ability and glass stability parameters. The glass transition temperatures was set up at T_g = -20 ℃ and liquidus temperature at T_l = -1.5 ℃, only ice crystallization temperature (T_x) was changed from T_l to T_g.
b) Normalized value of different glass-formation ability and glass stability parameters. The glass transition temperatures set up on the constant value Tg =-20 ℃ and temperature of melting was considered as constant T_l = -1.5 ℃, only ice nucleation temperature was cha nge from T_l to T_g. The equation $(k-k_{min})/(k_{max}-k_{min})$ for normalization of parameters was used.

4. Discussion

There are many other parameters which the authors expressed the relationship of the tree parameters (T_l, T_x and T_g) with the aim to express the GFA and GS. GS parameters for different oxide glass-forming system, give relatively good correlation with GFA. However, the best empirical correlations were found for those GS parameters that involved at least all three main DSC characteristic temperatures (T_l, T_x and T_g). The overall results of this research indicate that the GS parameters K_W, K_H and γ (Tab. 1), which are easy to measure, can be

used to compare the relative vitrification tendency of different oxide glass-forming systems (Nascimento *et al.* 2005). A new GFA parameter ω [Eq. 12, Tab.1] were compared together with other GFA parameters as suggested for bulk metaloid glasses together with proposed formerly, including T_{rg}, γ, α, δ and φ; ω was verified with alloying and oxide glasses. Results indicate the parameter ω is the most reliable and applicable approach to assess the GFA of various glasses (Lu *et al.* 2002). This ω parameter has a strong ability to measure and predict the GFA also of some cryoprotectant (Long *et al.* 2009). Kozmidis-Petrovic (2010) compare GFA/GA parameters (γ, KW, KH α, β, ω); and founded that the parameter β is the most sensitive to the changes, while γ is generally least sensitive.
In summary safekeeping parameter close to 1 during cooling means highest safeness, in contrary during warming approaching to the 0 representing the highest safeness. The Sk is linear within the temperatures, it can be expressed in percent. The safekeeping parameter is a good parameter to express both the dangerous zone in relative expression at which ice crystals growth and also the glass-forming ability and glass stability.

5. Acknowledgement: This research was partially supported by project MZe 2700604.

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Cryopreservation of *Hypericum perforatum* L.: multiple responses to cryogenic treatment

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1. Overview

The development of cryopreservation methodology and its transformation into a wide palette of different methods offered possibilities in addressing problems related to long term storage of unique plant genetic resources. Resolving problems of limited *in vitro* culture reinforced the acceptance of the low temperature preservation concept and cryopreservation became the essential component of each biotechnological facility. Though most of the theoretically proposed favourable attributes were feasible in liquid nitrogen environment a large group of inconveniences arose levelling the process of freezing to be more complex than expected, affected from both environmental and biological constrains. Subsequently a large scientific campaign started in order to meet the optimal conditions for a successful cryopreservation procedure.

Since the existence of generally accepted model organism suitable for elucidation of the broad cryopreservation traits became questionable, most of the researches focused on specific species with either medicinal or agro-economical importance. The increasing need for natural producers of biologically active metabolites with medicinal importance diverted our attention to one of the representatives of the genus *Hypericum*, *Hypericum perforatum* L. Its pharmacological significance resides in the production of secondary metabolites with large variety of pharmacodymanic activities reported over the last decades (Barnes *et al.* 2001). Recent interest is preferentially aimed on naphtodianthrones: hypericin and pseudohypericin, phloroglucinol derivates: hyperforin and adhyperforin largely applied for their antidepressive (Butterweck *et al.* 2000), anticancer (Blank *et al.* 2001), antiviral (Birt et *al.* 2009) and antimicrobal (Dall'Agnol *et al.* 2005) contributions. Selection and storage of highly biosynthesizing cultivars has therefore become an important issue. Although efficient *in vitro* culture and multiplication system have been established in our laboratory (Čellárová *et al.* 1992), somatic clones of the same genetic origin were recorded to have variations in total hypericin content (Čellárová *et al.* 1994), rendering the continuous subculture inappropriate for long term preservation. Furthermore, over the last years the culture collection of *Hypericum* spp. with differences in biosynthetic capability has been widened, thus the idea of *Hypericum* spp. genebank is prospective.

Among routinely used posthaw assessments, high recovery rates and sustainability of preserved plant material represent two of the basic evaluation parameters of a successful cryopreservation. There are few reports concerning successful cryopreservation of medicinal plant species, not only from the view point of recovery and regrowth but also of the bioactive metabolites stability (Ahuja *et al.* 2002, Dixit-Sharma *et al.* 2005). Unaltered productivity of pharmacodymanic secondary metabolites in *H. perforatum* is therefore of essential scientific value and was recorded for both slow cooling (Urbanová *et al.* 2002) and vitrification methods (Skyba *et al.* 2010). Furthermore, application of plant vitrification solutions (PVSs) 2 and 3 as cryoprotectants increased total hypericin content in almost 25% of recovered plants. Repeatedly observed maintenance of mitotic activity and chromosome number stability (Urbanová *et al.* 2002; 2004) with insignificant differences in DNA primary structure

analyses by RAPD (Random Amplified Polymorphic DNA) and VNTR (Variable Number Tandem Repeat) profiling highlighted the genetic similarity of the preserved plantlets. However, disputation about reliability of the DNA profiling has been discussed (Skyba *et al.* 2010). Despite the necessary prerequisites of maintenance the profiling characteristics in recovered plantlets, mean regeneration and regrowth rates were insufficient not exceeding 21%. In order to improve post thaw recovery critical processes were selected in each of the pre-cryogenic treatment steps and examined individually.

Large limitations are found in preservation of plant cell and organ cultures contrary to animal culture systems. The presence of rigid cell wall and vacuolar structures filled with freezable water predetermines the plant preservation to be a vastly more difficult task. Since water molecule has tendency to expand in volume during crystallization potentially damaging plant material and it influences the glass transition (Louaer *et al.* 2008), inputs were added in cryopreservation protocols to reduce the unbound water molecules. Dehydration of *H. perforatum* shoot tips from the initial state was performed by exposure to either standard cryosolution (0.56 M sucrose; 1.09 M glycerol; 1.28 M dimethyl sulfoxide) or PVSs depending on the applied method. Vitrification based protocols, where liquid phase is directly converted to metastable glassy state require increased attention in precryogenic treatments in order to dehydrate the plant material to an optimal water content. Resultant dehydration state consequentially affects survival and regeneration, increasing to almost 65 % after 180 min of PVS3 exposure. Interestingly the apparent correlation between time of exposure and recovery (Fig 1A) was probably caused by reduction of chemically bounded water molecules rather than freezable water (Bruňáková unpubl.). Explant hydration level can be modulated by the presence of plant growth regulators, their functional interconnections and longevity of treatment. We have shown the treatment necessity of both abscisic acid and 6-benzylaminopurine despite the assumed antagonistic effect (Reed 1993). On contrary, in conventional methods crystallization process appears alongside of freezing induced dehydration inserting another strongly impacting variable into the procedure. Although it was already depicted that recovery rate is cooling rate dependent by Peter Mazur in 1960s, we observed that cooling rate driven ice crystallization and growth were the superior detrimental processes affecting the recovery increase of undamaged *H. perforatum* shoot tips (Fig 1B) more than freezing induced dehydration (Skyba *et al.* 2010)

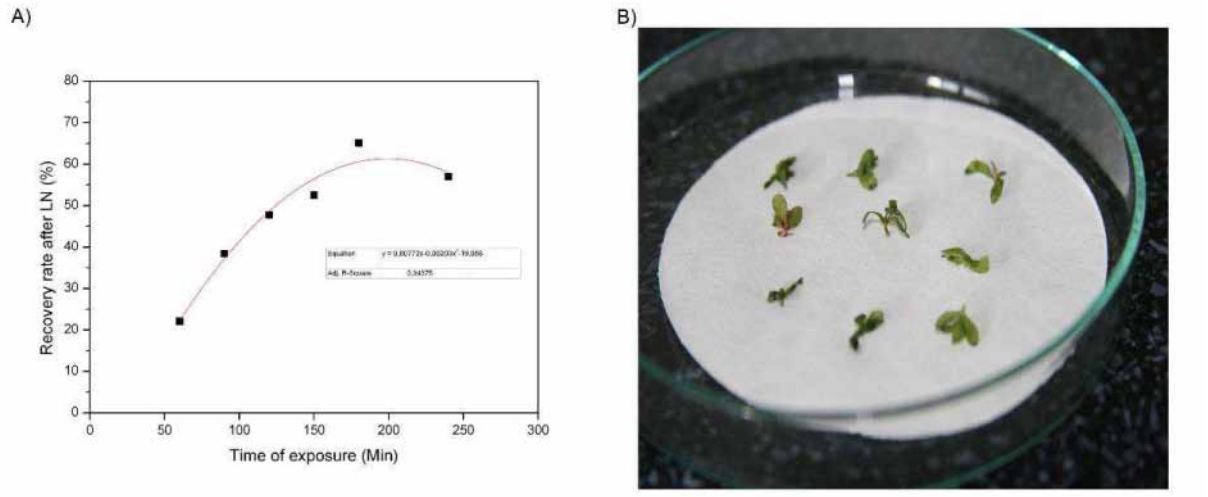

Fig. 1 Experimental data illustrating the influence of PVS3 exposure longevity on recovery (A) and structural integrity (B) of *Hypericum perforatum* L. shoot tips cryopreserved with vitrification and slow cooling respectively.

Apart from externally performed modulations of critical processes in terms of prolonged exposures, variation in compositions and concentrations, cooling regime manipulation in order to meet the superoptimal pre/treatments a large portion of effort was invested into examination of plant stress responses. Observations of Arora *et al.* (2002) and recent microarray studies (Fowler and Thomashow 2002) support consideration of oxidative stress as major temperature stress response in plants with increasing tendency during cold exposure. Concordant results were obtained by our laboratory underlining the significant activation of enzymatic antioxidant mechanism at various molecular levels as response to the production of active oxygen species during cryopreservation (Skyba, unpublished data). Additionally gradually increasing expression and activation of Cu/Zn superoxide dismutase (Fig 2A) located the stressors influence into chloroplasts that were observed to be severely damaged after cryotreatment (Fig 2B). Further antioxidant enzyme profiling classified *H. perforatum* to strong producers, presuming the antioxidant capacity as one of the plant genetic attributes of a biological foundation probably predisposing increased survival.

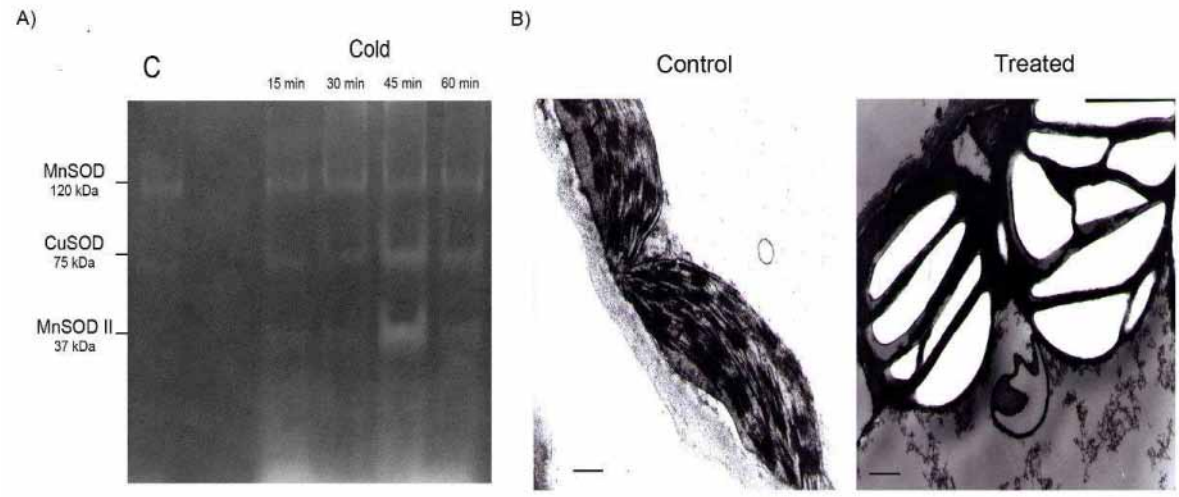

Fig. 2 Superoxide dismutase zymogram (A) illustrating severe generation of active oxygen species plants exposed to cold and ultrastructure of chloroplasts (B) indicating severe damage of thylakoids in the treated plants.

Although apparent improvement of survival and recoveries with a maximum of 65% appeared after alterations of several variables, further external inputs had mostly detrimental effect rendering the external modifications to have a saturated state. It is believed that further improvement is tightly dependent on the genetic architecture of the plant. These represent the very foundations predisposing the severity of external co-stimulatory effects some of which are extremely hard to modify. A group of naturally occurring processes was observed to depress the superoptimal externally altered conditions. Seasonal rhythm was recorded among survival of cryopreserved *Hypericum perforatum* shoot tips with highest survival during vernal period of the year despite previous *in vitro* cultivation (Skyba *et al.* 2010).

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This work was supported by the Slovak Research and Development Agency under the contract No. VVCE-0001-07 and contracts No. APVV-321-07, APVV-LPP-0015-07 and SK-BG 0008-08, by grant of the Scientific Grant Agency of Ministry of Education No. 1/0049/08 and COST 871 action Cryopreservation of Crop Species in Europe. The authors would like to

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The role of cryopreservation in the long-term conservation of vegetatively propagated plants

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1. Introduction

Vegetatively propagated species have a handicap for germplasm preservation, since seed storage, which is the easiest and cheapest method for long-term conservation, is not feasible. The usual approaches for these species are: field collections (also called field genebanks), the most traditional solution; *in vitro* conservation, especially as slow-growth cultures, and, most recently, cryopreservation. All these systems are, theoretically, able to guarantee genotype maintenance for long-term conservation.

Several economically important crops must be vegetatively propagated (e.g. banana, garlic, coffee, potato, sweetpotato, cassava, pineapple, sugarcane, mint, etc.). The most widely employed method for conservation of these species is as whole plants in field collections.

The three proposed methodologies for long-term conservation have advantages and, also, some disadvantages which have been summarized in Table 1. Probably the most valuable advantage of the field collections is that they can provide, in an easy way, source material for propagation and other purposes, as for example research works. However, this system presents some disadvantages as its requirement of ample space and constant labour force, and the risk of diseases, pests and natural hazards, together with unwanted mixing of accessions. The other two strategies, *in vitro* slow-growth and cryopreservation, reduce these risks and also allow the reduction in space requirements and labour costs for the maintenance of germplasm collections (Engelmann 1997), although the labour force needed is more qualified. Genetic stability is the main drawback attributed to *in vitro* conservation due to the possible occurrence of somaclonal variation associated to the stress conditions of the culture, which has been reported in many works (see review of Jain *et al*. 1998). Genetic variation has been also occasionally reported in cryopreserved material (Harding 2004, Kaity *et al*. 2008, Sánchez *et al*. 2008, Martín *et al*. 2011). The origin of this variation is usually attributed to the application of *in vitro* culture procedures, which are necessary before or/and during the cryopreservation process, instead of the cryopreservation treatment itself (Harding 1997).

Therefore, the most stable of the three long-term conservation methods would be the field collection, since the mutation rate expected in these individuals is the basic one, and it is not supposed to be increased due to stressful conditions of culture. However, it must be taken into account that conservation in field collections, like in the other *ex situ* methods, requires to bring genotypes from an environment in which they are adapted to a possible different one (Ghani Yunus 2001). According to Frankel (1970), there could be selection and increased opportunities for natural hybridization with alien material in field collections. Many factors will affect the population structure, as climate, soil, biotic components, life cycle length, breeding systems, competition and degree of care. These aspects may be associated to variations in the genetic composition of the original conserved sample.

2. A case study: the mint collection at IPK

Mint (genus *Mentha*) is an economically important crop with a wide distribution. It is a plant of medicinal and aromatic interest. Few species of mint and some hybrids are cultivated commercially, and they can be considered as 'difficult to conserve', since most of these mint crops produce short-lived seeds or have to be vegetatively propagated (hybrids).

At the Genebank of IPK (Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research) in Gatersleben, Germany, the three long-term conservation methods (field genebank, *in vitro* slow growth and cryopreservation) are used for mint germplasm. In fact, the plant material of the field genebank was the source to establish the slow-growth *in vitro* culture collection (Keller *et al.* 2006), from which the cryopreserved collection was also established (Fig. 1), using a vitrification-droplet protocol (Senula *et al.* 2007).

Accessions in the field collection are cultivated every year in well-defined plots (1m x 1.25m) (Figure 2). Slow-growth *in vitro* cultures were established using shoot apices from the plants of the field collection as starting material. Explants were cultured on MS medium without growth regulators and maintained at 2 ºC and 10 ºC. The cryopreserved collection was established from nodal segments obtained from the slow-growth cultures after a recovery period at 25 ºC, in order to guarantee their regeneration capability.

Material from the slow-growth as well as from the cryopreservation collections may be regenerated according to the protocols described by Keller *et al*. (2006) and Senula *et al.* (2007) to obtain plants that can be cultured again in field conditions (Figure 1). The existence of these collections offers a valuable opportunity to analyse and compare the utilization of the three long-term conservation strategies on plant material of the same origin.

As an initial approach, the genetic stability of plants of the same accession derived from the three different conservation methods was estimated. For this evaluation, RAPD (Random Amplified Polymorphic DNA) markers were the selected technique, and an accession of *Mentha x piperita* of German origin (MEN 198) the studied material. DNA was extracted from plants of the field collection, from the *in vitro* slow-growth cultures and regenerated from cryopreserved apices. Ten different RAPD primers were employed for the amplifications. The RAPD markers showed no genetic variation among the studied samples regardless their origin (García-Ginés *et al*. 2010).

A preliminary genetic analysis of ten individuals from the field collections of two other mint accessions (MEN 183 and MEN 208, *Mentha x piperita*), both of Cuban origin, was carried out. The same RAPD primers as in the previous study were employed. The analysis revealed that field genebank samples were not identical within accession. Three and five different genotypes were observed in MEN 208 and MEN 183, respectively, according to the RAPD patterns found. In the latter case, one genotype was quite different to the other nine samples (less than 85 % of similarity) of the accession. This difference could be attributed to a sample contamination from a different accession rather to mutation or diverse origin within the studied accession, MEN 183.

3. Discussion

Mentha x piperita is a hybrid without seed production. In clonal plant species it is frequent to find genetic diversity within a population. The origin of this genetic variation has been documented to come exclusively from either mutations or multiple origins of the clones detected (Ellstrand and Roose 1987). Genetic variation in field genebank accessions is usually not expected, however, the preliminary analysis of two Cuban accessions at the IPK have revealed a heterogeneous genetic composition which could be attributed to not only mutations or multiple origin, but also to mistakes and accidents in the handling. The analysis and

detection of different genotypes within an accession in vegetatively propagated species is fundamental for the appropriate management of *ex situ* conservation genebank.

Compared to cryopreservation, slow-growth may be more adequate for medium-term conservation, since it requires periodical subcultures (between 1.5 and 2 years in the mint cultures at the IPK), and longer culture periods are associated to a higher probability of somaclonal variation occurrence (Müller *et al*. 1990).

The preliminary results obtained in the genetic analysis of mint samples using RAPD markers point to cryopreservation as the most reliable technique for long-term conservation in mint. This procedure maintains the genetic stability of the starting material and, at the same time, avoids genetic contaminations that could arise from an inadequate management of the field genebank. An adequate management of a cryopreserved germplasm collection should include the study of the genetic profile of the starting material and of the recovered plants.

Apex cryopreservation is therefore an alternative to the classical methods for medium- and long-term conservation of mint genotypes. Optimization of this preservation system implies the development of protocols that can guarantee the regeneration ability of the preserved material together with maintenance of its characteristics. Further studies are needed for a complete evaluation of the different long-term strategies employed in mint collections.

4. Acknowledgments

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Table 1.- Advantages and disadvantages of the main long-term conservation strategies for plants.

Conservation strategy	**Advantages**	**Disadvantages**
Field collection	- Source material for propagation - Easy germplasm accessibility	- More space - More labour force - Risk of diseases, pests and natural hazards - Unwanted cross pollination or mixing by runners
Slow growth *in vitro* culture	- Safe exchange of plant material - Easy culture establishment - Less space - Better sanitary conditions	- Long-term genetic stability? - Specialized labour force
Cryopreservation	- Long-term conservation	- Higher initial cost - Specialized labour force - Genetic stability?

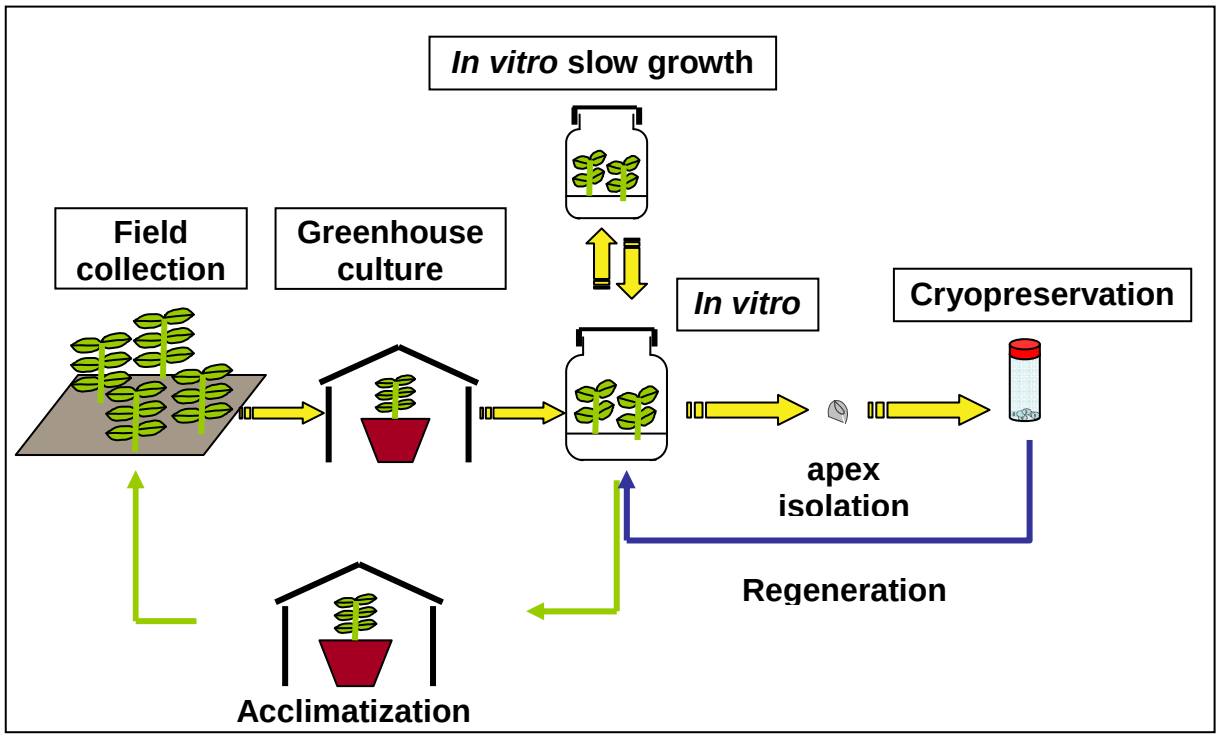

Figure 1. Scheme of the three main long-term conservation strategies for plants and their relationships for source material.

Figure 2.- Well-defined plot for a mint accession at the IPK field genebank.

Behaviour of regenerants of strawberry mericlones and chicory nodules after cryopreservation through droplet-vitrification

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1. Introduction

In order to establish protocols for long time conservation of strawberry and nodules regenerants of chicory, a droplet-vitrification method originally designed for the cryopreservation of apical meristems of *Musaceae* (Panis *et al.* 2005) has been applied.
By itself, the assessment of the plant conformity is very complex. Therefore, we decided to focus our study on the most susceptible traits to putative deviation: BAP cytokinin dependence of the strawberry flowering (Jemmali *et al.* 1995) and DNA variation associated with regeneration from undifferentiated tissue in chicory (Piéron, pers. communication).
Meristem tips of Elsanta and Gento nova strawberry cultivars and of chicory cultivar 77/5 have been cryopreserved. The regenerants from Gento nova and 77/5 have been observed during vegetative growth till flowering and their DNA submitted to flow cytometry analysis.

2. Material and methods

2.1. Cryopreservation

Strawberry. After two weeks of *in vitro* rooting, cultures of Elsanta and Gento nova were transferred at 4°C in dark conditions for 2 additional weeks. As pre-treatment, apical domes with 1-2 leaf primordia were then dissected and pre-cultured at 4°C in darkness for one week on a gelrite-solidified Murashige and Skoog (MS) medium supplemented with 0.1 mg/L BA, 1.0 mg/L IBA, 0.1 mg/L GA_3 and 0.1M sucrose (N medium) or 0.4 M sucrose (S medium). They were incubated into LS loading solution (2 M glycerol + 0.4 M sucrose) for 20 min at room temperature, then in PVS2 solution (Sakai et al 1990) for 45 min at 0°C and then transferred to droplets of PVS2 solution on a strip of aluminium foil (5 x 20 mm). The aluminium strip together with the shoot tips was plunged into liquid nitrogen. After 1 h, thawing took place by rinsing the strip in the recovery solution (1.2 M sucrose) at room temperature for 15 min. Subsequently, shoot tips were transferred onto two sterile filter papers on top of a gelrite-solidified hormone-free MS medium containing 0.1 M sucrose and 0.5 g/L of activated charcoal. After 24 hours, the explants were put to regenerate on N and Boxus (1974) (Knop's medium with 0.25 mg/L kinetin, 0.1 mg/L IBA, 0.1 mg/L GA_3 and 40 g/L glucose) media without filter paper. The first week of culture took place in the dark. Post-thaw survival and shoot regeneration rates were recorded after 4-6 weeks.
Chicory. *In vitro* wounded leaves of the cv 77/5 induce nodules formation followed by bud regeneration within 3-4 weeks of culture (Piéron *et al.* 1998). Shoot tips / buds together with small part of nodule tissue, were then dissected and pre-cultured at 4°C in darkness for one week. They were transferred to the loading solution and treated for cryopreservation as described for strawberry (see above). Regeneration took place on basic nodule induction medium (Piéron *et al*. 1998).

2.2 Plant behaviour

Growth of strawberry plants of both cultivars were grown in the greenhouse directly after regeneration and *in vitro* rooting ("*Dir"* lines: 2 plants per line) while axillary buds of Gento nova lines were propagated in presence of BAP during 5 subcultures ("*Sub"* lines: 4 plants per line) before acclimatisation. The final observations were made 3 months later: leaves (number, petiole length, limb width), inflorescences (number, shape, basal or elongated, number of flowers) per plant, presence of additional cores, shape. Flower induction has been performed by exposing the plants to 15°C and 9h photoperiod for 14 days followed by 20°C and 16h photoperiod.

The chicory 77/5 regenerants were propagated through axillary branching (Piéron *et al*. 1998). Ten plants per regenerant from basic and sucrose enriched chicory media (Piéron *et al*. 1998) were collected randomly from the greenhouse acclimatised material and transferred to the field for observation (plant growth, habit and homogeneity, leaves shape, hairiness and anthocyanin production).

2.3 Flow cytometry analysis

'Plant Cytometry Services' (Schijndel – The Netherland) performed the DNA Indexes determinations. Number of the chicory lines submitted to the analysis: 11 from nodules and 18 from shoot tips material, 29 and 62 lines belonged to control and cryo treated explants respectively.

3. Results and discussion

3.1 Plant regeneration

Strawberry. The post-thaw survival rate (callus + shoot formation) of both cultivars reached more than 90% on MS basic medium. Forty-five minutes of PVS2 treatment revealed no toxicity. A pre-culture phase in the presence of a higher sucrose concentration (0.4 M instead of 0.09 M) didn't improve survival after cryopreservation. Using the Boxus (1974) regeneration medium, most meristems gave rise to direct shoot regeneration without an intermediate callus phase.

Chicory. Cryopreservation resulted in 64.1 – 76.2% post-thaw shoot regeneration rates. The PVS2 treatment (=dehydration) was slightly toxic. Pre-treatment on sucrose enriched chicory medium (see above) has again not significant effect and no callus formation was recorded.

3.2 Plant behaviour

Strawberry. The cryopreservation protocol did not influence significantly the plant development of both « *Dir » and* « *Sub »* lines. Regenerants of both cultivars revealed normal aspect and the growth differences measured in the greenhouse were not significant. The «*Sub»* lines of Gento nova didn't show hyperflowering often observed as a putative deviation due to repeated BAP exposures (Table 1).

Table 1 Flowering of strawberry Gento nova « *Sub* » lines

Treatment	Number of lines	Average number of plants/line	Number of inflorescences/plant	Number of flowers /inflorescence
Control	8	4.6 -± 1.6	1.4 ± 0.5	3.6 ± 1.3
Cryopreservation	11	4.7 ± 1.4	1.4 ± 0.2	3.6 ± 0.7
S(1) Control	6	5.2 ± 1.2	1.4 ± 0.3	3.2 ± 1.2
S(1) cryopreservation	9	4.3 ± 1.1	0.9 ± 0.3	3.2 ± 1.1

(1): pre-culture in presence of 0.4 M sucrose

Chicory. The variability of the plant growth parameters between the lines and within several of these lines has been analysed. Plant vigour of the lines was medium to high, plant growth habit was upright, plant homogeneity was superior after cryo treatment, leaf shape was serrate to lobed with medium hairiness and the presence of anthocyanin was only noted from higher sucrose regeneration media. In comparison, the plant vigour within the lines was low to high, plant growth habit upright to oblique, with a medium plant homogeneity, entire to lobed leaf shape with slight hairiness but without or with a low level of anthocyanin. In general, cryopreservation did not affect the growth of the plants nor flowering that occurred during the second year in the field, flowers looking homogeneous. That confirms the cryo treatment did not substitute the normal vernalisation conditions.

3.3 Flow cytometry analysis.

DNA indexes of the chicory and strawberry regenerants did not differ from the control plants.

4. Conclusions

The absence of significant differences between control and cryopreserved plants confirmed that the cryopreservation using the droplet-vitrification protocol adapted from *Musaceae* can be applied for long term conservation of meristem-tips of strawberry and chicory.

5. Acknowledgements: The authors are very grateful to M. Delcorps for her technical contribution.

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Evaluation of dehydration stress response in potato and its use in cryopreservation

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1. Introduction

Plant productivity is strongly influenced by dehydration stress induced by high salinity, drought and low-temperature conditions. Plants respond to these stresses by displaying complex, quantitative traits.

Potato (*Solanum* spp.) is one of the most commonly cultivated species and is globally used for food, feed, medicine, and as base material for industrial products. However, only one of the eight cultivated potato species, *Solanum tuberosum* subsp. *tuberosum*, is widely commercialized, while 228 wild *Solanum* species are known. Potato yield is limited by the occurrence of different abiotic constraints, among them frost. In the huge genetic diversity of potato resources lies an enormous breeding potential that can be used to solve present and possibly future restriction of potato production worldwide. It is therefore necessary to preserve the existing genetic diversity of potato for current and future generations.

Although plant germplasm collections of vegetatively propagated species are traditionally conserved in field collections, the method of choice for long-term germplasm conservation is cryopreservation (at -196°C) (Keller *et al*, 2008). However, the large-scale use of cryopreservation is hampered by the lack of standardized methods. Hence, currently only a fraction of existing plant germplasm collections can be conserved by cryopreservation. The critical point when developing a cryopreservation procedure is the control and/or avoidance of intracellular ice nucleation, since ice crystals will damage membranous cell structures thereby destroying their semi permeability. The formation of ice crystals can be prevented by removing cell water through freeze induced dehydration, osmotic dehydration and evaporative desiccation.

One of the most common acclimation mechanisms associated to freezing tolerance is the accumulation in the plant tissues of osmoactive compounds, which can be induced by drought or alternatively by exposure to cold (Bray, 1993). Because plant tissues processed for cryopreservation are submitted to stress conditions similar to drought, salinity and frost, it is expected that studies on mechanisms in response to dehydration stresses are closely linked with resistance towards cryopreservation. During the steps preceding cryopreservation, the metabolism of donor plants or explants is affected, leading to changes that can be monitored.

In the present work, a study of abiotic stress response in potato was realized; a better understanding of this response will be useful for improving the existing cryopreservation protocols.

2. Material and methods

Apical shoots from 3-weeks-old *in vitro* plantlets of two varieties of potato (the commercial race *S. tuberosum* L. cv. Désirée and the wild potato *S. commersonii*) were submitted to

osmotic stress at 22°C: MS0 medium (MS with vitamins and 0.09M sucrose) as control (Murashige & Skoog, 1962), MS0 medium with 0.3M of sucrose and MS0 medium plus 0.21M of sorbitol (total sugar concentration of 0.3M). The plantlets were also cultivated in MS0 medium at 6°C in order to evaluate the changes occurring in the plant tissues after exposure to chilling. After 14 days of exposure different growth parameters were measured: length of shoots, number of leaves and fresh and dry weight.
Data presented are the means of relative values (calculated by division between absolute values in the treatments and values in the controls) of at least five independent experiments, with 12 explants per treatment (n=60). Data were analyzed using ANOVA, and comparisons among the mean values were evaluated by the least significant different test at $p<0.05$ (indicated with the letters above the bar in the figures).

3. Results

In order to compare both cultivars, relative values were calculated for growth and water content.

3.1 Relative increase in shoot length

Plant growth is affected by all the stress conditions currently investigated (Fig. 1). There are significative differences between cultivars in both osmotic stress treatments; sucrose and sorbitol seem to compromise shoot growth more in *S. commersonii*. On the other hand, the relative increase in shoot length in both cultivars seems to be equally affected by the cold treatment.

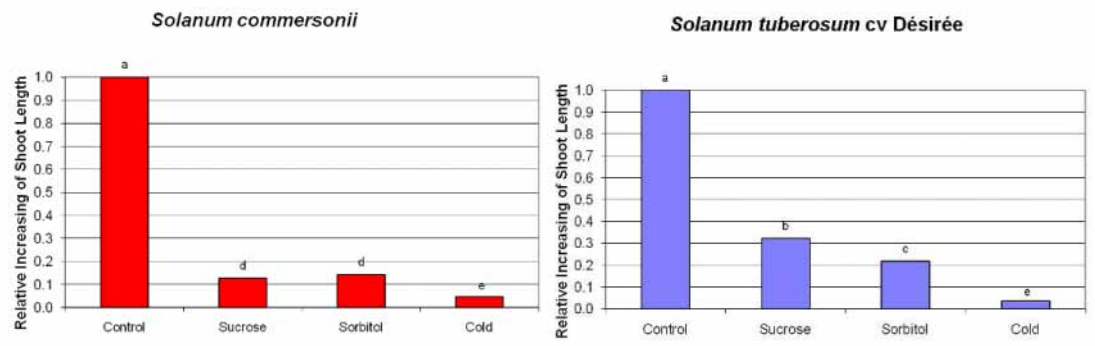

Fig 1. Shoot length increase of 3-weeks-old *in vitro* plants of two potato cultivars (*S. tuberosum L.* cv. Désirée *and S. commersonii*) submitted to different stress treatments for two weeks. N=60.
*Different letters indicate values significantly different at p≤0.05 (ANOVA test).

3.2. Number of leaves relative to the control

When potato plantlets are treated with sorbitol, no significant differences can be observed between the cultivars (Fig. 2). Although sucrose treatment induces a significant negative effect on the number of leaves in *S. commersonii*, the number of leaves of *S. tuberosum* cv Désirée is not influenced by growth on sucrose-supplemented medium.
Out of the three treatments, cold has the most negative effect on the number of leaves for both cultivars and this reduction is more pronounced in *S. commersonii*.

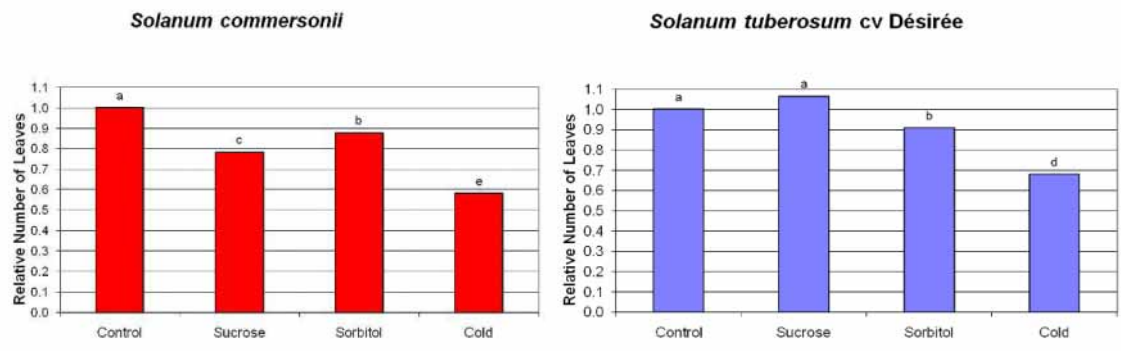

Fig 2. Number of leaves from 3-weeks-old *in vitro* plants of two potato cultivars (*S. tuberosum L.* cv. Désirée *and S. commersonii*) exposed to different stress treatments for two weeks. n=60. *Different letters indicate values significantly different at p≤0.05 (ANOVA test).

3.3. Relative water content

The sucrose treatment results in a more important decrease in water content compared with the sorbitol treatment (Fig. 3). However, when the two cultivars are compared in relation to their responses on the treatment with sucrose or sorbitol no significant differences were found. After the cold treatment, the water content decreases more in Désirée that in *S. commersonii.*

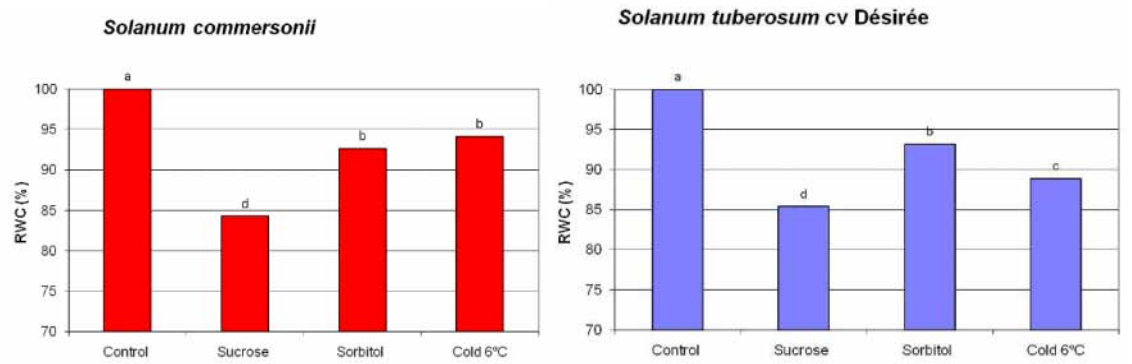

Fig 3. Water content from 3-weeks-old *in vitro* plants of two potato cultivars (*S. tuberosum L.* cv. Désirée *and S. commersonii*) submitted to different stress treatments for two weeks. n=60. *Different letters indicate values significantly different at p≤0.05 (ANOVA test).

4. Discussion

The presented data show that *in vitro* growing plantlets of *S. commersonii* are more affected by the different osmotic stress treatments. The cold treatment used in the present study affects shoot length increase in both cultivars with the same intensity. However the number of leaves and the water content are differently influenced by cold in both cultivars; while number of leaves is more affected in *S. commersonii*, the water content decreases more in Désirée.

This morphological study is the first part of a study aimed at increasing our knowledge on the molecular responses of potato to abiotic stress. In order to understand these responses at a biochemical level, samples have been collected and are currently analysed. Since proteomics is a powerful tool to analyse biochemical pathways and the complex response of plants to environmental stimuli (Renaut et al 2006), a proteomic approach will be executed that will

lead to new insights on the pathways implicated in the physiological responses under these stress conditions. Furthermore, the comparison between cryopreservation and abiotic stress responses of the potato cultivars involved in this study will help us to increase the number of accessions that can be cryopreserved, for potato and other plants.

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Clonal fidelity of *Iris pumila* plants regenerated after cryopreservation by vitrification

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1. Introduction

Irises are monocotyledonous perennial plants which grow in the temperate regions of the Northern Hemisphere. Many species of genus *Iris* are horticultural plants that are cultivated because of beauty of their flowers. In addition to the ornamental value of many irises, in recent years a lot of attention is paid on phytochemical research which resulted in the isolation of pharmacologically valuable secondary metabolites (Williams *et al*. 1997). *Iris pumila* (2n=32) is endemic, endangered and protected, which belongs to series of dwarf bearded irises. It has a great ornamental value and is also the progenitor in most breeding programs of dwarf irises. *I. pumila* plants produce xanthones, pharmacologically valuable substances, such as mangiferin. Protocol for cryopreservation of *I. pumila* shoot tips by vitrification was developed during the COST Action 871 "CryoPlanet" (Jevremović *et al* 2009).

The main aim of this work is to investigate clonal fidelity of regenerated *I. pumila* plants after cryopreservation, in comparison with plants both regenerated in tissue culture and collected from nature.

2. Material and methods

2.1. Plant material

Plant material used in the experimental work was (i) leaves of *I. pumila* plants collected from nature (ii) *in vitro* grown shoot cultures from which explants for cryopreservation were collected, and (iii) plants regenerated after cryopreservation procedure and acclimatized in greenhouse conditions.

2.2. Cryopreservation by PVS2-vitrification

For cryopreservation, the vitrification procedure based on PVS2 (Plant Vitrification Solution n°2) was applied. In short, the procedure steps were: (i) 2 weeks of cold hardening of shoot cultures at 4 °C, followed by other 2 days of the same hardening for the excised shoot tips, (ii) shoot tip loading with 2 M glycerol and 0.4 M sucrose (30 min), (iii) PVS2 treatment at 0°C for 20 min, (iv) direct immersion of cryovials containing 10 shoot tips, in liquid nitrogen, where they remained for at least one hour, (v) re-warming of cryovials at 40°C for 1 min, (vi) unloading of explants in MS (Murashige and Skoog, 1962) medium containing 1.2 M sucrose (20 min).

2.3. Plant regeneration

After cryopreservation, shoot development and proliferation were achieved on solid MS medium, supplemented with benzyl-adenine and gibberellic acid (4.4 and 0.3 μM, respectively). Shoot rooting was achieved on hormone-free MS medium. Fully developed plantlets were potted in a mix of peat and perlite (3:1) and acclimatized in greenhouse conditions.

2.4. Flow cytometry analysis

Relative genomic size is determined according Dolezel *et al.* (1992) using staining of nuclei with 4,6-diamidino-2-phenylindole-2HCl (DAPI) and *Vicia faba* as standard.

3. Results

I. pumila shoot tips survived to cryopreservation by vitrification in a percentage of 55%, three months after cryopreservation. The shoot multiplication index (3.2), measured after 4 weeks subculture, was similar to the one of iris shoots in standard culture conditions. Rooting of shoots regenerated after cryopreservation was very successful (90%). No significant differences were observed in morphological parameters (shoot length, number and length of formed roots) between plants from shoot tip cryopreservation and control plants.

Flow cytometric analyses showed that all the tested *I. pumila* plants regenerated after cryopreservation by PVS2-vitrification had the same genomic size of plants both regenerated by tissue culture, and collected in nature (Fig. 1).

4. Discussion

Today, cryopreservation is an important tool for long term conservation of plant genetic resources. Among the numerous techniques, shoot tip cryopreservation by PVS2-vitrification has repeatedly proven to be a very reliable and effective option for the *ex situ* conservation of a wide range of plant species (Panis and Lambardi 2006). It was previously reported that iris plants can be successfully regenerated after cryopreservation of shoot tips, following a PVS2-based vitrification procedure (Jevremović *et al*., 2009).

Genetic stability of plants regenerated after shoot tip cryopreservation has been already reported. For instance, Liu *et al.* (2008) used different molecular techniques to show the genetic fidelity of apple shoot from cryopreservation by a vitrification procedure. Some alterations in genomic size of *I. pumila* plants regenerated by somatic embryogenesis were reported earlier (Jevremović *et al*., 2010). In this study we showed that *I. pumila* plants regenerated after cryopreservation of shoot tips by PVS2-vitrification had the same genomic size of plants regenerated by *in vitro* organogenesis. Moreover, also the flow cytometry profile of plants collected in nature was comparable with the one of plants from shoot tip cryopreservation. Hence, cryopreservation by shoot tip vitrification is an affordable technique, in terms of ploidy stability, for the long-term *ex situ* conservation of iris genetic resources.

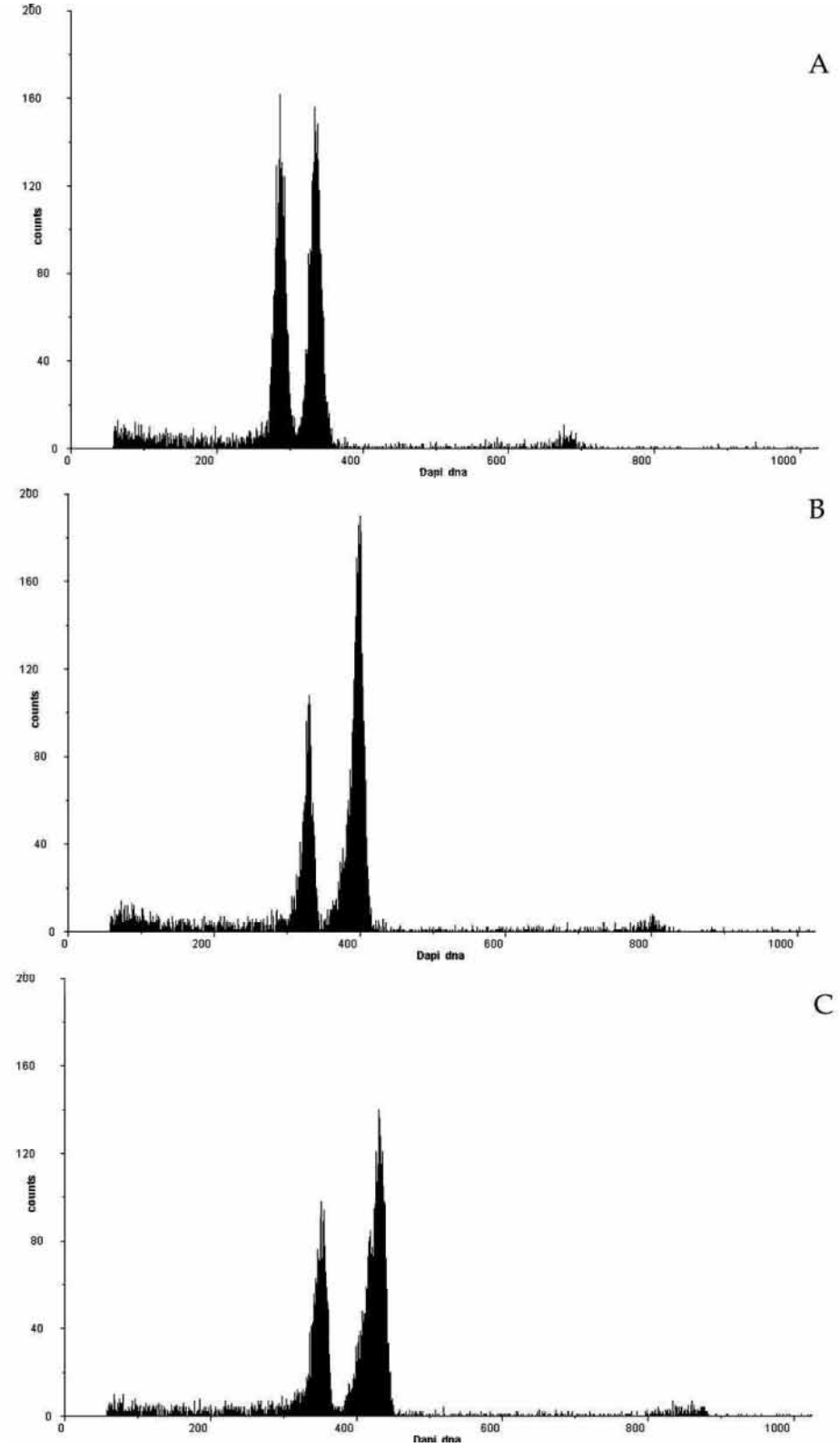

Fig. 1 Flow cytometry profile of *I. pumila* plants collected from nature (A), regenerated by organogenesis (B) and after cryopreservation (C). *I. pumila*, left *V. faba*, right peak.

5. Acknowledgements

The study was supported by Ministry of Education and Science of Serbia (Project TR31019).

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Bioenergetic parameters during cryopreservation of two *Abies cephalonica* embryogenic cell lines

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1. Introduction

Somatic embryogenesis has been proved to be the most promising method for the rapid propagation of *Abies* species and their hybrids. The long-term storage of germplasm is needed during progeny trials for tree breeding purposes. The aim of the present study was to investigate the occurrence of oxidative stress and determine the bioenergetic parameters (cellular content of ATP and glucose-6-phosphate, glu-6P) in embryogenic cell lines of *A. cephalonica* during the pre-treatment phases of cryopreservation and the early steps after thawing.

2. Materials and Methods

2.1. Cryopreservation procedure

Two embryogenic cell lines of *A. cephalonica*, 6 and 8, were cryopreserved as described in Aronen *et al.* (1999), except for the freezing of the samples. The freezing of the samples was conducted with NALGENE^{TM} Cryo 1ºC Freezing Containers at a cooling rate of -1 °C/minute to the pre-freezing temperature of -70 °C. The samples were kept for 24 hours in deep freezer before transferring to liquid nitrogen for 7-day period.

2.2. The proliferation rate

The proliferation of the embryogenic cell masses (ECM) was evaluated after cryopreservation by measuring the proliferation ratio (w_0/w_i) in which w_i is the initial fresh weight (FW) of the sample and w_0 is the FW at the time of sub-culturing either 3 or 5 weeks after the thawing.

2.3. Determination of ATP, glu-6P and histochemical localization of H_2O_2

The samples were collected for the determination of ATP, glu-6P and H_2O_2 localization at different time points during pre-treatments, thawing and proliferation stage.

For the analysis of ATP and glu-6P, samples were prepared as described in Petrussa *et al.* (2009). The cellular ATP content was determined by means of the luciferin-luciferase, luminometric method with ATPlite Luminescence ATP Detection Assay System (Perkin-Elmer). The cellular glu-6P content was evaluated using the enzymatic reduction of β-$NADP^+$ by glu-6P dehydrogenase (Bergmeyer *et al.* 1974). The measurements were performed by means of a Multilabel Counter (WALLAC, model 1420, Perkin-Elmer).

Histochemical localization of H_2O_2 was conducted using 3,5,3'5'-tetramethylbenzidine (TMB)-HCl as described in Barcelo *et al.* (1998). The staining intensity was classified into 5 categories: 1) no blue cells detected; 2) 1- 5 blue cells; 3) 5 - 20; 4) 20 - 40; 5) more than 40 blue cells detected.

2.4. Data analysis

The statistical examination of the proliferation rates and cellular levels of ATP and glu-6P were analyzed with factorial ANOVA (all variants effects were considered fixed) using the GLM procedure (SAS Institute 2004).

Staining intensity (color scores for the presence of H_2O_2) represents an ordinally scaled Poisson-distributed response variable. To normalize the data and to make the mean independent of variance, we used logarithmic transformation (Quinn and Keough 2002). Transformed data were analyzed with factorial ANOVA (the effects of both cell line and cryopreservation stage were considered fixed) using the GLM procedure (SAS Institute 2004), pairwise differences were tested using the Duncan's tests.

3. Results

Both tested embryogenic cell lines of *A. cephalonica* were recovered after 7-day cryostorage and proliferated intensively 5 weeks after thawing. The embryogenic cell line 8 had considerably slower proliferation rate than cell line 6 (Fig. 1).

Based on the histochemical H_2O_2 analyses both cell lines produced H_2O_2 during the cryoprotection protocol and after cryostorage. The highest intensity of H_2O_2 was detected immediately after thawing which was significantly different from the pre-treatment and cryoprotection. The response of tested embryogenic cell lines was not significantly different (Fig. 2).

The occurrence of oxidative stress during the cryopreservation procedure did not change the biochemical potential of cryopreserved embryogenic cell lines (Fig. 3A, B). Interestingly, the cellular glu-6P levels detected after recovery were significantly higher than those observed before the cryopreservation pre-treatments (Fig. 3B). The highest cellular levels of ATP were detected after the cryopreservation pre-treatment on 0.4 M sucrose media. However, these levels were not statistically different from the levels observed after the recovery (Fig. 3A).

Detected cellular ATP and glu-6P levels did not differ significantly between two tested cell lines.

4. Conclusions

Both cryopreserved embroygenic cell lines of *A. cephalonica* were recovered after 7-day cryostorage and started intensive proliferation. The presence of H_2O_2 in the embryogenic cell lines may indicate that the cells were exposed to oxidative stress immediately after the thawing. The steadily increasing trend of the presence of H_2O_2 (although not significant) is an indication that oxidative stress may appear already at earlier stages of cryopreservation and deserves further study.

5. Acknowledgements

Dr. A. Bertolini is acknowledged for excellent technical assistance and JK is grateful to COST 871 for providing her with STSM.

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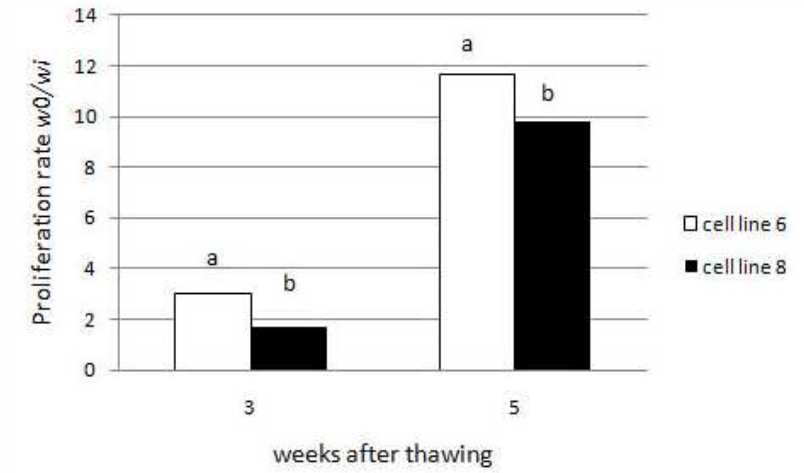

Fig. 1. The proliferation ratios (w_0/w_i) of *A. cephalonica* embryogenic cell lines 6 and 8. w_0 = FWs of ECMs at the time of subculturing, w_i = initial FW of ECMs. Columns with different letters are significantly different ($P < 0.05$)

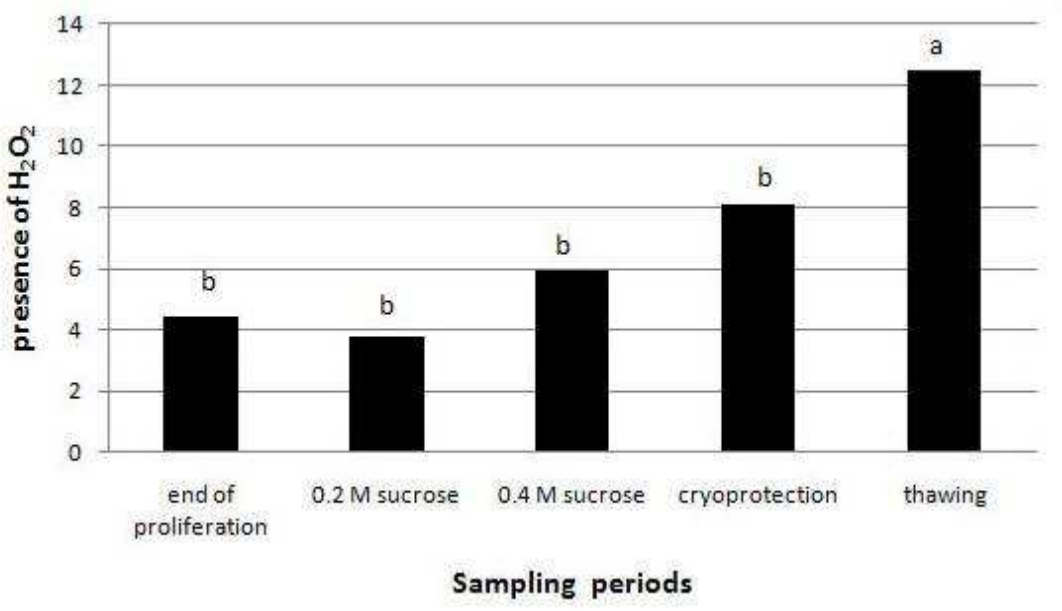

Fig. 2. The production of H_2O_2 (a mean number of blue dots observed) at the end of proliferation cycle, after pre-treatment lasting 24 h on 0.2 M sucrose medium, after 24 h on 0.4 M sucrose medium, after cryopreservation, and immediately after thawing. Columns with different letters are statistically different ($P < 0.05$)

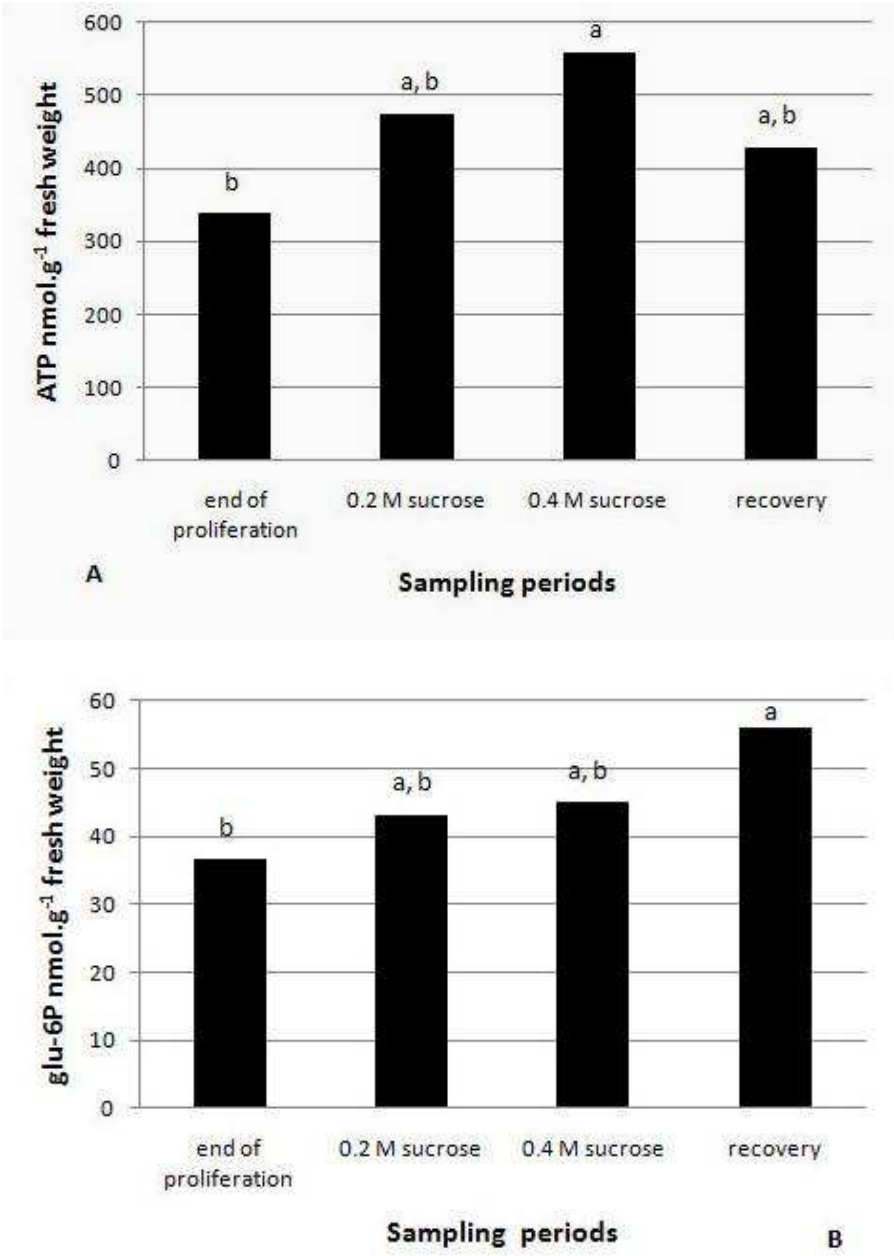

Fig. 3. The ATP (A) and glu-6P (B) cellular contents of ECMs (nmol g^{-1} FW) at the end of proliferation cycle, after pre-treatment lasting 24 h on 0.2 M sucrose medium, after 24 h on 0.4 M sucrose medium and after 3 weeks of subculturing. Columns with different letters are statistically different ($P < 0.05$)

Proteomic changes in *Gentiana cruciata* cell suspension during cryopreservation protocol

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1. Introduction

Tissue culture of gentians has shown the tremendous morphogenic potential of different explants. The embryogenic response of young seedling explants was significant in allowing the somatic cell genetic manipulation of these plants. Additionally the ability of cell suspension cultures of gentians to maintain this embryogenic character has facilitated cryopreservation experiments (Mikuła *et al*. 2005). Interestingly cryopreservation treatments have been shown to enhance the number of gentian somatic embryos regenerated.

Cryopreservation provides an effective means for the long-term storage of vegetatively propagated plant species and *in vitro* tissues. Previously reported improvements of the encapsulation/dehydration cryopreservation procedure for gentians embryogenic cultures and the lack of genetic alternations in regenerants derived from cryogenically stored cultures (Mikula *et al* 2011), has been decisive for the study of protein changes associated with the osmotic stress associated with sucrose treatments**.**

Safe storage of plant material at ultra-low temperatures requires careful optimisation of cryopreservation techniques. It includes the range of procedures aimed to the protection of the plant material against dehydration stress, chilling, storing at ultra-low temperatures and rewarming. The tolerance to dehydration and chilling, which is induced during the pre-treatment stages, is the requirement to maintain high viability of the plant material after re-warming (Mikuła *et al*. 2011). Changes related to acclimatization to such stressful conditions result in changes at the molecular level of the plant material. Proteomic analysis allows for a greater understanding of the physiological responses of cells manifested in the post-translational level (López 2007). Identification of protein expression changes during can help determine the metabolic processes induced at the different stages of a cryopreservation protocol. This permits the more targeted optimization of the given protocol to assure successful long-term storage of plant material at ultra-low temperatures.

2. Material and methods

2.1. Cryopreservation method used

Cryopreservation by encapsulation/dehydration is the most reliable procedure for preserving the viability and embryogenic competence of *Gentiana crucita* suspension cultures. Embryogenic cell suspension cultures were grown in MS medium supplemented with 2.0 mg/L BAP, 1.0 mg/L Dicamba, 0.1 mg/L NAA and 6% (w/v) sucrose were encapsulated in 1.3% (w/v) calcium alginate beads. Beads were incubated in the same medium containing 0.3 M, 0.5 M or 0.75 M sucrose, for 48hrs in each concentration and finally transferred to 1M sucrose for 24h. After this pre-treatment beads were harvested and dried by air in laminar-flow chamber for 5 h at room temperature. After air drying the beads were transferred to cryotubes and directly cooled in liquid nitrogen (Mikuła *et al*. 2008).

2.2. Proteomic analysis

Proteomic patterns were determined for each stage of the sucrose treatment, i.e. at each sucrose concentration stage. Proteins were isolated according to the procedure of Wang at al. (2006), but with some modifications introduced by us. The protein content was determined by the Bradford assay. The protein samples were focused using 3-10 nonlinear IPG strips for the first dimension, for the second dimension separation was achieved using a 12.5% (w/v) acrylamide gel and stained with CBB. The image comparative and statistical analyses (ANOVA) were carried out with the Image Master 2D Platinum 7.0 software. Protein spots showing a statistical significance ($p<0.05$) in abundance were considered as up or down regulated. These protein spots of interest were selected for identification by mass spectrometry. Identification was performed on LC-MS/MS, technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. The resulting peptide sequences were analyzed in Maskot based on a database.

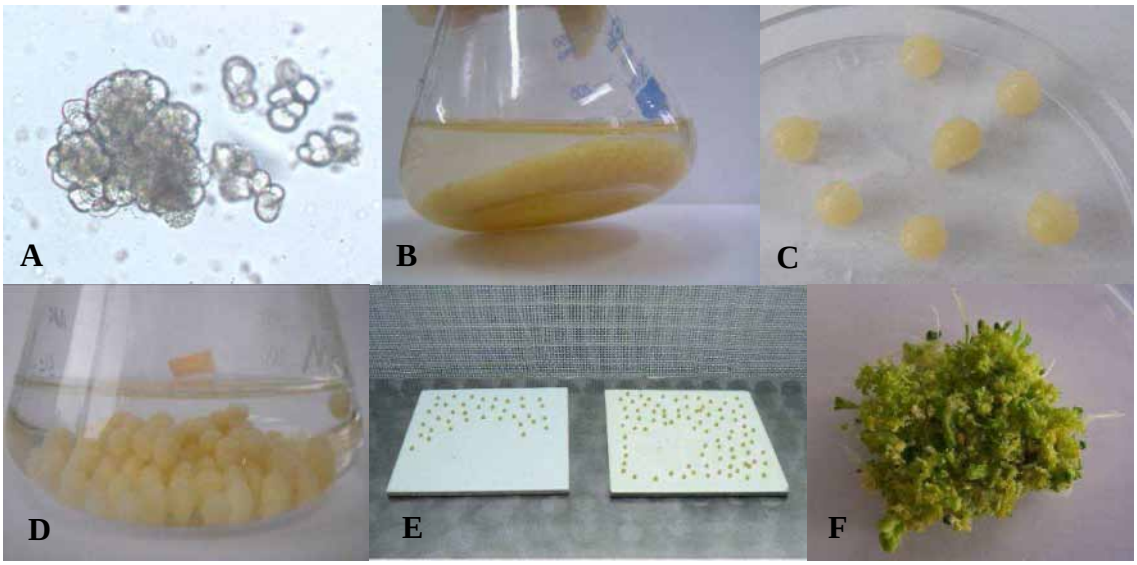

Fig.1. Stages of procedure used for embryogenic cell suspension cryopreservation and result of post-cryostorage culture

A) Non-stained specimen of embryogenic cell suspension
B) Embryogenic cell suspension culture in MS medium supplemented with 2.0mg/l BAP, 1.0 mg/l Dicamba 0.1 mg/l NAA + 6% sucrose in conical flask of (preculture period: two weeks)
C) Beads of cell suspension encapsulated with 1.3 % alginate
D) Incubation of beads in increasingly concentrated sucrose solutions (from 0.3M to 1.0M over 7 days)
E) Air flow desiccation in laminar flow chamber before direct cooling in liquid nitrogen
F) Substation somatic embryo formation from cryogenically stored embryogenic cell suspension in post-thaw regeneration agar culture

3. Results

Figure 1 presents the main steps of encapsulation/dehydration procedure used to cryopreserve *Gentiana* cell suspension cultures. Additionally Fig. 1.E shows very high embryogenic capacity of the tissue after cryopreservation. Alginate encapsulated embryogenic cell suspensions have never been used before for isolation of *Gentiana* protein, but it was shown that alginate did not influence the isolation of proteins of embedded tissue. A very rich profile

with a large number of protein spots was obtained (Domżalska and Rybczyński 2010) (Table 1). Protein profiles of encapsulated cell suspension showed almost lack of smears (Fig. 2).
Three types of proteins were found: some of them presented up-stream growth of activity, other proteins were connected with decreasing, but not much had been formed *de novo* during sucrose treatments. It is thought that the last of these groups will be very important for cell acclimatization to ultra-low temperature.

Table.1 Comparative analysis of 2D-PAGE proteomic images from each stage of the progressive sucrose treatment

	Encapsulated cell suspension without sucrose treatment	After sucrose treatment up to			
		0.3M	0.5M	0.75M	1.0M
No. of spots in gel	851	918	869	839	876
% of matches	94.00	91.67	95.67	95.67	88.67

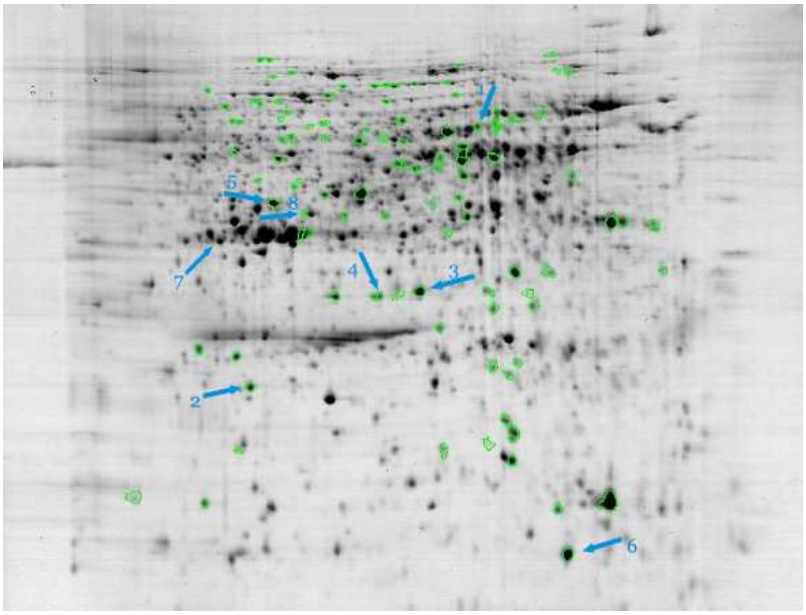

Fig. 2 An example of second dimension electrophoresis of proteomic analysis of embryogenic cell suspension of *Gentiana cruciata*

4. Discussion

Our previous studies have shown that gentian embryogenic cell suspension were an excellent source of plant material for somatic cell genetic manipulation including as small aggregates, cells and protoplasts which consistently maintained their embryogenic character. The effect of the gradually increasing the sucrose concentration up to 1.0 M on the morphogenic potential of the cell suspensions as part of the encapsulation/dehydration cryopreservation procedure, might be monitored by the changing number of regenerated somatic embryos and growing plantlets in post-thaw culture. The genetic uniformity of post-thaw regenerants predisposes the system for studies on proteomics on post-translation level of stress treated cell (Mikuła *et al*. 2008, 2011).
The experiments show that proteomic analysis has the potential to be a very useful tool to register changes in protein expression as a result of adaptations during cryopreservation

pretreatment and help to understand acquisition of freezing tolerance. Our primary results confirmed earlier published data that protein profiles undergo various changes associated the cryopreservation protocols (Carpentier *et al*., 2007). The results proved that post-translational changes are important in molecular analysis of plant material reaction connected with cryopreservation pretreatments.

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Cryopreservation of Norway spruce embryogenic cultures: levels of polyamines

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1. Introduction

Cryopreservation offers the potential for the economical and reliable long-term storage of genetic resources (Cyr 1999). The embryogenic suspensor mass (ESM) is well suited for cryopreservation because of its fast growth rate and its large population of meristematic cells. Moreover, partially differentiated cultures, such as those produced in conifer somatic embryogenesis, exhibit less variation than callus-type cultures (Wang *et al.* 1993). The ESM of some coniferous species, such as *Pseudotsuga menziesii*, *Picea glauca* and *Pinus pinaster* are quite tolerant to cryopreservation, while other species show marked differences in their cryotolerance (Norgaard *et al.* 1993, Cyr 1999). Polyamines (PAs) stabilize the molecular composition of plasma membranes and suppress lipid peroxidation, thereby preventing membrane injury, retaining membrane permeability, and reducing leakage under stress conditions. The biological activity of PAs is attributed to their cationic nature, which enables interactions with anionic biomolecules and protects them from oxidative damage (Zhang *et al.* 2009). The high flexibility of PA metabolism in response to stress, the metabolic link between PA and ethylene synthesis, and their inevitable role in cell division and proliferation, strongly suggest that PAs may play a significant role in cell survival after cryopreservation.

We focused on the cryopreservation of Norway spruce ESM. The specific aims of the present study are: (i) to relate the anatomical structure of ESM to cryotolerance; (ii) to compare PA contents in the ESM of different spruce genotypes and relate them to cryotolerance; and (iii) to relate changes in the anatomical structure of ESM with changes in the pool of PAs in the course of cryoprotectant-treatments and during regeneration after cryostorage.

2. Materials and Methods

2.1. Plant material

The embryogenic cultures of *Picea abies* (L.) Karst. were obtained either as a gift (cell line AFO 541 from AFOCEL, Nangis, France), or induced in our lab from zygotic embryos of mature seeds (Vágner *et al.* 2005) on GD medium (Gupta and Durzan 1986).

2.2. Cultivation protocol

The embryogenic cultures were grown on GD medium (Gupta and Durzan 1986) solidified by 0.75% agar and supplemented with 5 μM 2,4-D, 2 μM kinetin, 2 μM BAP and 30 g L^{-1} sucrose and incubated in darkness at 24 ± 1 ºC (Vágner *et al.* 2005). For maturation of embryos, the cytokinins and auxin were substituted with 20 μM abscisic acid (ABA) and 3.75% (w/v) polyethylene glycol 4000. The cultures were sub-cultured weekly into the fresh liquid medium and incubated in the dark at 24 ± 1 ºC for 5–6 weeks (Vágner *et al.* 2005).

2.3. Cryopreservation

Cultivation of ESM in liquid GD media (Gupta and Durzan 1986) was necessary for the treatment of ESM with gradually increasing concentrations of cryoprotectants. The cultures

were treated with sorbitol, which resulted in a 0.4 M sorbitol concentration. The flasks with cultures were then cooled on ice and DMSO was gradually added to reach the final 2% concentration. The cooled ESM was then filtered and transferred into 2 ml cryovial tubes. Freezing was performed in a commercial freezing container Mr. Frosty, Nalgene. Thereafter the cryovials were stored in liquid nitrogen (for details of cryoprotocol see Vágner *et al.* 2005).

2.4. Microscopy and anatomical study

The viability of the ESM was determined by double staining with fluorescein diacetate (FDA) and propidium iodide (PI) according to modified protocols of Jones and Senft (1985). All images were processed using the computer image analysis.

2.5. Polyamine analysis

The concentrations of endogenous free polyamines (putrescine, spermine and spermidine) and their bonded and conjugated forms were analyzed by HPLC in the ESM of Norway spruce (for details see Vondráková *et al.* 2010).

3. Results

3.1. Precultivation of cultures prior cryopreservation

The growth characteristics and alterations in PA contents during the process of cryopreservation were studied in detail in AFO 541 and C110 cell lines, since these cultures have comparable ESM anatomy. However, these two cultures differ in their rate of growth, their yield of matured somatic embryos, and particularly in their regeneration ability after cryopreservation. In both cultures, cultivated in liquid media prior to cryopreservation, the highest PA concentration in ESM was of Spd; its concentration rose and fell periodically during the two weeks in culture, with the maximum always occurring on the 2nd and/or 3rd day after changing the media (Table 1).

The treatment of the ESMs with sorbitol caused the continual disintegration of polyembryogenic centers and suspensors. The long vacuolized cells of suspensors are much more sensitive to osmotic changes elicited by sorbitol treatment than are the small cells of embryonal meristems. However, the stress reaction of treated cells was not only manifested in structural changes: starch accumulation further intensified during the cryoprotectant treatments; nuclei of suspensor cells in both cell lines were enclosed with starch grains; and free starch grains were observed to be present in their cytoplasm. The cryoprotectant-treatment influenced the level of percloric acid (PCA)-soluble free PAs. Marked decreases in free Put and Spd after sorbitol applications were observed in the ESM (Table 1). The level of free Spm did not change, and its content in DMSO treated cells, before freezing, was comparable with that in cells before receiving the cryoprotectant treatments. A continual decrease in the level of amines was observed during the cell osmotic pre-treatment. However, while the content of total PCA-soluble free PAs was double that of PCA-soluble conjugates in cells before the cryoprotectant treatments, in DMSO treated ESM the levels of both PA forms were approximately equal.

3.2. Regrowth of cultures after cryopreservation

Freezing the ESM before cryostorage resulted in massive selective damage to cells. All suspensor cells, long and vacuolized, were destroyed. The character of the ESM changed, as only the scattered small cells of embryonal meristems survived. The first newly developed early somatic embryos were observed on days 4–5 after thawing. Enlarged embryos, with

more robust embryonic heads connected with large suspensors, occurred in the AFO 541 culture on the 11th day after thawing. The embryogenic capacity remained constant after cryostorage.

The levels of free Put and Spd determined in the ESM on day 6 were low due to there still being a high proportion of dead cells present. However, a marked increase in Put and particularly Spd, two weeks after thawing, correlated well with the observed anatomical changes of the culture. The level of free Put and especially the high amount of Spd two and three weeks after cryostorage corresponded well with the PA values in the ESM cultivated in liquid medium before freezing. The fractional composition of the PA pool in the ESM of AFO 541 showed significant changes after thawing. A level of PCA-insoluble PA conjugates observed on days 6 and 11, more than two times higher than was observed in the tissue before freezing, was probably due to the decompartmentation of dead cells still present in tissue samples. A relatively high proportion of soluble PA conjugates occurred in the ESM, especially on day 6 after thawing, which reflects the unsatisfactory state of embryogenic tissue after freezing. The successful re-growth of AFO 541 ESM on days 15 and 21 was characterized by an increase in the total content of free PAs.

Table 1: PCA-soluble free putrescine (Put), spermidine (Spd) and spermine (Spm) in the course of 14-day cultivation of two Norway spruce embryogenic cell lines (AFO 541 and C110) in liquid proliferation medium. PAs are expressed as μmol.g^{-1} DW.
Day 0 and day 7: transfer of ESM to the fresh media, day 11: start of sorbitol treatment.

	AFO 541			C110		
day	Put	Spd	Spm	Put	Spd	Spm
0	2,7	3,9	0,8	1,8	1,9	0,3
1	3,0	4,0	08	1,6	2,0	0,2
2	3,2	4,2	0,8	1,9	2,7	0,5
3	3,0	4,8	0,7	1,6	2,2	0,3
4	3,1	4,3	0,7	1,5	2,3	0,3
5	3,4	3,8	0,6	1,5	2,3	0,3
6	2,9	3,4	0,5	1,4	1,9	0,3
7	3,1	3,6	0,5	1,6	2,0	0,2
8	3,3	4,2	0,6	1,4	2,3	0,3
9	3,2	4,2	0,5	1,7	2,4	0,3
10	2,8	3,8	0,6	1,2	1,7	0,2
11	2,1	3,8	0,5	1,3	1,5	0,2
12	1,8	3,8	0,6	1,3	1,4	0,2
13	1,7	3,5	0,5	1,4	1,4	0,2
14	1,6	3,0	0,4	1,3	1,2	0,1

4. Discussion

The involvement of PAs in the stress responses of plants indicates their importance for plant survival. The results presented here have shown that: (i) the size and structure of polyembryogenic complexes determines the quality of somatic embryos, but does not correlate with their cryotolerance; (ii) growth rate is the only character shown to have some association with cryotolerance; and (iii) a clear connection between the total content of PAs and cryotolerance was not determined.

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Physiological evaluation of *Hypericum rumeliacum* Boiss. plant regenerated after cryopreservation

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1. Introduction

Preservation of the world's genetic resources is currently at the forefront of conservation activities and biotechnology can play an important role in international plant conservation programs. The storage at ultra-low temperatures (-196°C) has a practical application for the preservation of plant cells and tissues, characterized by a high production of important secondary metabolites. The Balkan endemic *Hypericum rumeliacum* Boiss. is characteristic for the Balkan flora. The species is rare and with conservational value for Bulgaria. A number of studies report on its phytochemical composition and valuable pharmacological properties (Kitanov 2001; Galati *et al*. 2008). Our preliminary investigations have shown that *in vitro* culture *H. rumeliacum* possesses a high regeneration capacity and produces high levels of phenolics and flavonoids commensurable to the levels in the intact plant (Danova *et al*. 2010). The aim of the present work was to evaluate the physiological status of *Hypericum rumeliacum* Boiss. plants regenerated from shoot tips, cryopreserved by vitrification.

2. Materials and Methods

2.1. Plant material

Hypericum rumeliacum Boiss. was collected at its natural habitat in the Rhodopes Mountain, Bulgaria. *In vitro* shoot cultures were induced from sterilized mono-nodal stem segments of the *in situ* growing wild plant. Shoots were maintained on MS culture medium at 25°C, 16/8 h photoperiod and 60 μmo.$m^{-1}s^{-1}$ with a 45-day period of regular subculture. Before preculture shoot-tips were isolated and grown on the basal RM medium (Linsmaier-Skoog's salt mixture (Linsmaier and Skoog 1965), Gamborg's B5 vitamins (Gamborg *et al*. 1968), 30 g/l sucrose, 2 mg/l glycine, 100 mg/l myo-inositol) supplemented with 0.5 mg/l 6-benzylaminopurine (BA) ($RMB_{0.5}$ medium). Control plants were cultivated on RM basal medium. After 1 month cultivation shoot-tips were subjected to cryopreservation procedure described below. After thawing from liquid nitrogen, a 2-week cultivation in the dark and 1-week cultivation in half-intensity light, the cryopreserved shoot-tips were grown at the same environmental conditions as control unfrozen plants and propagated on the basal RM medium and RM medium supplemented with 0.1 mg L^{-1} BA.

2.2. Cryopreservation of Hypericum rumeliacum shoot tips

The preculture treatment performed to *H. rumeliacum* was based on 0.076 μM ABA exposure of shoot tips in $RMB_{0.5}$ liquid culture medium for 3, 7 and 10 days periods. Further explants were treated for 20 minutes in LS solution (2M glycerol and 0.4M sucrose) at room temperature. Plant shoot tips were dehydrated in PVS3 (50% w/v sucrose and 50% w/v glycerol) for 90, 120, 150 and 180 minutes on ice and finally directly immersed into liquid

nitrogen. After one week of storage, thawing was performed in water bath at 40ºC for 1 minute. Tips were rinsed in liquid $RMB_{0.5}$ containing 1.2 M sucrose. Afterwards shoot tips were cultivated on semi-solid $RMB_{0.5}$ for regeneration. The survival rate was determined as the percentage of green growing meristems with differentiating shoots 4–6 weeks after cryopreservation compared to the initial number frozen.

2.3. MDA determination and ROS imaging

Malondialdehyde (MDA) was determined according to Dhindsa *et al*. (1981) including TCA/TBA (thiobarbituric acid) addition and a heat/cool cycle. Absorption was read at 532 nm and 600 nm and MDA concentration was calculated using its molar absorptivity 155 µmol/cm. Intracellular ROS were detected using 2',7'-dichlorofluorescein diacetate (DCF-DA), following the protocol of Sakamoto *et al*. (2005). The fluorescence of DCF-DA stained samples was determined by Nikon Eclipse microscope, TS 100, filter B-2A, exciter 450–490, and magnification 200x.

2.4. Total phenols and flavonoids determination

Total phenolic content was determined according to Singleton *et al*. (1999). The absorbance was read at 765 nm. Total phenolic content was expressed as milligrams gallic acid equivalents per gram of DW of the sample. Total flavonoid content was performed according to Chang *et al*. (2002). The absorbance was measured at 415 nm. Total flavonoid content was expressed as milligrams quercetin equivalents per gram of DW of the sample.

3. Results

The recovery rate of *H. rumeliacum* plants cryopreserved by vitrification varied between 3.3 % and 14 %. The shoot tips which showed higher recovery rate were pre-cultured for 10 days with 0.076 µM abscisic acid and equilibrated on ice for 90 min and 180 min. The plants regenerated from these explants were subjected to physiological evaluation and compared with unfrozen controls. Increased accumulation of MDA (Fig.1A) and ROS determined by staining with DCF-DA occurred in regenerated plants after cryopreservation (Fig.1 B-G). Longer equilibration time on ice caused higher level of stress metabolites.

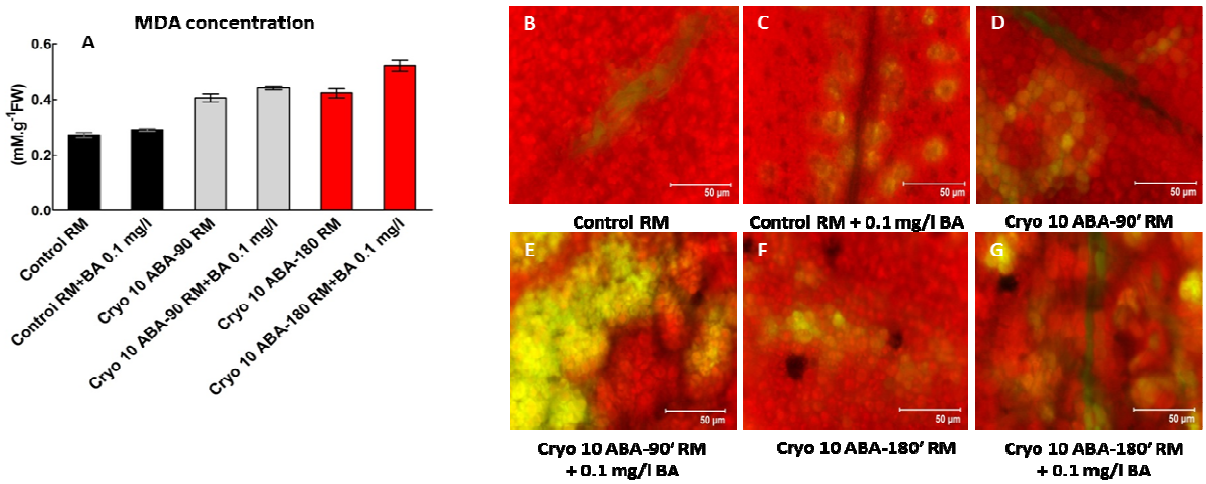

Fig.1. Concentration of MDA (A) and ROS accumulation in regenerated *H. rumeliacum* plants after cryopreservation by vitrification. (B-G) epifluorescence of ROS after staining with DCF-DA. The green fluorescence indicates ROS; chlorophyll fluorescence appears in red. Scale bar = 50 µm. Error bars indicate ± SEM $_{(n-1)}$

The biosynthetic capacity of the regenerated plants is not impaired after cryopreservation. Moreover cryopreserved plants showed higher levels of phenolic and flavonoid contents and possess increased antioxidant capacity than those of the unfrozen controls (Fig. 2 A-C). Regenerated plants cultivated on RM medium, supplemented with 0.1 mg/l BA showed increased accumulation of ROS and MDA and decreased level of phenolic content and antioxidant activity in comparison with plants propagated on cytokinin-free medium (Fig. 1A-G; 2 A-C). This suggests for possible antagonism between ABA and BA or delay of the protective effect of ABA under presence of BA.

4. Discussion

Cryopreservation in LN is a three-step process comprised of pre-culture, cryoprotection and cooling/freezing with recovery of the plant (Harding and Benson 1994). Each of the steps can have impact on the survival rate and on the genetic stability. Successful cryopreservation of shoot-tip meristems of *H. perforatum* has been performed showing high recovery rates and preserved biosynthetic capacity of the regenerants (Urbanova *et al*. 2002).The recovery rate of *H. rumeliacum* plants cryopreserved by vitrification varied between 3.3 % and 14 % what indicates an insufficient precryogenic preparation. Despite the promising results achieved in the presented work, further experiments aimed at optimization of the cryopreservation procedure and assessment of interaction between the pre-culture additives and post-thaw physiological status are required. Nevertheless cryopreservation seems to be prospective method for maintenance of biodiversity of threatened and endemic plant species.

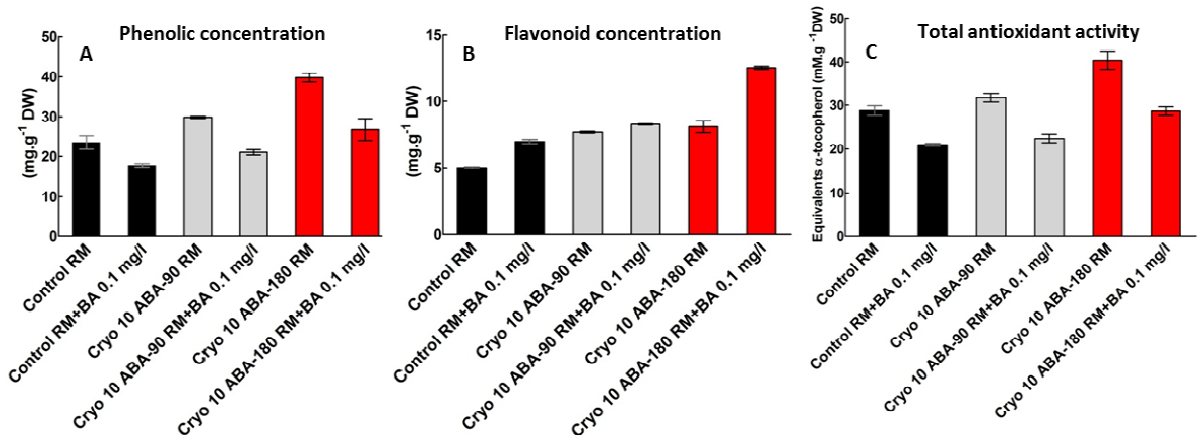

Fig.2. Influence of cryopreservation on the secondary metabolites accumulation in regenerated *Hypericum rumeliacum* plants. Error bars indicate ± SEM $_{(n-1)}$

5. Acknowledgements

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Part III
Working Group 2: Technology, Application and Validation of Plant Cryopreservation

Development and implementation of droplet-vitrification protocol for cryopreserving clonal germplasm in Korea

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1. Introduction

During the past decades, many facts have evidenced the significant improvement of cryopreservation methodology (Reed 2001; Towill 2002; Engelmann 2004; Reed 2008; Gonzalez-Arnao *et al.* 2008). The "plant vitrification revolution" (Benson 2004) promoted cryopreservation techniques based on a new concept combining dehydration in highly concentrated cryoprotectant mixtures with subsequent ultra-rapid cooling and rewarming. Plant vitrification solutions 2 and 3 (PVS2 and PVS3) which have been formulated with a balanced ratio of cryoprotectants, proved effective for cryopreserving a diverse range of plant materials derived from more than 200 plant species (Sakai *et al.* 1990; Nishizawa *et al.* 1995; Sakai and Engelmann 2007).

However, there are several important shortcomings that hamper routine utilization of cryobanking for conserving plant germplasm, particularly on a large-scale. All vitrification-based methods are basically multi-step procedures with limited options for standardization and unification. Obviously, there is no effective universal protocol, *i.e.* a predetermined sequence of treatments, which could be successful with all types of materials. Well-tested protocols are available for certain crops, whereas setting up, or at least adaptation, of the procedure would be necessary for many others. Adaptation normally requires experimental screening of numerous preliminary treatments such as cold hardening, preculture of explants with osmolites, variations in temperature and duration of cryoprotectant treatment, type and concentration of growth regulators in recovery medium, etc.. Numerous cryopreservation techniques that are currently available through publications may confuse curators of plant collections and newcomers who aim at cryopreserving their genetic materials with minimum efforts and highest efficiency.

The strategy for cryopreserving plant genetic resources at RDA was meant to achieve two main goals: to cryopreserve Korean the national collection of *Allium* and to simplify the process of protocol development for diverse clonal propagules like shoot apices, hairy roots, somatic embryos and undifferentiated cell cultures to assist curators in local areas in cryopreserving their genetic collections.

2. Cryobanking the *Allium* collection (Implementation)

The genus *Allium* comprises about 750 species. The most widespread is garlic, *Allium sativum* L., which is well known for its antimicrobal activities and strong flavor. For centuries, garlic has been remaining an essential component of Korean diet and one major source of bioactive compounds utilized in herbal medicine. The national collection of garlic germplasm comprises a total of 1,178 accessions including both hard- and soft-neck genotypes. All accessions are maintained as clonal propagules in the fields in four geographically isolated locations: two repositories are located in the south of the country and two in temperate and cold regions. In 2005-2010, the whole *Allium* collection has been cryopreserved using PVS3-based droplet-vitrification methods with the emphasis on the following steps:

1. Studies on the effect of explant size and origin (2001 – 2002);
2. Studies on the effect of plant material storage before cryopreservation and on the effect of cold-hardening. Anti-contamination treatments before cryopreservation (2001 – 2003).
3. Development of the "standard protocol" based on explant response to dehydration, rewarming, unloading and regrowth conditions (2002-2003);
4. Evaluation of critical factors in the cryopreservation protocol. Using Differential scanning calorimetry (DSC) and HPLC for analyzing thermal behavior of samples and the dynamics of influx/efflux of cryoprotectants (2002-2003);
5. Testing the newly emerged droplet-vitrification protocol. Applying the droplet-vitrification technique instead of vitrification (2005);
6. Adaptation of the standard protocol to diverse *Allium* genotypes. Implementation of the cryopreservation protocol to various accessions (2005-2010);
7. Evaluation and screening the collection for the duplicated accessions; filling the gaps in the collection. Adjusting the protocol to be implemented to very sensitive and non-bolting varieties (2009-present).

From 1,651 accessions cryopreserved, 1,008 accessions met the predetermined minimum requirements, *i.e.* showed post-cryo plant regeneration above 40% and were present with at least three cryovials without duplication with 10 samples per vial (minimum 30 explants). The most challenging factors in implementation were the following:

1. Geographical isolation and, sometimes, poor organization of local collections caused problems for timely harvesting (particularly important when using bulbil primordia as material for conservation).
2. Different sources of material should be used depending on genotype origin, ability to produce inflorescences, bulb morphology, etc.
3. Availability and physiological conditions of samples varied significantly from season to season.
4. Inner microbal contamination was one of major problems to overcome.

2.1. Plant material

Plant material was nearly the most important factor for successful cryopreservation of garlic germplasm. Different types of materials were tested, *i.e.* 1. bulb cloves; 2. matured bulbils (topsets); 3. large single bulbs not divided into cloves; and 4. bulbil primordia (asexual bulbs formed on unripe flower stalks). The first experiments employed shoot apices excised from single bulbs and cloves (Baek *et al.* 2003; Kim *et al.* 2004). Later on, however, the high level of contamination and long (up to 3 months) dormancy period in mature bulbs, prompted us to switch to immature bulbil primordia (Kim *et al.* 2006; 2007). The regrowth of bulbil primordia after cryopreservation depended on the size of initial bulbils, which was strongly genotype-specific and on the developmental stage of flower stalks. By contrast, the initial size of cloves and bulbs had no significant effect on the post-cryopreservation regrowth. In Korea, about 40% of *Allium* accessions, mostly of tropical origin, lost the ability to form inflorescences. For those accessions, using cloves as starting material was the only option available (Kim *et al.* 2007). Garlic bulbs are normally highly dormant and thus required 3 to 6 months of post-harvest storage prior to sterilization and explant excision (Kim *et al.* 2004). Barely 10% of the explants excised from bulbs shortly after harvesting were able to produce plantlets *in vitro* even without cryopreservation. By contrast, more than 85% explants excised from bulbs that were stored for at least 3 months showed normal regrowth after exposure to liquid nitrogen temperature (Kim *et al.* 2004).

It is well known that severe stress like cryopreservation may cause the outbreak of inner contamination (Keller 2002). In this regard, cloves are disadvantageous compared with

bulbils since 20 to 50% of them are normally infected with soil microorganisms, and this infection is difficult to eliminate (Kim *et al.* 2007). In contrast to cloves, less than 10% unripe inflorescences containing bulbil primordia were contaminated (Keller 2002; Ellis *et al*. 2006). Together with high and rapid post-cryopreservation regrowth, this made bulbil primordia the more adapted material for cryopreservation.

2.2. Protocol development.

The development of the cryopreservation protocol for garlic was based on comparative studies of regrowth, moisture content (MC) and thermal behavior of cryoprotectant solutions and dehydrated samples (Baek *et al.* 2003; Kim *et al*. 2004a; 2005). In addition, the evolution of DMSO, glycerol and sucrose concentrations in clove apices were monitored during the cryoprotectant and unloading treatments (Kim *et al*. 2004b). As a result, the "standard" cryopreservation protocol has been developed consisting of the following steps:

1. Material sterilization and extraction of explants. Please refer to Kim *et al.* (2007) for details.
2. Preculture of explants on solid medium containing 0.3 M sucrose for 2-6 days at 10°C. This step was essential for both clove apices and bulbul primordia (Kim *et al.* 2003).
3. Dehydration in PVS3 for 150-180 min at ambient temperature. For precultured explants, loading was unnecessary. In total, seven and 14 vitrification solutions of different compositions were tested in the vitrification and droplet-vitrification procedures, respectively. Both clove apices and bulbil primordia were highly tolerant to vitrification solutions, both PVS2- or PVS3-based. However, for cryopreservation, PVS3 showed highest efficiency in terms of dehydration and protective actions. MC of dehydrated clove apices varied from 57 to 67% FW after incubation in PVS2-based VSs compared to 37-54% FW after treatment with PVS3-based VSs. The sharp decrease of apex MC within the first 30 min of PVS3 treatment correlated with rapid the increase of sucrose and glycerol concentrations in samples, from 14.0 to 100.2 mg/g for sucrose and from 1.1 to 117.4 mg/g for glycerol, respectively. Afterwards, the concentration of sucrose and glycerol increased slowly, reaching 128.0 mg/g and 175.3 mg/g, respectively, after 150 min in PVS3 (Kim *et al.* 2004b). The equilibrium MC around 30% was reached after 120 min of incubation.
4. Cooling in drops of PVS3 attached to aluminum foil strips.
5. Rewarming in 10 ml pre-heated liquid MS medium with 0.8 M sucrose for 30 s.
6. Unloading in liquid MS medium with 0.8 M sucrose at ambient temperature for 40 min. Sucrose content in the unloading medium could be increased to 1.2 M, or substituted with up to 1.76 M sorbitol without decrease in regrowth. Unloading duration could be prolonged to 90 min without significant effect on regrowth. Sucrose content in explants declined sharply during the first 10 min of unloading while glycerol concentration decreased steadily within 90 min.

2.3. Critical factors

The systematic approach to cryopreservation of garlic described above revealed the following critical factors which should be carefully controlled to ensure rapid post-storage regrowth of samples: size and source of explants; post-harvest storage duration; preculture duration and sucrose concentration in preculture medium; composition of VS and dehydration period; unloading. The experiments showed that droplet-vitrification using aluminum foil strips produced significantly higher post-cryo regrowth than vitrification in cryovials (Kim *et al.* 2006).

3. Development of cryopreservation protocols for clonal plant germplasm

3.1. Protocol development - theory

Cryopreservation is, in principle, applicable to any type of plant tissues, which possess regeneration potential and are composed of actively dividing meristematic cells (Panis 2009). The most important barriers for the success are the high toxicity of concentrated vitrification solutions PVS2 and PVS3, and the low desiccation tolerance of species originated from (sub)tropical zones (Fahy *et al.* 1990; Sakai and Engelmann 2007). Another difficulty is that the optimal combination of treatments has to be found experimentally, after laborious screening of numerous variants.

After experimenting with shoot apices, bulbs, somatic embryos, hairy roots and embryogenic cell cultures of different plant species, we have proposed a simplified approach allowing to develop a cryopreservation protocol for various types of materials. Following this approach, explants are first subjected to one of four predetermined sets of treatments utilizing alternative loading (LS) and vitrification (VS) solutions with altered concentrations of cryoprotectors (Kim *et al.* 2009a and b). Each set consists of up to 10 treatments specifically developed to reveal explant response to dehydration and cryoprotectant toxicity. Treatments from one set might be performed simultaneously the same day (better) or can be divided into three to four independent groups, e.g. "preculture treatments", "loading", "treatments with VS", etc. The main question to answer at this stage is what treatment during the procedure produces the most harmful effect on explant regrowth, *i.e.* if the toxicity is mainly due to chemical or osmotic action.

During the second step, optimal treatments that have been chosen from preliminary experiments are further modified and combined into the "standard cryopreservation protocol". This protocol normally results in high regrowth after cryopreservation. Moreover, it can be easily adapted to a range of accessions.

Table 1. Preliminary scheme of VS treatments depending on the structure and size of plant materials and on their sensitivity to chemical and osmotic stress of VSs.

Cyto-toxicity		Explant size		
Osmotic	Chemical	Small (callus, sperm)	Medium (meristem, shoot)	Large/non-permeable (bulb, rhizome)
S[♪]	**S**	A3 (60~70%, ice), B6	A3 (70~80%, ice), B5	??
	T	A3 (60~70%)	A3 (80~90%)	A3 (90%)
T	S	B5	B3	B1
	T	A3(70~80%), B5	A3(80~90%), B3, B1	B1

[♪] **S** – sensitive; **T** – tolerant

Composition of vitrification solutions (% w/v): A3 (90%): glycerol 37.5% + DMSO 15% + EG 15% + sucrose 22.5%; B1: glycerol 50% + sucrose 50%; B3: glycerol 45% + sucrose 45%; B5: glycerol 40% + sucrose 40%; B6: glycerol 35% + sucrose 35%.

The major difference of this approach compared with PVS2-based vitrification methods is that the composition of LS and VS as well as the duration and temperature of VS treatment are variable depending on explant origin, size, structure and physiological state (Table 1). This method allowed to achieve post-cryopreservation regrowth above 70% with most materials tested within 4-6 months (Table 2).

Table 2. Current status of cryopreservation protocol development for various species and plant materials at the National Agrobiodiversity Center, RDA, Korea.

Plant material	Species	Regrowth after cryopreservation	Current status of protocol development
Hairy roots	*Rubia akane* *Scrophularia buergeriana* *Angelica gigas* *Nepeta cataria* *Fagopyrum tataricum* *Phytolacca esculenta*	75-98% depending on species	Completed
Shoot apices	*Chrysanthemum morifolium* *Solanum* spp.	Above 85%	Completed In progress
Bulbs	*Allium* spp. *Lilium* spp.	Above 80%	Completed, applied to 1,651 accessions In progress
Rhizomes	*Cymbidium kanran*	90-96%	Completed
Embryogenic callus, somatic embryos	*Kalopanax septemlobus*	~100%	Completed

3.2. Protocol development - examples

The most typical explants representing the "*tolerant*" (TT) group were immature bulbs of lily and garlic, as well as orchid rhizomes. Those explants were comparatively large (>3 mm), with a very compact cell structure. Influx/efflux of glycerol and sucrose in samples was slow due to the low permeability of the cell wall. Explants from this group were normally very tolerant to both osmotic and chemical toxicity produced by VSs. Poor regrowth after cryopreservation was mostly due to insufficient dehydration of samples before immersion in liquid nitrogen. Therefore, the cryopreservation strategy employed VSs with high glycerol and sucrose concentrations (PVS3). Treatment for 3 to 7 h at ambient temperature ensured dehydration of samples to optimal MC, which was comprised between 35 and 45% FW. For lily bulbs, a short longitudinal cutting allowing cryoprotectant to permeate into the inner parts of the explants showed some beneficial effect. Two-step preculture with 0.3-0.7 M sucrose for 1-3 days provided high regrowth after VS treatment and cryopreservation. No loading step was needed.

By contrast, explants belonging to the "*sensitive*" group (SS) were highly susceptible to both osmotic and chemical toxicity of VSs. Those explants required prolonged step-wise preculture with sucrose concentrations gradually increasing from 0.3 to 0.7 M to induce desiccation tolerance.

Loading step was found essential and often crucial. With most materials tested, alternative loading solutions C4 (17.5% glycerol + 17.5% sucrose, w/v) and C6 (20.0% glycerol + 20.0% sucrose, w/v) produced higher regrowth as compared with "classical" LS containing (w/v) 18.4% glycerol + 13.7% sucrose (Nishizawa *et al.* 1993).

In most experiments, PVS2 resulted in the lowest post-cryopreservation regrowth compared with the other VSs tested. This was mostly due to the high concentrations of DMSO and EG, which have been identified as the most toxic cryoprotectants (Fahy *et al.* 1990). Strong chemical toxicity allowed only short (up to 25 min) incubation with PVS2 at ambient

temperature, which resulted in insufficient dehydration of medium-sized explants like shoot apices of chrysanthemum and sweet potato. Increasing glycerol and sucrose content compared with the original PVS2 formula (solution A3) resulted in lower water potential and enthalpy of the VS and improved dehydration and cryoprotectant efficiency (Kim *et al.* 2009b). For those explants that appeared to be extremely sensitivity to both chemical and osmotic stress (hairy roots), exclusion of DMSO and EG (solutions B3, B5 and B6) and/or decreasing the total concentration of cryoprotectants (diluted A3 solution) proved beneficial (Table 1). These data confirmed that sensitive species require a balance between the cryoprotectant and the toxic effects of the VS employed to be successfully cryopreserved.

In conclusion, post-cryopreservation regrowth above 70% was achieved for most species tested using a new approach to protocol development (Table 2). Explants were subjected to specific preculture treatments followed by dehydration in alternative loading and vitrification solutions. The condition and duration of treatments as well as solution composition were optimized depending on material type and its tolerance to osmotic and chemical stress. This process was significantly simplified and facilitated due to the use of the already developed sets of treatments, each consisting of up to 10 variants.

For hairy roots and embryogenic cell cultures, cryopreservation protocols were developed within 4-6 months, excluding the time required for culture propagation. Cryopreservation protocols for lily and sweet potato are currently at the final steps of optimization.

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An overview of dormant-bud cryopreservation for the *ex situ* conservation of woody species in a maritime climate, based on experience with *Malus* cultivars

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Cryopreservation of winter-dormant buds for the *ex situ* conservation of woody species provides significant resource-based advantages, as field-harvested material is taken directly into the preservation protocol without additional pretreatment, and after recovery direct grafting replaces regrowth via *in vitro* culture. A protocol for such preservation was developed by Forsline *et al*. (1998) using material field-hardened under the relatively severe winters of a continental climate at Fort Collins, USA. Latterly, the protocol has been widely adopted and applied in countries such as Denmark (Vogiatzi *et al*. 2011a,b), Germany (Höfer *et al*. 2010), Italy (Lambardi *et al*. 2009) and Spain (Revilla *et al*. 2010), where the milder winters of a maritime climate limit the acquisition of natural hardiness (for example, see Fig.1). Despite the less severe winters, survival often exceeds the widely-accepted 40 % threshold for conservation (Reed *et al*. 1998), yet results can vary significantly with season and cultivar, and can fall to zero in extreme cases.

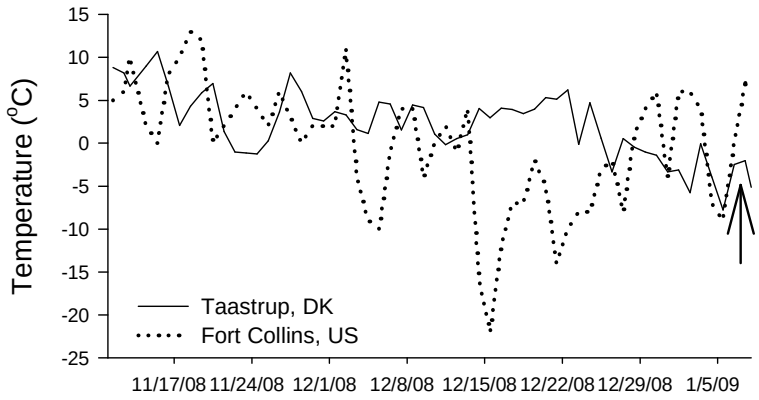

Figure 1 Mean daily temperatures for the winter of 2008-9 at Fort Collins, USA and Taastrup, in eastern Denmark, to illustrate differences between continental and maritime conditions. The arrow indicates a typical sampling date for cryopreservation in Taastrup.

Individual winters will, inevitably, vary with regard to their pattern of low temperatures and to achieve the best possible survival in a particular year it is necessary to understand, in detail, the function of each step of the cryopreservation protocol. In this way the procedures might be manipulated to optimise survival. Using apple (*Malus domestica*) growing in Taastrup, eastern Denmark, as a test species, the study of the past three years has looked at the cryopreservation procedure to better understand where and how viability is lost, and has identified processes critical for optimal recovery. This overview summarises much of the collected data from this apple study to provide a background that may be of value in improving the techniques for the cryopreservation of winter-dormant buds.

The protocol by Forsline *et al*. (1998) is shown in Figure 2. It requires that winter-dormant buds are gathered at a point where natural hardiness will ensure deep ecodormancy, as a minimum. For Taastrup, Denmark this will occur in January (the coldest part of the winter) and between 2008 and 2010 buds were taken when mean, daily field temperatures were below -4 ºC for four consecutive days. Explants were prepared as a population of 3.5 cm stem segments of comparable diameter holding a single bud and were taken through the processing as soon as possible after harvest. However, storage at +4 °C, tightly wrapped in polythene to limit water loss, for as long as two weeks had no significant effect on eventual, post-cryopreservation recovery.

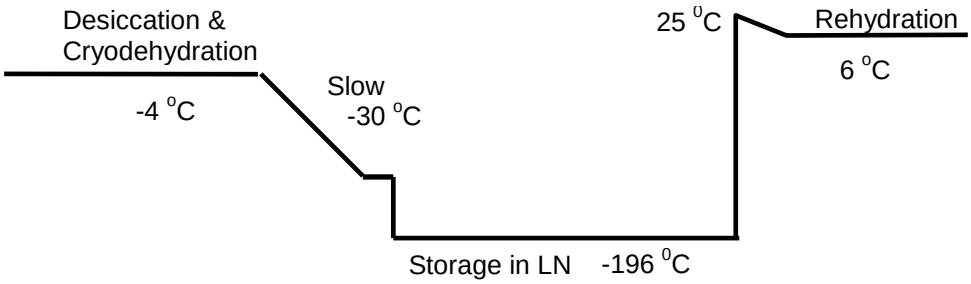

Figure 2. Schematic diagram of the freezing protocol developed by Forsline *et al*. (1998)

As no additional pretreatment is applied to the harvested branches, or isolated explants, prior to cryopreservation the extent of natural hardening will have a major impact on survival, and variation between years must be expected. Growing season conditions will also have an effect as they influence the physiological condition of the trees, their yield and their response to winter hardening conditions (Kahnizadeh *et al*. 1992). Such variation can be seen for three apple cultivars (Table 1), and the data also indicate that even when cortical tissues make a successful graft union, the associated buds may fail to grow due to lethal damage suffered during cryopreservation. Additionally, and despite significant efforts being put to achieving uniformity between replicates, an unwelcome degree of unexplained variation can still be found.

Table 1. Survival (survivors/total explants) after cryopreservation of three sets of dormant bud explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima'. Explants frozen in January, and grafted in March, in Taastrup, eastern Denmark

Winter 2008-09	Holsteiner Cox		Maglemer		Prima	
	Graft ok	Bud growth	Graft ok	Bud growth	Graft ok	Bud growth
unfrozen control	14/14	14/14	13/13	13/13	12/13	11/13
Set 1	14/14	13/14	15/15	15/15	15/15	11/15
Set 2	14/15	8/15	15/15	14/15	15/15	9/15
Set 3	10/10	7/10	9/9	9/9	10/10	4/10
Total	38/39	28/39	39/39	38/39	40/40	24/40
Winter 2010	46/51	14/51	34/34	30/34	49/49	41/49

The success of the protocol for apple has to be viewed in the light of the contribution of secondary buds, for it is not uncommon as the meristematic region of the primary bud to be lethally injured and for regrowth to come from a secondary bud (Fig. 3). In this *Malus* study up to 50 % of the survival of apple cultivars has been shown to come from secondary buds in some instances. Whilst this makes little difference from a conservation viewpoint it does influence scientific assessment of the variables within the procedure

The initial, prolonged incubation at -4 ^{o}C provides an opportunity for tissue water content to be reduced by evaporative loss and also by cryodehydration. This latter effect is a consequence of a freezing event that occurs, reproducibly, in the bud and stem tissues of the explant, within the first hour of incubation (Fig. 4). This was unexpected, as a much lower nucleation point might be expected, based on field observations (Burke *et al*. 1976). During the -4 ^{o}C incubation the water content of the explants is reduced from 45 to 30 % of fresh weight (Table 2), and is consistent between explants regardless of cultivar or eventual survival.

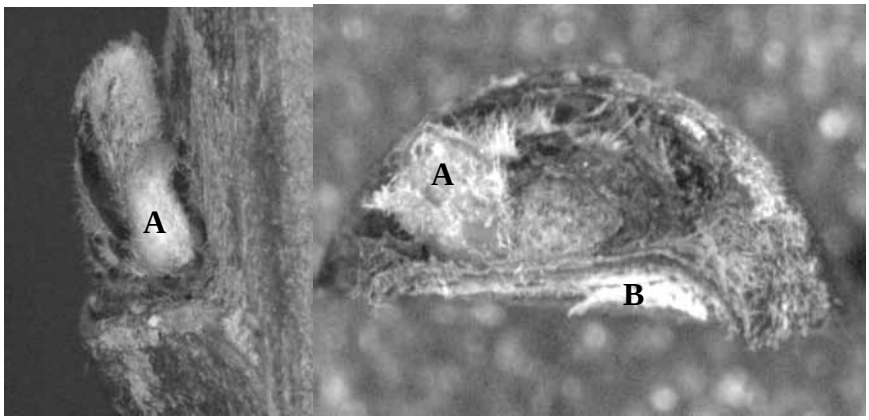

Figure 3. A lateral view (left) of an apple bud recovered from cryopreservation, showing the actively-growing secondary bud (A). The secondary bud is clearly visible in a median section (right) where the dead primary bud (B) can also be seen.

Table 2. The mean water content (% fwt ± sem) of 3.5 cm explants of *Malus* cultivars 'Holsteiner Cox', 'Maglemer' and 'Prima' from winter 2009-10 at the start, and end, of -4 ℃ desiccation (n = 5). Survival after cryopreservation (%) for this particular season is also shown (from Vogiatzi *et al*. 2011 b).

	Water content (% fwt)		
	Holsteiner Cox	Maglemer	Prima
Start -4 ℃	46.4±0.3	43.5±0.4	47.1±0.4
End -4 ℃*	31.6	30.4	31.6
Survival (%)	28	88	84

In this Danish study we have found that the inclusion of the -4 ^{o}C incubation step is essential for survival, at any level, yet Towill *et al*. (2005) claim that this can be omitted. However, in their study there was a storage period at -4 ^{o}C for several weeks prior to the experiments, which would allow cryodehydration to occur even if evaporative water loss were somewhat limited by wrappings around the explants.

The slow cooling (1 ^{o}C/h) to -30 ^{o}C that follows the -4 ^{o}C incubation provides an extended opportunity for further cellular cryodehydration and relocation of water, and there is no significant, further loss of viability as a result of this treatment.

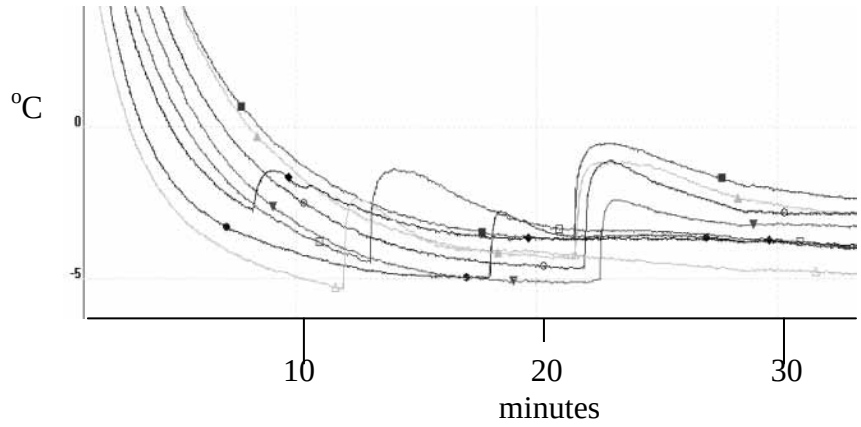

Figure 4. Typical exotherms indicating ice nucleation during desiccation at -4 °C in the buds of eight acclimated explants of the *Malus* cultivar 'Prima', harvested in the winter of 2009-10.

In the current study survival has not been recorded in any instance where the -30 °C step was omitted (Table 3). There may be a survival benefit, or disadvantage, if the 24 h holding period at -30 C is employed, with the outcome of this dependent upon season and, as yet, being unpredictable.

Table 3. Survival of 3 *Malus* cultivars following freezing into liquid nitrogen, after the indicated steps from the protocol derived by Forsline *et al*.(1998)

	% Bud Survival					
	Holsteiner Cox		Maglemer		Prima	
	Rep. 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
-4 °C;LN	0	0	0	0	0	0
-4 °C; -30 °C; LN	30	10	90	80	87,5	20
-4°C; -30 °C; 24 h at -30 °C; LN	20	0	86,7	80	100	80

Commonly, during the transfer from a -4 °C incubator to a programmable chamber to continue cooling to -30 °C, the amount of warming that can take place will cause limited thawing in some explants, particularly those next to the outer walls of their container. This thawing can be recognised subsequently by the exotherm that is clearly visible during cooling to -30 °C. However, if the explants are cooled from -4 to -30 °C in the same machine, without transfer and associated warming, then this freezing event is not observed. Consequently, it can be regarded as an artefact that does not appear to influence subsequent survival, and is not a critical, biological event associated with the cryopreservation protocol

An ongoing element of this study is to investigate more conveniently achieved temperatures to follow -4 °C e.g. -20 °C, with both controlled and uncontrolled cooling. Holding times will be prolonged, where necessary, to achieve the same level of cryodehydration achieved at -30 °C

The most significant losses during the cryopreservation process occur when the explants are transferred, by direct immersion, from -30 ^{o}C into liquid nitrogen and then recovered (Table 3), indicating difficulties with vitrification of cellular contents to withstand the stresses of cryopreservation, recrystallisation during thawing in critical regions of the bud and/or cortical tissue or problems with the repair of cellular injuries during rehydration.

Table 4. Loss of viability for 3 *Malus* cultivars following direct immersion in liquid nitrogen, after the indicated steps from the protocol derived by Forsline *et al*.(1998)

	% Bud Survival					
	Holsteiner Cox		Maglemer		Prima	
	2009	2010	2009	2010	2009	2010
-4 ^{o}C; -30 ^{o}C; 24 h at -30 ^{o}C	100	100	93	80	75	90
-4 ^{o}C; - 30 ^{o}C; 24 h at -30 ^{o}C; LN	72	12	97	83	60	90

The thawing process from the original Forsline *et al*.(1998) protocol is slow (in this study 0.8 ^{o}C/min to 6 ^{o}C) and the potential benefits of imposing a more rapid warming regime have not, to date been part of the investigation. Initial data indicates that the water content of the explants is restored to pre -4 ^{o}C levels after 24h incubation in a suitably moist substrate such as sand or peat. However, the benefits of an extended period in the moistened substrate are, as yet, unclear. In some instances, as for the high surviving cultivar in Fig. 5, there is a clear benefit, possibly due to the completion of repair of cellular damage. There is no comparable benefit for the low-surviving cultivar where, perhaps, the process of repair was less significant, as the explants were either viable or lethally damaged with few intermediate cases where repair might occur.

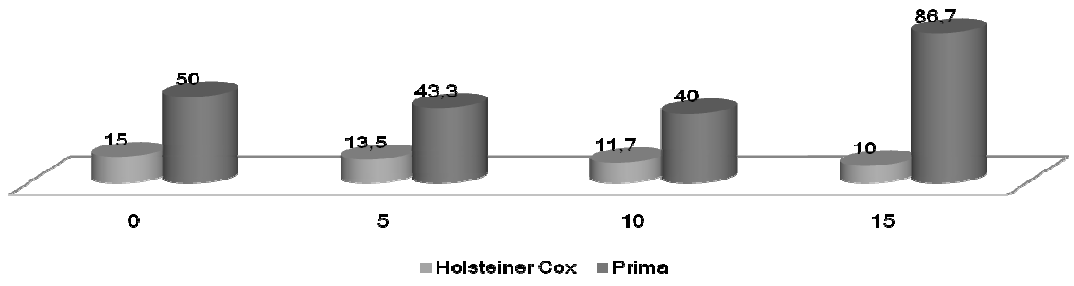

Figure 5. Survival % of two *Malus* cultivars, one with high and one with low viability, over 15 days of incubation in moist peat, following dormant bud cryopreservation.

This investigation of dormant bud cryopreservation in *Malus* is ongoing but it is apparent that the critical steps are concerned with the induced reduction, and eventual restoration, of cellular water content, as in any other cryopreservation protocol. There is a minimal degree of natural hardening required in the population of explants to allow for such manipulations

without lethal injury, and this hardening will vary, unavoidably, with growing conditions, the pattern of winter temperatures and cultivar. A conservation strategy based on dormant bud cryopreservation will need to be designed to accommodate such variation. The data gathered thus far from this study, and related investigations, suggest that survival of at least some explants can be ensured, even for material growing in relatively mild, maritime winters where acquired winter hardiness is limited. This may require further experimental study to determine the appropriate manipulations of cooling required to optimise cryodehydration and would widen access to the technique to include a range of locations in maritime Europe.

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Cryopreservation of *Malus* and *Fragaria*

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1. Introduction

The 'National Program for Genetic Resources of Agricultural and Horticultural Plants' in Germany is designed to provide long-term conservation, utilization, research and development for these species. The Institute for Breeding Research on Horticultural and Fruit Crops in Dresden-Pillnitz hosts the Fruit Genebank, which comprizes with 2,800 accessions. Currently the Genebank keeps the collections in the field as the active collections, with two trees per cultivar and wild species accession in *Malus* and six potted plants in *Fragaria*. In addition to the cultivar collection, the Genebank of the Institute also conserves 527 *Malus* wild species accessions and 310 *Fragaria* accessions, which are the largest collections in Europe. The conservation strategy requires the application of several conservation methods for safety duplication.

One approach is to establish the German Fruit Genebank as a network of collections held at several locations. Based on an assessment performed in several institutions, 950 apple cultivars were selected to be included in the National German Apple Genebank and 341 strawberry cultivars in the Strawberry Genebank (Flachowsky and Höfer 2010; Höfer 2010).

Maintaining the genetic diversity of vegetatively propagated crops is more demanding than for most seed-producing plants. The specific genotype must be maintained and backups for these materials are needed to provide security in case of a disease or environmental disaster (Engelmann 1997.)

Malus germplasm can be cryopreserved using buds from either *in vitro* or *in vivo* plants. Cryopreservation using a winter vegetative bud method, according to the USDA-ARS National Centre for Genetic Resources Preservation, Fort Collins (Towill *et al.* 2004), was successfully applied to the *Malus* accessions maintained at the Institute of Breeding Research on Horticultural and Fruit in Dresden-Pillnitz (Höfer 2007).

For strawberry, *in vitro* cold storage was tested and adapted as a safety duplication method. The average storage duration at 4°C was 22 months for a range of strawberry cultivars and 9 months for wild species accessions using five chamber bags as storage containers and a culture medium devoid of plant growth regulators and vitamins. A calculation of the costs related to the establishment and maintenance of a safety duplication of the whole collection demonstrated that the application of *in vitro* cold storage is too labour-intensive to be employed (Höfer 2011). The development of an effective method for cryopreservation is required for cost-effective long-term storage. An overview of the available cryopreservation methods for *Fragaria* was given by Reed (2008).

The establishment of a backup collection under cryogenic storage can protect against loss and induce some financial benefits by allowing repositories to hold fewer trees or plants in the field per accession. In this paper, we summarize the results achieved with cryopreservation of *Malus* and *Fragaria* and draw conclusions on the possible role of cryopreservation for the management of fruit genetic resources in Germany.

2. Materials and Methods

2.1. Materials

Four apple cultivars and 11 *Malus* wild species accessions were included in the experiment. Scion wood containing the current season's growth was cut from the field trees in Dresden-Pillnitz in January.

For *Fragaria*, 31 well established *in vitro* cultures of 22 cultivars and nine accessions of eight species were selected for the experiments from the genebank in Dresden-Pillnitz.

2.2. Cryopreservation Methods

Dormant buds: For *Malus*, scions were cut into 35 mm long, single node sections. Moisture content of the sections was determined gravimetrically and expressed on a fresh and dry weight basis (g H_2O/g dry matter). The sections were desiccated to 30 % moisture by placing them in a -5 °C cold chamber. When the sections reached the required moisture level they were placed in cryovials (two scioons per 4.5 ml tube, Nunc, Denmark) and cooled using a controlled rate freezer (Kryo 360-3.3, Messer Cryotherm, Germany) at 1°C/ h to -30°C. Following a hold at -30°C for 24 h, the cryovials were quickly transferred to the vapor phase of a liquid nitrogen (LN) cryotank (Biosafe, Messer Cryotherm, Germany) for storage for 2 months. Chip grafting was performed after a 15 day rehydration period at 4°C in moist sand. Bud emergence and growth were evaluated in the orchard in autumn and spring.

In vitro shoot tips: For *Fragaria*, the PVS2-based vitrification protocol of Majathoub (2005) adapted by Höfer (2011) was used. The dissected shoot tips from *in vitro* cultured plants were incubated in loading solution (2 M glycerol + 0.5 M sucrose in MS medium) for 15 min at room temperature. Explants were transferred to 1.8 ml cryovials (Nunc, Denmark), 0.75 ml PVS2 solution (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO), 0.4 M sucrose in MS medium; Sakai *et al.* 1990) were added and the explants were incubated on ice for 2.5 h. Then the cryovials were plunged directly in LN and stored in LN for a minimum of 1 day. The vials were rewarmed by plunging them in sterile water at 40°C for 2 min. The PVS2 solution was removed from the vials and unloading solution (1.2 M sucrose) was added and applied for 20 min. Finally, the shoot tips were transferred to normal proliferation medium in Petri dishes and placed in the growth room for 1 week in the dark, and then transferred to standard light conditions. Assessment of shoot tip recovery was performed 9 weeks after rewarming.

MS medium (Murashige and Skoog, 1962) with 0.44 µM benzylaminopurine, 0.049 µM indole-3-butyric acid, 30 g/l sucrose and 7.5 g/l agar in 200 ml glass jars (40 ml/jar) was used for multiplication. Growth room conditions were 23±1°C with a 16 h light/8 h darkphotoperiod under 60-65 µmol m^{-2} s^{-1} photon flux.

For statistical analysis in *Fragaria*, ANOVA and Duncan's Multiple Range Test ($P \leq 0.05$) using SAS Enterprise Guide 4.2. were applied.

3. Results and Discussion

Dehydration period of scion pieces at -5°C required for them to reach about 30% moisture content (app. 0.4 g H_2O/g dry matter) varied between 2 and 30 days depending on the genotype (data not shown). Furthermore, differences in the dehydration time were also observed between different years. In general, *Malus* wild species accessions required the shortest dehydration time (data unpublished). Data of dehydration time estimated in USDA-ARS National Centre for Genetic Resources Preservation, ranged from 4 to 6 weeks (Forsline *et al.*, 1998), indicating a strong effect of climatic conditions.

Results of our preliminary cryopreservation experiments with dormant apple buds showed successful recovery but also a large genotypic dependence. There was a particularly broad range of responses in the wild species (Table 1). The highest recovery was obtained for cv. Idared with 96 % bud recovery. Genotype dependence and difficulties in using *Malus* wild species were emphasized by Towill *et al.* (2004), who tested 1,915 accessions representing 30 species and 16 interspecific hybrids. For *M. x domestica*, 95 % of the accessions tested were successfully cryopreserved. For species other than *M. x domestica,* 83 % of genotypes met the criterion of a minimum of 40 % viable buds. A comparison of the same species tested in Fort Collins and in our laboratory revealed differences in both directions. For detailed analysis, genotypic screening will be performed. Further modifications of the cryopreservation protocol are necessary to adapt the method to mild winter conditions of Central Europe. The modifications include dehydration treatments, specific cooling conditions and grafting method. Our aim is to establish a routine method using the winter vegetative bud method for a large number of germplasm accessions. The *Malus* gene bank of the Institute for Fruit Breeding maintains 527 accessions of 26 primary species and 20 hybrid species. Applying this cryopreservation method to apple germplasm may markedly increase the efficiency of maintenance and should provide a way to duplicate the material of wild *Malus* species. The 950 apple cultivars of the National German Apple Genebank are conserved in two or more field collections in the network (Flachowsky and Höfer 2010; Höfer 2010).

Table 1. Bud recovery of chip buds of four apple cultivars and 11 different *Malus* wild species accessions after cryopreservation based on 20 buds (one time trial).

Genotype	Recovery (%)
Idared	96
Lord Lambourne	50
Piros	30
Golden Delicious	25
Malus x zumi	55
Malus fusca	50
Malus sylvestris	45
Malus sieboldii	35
Malus baccata	30
Malus sieversii I	25
Malus sieversii II	5
Malus sieversii III	5
Malus floribunda	0
Malus x spectabilis	0
Malus sagentii	0

A total of 31 strawberry cultivars and *Fragaria* wild species accessions selected from the Genebank in Dresden-Pillnitz were cryopreserved using PVS2 vitrification without cold acclimation (Table 2). Significant differences in mean regrowth were observed between cultivars (73 %) and wild species (39 %). Cultivars had good recovery (48 % to 84 %) while species were more variable (3 % to 80 %). The only other study on a range of strawberry accessions was performed at the National Clonal Germplasm Repository, Corvallis, Oregon (Reed and Hummer 1995); it included cold acclimation and controlled rate cooling of 56 accessions. Successful cryopreservation protocols for individual strawberry cultivars were reported using modifications of PVS2 vitrification (Niino *et al.* 2003, Pinker *et al*. 2009), and

encapsulation dehydration (Clavero-Ramirez *et al.* 2005, Hirai *et al*. 1998). We also tested four distinct cryopreservation protocols for strawberry: two PVS2 protocols, encapsulation-dehydration and controlled rate cooling (Höfer and Reed 2011). The PVS2 vitrification method with a 14 day cold acclimation (16 h dark at -1°C/8 h light at 22°C) (Luo and Reed 1997) gave significantly better regrowth compared with the other protocols.

There will be no further duplication of the two existing collections in the National German Strawberry Genebank because of high operating expenses for field collections and budget limitations. The PVS2 vitrification method with cold acclimation will be applied as the standard method for most of the 341 strawberry cultivars belonging to the National German Strawberry Genebank and for the 310 accessions of *Fragaria* wild species of the genebank collection in Dresden-Pillnitz. Twenty eight genotypes are already stored under cryopreservation.

The aim for the future is to develop an effective genetic resource conservation strategy for *Malus* and *Fragaria* in Germany and to transfer this management plan to the cultivars of the National German Fruit Genebank and the accessions of *Malus* and *Fragaria* wild species held at the Institute for Breeding Research on Horticultural and Fruit Crops Dresden-Pillnitz.

Table 2: Mean recovery (%) after cryopreservation using PVS2 vitrification without cold acclimation for a range of *Fragaria* genotypes (n = 20-100 explants), acc. to Höfer and Reed (2011). Means with the same letters are not significantly different P ≤ 0.05.

Recovery (%)			
Strawberry cvs.		Wild species acc.	
Herzbergs Triumph	84 ± 12	*F. nilgerrensis (81)*	80 ± 28
Anneliese (neu)	84 ± 13	*F. corymbosa 28*	68 ± 19
Aurora	82 ± 17	*F. mosch (62-11)*	61 ± 15
Carolina Superba	82 ± 13	*F. nilgerrensis (78)*	58 ± 22
Aprikose	81 ± 21	*F. gracilis (33)*	23 ± 18
Confitura	80 ± 20	*F. chiloensis (10)*	22 ± 15
Astino	79	*F. iinumae (39)*	21 ± 29
Dresden	79 ± 19	*F. vesca (131)*	19
Cambridge Late Pine	76 ± 18	*F. moupinensis (76)*	3 ± 5
Cambridge Early Pine	75		
Raveno	75 ±12		
Weiße Ananas	73 ± 21		
Deutsch Evern Solveta	73 ± 18		
Höltges Rheingauperle	72 ± 23		
Cambridge Favourite	70 ± 25		
Aphrodite	70 ± 15		
Hansa	69 ± 12		
Panther A	67 ± 13		
Merton Dawn	66 ± 25		
Sparkle	60		
Dana	55		
Georg Soltwedel	48 ± 22		
Average cvs.	73a	**Average wild species**	39b

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Cryopreservation of *Populus* species by PVS2 vitrification: influence of explant type, PVS2 treatment time, and preculture/pretreatment on shoot regeneration after rewarming

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1. Introduction

The different protocols available for cryopreservation of forest tree germplasm of deciduous trees have been reviewed by Häggman *et al.* (2008). Cryopreservation by PVS2 vitrification technique (Sakai *et al.* 1990) is a widely applied method for *in vitro* plant material of herbaceous as well as woody plants. Vitrification and the two derived protocols, encapsulation-vitrification and droplet-vitrification have been applied to different tissues of over 100 plant species (Sakai & Engelmann 2007) including several hardwood tree species (Panis and Lambardi 2006). An advantage in comparison to slow freezing or encapsulation-dehydration techniques is that no sophisticated devices or skills are needed so that this technique can be used in a normally equipped tissue culture laboratory. Until today, cyopreservation has been applied to the genus *Populus* only by a few working groups. Lambardi *et al*. (2000) and Lambardi (2002) reported the successful cryopreservation of *in vitro*-grown shoot tips of *Populus alba* and *Populus canescens* by one-step vitrification. In an experiment with axillary and apical buds from *in vitro*-grown shoots of three hybrid aspen clones (*Populus tremula* x *Populus tremuloides*) cryopreserved with the slightly modified vitrification method of Lambardi *et al.* (2000) regrowth of 3 to 75 % was achieved (Jokipii *et al*. 2004). In the laboratory of NW-FVA the vitrification technique was adapted and applied successfully to a range of forest tree species as *Fraxinus excelsior* (Schoenweiss *et al*. 2005) and – in the frame of the COST Action 871 – *Betula* spp. (Meier-Dinkel 2007), *Prunus avium* and *Populus tremula* (Meier-Dinkel 2010).

In this paper, the application to further *Populus* species and attempts to simplify the PVS2 protocol are reported. This protocol comprises range of steps from cold hardening of shoot cultures over dissection, preculture and re-treatment of explants to the final PVS2 application which were modified in order to save time and work.

The standard explants used here for cryopreservation are apical shoot tips. Since one *in vitro* shoot gives only one shoot tip explant, a high amount of shoots have to be produced before a clone can be cryopreserved with the standard number of 70 explants. So far, unused explants with a theoretical capacity for regeneration are quiescent axillary buds of microshoots. Since one shoot can yield up to 10 axillary buds, less plant material has to be propagated before a clone is ready for cryopreservation. Furthermore, axillary buds are easier and faster to dissect than apical shoot tips. The use of axillary buds could save time and work and was therefore tested for cryopreservation of *Populus* material.

2. Materials and Methods

2.1. Plant Material

The plant material used consisted of three new *Populus* species and hybrids, respectively: grey poplar (*Populus* x *canescens*), a natural hybrid between *Populus alba* and *Populus*

tremula, a grey polar backcross (*Populus* x *canescens* x *P. tremula*) and Euphrates poplar (*Populus euphratica*). Since the shoot regeneration with aspen (*P. tremula*) was generally high in several former experiments with a range of genotypes, an experiment to simplify the standard cryo-protocol for aspen was performed. Further modifications of the method were tested with grey poplar.

2.2. Cryopreservation protocol

The standard PVS2 cryopreservation protocol comprises the following steps:

1. Cold hardening of shoot cultures (begin 2 to 3 weeks after the last subculture, duration 3 weeks, 8 h light 37 $\mu Em^{-2}s^{-1}$ at 20°C, 16 h dark at 3°C)
2. Dissection of shoot tip explants (size 2 mm)
3. Preculture of the explants on WPM (Lloyd and McCown 1980) containing 0.8 M glycerol at 4°C for 3 days
4. Pretreatment (loading) of the explants in liquid WPM containing 2 M glycerol and 0.4 M sucrose for 25 min at room temperature
5. PVS2 treatment of the explants with PVS2 solution (w/v 30 % glycerol, 15 % ethylene glycol, 15 % DMSO) for 0.5 to 3 h on ice
6. Freezing and storage of cryovials in liquid nitrogen

The standard rewarming and recovery protocol is based on rapid rewarming:

1. Cryovials are placed in a 42°C water-bath for 1 min, followed by 20°C water for 10 s; then immediate replacement of PVS2 with liquid WPM containing 1.2 M sucrose, three rinses, last rinse for 3 min
2. For recovery the explants are placed on micropropagation medium according to the species and kept 2 days in the dark and then at 80 $\mu Em^{-2}s^{-1}$ (16 h light / 8h dark at 24 / 21°C)

Survival and regeneration of the explants was assessed after 2, 4 and 6 weeks.

2.3. Application to further Populus species

The first part of the experiments focused on the application of our standard protocol to further *Populus* species. With other tree species, survival and shoot regeneration could be improved considerably by determining the optimal duration of the PVS2 treatment. In order to adapt the protocol to two clones of a grey poplar backcross and one grey poplar clone PVS2 treatment times of 1, 2, 3 and 4 h were tested.

With Euphrates poplar, the standard protocol was tested using four PVS2 application times (1, 2, 3 and 4 h). In a second experiment, two explants types, apical shoot tips and axillary buds were compared applying 2, 3 and 4 h PVS2 treatment times.

2.4. Simplification of the standard protocol

Experiments aiming at simplifying the cryopreservation protocol were carried out with grey poplar (backcross) and aspen. The standard explants, which show up to 100 % shoot regeneration after cryopreservation are apical shoot tips. The aim was to identify explants which were easier and faster to dissect and/or available in higher quantity from *in vitro* shoot cultures. In an experiment with grey poplar backcrosses, three different explants types were compared to the standard 2 mm shoot tips: smaller (ca. 1 mm) and bigger (ca. 3 mm) shoot tips and axillary buds from microshoots were cryopreserved after 4 h PVS2 treatment. With aspen, three different explant types from *in vitro* shoot cultures were compared: apical shoot tips of 2 mm length from which most of the young leaves were removed, complete apical shoot tips of 2 mm length, and short apical shoot tips of 1 mm length.

Some modifications of the standard protocol aiming at its simplification were carried out with aspen. Shoot tip explants were cryopreserved a) without preculture for 3 days on WPM

containing 0.8 M glycerol at 4°C; b) without pretreatment with liquid WPM containing 2 M glycerol and 0.4 M sucrose for 25 min at room temperature; and c) with a reduced pretreatment time of 10 min with liquid WPM containing 2 M glycerol and 0.4 M sucrose.
Each treatment was carried out with 8 to 12 explants per clone. The experiments were not repeated. The presented results are therefore preliminary and have to be confirmed in further experiments.

3. Results and Discussion

3.1. Application to further Populus species

With two genotypes of a grey poplar backcross the standard protocol with PVS2 application times of 1, 2, 3 and 4 h resulted in shoot regeneration in all tested treatments. Six weeks after rewarming, shoot formation increased from 50 and 63 %, respectively, after 1 h PVS2 to 100 % after 4 h for the two clones (Fig. 1 and Fig. 2). Most shoots looked healthy and vigorous, and only a few shoots showed some signs of hyperhydricity (Fig. 3). For white poplar shoot tip explants exposed to PVS2 for 1 h on ice, Lambardi *et al*. (2000) reported similar shoot formation after 7 weeks from surviving shoot tips, varying from 47 to 62 % depending on the sucrose concentration in the preculture medium. However, exposure to PVS2 for more than 1 h led to injuries of the shoot tips after rewarming. The two grey poplar backcrosses investigated here were less sensitive to PVS2, showing 100 % survival and 100 % shoot formation after 4 h PVS2 treatment.

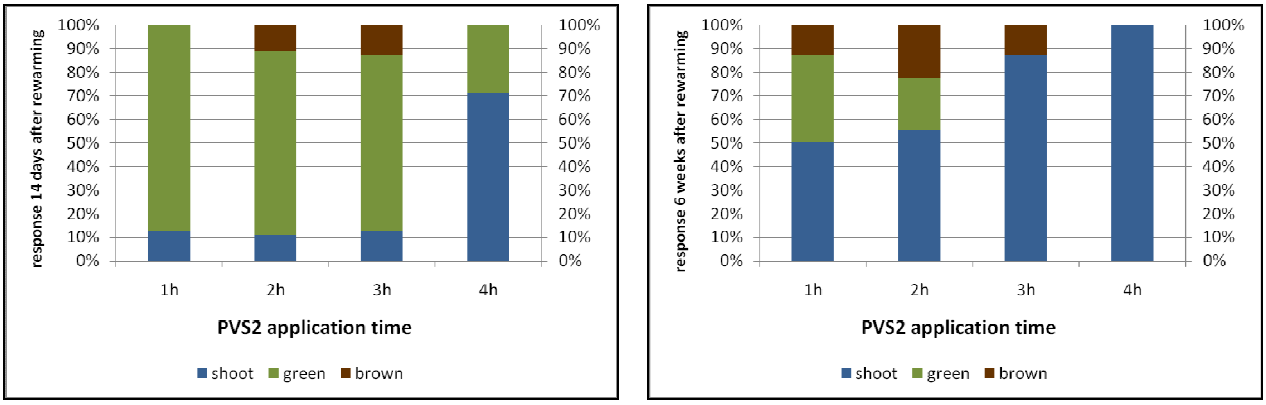


Figure 1. Effect of PVS2 application time on survival and shoot regeneration (%) of the grey poplar backcross (*Populus* x *canescens* x *P. tremula*) NW9-741 2 and 6 weeks after rewarming.

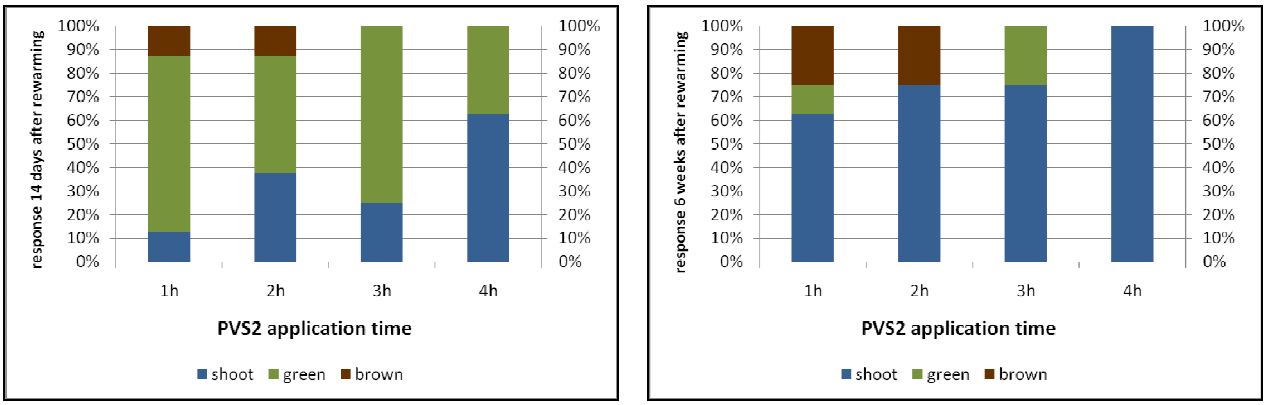


Figure 2. Effect of PVS2 application time on survival and shoot regeneration (%) of the grey poplar backcross (*Populus* x *canescens* x *P. tremula*) NW9-743 2 and 6 weeks after rewarming.

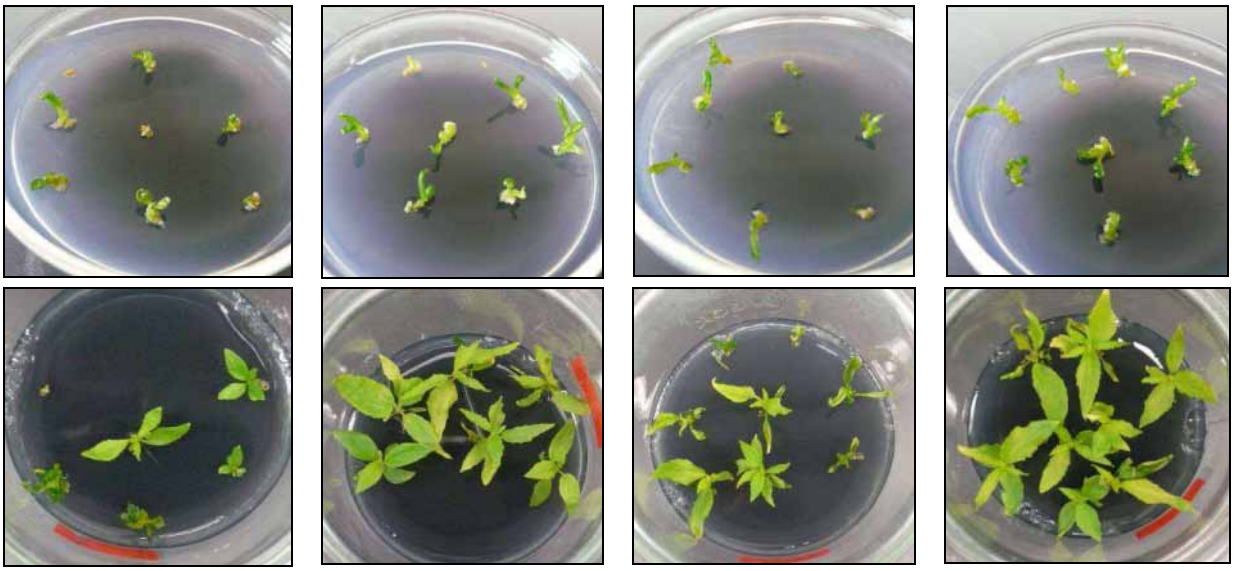


Figure 3. Effect of PVS2 application time on survival and shoot regeneration (%) of shoot tip explants of the grey poplar backcross (*Populus* x *canescens* x *P. tremula*) NW9-743 2 (top) and 6 weeks (bottom) after rewarming.

With the tested grey poplar clone no shoot regeneration could be achieved until six weeks after rewarming. The survival rate of the explants varied from 33 to 63 % with no clear effect of the PVS2 treatment time. The remaining explants were dead. Lambardi (2002) reported 54 % survival of shoot tips of grey poplar treated with PVS2 for 60 min at 0 °C. Shoot formation was not described.

The application of the standard protocol to Euphrates poplar with PVS2 treatment times of 1, 2, 3 and 4 h resulted in high survival of shoot tip explants, between 88 and 100 % 6 weeks after rewarming. The explants showed limited growth and some leaf-like structures. However, no complete shoots were regenerated. Axillary buds used in a second experiment treated with PVS2 for 2, 3 and 4 h were all dead after 4 weeks. There are no data available from the literature on cryopreservation of Euphrates poplar.

3.2. Simplification of the standard protocol

Four different explant types tested with two genotypes of a grey poplar backcross led to distinct regeneration results. Axillary buds proved to be unsuitable, with a low shoot regeneration percentage of 18 % with one clone and no shoot formation at all with the second clone (Fig. 4), although survival after 4 weeks was high with clone NW9-741 (Fig. 5). The short shoot tips showed a shoot regeneration of 88 and 50 %, depending on the clone. The shoot regeneration of standard shoot tips and long shoot tips was 100 % for genotype NW9-741 and 75 and 80 % for NW9-743 (Fig. 4). Over all explants, clone NW9-741 was less sensitive to cryopreservation-induced stress than NW9-743. However, a common tendency of both genotypes was that bigger explants resulted in better shoot formation than smaller ones. As in the experiment on PVS2 application time, grey poplar was more sensitive to cryopreservation. However, differences between explants were similar to the grey poplar backcrosses. Axillary buds were all dead after 4 weeks, whereas long shoot tips showed 75 % shoot formation (Fig. 6). Standard shoot tips and small shoot tips survived to a high proportion with only little shoot formation of the short shoot tips.

In *Vitis vinifera*, axillary buds of four- and five-month-old *in vitro*-grown plantlets were used as explants source for cryopreservation (Matsumoto and Sakai 2003). Axillary shoot tips, about 1 mm in length, consisting of the apical dome and a few primordial leaves, were

excised from the buds, precultured, pretreated and dehydrated with PVS2 for 80 min at 0°C. Shoot formation after 2 months was 65 % of the total number of shoot tips. In the case of grey poplar and the backcrosses, the cryopreserved complete axillary buds were much younger and less developed, as they were excised from *in vitro* shoots 4 to 5 weeks after the last subculture. This might be the reason for low survival and recovery of this explants type. The unpeeled axillary (and apical) buds of hybrid aspen used by Jokipii *et al*. (2004) were 2 to 4 mm long and thus much bigger and not comparable to the grey poplar axillary buds. The 'axillary buds' of white poplar used by Lambardi *et al.* (2000) as well as Tsvetkov *et al*. (2009) were no longer in a quiescent state but already flushing, so that growing shoot tips of 1 to 2 mm in length consisting of the apical meristem and some leaflets were excised.

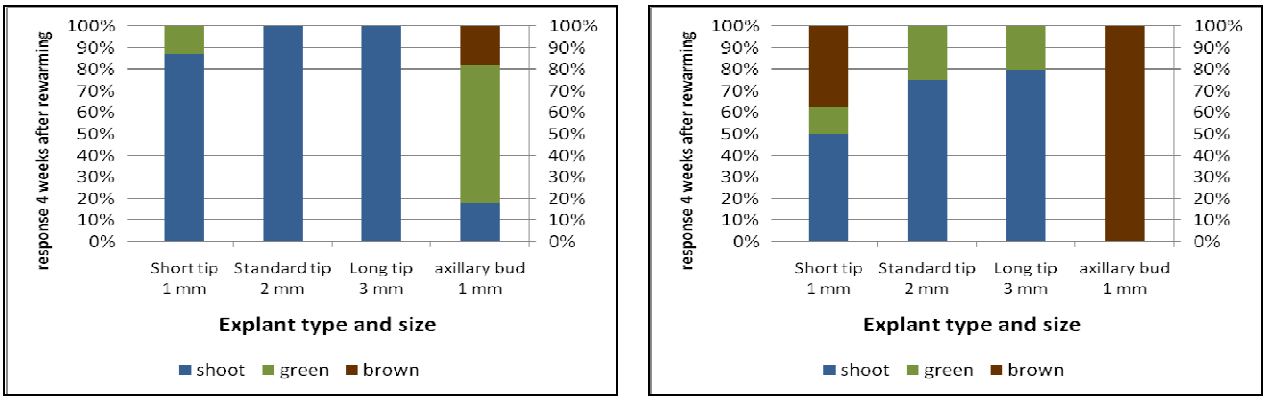
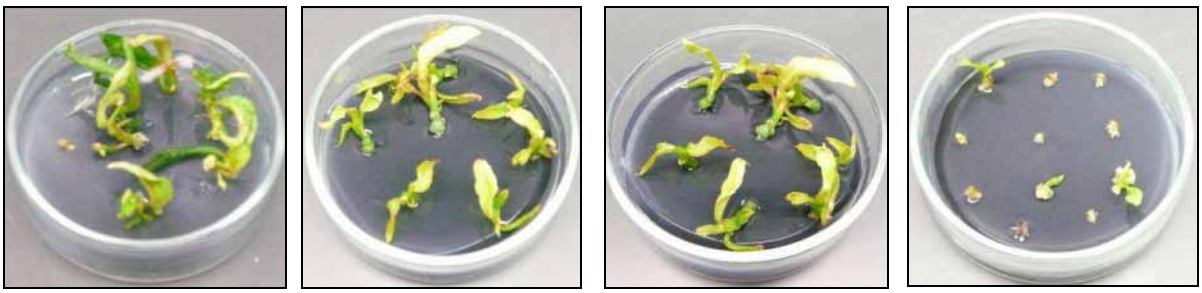


Figure 4. Effect of explant type and size on survival and shoot regeneration (%) of the grey poplar backcross (*Populus* x *canescens* x *P. tremula*) NW9-741 (left) and NW9-743 (right) 4 weeks after rewarming.



Short shoot tips 1mm | Standard shoot tips 2mm | Long shoot tips 3 mm | Axillary buds 1mm

Figure 5. Effect of explant type and size on survival and shoot regeneration of the grey poplar backcross (*Populus* x *canescens* x *P. tremula*) NW9-741 4 weeks after rewarming.

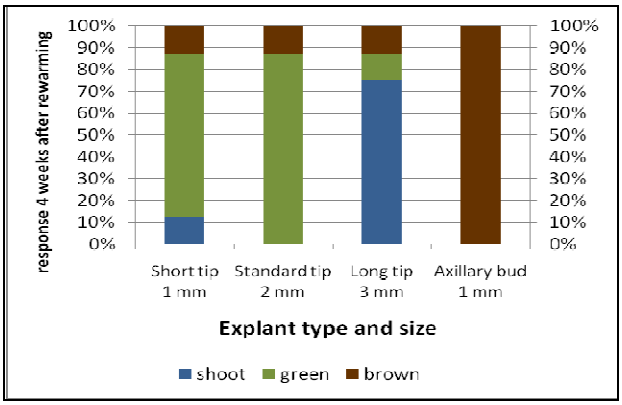


Figure 6. Effect of explant type and size on survival and shoot regeneration (%) of the grey poplar (*Populus* x *canescens*) NW9-744 4 weeks after rewarming.

With aspen, three different explant types from *in vitro* shoot cultures (apical shoot tips with most of the young leaves removed of 2 mm length, complete apical shoot tips of 2 mm length, and short apical shoot tips of 1 mm length) were cryopreserved according to the standard protocol. Shoot regeneration of all explants was 100 % after 6 weeks. The results show that: a) the size of the shoot tips up to 2 mm does not influence the shoot regeneration; and b) a time-consuming dissection of the shoot tips is not necessary. Lower regrowth of 3, 25 and 75 % after 30 min PVS2 incubation at 0°C was obtained with three hybrid aspen clones using comparatively large explants, 2 to 4 mm long unpeeled axillary and apical 'buds' from *in vitro* grown shoots (Jokipii *et al.* 2004). Shoot regeneration of standard shoot tips from 15 clones of aspen and hybrid aspen ranged from 50 to 100 % after 6 weeks (Meier-Dinkel 2010).

Three modifications of the standard protocol with the aim to make it simpler where compared to the standard protocol as control. Shoot tip explants were cryopreserved a) without preculture; b) without pre-treatment; and c) with a reduced pretreatment time of 10 min. Shoot formation of all simplified modifications 14 days after rewarming was as fast as of the control (Fig. 8, at the top). Shoot regeneration after 42 days of a) without preculture was 90 %. The control as well as the treatments b) and c) without and with reduced pretreatment time showed 100 % shoot formation after 6 weeks (Fig. 7). These first results show that a simplification of the standard cryo-protocol for aspen is possible without loss of regeneration capacity.

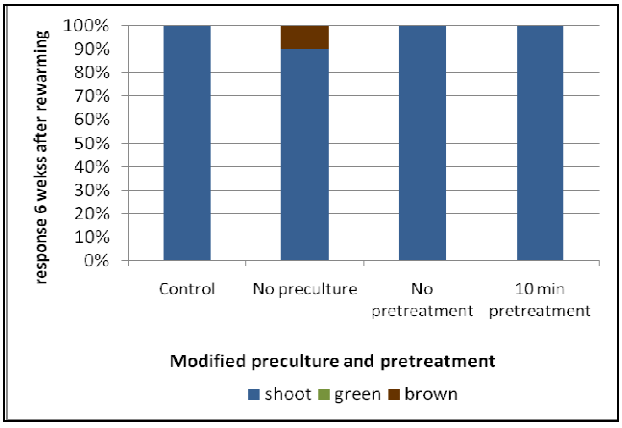
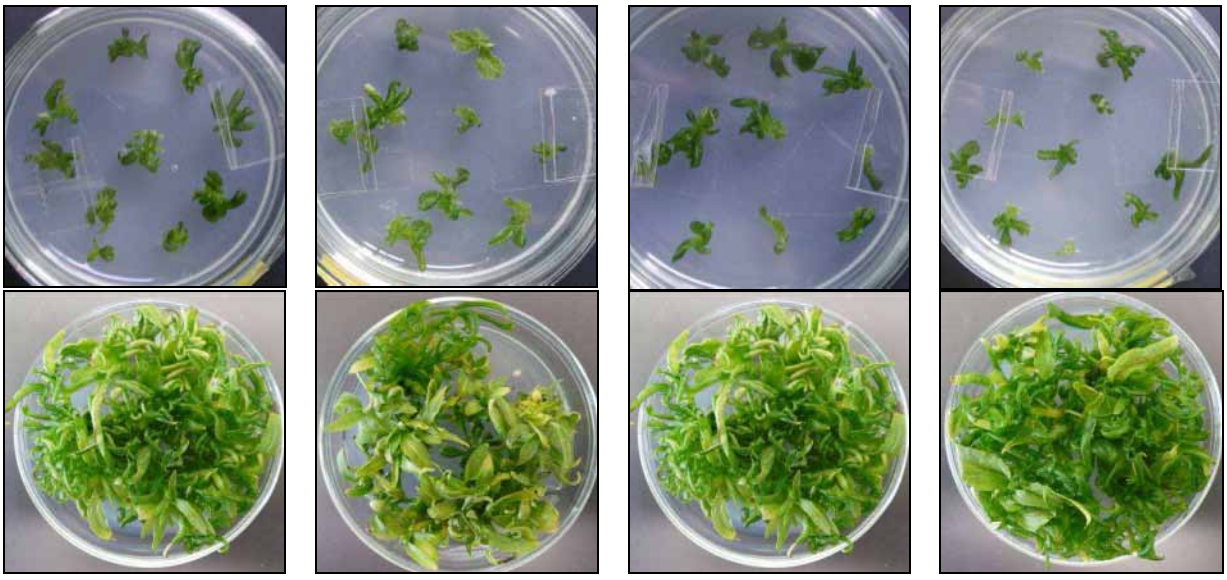


Figure 7. (left). Effect of simplification of the standard protocol on survival and shoot regeneration (%) of shoot tip explants of aspen (*Populus tremula*) 2 and 6 weeks after rewarming.

Figure 8. (below). Effect of simplification of the standard protocol on survival and shoot regeneration of shoot tip explants of aspen (*Populus tremula*) 2 (top) and 6 weeks (bottom) after rewarming.



Control (standard) | Without preculture | 10 min pretreatment | Without pretreatment

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Comparison of different PVS2-based procedures for cryopreservation of *Thymus* spp.

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1. Introduction

Thyme (*Thymus* spp.) is an important genus of the Lamiaceae family, covering more than 400 perennial species of aromatic and medicinal plants (Morales 2002), all being native to Mediterranean region (Loziene *et al*. 2007). Most of them are resistant to extreme conditions, with dense and tomentous hairs on acicular leaves and stems, allowing adaptation to dry and hot regions. Production of volatile secondary metabolites, which evaporate and produce a saturated atmosphere around the plant, contributes to its adaptation by limiting the water loss (Morales 2002).

Use of *Thymus* spp. dates back to ancient Egyptians, and still today they are used extensively all around the world as a natural source of phenolic oils, oleoresins, fresh and dried herbs, both for medicinal and non-medicinal (i.e., in cosmetic and alimentary industries, as well as for ornamental use) purposes (Lawrence and Tucker 2002). However, natural *Thymus* populations are unfortunately far from being adequate to support such a great and even growing demand for its products. Uncontrolled harvesting and destruction of forest land (*Thymus* spp. grow under forest cover, on mountainous and rocky lands, where the temperature of soil is relatively high, Rao *et al*. 2004) for agriculture or urbanization are also contributing to thyme genetic erosion (Marco-Medina *et al*. 2010).

In this sense, cryopreservation provides a unique alternative for preservation of such valuable germplasm, making available the access to a wide range of genetic diversity to be used as possible source of natural products. Present study is focused on development of cryopreservation procedures for *Thymus* spp. shoot tips, comparing three different PVS2-based methods, namely PVS2 vitrification, encapsulation-vitrification and droplet-vitrification, followed by direct immersion in liquid nitrogen (LN). Results have been evaluated in terms of shoot tip regeneration and their capacity to produce multiple shoots.

2. Materials and Methods

2.1. Plant Material

In vitro shoot cultures of *Thymus vulgaris* L. were established by transferring shoot tips of *in vitro*-germinated seedlings on semi-solid MS medium (Murashige and Skoog 1962), supplemented with 1 mg l^{-1} kinetin (Kin) and 0.3 mg l^{-1} gibberellic acid (GA_3) (regeneration medium), and maintained *in vitro* under standard culture conditions (i.e., 23±2 °C, 16 h photoperiod, provided by cool daylight fluorescent lamps, at 36 µmol m^{-2} s^{-1}) by periodically subculturing in 4-week intervals (Ozudogru *et al*. 2011). Shoot tips (1-1.5 cm long) excised from *in vitro* shoot cultures were utilized in cryopreservation trials. All semi-solid media used in the study were supplemented with 30 g l^{-1} sucrose and gelled with 3 g l^{-1} gelrite. The pH was adjusted to 5.8 prior to the inclusion of gelrite and autoclaved for 20 min at 121 °C.

2.2. Long-term conservation of shoot tips by PVS2 vitrification method

Prior to cryopreservation, shoot cultures were cold-hardened for 2 weeks at 4 °C in darkness, and excised shoot tips were precultured on semi-solid MS medium, supplemented with 0.25 M sucrose, for 48 h, at 4 °C in darkness. Excised shoot tips (1.5 mm, on average) were transferred to 2-ml Nalgene® cryovials (15 explant per cryovial) and incubated with a loading solution (LS; 2.0 M glycerol and 0.4 M sucrose; Matsumoto *et al*. 1994) for 30 min at 25 °C. LS was then discarded and cryovials were loaded with PVS2 (30 % glycerol, 15 % ethylene glycol and 15 % DMSO, all w/v, in MS medium supplemented with 0.4 M sucrose; Sakai *et al*. 1990), where shoot tips were treated with the solution for 15, 30, 45, 60, 75, 90, 105 or 120 min at 0 °C. Following PVS2 treatment, group of explants (LN +) was suspended in 0.6 ml of fresh PVS2 and rapidly immersed in LN. After at least 1 h of storage, they were thawed in a water bath at 40 °C for 2 min and washed in a washing solution (liquid MS medium, containing 1.2 M sucrose, Sakai *et al*. 1990) for 20 min at 25 °C before placing on regeneration medium. Second group (control group, LN -) was washed immediately after PVS2 treatment and plated on medium as described above.

2.3. Long-term conservation of shoot tips by encapsulation-vitrification method

Synthetic seeds were obtained by encapsulating shoot tips in 3 % Na-alginate solution and 100 mM $CaCl_2$ solution. They were then transferred to 2-ml Nalgene® cryovials (five synthetic seeds per cryovial) and incubated with LS for 30 min at 25 °C. Subsequent steps of the method were performed as described for PVS2 vitrification method.

2.4. Long-term conservation of shoot tips by droplet-freezing method

Sterile aluminium foil strips (~ 5 x 15 mm) were placed in an open Petri dish, resting on a frozen cooling element (temperature around 0 °C), and 3 drops of 4-5 µl PVS2 were dropped on each aluminium foil strip. Shoot tips were placed into PVS2 drops, each drop containing one explant, and treated with the solution for 15, 30, 45, 60, 75, 90, 105 or 120 min. After PVS2 treatment, the aluminium foils were transferred into chilled 2-ml Nalgene® cryovials (one aluminium foil per cryovial) and directly plunged into LN. Thawing was done at room temperature by immersing aluminium foils in washing solution. When the explants were totally melted, they were transferred on medium and evaluated for regeneration. A group of samples, treated with PVS2 but not frozen in LN (control group, LN -), were washed accordingly and plated on medium for regeneration, as well.

2.5. Data collection and statistical analysis

Two replications of 30 shoot tips were used for each treatment and each experiment was repeated twice. Following PVS2 incubation (followed or not by freezing in LN), data of post-thaw survival rate (i.e., percent of viable shoot tips, related to number of originally introduced explants) was recorded 2 weeks after being plated on regeneration medium, while regeneration rate (i.e., percent of regenerating shoot tips, related to the number of surviving explants), mean shoot number (no ± S.E.) and mean shoot length (cm ± S.E.) were recorded 4 weeks after being plated on medium. At this time, the Shoot Forming Capacity (SFC) Index (= average no. of shoots per regenerating explant) x (% of regenerating explant) / 100; Lambardi *et al*. 1993) was calculated as well. Statistical analysis of percentages was carried out by the X^2 test or by Post Hoc Multiple Comparisons test (Marascuilo and McSweeney 1977). Discrete data were subjected to ANOVA, followed by the least significant difference (LSD) test at $P \leq 0.05$ to compare means.

3. Results

3.1. Cryopreservation by encapsulation-vitrification method

When shoot tips were encapsulated and directly plated on medium (LN -), only 23.3 % of survival and recovery rates were achieved, with an SFC Index of 6.1 (Table 1). In previous trials, the regrowth rate of naked shoot tips at standard culture conditions was 96.7 % and SFC Index was 8.3 (Ozudogru *et al*. 2011), indicating a strong detrimental effect of encapsulation on thyme shoot tip regeneration. Differently, the mean shoot number of encapsulated shoot tips was much higher than that of naked shoot tips in standard culture conditions (26.4 vs. 8.6). When encapsulated shoot tips were treated with PVS2 and not frozen, shoot tip survival and regeneration rates were decreased even further, 3.3 % being the maximum regeneration rate (following 120 min-PVS2 treatment). Similarly, maximum post-thaw regeneration rate, obtained following 105 min PVS2 treatment and direct immersion in LN was also 3.3 %, providing SFC Index minor than 0.1 and single shoots of 0.3 cm, on average.

3.2. Cryopreservation by PVS2 vitrification method

Survival rates of 49.1-75.0 % and regeneration rates of 47.4-71.7 % were obtained when shoot tips were treated with PVS2 solution for up to 90 min (Table 2). Those explants that could tolerate the toxic effects of PVS2 solution possessed significantly high mean shoot numbers (ranging between 14.8 to 30.1 shoots). However, when shoot tips were immersed in LN following PVS2 treatment, maximum post-thaw survival and regeneration rates were only 24.5 % (15-min PVS2 treatment), although SFC index remained high (11.9) due to the very high mean shoot number (22.2) achieved. PVS2 applications longer than 15 min resulted with a decline in their survival and post-thaw regeneration rates. However, mean shoot number of the recovered shoot tips remained high also after PVS2 incubation up to 75 min and storage in LN.

3.3. Cryopreservation by droplet-vitrification method

Similar to the vitrification method, also shoot tips treated with PVS2 treatment up to 90 min in droplets (LN -) seemed to tolerate well the possible toxic effects of the solution, providing even better survival and regeneration rates, and reaching 90.7 % shoot tip regeneration following 90-min treatment (Table 3). The mean shoot number obtained by this method, and thus the SFC Index, were less than what was observed with the PVS2 vitrification method. The only result comparable to PVS2 vitrification method was the 20 shoots per explants obtained after 90-min PVS2 treatment, providing an SFC Index of 18.4. It is worth mentioning that PVS2 incubations longer than 90 min have been very toxic also when applied in droplets, regardless of being immersed or not in LN. When samples were treated with PVS2 up to 75 min and immersed in LN, majority of them showed low regeneration rates. However, 90-min treatment permitted 85 % survival and 80 % post-thaw regeneration rates, providing also more than 15 shoots per explant and 12.6 SFC Index.

4. Discussion

Cryopreservation enables the maintenance of the plant material for theoretically unlimited periods of time, once the protocols are optimized. Several reports are available in literature, dealing with cryopreservation of *Lavandula* and *Mentha* species, both belonging to Lamiaceae family. As regards *Lavandula* spp., the only report available deals with cryopreservation of cell suspension cultures (Tanaka and Takahashi 1995). *Mentha* spp., however, have been the object of numerous cryopreservation studies. In 1990, Towill

cryopreserved shoot tips of *Mentha aquatica* x *M. spicata* by vitrification method. Vitrification solution used in that study contained 15% ethylene glycol (w/v), 10 % polyethylene glycol-8000 and 1 M DMSO. Nine years after, Hirai and Sakai (1999) encapsulated *in vitro*-grown nodal buds of *Mentha spicata* and successfully cryopreserved them by vitrification method. Possible toxic effects of PVS2 and potential damages that may occur during vitrification were also studied by using *Mentha* spp. (Towell and Bonnart 2003; Volk and Walters 2006). Senula *et al*. (2007) were the first using droplet-vitrification method for the cryopreservation of *Mentha* spp. and they obtained 89 % post-thaw regeneration rate. In 2008, Uchendu and Reed compared slow cooling, encapsulation-dehydration and vitrification techniques to cryopreserve *M. x piperita* subsp. *citrata*, *M. canadensis*, *M. mistralis* and *M. cunninghamii*. The authors obtained 93 % post-thaw regeneration by slow cooling method, 73 % by vitrification, and 71 % by encapsulation-dehydration method. As for *Thymus*, the only report available in literature dealed with droplet-vitrification of *T. moroderi in vitro*-grown shoot tips (Marco-Medina *et al*. 2010). The authors tested PVS2 solution for 30, 60, 90, 120 and 180 min at around 0°C, and they obtained the highest post-thaw survival rate (around 60 %) following 30-min PVS2 treatment. The droplet-vitrification showed to be the best cryopreservation method also for the conservation of *T. vulgaris*. Indeed, in the present study, working with *T. vulgaris in vitro* grown shoot tips, 85 % survival and 80 % regeneration rate following 90 min PVS2 treatment and immersion in LN were achieved. The difference with the results obtained in *T. moroderi* can be attributed, not only to the use of different species, but also to the application of cold hardening and sucrose preculture preceding PVS2 incubation, probably providing a better conditioning of the *Thymus* material before immersing in LN. Optimized protocol of droplet-vitrification is currently being tested, and already providing promising results, by using shoot tips of *T. longicaulis* and *T. cariensis*, latter being an endemic and endangered species of Turkey.

5. Acknowledgements

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6. Tables

Table 1. Cryopreservation of thyme shoot tips by encapsulation-vitrification method.

PVS2 Exposure (min)	Survival Rate (%)	Recovery Rate (%)	Mean Shoot Number (n° ± S.E.)	Mean Shoot Length (cm ± S.E.)	SFC Index
LN -					
0	23.3 a	23.3 a	26.4 ± 7.4 a	0.8 ± 0.1 a	6.1
15	1.7 c	1.7 c	6.0 ± 0.0 b	0.6 ± 0.0 a	0.1
30	0.0 c	0.0 c	-	-	-
45	0.0 c	0.0 c	-	-	-
60	0.0 c	0.0 c	-	-	-
75	5.0 b	3.3 b	2.5 ± 1.5 b	0.9 ± 0.2 a	0.1
90	1.7 c	1.7 c	1.0 ± 0.0 b	0.3 ± 0.0 a	< 0.1
105	1.7 c	1.7 c	2.0 ± 0.0 b	0.5 ± 0.0 a	< 0.1
120	3.3 bc	3.3 b	2.5 ± 0.5 b	0.4 ± 0.1 a	0.1
LN +					
0	0.0 c	0.0 b	-	-	-
15	0.0 c	0.0 b	-	-	-
30	0.0 c	0.0 b	-	-	-
45	0.0 c	0.0 b	-	-	-
60	1.7 c	0.0 b	-	-	-
75	0.0 c	0.0 b	-	-	-
90	0.0 c	0.0 b	-	-	-
105	11.7 a	3.3 a	1.0 ± 0.0	0.3 ± 0.0	< 0.1
120	5.0 b	0.0 b	-	-	-

Table 2. Cryopreservation of thyme shoot tips by vitrification method.

PVS2 Exposure (min)	Survival Rate (%)	Recovery Rate (%)	Mean Shoot Number (no ± S.E.)	Mean Shoot Length (cm ± S.E.)	SFC Index
LN -					
15	55.6 b	53.7 b	22.2 ± 3.8 ab	1.0 ± 0.1 a	11.9
30	53.7 b	51.9 b	30.1 ± 3.9 a	1.0 ± 0.0 a	15.6
45	49.1 bc	49.1 c	17.8 ± 3.2 ab	0.9 ± 0.1 ab	8.7
60	71.7 a	71.7 a	18.3 ± 3.0 ab	0.8 ± 0.1 b	13.1
75	75.0 a	75.0 a	16.6 ± 2.7 ab	0.8 ± 0.0 b	12.7
90	50.9 b	47.4 c	14.8 ± 2.5 ab	1.0 ± 0.1 a	7.0
105	1.9 d	1.9 d	1.0 ± 0.0 b	0.3 ± 0.0 b	< 0.1
120	10.5 c	8.8 d	6.8 ± 4.9 b	0.6 ± 0.1 b	0.6
LN +					
15	24.5 a	24.5 a	15.3 ± 5.2 a	0.7 ± 0.1 ab	3.7
30	20.4 a	20.4 a	13.5 ± 5.3 a	0.5 ± 0.0 ab	2.7
45	5.3 b	5.3 b	18.7 ± 9.0 a	0.7 ± 0.1 a	1.0
60	18.3 a	18.3 a	11.2 ± 5.7 a	0.4 ± 0.0 b	2.0
75	3.3 b	3.3 b	14.5 ± 13.5 a	0.5 ± 0.2 ab	0.5
90	3.3 b	1.7 b	4.0 ± 0.0 a	0.8 ± 0.0 a	0.1
105	1.8 b	1.8 b	5.0 ± 0.0 a	0.4 ± 0.0 b	0.1
120	1.8 b	1.8 b	7.0 ± 0.0 a	0.5 ± 0.0 ab	0.1

Table 3. Cryopreservation of thyme shoot tips by droplet-vitrification method.

PVS2 Exposure (min)	Survival Rate (%)	Recovery Rate (%)	Mean Shoot Number (no ± S.E.)	Mean Shoot Length (cm ± S.E.)	SFC Index
LN -					
15	73.3 b	76.7 b	4.8 ± 0.8 b	0.7 ± 0.1 ab	3.7
30	70.0 b	80.0 b	4.8 ± 0.4 b	0.8 ± 0.1 a	3.8
45	66.7 bc	66.7 c	2.4 ± 0.2 b	0.4 ± 0.0 b	1.6
60	50.0 bc	66.7 c	2.7 ± 0.3 b	0.5 ± 0.0 b	1.8
75	70.0 b	70.0 bc	2.7 ± 0.2 b	0.4 ± 0.0 b	1.9
90	88.9 a	90.7 a	20.3 ± 2.7 a	0.9 ± 0.0 a	18.4
105	26.7 c	46.7 c	3.3 ± 0.4 b	0.7 ± 0.0 ab	1.5
120	1.7 d	0.0 d	-	-	-
LN +					
15	13.0 c	7.4 c	2.5 ± 0.6 c	0.4 ± 0.0 b	0.2
30	11.7 c	5.0 c	5.0 ± 4.0 bc	0.6 ± 0.2 a	0.2
45	17.6 bc	9.8 b	6.0 ± 1.7 b	0.6 ± 0.0 ab	0.6
60	25.9 b	11.1 b	6.0 ± 1.4 b	0.7 ± 0.1 a	0.7
75	16.7 bc	10.0 b	13.3 ± 4.4 a	0.7 ± 0.1 a	1.3
90	85.0 a	80.0 a	15.8 ± 2.4 a	0.7 ± 0.0 a	12.6
105	5.9 bc	3.9 d	7.5 ± 0.5 b	0.6 ± 0.1 ab	0.3
120	0.0 d	0.0 d	-	-	-

Cryopreservation of horticultural plants at MTT

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1. Introduction

1.1 Gene preservation and propagation of horticultural plants at MTT

MTT Agrifood Research Finland (MTT) is responsible for the coordination of the National Plant Genetic Resources Programme in general and for the execution of the programme on horticultural and field crop genetic resources. MTT was named as the main location of the preservation of vegetatively propagated agricultural and horticultural plants, as the programme was established in 2003 to facilitate the conservation of agricultural and forest genetic resources. On top of gene resource work, MTT Plant Production Research unit at Laukaa is operating nuclear plant maintenance and certified plant production of horticultural plants. Virus-free mother plants, being used for propagation in the active vegetative nursery propagation, are mainly maintained in an isolated greenhouse or as *in vitro* cultures at normal growth conditions.

1.2 Introducing cryopreservation to MTT work scheme

Since Laukaa Cryobank was established in 2006 to enable the use of cryopreservation at MTT, both cryopreservation research and actual long-term preservation of horticultural plants have been carried out at MTT Plant Production Research (Nukari et al. 2009). Cryopreservation methods have been optimised for *in vitro* derived shoot tips of various plant species. Preservation of dormant buds has been tested using a controlled rate cooling method (Ryynänen et al. 2008). Actual deposits to the Laukaa Cryobank have been started using mainly *in vitro* cultured materials and recently also controlled rate cooling of dormant buds. Cryopreservation has been utilised for depositing both plant genetic resources and certified nuclear stocks (Nukari and Uosukainen 2007; Uosukainen et al. 2007). Cryotherapy was also introduced as one additional method to the virus eradication procedure of the plant genetic resources and of the nuclear plants of certified production.

2. Materials and Methods

2.1 Plant material

Cryopreservation methods were optimised for *in vitro* derived shoot tips of raspberry (*Rubus idaeus* L.), strawberry (*Fragaria* × *ananassa* L.), blackcurrant (*Ribes nigrum* L.) and shrubby cinquefoil (*Dasiphora fruticosa* (L.) Rydb.). Also methods for hop (*Humulus lupulus* L.) have been tested. Most recently methods for lilacs (*Syringa* L.), lowbush blueberries (*Vaccinium* Angustifolium group) and rhododendrons (*Rhododendron* L.) were studied. Preservation of dormant *Ribes* buds was tested.

2.2 Cryopreservation techniques employed

Modified droplet-vitrification was used as the basic cryopreservation method for *in vitro* culture derived shoot tips or buds. For cryopreservation of different plant species either mainly 1-3 mm long apical shoot tips or mainly 0.3-2 mm long lateral buds were excised.

Cold pretreatments were used if necessary. Activated charcoal was used in preculture medium after isolation of the meristems for 3 days in the case of phenol producing plant species like raspberry. Stepwise or stable sucrose pretreatments from one to three days on either 0.25 M, 0.50 M or 0.75 M sucrose were utilised. Buds were vitrified using loading solution (LS, Sakai et al. 1991a, Nishizawa et al. 1992) treatment for 30 minutes and plant vitrification solution 2 (PVS2, Sakai et al. 1990, 1991b) treatments for mostly either 45 or 60 minutes, depending on the size of the buds. The LS consisted of 0.4 M (or occasionally 0.8 M) sucrose and 2 M glycerol and the PVS2 consisted of 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulphoxide, in liquid MS (Murashige and Skoog 1962) basal medium (pH 5.8). Buds were frozen on aluminium foil strips in cryovials. Also optimised encapsulation-dehydration method modified from the method of Revilla and Fernández (2008) was utilised for *in vitro* culture derived shoot tips of hop. Preservation of dormant buds was tested using a controlled rate cooling method (Ryynänen et al. 2008). The long-term preservation was carried out in outer threaded cryovials filled with air, in vapour phase of liquid nitrogen. True-to-type testing on the field was included, but only fenological methods were applied.

3. Results

3.1 Comparison of cryopreservation techniques

Modified droplet-vitrification method was optimised for *in vitro* derived shoot tips of raspberry, strawberry, blackcurrant and shrubby cinquefoil. Methods for hop were tested but the droplet-vitrification did not show especially good regrowth rates (data not shown) after the freezing treatment. Thus modified encapsulation-dehydration was chosen to be applied in the future cryopreservation work with hop. Methods for lowbush blueberries and rhododendrons were studied, but would still require research. In general the different plant species treated with modified droplet-vitrification method showed good or intermediate response to cryopreservation and there were only some remarkable exceptions. The damson plum (*Prunus domestica* subsp. *insititia* (L.) C.K.Schneid.) 'Yleinen Sinikriikuna' almost failed to show regrowth as the droplet-vitrification method was tried to be optimised for it. In the case of lilacs, preservation by the modified droplet-vitrification method neither succeeded as wished. The shoot formation started well, but the problems in the regeneration occurred first after it (Nukari et al. 2011). True-to-type testing did so far not show abnormalities caused by cryopreservation. The results of the preservation of dormant *Ribes* buds using a controlled rate cooling method are still preliminary.

3.2 Progress of research activities and long-term preservation by cryopreservation

The mastery of cryopreservation methods suitable for the plants in question is a prerequisite for successful long-term preservation. Progress did not only correlate with the cryopreservation efficiency of the laborants but also with the research activities involved to run in and optimise the methods (Table 1). Five Bachelor's Thesis works were executed on cryopreservation methods by students of the JAMK University of Applied Sciences (Flyktman 2007; Järvinen 2010; Laurén 2011; Miettinen 2009; Pantsu 2010). Actual deposits to the Laukaa Cryobank were started with raspberries, strawberries, hops, shrubby cinquefoils and blackcurrants, using *in vitro* cultured materials (Table 2). At the beginning of the year 2011, dormant *Ribes* buds were cryopreserved as a part of the Ribesco - Core collection of Northern European gene pool of *Ribes* EU project.

Table 1. Amounts of *in vitro* culture derived cryopreservation lots including cryopreservation experiment lots (in general about 1–20 *in vitro* derived explants per cryovial and 2–12 cryovials per lot) and long-term preservation lots (in general about 10 *in vitro* derived explants per cryovial and 2–12 banked cryovials per lot) and the amounts of experimental vials and cryobanked vials grouped by year from the year 2006 to the year 2010 at MTT Laukaa Cryobank.

Year	Amount of experimental lots	Amount of experimental cryovials	Amount of routine storage lots	Amount of banked cryovials
2006	63	483	45	174
2007	29	109	121	713
2008	35	250	50	279
2009	59	271	39	189
2010	78	371	84	356
Total 2006-2010	**264 lots**	**1 484 cryovials**	**339 lots**	**1 711 cryovials**

Table 2. Amounts of accessions that were used in cryopreservation activities and amounts of accession taken successfully into long-term cryopreservation, grouped by plant species, by the end of the year 2010 at the MTT Laukaa Cryobank. (Number of explants per banked cryovial was about 10 and number of cryovials per accession is shown in brackets).

Plant species	Amount of all accessions, for which cryopreservation was tested at MTT Laukaa	Amount of accessions in long-term preservation (number of cryovials per accession)
Raspberries	40	32 (5-42)
Strawberries	21	21 (6-218)
Plums	5	4 (1-3)
Cherries	3	-
Currants	18	3 (3-22)
Hops	11	6 (9-18)
Lilacs	7	-
Roses	2	-
Apples	1	-
Cloudberry	1	-
Shrubby cinquefoils	4	2 (5-10)
Lowbush blueberries	3	-
Rhododendrons	3	-
Potatoes	10	-
Altogether 14 different groups	**In total 129 accessions**	**In total 68 accessions**

4. Discussion

Droplet-vitrification showed to be quite a universal method to be used at our cryobank. However, other cryopreservation practices might be advantageous for some particular plant species like hop and damson plum. Also inside one plant species like blackcurrants use of

different cryopreservation methods can be considered depending on the utilisation purpose of the material. For example nuclear plant material of the certified production is worthy of use either from *in vitro* derived source materials or materials derived from an isolated greenhouse. Although the results of freezing dormant *Ribes* buds using a controlled rate cooling method (Ryynänen et al. 2008) are still preliminary, this method seemed beneficial in cryobanking big amounts of material in a short time period. The resources are limited and have to be shared between cryopreservation method optimisation experiments and storage routine. Activity in one area might affect the efficiency of the other. However, co-operative activity on both fields is necessary in order to intensify the cryopreservation work.

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Cryopreservation studies on bilberry (*Vaccinium myrtillus* L.)

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1. Introduction

Bilberry (*Vaccinium myrtillus* L., Ericaceae) is a perennial shrub found on acid soils, from mineral heath of mountains to organic soils of forests and old peat bogs throughout Europe (Ritchie 1956). As a characteristic field layer species of boreal forests, bilberry is a key species in Northern ecosystems. It is also economically one of the most significant wild berries in Northern and Eastern Europe, and deserves particular attention for its high antioxidant content, which plays an important role in human health care (Jaakola *et al.* 2001; Katsube *et al.* 2003).

Bilberry is one of the richest natural sources of flavonoids. The synthesis and the accumulation of flavonoids in plants are both developmentally and environmentally regulated (Taylor and Groteworld 2005). In bilberry fruit tissues, the biosynthesis of flavonoids, and in particular of anthocyanins, is regulated at a developmental level (Jaakola *et al.* 2002, 2010). Solar radiation has also been shown to influence the expression of the flavonoid pathway genes in bilberry leaves (Jaakola *et al.* 2004; Martz *et al.* 2010) and there is a strong positive latitudinal effect on the flavonoid content in bilberry. In common garden experiments, higher anthocyanidin concentrations have been found in fruits originating from Northern than from Southern latitudes (Martinussen *et al.* unpublished). The same pattern has also been found in wild populations of bilberry in Finland (Lätti *et al.* 2008). Thus, plant breeding efforts might lead to even higher nutritional or health value of the berries.

Large-scale breeding programs do not exist for bilberry at the moment, but *in vitro* techniques have already been established for rapid mass production of high quality plant material for large-scale cultivation, germplasm improvement, and gene conservation (Jaakola *et al.* 2001; Cao *et al.* 2003, Gajdosova *et al.* 2006). In bilberry, it takes around 3–4 years to flower, and the future breeding efforts for the species require the preservation of a rich genetic diversity. Therefore, the general aim of the study was to establish an efficient protocol for the (long-term) germplasm preservation of bilberry. Several bilberry genotypes covering different latitudes in Northern Europe were cryopreserved. In particular, shoot tips excised from *in vitro* plants were cryopreserved through the droplet-vitrification technique and the controlled cooling method was applied to seed material.

2. Materials and methods

2.1. Plant material

In vitro plants and seeds of bilberry (*V. myrtillus* L.) were used as starting material to test two different cryopreservation techniques. All the accessions were obtained from the Botanical Gardens of the University of Oulu (Finland).

*In vitr*o clones from five different wild populations in Northern Europe were used as starting material for the droplet-vitrification technique. The clones used were M1 (64º 48´ N, 25º 59´ E), P1 (62º 02´ N, 23º 02´ E) and S3 (67º 25´ N, 26º 35´ E) from Finland, clones R1 and R3 (66º 57´ N, 17º 43´ E, both) from Sweden, and clone N7 (60º 54´ N, 10º 44´ E) from Norway. Shoot tips derived from cold-stored (at 4 °C) *in vitro* plants were multiplied on a modified

MS medium (Jaakola *et al.* 2001), solidified with 8 g/L agar. The pH was adjusted to 4.8 prior to autoclaving. Plants were cultured at 22 °C with a 16 h light (40 µE•m-2•s-1) / 8 h dark photoperiod.
The seeds used were from five wild populations including Inari-Kaamassaari (69º 90´ N, 27º 98´ E), Inari-Vartasaari (69º 55´ N, 27º 48´ E) and Hailuoto (65º 24´ N, 24º 42´ E) from Finland, Kåfiord (69º 24´ N, 21º 00´ E) from Norway and Akureyri (66º 07´ N, 18º 38´ E) from Iceland. The seeds were collected between 2007 and 2009, and were stored at 4 °C. Before cryopreservation, viability of the seeds was tested by germination test. For each population 50 seeds were placed on top of moist filter paper in plastic Petri dishes and cultured at 20 °C in the dark for at least 2 weeks. Cryopreservation of the seed material was conducted by the controlled cooling method.

2.2. Droplet-vitrification method

In vitro plants of all five clones were exposed to three different light/temperature conditions for two weeks. The light/temperature conditions were (a) cool white (40 µE•m-2•s-1), in which plantlets were incubated throughout the *in vitro* culturing period, (b) LED light, in which plantlets were cultured at 22 °C and exposed at LED light (650 nm, 5 µE•m-2•s-1) and cultured at 22 °C, (c) cold acclimation (CA), in which plantlets were incubated at 22 °C with 8 h low light (10 µE•m^{-2}) / 5 °C 16 h dark. Shoot tips, 1-2 mm, with attached leaf primordia were excised from treated plantlets and further exposed to the same light/temperature conditions for 24 h on modified MS medium (Jaakola *et al.* 2001), supplemented with 135 g/L sucrose, 5 % dimethyl sulfoxide (DMSO) and 8 g/L agar, before cryopreservation.
The protocol described for potato shoot tips (Schäfer-Menuhr *et al.* 1994) was used with some modifications to improve shoot tips survival (see Results). Shoot tips were excised and pre-treated on modified MS medium (Jaakola *et al.* 2001) supplemented with 135 g/L sucrose, 5 % v/v DMSO and 8 g/L agar for 24 h at different light/temperature conditions (see above). Shoot tips were transferred on filter paper into a Petri dish with 2 ml of loading solution (liquid MS media with 135 g/L sucrose and 10 % DMSO, pH 5.7) at room temperature for 1 h. For each clone, 25 shoot tips were excised, 20 to be cryopreserved and 5 as controls, unfrozen but cryoprotected. Different combinations of the sucrose concentration of the pretreatment medium (75 g/L, 135 g/L) and of the loading solution (30 g/L, 75 g/L, 135 g/L), and moreover the exposure time to loading solution (1h, 3h) were tested in a preliminary experiment using shoot tips from a single clone (S1, from Finland).
Shoot tips were transferred to a droplet of about 2 µL of loading solution on a strip of aluminium foil (5 mm x 20 mm), which was plunged into a 2 ml cryovial previously filled with LN and placed in a polystyrene box with LN. Cryovials, once closed, were stored for 1 h in LN. The shoot tips of each clone were rewarmed plunging rapidly the aluminium foils into 30 ml of liquid MS medium with 30 g/L sucrose at room temperature. Shoot tips were dried on filter paper and placed on solid modified MS medium (Jaakola *et al.* 2001) for regrowth, at the same light conditions of plant *in vitro* culturing. In a preliminary test, the applicability of MS solid media with zeatinriboside (0.5 mg/L), IAA (0.5 mg/L) and GA_3 (0.2 mg/L) were tested as regrowth media. Both post-thaw survival and regeneration were determined 2-6 weeks after cryopreservation.

2.3. Controlled cooling method

Dried seed material was at dormant state and was proposed to be cold acclimated due to the long-term storage at 4 °C in the dark. For each origin, 180-240 seeds were placed in 2 ml cryovials (60-80 seeds/vial). The slow freezing was achieved by placing the cryovials in the Nalgene® Cryo 1 °C Freezing Container at -80 °C for 4 h (reaching a controlled cooling rate of -1 °C/min) before storing them in the LN. For recovery, the cryopreserved seeds were

taken out of the LN containers, thawed rapidly at 37 °C water bath for 2 min and placed on ice for 1 min. After rewarming, seed viability was tested through the germination test as described above.

3. Results

3.1. Droplet-vitrification method

Initially, the original protocol described for potato (Schäfer-Menuhr *et al.* 1994) was applied for cryopreservation of bilberry shoot tips. Only one shoot tip out of 21 survived after two weeks from rewarming. In addition, in the presence of the MS solid media with zeatinriboside (0.5 mg/L), IAA (0.5 mg/L) and GA_3 (0.2 mg/L), both control and cryopreserved shoot tips produced callus. Thus, in a preliminary experiment, several modifications of the protocol were tested and, based on the best regrowth after cryopreservation, a modified MS medium (Jaakola *et al.* 2001) was selected for further experiments.

Only one treatment resulted in the survival of shoot tips excised from *in vitro*-grown plantlets. When the sucrose concentration of the pretreatment medium and of the loading solution was 135 g/L, and moreover when the exposure time to loading solution was 1 h, two shoot tips out of seven were able to survive two weeks from rewarming. These conditions were used to cryopreserve *in vitro* plants, pretreated with different temperature/light combinations. In all the other treatments, the shoot tips turned brown and died.

All the clones tested showed different behaviour after rewarming. Clones M1, N7 and S3 were not able to survive after the pretreaments. In the case of clone P1 four and eight percent of the shoot tips were viable after cool white illumination (control) and cold acclimation treatments, respectively. The two clones R1 and R3, which were derived from the same bilberry population, had the same percentage of survival (4 %) after the cold acclimation treatment. From the cryopreserved clones only the shoot tips of clone R3 survived after LED light pretreatment. All the clones tested showed a low percentage of survival compared to the unfrozen controls with 100 % regeneration. However, all the survived shoot tips started to re-grow and after four weeks they were able to regenerate shoots.

3.2. Controlled cooling method

Seed lots collected from different origins showed different percentages of germination before cryopreservation. The germination percentage was high in seeds collected from Inari-Kaamassaari, Hailuoto and Kåfiord, varying between 63 and 71 %. The Inari-Vartamasaari seed lot germinated poorly, only 27 % of the seeds were able to germinate. The lowest percentage (two percent) was recorded for the seeds collected in Akureyri.

After cryopreservation, the percentages of germination for the seeds collected in Inari-Kaamassaari, Hailuoto and Kåfiord were ranging between 56 and 70 %, leading to over 80 % regrowth.

4. Discussion

At present specific cryopreservation techniques are available for a wide range of crop species, and most of the existing cryopreservation techniques are effective for temperate berry crops (Razdan and Cocking 1997; Reed 2008). Some of these have been applied also to *Vaccinium corymbosum* L., *V. uliginosum* L. and *V. ovatum* Pursh. (Reed 1989), *V. pahalae* Skottsberg (Shibli *et al.* 1999), *V. corymbosum* L. and *V. macrocarpon* Aiton (Uchendu and Reed 2009). In the present study, the development of *ex situ* germplasm preservation of bilberry was successful when two cryopreservation techniques, droplet-vitrification and control rate

cooling, were applied to *in vitro* shoot tips and seeds, respectively. The controlled rate cooling method on bilberry seeds turned out to be the most promising application.

The regowth ability of the cryopreserved seeds, collected from different locations in Northern Europe varied from 67 to 98 %. This indicates that the controlled cooling rate technique is applicable for the preservation of a wide range of bilberry populations. Seeds are a great source of genetic variability, and a large amount of bilberry seeds can easily be collected throughout Northern Europe, where it grows abundantly. Thus, bilberry seed cryopreservation technique allows storage of a wide range of genetic diversity of the species. Moreover, germinating seeds of bilberry are excellent explants for starting *in vitro* cultures, offering the possibility to select specific clones of particular interest, once seeds are rewarmed and regenerated after cryopreservation.

On the other hand, the preservation of particular clones, especially of some elite genotypes, requires the cryopreservation of *in vitro* propagated plants. The droplet-vitrification technique is a very recent cryopreservation method which has been, so far, applied to a limited number of plant species, despite the very promising results achieved from its application (Sakai and Engelmann 2007). This technique has been successful for the cryopreservation of *Prunus* (De Boucaud *et al.* 2002), yam (Leunufna and Keller 2003), *Chrysanthemum* (Halmagyi *et al.* 2004) and banana (Panis *et al.* 2005), and with some modifications to the original protocol, also for bilberry. In the present study, bilberry shoot tips were able to regrow after thawing if preculture steps were added before cryopreservation even if the regrowth percentages were lower than in the case of seed material.

Resistance to freezing and desiccation develops also after cold acclimation, which is usually applied as treatment before cryopreservation to minimize freeze-induced desiccation stress during freezing and increasing survival following cryopreservation (Uchendu 2009). The development of cold acclimation is a complex phenomenon involving changes in gene expression that result in the alteration in metabolism and composition of lipids, protein and carbohydrates (Guy 1990; Thomashow 1999). In the genus *Vaccinium*, survival rates after cold acclimation have been shown to vary greatly among species (Reed 1989). Anyway, in blueberry, cold acclimation is known to enhance accumulation of polyamines and special proteins including dehydrins, and to up-regulate the expression of low temperature genes induced by the CBF (CRT binding factor) transcription factor (Naik *et al.* 2007; Polashock *et al.* 2010). In fact, blueberry survival increased from 6% to 58% following three or more weeks of cold hardening (Reed 1989). Also in the present study with bilberry, the cold acclimation treatment led to survival and regrowth of 50% of clones tested, while when exposed to cool white illumination only one out of six tested clones (18%) survived and was able to regrow.

Nevertheless, none of the bilberry clones cryopreserved with the droplet-vitrification technique reached the 40 % recovery recommended for germplasm storage (Reed 2001; Reed *et al.* 2005), since recovery ranged only from 4 % to 8 %. There might be several reasons for the low recovery percentages. First of all, recovery is known to be dependent on genotype (Uchendu 2009), but also the age of the shoot tips seemed to affect the survival after cryopreservation since, in the present study, the shoot tips excised from the short lateral branches of the bilberry shrub were the ones that regenerated after cryopreservation. The physiological state of cells and tissues must be optimal for the acquisition of maximum possible dehydration tolerance and for producing vigorous recovery growth (Withers 1979; Dereuddre *et al.* 1988). Generally, explants from rapidly growing cultures are preferred, since actively dividing cells have small vacuoles and a high nucleo-cytoplasmic ratio, which makes the explants more likely to withstand desiccation and freezing (Ashmore 1997). Anyhow, bilberry is a rhizomatous plant that grows vegetatively, thus ageing acts not on the individual

as a whole, but on the separate parts of the plants which inevitably become senescent and displays decreased growth rates (Ritchie 1956; Wijk 1986).

To conclude, both controlled cooling rate and droplet-vitrification cryopreservation techniques were applied for seeds and *in vitro* shoot tips of bilberry, respectively. Both of the techniques were successful at least for some bilberry clones or seed origins but generally seed material had higher re-growth percentages than *in vitro* shoot tips. This indicates that, the droplet-vitrification protocol which was for the first time applied to bilberry shoot tips still needs further optimization.

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Development of droplet-vitrification protocols for tropical vegetatively propagated crops

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1. Introduction

Storage of desiccated seeds at low temperature, the most convenient method to preserve plant germplasm, is not applicable to plant species that are propagated vegetatively. For such species field collection was developed. Although clonal orchards play a pre-eminent role in assisting conservation programmes, their maintenance requires large areas of land and high running costs. Further, they are prone to adverse weather conditions and to hazards such as pests, diseases and genetic alterations. *In vitro* collections, established for some vegetatively propagated species and maintained by means of traditional micropropagation, is labour-intensive as well, and there is always the risk of losing accessions due to contamination, human error or somaclonal variation (i.e. mutations that occur spontaneously in tissue culture, with a frequency that increases with repeated subculturing). Cryopreservation, or freeze-preservation at ultra-low temperature (–196°C, the temperature of liquid nitrogen), is a sound alternative for the long-term conservation of plant genetic resources since biochemical and most physical processes are completely arrested under these conditions. The advantage of cryopreservation over other plant conservation methods is thus that the material is stored (i) in a stable way, (ii) for the long term at very low costs and (iii) in disease-free conditions. As such, plant material can be stored for unlimited periods.

This study is executed in the framework of the project "Development and refinement of cryopreservation protocols for the long-term conservation of vegetatively propagated crops" funded by the Global Crop Diversity Trust. This project aims to develop cryopreservation protocols for several vegetatively propagated crops, viz. sweet potato, cassava, yam, taro and other edible aroids. K.U.Leuven's responsibility is to apply and optimize the droplet-vitrification protocol that was originally designed for banana (Panis *et al.* 2005) to (i) sweet potato, (ii) some cryopreservation-recalcitrant cassava cultivars and (iii) edible aroids like *Colocasia, Alocasia, Cyrtosperma* and *Xanthosoma*.

The different steps that were followed in developing a droplet-vitrification protocol for the species under investigation are;

1. Define accessions representative for the collection
2. Check accessions for the absence of contamination
3. Define optimal shoot multiplication medium
4. Define meristem outgrowth medium
5. Define toxicity level for LS solution
6. Define toxicity level (against PVS2 at 0°C) for LS treated tissues
7. Define optimal length of PVS2 treatment after cryopreservation
8. Redefine regrowth conditions (culture media as well as meristem size)

Some of these improvement steps will be discussed for the three plant species under investigation, but focus will be mainly put on the experiments executed with sweet potato.

2. Materials and Methods

2.1. Plant material

Sweet potato *in vitro* material of 10 accessions ('Tanzania', 'Camote Mata Serrano', 'Cinitavo', 'Espelma', 'Trujillano', 'Jewel', 'TIS 87/0029', 'Ibarreno', 'Manchester Hawk' and 'CMR 1112') was received from CIP (Centro Internacional de la Papa, Peru).

Cassava *in vitro* material of six6 accessions was received from CIAT (Centro Internacional de Agricultura Tropical, Colombia). The selection of these cultivars was based on the cryopreservation behaviour that was observed in CIAT. 'BRA856' and 'COL1667' are good responding cultivars, 'CM 3306-4' and 'CR113' are intermediate, while 'CM507-37' and 'COL 1468' are not surviving the CIAT protocols.

In case of edible aroids the following numbers of cultivars were obtained from SPC (Secretariat of the Pacific Community, Fiji): 5 *Alocasia* (Giant taro), 3 *Xanthosoma* (Tannia), 3 *Colocasia* (Taro) and 2 *Cyrtosperma* (Swamp taro).

2.2. Check accessions for the absence of contamination

Upon arrival the cultures were screened for the presence/absence of endophytic bacteria on a bacterial growth medium (BACT medium) containing 23 g/L Difco® Bacto nutrient broth, 10 g/L glucose and 5 g/l yeast extract (Van den Houwe and Swennen 2000). The plates are incubated at least for 3 months in an incubator (under dark conditions) at 28 °C.

2.3. Define optimal shoot multiplication medium

For sweet potato, 4 media were compared: (i) MS medium (Murashige and Skoog 1962), (ii) half-strength MS medium, (iii) sweet potato culture medium commonly used by CIP (containing 0.25 mg/l GA_3) and (iv) Hirai and Sakai (2003) medium, while for the edible aroids, MS medium as well as taro elongation and taro multiplication media (MS media containing respectively 0.005 and 0.5 mg/l TDZ, (SPC, personal communication) were used. For cassava, MS medium was compared with 4E medium (MS with 0.04 mg/l BAP, 0.02 mg/l NAA, 0.05 mg/l GA_3, 1 mg/l thiamine-HCl, 100 mg/l m-inositol, agar 0.45 %; Roca 1984).

2.4. Define optimal meristem outgrowth medium

For all species under investigation, the following 5 meristem recovery media were compared; (i) Taro elongation medium (MS + 2.27 µM TDZ), (ii) MS medium supplemented with 2.22 µM BA for 1 week, afterwards transferred to hormone free MS, (iii) continuous hormone free MS (iv) MS medium with Kin (2.32 µM) and GA_3 (0.144 µM) and (v) Hirai and Sakai medium (2003) (MS + 1.44 µM GA_3).

2.5. Cryopreservation technique employed

The cryopreservation protocol was based on the droplet-vitrification protocol that was developed for banana meristems (Panis et al . 2005) and later applied to taro (Sant *et al.* 2008), pelargonium (Gallard *et al.* 2008), potato (Panta *et al.* 2009), thyme (Marco-Medina *et al.* 2010) and apple (Condello *et al.* 2009). Also embryogenic culture of olive (Sánchez-Romero *et al.* 2009) could be successfully cryopreserved with this method. This method involves the following steps.

- loading of explants for 20 min with 2 M glycerol and 0.4 M sucrose dissolved in MS medium at room temperature
- dehydration with PVS2 (Sakai *et al.* 1990) (30 % (3.26.M) glycerol, 15 % (2.42 M) ethylene glycol (EG) and 15 % (1.9.M) DMSO dissolved in MS medium that contained 0.4 M sucrose) at 0°C for periods varying between 10 minutes and 2 hours

- transfer of 6-10 explants to a droplet of PVS2 (of about 15 µl) on a strip of aluminium foil and rapid plunge in liquid nitrogen
- transfer of frozen strip to 2 ml Cryovial filled with liquid nitrogen for storage
- after storage, rapid thawing by rinse the aluminium foil with explants in recovery solution that 1.2 M sucrose dissolved in MS medium at room temperature and keep for 15 minutes
- transfer of explants to regeneration medium with elevated sucrose concentration (0.3) for 1 to 2 days

2.6. Redefinition of regrowth conditions

The best two media resulting from the meristem outgrowth test (see 2.4.) are compared after cryopreservation. Also the size of the meristems should be reconsidered; how many leaf primordia are still surrounding the apical dome and how much stem tissue is still present below the meristem.

3. Results

3.1. Check accessions for the absence of contamination

Since material that is used for cryopreservation should be free of fungi and bacteria, all the material is screened using the methods that are developed for banana at the laboratory for Tropical Crop Improvement. This includes careful observation of material during subculturing and streaking cut ends onto bacteria growth medium. Special attention is given to slow growing endogenous bacteria that often do not interfere with normal *in vitro* multiplication but only appear (and interfere) with cryopreservation. In case material was contaminated, it was removed from the culture room and requested again from the source institute.

3.2. Definition of optimal multiplication medium

In order to be able to execute enough cryopreservation experiments, access to a big quantity of high quality plant material is necessary. Out of the different media tested for both sweet potato (Figure 1) and cassava, the most simple medium, hormone free MS medium, proved to result in the best growth and the highest amount of new axillary buds. For the edible aroids, the taro multiplication medium containing thidiazuron, a plant growth regulator with a cytokine-like effect was optimal. This difference might lie in the fact that edible aroids are monocots and the other 2 species are dicotyledonous plants. Banana, another monocotyledonous species also needs high concentrations of cytokinines (BA or thidiazuron) for multiplication.

3.3. Definition of optimal meristem outgrowth medium

The optimal regrowth medium that results in the highest regeneration frequency of non-cryopreserved meristems is very species dependent. Hirai and Sakai medium proved to be best for sweet potato, while MS medium containing Kin (2.32 µM) and GA_3 (0.144 µM) was optimal for cassava. Taro elongation medium (containing a low amount of thidiazuron) is used for excised meristems of edible aroids such as *Xanthosoma* spp (Figure 2).

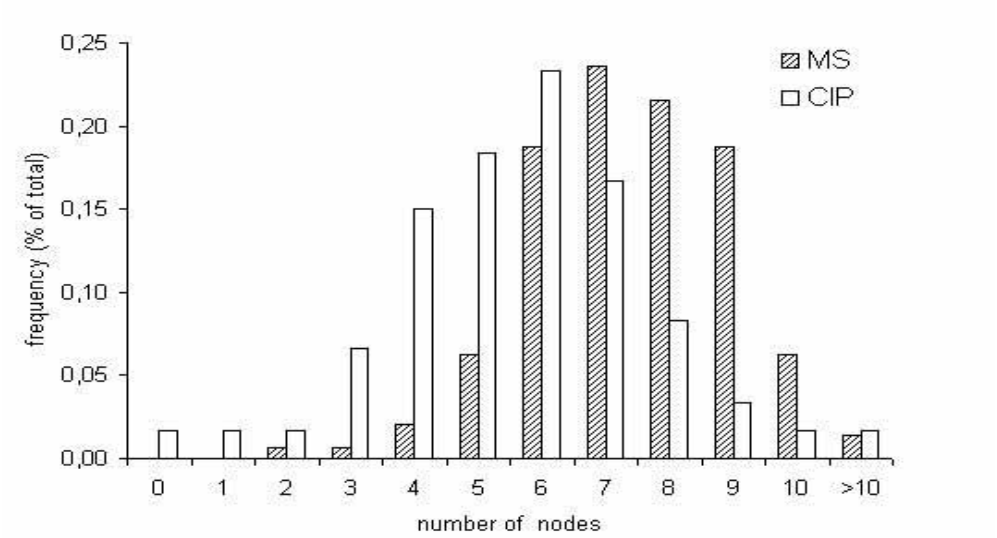

Figure 1. Number of nodes in shoots of the sweet potato 'Tanzania' developed after six weeks of culture on MS medium and CIP medium (MS containing 0.25 mg GA_3).

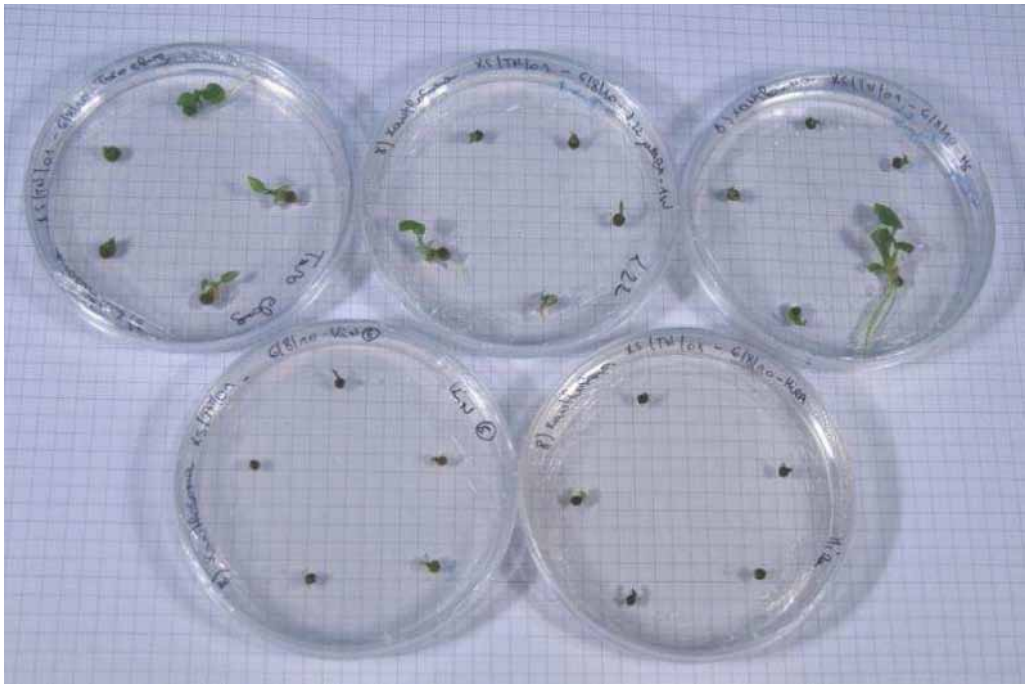

Figure 2. Outgrowth of non cryopreserved meristems of *Xanthosoma*, two months after excision. Upper row from left to right; Taro elongation, MS with 2.22 µM BA one week afterwards MS, MS without PGRs. Lower row from left to right; MS with Kin and GA_3 and Hirai and Sakai medium.

3.4. Definition of conditions for cryopreservation

The most important step to be optimized in the cryopreservation protocol is the length of the PVS2 treatment at 0 °C. When the treatment is too short, insufficient amounts of water are extracted from the tissues leading to irreversible freezing damage caused by the formation of ice-crystals. On the contrary, when the treatment is too long, toxicity effects of PVS2 might occur. The main difficulty is to determine the, often very narrow, window where vitrification occurs without the treatment being toxic. Our experiments have shown that for sweet potato and edible aroids shorter PVS2 treatments (30 minutes) are needed compared to cassava (45-60 minutes). This might be linked to the fact that cassava is much more drought resistant. We

distinguish four response after cryopreservation (i) no growth, the tissue becomes completely white or black, (ii) survival, green or yellow-white callus growth is observed, (iii) shoot regeneration, a growing shoot can be observed, often even up to 3-4 mm, but then growth stops (iv) plant formation, a normal rooted plant can be regenerated (Figure 3 and 4).

Figure 3. Plant formation, shoot regeneration and survival of meristems of the sweet potato 'Jewel'. Explants were treated with LS and PVS2 with (Frozen, left of line on Petri dish) and without exposure to liquid nitrogen (Control, right of line on Petri dish).

3.5. Redefinition of regrowth conditions

Composition of the regrowth medium (results not shown) as well as the size of the meristems influence both post-thaw regeneration. In case of sweet potato, cutting back the explant until there are only two primordia left, results in the highest post thaw plant formation (Figure 4).

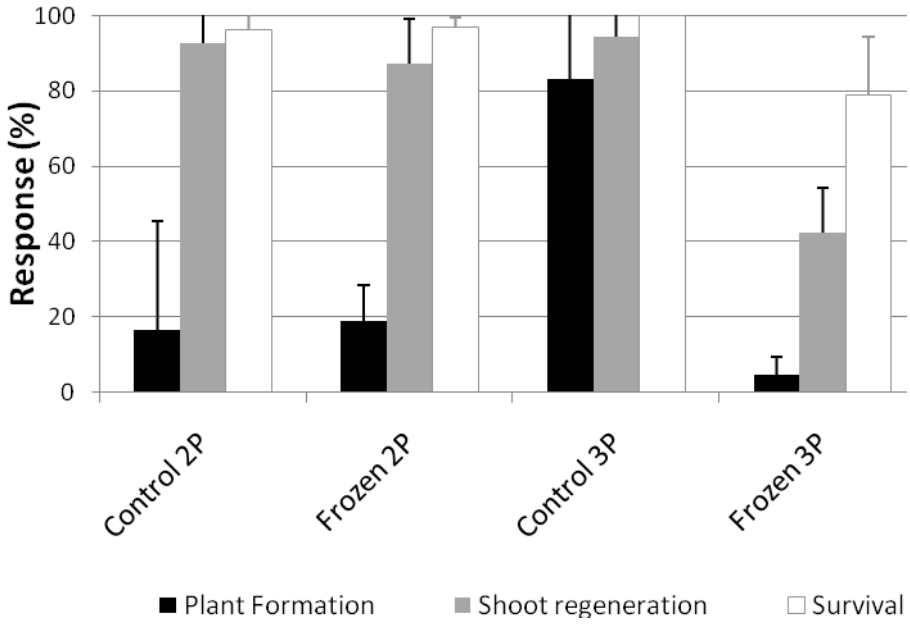

Figure 4. Plant formation, shoot regeneration and survival of meristems of different sizes containing 2 primordia (2P) or 3 primordia (3P) from the sweet potato 'Jewel'. Explants were treated with LS and PVS2 without (Control) and with exposure to liquid nitrogen (Frozen).

4. Discussion

In this paper, we show that by making some adaptation to the existing droplet-vitrification protocol, a wide variety of plants can be cryopreserved. For sweet potato, edible aroids and cassava, respectively 4 to 51 %, 0 to 95 % and 15 to 84 % of the explants can form normal plants after cryopreservation. Further improvement is especially expected from the optimization of regrowth conditions with special emphasis on the medium composition.

5. Acknowledgements

The authors acknowledge the Global Crop Diversity Trust for funding support through the project "Development and refinement of cryopreservation protocols for the long-term conservation of vegetatively propagated crops". Also CIP (Centro Internacional de la Papa, Peru), SPC (Secretariat of the Pacific Community, Fiji) and CIAT (Centro Internacional de Agricultura Tropical, Colombia) are gratefully acknowledged for providing the plant material.

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Ways of collaboration – COST Short-Term Scientific Missions on three crops and their outcomes – potato, garlic and mint

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1. The cryopreserved collections of IPK

The genebank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) is one of the oldest living plant collections of Europe, founded already in the year 1943. It works as the Central German Genebank for cereals, potato, vegetables, technical crops as well as medicinal and aromatic plants. Some vegetatively propagated crops are routinely maintained in cryopreservation on the basis of empirically elaborated protocols. The main species are potato, garlic and mint. The genebank is surrounded by several departments performing basic research on crop plants, thus embedded in a favourable scientific situation. From its beginning, there was a continuous completion of the methodical arsenal used for germplasm conservation, starting with the establishment of a store in 1976. *In vitro* storage started in 1989, and since 1997, cryopreservation has been developed. After general renovation of the technical cryopreservation facilities in 2005, there are now good facilities to hold training courses in the framework of COST STSMs (Short-Term Scientific Missions). These STSMs were used for two purposes: 1) to teach young scientists and engineers; and 2) to get some new insight in the systems used for cryopreservation, both at practical and theoretical levels. We need to state that STSMs have also some limitations because the duration of visitors' stays in the receiving institute is usually too short for a final result. This requires continuation of the experiments by the staff of the host after the visit. Thus, the final result can be obtained only some time after the stay. This was managed by continuous further contacts and ongoing collaboration, which is, in principle, one of COST's objectives. We have also to highlight that the range of the experiments is usually too small to get results which can be secured statistically. Thus, they are thought to set an initial benchmark for further experimental programmes.

At present (February 2011), in IPK's cryobank there are 1,244 accessions of potato, 79 of garlic, five of other *Allium* genotypes and 56 of *Mentha*. These three crop groups were the targets of STSM actions. Two STSMs were performed per crop group. Their working periods differed from 2 weeks to 2 months.

2. Survey of STSMs held for various crops

2.1. Potato

The cryopreserved potato collection is the most advanced one at IPK. Despite the fact that already over 1,180 accessions have been cryopreserved using the DMSO droplet method (Schäfer-Menuhr *et al.*, 1996), some other laboratories use vitrification and/or droplet-vitrification (Kryszczuk *et al.*, 2006; Panta *et al.*, 2006; Sarkar and Naik, 1998). Therefore, it is of interest for colleagues newly entering this field to compare these basic methods. The DMSO droplet method was compared with droplet-vitrification using PVS2, modified for potato based on the protocol described for mint by Senula *et al.* (2007). The practical comparison and its results should be a help for decision-making in the collections which will be established in Finland and Estonia. A comparative experiment was conducted with six accessions. As demonstrated (Fig. 1), there was prevalently higher regeneration with the DMSO droplet method compared with the droplet-vitrification. This result was confirmed by a second comparison of accessions (Fig. 2).

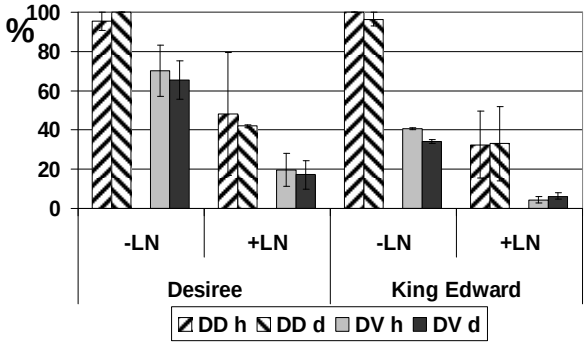
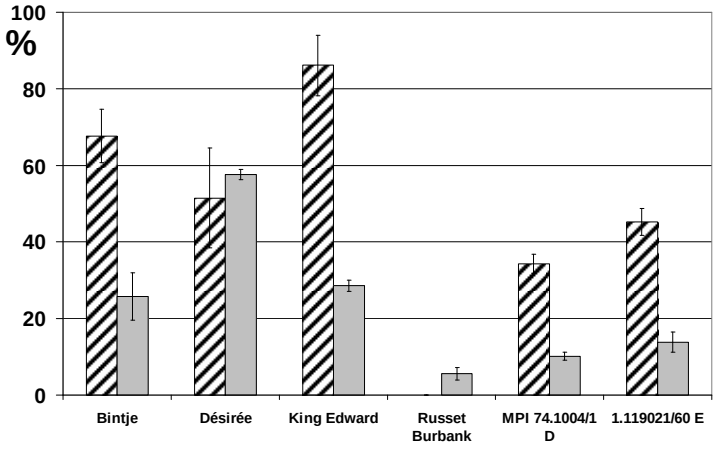


Figure 1. Regeneration (%) of two potato accessions of IPK's genebank cryopreserved using DMSO droplet (DD) and droplet-vitrification (DV) and two different light intensities in the recovery phase 8 weeks after rewarming. h - light intensity 40 µmol m^{-2} s^{-1}, d - 20 µmol m^{-2} s^{-1}. Sample size per repetition: 20 (-LN – control, not cryopreserved) and 54-90 (+LN - cryopreserved) explants. Bars mark the standard error of two repetitions.

Figure 2. Regeneration (%) of a comparative experiment using six potato accessions of IPK's genebank cryopreserved using DMSO droplet (hatched) and droplet-vitrification (filled columns). 10 explants/repetition. Bars: standard error of 3 (in 4 varieties) and 6 (in 2 breeding lines) repetitions



Comparison of the water status using Differential Scanning Calorimetry (DSC) showed that the DMSO droplet method resulted in higher contents of freezable water within the shoot tips compared to those treated with the droplet-vitrification protocol (Fig. 3). Glass transitions were measured between -114 and -123 °C for both cryopreservation protocols. Whereas there was a small amount of water still present in the first test with 'Bintje' and 'Desirée' (30 min cryoprotection with PVS2), longer incubation (60 min) in the cryoprotectant resulted in absence of ice formation in 'Desirée' (data not shown). Some other factors were tested without significant differences. Both the light conditions (dark vs. light; different illumination intensities) and the filling method of the cryovials before adding the aluminium strips bearing the explants (filled with liquid nitrogen vs. filled with air) did not give remarkable changes.

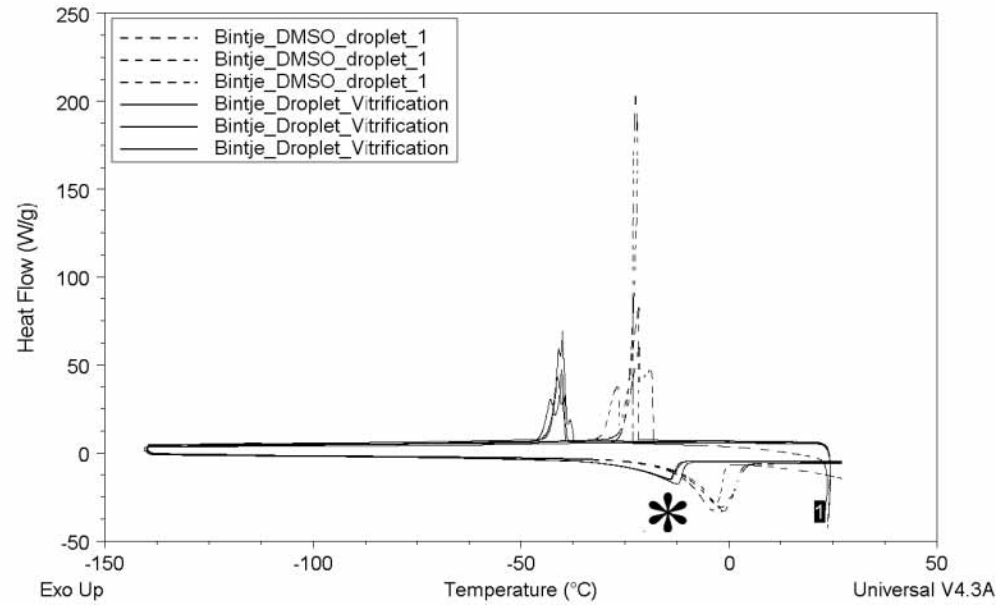


Figure 3. Examples of two measurements using DSC showing that the amount of freezable water is lower (characterised by smaller melting peaks, marked by an asterisk*) in droplet-vitrification (full lines) as compared with DMSO droplet (broken lines) method. Three measurements are represented, each showing the state before cooling to liquid nitrogen temperature.

Table 1. Survival ([S, %] and regeneration [R, %] in three clones of garlic as a function of the cryopreservation method. 20 (vitrification) and 10-20 (droplet-vitrification) explants per experimental condition.

	Weeks after rewarming	Vitrification (PVS3)			Droplet-vitrification (PVS2)		
		All 0232	Gros Bleu	Vacoas	All 0232	Gros Bleu	Vacoas
-LN	1 [S]	95.0	68.2	58.8	82.4	72.7	50.0
	2 [S]	95.0	90.9	100.0	94.1	81.8	42.9
	4 [R]	95.0	90.9	76.5	82.4	81.8	35.7
	8 [R]	100.0	90.9	82.4	64.7	81.8	35.7
+LN	1 [S]	100.0	71.4	33.3	90.0	40.0	33.3
	2 [S]	100.0	90.5	71.4	85.0	40.0	46.7
	4 [R]	88.9	90.5	52.4	40.0	40.0	33.3
	8 [R]	50.0	85.7	42.9	10.0	20.0	26.7

2.2. Garlic

Garlic is, in comparison to cultivated potato, more heterogeneous because of various abilities to form scapes and inflorescences and major differences in bulb morphology and climatic adaptation. Therefore, it was interesting to observe whether tropical garlic from Réunion Island was also suitable for cryopreservation. This was tested using two tropical genotypes ('Gros Bleu', 'Vacoas') in comparison with one from the Gatersleben collection (All 0232). Two methods were compared, namely vitrification with PVS3 (Nishizawa *et al*., 1993) and droplet-vitrification with PVS2 (Sakai *et al*. 1990). Even though survival after one and two weeks did not yet give clear differences, further development was clearly better after vitrification using PVS3 compared with droplet-vitrification using PVS2 (Table 1), thus confirming some of our earlier findings (Makowska *et al*., 1999).

Since in garlic, there are still several genotypes with rather poor performance, antioxidants could presumably improve the results. Therefore, vitamin C was added to the cultures at various steps of the procedure. Table 2 shows the results for one accession, which were confirmed by similar results with another one (data not shown). So far, it seems that some experimental conditions led to better regeneration, even though differences were not significant.

Table 2. Regeneration (%) of garlic '444K' after application of vitamin C (VC) at various steps of the cryopreservation protocol. Sample size per replicate: 20 (experiments), 10 explants (growth controls).

	- LN				+ LN			
	vitrification			droplet vitrifi-cation	vitrification			droplet vitrifi-cation
Concentration VC	0.14 mM	0.28 mM	0.43 mM	0.28 mM	0.14 mM	0.28 mM	0.43 mM	0.28 mM
Growth control +VC	100.0	100.0	100.0	85.7				
Growth control –VC	100.0	100.0	100.0	100.0				
Standard protocol	86.4	65.0	60.9	100.0	54.5	30.0	50.0	72.0
VC to preculture	70.0	70.0	52.4	88.2	19.0	47.4	52.4	65.2
VC to loading solution	81.0	90.0	72.7	100.0	61.9	36.8	50.0	86.4
VC to washing solution	81.0	45.0	68.2	100.0	85.7	23.8	70.8	72.7
V to regrowth medium –Fe	59.1	25.0	45.8	95.2	34.8	20.0	54.2	58.3
V to regrowth medium + Fe	54.5	70.0	56.5	90.5	40.9	10.0	33.3	57.1

2.3. Mint and sage

After general training using various accessions, some parameters were tested. In order to simplify the method, the droplet-vitrification protocol using tubes filled with liquid nitrogen prior to inserting foils with the explants was compared with tubes only filled with air ("non-filled"). Despite the fact that the latter variant resulted in lower cooling rate, results were not significantly lower compared with those obtained with liquid nitrogen in the tubes. It seemed also not consistently different, whether the PVS2 dehydration pretreatment was performed at room temperature or on ice (Fig. 4). In conclusion, the simpler protocol (room temperature and 'empty' tubes) should be used, because the simpler the protocol is,the lower is the risk to damage the material. Another factor seems to be important for further progress in cryopreservation. This is the presence of endophytes marked by asterisks in Fig. 4. Since they may cause unpredictable changes, they seem to be responsible for the decline in regeneration percentages in many cases, as published by Senula and Keller (2011).

Vitamins were also added to the cultures in a similar way as for garlic (see above). Also here results are still preliminary. Fig. 5 shows that when vitamin C was present in the regrowth medium, the proportion of fully developed plants was higher compared with other variants. This indicated again that experiments needed to be conducted beyond the period of the STSM to get a conclusive picture of the results.

Finally, we were interested to observe whether the methods used for mint could be adapted to another species of the same family Lamiaceae, namely sage, *Salvia officinalis* L. This is, in principle, possible depending on the genotype. A preliminary test of encapsulation-vitrification, published by Hirai and Sakai (1999) for mint, modified by using PVS3 resulted also in good regeneration (Fig. 6).

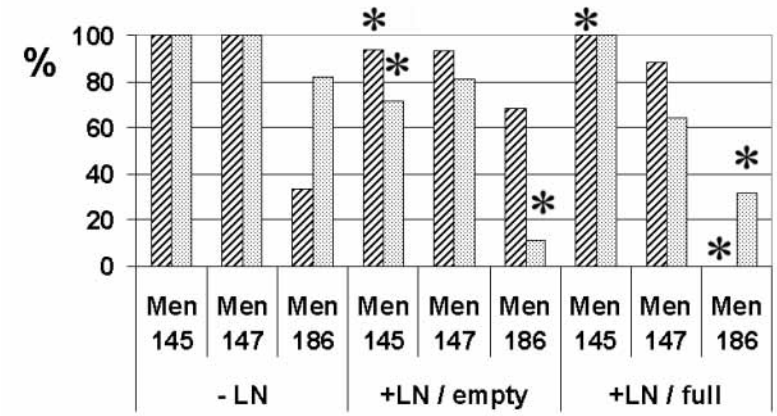


Figure 4. Regeneration (%) in droplet-vitrification of three mints using PVS2 dehydration at room temperature (hatched) or on ice (dotted columns). The tubes were not filled (empty) or filled (full) with liquid nitrogen prior to inserting the foils into tubes. Sample sizes per variant: 15 (-LN - control not cryopreserved) and 30 (+LN - cryopreserved) explants. Asterisks: visible presence of endophytes after rewarming.

Figure 5. Regeneration (%) of mint Men 154 after addition of 0.12 mM ascorbic acid to various phases of the droplet-vitrification protocol. Control – no vitamins, additions to the preculture medium (PC), loading solution (LSA), washing solution (W) and regeneration medium (R). P – fully developed plants counted only. Sample sizes per variant: 10 (-LN – control, not cryopreserved) and 20 (+LN - cryopreserved) explants.

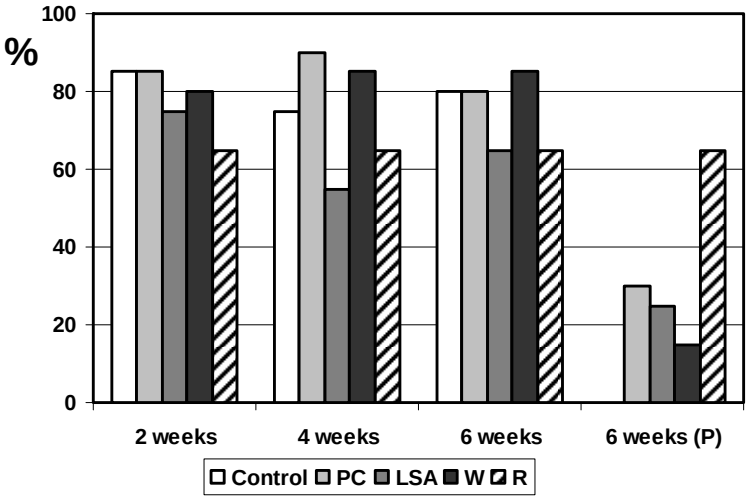
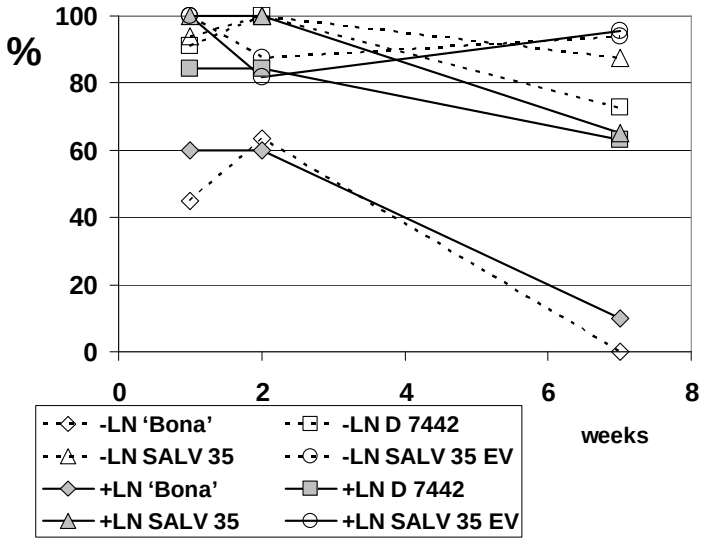


Figure 6. Survival after 1 week and regeneration (%) after 2 and 7 weeks of three accessions of sage using the standard droplet-vitrification and a protocol of encapsulation-vitrification (EV). Sample size per variant: 10-20 explants.



3. Conclusions from the STSMs

All STSMs gave valuable impulses for further application and research. It seems not realistic to always expect direct continuation of the initiated programmes, because many local factors have to be considered which influence them. Thus, because of the local funding situation, routine cryopreservation of potato could not be started in the countries from which participants came to perform STSMs (Finland and Estonia). In the case of tropical garlic, some additional activities need to be performed to test the reliability of the methods on a larger number of genotypes under the local conditions of the partner. So far, increase in regeneration using vitamin C was not significant, which calls for further research and selection of proper genotypes. Indeed, in the present study, it was found that the genotypes used had a high regeneration percentage so that the application of vitamin C could not significantly improve results. Weakly reacting genotypes should be better suited to express a vitamin effect. The confirmation of the importance of endophytes will lead to even stronger engagement of the partners to elucidate their effects. Most promising is the extension of the methods used for mint to another species, which induced actions to expand the activities to other species in the partner's team. Altogether, the conclusion can be drawn that this type of activities was a well-placed investigation for further strengthening and integration of cryopreservation throughout Europe.

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Cryopreservation of endemic *Hladnikia pastinacifolia* by encapsulation-dehydration

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1. Introduction

Hladnikia pastinacifolia Rchb. (Apiaceae) is a monotypic endemic genus with an extremely narrow distribution area (4 km^2; Trnovskigozd, Slovenia (Čušin 2004). Its recent distribution is a remnant of its pre-Pleistocene distribution area, so it is a tertiary relic. It also has a unique position within the Apiaceaefamily (Šajna *et al.* 2009b) and low genetic variability - RAPD (Šajna *et al.* 2009b). Its rarity, despite the availability of habitats, calls for protection of this species not only by legislation but also by *ex situ* conservation and cryopreservation. Cryopreservation of this endemic species would be one of several conservation activities for this endangered species. In this contribution, we describe the development of an encapsulation-dehydration protocol for *in vitro* shoots of *H. pastinacifolia.*

2. Materials and Methods

2.1. Plant material

Cryopreservation experiments were conducted with *in vitro* shoots of *H. pastinacifolia* Rchb. (Apiaceae), multiplied on Murashige and Skoog (1962) medium (MS) with 2-20 µM BAP (6-Benzylaminopurine) and 2-5µM IBA (Indole-3-butyric acid) (Ciringer *et al.* 2008; Šajna *et al*. 2009a). The cultures were maintained in a growth chamber at 23±2°C, under a 16 h light/8 h dark photoperiod, with 37-50 $\mu molm^{-2}s^{-1}$ light intensity.

2.2. Encapsulation-dehydration

In vitro shoots were precultured at 4°C in the dark for 7-14 days. Shoot tips (3-5 mm long) were excised, placed in 3% Na-alginate with different sucrose or sucrose and glycerol concentrations (Table 1), encapsulated in MS medium without $CaCl_2$ for 30-60 min, and polymerized for 30-60 min in MS medium supplemented with 100 mM $CaCl_2$ with sucrose or sucrose and glycerol at room temperature. Encapsulated shoots were osmoprotected for 10-12 h in MS medium with sucrose or sucrose and glycerol, dried in the air current of a laminar flow cabinet from 47% to 28% moisture content (fresh weight basis) and frozen by direct immersion in liquid nitrogen (LN). The cryopreserved shoots were transferred to the multiplication MS medium in a growth chamber. Survival was evaluated 2 weeks after cryopreservation by counting the number of shoots showing signs of regrowth. It was expressed as the percentage of surviving shoots per number of treated shoots.

3. Results and Discussion

The excised shoots tips of *H. pastinacifolia* could be successfully cryopreserved using an encapsulation-dehydration protocol (Engelmann *et al.* 2008), although the procedure should

be improved. The pre-encapsulation and encapsulation steps were more or less optimized: preconditioning at low temperature, preculture of excised shoots, encapsulation with a higher concentration of sucrose or sucrose and glycerol, and osmoprotection with different concentrations of sucrose and sucrose and glycerol (Table 1). Especially useful was preconditioning in a refrigerator in the dark at 4°C for 4-14 days. During this treatment the shoots became slightly rejuvenated, weakly connected to the base of the shoots and with each other, thus allowing optimal manipulation (data not shown). Preculture with 0.75 M sucrose was not beneficial for excised shoots (data not shown), so we did not include this step in our cryopreservation protocol. Among the different combinations of cryoprotectants tested, encapsulation in 3% Na-alginate combined with 0.25 M sucrose and 1 M glycerol resulted in complete survival and regrowth of shoots. Dehydration of osmoprotected shoot tips was less successful for survival and regrowth. Whether we used one- or two- step osmoprotection, survival of cryopreserved shoots decreased to 19-22%. Furthermore, the majority of shoot tips failed to develop into fully-grown plants *in vitro*. To improve shoot regrowth after cryopreservation, the optimization of the final steps of the encapsulation-dehydration procedure is required. Further research is needed to optimize the encapsulation-dehydration protocol, and other cryopreservation procedure should be tested.

4. Acknowledgements

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Table 1. Details of the experiments performed to develop the encapsulation-dehydration protocol for *in vitro* shoot tips of *Hladnikia pastinacifolia.*

Experiment	Encapsulation (1-2[h]): MS, Na-alginate with sucrose and with/without glycerol; $CaCl_2$ with sucrose and with/without glycerol	Osmoprotection (each step10-12[h]) liquid MS with sucrose and with/without glycerol	Recovery before dehydration [%]	Water content after dehydration [%]	Recovery after dehydration [%]	Survival after cryopreservation and rehydration (20 min) [%]
1a*	Na-alginate + 0.25 M sucrose + 2 M glycerol $CaCl_2$ + 0.1 M sucrose	0.75 M sucrose	33			
1b*				28	13	
1c*				28		10
2a*	Na-alginate + 0.25 M sucrose + 2 M glycerol $CaCl_2$ + 0.25M sucrose + 2 M glycerol	0.37 M sucrose + 3 M glycerol; 0.75 M sucrose + 4 M glycerol	76			
2b*			56			
3a*	**Na-alginate + 0.25 M sucrose + 2 M glycerol $CaCl_2$ + 0.25 M sucrose + 2 M glycerol	-	67			
3b*		-		32	17	
3c*		-		32		0
3d*		0.37 M sucrose + 3 M glycerol	12			
3e*				49	0	
3f*				49		0
4a*	Na-alginate + 0.25 M sucrose + 2 M glycerol and $CaCl_2$ + 0.25 M sucrose + 2 M glycerol	-	76			
4b*		0.37 M sucrose + 2 M glycerol	40			
4c*				36	8	
4d*				36		0
4e*	Na-alginate + 0.25 M sucrose + 2 M glycerol $CaCl_2$ + 0.25 M sucrose + 2 M glycerol	0.37 M sucrose + 2 M glycerol; 0.75 M sucrose + 2 M glycerol	40			
4f*				47	0	
4g*				47		0
5a*	Na-alginate + 0.25 M sucrose + 1 M glycerol and $CaCl_2$ + 0.25 M sucrose + 1 M glycerol	-	100			
5b*		0.37 M sucrose + 1 M glycerol	92			
5c*				28	21	
5d*				28		0
5e*		0.37 M sucrose + 1 M glycerol; 0.75 M sucrose + 1 M glycerol	84			
5f*				42	17	
5g*				42		0
6a*	Na-alginate + 0,25 M sucrose + 1 M glycerol $CaCl_2$ + 0.1 M sucrose	0.75 M sucrose		32		***19
6b*		0.37 M sucrose + 1 M glycerol		22		***22
6c*		0.37 M sucrose + 1 M glycerol; 0.75 M sucrose + 1 M glycerol		40		***22

*Preconditioning: dark, T=4℃, 4-14 days; **Encapsula tion 3 h; ***Survival after 7 days in the dark.

Cryopreservation of sugarcane (*Saccharum* sp.) shoot tips using encapsulation-dehydration and droplet-vitrification

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1. Introduction

Over last years cryopreservation has been widely studied for its potential for long-term storage of plant germplasm. It allows overcoming the problems linked with field conservation (necessity of space and maintenance, risks of abiotic and biotic stresses) and with *in vitro* slow growth storage (high maintenance costs, risks of contaminations and somaclonal variation). Cryopreservation of sugarcane shoot tips, has already been successfully achieved using the encapsulation-dehydration technique (E-D, Gonzalez-Arnao *et al*. 1993; Paulet *et al*. 1993; Gonzalez-Arnao *et al*. 1999). New vitrification-based techniques have been developed recently. They could represent an opportunity to improve the efficiency of sugarcane cryopreservation. We thus compared E-D with the new droplet-vitrification technique (D-V, Panis *et al*. 2005).

2. Materials and methods

2.1. Plant material

Two sugarcane commercial clones were used in our experiments, H70-144, from Hawaii, and CP68-1026, from Canal Point (USA). Mother plants were transferred monthly on semi-solid Murashige and Skoog (1962) medium, enriched with 60 g L^{-1} sucrose and 7 g L^{-1} agar, at 27±1 °C, under a 12 h d^{-1}photoperiod, with a photon dose of 50 µmol m^{-2} s^{-1}.

2.2. Cryopreservation

For cryopreservation experiments we used explants of 0.5-1 mm in length consisting of the apical meristem, one or two leaf primordia and a basal part, which were dissected from *in vitro* plantlets 30-40 days after the last transfer. Dissected shoot tips were maintained overnight on standard medium in the dark to minimize the dissection stress.

During the E-D protocol, apices were encapsulated in 3 % calcium alginate beads (diameter of 4-5 mm) and cultured for 24 h in 0.75 M sucrose liquid medium. These pretreatment conditions had been established by Gonzalez-Arnao *et al*. (1993). The encapsulated shoot tips were dehydrated in containers (10 apices per container) filled with 80 g silica gel to moisture contents (MC, fresh weight basis) between 35 and 20 %, and then placed in 2 ml sterile polypropylene cryovials and plunged directly in liquid nitrogen (LN), where they were kept for a minimum of 15 min. Beads were rewarmed by transferring them directly on recovery medium in Petri dishes.

For D-V, after overnight recovery from dissection, explants were pretreated for 20 min in loading solution (2 M glycerol and 0.4 M sucrose) and osmotically dehydrated with PVS2 (30 % glycerol, 15% DMSO, 15% EG, 13.7% sucrose; Sakai *et al*. 1990), at 0 °C for 20 to 80

min or PVS3 (50 % glycerol and 50 % sucrose; Nishizawa *et al*. 1993) at room temperature for 20 to 100 min. Cooling was performed placing explants in 10 µL PVS droplets on aluminum foil strips and plugging them directly in LN. For rewarming, the aluminum foils were plunged rapidly in unloading solution containing 1.2 M sucrose for 20 min; explants were then transferred on recovery medium.
Recovery medium consisted of semi-solid MS medium with 30 g L^{-1} sucrose, 0.2 mg L^{-1} 6-benzylaminopurine (BAP), 0.1 mg L^{-1} kinetin, 7 g L^{-1} agar and 1 g L^{-1} Plant Preservative Mixture (PPM) to avoid the proliferation of endophytic bacteria. Shoot tips were kept in the dark for seven days after cryopreservation, then transferred under standard culture conditions.

2.3. Viability and plant growth
Results were collected by recording survival and recovery. Survival was evaluated after 10 days, by counting the explants showing green pigmentation and swelling. Recovery was measured after 40 days on regrowth medium, by counting the explants producing normal shoots through direct organogenesis. Results, presented in percent of plants surviving/recovering over the total number of plants treated per experimental condition, were analyzed using ANOVA following arcsin transformation, with Duncan's multiple range test (DMRT), using the SPSS 14.0 software.

3. Results

Explants of both clones tested were able to withstand cryopreservation. With E-D, apices of both clones displayed high survival and recovery, 60 % for H70-144 and 53 % for CP68-1026, after dehydration to around 20 % moisture content (Table 1). Regrowth of cryopreserved apices was rapid and direct (Fig. 1).
Following the D-V protocol, the highest recovery percentages were achieved for treatment durations of 20 and 40 min with PVS2 and PVS3, respectively (Table 1). The two clones reacted differently to the vitrification solutions employed. Following the treatment with PVS2, recovery was 37% for H70-144 and only 20% for CP68-1026. By contrast, following treatment with PVS3, recovery was similar for both clones, reaching 33% for H70-144 and 27% for CP68-1026. Regrowth of cryopreserved apices was also rapid and direct (Fig. 2).
When comparing E-D and D-V with the two clones tested (Table 1) it appeared that E-D ensured higher recovery after cryopreservation.

Table 1: Survival and recovery (%) of control and cryopreserved shoot tips of both clones treated by D-V (using PVS 2 and PVS 3) and E-D.

Clone/ treatment	Parameter	Protocol		
		PVS2	PVS3	ED
H70-144/ -LN	Survival	77 a	83 a	50 b
	Recovery	67 ab	73 a	47 b
H70-144/ +LN	Survival	43 a	53 a	60 a
	Recovery	37 b	33 b	60 a
CP68-1026/ -LN	Survival	50 b	87 a	83 a
	Recovery	50 b	87 a	83 a
CP68-1026/ +LN	Survival	23 b	40 ab	53 a
	Recovery	20 b	27 b	53 a

Values followed by the same letter in the same row are not significantly different at the 0.05 probability level.
In D-V samples were treated with VSs for 20 min for clone H70-144 and 40 min for clone CP68-1026; In E-D freezing was performed after dehydration to 20 % MC.

4. Discussion

Our results showed that sugarcane apices could be successfully cryopreserved using both E-D and D-V. They are in accordance with the results of Gonzalez-Arnao *et al*. (1993, 1999) and of Paulet *et al*. (1993) concerning the use of E-D. However, it is the first report of cryopreservation of sugarcane apices using D-V. E-D appeared more efficient compared to D-V, since shoots could be recovered from almost all explants which withstood cryopreservation. With D-V, the decrease noted between survival and recovery was due to the high toxicity of the PVSs employed (Kim *et al*. 2009). However, both techniques can be employed for cryopreservation of sugarcane, as they ensure rapid regrowth of phenotypically normal *in vitro* plantlets without callus formation from cryopreserved apices; this should be linked to the histological studies carried out by Gonzalez-Arnao *et al*. (1993), which showed that a high percentage of cells in the meristematic area of apices were still alive after cryopreservation. In conclusion, optimization of the successive steps of both protocols is still required to improve recovery of apices after cryopreservation, especially in the case of D-V, for which only preliminary investigations have been performed.

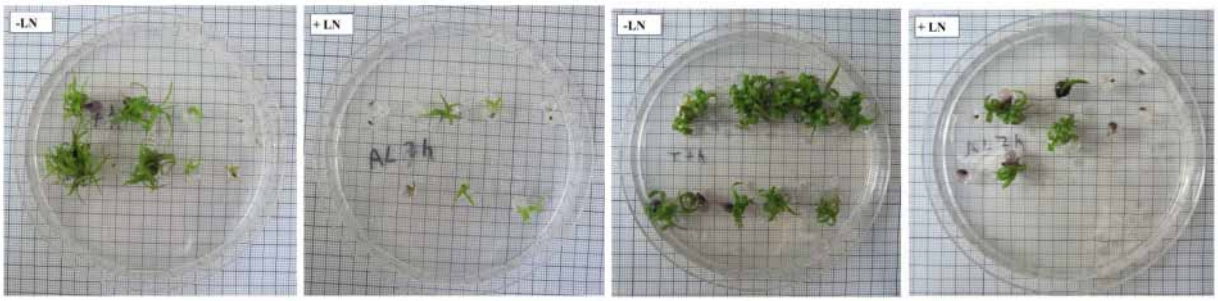

Figure 1: Survival and recovery of control (-LN) and cryopreserved (+LN) shoot tips of clone H70-144 (left) and CP68-1026 (right) treated by E-D.

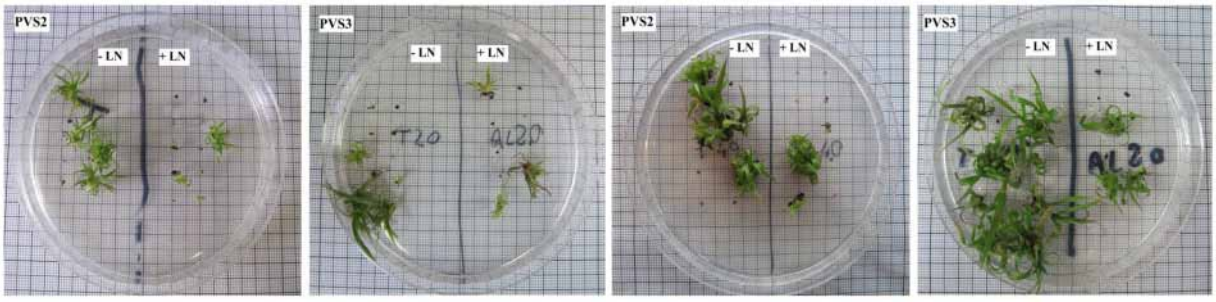

Figure 2: Recovery of control (-LN) and cryopreserved (+LN) shoot tips of clone H70-144 (left) and CP68-1026 treated by D-V using PVS 2 and PVS 3.

5. Acknowledgements

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Progress in cryopreservation by droplet-vitrification at the C.R.A. Fruit Tree Research Centre of Rome, Italy

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1. Introduction

The CRA Fruit Tree Research Centre of Rome is the repository of the *in vivo* national collection of fruit tree germplasm with about 8,000 genotypes planted by now (www.fru.entecra.it/rgv/inventario_nazionale). Programs for the application of cryopreservation at the experimental level were developed in the CRA-FRU in the framework of the National Project RGV FAO financed by the Italian Agricultural Ministry, of the COST Action 871 and of the UE Project Crympcet to several fruit species applying encapsulation-dehydration, in collaboration with F. Engelmann (Institut de Recherche pour le Développement, Montpellier, France). Recently, the droplet-vitrification technique was also applied in collaboration with B. Panis (Laboratory of Tropical Crop Improvement, Leuven, Belgium) in the framework of the COST Action 871. Among fruit species, droplet-vitrification was previously applied to *Musa* spp. (Panis *et al.* 2005), *Prunus* spp. (De Boucaud *et al.* 2002), *Carica papaya* (Ashmore *et al.* 2001) and, recently, to apple (Halmagyi *et al.* 2010; Condello *et al.* 2011). Here we report some of the results obtained at the CRA-FRU applying this method to apple, hazelnut and peach cultivars.

2. Materials and Methods

2.1. Plant material

Apple: Nodal segments, consisting of the axillary bud (AB) with a portion (0.2-0.4 cm in length) of the stem (Fig. 1A), were collected from *in vitro* grown shoots that remained on the same medium for 4 months of cv Pinova, Jonagold. The leaf was removed from each segment, which was further dissected longitudinally.
Hazelnut and peach: shoot tips from *in vitro* grown shoot, regularly subcultured of cultivar Tonda Gentile and Rich Lady, respectively, were used.

2.2. Cryopreservation

Excised explants were transferred to a filter-sterilized loading solution (LS) and kept in the dark for 20 min at room temperature. The LS consisted of 2 M glycerol and 0.4 M sucrose in liquid growth medium (pH 5.7) according to the species without growth regulators. LS was replaced by ice-cooled filter sterilized PVS2 solution (Sakai *et al.* 1990). ABs of cv Pinova were immersed in PVS2 solution for 15, 30, 45, 60, 80 or 100 min at 0°C, while ABs of cv Jonagold were treated with PVS2 for 15, 30, 45 or 60 min, also at 0°C. For hazelnut and peach, the duration of PVS2 exposure at 0°C was 60 or 90 min. Afterwards, explants were individually transferred to a droplet of PVS2 solution (Fig. 1B) and placed on a strip of aluminium foil (5 mm x 20 mm), maintained at 0°C and plunged in liquid nitrogen (LN). Then the strips were rinsed in unloading solution (US) at room temperature. Explants were maintained for 15 min in US, and then placed on regrowth medium according to the species.

3. Results

Apple: four weeks after rewarming it was possible to obtain ABs resuming growth and 2 months later regrowing explants already showed shoot development (Fig. 1B). Regrowth percentages after immersion in LN were significantly affected by the length of PVS2 exposure in both cultivars Pinova and Jonagold. Regrowth improved with increasing PVS2 exposure times from 15 min (3.3%) to 60 min (46.7%) in cv Pinova and from 30 min (6.7%) to 60 min (40.0%) in cv Jonagold.

Hazelnut: survival (35%) was obtained only with the 90 min PVS2 treatment (Fig. 2).

Peach: survival percentages after immersion in LN were higher (43%) with 90 min PVS2 treatment than with 60 min (20%).

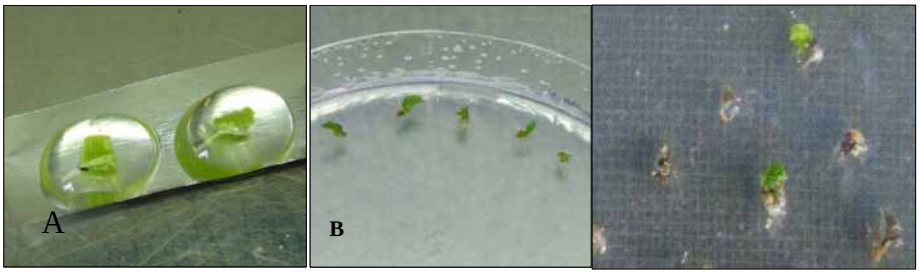

Figure 1. Apple, cv Pinova. A. Axillary buds in droplets of PVS2 on aluminium foil. B. Recovery of axillary buds after cryopreservation.

Figure 2. Hazelnut, cv Tonda Romana. Response of shoot tips 4 weeks after re-warming.

4. Discussion

The droplet-vitrification method, based on direct immersion of explants on aluminium foil strips in LN, allows to reach cooling rates higher than in vitrification-based protocols, reducing the formation of lethal intracellular ice crystals (Panis *et al.* 2005). We successfully applied droplet-vitrification to apple cv Pinova and Jonagold, confirming the potential of this method to be used on apple genotypes. In addition, the preliminary results obtained with hazelnut and peach suggest that the method can be also suitable for these fruit species.

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Shoot tips cryopreservation of *Solanum* spp. varieties

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1. Introduction

The French "potato and wild tuber-bearing relatives" collection is located in west Brittany (Ploudaniel, France) and comprises approximately 10,000 accessions. The material is maintained vegetatively either by means of tubers multiplied in the fields or as *in vitro* plantlets. However, to save money and time, it would be worth cryopreserving some of these genotypes for long term storage. We therefore developed a way of cryopreserving potato shoot tips derived from the method developed by Fabre and Dereuddre (1990), which combines apex encapsulation, controlled dehydration and cryopreservation.

2. Materials and Methods

2.1 Materials

Potato shoot tips of *Solanum tuberosum* ('Desiree', 'Bintje', 'Europa', 'Franceline', 'Noisette') and *Solanum phureja* (IVP48) from the INRA gene bank in Ploudaniel were grown *in vitro*.

2.2 Methods

Three week-old apical sections (2.5-3 cm in length) were transferred on medium modified after Tendille and Lecerf (1974) containing 25 g/l sucrose and 7 g/l agar (Univar) in aerated jars with cotton lids. Plantlets were cultivated at 20 ± 2 °C with a photoperiod of 16 h/day for 21 days. Potato meristems were sampled under the microscope and then pre-cultured for two days in a Tendille and Lecerf modified medium at 21 °C in the dark. For encapsulation, the meristems were suspended in a liquid calcium-free MS medium (Murashige and Skoog, 1962) with 3 % (w/v) sodium alginate (Sigma). Beads containing one apex were encapsulated for 90 min polymerisation in a liquid MS medium with 100 mM $CaCl_2$ before transfer for four days on a Tendille and Lecerf modified medium supplemented with 0.75 M sucrose at 21 °C in the dark. The alginate beads were then dried for three hours in a flow cabinet and plugged in liquid nitrogen in cryovials.

After a few days in liquid nitrogen, the meristems were thawed in liquid MS medium at room temperature and then cultured on a Tendille and Lecerf modified medium with different concentrations of IAA, zeatin riboside and GA_3 (table 1).

The effect of pre-conditioning treatment of the mother plant was evaluated by comparing the two treatments:

- prior to meristem sampling, the plantlets were cultivated for three weeks on a propagation Tendille and Lecerf modified medium containing either 75 g/l, 50 g/l or 25 g/l sucrose in aerated jars with cotton lids;
- after 3 weeks subculture and prior meristem sampling, stem sections with one axillary bud were sampled and subsequently subcultured for seven days in Petri dishes on a Tendille and Lecerf modified medium.

Table 1: Hormonal concentrations tested in the regeneration medium of cryoconserved potato shoot tips.

	IAA mg/l	Zeatine riboside mg/l	GA_3 mg/l
MEDIUM			
MSD	100	200	1000
MSE	100	400	1000
MSH	100	600	1000
MSI	100	200	200
MSJ	100	400	200
MSG	100	600	200

3. Results

3.1 Effect of pre-conditioning treatment of the mother plant

The plantlets cultivated for three weeks on the propagation Tendille and Lecerf medium modified with 75, 50 or 25 g/l of sucrose, differed in size and in the colour of their foliage (Fig 1). The regeneration rate of the control 'Bintje' (meristems not plugged in liquid nitrogen) was 90 %, 100 %, and 81 % at sucrose concentrations of 25, 50 and 75 g/l, respectively, while the regeneration rate of the cryopreserved meristems of 'Bintje' was 31 %, 42 %, and 18 % at same concentrations (Fig 2). With a concentration of 75 g/l in the mother plant culture medium, the reduction of meristem regeneration ability after cryopreservation is significant for 'Bintje'.

The regeneration rate of the control 'Desiree' was 100 %, 90 %, and 90 % at sucrose concentrations of 25, 50 and 75 g/l, respectively, while the regeneration rate of cryopreserved meristems of 'Desiree' was 59 %, 40 %, and 52 % at same concentrations (Fig 2). No significant difference in meristem regeneration ability was observed according to sucrose concentration in the mother plant culture medium for 'Desiree'.

Fig 1. Three-week old plantlets on propagation medium with sucrose concentrations of 75 (left), 50 and 25 g/l (right)

During the preparation of the mother plant, adding seven days of pre-culture of the stem sections to homogenize the meristem state prior to sampling significantly decreased survival and regeneration rates of meristems in both 'Bintje' and 'Desiree' ($p < 0,05$; $\chi 2$ tests, Table 2).

3.2 Effect of the composition of the regeneration medium

Even within the same species (i.e. *Solanum tuberosum*), genotype had the strongest effect on the regeneration rate, the 'Bintje' and 'Europa' usually being the most reactive and 'Desiree' and 'Franceline' the least.

Globally, the best results were obtained with low GA_3 and high or medium zeatin concentrations for almost all *S. tuberosum* varieties while IVP 48 regenerated better with high levels of GA_3 in the medium. No effect of GA_3 or of zeatin was observed on regeneration rates of the 'Noisette'.

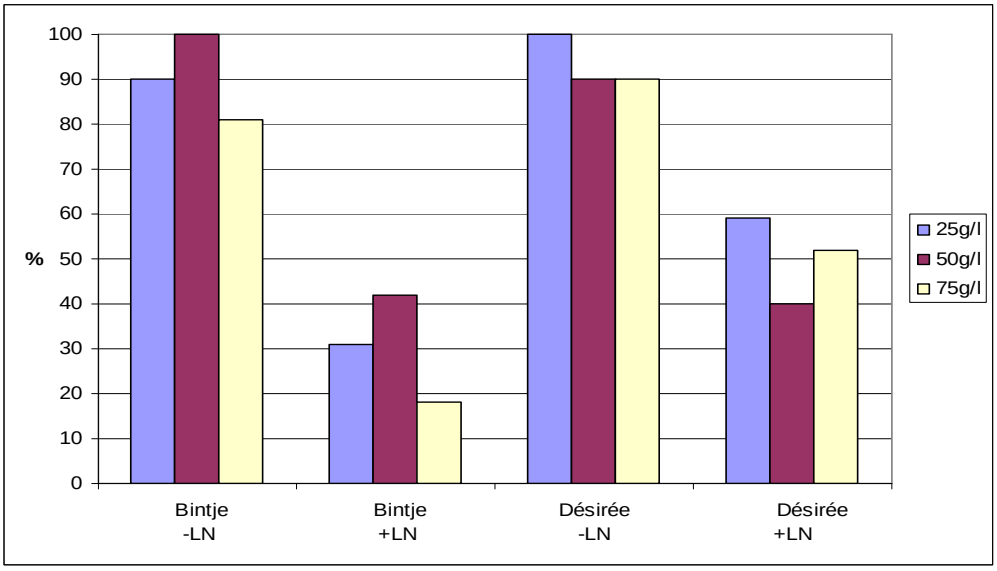

Fig 2. Percentage of plant regeneration from control (-LN) and from cryopreserved (+LN) potato shoot tips as a function of the sucrose concentration of the initial mother plantlet propagation medium. For each treatment 10 shoot tips for –LN and 20 shoot tips for +LN were sampled and cultured. Differences of less than 20% are not significant ($p < 0,05$; $\chi 2$ tests).

Table 2. Effect of the pre-culture period on homogenization of the meristem state during the preparation of the mother plant

Meristem excised on	Rates (%)	Desiree	Bintje
Stem section with one axillary meristem subcultured for 7 days	Numbers of explants	123	124
	Survival	33	34
	Regeneration	27	20
Plantlets subcultured for 3 weeks (control)	Numbers of explants	33	30
	Survival	65	82
	Regeneration	51	67

4. Discussion

After having used the droplet freezing method and observing abnormalities in regenerated plants in the field (data not shown), we decided to use the encapsulation-dehydration method.
Concerning pre-conditioning treatments, the results we obtained show that by increasing the sucrose concentration from 25 to 75 g/l in the medium, there was no significant increase in the regeneration rate of 'Bintje' nor 'Desiree'. This is in contradiction with the results obtained by Fabre and Dereuddre (1990) who showed that increasing the pre-culture period on high sucrose medium could be beneficial.
The importance of the physiological state of the plant material was already shown by Bouafia *et al*. (1996). To try to homogenize the state of the meristems, stem sections with one axillary bud were subcultured for seven days before meristem sampling. This resulted in a significant decrease in the regeneration rate in both varieties.

We tested different compositions of the regeneration medium to try increasing the regeneration rate and to obtain plants without calli. Significant “genotype x hormone concentration” effects were observed.
All the factors studied proved to have an effect (mainly negative) on plant regeneration after meristem immersion in liquid nitrogen but a consistent variety effect and significant interactions ‘genotype x medium’ were also observed, which made it difficult to identify the best method.

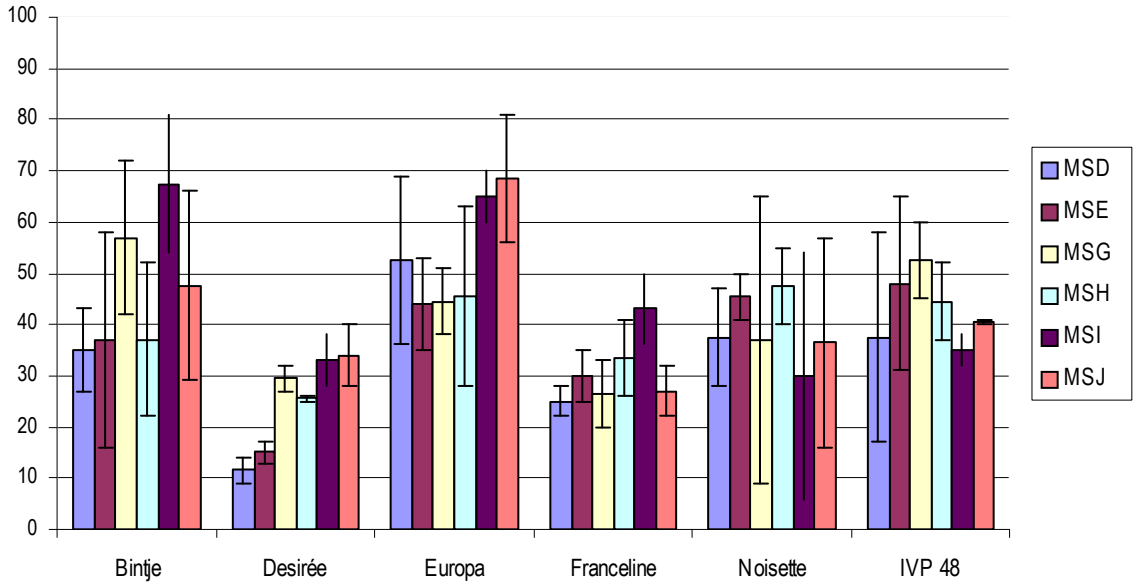

Fig 3. Effect of the hormone concentration (GA_3 and zeatin) in the regeneration medium on the regeneration rate of cryopreserved potato shoot tips of six potato varieties (number of cultured meristem is between 54 and 74). Vertical bars are standard deviations.

5. Acknowledgements

We gratefully thank Dr Florent Engelmann (IRD, Montpellier) and Dr Agnès Grapin (Agrocampus Ouest, Angers) for insightful comments and help on our work. Part of this study was financially supported by the French ANR-Ibisa CRYOVEG project.

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Integration of cryopreservation in French plant genetic resource collections: the CRYOVEG project

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1. Introduction

Cryopreservation (liquid nitrogen, -196 °C) is currently the only technique ensuring long-term, safe and cost-effective conservation of vegetatively propagated plants and of non-orthodox seed species (Engelmann 2011). In France, cryopreservation is presently used only for the conservation of genetic resources of a limited number of forestry species but it is not used for food crops. With the aim of improving the long-term safety and security of national plant germplasm collections through the increased utilisation of cryopreservation, the Consultative Committee for Biological Resources / Infrastructures in the Biology, Health and Agronomy sectors (CCRB/IBiSA) opened in 2008 the Call for Projects "Biological Resource Centers" and funded the CRYOVEG (Cryopreservation of French plant genetic resources collections) project, which had been submitted to this call by a group of French researchers and curators of plant germplasm collections.

The CRYOVEG project aims at 1) developing or optimizing cryopreservation techniques in a range of selected species; 2) establishing a national scientific and technical network of plant biological resource centers (BRCs) using cryopreservation. The project has a network organization, with IRD/INRA Montpellier as the cryopreservation expertise centre and partners in continental France and overseas departments in charge of genetic resource conservation for various species: INRA Petit Bourg, Guadeloupe (yam); INRA San Giuliano, Corsica (*Citrus*); INRA Bordeaux (*Prunus*); INRA Angers (apple and pear); INRA Montpellier (grapevine); INRA Ploudaniel (potato, *Brassica*); IRD La Réunion (coffee); CIRAD Roujol, Guadeloupe (sugarcane); and CIRAD La Réunion (vanilla, garlic).

The project started in September 2009. After a launching meeting held in IRD in October 2009, participants from all BRCs involved performed a training period in Montpellier on cryopreservation of seeds, *in vitro* shoot tips and/or dormant buds, depending on their species of interest, and then implemented the experimental programme established with Montpellier colleagues in their respective laboratories. Several participants also benefited from STSMs funded by COST Action 871, which allowed them to receive additional training in laboratories of European partners. In this paper, we present a brief summary of the results obtained by project participants regarding cryopreservation of dormant buds, *in vitro* cultures and seeds during the first year of the project.

2. Results

Dormant buds

Apple and pear: For the first set of experiments, the cryopreservation technique developed by the NCGRP (National Centre for Genetic Resources Preservation, Fort Collins, USA) was employed. A total of 15 *Malus* and 15 *Pyrus* accessions have been tested. With apple, the mean regeneration percentage was 32 %, with results varying between 0 and 78 %, depending on the accession. With pear, the mean regeneration percentage was 28 %, with results between 0 and 92 %. These results are extremely satisfactory, particularly with, pear, which is considered very difficult to cryopreserve.

Prunus: The experiments have been performed with sweet (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.), using the protocol established by Towill and Forsline (1999). Dormant buds of five *Prunus* genotypes were first dehydrated to various moisture contents, cooled slowly or rapidly and regenerated either through direct grafting on rootstocks or through *in vitro* culture of apices extracted from rehydrated dormant buds. Until now only a limited percentage of regeneration has been obtained, only from *in vitro* cultured apices.

Grape: Experiments were performed with dormant buds of the variety 'Muscat', using various combinations of dehydration (slow or rapid) and cooling (slow or rapid) methods. After rewarming and rehydration, apices were extracted from the buds and introduced *in vitro*. Only apices sampled from buds dehydrated to 25 % moisture content and cooled slowly showed signs of regrowth. This may indicate that there is a difference in the reactivity of shoot tips, depending on the dehydration and cooling procedure.

Citrus: preliminary experiments were performed with *Poncirus* dormant buds. No regrowth has been obtained yet after cryopreservation.

In vitro cultures

Potato: Experiments focused on several parameters of the encapsulation-dehydration technique, including the size of the apices used for cryopreservation, pre-treatment of mother-plants and composition of regeneration medium. The optimal stage of development of shoot tips was between "open leaf primordia" and "closed leaf primordia". As regards pretreatment, there was no positive effect on post-cryopreservation recovery of sampling shoot tips on single node cultures. A culture of mother-plants on medium with high sucrose content had different effects on recovery, depending on the cultivar. Finally, recovery was generally better on medium containing Tendille and Lecerf (1974) mineral elements.

Sugarcane: the encapsulation-dehydration technique was tested on two sugarcane varieties. Recovery of cryopreserved shoot tips varied between 25-54 % for one variety and between 10-30 % for the other.

Yam: the encapsulation-dehydration and droplet-vitrification were compared using *in vitro* shoot tips of one yam variety. Survival was higher with encapsulation-dehydration, reaching

30 %, and less than 20 % with droplet-vitrification, due to the high toxicity of the vitrification solutions employed.
Garlic and vanilla: positive results were achieved with garlic during an initial training period in IPK, Germany. However no positive results were obtained during additional experiments performed in Réunion island, because the plant material employed was not at the right physiological stage. Only preliminary results were performed with vanilla, which did not produce positive results.

Seeds
Traditional vegetable species from Réunion Island: seeds of accessions belonging to various families including Fabaceae, Cucurbitaceae and Solanaceae and of several maize accessions could be successfully cryopreserved using the protocol developed by Dussert *et al.* (1997) for coffee seeds.
Citrus: seeds of 33 varieties belonging to three genera and 14 species were cryopreserved. The materials tested displayed different degrees of tolerance to desiccation and cryopreservation, with some species showing high seed germination after cryopreservation (>80%), others intermediate germination (40-70 %), and others low germination (<18 %).
Brassica: seeds of 17 *Brassica* varieties were employed for cryopreservation experiments. Germination of cryopreserved seeds could be achieved with all materials tested, after partial desiccation of seeds using saturated salt solutions and slow or rapid cooling.
Coffee: seeds of 138 accessions belonging to four species, *C. arabica* (117 accessions), *C. pseudozanguebariae* (5), *C. costatifructa* (6) and *C. racemosa* (5) were cryopreserved. The results showed that processing of the seeds after harvest (moisture content, duration of storage) is of critical importance. They also indicated that it is possible to establish a cryobank of *C. arabica* and of a range of wild coffee species.

3. Conclusion
Very encouraging results have already been achieved during the first year of the project. The experimental programme of all participants for the second year of the project has been established, which should lead to improved results at the end of the project. The establishment of cryopreserved collections in a near future appears as a foreseeable reality for many species included in the CRYOVEG project.

4. Acknowledgements
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Cold hardening and sucrose pretreatment to improve cryopreservation of date palm highly proliferating meristems using vitrification, encapsulation- vitrification and droplet-vitrification protocols: a biochemical investigation

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1. Introduction

Socio-economically, one of the most important perennial plants in Sub-saharan and hot regions is date palm (*Phoenix dactylifera* L.). This is why extensive efforts have been undertaken by the scientific community to overcome constraints hampering the extension of date palm plantations (El Hadrami and El Hadrami 2009). Biotechnological tools are effective to propagate, improve and conserve plant genetic resources (Pati *et al*. 2006; Parveez *et al*. 2000; Engelmann 2004; Panis 2008). In the case of date palm, biotechnologies have already been fully employed for large scale propagation (Fki *et al*. 2003; Fki *et al*. 2010). Nevertheless, biotechnological approaches for date palm improvement and conservation still need additional investigations. This study aimed at producing and cryopreserving highly proliferating meristems which have the capacity to generate true-to-type *in vitro* date palms. A biochemical study was carried out to explain the use of the sucrose preculture and cold hardening phases in the cryopreservation protocol.

2. Materials and Methods

2.1. Plant material

In vitro shoot cultures were established using date palm 'Kenizi' *in vitro* tissue cultures. Explants were a mixture of organs consisting of chlorophyll-free leaves, green leaves and roots.

2.2. Methods

Explants were inoculated on MS medium (Murashige and Skoog 1962) supplemented with 30, 50 and 70 g l^{-1} sucrose to produce highly proliferating meristems. Prior to cryopreservation, meristems were cultured on MS medium enriched with 180 g l^{-1} sucrose or incubated at 4°C for 2, 5 and 10 days. For cryopreservation, standard vitrification, encapsulation-vitrification and droplet-vitrification protocols were applied (Panis *et al*. 2005). For encapsulation, meristems were placed in autoclaved 3 % sodium alginate solution dissolved in MS medium without calcium, then sucked up into a micropipette and dropped in 75 mM $CaCl_2$ $_2$ H_2O (Lakshmana and Singh 1990). Meristem proliferation was performed employing temporary immersion system (TIS). Total soluble proteins were extracted and then electrophorized on SDS-PAGE. Proline content was determined according to Bates *et al.* (1973).

3. Results

Murashige and Skoog medium supplemented with 70 g l^{-1} sucrose was effective to produce highly proliferating meristems from *in vitro* tissue culture. Hypertrophied chlorophyll-free leaves showed the highest morphogentic capacity as they produced numbers of caulogenic meristems after 3 months (Fig. 1). Both sucrose preculture and cold hardening considerably improved post cryopreservation recovery after vitrification. At the biochemical level, these treatments modified the soluble proteins profiles and increased proline content (Fig. 2 and Table 1).

Compared to the standard vitrification protocol, the encapsulation-vitrification and particularly the ultra-rapid droplet freezing techniques proved their high efficiency for cryopreservation of date palm 'Kenizi' highly proliferating meristems. Thus, the highest survival percentages using these techniques were 28, 39 and 61 %, respectively. We also showed that cryopreservation did not affect the morphogenetic capacities of the plant material, and multiple bud cultures were established employing temporary immersion system (TIS). Morphological studies showed the genetic stability of clonal material following cryopreservation.

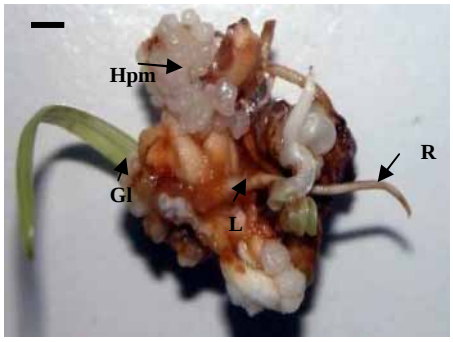

Figure 1. Highly proliferating meristems initiated from date palm 'Kenizi' *in vitro* tissue culture. *Hpm* highly proliferating meristem, *R* root, *Gl* green leaf, *L* hypertrophied chlorophyll-free leaf. Scale bar 2 mm

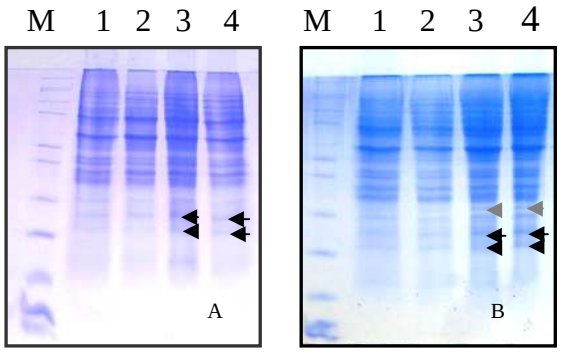

Figure 2. Effects of sucrose preculture (A) and cold hardening (B) on the total soluble proteins profiles of highly proliferating meristems. M: marker; Lane A1: control; Lane A2: 2days 180 g/l sucrose; Lane A3: 5 days 180 g/l sucrose; Lane A4: 10 days 180 g/l sucrose; Lane B1: control; Lane B2: 2 days 4℃; Lane B3: 5 days 4℃; Lane B4: 10 days 4℃.

Table 1. Effect of sucrose (180 g l^{-1}) and cold (4 ℃) treatments on proline content in date palm caulogenic cultures. Experiments were replicated three times.

Duration of treatment (days)	Proline content (µg proline per g FW)	
	Sucrose (180 g l^{-1}) treatment	Cold (4 °C) treatment
0	105.3 a	96.0 a
2	390.0 b	386.0 b
5	383.3 b	395.0 b
10	378.3 b	398.3 b

Data followed by the same letter within the same column are not significantly different according to Duncan's test ($P < 0.05$).

4. Discussion

In this paper, we show that cryopreservation of highly proliferating meristems is a promising tool to establish date palm cryobanks. We proved the benefits of sucrose preculture and cold hardening on post-rewarming regeneration. Both treatments seemed effective to activate genes coding for resistance towards severe osmotic stress and ultra-low temperature. Furthermore, alginate was not toxic to date palm meristems and protected them against cryo-damages. This is in accordance with results obtained by Daikh and Demarly (1987) and Bekheet *et al*. (2002) who confirmed that alginate does not affect date palm somatic embryo germination. Many reports showed the efficiency of the vitrification technique and its two derived protocols, encapsulation-vitrification and droplet-vitrification (see Sakai and Engelmann 2007, for a review). From this study, we conclude that droplet-vitrification is the best technique for date palm germplasm cryobanking.

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Cryopreservation of potato landraces using droplet-vitrification

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1. Introduction

Presently, VIR holds the oldest and one of largest potato collections, consisting of approximately 8,700 accessions including cultivated and wild species. The VIR potato collection has a high practical importance, as it serves as the base for national breeding programs. One of the most important parts of the collection are landraces representing native cultivated species collected by VIR expeditions. The most important task today is to develop safety duplicates for the large field-maintained collection of cultivated potato species.

At present about 250 potato accessions of cultivated species are conserved *in vitro* (Gavrilenko *et al.* 2007). The *in vitro* collection was established with the following objectives: to conserve genetic diversity, to maintain a duplicate of the most important part of the field collections, to avoid losses of plant material maintained in the field collections and to conserve pathogene-free accessions under controlled conditions. Recently we have initiated a cryopreservation program for this *in vitro* material.

2. Materials and Methods

2.1. Plant material

The plant material used consisted of 15 accessions of Andean potato landraces (*Solanum tuberosum* ssp. *andigenum*) and of five accessions of Chilean potato landraces (*S. tuberosum* ssp. *tuberosum*). Each accession was represented by one clonal genotype. For cryopreservation we isolated both apical and axillary buds of 20 accessions (Table 1).

2.2. Cryopreservation techniques employed

In vitro plants were cultivated on MS (Murashige and Skoog 1962) medium at 22ºC, with a photoperiod of 16 h day/8 h night (Fig. 1A). For cryopreservation we used the droplet-vitrification method of Panis *et al*. (2005). Apical and axillary buds (from the upper part of microplants) were transferred in liquid LS medium for dehydration and after 20 min they were transferred in PVS2 solution (at 0°C). Buds were placed in small droplets of PVS2 medium on pieces of aluminum foil (Fig. 1B) and directly immersed in liquid nitrogen. Buds were rewarmed in unloading solution at room temperature.

In total 270 buds per accessions were isolated. Three repetitions were executed for control buds (cryoprotected, not cryopreserved) with 10 buds per repetition for each type of explant and 60 buds per accessions (30 - for control apical buds and 30 - for control axillary buds). 210 buds (both apical and axillary) per accession were cryoprotected and cryopreserved. Three repetitions for each type of explant were executed from the cryopreserved material with 20 buds per repetition to examine regeneration both for apical and for axillary buds – in total 120 buds per accession. Survival and regeneration percentages were determined for this material on week 3, 6 and 8 after rewarming (Fig. 1C). Besides that, 90 explants per accession were left in the cryotank for long-term conservation.

3. Results

As expected, a high correlation was observed between the survival and the regeneration percentages, both for apical and axillary buds (Table 1). Depending on the genotype, regeneration after cryopreservation (scored on week 8) varied from 15% to 86% with apical buds and from 15% to 77% with axillary buds. More than half (11 of 20) accessions had regeneration percentages higher than 50%. The percentage of regenerated plants using apical buds was significantly ($p \leq 0,05$) higher compared to axillary buds in six of 20 accessions: k-2084, k-3231, k-1751, k-1697, k-634, k-4499. The opposite situation was observed only in two accessions (k-3987, k-9002); however in this case differences in regeneration percentages were not significant (Table 1). No statistical differences in regeneration percentages were found between potato landraces belonging to different subspecies - *S. tuberosum* ssp. *andigenum* and *S. tuberosum* ssp. *tuberosum*. Cryopreservation of endangered potato landraces at VIR is in progress.

4. Discussion

The large field-maintained potato collections at VIR require the application of modern techniques for safety duplication. Cryopreservation could ensure the secure and reliable long-term conservation of the germplasm collection. In this study a droplet-vitrification procedure based on the work of Panis *et al.* (2005) was applied to 20 accessions of tetraploid potato landraces from the VIR *in vitro* collection. We showed that the application of this protocol is promising for cryopreservation of diverse tetraploid landraces of *S. tuberosum*, although survival and regeneration percentages were significantly affected by genotype. The regeneration of plants from apical buds was statistically higher than from axillary buds only in six of 20 accessions. In most cases, differences in regeneration between these two types of explants were not statistically significant. These results support the use of axillary buds of potato microplants in practical routine genebank cryopreservation.

5. Acknowledgements

This study was supported by a collaborative project between the CRPGL, Luxembourg, the VIR, Russia and Bioversity International.

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Table 1 Results of application of droplet-vitrification method for 20 potato accessions and two types of explants (apical and axillary potato buds).

№	*S. tuberosum* subspecies	VIR catalog number	Number of apical/axillary buds[+]	Surviving,% apical / axillary buds	Regeneration % apical / axillary buds
1	*ssp. tuberosum*	k-2084*	60/60	31.7±1.4 / 21.0±2.5	21.7±2.0 / 15.1±2.5
2	*ssp. tuberosum*	k-3456	60/60	78.3 ±3.8 / 76.0 ±5.2	78.3 ±3.8 / 76.0 ±5.2
3	*ssp. tuberosum*	k-7573	60/60	22.5±1.4 / 23.0±1.4	15.0±1.4 / 17.9±1.4
4	*ssp. tuberosum*	k-7583	60/60	64.7± 7.1 / 79.3± 7.1	62.7±5.7 / 77.0±6.3
5	*ssp. tuberosum*	Juz-8969	60/60	23.3±1.9 / 21.3±1.8	18.3±1.9 / 20.0±5.8
6	*ssp. andigenum*	k-1688	60/60	21.6±2.0 / 20.0±2.5	16.7±1.4 / 15.0±2.5
7	*ssp. andigenum*	k-1697*	60/60	67.0 ±6.5 / 62.0±5.2	61.7 ±2.2 / 49.1±9.9
8	*ssp. andigenum*	k-1714	60/60	35.0±6.2 / 28.3± 5.1	28.3±5.1 / 23.3±2.7
9	*ssp. andigenum*	k-1751*	60/60	45.0±10.6 / 25.0±2.5	41.8±1.4 / 22.5±1.4
10	*ssp. andigenum*	k-1775	60/60	72.7±9.8 / 78.0±9.3	72.7±9.8 / 76.3±8.5
11	*ssp. andigenum*	k-3231*	60/60	86.3±2.5 / 48.3±1.8	86.3±2.5 / 48.3±1.8
12	*ssp. andigenum*	k-3987	60/60	66.3±3.8 / 75.7±5.2	66.3±3.8 / 75.7±5.2
13	*ssp. andigenum*	k-4617	60/60	27.5±8.6 / 52.5±10.1	17.5±2.8 / 36.2±13.7
14	*ssp. andigenum*	k-4634*	60/60	31.7±1.4 / 20.0±2.5	21.7±2.0 / 15.0±2.5
15	*ssp. andigenum*	k-4499*	60/60	76.0±2.1 / 47.0±2.1	76.0±2.1 / 47.0±2.1
16	*ssp. andigenum*	k-5588	60/60	72.0±2.2 / 67.0±5.3	72.0±2.2 / 67.0±5.3
17	*ssp. andigenum*	k-8201	60/60	62.0±3.5 / 54.0±2.8	62.0±3.5 / 54.0±2.8
18	*ssp. andigenum*	k-9002	60/60	59.3±3.6 / 63.0±1.4	59.3±3.6 / 63.0±1.4
19	*ssp. andigenum*	k-9571	60/60	20.0±5.8 / 22.5±1.4	20.0±5.8 / 17.5±1.4
20	*ssp. andigenum*	k-20579	60/60	68.0±5.0 / 51.0±7.7	68.0±5.0 / 51.0±7.7

*There were significant ($p \leq 0,05$) differences in regeneration between apical and axillary buds.
[+] In addition three repetitions for each type of explant with 10 buds per repetition were used as control for each accession.

Fig. 1 Steps of cryopreservation: *in vitro* plants (**A**), explants in the droplets of the PVS2 on pieces of aluminium foil (**B**) and plant regeneration (k- 1775) after cryopreservation (**C**).

Cryopreservation: an efficient tool for *Pelargonium* species long-term conservation

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1. Introduction

The *Pelargonium* genus (Geraniaceae) plays an important economic role in ornamental horticulture. A *Pelargonium* collection, located in an AGROCAMPUS-OUEST greenhouse has been vegetatively maintained for decades. It contains nearly 500 accessions, including a majority of *P.* x *hortorum* (60%) and *P.* x *peltatum* (25%) cultivars. In order to guarantee this collection, apex cryopreservation studies have been undertaken since 2000 with the following objective: to set up a reproducible and efficient protocol which could be applicable to a large range of genotypes and could produce healthy true-to-type plants.

2. Protocol set up

To set up an efficient protocol *P.* x *peltatum* 'Balcon Lilas' was chosen as model accession. The encapsulation-dehydration approach was first carried out, mainly studying 3 steps of the process: preculture, evaporative dehydration and thawing (Grapin *et al.*, 2001; Dumet *et al.*, 2002; Grapin *et al.*, 2003). Only sucrose, among the osmotica tested at the same osmolarity (glucose, sorbitol), allowed us to obtain an apex tolerance to desiccation which could be compatible with survival after exposure to liquid nitrogen (Grapin *et al.*, 2007).

Then, a droplet-vitrification procedure was developed, adapting a *Musa* protocol (Panis *et al.*, 2005). The optimised durations in the loading solution and in plant vitrification solution 2 were 20 and 15 min respectively (Gallard *et al.*, 2008). This new procedure gave better and more reproducible survival and regeneration results than the droplet-vitrification one. Testing it on 28 accessions, representative of the genus diversity, an average of 65% survival rate was obtained and plants were regenerated for each genotype, except for *P.* x *peltatum* 'Papa Crousse'. The establishment of a cryobank could be then conceivable.

3. Genetic stability of Pelargonium cultivars

As *Pelargonium* is an ornamental plant, the phenotypic stability of the recovered plants is essential. Plants regenerated from control and cryopreserved apices were observed for seven cultivars, using preferentially quantified characters. The regeneration of both cryopreserved and non-cryopreserved apices produced true-to-type plants, except for some variegated cultivars which are periclinal chimeras. This can be corroborated by the fact that apex regrowth was mostly direct, as demonstrated by histological studies.

4. Cryotherapy

The possibility of eradicating the pelargonium flower break virus (PFBV) and pelargonium line pattern virus (PLPV), two viruses widely present in the plants of our collection, by the cryotherapy of axillary shoot apices was investigated.

Immunolocalisation demonstrated the presence of PFBV and PLPV in *Pelargonium* apices, even in the meristematic dome. Apex culture did not eliminate PFBV and only 15 %

regenerated plants of ‘Stellar Artic’ were ELISA PLPV-negative. Cryotherapy was more efficient in producing ELISA-negative plants: 25% and 50% of the plants tested, for PFBV and PLPV respectively. However, immunolocalisation undertaken on apices from the ELISA-negative cryoregenerated plants showed that they were still virus-infected, demonstrating that cryopreservation can only partly reduce the quantity of these viruses in *Pelargonium* plants but not eliminate them totally (Gallard *et al.*, 2011).

5. Dynamic study of cellular events during cryopreservation

Over the last few years, new imaging techniques have been developed, mainly in animal sciences, creating real-time or 3D images. Real-Time Microscopy (RTM) allows us to observe living cells without stain or contrast agents. Confocal Laser Scanning Microscopy (CLSM), combined with special software, allows us to reconstruct tissues or organs three-dimensionally. We have adapted these two techniques in order to better understand *Pelargonium* apex evolution during some steps of a droplet-vitrification protocol, focusing on the structural modifications due to the addition of LS and PVS2 (Gallard *et al.*, 2009). We have demonstrated that it is possible to have a sequence of the events in real time. We have characterised and quantified the LS protecting effect.

6. Conclusion

The best results for *Pelargonium* cryopreservation were obtained with a droplet-vitrification procedure. The protocol is very simple, without any pretreatment or prior *in vitro* step. It was successfully applied on 10 different *Pelargonium* species. For non-chimeral cultivars, the regenerated plants were phenotypically true-to-type.
Recently, cryopreservation on *Rosa* has been undertaken with promising results.

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Table 1: Apex survival and number of regenerated plants of *Pelargonium* accessions after cryopreservation by encapsulation-dehydration or droplet-vitrification.

Cryopreservation procedure		Encapsulation-Dehydration		Droplet-Vitrification				
Species	Cultivars	Nb of apices	Survival (%)	Nb of apices	Survival (%)	Nb of surviving apices	Nb of regrowing plants	Nb plants / Nb apices
Section Ciconium								
x *hortorum*	Alain	24	42	31	58.1	18	11	0.4
	Belle de Granges			39	46.2	18	6	0.2
	Bicolor	24	84	35	71.4	25	13	0.4
	Cahors	24	49	36	88.9	32	12	0.3
	Distinction	8	12	36	72.2	26	22	0.6
	Féerie orange			39	56.4	22	18	0.5
	Fortuna			30	66.7	20	12	0.4
	Isabell	24	33	33	87.9	29	17	0.5
	Neurot			31	90.3	28	20	0.6
	Panaché sud			48	60.4	29	9	0.2
	Renard bleu	24	28	41	78.0	32	15	0.4
	Stellar artic	24	46	34	70.6	24	15	0.4
	Ville d'Ostende			44	75.0	33	32	0.7
x *peltatum*	Avalanche			37	37.8	14	4	0.1
	Balcon lilas	>500	30 to 90	72	66.7	48	31	0.4
	Balcon rouge	80	73	45	64.2	30	16	0.4
	La France	9	44	40	70.0	28	13	0.3
	Martine			33	81.8	27	8	0.2
	Papa Crousse	9	22	32	34.4	11	0	0.0
	Raymonde	10	10	36	55.6	20	4	0.1
acetosum				30	70.0	21	17	0.6
Section Glaucophyllum								
domesticum	Pansy	24	70	29	82.8	24	21	0.7
Section Jenkinsonia								
tetragonum				30	40.0	12	25	0.8
Section Pelargonium								
capitatum				30	90.0	27	19	0.6
crispum				24	70.8	17	11	0.5
cucullatum				40	72.5	29	14	0.4
Section Polyactium								
gibbosum				32	40.6	13	4	0.1
Section Reniformia								
x *fragrans*		8	37	27	14.8	4	1	0.03

First results on cryopreservation by dormant bud technique of a set of *Malus* and *Pyrus* cultivars from the INRA Biological Resources Centre

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1. Introduction

The Pip Fruit Biological Resources Centre of INRA (National Institute for Agricultural Research) located in Angers (France) is in charge of the preservation, management, characterization and promotion of traditional and scientific genetic resources of apple (8,493 accessions), pear (825 accessions), quince (64 accessions) and related species. The creation of a cryobank should allow us to optimize the security of long term preservation of these germplasm collections.

The development of a cryopreservation program for apple and pear germplasm was initiated in 2010 in the UMR GenHort (Angers) thanks to our participation in the national project CRYOVEG, funded by the French public body IBISA (Biology Infrastructure Health and Agronomy), which aims at developing or optimizing cryopreservation techniques for different plant species maintained in French Biological Resources Centres, and at establishing at the national level a scientific and technical cryopreservation network.

Two training periods in USDA NCGRP, Fort Collins USA (25th January to 29th January 2010) and in JKI Dresden Germany (1st February to 5th February 2010) funded by IBISA and COST respectively allowed us to improve our skills and knowledge about this technique.

The aims of this project were two-fold:

- to validate the reference protocol in our experimental environment (equipment, plant material and climatic aspects),
- to evaluate the response of different genotypes of our germplasm collections.

2. Materials and Methods

2.1. Plant material

The plant material used was a set of diverse genotypes of *Malus* and *Pyrus* from our germplasm collections:

- 15 *Malus* varieties: ancient (11) and modern (2) varieties of dessert apple, ancient varieties of cider apple (2).
- 15 *Pyrus* varieties: ancient varieties of European pear (12), varieties of nashi (3).

2.2. Cryopreservation protocol employed at INRA UMR Genhort

The experimental protocol used in Angers was adapted from reference protocols developed in USDA NCGRP, Fort Collins (Towill *et al*, 2004; Towill and Ellis, 2008). The different steps of protocol were:

- Sampling of graftwoods: the budsticks were harvested in January 2010 in cold conditions (-3 °C). Three consecutive days of negative temperatures before harvesting were observed, which corresponds to the optimal conditions for the reference protocol.
- Storage of graftwoods at -0.5 °C in airtight bag during 4 to 12 weeks.
- Preparing of samples: single nodal sections, 3.5 cm long, with the bud in central position were prepared from budsticks at -0.5 °C.
- Desiccation of nodal sections at -5 °C in an incubator (Sanyo ® MIR 254). The objective was to reach 30 % moisture content (fresh weight basis).
- Slow cooling: Sections were packaged in plastic tubes for slow cooling in a climatic chamber (Binder ® MK53) at 1 °C/h to -30 °C with an additional step at -30 °C for 24 h.
- Storage in plastic tubes in the vapour phase over liquid nitrogen in a liquid nitrogen freezer (Taylor-Wharton ® 750 RS) for 72 h.
- Slow rewarming in plastic tubes at +3 °C for 24 h and rehydration in plastic bags filled with moist peat moss at +3 °C for 8 to 15 days, depending on the variety.
- Regeneration: grafting of the buds by the chip budding technique on MM106 rootstocks for apple and Kirschensaller rootstocks for pear. For each test date and variety, at least 12 buds from cryopreserved material and 6 buds from fresh material were grafted. The rootstocks were planted in pots in greenhouse for 4 to 6 weeks before grafting.

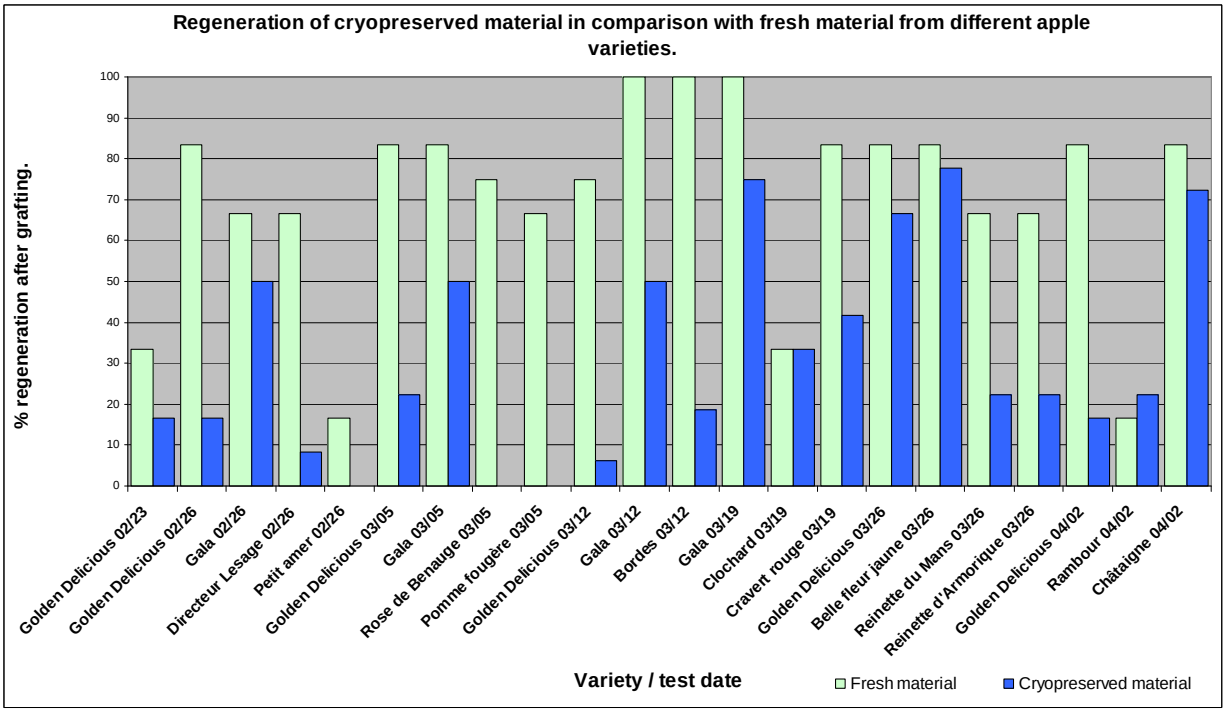

Figure. 1: Regeneration of cryopreserved material in comparison with fresh material from different apple varieties (percentages were calculated from at least 12 buds from cryopreserved material and 6 buds from fresh material).

3. Results

Considering all the experiments, the average regeneration percentage after grafting of fresh material was 69.6 % for apple and 54.6 % for pear, while the average regeneration percentage after grafting of cryopreserved material was 31.8 % for apple and 26.9 % for pear.

For apple (Figure. 1), the regeneration percentages of cryopreserved material ranged from 0 to 77.8 %. Three genotypes ('Petit amer', 'Pomme Fougère', 'Rose de Benauge') did not respond to the technique. When using the same protocol in different series of tests at different dates, regeneration after cryopreservation fluctuated between 6.3 % and 66.7 % for 'Golden Delicious' and between 50.0 % and 75.0 % for 'Gala'.

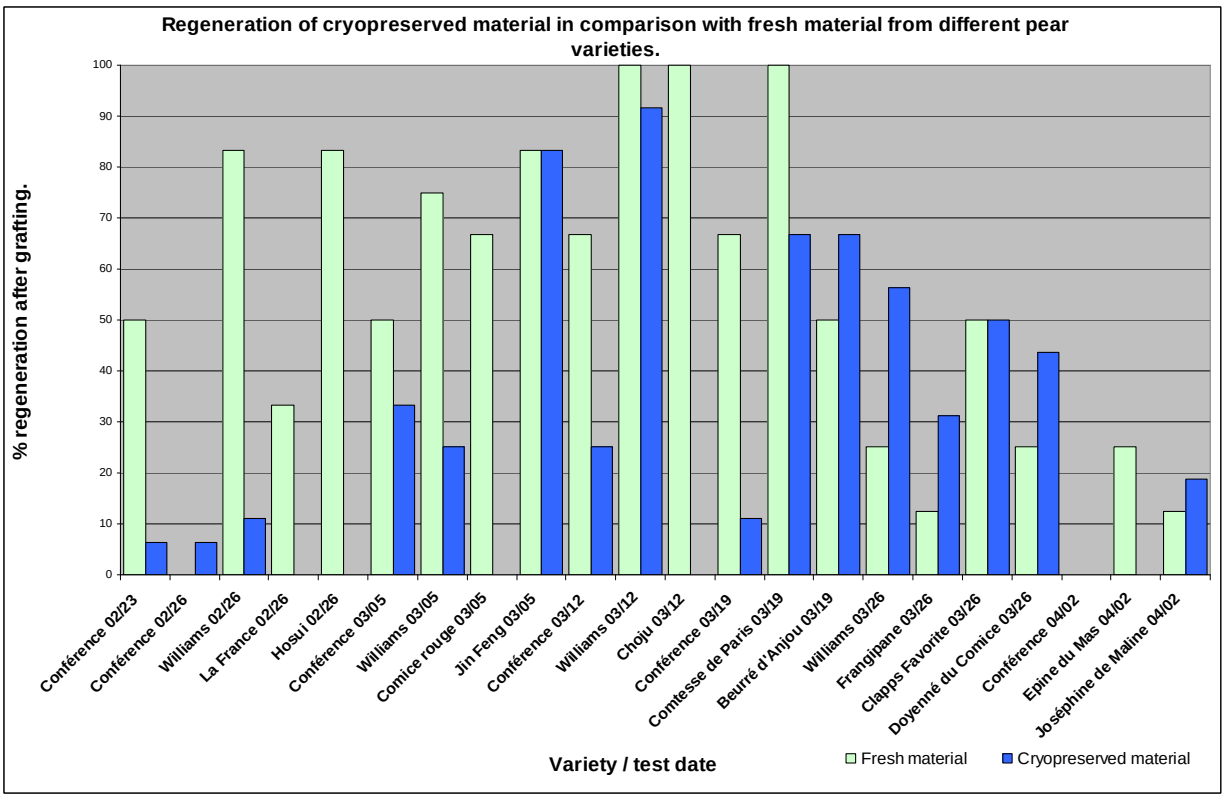

Figure. 2: Regeneration of cryopreserved material in comparison with fresh material from different pear varieties (percentages were calculated from at least 12 buds from cryopreserved material and 6 buds from fresh material).

For pear (Figure. 2), the regeneration percentages of cryopreserved material ranged from 0 to 91.7 %. Five genotypes ('La France', 'Hosui', 'Comice rouge', 'Choju', 'Epine du Mas') did not respond to the technique. When using the same protocol in different series of tests at different dates, regeneration after cryopreservation fluctuated between 11.1 % to 91.7 % for 'Williams'.

4. Discussion

The dormant bud cryopreservation protocol could be successfully applied in our experimental conditions and on our plant material, both with *Pyrus* and *Malus*. These results are very encouraging, especially for *Pyrus* which is reportedly more recalcitrant to the method. However, the current protocol does not yet guarantee a satisfactory regeneration percentage for all genotypes, nor a satisfactory reproducibility of the results for a given genotype. Several factors, which seem to significantly influence the results have been identified: bud

morphotypes, rehydration phase (technique used and duration), rootstock calibre, grafting technique, etc. In 2011, the key points which need to be further examined are the optimal bud residual moisture content, the slow cooling, rewarming and rehydration phases, as well as some technical questions related to grafting.

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Duration of culture of grapevine (*Vitis vinifera*) microcuttings on medium with zeatin riboside affects shoot tip recovery after cryopreservation

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1. Introduction

Cryopreservation is the only method currently available for the safe, cost-effective, long-term conservation of vegetatively propagated plant species such as grapevine. Cryopreservation protocols have been established for shoot tips sampled from grapevine *in vitro* plantlets (Plessis *et al.* 1993; Wang *et al.* 2000; Zhao *et al.* 2001; Matsumoto and Sakai 2003).
An important parameter for successful cryopreservation is the physiological state of the plant material, and buds taken at different levels on the shoots of the stock plants could differ in this regard. It can then be advisable to preculture microcuttings to obtain axillary shoots from which terminal buds can be sampled, constituting a more homogeneous explant population. A cytokinin can be used at this stage to achieve quicker and more homogenous shooting from the microcuttings. Zeatin riboside (ZR) has been shown to stimulate bud proliferation in grapevine (Goussard 1987). As culture duration on ZR medium could affect the physiological state of the plant material, we investigated the effect of this parameter on grape shoot tip recovery after cryopreservation.

2. Material and methods

2.1 Plant material

The plant material employed in this study consisted of *in vitro* plantlets of grapevine (*Vitis vinifera*) cultivar 'Portan', (initial material courtesy of the late Dr A. Bouquet) obtained from the INRA grapevine germplasm field repository in Vassal, France.

2.2 Methods

In vitro culture. *In vitro* plantlets were cultured on half-strength MS medium (Murashige and Skoog 1962) containing 20 g/l sucrose and 7 g/l agar. They were kept without subculture for 2 months at 26 ± 1 °C under a 16 h light/8 h dark photoperiod, with a light intensity of 3000 lux until they were approximately 12 cm long. The plantlets were then dissected in microcuttings consisting of stem fragments of approx. 1.5 cm with one bud, which were placed on culture medium containing 1 µM ZR, and kept on this medium for 4 to 16 weeks with transfer to new medium every 4 weeks. Control samples consisted of shoot tips, which were not cultured on medium with ZR. Mean explants number per treatment was 10.

Cryopreservation. The droplet-vitrification technique was employed for shoot tip cryopreservation. After excision, shoot tips were placed for 24 h on a medium containing 0.3 M sucrose, treated for 20 min with a loading solution containing 1.2 M glycerol + 0.4 M

sucrose, then with cold (0 °C) half-strength PVS2 vitrification solution (Matsumoto and Sakai 2003) for 30 min, dehydrated with full-strength PVS2 for 25, 50 or 75 min, cooled rapidly in liquid nitrogen in PVS2 droplets placed on aluminium foils, rewarmed rapidly by immersion of foils in an unloading solution containing 1.2 M sucrose for 20 min, then transferred for recovery on half-strength MS based with 1 µM BA (Wang *et al.* 2003).

3. Results

Recovery of cryopreserved shoot tips was achieved after up to 8 weeks of culture on ZR medium (Table 1). No regrowth was obtained after longer culture durations. The highest regrowth percentages (40-45 %) were noted with shoot tips cultured on ZR medium for 4 or 8 weeks, which had been treated with PVS2 for 75 min. Regrowth of cryopreserved shoot tips was rapid and direct (Fig. 1). Extended culture duration on ZR medium induced callogenesis during the development of microcuttings (Fig. 2).

Table 1. Effect of culture duration on medium with ZR and of duration of exposure to PVS2 on recovery (%) of grapevine shoot tips after cryopreservation.

Culture duration on ZR medium (weeks)	Recovery (%) **Duration of exposure to PVS2 (min)**		
	25	**50**	**75**
0	33	6	20
4	18	20	40
8	0	30	45
12	0	0	0
16	0	0	0

Figure 1: Regrowth of cryopreserved shoot tip treated with PVS2 for 75 min.

Figure 2: Extended culture duration of shoot tips on ZR medium induced callogenesis.

4. Discussion

This study demonstrated that microcutting culture duration on ZR affected shoot tip recovery percentage after cryopreservation. Shoot tips should be sampled on microcuttings cultured on ZR medium for 0-8 weeks. Although a stimulatory effect of preculture cannot be undoubtedly demonstrated from these experiments, it can be remarked that the highest recovery percentages obtained in this study were achieved after one or two preculture cycles on ZR-containing medium.

Only intermediate recovery was obtained after cryopreservation in these preliminary experiments. Improved results may be achieved notably by modifying PVS2 treatment duration, or by using other vitrification solutions such as PVS3 or recently developed alternative solutions (Kim *et al.* 2009).

5. Acknowledgements

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Cryopreservation of *Prunus cerasifera* Ehrh. shoot tips by encapsulation-dehydration

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1. Introduction

The Fruit Research Institute is a leading scientific institution in the field of fruit growing in Serbia. Besides the long-term scientific research in the field of fruit breeding, the Institute has long tradition of collecting, evaluating and utilizing autochthonous genotypes. The *ex situ* collection of the Institute comprises 1,136 genotypes of different fruit species (cultivars developed at the Institute, autochthonous and introduced cultivars). Therefore, it is of vital importance to prevent the extinction of this extremely valuable germplasm. In recent times, the tissue culture group of the Institute has initiated the application of cryopreservation techniques for long-term conservation of temperate fruit species, which adds substantially to traditional germplasm conservation (Ruzic *et al.* 2010; Condello *et al.* 2010).

2. Materials and Methods

2.1. Experimental design

Twelve combinations were monitored and five apices (explants) were used for each experimental condition (Table 1).

Table 1. Experimental design.

No	Alginate %	Sucrose concentration (M)	Desiccation (h)	Combination mark
1	3	0.75	4	0.75-3/4
2	3	1	4	1-3/4
3	5	0.75	4	0.75-5/4
4	5	1	4	1-5/4
5	10	0.75	4	0.75-10/4
6	10	1	4	1-10/4
7	3	0.75	8	0.75-3/8
8	3	1	8	1-3/8
9	5	0.75	8	0.75-5/8
10	5	1	8	1-5/8
11	10	0.75	8	0.75-10/8
12	10	1	8	1-10/8

2.2. Plant material used

In the present study, *in vitro* grown shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) were tested for regrowth after cryopreservation using a slightly modified encapsulation-dehydration method described by Dereuddre *et al.* (1990).

2.3. Cryopreservation technique employed

For cryopreservation, the encapsulation-dehydration method (E-D) was used. Excised shoot tips, 2–3 mm long (leaves excluded), were encapsulated in alginate beads composed of 3%, 5% and 10% (w/v) low viscosity alginic acid – sodium salt (ACRŌS Organics, Belgium) in liquid Murashige and Skoog medium (MS) (1962) without $CaCl_2$, supplemented with benzyladenine (BA) 1 mg l^{-1}, indol 3-buturic acid (IBA) 0.1 mg l^{-1} and gibberelic acid 0.1 mg l^{-1}, (pH 5.7) and were allowed to polymerise for 30 min at room temperature in MS medium supplemented with 100 mM $CaCl_2$ and 0.06 M sucrose. Encapsulated shoot tips were pre-treated in liquid MS medium with 0.75 or 1 M sucrose for 24 h in the growth room (Fig. 1). Desiccation included placing beads in air-tight containers (3.5 x 4.5 cm; five beads per container) with 8 g silica gel for 4 and 8 h (moisture content ~29% and 20% respectively). Desiccation curves were previously drawn to calculate the moisture content after desiccation between 0 to 24 h. Dried beads were placed in 2 ml polypropylene cryovials (five beads/cryovial) and plunged directly into liquid nitrogen (LN) for at least 1 h. Rewarming involved placing the cryotubes in the air current of the laminar flow cabinet for 2 min. White beads bearing ice were not well dehydrated and were therefore rejected. The beads were then transferred to Petri dishes containing standard medium (MS medium supplemented with BA 1 mg l^{-1}, IBA 0.1 mg l^{-1} and GA_3 0.1 mg l^{-1}, pH 5.7, 0.06 M sucrose and 7.2 g l^{-1} agar and kept in the growth room in the dark for 7 days. Explants which resumed normal development (production of new leaves and/or expansion of small shootlets) 28 days after cryopreservation were considered as regrowing.

Table 2. Regrowth of shoots 28 days after LN treatment.

Combination mark	% beads with ice after immersion in LN	Non-regenerated explants (%)	Regrowth (%)	Average nbr of shoots per explant
0.75-3/4	40	20	40	2.5
1-3/4	0	60	40	2.5
0.75-5/4	0	100	0	-
1-5/4	0	100	0	-
0.75-10/4	100	0	0	-
1-10/4	40	60	0	-
0.75-3/8	0	40	60	2.3
1-3/8	0	100	0	-
0.75-5/8	0	40	60	1.0
1-5/8	0	100	0	-
0.75-10/8	0	100	0	-
1-10/8	0	80	20	1.0

Fig. 1. Encapsulated shoot tips of cherry plum after treatment with 1 M sucrose.

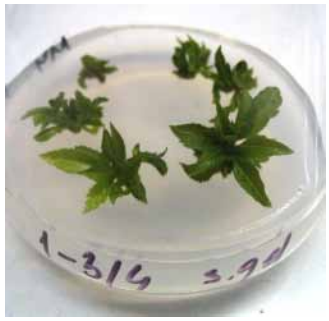

Fig. 2. Shoots after 1st subculture after regrowth from LN.

3. Results

It was only 8 days after the transfer of explants in the light that the first alginate bead was observed to burst. After 1 h immersion in LN some beads contained ice, particularly after a 4 h desiccation period, regardless of alginate concentration. The highest regrowth, amounting up to 60%, was obtained with 8 h desiccation and 0.75 M sucrose pretreatment, regardless of alginate concentration (Table 2). In the first subculture after regrowth, shoots had short stems with tiny, emerging buds, but after two successive subcultures they regained the morphology of *in vitro* cherry plum and had longer stems and a higher multiplication index (Fig. 2).

4. Discussion

The encapsulation-dehydration technique display several advantages: easy handling of samples, simplification of cryoprotective media, elimination of costly programmable freezers, and increased sized of explants withstanding LN storage (Gonzales-Arnao and Engelmann 2006). In this paper we demonstrated that shoot tips of cherry plum could be cryopreserved by E-D technique. Osmotic dehydration in 0.75 M sucrose followed by 8 h desiccation gave the highest regrowth after LN (60%) for explants encapsulated in both 3% and 5% alginate beads. Reed *et al.* (2008), with same cryotechnique, obtained similar regrowth for *Rubus* sp. plantlets following LN. Cryopreserved shoot tips multiplied in successive subcultures had a normal morphology in the second subculture after regrowth, with a similar multiplication capacity compared with non-cryopreserved shoots. Considering our aim, which is to establish a national *in vitro* fruit genebank, these results represent a contribution to the development of standard protocols for maintenance of *in vitro* fruit germplasm, as well as for the introduction of cryopreservation as a new research area in our country.

5. Acknowledgements

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Cryopreservation of *Asplenium cuneifolium* gametophyte and regeneration of plant material in post-rewarming culture

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1. Introduction

The aim of this study was to determine the effect of cryopreservation methods on the viability of *Asplenium cuneifolium* gametophytes, their recovery ability in post-rewarming culture and reproduction through sporophyte generation.

Culture of gametophytes, initially only tree ferns (Goller and Rybczynski 1995; 2007) and later on herbaceous ferns, helped us increasing our garden collections. Later on our experience on how to initiate and carry out gametophyte *in vitro* culture was used for cryopreservation experiments in the framewotk of COST Action 871.

In the sexual life cycle, independent sporophyte and gametophyte generations alternate with each other. The life span of gametophytes (i.e haploid generation) is limited, independent of the systematic position of the species. Gametophytes could complete their whole life cycle in *in vitro* conditions form germinating spore to zygote of sporophyte (Rybczyński and Mikuła 2011). However, their *in vitro* multiplication is quite easy and provides unlimited numbers of explants for various experiments. Such single cell layer explants appeared very useful for cryopreservation experiments. Cryopreservation is actually recognized as the safest method for long-term conservation of plant material in liquid nitrogen. In the Botanical Garden, the encapsulation-dehydration technique was developed. The technique effectively ensured the viability of gametophytes of seven tree fern species and two herbaceous ones. Despite significant differences in resistance to cold environment of sporophytes of the studied fern species, viability of their gametophytes after cryopreservation was high, and attained from 70 to 100% (Mikuła *et al.* 2011).

2. Material and Methods

2.1. Plant material

Gametophyte cultures were initiated from *Asplenium cuneifolium* spores, obtained from the Botanical Garden of Wroclaw University in Poland. The proliferation of secondary gametophytes occurred on ½ MS medium with 2 % sucrose, with pH adjusted to 5.8.

2.2. Cryopreservation techniques employed

Primary gametophytes at the heart stage were subjected to cryopreservation experiments as follows: encapsulation, 2-week long preculture (0.25 M sucrose and 10 µl ABA), 3-day long pretreatment with increasing sucrose concentration (0.5 M, 0.75 M, 1 M), 5-h air desiccation and 3-day long cryostorage. In a second experiment, the encapsulation–vitrification procedure was employed. In this method, encapsulated gametophytes were treated with PVS3 vitrification solution for 0.5 h, 1.0 h, 2.0 and 3 h, then immersed in liquid nitrogen (Fig.1). Encapsulated gametophytes treated with PVS3 solution but non-frozen were designated as controls.

3. Results

Depending on the method employed, gametophytes were encapsulated, thus resulting in the protection of their structural integrity against harmful high concentration of applied cryo-solutions (Mikuła *et al*. 2011). Non-cryostored, encapsulated gametophyte tissues of *Asplenium cuneifolium* withstood PVS3 treatment for 0.5-3.0 h, showing 100% survival (Fig. 2). Cryopreservation resulted in a decrease in survival to 80%. Survival of gametophytes was 100% after cryopreservation by encapsulation/dehydration (Fig. 3). Both methods guaranteed cell viability within individual explants from 80% to 100%. The formation of sporophytes from non-frozen gametophytes (controls) was achieved within 9 months of culture initiation. After cryopreservation, the regeneration process of gametophytes and the reproduction of sporophytes were delayed by about one month in comparison to controls. This period was needed to carry out the cryopreservation procedure. The cryopreservation experiments performed with gametophytes confirmed that the encapsulation-dehydration method developed is more effective than encapsulation-vitrification to ensure high viability and rapid recovery of sporophyte cultures (Fig. 3).

4. Discussion

The combination of gametophyte culture with cryopreservation allowed creating an active program of endangered fern species protection. These results confirmed earlier results achieved with selected species of tree ferns. In the case of encapsulated gametophytes of *Dicksonia fibrosa* and *Cyathea delgadii* (*C. schan-chin*), 100% survival after cryopreservation was achieved, independent of the preculture conditions. Gametophytes of five other species produced 60-80% survival. The application of a 2-week preculture with ABA gave better results compared with preculture without ABA for these species (Makowski *et al.* 2009).

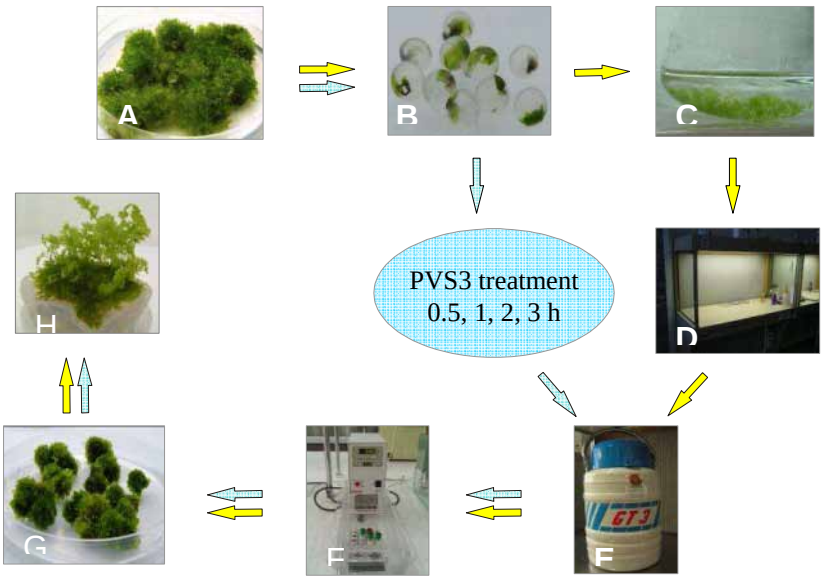

Figure 1. Cryopreservation procedure for *Asplenium cuneifolium* gametophytes using of two different cryotreatments: encapsulation-vitrification (blue) and encapsulation-dehydration (yellow). A) Initial gametophyte culture, B) Encapsulation and preculture, C) Osmotic dehydration, D) Air desiccation, E) Storage in LN, F) Rewarming 38°C/3 min.), G) Recovery culture of gametophyes, H) Sporophyte production in post freezing culture.

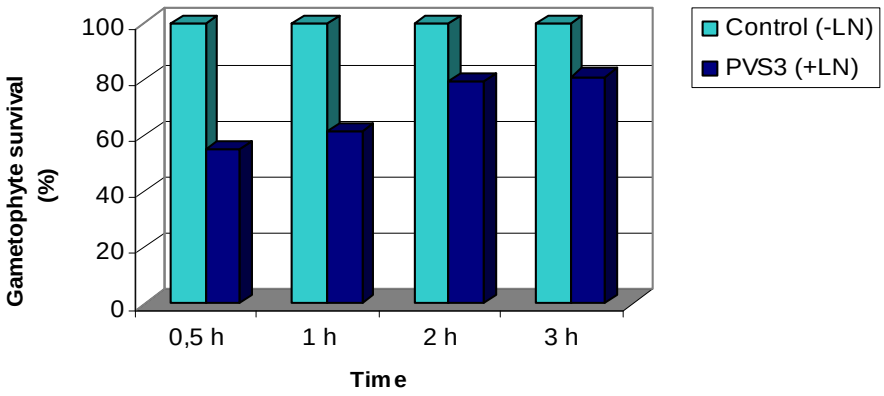

Figure 2. Effect of duration of PVS3 treatment on survival (%) of gametophytes of *Asplenium cuneifolium,* cryopreserved using an encapsulation-vitrification protocol.

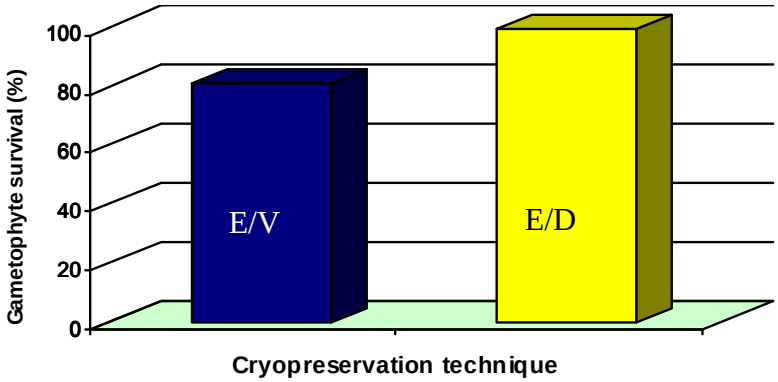

Figure 3. Efficiency of two cryopreservation techniques used with *Asplenium cuneifolium* gametophyte (E/V - encapsulation-vitrification, E/D - encapsulation-dehydration).

5. Acknowledgements

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Cryopreservation of olive embryogenic cultures

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1. Introduction

Olive (*Olea europaea* L.) is the second most important oil fruit tree crop worldwide after oil palm (Baldoni and Belaj 2009). Although it is a typical and widespread tree of the Mediterranean region, in the latest years it has expanded also in non-traditional producer countries such as the United States, Australia and Japan (Pinelli *et al*. 2003). According to the importance of this crop, a number of olive breeding programmes are carried out around the world to produce new varieties. However, different factors such as its long juvenile phase, the low level of fruit set and the erratic seed germination (Acebedo *et al*. 1997) limit the development of new cultivars. Biotechnological approaches will help overcome these problems, thus significantly reducing the time required to get results. The application of biotechnological tools for olive breeding, such as genetic transformation, somaclonal variation, *in vitro* mutagenesis or protoplast manipulation, relies on the availability of embryogenic lines.

Somatic embryogenesis has been successfully achieved in many olive cultivars from different explants (Rugini and Baldoni 2005). Nevertheless, induction of embryogenic cultures is time consuming and, in some cases, can only be carried out when explants are at the desirable stage. Routine maintenance of valuable embryogenic lines by periodic subculturing is also a laborious task and may lead to culture contamination, loss of embryogenic competence and loss of genetic stability. Therefore, embryogenic cultures represent a material of great biotechnological value which is important to adequately conserve. Cryopreservation allows the maintenance of valuable embryogenic lines utilized in bioengineering, avoiding the drawbacks due to repeated subculturing or allowing the storage of transgenic material carrying genes of interest while field trials are ongoing (Lynch 2000).

2. Cryopreservation of olive

Cryopreservation of olive embryogenic cultures was reported for the first time by Shibli and Al-Juboory (2000) who cryopreserved somatic embryos at the cotyledonary stage (1-2 mm long) using encapsulation-dehydration and encapsulation-vitrification. With encapsulation-dehydration, a maximum of 40% regrowth was obtained when beads were precultured for 4 days in hormone-free liquid medium containing 0.75 M sucrose and subsequently dehydrated under the laminar flow cabinet for 4 h (21.1% bead moisture content). Using the encapsulation-vitrification method, the best results (54% regrowth) were obtained when encapsulated embryos were incubated in a PVS2 solution at 0^{o}C for 3 h. A treatment of the embryogenic callus for 1 day at 30^{o}C before encapsulation effectively increased regrowth with both techniques, achieving 50 and 58% regrowth for encapsulation-dehydration and encapsulation-vitrification, respectively.

Lambardi *et al*. (2002) cryopreserved embryogenic tissues composed of an assortment of somatic embryos at different developmental stages, abnormal embryos and other teratomic forms. Both, controlled rate and vitrification/one step freezing procedures were applied in this investigation. The best results were obtained after a 90 min incubation period in the PVS2

solution at 0°C and direct immersion in liquid nitrogen. Under these conditions, 38% of samples showed post-rewarming regrowth.
More recently, Sánchez-Romero *et al*. (2009) studied the influence of the developmental stage of embryogenic explants on toxicity of cryoprotective solutions used in the vitrification-based methods. The results obtained revealed that organized embryogenic tissues were more sensitive to loading solution (LS) and PVS2 than non-organized tissues. Although recovery levels were not significantly affected by cryoprotective solutions for the time periods tested, significantly lower regrowth rates were obtained when somatic embryos were used as cryopreservation explants.
Sánchez-Romero *et al*. (2009) also compared three cryopreservation protocols using non-organized embryogenic tissues: a slow cooling method ($1^{o}C\ min^{-1}$), the "classical" vitrification protocol and droplet-vitrification on aluminium foil strips. The best results were obtained using the droplet-vitrification method after 60 min incubation in PVS2 at 0°C. Although 100% recovery was achieved in both vitrification-based protocols, regrowth rates were significantly higher when using droplet-vitrification. The application of a long-term preculture in basal medium containing 0.4 M sucrose had a significant influence on the initial response of cultures, protecting cells against the toxic effects of the vitrification solutions.
The ultra-fast freezing protocol using droplet-vitrification on aluminium foil strips was subsequently utilized to cryopreserve olive somatic embryos (Bradaï and Sánchez-Romero 2010). In order to optimize this technique for this type of explants, different times of incubation in PVS2 were tested with different cell lines. In agreement with previous observations (Sánchez-Romero *et al*. 2009), the high sensitivity of olive somatic embryos to PVS2 was a generalized response and could be observed in all cell lines tested. Consequently, although high recovery was generally obtained, regrowth rates, six weeks after cryopreservation, were very low in all cases. In order to improve cultures response after cryopreservation, Bradaï and Sánchez-Romero (2011) studied the effect of different sucrose precultures using the droplet-vitrification procedure. In general, sucrose pretreatment increased recovery after cryopreservation; culture regrowth was more vigorous and somatic embryos appearance significantly improved. A decrease in the time required for observing new proliferation was also evident.

3. Conclusion

In conclusion, cryopreservation of olive embryogenic cultures can be considered an achievable objective that can be addressed by using different techniques. Nevertheless, the best results are obtained with droplet-vitrification on aluminium foil strips. Using this technique, it is possible to successfully cryopreserve embryogenic tissues at different developmental stages. Nevertheless, further investigation is advised in order to improve somatic embryos response.

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Survival of *Rosa canina* and *Rosa rubiginosa* meristems after cryopreservation by the droplet-vitrification method

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1. Introduction

As a result of an intensive selection, the genus *Rosa* is today represented by over 30 000 cultivars of modern roses. In rose breeding, wild rose species are of great importance, e.g. *Rosa canina* and *R. rubiginosa* which are frequently found in natural habitats in Poland near Krakow. They are also used in horticulture as ornamental plants and *R. canina* can be used as a rootstock for grafting. The best method of protection of genetic resources and biological diversity is a long-term storage of valuable specimens in liquid nitrogen (Engelmann 2004; Reed 2008).

The aim of the present studies was to achieve high post-cryopreservation regeneration of the meristems isolated from apical buds of *Rosa canina* and *R. rubiginosa* using the droplet-vitrification method.

2. Material and Methods

2.1. Plant material

The bare apical meristems of *Rosa canina* and *R. rubiginosa* (about 0.1 mm) were isolated from apical rose buds with a fragment of the basal tissue from plants grown *in vitro*. Roses were cultivated on the basal MS medium (Murashige and Skoog 1962), supplemented with 1 µM BA and 1.5 µM GA_3, containing 0.087 M sucrose and 0.7 % agar, pH 5.7, under 16 h photoperiod, at photosynthetic photon flux density (PPFD) of 30 µmol m^{-2} s^{-1}, at a temperature of 23/25 °C (night/day) and relative humidity of 80 %.

2.2. Cryopreservation techniques

The meristems were placed in the loading solution LS (2 M glycerol and 0.4 M sucrose in MS medium, pH 5.7) for 20 minutes. Then, meristems were treated for 10-30 minutes with PVS2 solution (0.4 M sucrose, 30 % v/v glycerol, 15 % v/v ethylene glycol and 15 % DMSO in MS medium, pH 5.7). Subsequently, the explants were transferred to aluminum foil strips (0.5 x 2 cm) with a drop of PVS2 (Panis *et al.* 2005). The foil was placed in 2 ml cryotubes (Sigma) filled with liquid nitrogen and then the latter were placed in a cryogenic Dewar.

2.3. Rewarming

Foils strips with frozen rose meristems were immediately transferred to the liquid medium (1.2 M sucrose in MS medium) at 23 °C for 20 minutes. Then they were placed on a sterile filter paper and transferred to a solid medium with the composition equivalent to 50 % MS and with agar concentration limited to 0.5 % for 24 h. Subsequently, explants were located on the basal medium.

2.4. Cultivation

Explants were cultivated at PPFD limited to 5 μmol m^{-2} s^{-1} for seven days after freezing. Afterwards, they were maintained under conditions used during multiplication.

All experiments were conducted in 3 replicates (12 per each), means 36 explants in each variant. Statistical analysis was performed by a combination method, with two independent variables, and confidence level α=0.05 using a Stat Soft Statistica, ANOVA test. Data were compared using Duncan's multiple range test.

3. Results and discussion

Tissue culture is an important approach to long-term conservation of valuable rose germplasms. Among plant materials, apical meristems are most frequently chosen for cryopreservation. There are only a few protocols previously published on *Rosa* cryopreservation (Lynch at al. 1996; Lambardi *et al.* 2002). Previati at al. (2008) obtained 10 % explant regeneration after cryopreservation of two Italian selections of roses using the encapsulation–dehydration method. When this method was applied in cryopreservation experiments with the rose 'New Dawn' by Pawlowska and Bach (2009), 20 % of successful regenerations were achieved. Better results for roses originating from *in vitro* cultures (25-67 % explant regeneration after cryopreservation) were obtained when sucrose preculture was combined with the droplet-vitrification method (Halmagyi and Pinker 2006; Pawlowska 2010).

In the present study, apical rose meristems originating from *in vitro* cultures of *Rosa canina* and *Rosa rubiginosa* apical buds have been used. Post-cryopreservation survival was evaluated by calculating a percent of explants which resumed development after cryopreseravtion and formed green sometimes degenerated leaves 7-21 days after rewarming. After cryopreservation by the droplet-vitrification method, explants untreated with a cryoprotectant did not survive cryopreservation and did not resume development. Some explants treated with PVS2 before cryopreservation, survived and developed green leaves (during 7-21 days after rewarming). The best post-cryopreservation survival was obtained for *Rosa canina* meristems when PVS2 treatment lasted 20 minutes (95 %) (Table 1). A thirty-minute exposure of *R. canina* germplasm to PVS2 significantly lowered post-cryopreservation survival (to 55 %) while a 10-minute PSV2 treatment resulted in 72 % survival. On the other hand regeneration of *Rosa rubiginosa* meristems was the best when PVS2 treatment lasted 30 minutes (83 %) (Table 1). Statistical analysis of the data for this germplasm revealed that a shorter PSV2 exposure (10, 20 min) significantly lowered post-cryopreservation survival. The droplet-vitrification procedure according to Panis at al. (2005) is the most promising technique for cryopreservation of in vitro-cultured *Rosa* meristems. However, further studies of other *Rosa canina* and *Rosa rubiginosa* germplasms and other rose species and varieties are necessary.

Table 1. The effect of genotype and PVS2 treatment time on survival (%) of the rose meristems after cryopreservation by the droplet-vitrification method.

Species	PVS2 treatment		
	10 min.	20 min.	30 min.
Rosa canina	72c*	95e	55b
Rosa rubiginosa	40a	50b	83d

* Mean values designated with the same letters do not differ significantly

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Development of procedure for immunolocalisation of *Tomato Spotted Wilt Virus* in *Impatiens walleriana* apices

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1. Introduction

Impatiens spp. are very popular ornamental plants which can suffer from *Tomato Spotted Wilt Virus* (TSWV) carried in the tissues. TSWV is considered to be widespread, with extremely broad host-range which includes over 1090 host plants, and responsible for numerous epidemics with substantial economic losses. *Impatiens* spp. plants infected with TSWV exhibit different symptoms including chlorotic and necrotic spots and rings on the leaves and necrotic lesions on the stems. Symptom appearance and intensity of virus depend on cultivar susceptibility, time of infection, nutritional and environmental conditions and aggressiveness of the TSWV isolates. Because of usual presence of distinctive symptoms, TSWV infected *Impatiens* plants are no longer marketable since demands of the ornamentals market require only perfectly looking plants. Virus-free plants of *Impatiens* spp. are possible to obtain through meristem-tip culture from plants infected with TSWV (Milošević *et al.* 2010). Using this system, 80 % of *in vitro* plantlets of *I. walleriana* are shown to be virus-free confirmed by DAS-ELISA and RT-PCR. But, only immunolocalization of viruses in the tissue can confirm presence of virus particles even in DAS-ELISA negative plants as it was shown for *Pelargonium* plants (Gallard *et al.* 2009).

The main objective of this study is to make a good protocol for immunolocalization of TSWV in infected *Impatiens* plants. The final goal of the study is to implement cryopreservation techniques as cryotherapy in order to build up an efficient system for elimination of TSWV in *Impatiens* species.

2. Materials and Methods

2.1. Plant material

The plant material consisted of shoot cultures of *I. walleriana*, propagated by tissue culture, which were DAS-ELISA positive for TSWV. DAS-ELISA-negative shoots were obtained by meristem culture and plants regenerated after cryopreservation (vitrification procedure). All shoot cultures of *I. walleriana* were multiplied by tissue culture on Murashige and Skoog (1962) medium supplemented with NAA and BAP (0.1 and 1.0 mgL^{-1}, respectively).

2.2. Histological study

For histological study shoot tips were fixed in glutaraldehyde (4 %) and dehydrated in a set of increasing ethanol solutions [50, 70, 80, 95, 100 % (v/v)] for 10 minutes in each step. Samples were than embedded in resin, Technovit 7100. Slices (3 μm) were cut at room

temperature using microtome Leica RM 2165 to obtain series of ultra-thin sections from the same shoot tips. Slices were stained with toluidin blue and then mounted in DPX before microscopic examination.
The second part of histological observation was the optimization of cryostat procedure. Several parameters have been determined: set box temperature, freeze object temperature and determination of section thickness. For setting the box temperature several temperature regime were analysed (-25, -30 and -40 °C) and corresponding freeze object temperature. To determine the best thickness of sections several size of cross-sections were investigated (8, 16, 18 and 20 μm).

2.3. Immunolocalisation study
Immunolocalization of TSWV was done by using two specific antibodies: primary (TSWV polyclonal chicken antibody which reacts with native TSWV particles) and secondary (Anti-goat anti-chicken Ig antibody with fluorochrome Alexa Fluor 488, Molecular Probes, Cat. number A-11039). Chlorophyll autofluorescence and virus labelling with fluorochrome Alexa Fluor 488 were excited by laser He-Ne (543 nm) and Ar (488 nm) and emission (610 nm and 525 nm, respectively). For each experiment two controls were used: a) sections without secondary antibody, in order to confirm the absence of autofluorescence at the fluorochrome wavelength and b) section with only secondary antibody, in order to demonstrate the lack of no specific hybridization of secondary antibody. Immunological labelled slides were examined under confocal light scanning microscope (Fluoview, Olympus).

3. Results
First, series of histological observations were done on shoot tips in order to determine the morphology of the meristem zone and first two leaves primordia of *I. walleriana* (Fig 1A). Optimization of cryostat procedure showed that sections with 20 μm thickness, cut at -25 ºC of cold chamber and objective temperature at -40 ºC are the best for immunolocalization experiments (Fig 1B).

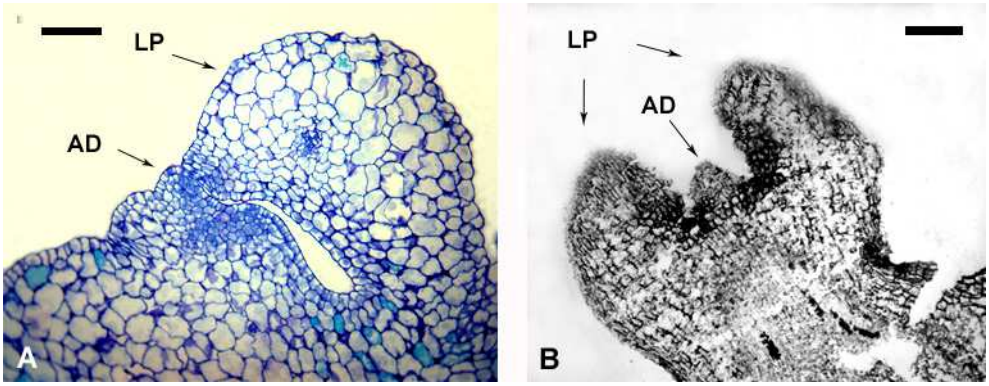

Fig 1. Histological observation of meristem zone with apical dome (AD) and leaves primordia (LP) of *I. walleriana* obtained by resin technique (A, bar 40 μm) and cutting on cryostat Leica CM 3050S on optimal temperature conditions (B, bar 50 μm.).

According to our only very preliminary results, it seems that TSWV was present in all plants samples that we analysed, even in plant material which was indexed negative by DAS-ELISA and, therefore, signed as negative control. Also, according these results virus eradication after cryopreservation, was not observed. However, a very small number of samples was so far analyzed. Further analyses are necessary. These preliminary results indicate that the spread of this virus in *I. walleriana* cultures may be underestimated when only concluded from DAS-ELISA.

4. Discussion

For cryotherapy research it is very important to know the spread of virus particles in plant tissue, especially in apical dome and first leaf primordium (Wang *et al.* 2009). There was no information about morphology of apical dome and first leaf primordium in *Impatiens* spp. and we started with histological study with tissue embedded in resin. Also, for immunolocalisation studies the crucial step is optimization procedure for cutting on cryostat. These investigations were necessary since there was no previously experience of cutting *Impatiens* tissue.

TSWV signals were detected by immunolocalization in all analysed samples, even in seemingly virus-free plants. The spread of this TSWS in *Impatiens* cultures is obviously underestimated due to DAS-ELISA results. Only immunolocalization of viruses can confirm presence of virus particles even in DAS-ELISA-negative plants. This was also recorded for two viruses in *Pelargonium* cultures (Gallard *et al.* 2009).

After these preliminary results we can not conclude that the developed protocol for immunolocalisation of TSWV is very efficient. Also, further research has to be done to improve elimination systems of TSWV in *Impatiens* species by means of cryopreservation.

5. Acknowledgements

This research was done as STSM grant of M. Trifunović at UMG HORTGEN, Angers in COST action 871. The study was supported by Ministry of Education and Science of Serbia (Project TR31019).

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Cryopreservation of cherry plum and blackberry shoot tips by droplet-vitrification

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1. Introduction

During a STSM performed in IRD Montpellier in the framework of COST Action 871, the droplet-vitrification technique was applied to *in vitro* grown cherry plum and blackberry cultivar 'Čačanska Bestrna'. The main advantage of this technique compared with vitrification is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotectant medium in which the explants are placed and the direct contact with liquid nitrogen (Sakai and Engelmann 2007). This paper presents a report on preliminary results with cryopreservation of cherry plum and blackberry cultivar 'Čačanska Bestrna' *in vitro* grown shoot tips using droplet-vitrification. The establishment of the droplet-vitrification protocol was performed by evaluating the effect of different vitrification solutions (VSs) and treatment durations on recovery after liquid nitrogen (LN) exposure.

2. Materials and Methods

2.1. Plant material

Cryopreservation experiments were performed with *in vitro* shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) and blackberry (*Rubus fruticosus* L. cv. 'Čačanska Bestrna'). Aseptic cultures of these species had been previously established at the Tissue Culture Laboratory of Fruit Research Institute, Čačak, Serbia. Shoot tips (1–2 mm in length) were sampled from nodal segments maintained for 3 weeks on MS medium (Murashige and Skoog 1962) containing 0.1 mg l^{-1} N^6-benzyladenine (BA), 0.1 mg l^{-1} indole-3-butyric acid (IBA), 0.1 mg l^{-1} gibberellic acid (GA_3), 20 g l^{-1} sucrose and 7 g l^{-1} agar. Cultures were maintained in growth chamber at 23 ± 1°C, under a 16 h light/8 h dark photoperiod and light intensity of 54 µmol m^{-2} s^{-1}.

2.2. Cryopreservation technique employed

Isolated shoot tips were precultured in the dark at 23°C, in liquid MS medium with progressively increasing sucrose concentration (0.3 M for 15 h, then 0.7 M for 5 h). Loading involved 30 min incubation of explants in a solution comprising 1.9 M glycerol and 0.5 M sucrose (Kim *et al*. 2009a). Explants were dehydrated at room temperature using a modified PVS2 solution (solution A3, Kim *et al*. 2009b) for 10, 20 and 30 min and the PVS3 solution (Nishizawa *et al.* 1993) for 60, 90 and 120 min. Explants were cooled by direct immersion in LN in 10 µl droplets of vitrification solution placed on aluminium foil strips. Foil strips were retrieved from LN and plunged in a preheated (37°C) unloading solution (0.8 M sucrose) for 30 s, then an equal volume of unloading solution at room temperature was added for a further 30 min incubation (Kim *et al*. 2009b). Dehydration controls refer to replicates carried out under the same conditions as cryopreservation experiments but without immersion in LN. Shoot tips were transferred on regrowth medium, cultivated in the dark for 7 days, and then transferred to standard conditions. Survival was evaluated 3 weeks after cryopreservation by

counting the number of shoots that showed any kind of growth, while regrowth was defined as further development of apices into shoots with developed leaves 4–8 weeks after rewarming.

2.3. Statistical analysis

The experiments were replicated twice in two different laboratories (IRD Montpellier, France and Fruit Research Institute, Čačak, Serbia) and 10–15 shoot tips were used per experimental condition. Data presented in the form of percentages were subjected to arcsin transformation and subsequently analysed by ANOVA, followed by Duncan's Multiple Range Test for mean separation.

3. Results

3.1. Cryopreservation of Rubus fruticosus shoot tips

There was no significant effect of PVS treatment (both nature of solution and treatment duration) on regrowth of non-cryopreserved shoot tips of *R. fruticosus* (Fig. 1a). Regrowth of control shoot tips dehydrated with PVS3 ranged between 40% and 77.5%, and 45% and 60% for those dehydrated with modified PVS2 (A3). As for regrowth of cryopreserved shoot tips, PVS3 was better than the modified PVS2, but the difference was significant ($P< 0.05$) only for the shortest treatment duration. Also, PVS3 treatments provided fast-growing and more vigorous plantlets after cryopreservation (Fig. 2) in comparison with PVS2 (A3) treatments. The duration of PVS3 treatment had no significant effect on regrowth of cryopreserved shoot tips (45.8–70%), while the longest (30 min) exposure of explants to A3 solution resulted in a significant increase in regeneration percentage (30%) in comparison with a 10-min treatment with the same solution (5%).

3.2 Cryopreservation of Prunus cerasifera shoot tips

No significant influence of PVS treatment on regrowth of both dehydrated controls and cryopreserved shoot tips was observed in *P. cerasifera*. Regrowth of control explants ranged between 15% and 45.6%, and between 5% and 20% for cryopreserved samples (Fig. 1b). In addition, control shoot tips (both non-dehydrated and non-cryopreserved) loaded with C4 solution showed poor regrowth (37.3%). All explants (both dehydrated controls and cryopreserved shoot tips) grew slowly and showed pronounced signs of hyperhydricity (their stems and leaves were thick, rigid and fragile) (Fig. 3). Very often, regenerating explants formed clusters of small bud rudiments. The same signs were also visible on loading controls.

4. Discussion

One of the most critical steps in developing vitrification-based cryopreservation protocols is dehydration with highly concentrated cryoprotectant solutions. Application of VSs is usually highly species-specific and the duration of dehydration has to be determined very precisely in order to find a correct balance between toxicity and an adequate dehydration to reduce possibility of ice crystal formation in cryopreserved tissues (Panis *et al*. 2005). Our preliminary experiments, conducted in two different laboratories, demonstrated that it is possible to cryopreserve both *Rubus* and *Prunus* genotypes using droplet-vitrification including treatment with both PVS3 and PVS2 (A3) solutions. However, the regrowth percentages achieved and the qualitative characteristics of regrowing shoots were lower in *P. cerasifera*. Although the utilization of PVS3 is limited due to the high osmotic stress it may induce (Kim *et al*. 2009b), in our experiments *R. fruticosus* explants withstood a broad range of treatments with PVS3 without the occurrence of significant decrease in regrowth

percentage. As for the qualitative aspects of different PVS3 treatment durations, a 60 min treatment produced the highest quality plantlets after cryopreservation. This indicates that further optimisation of the protocol for this genotype may be achieved by decreasing the duration of PVS3 treatment. As for droplet-vitrification experiments performed with PVS2, a slight increase duration of PVS2 (A3) treatment and/or the use of other PVS2-based solutions with lower concentrations of dimethylsulfoxide (DMSO) and ethylene glycol (EG) may improve regrowth of cryopreserved shoot tips. The low regrowth of non-frozen controls and the low quality of regenerated shoots of *P. cerasifera* indicate the necessity of identifying the step(s) of the protocol which is/are harmful to shoot tips. Further research should focus on optimizing the preconditioning step (suppression of sucrose pretreatment and/or addition of cold acclimation) and comparing the effect of other loading and vitrification solutions and of shorter treatment durations.

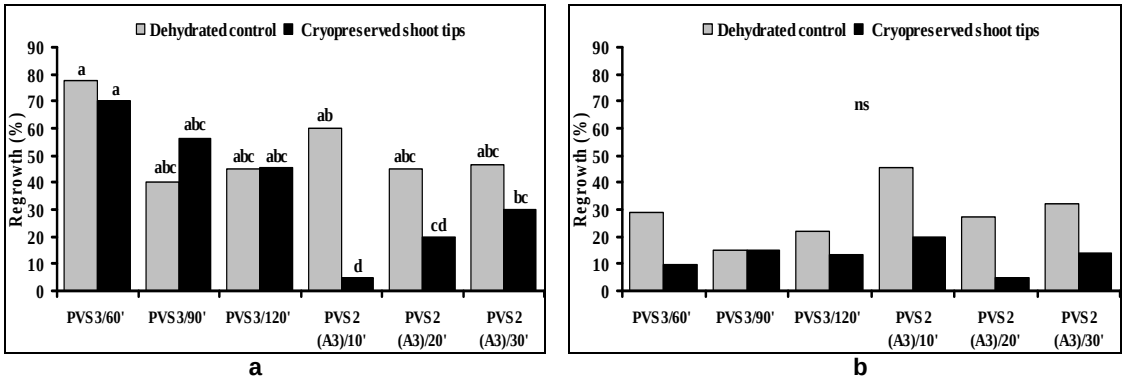

Figure 1. Regrowth of dehydrated controls and cryopreserved shoot tips of *R. fruticosus* (a) and *P. cerasifera* (b) dehydrated with PVS3 and modified PVS2 solution (A3). Mean values followed by the same letter are not significantly different ($P < 0.05$); ns – non significant.

Figure 2. Regrowth of cryopreserved shoot tips of *R. fruticosus* dehydrated for 60 min with PVS3.

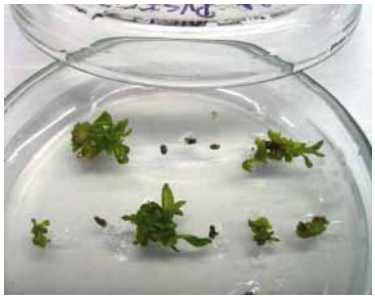

Figure 3. Regrowth of cryopreserved shoot tips of *P. cerasifera* dehydrated for 120 min with PVS3.

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Cryopreservation as a tool in conservation of orchids in Northern Poland

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1. Introduction

All together 46 orchid species belonging to 22 genera are known to inhabit wild sites in Poland. If we consider only the region of Pomerania, 11 species are already extinct and 16 are in direct danger of extinction. Two orchid species, the lady's slipper orchid (*Cypripedium calceolus* L.) and the fen orchid (*Liparis loeselii* L. C. Rich.) are considered to be among the most threatened in Poland and in Europe, therefore recovery plans for these species have been developed. An effective plant conservation program involves a range of various strategies including ecological and genetic studies of plants, habitat management and *ex situ* conservation. Several projects addressing these issues have been undertaken in our laboratory. These include seed banking, asymbiotic germination and identification of mycorrhizal fungi associated with certain orchid species, as terrestrial orchids require the presence of a compatible fungal partner for seed germination and continued growth. The present project focuses on two species, the lady's slipper and the fen orchid. The study includes estimation of genetic diversity among wild populations in Pomerania, hand pollination and collection of seeds ensuring high genetic diversity of *in vitro* raised seedlings. Recently our efforts focused on cryopreservation of orchid seeds and mycorrhizal fungi.

2. Materials and Methods

2.1. Seed collection

Mature *L. loeselii* seed capsules resulting from natural pollination were collected from wild plants in natural habitats in Pomerania. Seed capsules were collected just prior to seed release. Contents from seed capsules were combined and thoroughly mixed. Two accessions of *L. loeseli* seeds were used in the present study: a) seeds from the year 2007 (collected in 2008 after 1 year on site) and stored in 4°C for 2.5 years; and b) fresh seeds collected in 2010. All the seeds were dried over calcium chloride hexahydrate for 3 days prior to storage.

2.2. Seed viability

Assessment of seed viability was undertaken by a modified version of the tetrazolium method (Van Waes and Debergh 1986). Orchid seeds were soaked for 15 min in 5% $Ca(OCl)_2$ and 1% Tween 80 before being washed for 24 h in water and allowed to stain in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 24 h. Completely stained embryos were scored as viable.

2.3. Seed cryopreservation

Only the seeds that proved viable with the tetrazolium test were used for cryopreservation. Seeds were placed in cryovials, plunged in liquid nitrogen (LN) and stored for 1 week. A rapid rewarming method (40°C for 2 min) was used to recover seeds.

2.4. Seed germination

Two methods were applied: asymbiotic and symbiotic. Orchid seeds were sterilised for 15 min in 5% $Ca(OCl)_2$ with a drop of Tween 80, rinsed in water and either placed on oats medium (Clements and Ellyard 1979) for symbiotic germination or on Fast medium (Fast 1974) for asymbiotic germination. Plates intended for symbiotic germination were inoculated with 3 mm agar cubes colonised by symbiotic fungus and incubated in the dark at 18°C. Asymbiotic germination was conducted in dark at 23°C. Germination was assessed after 9 weeks in culture. Germination stages were scored according to Ramsay *et al*. (2003). Each treatment was replicated three times.

2.5. Fungal isolations

Hyphal coils (pelotons) isolated from wild adult plants of *L. loeseli* were cultured *in vitro*. Once putative mycorrhizal fungi were free of contamination, their efficacy was tested by symbiotic germination with *L. loeseli* seeds. After successful germination, the identity of endosymbiont was determined by sequencing of ITS1F (Gardes and Bruns 1993) and ITS4 genes (White 1990) as *Tulasnella calospora* (97% of identity).

2.6. Fungal cryopreservation

Agar cubes colonised by mycelia from cultures of *L. loeseli* mycorrhizal fungus were used for testing storage ability in LN. Three cryoprotectants (10% DMSO, 10% glycerol, 10% glucose) were tested. Fungal isolates were exposed to treatments for 1 h before being plunged in LN. Cultures were cryostored for 10 days before being rewarmed rapidly in a water bath at 40°C for 2 min. Control treatments were treated in the same manner, except that these remained non-frozen. Cultures were revived on two media: PDA and modified FIM (Mitchell 1989) and incubated in the dark at 18°C. Growth rates were recorded by measuring the radius of colonies daily.

2.7. Statistics

Data were analysed by U Mann-Withney Test implemented in Statictica v.9 to test for significant differences in seed viability between treatments.

3. Results

3.1. Seed viability

Unsuccessful asymbiotic and symbiotic germination revealed that seeds collected in 2008 were no longer viable. These results were confirmed by TTC staining and therefore seeds form 2008 were excluded from cryopreservation tests. Viability of seeds collected in 2010, measured by TTC staining, equalled 60% for non-frozen seeds and 47% for frozen ones.

3.2. Seed cryopreservation and germination

Differences in seed germination percentages, between control and cryopreserved seeds, were statistically insignificant ($U=12$, $p=0.58$). Seed viability tested by TTC staining was comparable with the percentage of symbiotic seed germination. Asymbiotic germination,

scored after 9 weeks of culture, was less efficient (mean value14.6%) than symbiotic germination (mean value 50.4%).

3.3. Fungal cryopreservation

Growth rates observed on two media, PDA and FIM, varied and were significantly lower on FIM medium. Also the morphology of hyphae varied depending on the medium (Fig. 1). In control treatments, exposure of fungal isolates to cryoprotectants without freezing revealed no apparent deleterious affects on growth of fungal isolates. Following LN immersion, cultures from all treatments, except for 10% glycerol, survived and revived on FIM (Fig. 2).

4. Discussion

Methods ensuring efficient cryopreservation of *L. loeselii* seeds and its mycorrhizal fungus were described. Orchid seeds are ideal for long-term conservation (seed banking) in LN as seeds are minute, which makes the method very economical. Symbiotic germination levels within the given timeframe, were much higher than asymbiotic ones, as asymbiotic germination is generally slower. Thus, to enable direct comparison between the two germination methods, the experiment should be prolonged, in the case of some species even up to 6 months (Pritchard and Prendergast 1990). For this reason an alternative method for rapid estimation of viability would be advisable. In the case of *L. loeselii*, TTC staining proved to be a good predictor for actual germination efficiency. In the current study, the fungal endosymbiont was found to store well under cryogenic conditions, even without the use of cryoprotectants. Similar findings were already reported by Batty *et al.* (2001) for fungal symbionts associated with Western Australian orchid species. Our results show that *L. loeselii* seeds and fungal endosymbiont associated with this species are suitable for long-term conservation in LN.

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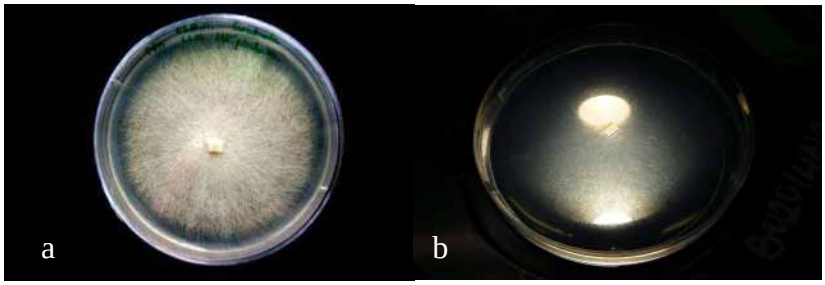

Figure 1. Growth of *Tulasnella calospora* on a) PDA and b) FIM medium.

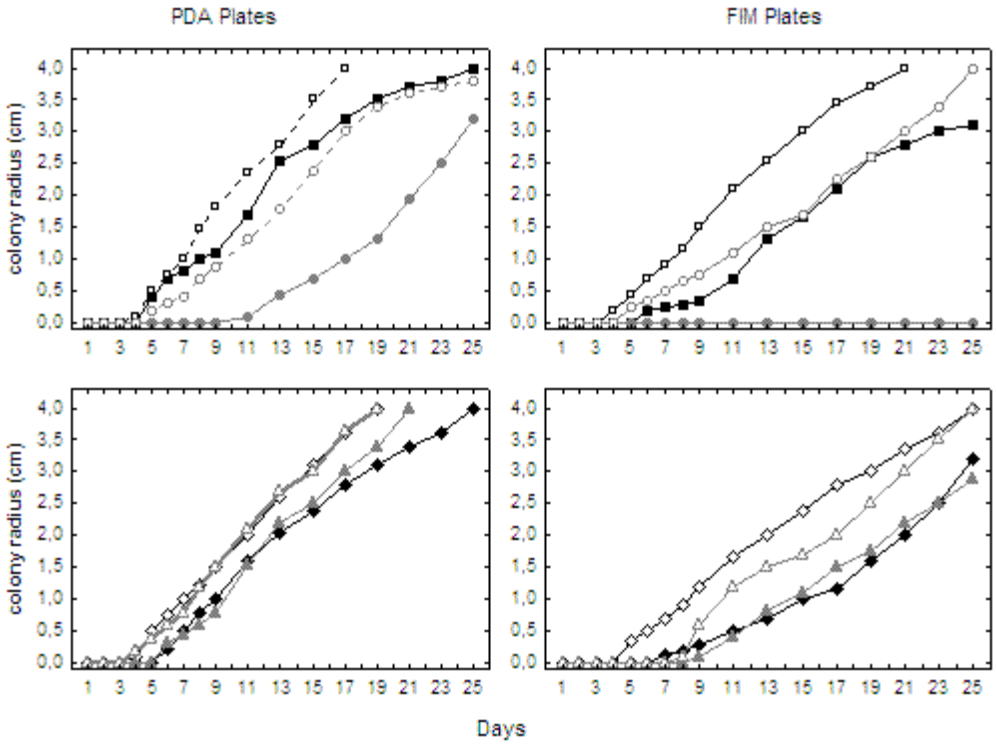

Figure 2. Growth responses of orchid mycorrhiza following either storage in LN (■ - unprotected/frozen, ● - glycerol/frozen, ♦ - glucose/frozen, ▲ - DMSO/frozen) or as non-frozen controls (□ - unprotected control/non-frozen, ○ - glycerol/non-frozen, ◊ - glucose/non-frozen, Δ - DMSO/non frozen).
Isolates were recovered on two media: FIM and PDA.

Part IV
Country Reports

Country Report: Belgium

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1. Major achievements in Belgium related to plant cryopreservation

1.1. Working Group 1

As regards Working Group 1 (Fundamental Aspects of Cryopreservation/Cryoprotection and Genetic Stability), proteomic research using 2D-gelelectrophoresis has been executed at K.U.Leuven, to unravel the mode of action of cryoprotection in banana and to explain cultivars differences after cryopreservation (Carpentier et al; 2007). In collaboration with CIP (International Potato Centre, Lima, Peru), membrane components (sterols and phospholipids) and polyamines were analysed and correlated with the cryopreservation ability of 4 different potato cultivars. The latter research will lead to the PhD degree of Ana Panta, germplasm curator at CIP.

Additionally within this work package, the field behaviour of strawberry and chicory plants issued from cryopreserved samples was compared to controls at the CRA-W in Gembloux.

Table 1. Summary of WG1 research activities performed in Belgium during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
CRA-W, Gembloux	Ph. Druart	• Strawberry • Chicory	Phenotyping and flow cytometry of plants after cryopreservation	2006-2008
K.U.Leuven	B. Panis (in collaboration)	• Banana • Potato	Fundamental aspects of plant cryopreservation (Proteomics and biochemical analyses)	2006-2011

1.2. Working Group 2

Related to WG 2 (Technology, Application and Validation of Plant Cryopreservation), an encapsulation-dehydration protocol was established for hairy roots of the saponin producing plants *Maesa lanceolata* and *Medicago truncatula* at the University of Ghent (Lambert *et al.*, 2009).

Moreover, a start was taken with the long-term storage through cryopreservation of largest *in vivo* germplasm of *Rhododendron simsii* (350 different accessions) at the Institute for Agricultural and Fisheries Research (Melle, ILVO).
Activities at K.U.Leuven where (i) the continuation of cryopreservation of the world banana collection (819 accessions are currently safely stored) and (ii) the development of droplet-vitrification based cryopreservation protocols for a wide variety of plant species. Some of these were developed in collaboration with partners within the COST action

- Potato (CRPGL, Luxemburg, VIR, Russia)
- Chicory and Strawberry (CRA, Gembloux, Belgium)
- Pelargonium (INH, Angers, France)
- Date Palm (Université de Sfax, Tunesia)
- Thyme (Univ Alicante, Spain)
- Olive (IFAPA, Malaga, Spain)
- Hop (Univ Oviedo, Spain)
- Photinia (CNR, Firenze, Italy; Gebze, Turkey)
- Vitis (CNR, Palermo, Italy, PFR, Palmerston, NZ)
- Apple (Fruit Tree Research Institute, Italy)
- Tomato and Bituminaria (University of Valencia, Spain)
- Narcissus (Daffodil) and Galanthus (Snowdrop) (University of Krakow, Poland)

while others were developed in collaboration with partners from non-COST member countries

- Ulluco (CIP, Peru)
- Sweet potato (CIP, Peru)
- Taro and other edible Aroids (SPC, Fiji)
- Cassava (IITA, Nigeria, CIAT, Colombia)

For cryopreservation of embryogenic cultures, the classical (slow) freezing protocol was applied to banana embryogenic cell suspensions, *Fraxinus excelsior* L. embryogenic callus (in collaboration with CNR, Firenze, Italy; Gebze, Turkey) (Ozudogru *et al.*, 2010) and embryogenic tissues of *Pinus nigra* Arn. and hybrid firs (*Abies alba* x *A. cephalonica*, *Abies alba* x *A. numidica*) (In collaboration with the Slovak Academy of Science) (Salaj *et al.*, 2007; 2010).

2. Collaborations initiated during the 4 years of the COST Action

Belgium participants were actively involved in the COST Action; some examples;

- Evelyne Vanvlasselaer (K.U.Leuven) participated at the 2008 DSC workshop in Prague
- Philippe Druart (CRA Gembloux) participated at dormant bud cryopreservation workshop in Kopenhagen:
- Raquel Folgado, University of Oviedo (Spain), Emillano Condello, Fruit Tree Research Institute, Rome (Italy) and Malgorzata Maslanka, University of Agriculture in Krakow (Poland) executed an STSM at K.U.Leuven
- Annelies Vertommen (K.U. Leuven) executed and STSM at Technical University of Denmark, Kgs, Lyngby.
- A WG 2 Meeting was held on the 9th of April, 2009 in Leuven, Belgium. This was a co-organization with ISHS (International Society for Horticultural Sciences)

Many new collaboration were established within the framework of the COST action 871 (see Section 1), many of them resulted in publications in peer reviewed journals.

Table 2. Summary of WG2 research activities performed in Belgium during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
CRA-W, Gembloux	Ph. Druart	• Strawberry • Chicory	Development of cryopreservation protocols using droplet-vitrification	2006
ILVO	J. Van Huylebroeck	• *Rhododendron simsii*	Long-term storage of the largest *in vivo* germplasm collection of Rhododendron	2006-2010
Ghent University	D. Geelen	• *Maesa lanceolata* • *Medicago truncatula*	Establishing a cryopreservation protocol for hairy roots of saponin producing plants	2006-2010
K.U.Leuven	B. Panis	• *Musa* spp.	Cryopreservation of the world banana collection	2006-2011
K.U.Leuven	B. Panis (in collaboration)	• Banana • *Fraxinus excelsior* L. • *Pinus nigra* Arn. • hybrid firs	Cryopreservation of embryogenic cultures using classical (slow) freezing	2006-2011
K.U.Leuven	B. Panis (in collaboration)	• Potato • Ulluco • Sweet potato • Chicory • Strawberry • Taro and other edible Aroids • Pelargonium • Date Palm • Thyme • Olive • Hop • Photinia • Vitis • Apple • Cassava • Tomato • Bituminaria • Narcissus • Galanthus	Development of cryopreservation protocols for shoot tip cultures using droplet-vitrification	2006-2011

3. Future of plant cryopreservation in Belgium

The Laboratory for Tropical Crop Improvement (K.U.Leuven) will make all necessary efforts to keep on playing a leading role in plant cryopreservation research through established contacts with Bioversity International (Rome), The Global crop Diversity Trust (Rome), ISHS (International Society for Horticultural Sciences).

4. Most important publications (2006-2010)

Feki L., Bouaziz N., Sahnoun N., Swennen R., Drira N. and Panis B., (in press). Droplet-vitrification of embryogenic masses for date palm cryobanking. CryoLetters

Carpentier S., Vertommen A., Swennen R., Witters E., Fortes C., Souza Junior M. T. and Panis B., 2010. Sugar-mediated acclimation: the importance of sucrose metabolism in meristems. Journal of Proteome Research 9:5038-5046

Marco-Medina A., Casas J. L., Swennen R. and Panis B., 2010. Cryopreservation of *Thymus moroderi* by droplet-vitrification. CryoLetters 31:14-23

Ozudogru E. A., Capuana M., Kaya E., Panis B. and Lambardi M., 2010. Cryopreservation of *Fraxinus excelsoir* L. embryogenic callus by one-step freezing and slow cooling techniques. CryoLetters 31:63-75

Salaj T., Matusikova I., Panis B., Swennen R. and Salaj J., 2010. Recovery and characterisation of hybrid firs (*Abies alba* x *A. cephalonica* and *Abies alba* x *A. numidica)* embryogenic tissues after cryopreservation. CryoLetters 31:206-217

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Sánchez-Romero C., Swennen R. and Panis B., 2009. Cryopreservation of olive embryogenic cultures. CryoLetters 30:359-372

Wang Q. C., Panis B., Engelmann F., Lambardi M. and Valkonen J., 2009. Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. Annals of Applied Biology 154:351-363

Gallard A., Panis B., Dorion N., Swennen R. and Grapin A., 2008. Cryopreservation of Pelargnonium apices by droplet-vitrifications. CryoLetters 29:243-251

Sant R., Panis B., Taylor M. and Tyagi A., 2008. Cryopreservation of shoot-tips by droplet-vitrification applicable to all taro (*Colocasia exculenta* var. esculenta) accessions. Plant Cell Tissue and Organ Culture 92:107-111

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Panis B., Van den houwe I., Piette B. and Swennen R., 2007. Cryopreservation of the banana germplasm collection at the International Transit Centre - Bioversity International. Advances in Horticultural Science 21:235-238

Salaj T., Panis B., Swennen R. and Salaj J., 2007. Cryopreservation of embryogenic tissues of *Pinus nigra* Arn. by a slow freezing method. CryoLetters 28:69-76

Misson J.-P., Druart Ph., Panis B. and Watillon B., 2006. Contribution to the study of the maintenance of somatic embryos of *Abies nordmannian*a LK: culture media and cryopreservation method. Propagation of Ornamental Plants 6:17-23

Zhu G. Y., Geuns J., Dussert S., Swennen R. and Panis B., 2006. Change in sugar, sterol and fatty acid composition in banana meristems caused by sucrose-induced acclimation and its effects on cryopreservation . Physiologia Plantarum 128:80-94.

Country Report: Bulgaria

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1. Major achievements in Bulgaria related to plant cryopreservation

1.1. Working Group 1

Several young Bulgarian researchers took part in the activity of WG1. In 2010, a PhD thesis partly devoted on using plant cryopreservation methods was successfully defended (Danova 2010). In the course of the PhD studies parts of the dissertation were presented at different scientific events and subsequently published (Danova et al 2009a, 2009b, 2009c, 2009d). The same PhD student also attended a training course on application of Differential Scanning Calorimetry (DSC) in cryopreservation research hosted by the Crop Research Institute, Prague during September 2008. Two other early stage researchers from Sofia University benefited from their participation in COST 871. Some post freeze ultrastructural responses of photosynthetic apparatus in *H. perforatum* L. and *O. stamineus* Benth. were reported (Ganeva *et al.* 2009, Koleva *et al.* 2010). It was also found that cryopreservation affected some basic physiological indices in *Hypericum rumeliacum* Boiss. and *Orthosyphon stamineus* Benth. (Yordanova *et al.* 2009, Yordanova *et al.*, 2009; 2011).

1.2. Working Group 2

Within the thematic area of this working group a short-term scientific mission was successfully realized as result from the collaboration between Forest Research Institute, Sofia and Tree and Timber Institute (IVALSA), Florence. During the research visit the applicability of different vitrification–based cryotechniques for long-term storage of valuable *Populus* spp. germplasm was tested (Tsvetkov et al 2009). It is important to note that quite promising results were obtained after application of droplet-vitrification technique, with data being comparable to those achieved with classical vitrification methods. One Bulgarian representative attended the 2nd Workshop on cryopreservation by using 'Dormant Bud Technique' held from 27 to 28 May 2010 in Florence.

Data about the general Bulgarian activity within the COST 871 action are shown in Tables 1 and 2.

Table 1. Summary of research activities performed with participation of Bulgarian scientists during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
Forest Research Institute, Sofia (FRI)	I. Tsvetkov (Principal foreign collaborator Dr. Lambardi)	*Populus* spp.	Cryopreservation of valuable germplasm	2007
"St. Kliment Ohridski" Sofia University (SU)	V. Kapchina-Toteva (Principal foreign collaborator Prof. Čelárová)	*Hypericum* spp. *Orthosyphon stamineus* Benth.	Evaluation of post-freeze effects on physiological status and photosynthetic apparatus	2007 - 2010

Table 2. General Bulgarian cryopreservation-related activities within the COST 871 action.

Total number of participants	Early Stage Researchers	Defended PhD theses	Number of attended scientific events	Participants in Training courses/Work-shops	STSMs
4*	3	1	8	2	1

*Gender balance: 1M/3F

2. Collaborations initiated during the 4 years of the COST Action

In the time course of COST 871 fruitful collaborations were established between the Forest Research Institute, Sofia and the Tree and Timber Institute (IVALSA), Florence, Italy as well as between "St. Kliment Ohridski" University of Sofia and "P.J. Šafárik" University of Kosice, Slovaklia. The first collaboration resulted in realization of a Short Term Scientific Mission devoted on application of cryopreservation techniques for long-term storage of valuable *Populus* spp. germplasm. The second collaboration started in 2007 with initiation of a bilateral Slovak-Bulgarian research project on "Estimation of cryopreservation impact on basic physiological and genetic characteristics in *in vitro* regenerated plants" (Bg-Sk 101/07). The mutually profitable partnership was extended by new successive project "Cryopreservation and physiological status of the Balkan endemic species *Hypericum rumeliacum* Boiss." (Bg-Sk 209/08).

3. Future of plant cryopreservation in Bulgaria

The general advantages related to participation of the country in the COST 871 could be summarized, as:

- Successful initial steps in using cryotechniques for preservation of plant germplasm have been made;
- Chronologically, the cryo-experiments were the first attempt for long-term storage of valuable plant genetic resources of medicinal plants and forest trees;
- Productive internal and international collaborative links were established which is a good basis for further efficient networking;
- Some Early Stage Researchers/PhD students were either trained or given opportunity to actively take part in various scientific events related to plant cryopreservation;
- Some reasonable number of presentations/publications with participation of Bulgarian researchers was produced during the action.

The common perspectives of plant cryopreservation to be taken forward are:

- The promising results obtained within COST 871 open new horizon towards potential integration of cryotechnologies in to the traditionally strategies used for plant germplasm conservation;
- There is a need for a new global vision of the management of plant genetic resources at a national level consistent with the present and forthcoming challenges. From this point of view, cryotechnologies are supposed to play a key role as an essential tool of current plant biotechnology;
- All institutions interested in the development and application of cryotechnologies for plant germplasm conservation are faced with the need to consolidate their efforts and

take concerted actions, e.g. looking for opportunities of preparing common project proposals covering both fundamental and practical aspects of plant cryopreservation;

- There is a need to initiate local activities oriented to promotion of public awareness about the opportunities of cryotechnologies for long-term preservation of valuable plant germplasm;
- Emphasis should be given on maintaining and promoting the established useful collaborations within the COST Action 871. The national and international network partnerships should be continued and extended in the years to come. A good example of the above is the new proposal for bilateral Slovak-Bulgarian project "Structural and functional changes of *Hypericum* spp. tissues subjected to cryopreservation" submitted in 2010.

4. Most important publications

Danova K, Kapchina-Toteva V (2009a) Cryopreservation – a new method for conservation of *Hypericum rumeliacum* Boiss International Conference 'Economics and Society Development on the Base of Knowledge', 4-5 June, St. Zagora, Bulgaria, Vol. Medical Biology Studies, pp. 90-95. (In Bulgarian, abstract in English)

Danova K, Urbanová M, Skyba M, Čellárová E, Kapchina V (2009b) Evaluation of some physiological markers in Balkan endemic *Hypericum rumeliacum* Boiss. regenerated after cryopreservation, 1st International Symposium on Cryopreservation in Horticultural Species, 5-8 April 2009, Leuven, Belgium, Book of abstracts, p. 73

Danova K, Urbanová M, Skyba M., Čellárová E, Kapchina V (2009c) Utilization of the methods of plant biotechnology for conservation of medicinal plant species. XI Anniversary Scientific Conference "Biology – Traditions & Challenges", 27-29 May, 2009, Sofia, In: http://biofac-conference.com

Danova K, Urbanová M, Skyba M, Čelárová E, Stefanova M, Koleva D, Kapchina-Toteva V (2009d) Impact of cryopreservation on biochemical parameters of *in vitro* cultured *Hypericum rumeliacum* Boiss., International Symposium "New Research in Biotechnology", Series F, Special Volume, ISSN 1224-7774, 19-20 November, USAMV Bucharest, pp. 78-85

Danova, K (2010) *In vitro* cultivation and secondary metabolites in species from genera *Hypericum* and *Pulsatilla*. Cryopreservation of *Hypericum rumeliacum* Boiss., Dissertation, University of Sofia

Ganeva Ts, Stefanova M, Čellárova E, Uzunova Kr, Koleva D (2009) Structural responses of the photosynthetic apparatus of *Orthosyphon stamineus* Benth. to temperature stress after cryopreservation, Botanica Serbica, 33: 163-167

Koleva D, Ganeva Ts, Stefanova M, Skyba M, Čellárova E, Kapchina-Toteva V (2010) Ultrastructural responses of photosynthetic apparatus in *in vitro* cultured *Hypericum perforatum* L. to cryopreservation. COST 871 WG1 meeting, 28-30 April, Poznan, Poster abstract, p. 27

Tsvetkov, I, Benelli C, Capuana M, De Carlo A, Lambardi M (2009) Application of vitrification-derived cryotechniques for long-term storage of poplar and aspen (*Populus* spp.) germplasm, Agricultural and Food Science, 18: 160-166

Yordanova V, Yordanova Zh, Encheva S, Ganeva Ts, Čellárova E, Kapchina-Toteva V (2009) Impact of cryopreservation on basic physiological indices in *Orthosyphon stamineus* Benth., International Conference "Economics and Society Development on the Base of Knowledge", 4-5 June, St. Zagora, Bulgaria, Vol. "Medical Biology Studies", pp. 38-42. (In Bulgarian, abstract in English)

Yordanova Zh, Urbanova M, Čelálrova E, Ganeva Ts, Koleva D, Kapchina-Toteva V (2009) Influence of cryopreservation on the basic physiological indices in propagated *in vitro Orthosyphon stamineus* Benth. Proceedings of the 2nd International Symposium 'New Research in Biotechnology', Series F, Special Volume, ISSN 1224-7774, 19-20 November, USAMV Bucharest, pp. 165-171

Yordanova Zh, Dimitrova M, Čelálrova E, Kapchina-Toteva V (2011) Physiological evaluation of *Hypericum rumeliacum* Boiss. plants regenerated after cryopreservation, COST Action 871 Final Meeting, 8-11 February 2011, Angers, France, Poster abstract, p. 17

Country Report: Czech Republic

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1. Major achievements in the Czech Republic related to plant cryopreservation

1.1. Working Group 1

In the Crop Research Institute (CRI), thermal characteristics of cryopreserved plant material were studied in several crops. The first order transition (crystallization, melting) and the second order transition (glass transition) characteristics were defined in the plant samples. The amount of freezable water, onset of melting temperature and the glass transition temperature were measured by differential scanning calorimetry in garlic, apples, potatoes and grape vine. The results were utilized for cryoprotocol improvement in the Working Group 2 activities.

Cryopreservation protocols of Norway spruce and silver fir embryogenic cultures (Table 1) were elaborated in the Institute of Experimental Botany (IEB). Cryopreservation techniques are currently being used for the cryostorage of more than 100 embryogenic cell lines of these conifers in the IEB. Under the project, the role of endogenous polyamines during cryopreservation was studied and the results published.

1.2. Working Group 2

The activities related to WG2 were focused on cryoprotocol improvement and following routine cryopreservation of several crops in the CRI (Table 1). The CRI participated on EURALLIVEG, the AGRI GEN RES project, focused especially on garlic and shallot cryopreservation in the EU. Altogether 108 accessions which originated in the Czech Republic were successfully cryopreserved. The mean plant recovery of cryopreserved *Allium* plants was 51 %. Selected apple cultivars were conserved by the two-step cryopreservation method using apple dormant buds. The mean shoot regeneration after cryopreservation was 87% for 34 cultivars. Potato and hop germplasms have been routinely cryopreserved in collaboration with the Potato Research Institute Havlickuv Brod and the Hop Research Institute Zatec, respectively. To present, 55 accessions of potato and 50 accessions of hop have been stored in liquid nitrogen. The mean plant recovery was 39% for hop and 29% for potato. Effect of ultra-low temperature on virus elimination has been tested in garlic, potato and hop. A significant effect of cryotherapy on virus elimination was found in garlic and potato. Preliminary results indicated a possible effect on virus elimination in hops as well.

2. Collaborations initiated during the 4 years of the COST Action

2.1. Training Workshops

The CRI organized annual training workshops on thermal analysis utilization for plant cryopreservation for three years during COST Action 871. The workshops were focused on differential scanning calorimetry measurement of real biological samples treated by three different cryopreservation methods. Altogether 18 young scientists from 10 European countries and 1 near neighbor country participated in the workshops.

2.2 Short Term Scientific Missions

CRI hosted Dr. Katarina Brunakova from PJ Safarik University in Kosice, Faculty of Science, Institute of Biology and Ecology, Slovakia. The topic of the STSM was "Thermal analysis of *Hypericum perforatum* L. shoot tips prior to and post vitrification" (Reference Code: COST-STSM-871-05554).

2.3 Other collaborations

Crop Research Institute:

Dr. Matus Skyba (PJ Safarik University in Kosice, Faculty of Science, Institute of Biology and Ecology, Slovakia)

Institute of Experimental Botany:

Dr. Jana Krajnakova (University of Udine, Italy and MENDELU Brno, Czech Republic)

Dr. Marie-Anne Lelu (Nangis, France)

Table 1. Summary of research activities performed in the Czech Republic during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
CRI	Zamecnik	Garlic	Thermal analysis	2007-2010
CRI	Faltus	Potato, Grape vine	Thermal analysis	2009-2010
IEB	Vagner	*Picea abies*	Fundamental research	2007-2010
IEB	Vagner	*Abies alba*	Fundamental research	2007-2010
IEB	Vagner	*Abies nordmanniana*	Fundamental research	2007-2010
IEB	Vagner	*Abies cephalonica*	Fundamental research	2007-2010
CRI	Zamecnik	*Allium*	Cryoconservation	2007-2010
CRI	Jadrna	Apple	Cryoconservation	2008-2010
CRI	Faltus	Potato, Hop	Cryoconservation	2007-2010
CRI	Zamecnik	Garlic	Cryotherapy	2009-2010
CRI	Faltus	Potato, Hop	Cryotherapy	2009-2010

3. The future of plant cryopreservation in the Czech Republic

The dormant bud cryopreservation method showed the most satisfactory results As compared with other cryopreservation approaches used to conserve apple germplasm. Consequently, the cryopreservation of apple dormant buds continues at the CRI. This method is to be tested for pear dormant buds as well. After the termination of the EURALLIVEG project, garlic cryopreservation will continue within the framework of a national project. Cryoconservation of potato and hop collections will continue as well. The research on cryotherapy effectiveness on virus elimination will continue in garlic, potato and hop. As a result of the positive results in potato, the method will be applied to elimination of Potato Leaf Roll Virus (PLRV) and Potato Virus Y (PVY). Based on the WG1 results of thermal analysis, cryoprotocol improvement will continue in potato and grape vine. Fundamental research on cryopreservation of conifer embryogenic cell lines continues in the IEB Prague and the MENDELU Brno.

4. Most important publications

Bilavcik A, Zamecnik J, Faltus M (2007) The use of differential scanning calorimetric analysis of plant shoot tips in cryopreservation, CRYOPLANET, COST Action 871 1st Meeting of the Working Group 1, Oviedo (Spain), April 12-14, 2007, "Fundamental Aspects of Cryopreservation /Cryoprotection and Genetic Stability", 26-29 ISBN AS/1414-2007

Faltus M, Bilavcik A, Zamecnik J (2007) Study of phytohormone composition of growth medium for hop plant recovery improvement after cryopreservation, In: Cryoplanet, 1st Meeting of Working Group 2 - Florence, May 10-12, 2007, pp 12-13

Faltus M, Bilavcik A, Zamecnik J, Svoboda P (2007) Effect of phytohormone composition of nutrient medium on in vitro plant regeneration in hop clones with different sensitivity to indole-3-butyric acid, ISSN 0394-6169, Advances in Horticultural Science, 21: 219-224

Vágner M, Špačková J, Eliášová K, Fischerová L, Vondráková Z (2007) Cryopreservation of Norway spruce embryogenic cultures. Proc. COST 871 Meeting, Oviedo, Spain, pp 34-35

Zamecnik, J, Faltus, M., Bilavcik, A (2007) Cryoprotocol used for cryopreservation of vegetatively prpagated plants in the Czech Cryobank, In: Cryoplanet, 1st Meeting of Working Group 2 - Florence, May 10-12, 2007, pp 50-51

Zamecnik J, Faltus M, Bilavcik A (2007) Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech cryobank, ISSN 0394-6169, Advances in Horticultural Science, 21: 247-250

Bilavcik A, Faltus M, Zamecnik J, Casal R A, Jandurova O M (2008) Dehydration of grapevine dormant buds in relation to cryopreservation. CRYOPLANET - COST Action 871. Agrifood Research Working papers 153. Oulu, Finland, 20-23 February 2008. pp 33-34. Poster abstract

Faltus M, Bilavcik A, Zamecnik J, Svoboda P, Domkarova, J (2008) Establishment of cryobank of potato and hop apices in the Czech Republic. CRYOPLANET - COST Action 871. Agrifood Research Working papers 153. Oulu, Finland, 20-23 February 2008. pp 46-47. Oral abstact

Zamecnik J, Bilavcik A, Faltus M (2008) Temperature Modulated Differential Scanning Calorimetry - a tool for evaluation of plant glass transition at low temperatures. Cryopreservation of crop species in Europe. CRYOPLANET - COST Action 871. Agrifood Research Working papers 153. Oulu, Finland, 20-23 February 2008. pp 26-27. Oral abstact

Cvikrová M, Vondráková M, Eliášová K, Martincová O, Vágner M (2009) Endogenous polyamines in Norway spruce embryogenic cultures during cryopreservation. Proc. 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, April 2009

Faltus M, Zamecnik J (2009) Thermal characteristics of some vitrification solutions. Fundamental Aspects of Plant Cryopreservation. 17-18 Feb 2009, Royal Botanic Gardens, Kew, Millennium Seed Bank, Wakehurst Place. COST Action 871 CryoPlanet. Oral abstract.

Faltus M, Zamecnik J (2009) Thermal characteristics of some vitrification solutions. EU COST Action 871 Abstracts 2009. CryoLetters 30: 382-397

Zamecnik J, Faltus M, Kotkova R. (2009) Glass transition determination in *Allium* shoot tips after dehydration. CryoLetters 30: 382-397 (2009).EU COST Action 871 Abstracts 2009.

Zamecnik J, Sestak J (2009) Biological glasses and their formation during overwintering and cryopreservation of plants. In: Some Thermodynamic, Structural and Behavioral Aspects of Materials Accentuating Non-crystalline States. eds. J. Šesták, M. Holeček and J. Málek, OPS Nymburk, ZČU Plzeň, pp 176-198. SBN 978-80-87269-06-0

Zamecnik J, Faltus M (2009) Evaluation of thermograms from Diffrential Scanning Calorimeter. Fundamental Aspects of Plant Cryopreservation. 17th-18th Feb 2009, Royal Botanic Gardens, Kew, Millennium Seed Bank, Wakehurst Place. COST Action 871 CryoPlanet. Oral abstract.

Zamecnik J, Faltus M (2009) Evaluation of thermograms from Diffrential Scanning Calorimeter. EU COST Action 871 Abstracts 2009. CryoLetters 30: 382-397

Zamecnikova J., Zamecnik J, Faltus M, Fernandez E, Viehmannova I (2009) Preparation of shoot tips by sucrose and dehydration pre-treatment of *Ullucus tuberosus* for cryopreservation. 1st International Symposium on Cryopreservation in Horticultural Species. Leuven, Belgium, 5-8 April 2009. p 133. Oral abstract

Schwarzerová K., Vondráková Z, Fischer L, Bellinvia E, Eliášová K, Havelková L., Fišerová J, Vágner M, Opatrný Z (2010) Actin role in plant embryogenesis: improvement of spruce somatic embryo maturation by the treatment with anti-actin drug latrunculin B. 5th EPSO Conference: 29 August - 2 September, 2010, Finland

Schwarzerová K., Vondráková Z, Fischer L, Bořiková P, Bellinvia E, Eliášová K, Havelková L., Fišerová J, Vágner M, Opatrný Z (2010) The role of actin isoforms in spruce somatic embryogenesis. 12th Conf. of Experimental Biology of Plants, Bull. CSEBR and Sect. of Physiol. of Slovak Bot. Soc., p. 86, Prague, September 14.-17., 2010

Eliášová K, Vondráková Z, Vágner M (2010) Cryopreservation of embryogenic cultures of conifers. 12th Conf. of Experimental Biology of Plants, Bull. CSEBR and Sect. of Physiol. of Slovak Bot. Soc., Prague, September 14.-17., p 164

Vágner, M., Vondráková, Z., Eliášová, K., Martincová O, Cvikrová M (2010) Cryopreservation of Norway spruce embryogenic cultures: levels of polyamines. Advances in Somatic Embryogenesis of Trees And Its Application for the Future Forests and Plantations, Suwon, Korea, August 18-21, 2010

Schwarzerová K, Vondráková Z, Fischer L, Bořiková P, Bellinvia E, Eliášová K, Havelková L., Fišerová J, Vágner M, Opatrný Z (2010) The role of actin isoforms in somatic embryogenesis in Norway spruce. BMC Plant Biology 10: 89

Vondráková M, Cvikrová M, Eliášová K, Martincová O, Vágner M (2010) Cryotolerance in Norway spruce embryogenic cultures: association with anatomical features and polyamines. Tree Physiol. 30: 1335-1348

Zamecnik J, Faltus M (2010) Thermal analysis of plant shoot behavior at ultralow temperature determined by classical and modulated differential scanning calorimetry. 4th Conference Working Group 1, COST Action 871. Fundamental aspects of plant cryopreservation - Cryoprotection and genetic stability. Poznan-Kornik, Poland, 29-30 April 2010. p 17. Oral abstract

Vondráková Z, Eliášová K, Fischerová L, Vágner M (2011) The role of auxins in somatic embryogenesis of *Abies alba.* Cent. Eur. J. Biol. *(in press)*

Vágner M, Vondráková Z, Eliášová K, Martincová O, Cvikrová M (2011) Cryopreservation of Norway spruce embryogenic cultures: levels of polyamines. Proc. of COST 871 Meeting, Angers, February, 2011

Zamecnik J, Faltus M (2011) Behavior of water in plants at low and ultralow temperatures. In: Handbook of Plant and Crop Stress. Ed. Pessarakli M., 3rd Edition. CRC Press, Taylor & Francis Publishing Company, Florida, pp 287-318

Country Report: Denmark

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1. Major achievements in Denmark related to plant cryopreservation

Denmark is a relatively small country and only the researchers from the University of Copenhagen, who were participants in COST 871, are active in plant cryopreservation. There is some interest from colleagues at the Botanic Garden in Copenhagen (also part of the University) which our group is keen to develop, but there is no active programme as yet. The summary details of the group are described below:

Institute	Team leader	Plant species	Thematic area	Period
Univ of Copenhagen	Brian Grout	*Malus* sp ; *Pyrus* sp. ; *Ribes* sp. ; *Phaseolus* sp.	Genetic conservation	2007-2011

The group's research efforts are largely targeted towards dormant bud cryopreservation and it application to the conservation of the genetic resources of fruit trees, in significant part as a direct response to the detail of the mandate for genetic conservation given to the Institute by the Danish government.

The Copenhagen team has benefited significantly from the networking provided by COST 871, taking an active part in all of the meetings held since Oviedo in April 2007. The city was also the venue for a Management Committee meeting, attached to a Symposium of the Society for Low Temperature Biology in September 2008, and. a successful workshop on dormant bud cryopreservation in February 2009.

1.1. Working Group 1

The Danish research activity in Work Group 1, essentially the more biologically fundamental studies, has centred on the issues of contamination of samples, and transport vessels, due to inadvertently cryopreserved micro-organisms in liquid nitrogen (LN), together with basic studies on the variation in response to cryopreservation due to cultivar differences.

1.2. Work Group 2

Work Group 2, containing the more applied studies, is where most of the Danish efforts have been concentrated. The experimental work has focused on understanding and optimising cryopreservation protocols for woody fruit species in the practical setting of working, *in situ* collections of genetic resources. The practical work has been conducted largely at the University Pometum, in Taastrup, which is a contributory collection of NordGen (the Nordic Gene Bank). This field-based collection holds some 760 accessions of apple, of which more than 200 are of Danish origin, and over 250 accessions of other top fruits, mostly pears, plums and cherries. It is the long-term intention to see all of these cryopreserved using dormant bud techniques. Additionally in WG2 there is an ongoing project concerned with the cryopreservation, and recovery, of isolated zygotic embryos from very large seeds, where there are practical and resource issues that complicate the use of LN storage. The intent is to achieve high levels of post-thaw survival without resort to conventional *in vitro* recovery techniques.

2. Collaborations initiated during the 4 years of the COST Action

The COST Action 871 collaboration has been of significant, and unique, value in providing a forum for the transfer of information, techniques and ideas into, and out of, Denmark. The COST Action 871 has provided the platform to strengthen collaboration with the group at the University of Reading (UK) led by Andy Wetten, where interests in woody plant cryopreservation are shared. The links with colleagues in Finland have been significantly strengthened as a result of COST Action 871meetings, and a PhD student exchange between Copenhagen and Oviedo has been arranged as a direct consequence of the COST Action 871network. Additionally, support for work in Differential Scanning Calorimetry at the Thermal Analysis Training Workshop in Prague, and subsequent Short Term Scientific Mission funding to continue this work, has made a significant difference to the scope of study undertaken by one of the Copenhagen-based PhD students and has permanently expanded the capabilities of the laboratory.

3. Future of plant cryopreservation in Denmark

The future for plant cryopreservation in Denmark, at least in the short- to medium- term, will rest with the Copenhagen group. Expansion of activity, inevitably, requires funding and the only likely source at present is via the national commitment to the Nordic Gene Bank, and this avenue is being actively pursued. However, high-quality, applied research in this area will continue at the Taastrup laboratories. There is the possibility of some funding via a new Scandanavian emphasis on healthy fruits and vegetables, and a consequent interest in yields of compounds such as antioxidants. This has generated concern over the available biodiversity in the geographical region and will, it is hoped, generate some practical support for genetic conservation in which cryopreservation might share.

4. Most important publications

As primary authors, members of the Copenhagen group have contributed the papers listed below to the overall portfolio of information generated by COST Action 871. A number of other publications were produced, as a result of external collaboration, where group members were not placed as the first author

Grout BWW (2007) Cryopreservation of plant cell suspensions. In “Methods in Molecular Biology 368 – Cryopreservation and freeze-drying protocols” edited by J.G. Day & G.N.. Stacey, pp 153-161, Humana Press, New Jersey

Grout BWW, Morris GJ. (2008) Contaminated liquid nitrogen storage vessels as potential vectors for pathogens. CryoLetters 29: 74-75 (Abst.)

Toldam-Andersen TB, Krogholm KS, Grout BWW (2008). Cryopreservation of dormant buds in a genebank collection of *Pyrus communis*. CryoLetters 29: 81-82 (Abst.)

Grout BWW, Green J, Benson EE, Harding K, Johnston JW (2008) Cryopreservation of encapsulated ribes meristems and variation between accessions within a cultivar. In “Agrifood Research Working Papers 153 - Cryopreservation of Crop species in Europe” edited by J. Laamamen, M. Uosukainen, H. Haggman, A. Nukari and S. Rantala, p.50, MTT Agrifood Research, Finland. [*www.mtt.fi/mtts/pdf/mtts153.pdf*]

Grout BWW, Morris GJ (2009) Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. Theriogenology 71: 1079–1082

Grout BWW, Green J, Toldam-Andersen TB (2009) Practical benefits of dormant bud cryopreservation for genetic resource conservation. CryoLetters 30: 389-390 (Abst)

Vogiatzi C, Grout B, Toldam-Andersen T. (2010) Understanding the cryosurvival of cold-hardened, winter apple buds – critical water content and the role of non-differentiated secondary primordial. Cryoletters 31: 182-183 (Abst)

Vogiatzi C, Grout BWW, Wetten A, Toldam-Andersen TB. (2010) Critical steps in cryopreservation of dormant winter buds collected under relatively mild winter conditions. Cryobiology 61: 368 (Abst)

Green, J, Grout B. (2010) Cryopreservation of winter buds of nine cultivars of blackcurrant (*Ribes nigrum* L.). CryoLetters 31**:** 341-346

Vogiatzi C, Grout BWW, Wetten A, Toldam-Andersen TB (2011) Cryopreservation of winter-dormant apple buds: I- Variation in recovery with cultivar and winter conditions. CryoLetters. *In press*

Vogiatzi C, Grout BWW, Toldam-Andersen TB, Wetten A (2011) Cryopreservation of winter-dormant apple buds: II - tissue water status after desiccation at -4°C and before further cooling. CryoLetters, *In press*

Country Report: Finland

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1. Major achievements in Finland related to plant cryopreservation

In Finland, cryopreservation research was started in 1990's at the Forest Research Institute (Metla) with the aim to preserve forest tree breeding and research material. In 2004 the Agrifood Research Finland (MTT) started to examine the possibility of utilizing cryo techniques for the long-term preservation of genetic resources of vegetatively propagated plants. Therefore we had great interest in participating COST 871 Action Cryoplanet.

1.1. Working Group 1

Compared with Working Group 2 in COST 871 Action, Finland has been active mainly in Working Group 1. Finland hosted a combined meeting of WG1 and WG2 at Oulu on 20- 23 February 2008. The meeting was organized by the University of Oulu and MTT. A book of abstracts was published by MTT (Laamanen *et al.* 2008). Additionally a special issue of Agricultural and Food Science was published after the meeting.

The active Finnish research institutes in this COST Action were MTT, Metla, the University of Oulu and the University of Helsinki. The cryobank was established at MTT Laukaa for the National Programme for Plant Genetic Resources in spring 2006. Several national research projects were performed during this action followed by long-term back-up preservation of the Finnish national core collections of horticultural plants. Cryopreservation techniques was studied on potatoes and several plant species in families *Betula, Fragaria, Humulus*, *Potentilla, Prunus, Malus, Syringa, Rhododendron, Ribes, Rubus*, and *Vaccinium*. (Häggman et al 2008; Nukari et al 2009; Nukari and Uosukainen 2007; Uosukainen et al 2007). MTT scientist Anna Nukari has undertaken her PhD studies on cryopreservation of raspberry and strawberry at the University of Helsinki. Internationally MTT coordinated the EU-project RIBESCO–Core Collection of Northern European Gene Pool of *Ribes*. This project included evaluation, DNA analysis, selection of plant material, virus indexing and cryopreservation of dormant buds of 53 blackcurrant varieties. Valuable contribution to RIBESCO-project was obtained from the two Small Group Meetings in the frame of COST Action 871 on dormant bud techniques at Copenhagen and Florence. At Metla the general aim of cryopreservation research was to develop and apply methods as a part of forest genetics and tree breeding research (Malabadi and Nataraja 2006; Ryynänen and Aronen 2007).

In the University of Oulu cryopreservation was mostly used as a research tool and also courses on cryopreservation were provided for the students. This COST action has led to new PhD and MSc projects, for example PhD studies of Jaanika Edesi on potato cryopreservation and Saija Rantala work on *Ribes* cryopreservation in collaboration with MTT and Laura Zoratti's MSc work on bilberry cryopreservation. In 2010 we established a cryofacility at the Botanical Gardens of the University of Oulu. Cryotherapy was the research topic at the University of Helsinki and also at MTT. This research activity was greatly inspired by the visiting scientist from China, Dr. Q. C. Wang (Wang et al 2009; Wang and Valkonen 2008; Wang and Valkonen 2009a; Wang and Valkonen 2009b).

The Finnish National Programme for Plant Genetic Resources was established in 2003 and organized under the Ministry of Agriculture and Forestry. The National Advisory Committee for Gene Resources, nominated by the Ministry, is in charge of the activities. MTT is responsible for the coordination of the programme and for the long-term preservation of field and horticultural crop genetic resources. Metla has the responsibility of preservation of forest tree genetic resources. During COST 871 our Programme has advanced outstandingly. On early stage of strategic planning, cryopreservation was included in the Programme. In the guidelines for long-term preservation of Finnish plant genetic resources of fruits, berries and woody ornamentals (Aaltonen *et al.* 2006a; Aaltonen *et al.* 2006b), cryopreservation was accepted as the first back-up preservation method for field collections. In 2006 the preservation of genetic resources of horticultural crop plants was integrated into certified propagation stock and plant propagation scheme. In this context the Ministry of Agriculture and Forestry (2006) accepted cryopreservation as a preservation method also for the certified nuclear stock material. The advantages were clear: (a) The core collection of vegetatively propagated horticultural plants was based on the nuclear stock plant collection, which was disease indexed and approved by plant inspection authorities. (b) the collection was safely preserved in cryobank, *in vitro* or in isolated greenhouses. (c) the knowledge and resources of plant disease testing and eradication techniques and *in vitro* propagation were utilized in both certified production and preservation of genetic resources.

Table 1. Summary of WG 1 research activities performed in Finland during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
MTT Agrifood Research Finland	Merja Veteläinen	Vegetatively propaga horticultural crops	Long-term preservation of gene resources	2006-2011
MTT Agrifood Research Finland	Marjatta Uosukainen	Raspberry, strawberry, potato	Cryopreservation techniques	2006-2008
MTT Agrifood Research Finland	Marjatta Uosukainen	*Dasiphora, Fraga Prunus, Ribes, Rubus, Syringa,*	Long-term cryopreservation of gene resources	2009-2011
MTT Agrifood Research Finland	Anna Nukari	*Malus,Prunus, Rhododendron, Ro Syringa, Vaccinium*	Cryopreservation techniques	2009-2011
MTT Agrifood Research Finland	Jaana Laamanen	*Allium sativum, Humulus lupulus*	Cryotherapy	2010-2011
University of Oulu	Hely Häggman	*Pinus, Populus, Betula*	Preservation research material	2006-2011
University of Oulu	Hely Häggman	Potato (*Solanum*) *Vaccinium*	Cryopreservation techniques	2008-2011
University of Helsinki	Jari Valkonen	*Raspberry, sweetpotato*	Cryotherapy	2006-2009
Metla, Fo Research Institute	Tuija Aronen	*Betula, Fraxinus, Sorbus*	Cryopreservation techniques	2006-2011
Metla, Fo Research Institute	Tuija Aronen	*Betula, Sorbus*	Preservation research and breeding material	2006-2011

1.2. Working Group 2

As regards genetic stability within vegetative propagation and cryopreservation studies at Metla, Malabadi and Nataraja (2006), performed a RAPD study on cryopreserved cultures of *Pinus roxburghii* after in vitro culture and cryopreservation and phenotypic expression of leaf variegation in *Betula pendula* was studied by Ryynänen and Aronen (2007). Genetic fidelity of the regenerated silver birch plants has also been evaluated using RAPD assays together with chromosome analysis. At MTT field testing of strawberry, raspberry and plum have been started.. The results of these studies will be obtained in 2012-2014.

Table 2. Summary of WG 2 research activities performed in Finland during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
Metla, Fo Research Institute	Tuija Aronen	*Betula*, *Pi roxburghii*	Genetic stability after cryopreservation	2006-2009
MTT Agrifood Research Finland	Marjatta Uosukainen	Raspberry, strawberry, plum	Genetic stabil field perfomance	2009-2011

2. Collaborations initiated during the 4 years of the COST Action

2.1. STSM- visits from Finland: In 2009 Anna Nukari from MTT and Jaanika Edesi from the University of Oulu visited in IPK laboratory in Gatersleben, Germany. The aim of these visits was to learn potato cryopreservation methods and DSC techniques. These visits were well appreciated and the collaboration will continue.

2.2. Small group meetings in the frame of COST action 871: Scientist Saija Rantala from MTT participated in Workshops on Cryopreservation by the Dormant Bud Technique in Copenhagen, (25 – 26 February 2009) and in Florence, (26 – 28 April 2010).

2.3. Other co-operation initiated within EU

In 2009, research scientist Veli-Matti Rokka from MTT visited IPK laboratory in Gatersleben to get training in cryopreservation of potato and Jaanika Edesi from the University of Oulu had a short training period on principles on cryopreservation at MTT laboratory at Laukaa. These visits have lead to continuous cooperation. COST Action 871 has also initiated collaboration between the Nordic countries. The NordGen organization arranged in autumn 2007 a workshop on cryopreservation at MTT Laukaa and seminars in Lygnaseter, Norway (2009) and Alnarp, Sweden (2011) on preservation of genetic resources and developments of cryopreservation techniques. Planning to utilize cryopreservation is on going within Scandinavia on different levels.

3. Future of plant cryopreservation in Finland

For the future, we will apply cryopreservation techniques as much as it is technically possible for the long-term preservation of plant gene resources as well as for the preservation of horticultural nuclear stock material and forest tree breeding and research material. MTT will focus on horticultural and agricultural plants and Metla will apply the technique to forest trees. The University of Oulu will in the coming years focus on conservation of native plants

germplasm. In addition we will look forward to provide cryofacility service also for other botanical gardens in Finland in the co-operation framework. Several proposals were given in autumn 2010 to organize and finance the National Programme in the future. The financial solutions are expected in 2011.

4. Most important publications

Aaltonen M, Antonius K, Hietaranta T, Karhu S, Kinnanen H, Kivijärvi P, Nukari A, Sahramaa M, Tahvonen R, Uosukainen M (2006a) Guidelines for long-term preservation of Finnish plant genetic resources. Fruits and berries. (In Finnish with an English abstract). Agrifood Res/Plant Prod 89. MTT, Jokioinen

Aaltonen M, Antonius K, Juhanoja S, Järvelin V, Laamanen J, Nukari A, Peräinen R, Sahramaa M, Uosukainen M, Uusitalo M (2006b) Guidelines for long-term preservation of Finnish plant genetic resources. Woody ornamentals. (In Finnish with an English abstract). Agrifood Res/Plant Prod 91. MTT, Jokioinen.

Häggman H, Rusanen, M, Jokipii S (2008) Cryopreservation of *in vitro* tissues of deciduous forest trees. In: Reed B (ed) Plant Cryopreservation. A Practical Guide. Springer, New York, pp 365-386

Laamanen J, Uosukainen M, Häggman H, Nukari A, Rantala S, (2008) (eds) Cryopreservation of crop species in Europe. CRYOPLANET – COST Action 871 20th - 23rd of February, Oulu, Finland. Agrifood Res Work Papers 153

Malabadi, R B, Nataraja K (2006) RAPD detect no somaclonal variation in cryopreserved cultures of *Pinus roxburghii* Sarg. Propag Ornam Plants 6: 114-120

Ministry of Agriculture and Forestry (2006) The statute of the Ministry of Agriculture and Forestry on certified propagation and plant material. Asetus 9/2006. (in Finnish), MMM Tietopalvelukeskus, Helsinki

Nukari A, Uosukainen M (2007) Cryopreservation in the Finnish national germplasm programme for horticultural plants. Adv Hort Sci 21: 232-234

Nukari A, Uosukainen M, Rokka V-M, Antonius K, Wang Q C, Valkonen J P T (2009) Cryopreservation and their application in vegetatively propagated crop plants in Finland. Agr Food Sci 18:117-128

Ryynänen L, Aronen T (2007) Phenotypic expression of leaf variegation in two *Betula pendula* Roth genotypes following micropropagation, cryopreservation and grafting. Propag Ornam Plants 7: 23-28

Uosukainen M, Laamanen J, Nukari A (2007) Cryopreservation in certified plant production. Adv Hort Sci 21: 258-260

Wang Q C, Cuellar W J, Rajamäki M-L, Hirata Y, Valkonen J P T (2009) Combined thermotherapy and cryotherapy for efficient virus eradication: relation of virus distribution, cellular changes, cell survival and viral RNA degradation in shoot tips. Mol Plant Path 9: 237-250

Wang Q C, Valkonen J P T (2008) Effiecient elimination of sweetpotato little leaf phytoplasma from sweetpotato by cryotherapy of shoot tips. Plant Path 57: 338-347

Wang Q C, Valkonen J P T (2009a) Recovery of cryotherapy-treated shoot tips following thermotherapy of *in vitro*-grown stock shoots of raspberry (*Rubus idaeus* L.). CryoLetters 30 :171-182

Wang Q C, Valkonen J P T (2009b) Cryotherapy of shoot tips: novel pathogen eradication method. Trends Plant Sci 14: 119-122

Country Report: France

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1. Introduction

France is one of the European countries, which has one of the longest histories in plant cryopreservation research. The first paper related to plant cryopreservation was published in 1973 by Coulibaly and Demarly. Research activities took off in the mid-eighties, thanks to a project supported by the French Ministry of Research and Technology involving several private companies and public research institutes. French researchers significantly contributed to the development of plant cryopreservation. The encapsulation-dehydration technique was developed in CNRS Meudon by the group of the late Jean Dereuddre (Dereuddre *et al.* 1990). This technique has been successfully applied to a large range of plant species (Gonzalez-Arnao and Engelmann 2006). The possibility of using cryopreservation to eliminate viruses from shoot tips (the process termed cryotherapy) was demonstrated for the first time by the group of Marthe Brison and Marie-Thérèse de Boucaud in Bordeaux (Brison *et al.* 1997). It was shown subsequently that cryotherapy could be applied to eliminate viruses from a number of plant species (Wang *et al.* 2009). French researchers were also involved in the implementation of the EU-funded CRYMCEPT project, by coordinating Workpackage 3 (Sugars, Stéphane Dussert, IRD Montpellier), which aimed at developing methods for analysing soluble sugars in plant tissues and at studying the relationship between sugar content and tolerance to desiccation and cryopreservation of plant tissues and organs, and Workpackage 9 (Dissemination of protocols and techniques, Florent Engelmann, IRD and IPGRI), through the organisation and implementation of specialized training workshops. Yet another achievement was the establishment of a cryopreserved collection of dormant buds of 444 elm European clones, jointly with German partners, in the framework of project RESGEN CT96-78 (Luc Harvengt, AFOCEL, Nangis; Harvengt *et al.* 2004).

The activity decreased at the beginning of the 2000's, with research activities taking place in a limited number of laboratories, including AGROCAMPUS OUEST Angers, INRA Orléans, IRD Montpellier and Nestlé Tours until the initiation in 2009 of the CRYOVEG project (Cryopreservation of French plant genetic resources collections), financed by the GIS IBiSA, coordinated by IRD Montpellier. The CRYOVEG project, in which 10 Biological Resource Centres (BRCs) participate, is aimed at developing or optimizing cryopreservation techniques for a range of selected species, and at establishing a national scientific and technical network of BRCs using cryopreservation (Engelmann *et al.* 2009).

2. Major achievements in France with respect to plant cryopreservation during the 4 years of the COST Action

The research activities, which took place in France during the 4 years of the COST Action are summarized in Table 1. They were related both to Working Group 1 (Fundamental aspects of

cryopreservation/cryoprotection and genetic stability) and Working Group 2 (Technology, application and validation of plant cryopreservation, chaired by F. Engelmann, IRD). Many of them were initiated in the framework of the CRYOVEG project. Another significant achievement was the joint organisation, between AGROCAMPUS OUEST and IRD, of the final meeting of the COST Action, which took place in Angers, France, in February 2011.

Table 1. Summary of the major research activities performed in France during the course of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
CIRAD Guadeloupe	D. Roques	Sugarcane	Protocol devlpt.	2009-2011
CIRAD la Réunion	M. Roux-Cuvelier	Vanilla, garlic	Protocol devlpt.	2009-2011
CIRAD-INRA Corsica	F. Luro	Citrus	Protocol devlpt.	2009-2011
INRA Guadeloupe	F. Gamiette	Yam	Protocol devlpt.	2009-2011
INRA Bordeaux	E. Balsemin	*Prunus*	Protocol devlpt.	2009-2011
INRA Angers	L. Feugey, A. Guyader	Apple, pear	Protocol devlpt.	2009-2011
INRA Ploudaniel	J.E. Chauvin, A. Label	*Brassica*, Potato	Protocol devlpt.	2009-2011
INRA Montpellier	P. Chatelet	Grape	Protocol devlpt.	2009-2011
IRD la Réunion/ Montpellier	S. Dussert	Coffee	Protocol devlpt.	2009-2011
AGROCAMPUS OUEST	A. Grapin	*Pelargonium*	Cryotherapy Genetic stability Cytological studies	2005-2010
AGROCAMPUS OUEST	A. Grapin	*Rosa*	Protocol devlpt	2010-

3. Collaborations initiated during the 4 years of the COST Action (including STSMs)

Several French scientists implemented STSMs and participated in specialized workshops, as follows:

STSMs:

- David Teyssedre (CIRAD Réunion) in IPK, Gatersleben, Germany, February 2009: cryopreservation of *Allium*
- Arnaud Guyader (INRA Angers) in JKI Dresden-Pillnitz, Germany, February 2010: cryopreservation of apple and pear
- Emilie Balsemin (INRA Bordeaux) in NW-FVA, Hann. Münden, Germany, April-May 2010: Cryopreservation of *Prunus avium*

Workshops:

- Workshop for the dormant bud method in cryopreservation, Copenhagen, 25-26 February 2009 (Agnès Grapin)
- 2nd Workshop on Cryopreservation by the Dormant-Bud Technique, Florence, 26-28 May 2010 (Laurence Feugey)

Foreign researchers also implemented STSMs in French laboratories:

- Tatjana Vujovic (Fruit Research Institute, Čačak, Serbia) in IRD Montpellier, Nov. 2010: Cryopreservation of *Prunus cerasifera* and *Rubus fruticosus*

- Milana Trifunovic (Institute for Biological research, Belgrade, Serbia) in AGROCAMPUS OUEST, Angers, April-May 2010: cryotherapy of *Impatiens*

In addition, collaborative research activities in cryopreservation were initiated between:
- INRA/IRD and Czech and Croatian colleagues, on grape, as follows :
  - Visit of Petra Jardna (Crop Research Institute, Prague, Czech Republic) in IRD/INRA Montpellier, 2009.
  - Thesis of Zvjezdana Markovic (Univ. Zagreb, Croatia), co-tutelle between Univ. Zagreb and Montpellier SupAgro, 2010.
- AGROCAMPUS OUEST Angers and KUL (Belgium) on *Pelargonium* droplet-vitrification.

4. Future of plant cryopreservation in your country (including new initiatives)

The future activities related to cryopreservation in France will cover different topics:
- Fundamental research, such as the understanding of some of the mechanisms involved in tolerance of plant tissues and organs to desiccation and cryopreservation;
- Applied research, involving the development of cryopreservation protocols for additional species;
- Large scale application of protocols for the establishment of cryopreserved collections in Biological Research Centres;
- Establishment and strengthening of collaborations with European and non-European partners.

5. Most important publications related to the COST Action

The most important publications related to the COST Action are listed below.

Dussert S, Engelmann F (2006) New determinants of coffee (*Coffea arabica* L.) seed tolerance to liquid nitrogen exposure. CryoLetters 27:169-178

Engelmann F (2009) Use of biotechnologies for conserving plant biodiversity. Acta Hortic 812:63-82

Engelmann F (2009) Plant germplasm cryopreservation: progress and prospects. Cryobiology 59:370-371

Engelmann F (2011) Encapsulation-dehydration: past, present and future. Acta Hort, in press.

Gallard A (2008) Etude de la cryoconservation d'apex en vue d'une conservation à long terme de collections de ressources génétiques végétales : compréhension des phénomènes mis en jeu et évaluation de la qualité du matériel régénéré sur le modèle *Pelargonium*. PhD Thesis, Univ Angers, France

Gallard A, Chevalier M, Dorion N, Mallet R, Filmon M, Grapin A (2011) Immulocalisation of two viruses (PFBV and PLPV) in *Pelargonium* apices and study of their potential eradication by cryopreservation. Acta Hortic: in press

Gallard A, Escoute J, Verdeil JL, Grapin A (2009) Dynamic study of cellular events during cryopreservation. CryoLetters 30: 391-392

Gallard A, Mallet R, Chevalier M, Grapin A (2011) PFBV and PLPV in *Pelargonium* apices: cryotherapy efficiency and virus immunolocalization. CryoLetters 32: in press

Gallard A, Panis B, Dorion N, Swennen R, Grapin A (2008) Cryopreservation of *Pelargonium* apices by droplet-vitrification. Cryo-letters. 29: 243-251

Gallet S, Gamiette F, Filloux D, Engelmann F (2007) Cryopreservation of yam germplasm in Guadeloupe (FWI). Adv Hortic Sci 21:244-246

Georget F, Engelmann F, Domergue R, Côte F (2009) Morpho-histological study of banana (*Musa* spp. Cv. Grande Naine [AAA]) cell suspensions during cryopreservation and regeneration. CryoLetters 30:398-407
Grapin A (2010) How to start a programme on *Rosa* cryopreservation? CryoLetters 31:84
Grapin A, Gallard A, Dorion N (2007) Cryopreservation at the 'Institut National d'Horticulture' (INH) of Angers: past, present and future. Adv Hort Sci 21: 255-257

6. References

Brison M, Boucaud MT de, Pierronnet A, Dosba F (1997) Effect of cryopreservation on the sanitary state of a cv *Prunus* rootstock experimentally contaminated with plum pox potyvirus. Plant Sci 123:189–196
Coulibaly Y, Demarly Y (1979) *In vitro* androgenesis in *Oryza sativa* cv. Cigalon from anthers preserved in liquid nitrogen (-196 deg C). Agr Trop 34:74-79
Dereuddre J, Scottez C, Arnaud Y, Duron M (1990) Resistance of shoot-tips from *in vitro*-cultured plantlets of pear (*Pyrus communis* L. cv. Beurré Hardy), coated in alginate, to dehydration then freezing in liquid nitrogen: effect of previous cold hardening. CR Acad Sci Paris, 310, Sér III:317-323
Engelmann F, Balsemin E, Barreneche T *et al.* (2009) Cryopreservation of French plant genetic resources collections (CRYOVEG) Cryobiology 53:411
Gonzalez Arnao MT, Engelmann F (2006) Cryopreservation of plant germplasm using the encapsulation-dehydration technique: review and case study on sugarcane. CryoLetters 27:155-168
Harvengt L, Meier-Dinkel A, Dumas E, Collin E (2004) Establishment of a cryopreserved gene bank of European elm. Can J Forest Res 34:43-55
Wang QC, Panis B, Engelmann F, Lambardi M, Valkonen JPT (2009) Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. Ann Appl Biol 154:351-363

Country Report: Germany

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4: Northwest German Forest Research Institute (NW-FVA), Dept. Forest Genetic Resources, Hann. Münden, Germany
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1. Major achievements in Germany related to plant cryopreservation

1.1. Working Group 1

Although the group in DSMZ was the only laboratory in Germany formally participating in Working Group 1 and the only one using a transgenic approach for fundamental research in cryopreservation some studies of fundamental value were investigated in other laboratories, such as IPK Gatersleben, where studies by DSC on aspects of cold effects on various parameters as well as regeneration studies have been undertaken. Additionally other scientists in Germany working on cold, osmotic and salt tolerance of plants have been not involved in COST 871. At DSMZ recombinant cell lines and plants from potato and tobacco were established acquiring salt and/or osmotic tolerance by over-expression of the AtNHX1 protein (tobacco) and homologous over-expression of the PR10A protein (potato plants and cell cultures). These cell lines have been used for the investigation of fundamental aspects of the influence of salt and osmotic tolerance on cryopreservation. Specific vector constructs have been used to facilitate expression monitoring of recombinant genes in the cell cultures. For future comparison of the results obtained from the cell cultures with those obtained for intact plants, transgenic potato plants have been raised transformed with the same vector systems. This allows future collaboration between IPK and DSMZ. Improvements of cryopreservation methods for cell cultures achieved during the project will be practically applied for the conservation of the cell culture collection maintained at DSMZ and offered as a service by DSMZ to establish methods for the safe deposit of cell lines or Patent Deposit under the Budapest treaty.

1.2. Work Group 2

In Working Group 2, several institutions were active. IPK Gatersleben maintains the world's largest potato cryo-collection using the DMSO droplet method now amounting to 1244 accessions. The method was modified resulting from studies on factors of preculture and recovery. Methylation studies were used for investigation of epigenetic variations in potato cryopreservation. Garlic and other alliums (84 accessions) are cryopreserved using vitrification, and mint cryopreservation was developed (56 accessions) with droplet-vitrification. A cost analysis was performed for garlic cryopreservation in comparison with

field maintenance. Use of alternating temperatures during preculture improved regeneration in all species cryopreserved by the IPK group. DSC is used for analysis of the water state in cryopreservation. The IPK group started investigations on pollen cryopreservation as a complementary method to store populations of outbreeding accessions together with shoot tips of mother plants in cases, where it is difficult to obtain large quantities of true-to-type seeds for storage. IPK organised a COST WG2 meeting. The JKI-ZGO Dresden-Pillnitz is working on two crops. In *Fragaria* methods of micropropagation were optimized and PVS2 vitrification is used for cryopreservation (21 accessions), optimization experiments are ongoing. The dormant bud method is adapted for *Malus.* The NW-FVA maintains the European collection of *Ulmus* species on the basis of dormant buds (444 accessions). Experiments were performed with *in vitro* shoots of forest trees using PVS2 vitrification (120 accessions). Regeneration frequencies of 100 % was obtained in *Populus*. *Betula, Prunus* and *Acer* are other successfully cryopreserved genera. At Humboldt University Berlin, two groups are interested in cryopreservation. The Institute of Biology at Faculty Mathematics and Natural sciences worked on cryopreservation of conifers for maintaining clonal varieties and orchid seeds, and the tissue culture laboratory of the Faculty Agriculture and Horticulture contributed to cryopreservation of strawberry, roses and Jatropha and is interested in using DSC.

Table 1. Summary of research activities performed in Germany during the 4 years of COST Action 871.

Institute	Team leaders	Plants	Thematic	WG	Period (Years)
DSMZ	H.-M. Schumacher	*Solanum tuberosum*	Cell cultures, using of GMO for analyses	1	2007-10
HUB	I. Pinker	*Fragaria*	Shoot tip droplet-vitrification	2	2006-09
		Rosa		2	2006
		Jatropha	Embryo axes droplet-vitrification	2	2009
HUB	K. Zoglauer	*Abies* spec.	Embryogenic cell cultures	2	2007-10
		Dahlia spec.	Seed cryopreservation	2	2007-10
		Orchidaceae		2	2007-10
IPK	E.R.J. Keller	*Solanum tuberosum*	DMSO droplet method, DSC	2	2007-10
		Allium spec.	Vitrification	2	2007-10
		Mentha spec.	Droplet-vitrification	2	2007-10
JKI	M. Höfer	*Fragaria*	Vitrification of shoot tips		2007-10
		Malus	Dormant-bud method		2007-10
NW-FVA	A. Meier-Dinkel	*Populus tremula*	Vitrification of shoot tips	2	2008-09
		Populus tremula x *P. tremuloides*		2	2008-09
		Populus x *canescens*		2	2010
		Betula pendula		2	2007-08
		Betula pubescens		2	2007-08
		Betula pubescens		2	2007-08
		Prunus avium		2	2009-10
		Acer pseudoplatanus		2	2010

2. Collaborations initiated during the 4 years of the COST Action

Eight STSMs were hosted specifically, IPK 6, JKI 1, NW-FVA 1. They were provided to teach young colleagues learning techniques or colleagues starting to work on new species or cryopreservation in general. Stable collaboration exists between Poland (Institute of

Horticulture Skierniewice) and Germany (IPK), which was supported by the STSM. Collaboration was planned between Finland (MTT) and Germany (IPK), but a project submitted in Finland, was unfortunately, not approved. Bilateral collaboration is in preparation between Spain (University Madrid) and Germany (IPK). The STSM were also used to initiate further joint activities of France (CIRAD La Réunion, INRA Stations Ploudaniel and Angers-Nantes) and Germany (IPK and JKI). Techniques learnt at IPK will be used in Poland (University of Warsaw) for a programme to introduce entirely new species into cryopreservation. Furthermore, there were mutual benefits from interactions of COST 871 with the European Project AGRI GENRES 050 EURALLIVEG and an AEGIS project, both coordinated by IPK, which covered collaboration in cryopreservation of garlic between IPK and Poland (Skierniewice) and Czech Republic (CRI Prague-Ruzyne) and the genebank at Braga, Portugal, respectively.

3. Future of plant cryopreservation in Germany

The importance of cryopreservation is acknowledged and fixed as a task in the German National Programme of Plant Genetic Resources, thanks to the active inputs of IPK and JKI. During the time of the COST 871, in JKI and NW-FVA, the establishment of new cryopreserved collections has been started and the collaboration between German institutes using cryopreservation have been improved. The practical implementation is however, still not satisfying, mainly because of lack of funds. Essential steps of all cryopreservation protocols have to rely on time-consuming manual work (especially explant excision) of experienced technical staff. This may cause bottlenecks which seem to be obstacles for quick expansion of this method of conservation. This is the case despite the fact that cryopreservation is by far the most cost-effective method to store vegetative germplasm in the long-term. Initiatives are underway, which analyse the usefulness of cryopreservation to provide more effective data supported arguments in favour of this approach to plant conservation.

4. Most important publications

Abdullateef S, Pinker I, Böhme M (2009) The current status of conservation of agricultural and horticultural genetic resources and its cryopreservation future in Syria: 1. Intern. Symposium on cryopreservation in horticultural species. Proc First Int. Symp Cryopreservation Hortic Spec, Heverlee, Belgium, 5-8 April 2009, Acta Hortic in press

Ali Z, Schumacher HM, Heine-Dobbernack E, El Banna A, Hafeez FY, Jacobsen HJ, Kiesecker H (2010) Dicistronic binary vector system - A versatile tool for gene expression studies in cell cultures and plants. J Biotechnol 145:9-16

El-Banna A, Hajirezai MR, Wissing J, Ali Z, Vaas J, Heine-Dobbernack E, Schumacher HM, Kiesecker H (2010) Over-expression of PR-10a leads to increased salt and osmotic tolerance in potato cell cultures. J Biotechnol 150:277-287

Höfer M (2007) Preliminary results of cryopreservation of *Malus* germplasm from the Gene bank collection of the Institute of Fruit Breeding Dresden. Adv Hort Sci 21:251-254

Höfer M (2010) Cryoconservation strategy of fruit genetic resources in Germany. CryoLetters 31:76-94

Kaczmarczyk A, Grübe M, Keller ERJ (2008) Influence of alternating cold preculture on cryopreservation results for potato shoot tips. CryoLetters 29:79

Kaczmarczyk A, Grübe M, Keller ERJ (2009) Present state of potato cryopreservation – new aspects reviewed (Abstr.). CryoLetters 30:81-82

Kaczmarczyk A, Grübe M, Keller ERJ (2010) History and development of the potato cryopreservation method and the cryopreserved collection in the IPK Gatersleben (Abstr.) CryoLetters 31:91-92

Kaczmarczyk A, Houben A, Keller ERJ, Mette F (2010) Influence of cryopreservation on the cytosine methylation state of potato genomic DNA. CryoLetters 31:380-391

Kaczmarczyk A, Rokka VM, Keller ERJ (2011) Potato shoot tip cryopreservation. A review. Potato Res 54:45-79.

Kaczmarczyk A, Rutten T, Melzer M., Keller ERJ (2008) Ultrastructural changes associated with cryopreservation of potato (*Solanum tuberosum* L.) shoot tips. CryoLetters 29:145-156

Kaczmarczyk A, Shvachko N, Lupysheva Y, Hajirezaei MR, Keller ERJ (2008) Influence of alternating temperature preculture on cryopreservation results for potato shoot tips. Plant Cell Rep 27:1551-1558

Kaczmarczyk A, Zanke C, Senula A, Grübe M, Keller ERJ (2011) Thermal Analyses by Differential Scanning Calorimetry for Cryopreservation of Potato Shoot Tips. Proc First Int. Symp Cryopreservation Hortic Spec, Heverlee, Belgium, 5-8 April 2009, Acta Hortic in press

Keller ERJ (2007) Cryopreservation for maintenance of plant germplasm in Germany. Adv Hort Sci 21:228-231

Keller ERJ, Kaczmarczyk A, Senula A (2008) Cryopreservation for plant genebanks - a matter between high expectations and cautious reservation. CryoLetters 29:53-62

Keller ERJ, Senula A, Büchner D, Kaczmarczyk A, Zanke C (2008) Different protocols – different situations – different genotypes from the research laboratory to application in genebanks. Some subjects of discussion. In: Laamanen J, Uosukainen M, Häggman H, Nukari A, Rantala S (eds) Cryopreservation of Crop Species in Europe, Abstr CryoPlanet COST Action 871, Meeting Oulu, Finland, February 20-23, 2008. p. 61

Keller ERJ, Senula A, Kaczmarczyk A (2008) Cryopreservation of herbaceous dicots. In: Reed BM (ed) Plant cryopreservation: a practical guide. Springer, New York. pp. 281-332.

Keller ERJ, Senula A, Zanke C, Kaczmarczyk A (2010) Practical implications to use different explant sources for routine cryopreservation (Abstr.) CryoLetters 31:81-82

Keller ERJ, Senula A (2010) Cryopreservation of plant germplasm. In: Davey MR, Anthony P (eds) Plant Cell Culture. Essential Methods. Wiley Chichester, UK. pp. 131-151

Keller ERJ, Senula A, Zanke C (2011) Alliaceae in cryopreservation, achievements and constraints. Proc First Int. Symp Cryopreservation Hortic Spec, Heverlee, Belgium, 5-8 April 2009, Acta Hortic in press

Meier-Dinkel A (2007) Cryopreservation of *Betula in vitro* shoot tips by vitrification. Adv Hort Sci 21:215-218

Pinker I, Halmagyi A, Olbricht K (2009) Effects of sucrose preculture on cryopreservation by droplet-vitrification of strawberry cultivars and morphological stability of cryopreserved plants. CryoLetters 30:202-211

Pinker I, Halmagyi A, Olbricht K, Ehrig F (2009) Cryopreservation of strawberry cv. 'Senga Sengana' by droplet-vitrification. Acta Hort 839:253-260

Senula A, Keller ERJ (2011) Cryopreservation of mint – routine application in a genebank, experience and problems. Proc First Int. Symp Cryopreservation Hortic Spec, Heverlee, Belgium, 5-8 April 2009, Acta Hortic in press

Senula A, Keller ERJ (2010) Cryopreservation of *Orthosiphon aristatus* – an attempt to transfer a droplet-vitrification method using PVS2 from mint to *Orthosiphon*. (Abstr.) CryoLetters 31:94

Senula A, Keller ERJ, Sanduijav T, Yohannes T (2007) Cryopreservation of cold-acclimated mint (*Mentha* spp.) shoot tips using a simple vitrification protocol. CryoLetters 28:1-12

Country Report: Greece

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1. Major achievements in Greece related to plant cryopreservation

Working Group 1

Greece joined the COST 871 with the aim of operating though Working Group 1 to foster research and development related to novel plant freezing assays, that would ultimately be used in various applications including research in cryopreservation. More specifically, the proposed assays would be utilized as a screening tool in order to rank plant genotypes according to their freezing tolerance. Moreover, the proposed assays may prove useful in dissecting the mechanisms that control freezing events, for example in cold hardy plants, as well as in measuring the effect of various cryoprotectants in freezing behaviour of freezing sensitive plants.

The proposed plant freezing assays are semi-high throughput and involve the use of microtiter plates. Since they are amenable to automation, they are suitable for both high and low tech labs.

Various trangenic and non-transgenic plant samples have been screened for freezing tolerance, from genera including Laurel, Nicotiana, Beta, Citrus, Cicer, Origanum and Arabidopsis, by using the above mentioned assays. In most cases, the plant samples used are either leaf discs or leaf strips; but also seeds, buds and seedling parts, hypocotyls, epicotyl and roots have also been employed.

Additionally, recent research on the use of microtiter plates (Zaragotas and Anastassopoulos, 2011) proved the non uniform freezing conditions in 96 well microtiter plates and proposed the use of experimental designs in order to eliminate the within microtiter plates environmental variability. That research outcome may prove useful for cryostorage of samples in 96 well microtiter plates.

Table 1. Summary of research activities performed in Greece during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
TEI Larissas	Anastassopoulos E.	*Origanum vulgare* *Cicer arietinum*	Screening for freezing resistance	2007-2011

2. Collaborations initiated during the 4 years of the COST Action

In 2007, Dr. Elias Anastassopoulos participated at the First Training Workshop on DSC and thermal analysis organized by Dr Milos Faltus at the Crop Research Institute in Prague (Czech Republic).

Moreover, opportunities for collaboration within the action have been explored in order to test the usefulness of the proposed assays, in plant cryopreservation.

3. Future of plant cryopreservation in Greece

For many years, plant germplasm conservation in Greece was limited mainly in cereal crops and did not involve the use of cryopreservation. The importance of plant cryopreservation, but also of conserving biodiversity, has been recently realised in Greece, particularly after the forest fires that broke in the country in August 2007 and despite the fact that Greece is a country rich in biodiversity. The total number of higher plant species known, in Greece is 4.992 according to data from the World Resources Institute for the year 2002. The agricultural area in Greece, according to FAO statistics, in 2007, was 4.689.500 ha, of which 2.118.600 ha was arable land and 1.125.000 ha permanent crops, whereas the forest area was 3.812.400 ha. The above figures prove that there is great potential for plant cryopreservation of crop species of interest to Greek farmers.

The first attempt to create an oregano cryobank is underway at the Laboratory of Plant Biotechnology at the TEI Larissa, in Greece, in collaboration with Dr. Jayanthi Nadarajan of the Royal Botanic Gardens, Kew. Oregano is an under-utilised crop that holds great promise for the global agriculture, since many mainly *in vitro* studies have proven its antibacterial and antioxidant activities. Greece and neighbouring countries are particularly rich in genetic variation within the Origanum genus and the establishment of such a cryobank will help explore further various applications and uses.

4. Most important publications

Anastassopoulos E (2007) Towards a microplate technology platform for plant breeding. "Technology, application and validation of plant cryopreservation." Florence, Italy, May 10-12, 2007. Book of abstracts p. 39

Anastassopoulos E (2009) Robotic plant breeding prospects expectations and prospects (in Greek). 1^{o} Hellenic Robotics Conference, 23-24 Febrauary 2009, Athens, Greece. Book of Abstracts pp. 1-6

Anastassopoulos E, Zaragotas D (2011) Freezing assays in microtiter plates and their use in plant breeding. International Conference on "Plant gene discovery technologies." Vienna, Austria, February 23-26, 2011. Book of abstracts p. 31

Anastassopoulos E, Zaragotas D, Vlachostergios D (2009) Assessment of an *in vitro* screening assay for the identification of chickpea plants tolerant to freezing. Integration of Cryopreservation in Genebank Strategies. 10-11 September 2009, Gatersleben, Germany Book of Abstracts p. 29

Zaragotas D, Anastassopoulos E (2011) Uniformity trials in plant freezing assays, involving microplates. Cryoletters 32: 21-27

Country Report: Italy

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1. Major achievements in Italy related to plant cryopreservation

1.1. Working Group 1

CRA-FRU carried out a study on somaclonal variation after cryopreservation of *Pyrus pyraster* shoot tips by RAPDs and SSRs. The results showed that cryopreservation using encapsulation-dehydration does not affect genetic stability of wild pear (Condello *et al.* 2009). Moreover, during cryopreservation of apple shoot tips by the encapsulation method, polyamines, transglutaminase activity and protein patterns were investigated, showing that polyamine intracellular levels, as well as transglutaminase activity, decreased in shoot tips during the dehydration pre-treatment with sucrose (Forni *et al.* 2010).

The University of Parma (A. Fabbri and co-workers), in collaboration with the CNR-IVALSA, carried out an osmometric and anatomical study on shoot tips of the grape rootstock 'Kober 5BB' (*Vitis Berlandieri* x *V. riparia*) during the preparatory steps preceding the direct immersion of explants in liquid nitrogen (LN). The observations concerned modifications at the cellular level, in order to understand the mechanisms of adaptation to dehydration treatments and to characterize the "key steps" on which successful cryopreservation depends (Ganino *et al.* 2011).

1.1. Working Group 2

At the CNR-IVALSA, a cryo-protocol for the conservation of citrus germplasm by dehydration and direct immersion in LN of polyembryonic seeds was developed (De Carlo *et al.* 2011). The technique is presently used to duplicate an ancient collection of *Citrus* spp., initiated by Cosimo I° de' Medici in the XVI° century in a cryo-bank (Lambardi *et al.* 2007b). Moreover, in the frame of a collaboration with Veneto Agricoltura, the duplication of an in-field collection of apple germplasm is in progress, using the dormant-bud method (Lambardi *et al.* 2009). The technique was optimized and evaluated for its advantage in terms of time and labour required for the preservation of apple germplasm in comparison with the classic tissue culture-based cryopreservation approach (Lambardi *et al.* 2011). The dormant-bud technique was also applied to persimmon (Benelli *et al.* 2009). Other studies developed effective cryo-procedures for embryogenic callus of common ash (Ozudogru *et al.* 2009a) and horsechestnut (Lambardi *et al.* 2008), embryonic axes of peanut (Ozudogru *et al.* 2009b, 2009c), shoot tips of redwood (Ozudogru *et al.* 2011) and rose (Previati *et al.* 2008; Ozudogru *et al.* 2010). As for the CRA-FRU, cryopreservation protocols by encapsulation-dehydration were developed using shoot tips of *Pyrus* spp. (Condello *et al.* 2009), apple, peach (Damiano *et al.* 2011), blackberry, strawberry and raspberry (Damiano *et al.* 2007a, 2007b; Caboni *et al.* 2010), as well as by droplet-vitrification in apple (Condello *et al.* 2011a) and raspberry (Condello *et al.* 2011b).

2. Collaborations initiated during the 4 years of the COST Action

In Italy, large part of the activity in the frame of the COST Action 871 "CryoPlanet" has been carried out by the CNR-IVALSA in Florence and the CRA-FRU in Rome. During the four years of the Action, the two institutes established numerous collaborations with other Italian scientific institutions, with the main aims to increase the interest in cryopreservation and to promote the technique as an innovative and strategic tool for the preservation of Italian plant germplasm. The main institutes that, under the guidance of IVALSA and FRU, initiated studies on cryopreservation are the Universities of Tor Vergata (apple), Milano (cherry), Palermo (hazelnut, citrus), Parma (grape), Reggio Emilia (orchids) and Florence (persimmon), the Santa Lucia Foundation of Rome (protein expression), the regional agency Veneto Agricoltura (apple, pear) and the Centre of Forest Biodiversity of Peri (forest seeds).

Table 1. Summary of main research activities performed in Italy during the 4 years of COST Action 871 (the column "Thematic area" reports on the tissue/organ used).

Institute	Research leader	Plant species	Thematic area	Period
CNR-IVALSA	A.E. Ozudogru	*Arachis hypogaea*	Excised embryos	2007-2009
CNR-IVALSA	A. De Carlo	*Citrus* spp.	Polyembrionic seeds	2007-2011
CNR-IVALSA	C. Benelli	*Diospyros kaki*	Dormant buds	2007-2009
CRA-FRU	C. Damiano	*Fragraria x ananassa*	Shoot tips, cell suspens.	2007-2009
CNR-IVALSA	A.E. Ozudogru	*Fraxinus excelsior*	Embryogenic callus	2008-2009
CNR-IVALSA	M. Lambardi	*Malus domestica*	Dormant buds	2008-2011
CRA-FRU	E. Caboni	*Malus domestica*	Axillary buds, shoot tips	2008-2010
CRA-FRU	C. Damiano	*Morus alba*	Shoot tips	2007-2009
CNR-IVALSA	C. Benelli	*Olea europaea*	Buds, embryog. callus	2007-2010
CNR-IVALSA	M. Lambardi	*Populus* spp.	Shoot tips	2007-2009
CRA-FRU	E. Caboni	*Pyrus* spp.	Shoot tips	2007-2009
CNR-IVALSA	A. De Carlo	*Rosa* spp.	Shoot tips	2007-2008
CRA-FRU	E. Caboni	*Rubus idaes*	Shoot tips	2008-2009
CNR-IVALSA	A.E. Ozudogru	*Sequoia sempervirens*	Shoot tips	2008-2011
Univ. of Parma	A. Fabbri	*Vitis* rootstock	Shoot tips	2007-2009

The CNR-IVALSA established also several International collaborations on specific topics. Thanks to a common programme with the University of Derby on olive cryopreservation, significant advances have been made in the cryopreservation of shoot tips from Italian cultivars (Lynch *et al.* 2007), as well as on the effects of osmotic pretreatments on oxidative stress, antioxidant profiles and cryopreservation of somatic embryos (Lynch *et al.* 2011). Worthy of mention also the collaboration established on cryotherapy with the Northwest Agricultural and Forest University of Yangling, China (Wang *et al.* 2009), and the Catholic University of Leuven on cryopreservation of ash embryogenic callus (Ozudogru *et al.* 2009a), adapting a slow-cooling procedure originally developed for banana. As for the CRA-FRU, the collaboration established with the Catholic University of Leuven on the cryopreservation by the droplet-vitrification method allowed to reach significant advances in cryopreservation of apple and raspberry.

Three Short Term Scientific Missions have been executed at the CNR-IVALSA: Ivaylo Tsvetkov from Bulgaria worked on the optimization of cryo-protocols on poplar and aspen shoot tips (2007; Tsvetkov *et al.* 2009), Sladana Jevremovic developed cryopreservation procedures for *Iris* spp. (2008; Jevremovic *et al.* 2011) and Elena Rios Thalmann applied different cryopreservation methods (droplet, encapsulation-based and dormant-bud methods) to apple and pear germplasm (2009). Moreover, Emiliano Condello of the CRA-FRU and Jana Krainakova of the University of Udine spent their STSMs at the Catholic University of Leuven (apple cryopreservation) and at the University of Oulu (cryopreservation of conifer

embryogenic callus), respectively. The CRA-FRU also hosted an STSM on droplet-vitrification (COST Action 863) and participated to the ECPGR *Prunus* WG8 meeting, promoting fruit tree cryopreservation.
In the frame of the Action, two important events have been organized by the CNR-IVALSA, i.e., the "1st Meeting of WG2: Technology, Application and Validation of Plant Cryopreservation" (Lambardi *et al.* 2007a) and the "2nd Workshop on Cryopreservation by the Dormant-Bud Technique" (2010).

3. Future of plant cryopreservation in Italy

Thanks to the intense activity of experimentation and promotion of the cryogenic technology made by the two Italian teams involved in the MC of "CryoPlan*et*" COST Action, numerous and diverse institutes (Universities, Research Centres, Regional Agencies) are today acquainted with the potential of cryopreservation for the safe long-term conservation of plant genetic resources. Some of them are also carrying out, alone or in collaboration with IVALSA or FRU, research on a wide range of plant species.
The future main aim for Italy is the establishment of active cryo-banks of plant germplasm. In this view, a project for the creation of a regional cryo-bank has been submitted by the CNR-IVALSA for funding to Veneto Agricoltura, the agriculture agency of the Veneto region. Moreover, most of the activities of the CRA-FRU were performed in the frame of the project RGV-FAO, granted by the Italian Ministry of Agriculture, aiming to introduce ancient fruit cultivars in *in vitro* culture and to establish a cryobank of Italian fruit germplasm. So far, hundreds of accessions are ready to be processed for cryopreservation and/or for exchange.

4. Most important publications

Benelli C, De Carlo A, Giordani E, Pecchioli S, Bellini E, Kochanova Z (2009) Vitrification/one-step freezing procedure for cryopreservation of persimmon dormant buds. Acta Hortic. 833: 163-168

Caboni E, Frattarelli A, Damiano C (2010) Cryopreservation of strawberry in vitro plant. In: Mezzetti B, Ruzic D, Gajdosova A (eds) A Guide to Some In Vitro Techniques – Small Fruits. Cost 863, Brussels, pp. 106-111

Condello E, Palombi MA, Tonelli MG, Damiano C, Caboni E (2009) Genetic stability of wild pear (*Pyrus pyraster*, Burgsd) after cryopreservation by encapsulation dehydration. Agricultural and Food Science. 18 (2): 136-143

Condello E, Caboni E, Andrè E, Piette B, Druart P, Swennen R, Panis B (2011a) Cryopreservation of *in vitro* axillary buds of apple following the droplet-vitrification method. Cryo Letters (*in press*)

Condello E, Ruzic Dj, Panis B, Caboni E (2011b) Raspberry cryopreservation by droplet-vitrification technique. Acta Hort. *In press*

Damiano C, Arias M, Frattarelli A (2007a) Cryopreservation of some mediterranean small fruit plants. Acta Hort. 760: 187-194

Damiano C, Arias M, Frattarelli A (2007b) Recent advances in cryopreservation of small fruit germplasm. Adv. Hort. Sci. 21(4): 225-227

Damiano C, Caboni E, Frattarelli A, Condello E, Palombi M, Engelman F, Arias M (2011) Cryopreservation of fruit tree species through the encapsulation-dehydration of in vitro shoot tips at the CRA-Fruit Research Centre of Rome. Acta Hort. (*in press*)

De Carlo A, Lambardi M, Ozudogru AE (2011) Cryogenic technologies for the long-term storage of Citrus germplasm. In: Yeung E. and Thorpe T.A. (eds) Plant Embryo Culture: Methods and Protocols. Molecular Biology Series. Springer, New York, pp. 185-200

Forni C, Braglia R, Beninati S, Lentini A, Ronci M, Urbani A, Provengano B, Frattarelli A, Tabolacci C, Damiano C (2010) Polyamine concentration, transglutaminase activity and changes in protein synthesis during cryopreservation of shoot tips of apple var. Annurca. CryoLetters 31(5):413-425

Ganino A, Silvanini D, Beghè C, Benelli C, Lambardi M, Fabbri A (2011). Anatomical and osmometrical studies on axillary shoot tips of rootstock Kober 5BB (*Vitis Berlandieri* x *V. riparia*) during cryopreservation treatment (vitrification). Biologia Plantarum (*in press*)

Jevremovic S, Benelli C, De Carlo A, Subotic A, Lambardi M (2011) Development of cryopreservation procedures for dwarf irises (Iris spp.). Acta Hort. (*in press*)

Lambardi M, Benelli C, De Carlo A, Ozudogru EA, Previati A, Ellis D (2011) Cryopreservation of Ancient Apple Cultivars of Veneto: A Comparison Between PVS2-Vitrification and Dormant Bud Techniques. Acta Hort. (*in press*)

Lambardi M, Benelli C, De Carlo A, Previati A (2009) Advances in the cryopreservation of fruit plant germplasm at the CNR-IVALSA Institute of Florence. Acta Hort. 839:237-243

Lambardi M, Benelli C, Rinaldelli E, Silori C (2007a) Special Issue on Technology, Application and Validation of Plant Cryopreservation (Proc. of the "COST Action 871, 1st Meeting of WG2). Adv. Hort. Sci. 21(4), pp. 189-292.

Lambardi M, Halmagyi A, Benelli C, De Carlo A, Vettori C. (2007b) Seed cryopreservation for conservation of ancient Citrus germplasm. Adv. Hort. Sci. 21(4):198-202

Lambardi M, Ozudogru AE, Benelli C (2008) Cryopreservation of embryogenic callus. In: Reed B. (eds) Plant Cryopreservation: A Practical Guide. Springer, Berlin, pp. 177-210

Lynch P.T., Siddika A, Mehra A, Fabbri A, Benelli C, Lambardi M (2007) The challenge of successful cryopreservation of olive (*Olea europaea* L.) shoot tips. Adv. Hort. Sci. 21(4): 211-214

Lynch PT, Siddika A, Johnston JW, Trigwella SM, Mehra A, Benelli C, Lambardi M, Benson EE (2011). Effects of osmotic pretreatments on oxidative stress, antioxidant profiles and cryopreservation of olive somatic embryos. Plant Science 181:47-56

Ozudogru EA, Capuana M, Kaya E, Panis B, Lambardi M (2009a) Cryopreservation of *Fraxinus excelsior* L. embryogenic callus by one-step freezing and slow cooling techniques. CryoLetters 31:63-75

Ozudogru EA, Kaya E, Kirdok E, Capuana M, Benelli C, Engelmann F (2011). Cryopreservation of redwood (*Sequoia sempervirens*) in vitro buds using vitrification-based techniques. CryoLetters (*in press*)

Ozudogru EA, Ozden-Tokatli Y, Gumusel F, Benelli C, Lambardi M (2009b) Cryopreservation for the safeguard of Turkish peanut germplasm. CryoLetters 30(1):87-88

Ozudogru EA, Ozden-Tokatli Y, Gumusel F, Benelli C, Lambardi M (2009c) Development of a cryopreservation procedure for peanut (*Arachis hypogaea* L.) embryonic axes and its application to local Turkish germplasm. Adv. Hort. Sci. 23(1): 41-48

Ozudogru EA, Previati A, Lambardi M (2010) In vitro conservation and cryopreservation of ornamental plants. In: S.M. Jain and S.J. Ochatt (eds) Protocols for In Vitro Propagation of Ornamental Plants. Methods in Molecular Biology, vol. 589. Humana Press-Springer, New York, pp. 303-324

Previati A, Benelli C, Da Re F, Ozudogru E.A., Lambardi M (2008). Micropropagation and *in vitro* conservation of virus-free rose germplasm. Prop. Ornam. Plants 8(2): 93-98

Tsvetkov I, Benelli C, Capuana M, De Carlo A, Lambardi M (2009) Application of vitrification-derived cryotechniques for long-term storage of poplar and aspen (*Populus* spp.) germplasm. MTT Agr. Res. Finl. 18: 160-166

Wang QC, Panis B, Engelmann F, Lambardi M, Valkonen JPT (2009) Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. Ann. Appl. Biol. 154(3): 351-363

Country Report: Luxembourg

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1. Major achievements in Luxemburg related to plant cryopreservation

There is only one institution dealing with plant cold hardiness in Luxembourg, namely, the Public Research Centre – Gabriel Lippmann through its Environment and Agrobiotechnologies department.

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). Different aspects of plant cold hardiness have been undertaken in Luxembourg so far. The first side of the research aims at understanding the effects of pretreatments on the cryopreservation ability of potato shoot tips. This work seeks as well to evaluate the effects of such pretreatments on the primary metabolism of potato. This is a thesis project undertaken by R. Folgado in collaboration with the University of Leuven, Belgium (Bart Panis). The second aspect of the research undertaken in Luxembourg concentrates on the application of cryopreservation, as a tool, for *ex situ* conservation of germplasm collections. The CRP – Gabriel Lippmann in Luxembourg is involved in a research project managed by Bioversity International aiming at preserving the huge collections of vegetatively propagated plant germplasm at the Vavilov Research Centre in Saint-Petersburg, Russian Federation (Tatjana Gavrilenko).

Besides, research projects developed in Luxembourg aim at understanding cold perception in trees as well as cold deacclimation in *Hydrangea sp*.

1.1. Working Group 1

Raquel Folgado's thesis (2009 - 2012)

The method of choice for long-term germplasm conservation is cryopreservation or freeze-preservation at ultra-low temperature (-196°C). At this temperature all biological, biochemical and physical processes are arrested. However the large-scale use of cryopreservation is hampered by the lack of standardized methods. Hence, only a fraction of plant germplasm is conserved by cryopreservation.

The aim of the project is to focus on the primary metabolism and the responses induced by the drastic environmental changes during the different steps of the cryopreservation methods. Among others, a proteomic and a metabolomic study related to the carbohydrate biochemical pathways will be undertaken. The research project focuses on proteomic changes associated with tolerance towards cryopreservation in potato. Proteomics is an emerging discipline in the post-genomic era. It focuses on the measurements, comparison and identification of protein patterns in cells and tissues under different physiological conditions. This project, being a collaboration between KULeuven, Belgium (Dr. B. Panis) and CRP-Gabriel Lippmann, Luxembourg (Dr. J.F. Hausman), will first lead to a better understanding of the mode of action of cryoprotection in potato and prepares the road for more efficient cryopreservation protocols for this crop. Second, new breeding tools for potato breeding will become available

for frost/dehydration tolerance because this is closely linked to cryoprotection. Through collaboration with CIP (International Potato Centre, Lima, Peru) access to the widest possible diversity of potato germplasm is facilitated.

1.2. Working Group 2

Ex situ conservation of germplasm collections

Fresh and processed products derived from small berries make important contributions to human nutrition and health, as well as offering economic opportunities for a decentralized and high value agricultural production. Berries in general are valuable sources of minerals, vitamins, dietary fibre, antioxidants and other nutrients, which make them an important commercial fruit crop. However, a wide diversity of phytochemical levels and antioxidant capacities exists within genotypes of these small fruits.

The N.I. Vavilov Institute of Plant Industry (VIR), one of the biggest and oldest germplasm collections worldwide, among others maintains raspberries, mountain ash, blackcurrant and honeysuckle. These collections are poorly characterised and there is a lack of information about genetic diversity and variability in terms of nutrient, micronutrient and non-nutrient phytochemicals with health functions of these berry species.

The project aims i) to investigate genetic diversity in subsets of the above-mentioned species, ii) to analyse their diversity in terms of biochemical compounds. Identification of berry accessions providing elevated concentrations of health promoting compounds can be of importance in the improvement of food quality in order to eliminate dietary deficiencies and reduce the risk of contracting chronic diseases. A major aim of the project is to demonstrate the relevance of the conservation of plant germplasm collections as a source of valuable genetic diversity for future generations.

Besides, this project also aims at developing new protocols for ex situ conservation of plant genetic resources, including *in vitro* slow growth techniques and cryopreservation after virus eradication (in collaboration with Dr. T. Gavrilenko, Vavilov Institute for Research, St Petersburg, Russian Federation).

Cold deacclimation in *Hydrangea sp*.

This work was realised in collaboration with Department of Horticulture, Aarhus University, Denmark (Dr. M. Pagter) and Department of Horticulture, Iowa State University, USA (Dr. R. Arora). Cold deacclimation and associated changes in soluble carbohydrates and water status of two Hydrangea species differing in susceptibility to frost injuries was followed under natural conditions. In fully cold hardy plants of *H. macrophylla* stem freezing tolerance fluctuated in parallel with changes in air temperature, while in a seasonal perspective increased temperatures caused a sigmoid deacclimation pattern in both *H. macrophylla* and *H. paniculata*. Timing of deacclimation was approximately synchronized in the two species, but *H. paniculata*, the hardier species based on mid-winter hardiness, deacclimated faster than *H. macrophylla*, indicating that deacclimation kinetics were not correlated with mid-winter hardiness. Experiments indicated that accumulation patterns of specific carbohydrates differed between the two species, suggesting that they utilize different strategies to overcome cold.(Pagter *et al*, 2011; in press Env. Exp. Bot.)

2. Most important publications

Criel B, Hausman JF, Oufir M, Swennen R, Panis B, Renaut J (2006) Proteome and sugar analysis of abiotic stress underlying cryopreservation in potato. Communications in Agricultural and Applied Biological Sciences, 71 (1)Ghent University:3-6

Criel B, Panis B, Oufir M, Swennen R, Renaut J, Hausman JF (2009) Primary metabolism of abiotic stress underlying cryopreservation in potato. 1st International Symposium on

Cryopreservation in Horticultural Species. Leuven, Belgium, 5-8 April 2009. 90. Poster abstract

Criel B, Panis B, Oufir M, Swennen R. Renaut J, Hausman JF (2008) Protein and carbohydrate analyses of abiotic stress underlying cryopreservation in potato. Cryopreservation of crop species in Europe. CRYOPLANET - COST Action 871. Agrifood Research Working papers 153. Oulu, Finland, 20-23 February 2008. 37-38

Criel B, Panta A, Carpentier S, Renaut J, Swennen R, Panis B, Hausman JF (2005) Cryopreservation and abiotic stress tolerance in potato: a proteomic approach. Proceedings 11th symposium on Applied Biological Sciences. Leuven, Belgium, 6 October 2005. Communications in Agricultural and Applied Biological Sciences 70 (2): 83-86

Folgado R, Hausman JF, Swennen R, Hoffmann L, Panis B (2010) Study of abiotic stress response in potato for improving cryopreservation protocols. 4th Conference Working Group 1, COST Action 871. Fundamental aspects of plant cryopreservation - Cryoprotection and genetic stability. Poznan-Kornik, Poland, 29-30 April 2010. 26. Poster abstract

Folgado R, Panis B, Renaut J, Sergeant K, Hoffmann L, Swennen R, Hausman JF (2011) Evaluation of dehydration stress response in potato and its use in cryopreservation. Cryopreservation symposium. Final meeting of the COST Action 871 "Cryopreservation of crop species in Europe". Angers, France, 8-11 February 2011. Poster abstract

Oufir M, Legay S, Nicot N, Van Moer K, Hoffmann L, Renaut J, Hausman JF, Evers D (2008) Gene expression in potato during cold exposure: Changes in carbohydrate and polyamine metabolisms. Plant Science 175:839-852.

Pagter M, Hausman JF, Arora R (2011) Deacclimation kinetics and carbohydrate changes in stem tissues of Hydrangea in response to an experimental warm spell. Pla,nt Science 180:140-148

Country Report: Poland

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5: Plant Genetic Resources of Vegetable Crops Lab, Institute of Horticulture, Skierniewice, Poland

1. Major achievements in Poland related to plant cryopreservation

In Poland, the number of institutes possessing plant cryo-facilities is limited, so the number of research projects have been carrying on in this field is most probably lower comparing to the other countries. In both Institute of Dendrology and Botanical Garden of the Polish Academy of Sciences the problem of plant cell cryopreservation was the subject of interest for latest years on the base of well established studies of cryoprotective activity of antifreeze proteins and somatic cell genetic manipulation. This is confirmed by numerous papers published so far. However, many related topics, such as: plant and seed response, reactive oxygen species and antioxidants to desiccation, osmotic and low-temperature stresses are very popular objectives of studies in various Polish research institutes and university plant physiology departments.

Since the beginning of COST Action 871 four new projects have been included in Action. They are mostly dedicated to the cryopreservation of endangered herbaceous and woody species that are native to Polish flora. These include ferns, gentians, orchids, roses, black alder, beech, cherry, elm, European hornbeam, firs, maples, oak, pines, spruce, yew and wild fruit trees.. International and national projects on genetic resources concern only *Allium* spp. and historical cultivars of apple trees, respectively.

1.1. Working Group 1

Studies of the relationship between plant cell and ultra-low temperature concern the description of this living structure response on structural, physiological and molecular levels of plant body organization. The N-terminal sequence of 27 kDa of cryoprotective activity protein, from cold-hardy coniferous species showed a high level of identity with class IV chitinases. This means that it is possible dual role as a cryoprotective and PR-protein. The ultrastructural study of embryonic axes, cryo-tolerant seeds, dehydrated and cryogenic stored, showed mild injury of the cell structures. Applying the 2DE proteomic studies of protein profile changes during the sucrose dehydration of cell suspension of gentian showed dynamic changes of the large set of proteins. The most important are that, which were recognized as newly formed during pretreatment with increasing of the sucrose concentration. The assessment of genetic stability of post-cryopreserved plant material with the help of various platforms of restriction enzymes is the priority of improving the cryo-protocols. True-to-typeness of cryoregenerants confirms their usefulness.

1) Biochemical and structure changes during procedures of ultra-low temperature treatments

- "Molecular and physiological changes in cryopreserved embryonic axes of *recalcitrant, suborthodox and orthodox* seeds" (Polish Ministry of Science and Higher Education (PMS&HE) No: N309 101836; 2009-2012; ID PAS, to P. M. Pukacki).
- "Biochemical and physiological studies on molecular mechanisms of cold tolerance of Norway spruce (*Picea abies* (L.) Karst.)" (PMS&HE No: 3P 6L021 24; 2003 -2006; ID PAS, to P.M. Pukacki)

2) Genetic stability assessment of *Gentiana* sp. cultures after cryopreservation with application of flowcytometry and molecular methods (PMS&HE) No:302 3595 33. Bot. Garden –CBDC PAS, to A. Mikuła).

1.2. Working Group 2

In a majority of Polish projects on cryopreservation, *in vitro* methods are applied before and/or after freezing in liquid nitrogen. Application of such material like embryogenic cell suspensions, protocorms of terriestal orchids, fern gametophyte, apical meristems of rose buds and isolated embryonic axis let us to have quit unique system for studying ultra-low temperature effects on plant body. Natural propagules: seeds of woody and herbaceous plants, dormant buds of apple trees, are the plant material cryostored in Polish seedbanks. The using of various type of plant material originated from different stage of plant development have resulted in the work out of numerous cryopreservation protocols.

1) Cryopreservation of plant material with application of *in vitro* regeneration technique for plant diversity conservation (PMS&HE No: 39/N COST/2007/0 Bot. Garden –CBDC PAS to J.J. Rybczyński).
2) Wild Polish roses cryopreservation for biodiversity protection, horticultural production and breeding (PMS&HE) No: N N310 142635, Dept of Ornamental Plants, University of Agriculture, to B. Pawłowska).
3) *Ex situ* conservation of Polish terrestrial orchids; from *in vitro* culture initiation to reintroduction. (PMS&HE of Poland No: 3984/B/P01/2010/39 Intercollegiate Faculty of Biotechnology UG-MUG, Biotechnology Department, Laboratory of Plant Protection and Biotechnolgy, University of Gdańsk. to J. Znaniecka)
4) Somatic embryogenesis and cryopreservation of embryogenic cultures of *Picea abies* (L.) Karst. and *P. omorika* (Pančić) Purk. by the vitrification method. (PMS&HE No: ID PAS, to T. Hazubska-Przybył).

2. Collaborations initiated during the 4 years of the COST Action

UE projects:

1) Vegetative *Allium*, Europe's Core Collection, Safe & Sound (EURALLIVEG), (AGREEMENT NUMBER 050 AGRI GEN RES 870/2004; Contract No AGRI-2006-0395; http://euralliveg.ipk-gatersleben.de ; Plant Genetic Resources Lab, Research Institute of Vegetable Crops, Skierniewice, T. Kotlińska and M. Olas-Sochacka)
2) Cryopreservation of young inflorescence bases in bolting garlic for germplasm storage in the frame of "A European Genebank Integrated System (AEGIS)" of European Cooperative Programme for Plant Genetic Resources (ECPGR), (Plant Genetic Resources Lab, Research Institute of Vegetable Crops, Skierniewice, T. Kotlińska and M. Olas-Sochacka)

Other projects involving cryo-methods:

1) Epigenetic stability of seeds and tissues of trees after cryogenic storage. (PMS and HE 0720/B/P01/2009/36, 2009-2012. ID PAS) ..
2) Cryogenic storage of genetic resources of endangered forest tree species in gene bank. (2005-2010), *Quercus, A. alba, Taxus baccata.* ID PAS.

3) Special Research Project „Europain net of seed preservation of native plant species". Botanical Garden - CBDC PAS
4) Application of cryopreservation methods for formation of gene bank of historical apple trees cultivars 2010- 2012 Botanical Garden – CBDC PAS
5) Long-term preservation of European vegetatively propagated *Allium* germplasm (PMS and HE No 523/AgriGenRes/2008/7; Plant Genetic Resources Lab, Research Institute of Vegetable Crops, Skierniewice, T. Kotlińska and M. Olas-Sochacka)

Short Term Scientific Mission

In 2008 to 2010, of Action, efforts were made to involve as much as possible early stage scientists: The following three applicants from Poland were selected by the MC

1. Malgorzata Maślanka, University of Agriculture in Krakow, working with: B. Panis, Catolic University, Leuven, Belgium, from April 14, 2010 to May 25, 2010.
2. Malgorzata Pelc, Warsaw University of Life Sciences, working with: J. Keller, IPK, Gatersleben, Germany, April 07.to – May 28. 2010
3. Marta Olas-Sochacka, Plant Genetic Resources Lab, Research Institute of Vegetable Crops, Skierniewice – working with: J. Keller, IPK, Gatersleben, Germany; October 19 to November 19, 2010

3. Future of Plant Cryopreservation in Poland

At present various projects as well as seven research grants were funded by the Ministry of Science and Higher Education, and Ministry of Environmental. We do hope that licentiates, graduated university MSc, six PhD and four habilitation projects confirm the personal progress in the field of cryopreservation in Poland.

PhD projects

1) Selected species of *Pteropsida* in *in vitro* culture and cryopreservation. (supervisor: A.Mikuła).
2) The analysis of proteom changes of *Gentiana cruciata* embryogenic cell suspension in reaction on dehydration treatments related to cryopreservation. (sup. J.J. Rybczyński).
3) Physical and chemical changes of plant tissues after cryopreservation treatment. (sup. P.M. Pukacki).
4) Cryopreservation of genetic resources of wild fruit trees of *Malus sylvestris, Pyrus communis, Prunus avium and Corylus avellana* growing in Poland. (sup. Chmielarz).
5) Cryopreservation of *Lilium martagon (*sup. A. Bach)
6) Optimization of long-term storage conditions of garlic germplasm (sup. K. Niemirowicz-Szczytt).

Habilitation projects

1) A. Mikula 2011. Induction, maintenance, and long-term preservation in liquid nitrogen of embryogenic potential culture of *Gentiana* spp.
2) P. Chmielarz 2011. Cryogenic storage of orthodox and suborthodox seeds of forestry broad trees.
3) B. Pawłowska 2012. Wild polish roses cryopreservation for biodiversity protection, horticultural production and breeding.
4. T. Hazubska-Przybył 2015. Vitrification-based methods for long-term storage of somatic embryos of *Picea omorika* and *P. abies* in liquid nitrogen.

4. Most important publications

Bujarska-Borkowska B, Chmielarz P (2010) Stratification, germination and emergence of mazzard seeds following 15 or 20 years storage. Forestry 83:189-194

Chmielarz P (2009) Cryopreservation of dormant European ash (*Fraxinus excelsior*) orthodox seeds. Tree Physiol. 29: 1279-1285
Chmielarz P (2009) Cryopreservation of dormant orthodox seeds of forest trees: mazzard cherry (*Prunus avium* L.). Ann. For. Sci. 66: 405p1-405p9
Chmielarz P (2010) Cryopreservation of conditionally dormant orthodox seeds of *Betula pendula*. Acta Physiol. Plant. 32:591-596
Chmielarz P (2010) Cryopreservation of dormant *orthodox* seeds of European hornbeam (*Carpinus betulus*). Seed Sci. Technol. 38: 146-157
Chmielarz P (2010) Cryopreservation of the non-dormant orthodox seeds of *Ulmus glabra*. Acta Biol. Hungar. 61: 224-233
Hazubska-Przybył T, Bojarczuk K, Chmielarz P, Michalak M (2010) Somatic embryogenesis and cryopreservation of ornamental *Picea* species: modern methods of propagation and long-term storage. Acta Horticulturae (in Press)
Hazubska-Przybył T, Chmielarz P, Michalak M, Bojarczuk K (2010) Cryopreservation of embryogenic tissues of *Picea omorika* (Serbian spruce). Pl.Cell Tiss.Org.Cult. 102: 35-44
Jarząbek M, Pukacki PM, Nuc M (2009) Cold-regulated proteins with potent antifreeze and cryoprotective activities in spruces (*Picea* spp). Cryobiology 58:268-274
Keller ERJ, Zanke C, Stavělíková H, Zámečník J, Kotlińska T, Miccolis V, Kik C, Esnault F, Kolodinska A, Fischer D, Astley D (2007) First Steps Of Integrating Europe's Genetic Resources In Garlic And Shallot In A New Genres Project. 5th International ISHS Symposium on Edible Alliaceae (ISEA), October 29-31, 2007. De Meerpaal, Dronten, The Netherlands. Acta Horticulture (in press)
Mikuła A, Jata K, Rybczyński JJ (2009) Cryopreservation strategies for *Cyathea australis* (R.BR.) DOMIN. CryoLetters 30(6), 429-439
Mikuła A, Makowski D, Walters C, Rybczyński JJ (2011) Exploration of cryo-methods to preserve tree and herbaceous fern gamethophyte. In: Working with ferns. Issues and applications. Ed. Fernandez H. et al. Springer. pp. 173-192
Mikuła A, Olas M, Śliwińska E, Rybczyński JJ (2008) Cryopreservation by encapsulation of *Gentiana* spp. cell suspension maintains re-growth, embryogenic competence and DNA content. CryoLetters 29:409-418
Mikuła A, Tomiczak K, Rybczyński JJ (2011) Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi) genetic uniformity of regenerants. Plant Cell Rep. 30: 565-574
Mikuła A, Tomiczak K, Wójcik A, Rybczyński JJ (2011) Encapsulation-dehydratation method elevates embryogenic abilities of *Gentiana kurroo* cell suspension and carrying on genetic stabilitiy of its regenerants after cryopreservation. Acta Horticulture (in press)
Pawłowska B (2008) Employment of encapsulation-dehydration method for liquid nitrogen cryopreservation of ornamental plants explants propagated *in vitro*. Folia Horticulturae 20/1: 61-71
Pawłowska B (2011) Cryopreservation of *Rosa canina* and *R. rubiginosa* apical buds by the droplet-vitrification method. Acta Horticulturae (in press)
Pawłowska B, Bach A (2009) Cryopreservation of in vitro grown shoot buds of rose 'New Dawn' using encapsulation-dehydration method. Abstracts 1^{2th} National Conference '*In vitro*'Cultures, Poznań 2009. Acta Biol. Cracoviensia 51 suppl. 1: 56-56
Pawłowska B, Bach A (2011) Cryopreservation by encapsulation-dehydration of *in vitro* grown shoot buds of rosa 'New Dawn'. Acta Horticulturae (in press)
Pukacki PM, Jarząbek M, Juszczyk K (2009) Cryoprotective activity of thermal hysteresis proteins of embryonic axes of orthodox and recalcitrant seeds. Acta Physiol. Plant. 31(1):107
Zanke C, Keller ERJ, Zámečník J, Kotlińska T, Olas M (2009) Cryopreservation of vegetative garlic for the establishment of a European Core collection. Acta Horticulture (in press)

Country Report: Portugal

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1. Introduction

The diversity of habitats and landscapes in Portugal, the result of many geographical and historical factors, has given rise to a great variety of natural life, particularly species with a restricted area of distribution within continental Europe, that needs to be preserved.
Although the first research studies on cryopreservation of plants in Portugal were initiated in 1999, at present cryopreservation has not yet been implemented as a strategy for germplasm preservation. With the exception of somatic embryogenic masses induced from plus trees of maritime pine, no collection is maintained by cryopreservation, and the ongoing research is related to academic work carried out at Universities and research institutes. The "Banco Português de Germoplasma Vegetal" created in 1977 maintains, in the form of seeds and vegetative propagation material, a collection of 41,009 accessions from 155 species (wild and cultivated), representing 70% of the total plant material preserved in Portugal.
Since 2005 only 5 papers from Portuguese groups were published on plant cryopreservation: one dealing with the cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration, two with the cryopreservation of seeds from the endangered species *Drosophyllum lusitanicum* and *Tuberaria major*, and two related to the stability during recovery of cryopreserved embryogenic cultures of maritime pine. These research works were all performed at university or research institutes.

2. Major achievements in Portugal related to plant cryopreservation

Since the beginning of the COST 871 and resulting directly from the collaboration between the COST 871 members, new research lines have been initiated, namely the cryopreservation of apices of the endangered species *Tuberaria major* and *Thymus lotocephalus*. One Short Term Scientific Mission (STMS) took place at the Polytechnic University of Madrid (Learning about cryopreservation techniques and their applications to the cryopreservation of seeds and shoot apices, beneficiary: Laura Fernandes, University of Algarve; host: M. Elena González-Benito). Another STMS made possible a visit of Liliana Marum (IBET, Oeiras, PT) to Oviedo University (R. Rodriguez) to learn about analyses of global methylation in maritime pine embryogenic cultures (which may help to detect variation putatively occurring due to cryopreservation). Additionally, a student from University of Algarve started her PhD work with the title "Cryopreservation as a tool for preserving genetic variability of three endangered species endemic from Algarve region". Some young researchers participated in several seminars organized by COST 871 and these participations will be very important for the future development of cryopreservation in Portugal. An example was the participation of Aida Reis from the "Banco Português de Germoplasma Vegetal" (Braga, Portugal) at the COST 871 WG1 meeting in Sussex (February 2008). This meeting was organized together with a visit to the Millenium Seed Bank that allowed learning about important strategies being used to store seeds, useful for the Portuguese Bank.

Country Report: Serbia

Đurđina Ružić[1] and Slađana Jevremović[2]

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2: Institute for Biological Research 'Siniša Stanković', University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

1. Major achievements in plant cryopreservation in Serbia

Members of the Management Committee for Serbia in COST Action 871 'Cryopreservation of crop species in Europe' were Slađana Jevremović (WG1) and Đurđina Ružić (WG2). Serbia signed the Memorandum of Understanding at the beginning of the Action and participated in all COST 871 activities starting from the Kick off meeting in Bruxelles in 2006. The activities was mainly carried out in two scientific institutions: Fruit Research Institute (FRI) Čačak and Institute for Biological Research 'Siniša Stanković' (IBISS), Belgrade. FRI is situated in the central part of Serbia, in the major fruit growing region. The institute was founded by the Ministry of Agriculture of Serbia in 1946 and has 67 employees organized in 7 scientific departments. IBISS, part of the University of Belgrade, is an interdisciplinary research and teaching center founded in 1947. It has 246 employees organized in 11 departments. The plant research is mainly executed in the department of Plant Physiology where 42 employees are working in different aspects of fundamental research.
COST action 871 had a great impact in plant cryopreservation activities in both the above Serbian research institutions. The initiation of cryopreservation activities was the major achievement since no plant cryopreservation research had been conducted in Serbia before this COST action. All COST action activities (meetings, STSMs) helped in terms of transfer and development of plant cryopreservation research in our country. As a final result, cryopreservation laboratories were established in both institutions and the first results applying most of developed cryotechniques with direct immersion in liquid nitrogen such as, encapsulation dehydration, vitrification and droplet-vitrification on fruits species and endemic, endangered and horticulturally valuable crops have been obtained.

1.1. Working Group 1

During the COST action 871, the research team from IBISS participated at WG1 activities which included development of methods for cryopreservation of *Iris* sp. and some ornamental species. i.e. *Impatiens* sp. and several *Chrysanthemum* sp. cultivars (see Table 1 and Figs 3-4). Presently, six researchers are involved in plant cryopreservation work in IBISS. Different fundamental aspects of cryopreservation like cryoprotection of endemic and endangered species, secondary metabolite production and genetic stability of plants are included in the cryopreservation research. Cryopreservation studies are extended to cryotherapy for the elimination of viruses in horticulturally valuable crops.

1.2. Working Group 2

As regards the WG2, the research team from FRI includes 2 researchers and 2 technicians working on 'cold storage' and technology development, application and validation of cryopreservation methods on different fruit species. This research includes application of methods on small and stone fruit species mainly, like raspberry, blackberry and autochthonous plums and the rootstocks (Table 1; Figs 1–2). Also, this team is working on the the dormant bud technique for the cryopreservation of valuable autochthonous fruit species.

Table 1. Summary of research activities performed in Serbia during the 4-year COST Action 871

Institute	Team leaders	Plant species	Thematic area	Period
FRI	Ružić Đ.	Raspberry	WG2 Encapsulation	2008
FRI	Ružić Đ.	Raspberry, Blackberry	WG2 Encapsulation dehydration; vitrification	2009
FRI	Ružić Đ	Raspberry, Blackberry, Autochthonous plums; Rootstocks	WG2 Encapsulation dehydration; droplet-vitrification; vitrification	2010–2011
IBISS	Jevremović S.	*Fritillaria* sp.	WG1 Oxidative stress	2007–2008
IBISS	Jevremović S.	*Iris* sp.	WG1 Secondary metabolism; clonal fidelity	2008–2011
IBISS	Jevremović S.	*Impatiens* sp.	WG2 Cryotherapy	2009–2011
IBISS	Jevremović S.	*Chrysanthemum*	WG2 Droplet-vitrification	2011

2. Collaborations initiated during the 4 years of the COST Action

Our participation in COST 871 Action has enabled us to initiate collaboration with institutions and researchers that are great experts in a field of plant cryopreservation, since cryopreservation was a new research area in Serbia. Instruments of Short Term Scientific Mission (STSM) and all other activities on COST 871 Action meetings have had a huge impact on initiating work in the field of cryopreservation. Researchers from Serbia were granted with three STSMs and the possibility to participate at one Small Work Group Meeting (SWM, Table 2). Collaboration was initiated with research groups from Italy (Florence, Roma), France (Angers, Montpellier) and Belgium (Leuven).

Table 2. Summary of collaboration during the 4-years of COST Action 871

Type		
STMS	Participant	Slađana Jevremović.
	Title	Development of effective procedures for the cryopreservation of iris germplasm (*Iris* spp.)
	Place	CNR, IVALSA, Sesto Fiorentino, Florence, Italy
	Supervisor	Maurizio Lambardi
	Time	6 May–2 July 2008
STMS	Participant	Milana Trifunović
	Title	Immunolocalization of *Tomato Spotted Wilt Virus* (TSWV) in *Impatiens* sp. apices toward eradication by cryopreservation
	Place	UMR GenHort, Angers, France
	Supervisor	Agnes Grapin
	Time	21 April–31 May 2010
STMS	Participant	Tatjana Vujović
	Title	Cryopreservation of autochthonous plum genotypes (*P. insititia* L. and *P. cerasifera* Ehrh.) using encapsulation-dehydration and droplet-vitrification techniques
	Place	IRD de Montpellier, Avenue, Montpellier Cedex 5, France
	Supervisor	Florent Engelmann
	Time	17–30 October 2010

SGM	Participant	Đurđina Ružić
	Title	Cryopreservation by the dormant-bud technique
	Place	Area di Ricerca CNR, Florence, Italy
	Organizer	Maurizio Lambardi
	Time	26–28 May 2010

3. Future of plant cryopreservation in Serbia

Plant cryopreservation research starts in Serbia with COST 871 Action and is to be continued in future. The training courses for young scientists will be conducted and the implementation of some cryopreservation methods such as, cryotherapy for eradication of some plant viruses are planned and approved in the framework of a new project period (2010–2014). Funds have been granted by the Ministry of Science of the Republic of Serbia. In addition, cryopreservation will be applied to a greater number of endemic and endangered plant species, as well as to autochthonous fruit species. Cryopreservation using the dormant bud technique is also planned for the near future on some fruit species valuable on the national scale.

Participation in COST 871 Action in the past four years has been a good base for the implementation of cryopreservation as a method for long-term conservation of important crops in gene bank in Serbia.

4. Most important publications

We started with cryoresearch only a few years ago and our results are now presented mainly on the national and international meetings. The most important publications are presented below.

Ružić Đ, Vujović T, Cerović R (2008) *In vitro* methods used in preservation of fruit germplasm in Serbia. Cryopreservation of crop species in Europe, Cryoplanet COST Action 871, Workshop, Oulu, Finland, pp. 56–57

Ružić Đ, Stikić R, Todić S, Veličković M (2008) The application of some theoretical knowledge in the field of fruits and grapes physiology and ecology. 13th Congress of Fruit and Grape Growers of Serbia with International Participation, Novi Sad, Serbia, Book of Abstracts, pp. 39–40

Jevremović S, Nikolić M., Mišić D, Maksimović V, Trifunović M, Subotić A (2008) The current status of conservation of plant genetic resources in IBISS and related cryopreservation activities. Cryopreservation of crop species in Europe, Cryoplanet COST Action 871, Workshop, Oulu, Finland, p. 30

Ružić Đ, Stikić R, Todić S, Veličković M (2008) The application of some theoretical knowledge in the field of fruits and grapes physiology and ecology. 13th Congress of Fruit and Grape Growers of Serbia with International Participation, Journal of Pomology, 43, 167/168: 67–79

Ružić Đ, Vujović T, Cerović R (2009) Viability and multiplication of blackberry and raspberry shoots upon encapsulation. 1st International Symposium on Cryopreservation of Horticultural Species, Leuven, Belgium, Book of Abstracts, p. 131

Ružić Đ, Vujović T, Cerović R (2009) Short-term *in vitro* cold storage of raspberry shoots. Journal of Mountain Agriculture on the Balkans 12, 4:883–899

Jevremović S, Subotić A, de Carlo A, Benelli C, Lambardi M (2009) Protokol za kriokonzervaciju vrhova izdanaka *Iris pumula* pomoću vitrifikacije. XVIII Simpozijum DFBS, Vršac, Knjiga apstrakata, p. 42

Jevremović S, Subotić A, de Carlo A, Benelli C, Lambardi M (2009) Development of cryopreservation procedures for dwarf irises (*Iris* spp) 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, Book of Abstracts, p. 45

Condello E., Ružić Dj, Panis B, Caboni E (2010) Raspberry cryopreservation by droplet-vitrification technique. 28th International Horticultural Congress. Lisboa, Portugal, Book of Abstracts, Volume II, p. 527

Ružić Dj, Condello E, Panis B, Caboni E (2010) Raspberry cryopreservation by droplet-vitrification technique: preliminary results. 4th Meeting Working Group 1, COST Action 871. Fundamental aspects of plant cryopreservation - Cryoprotection and genetic stability. Poznań-Kórnink, Poland, Book of Abstracts, p. 38

Jevremović S, Krstić-Milošević D, Janković T, Menković N, Subotić A, Benelli C, de Carlo A, Lambradi M (2010) Detremination of mangiferin content in *Iris pumila* L. shoot cultures before and after cryopreservation. 4th Meeting Working Group 1, COST 871, Fundamental Aspects of Plant Cryopreservation – Cryoprotection, Poznań-Kórnink, Poland, Book of Abstracts, p. 8

Some results – photo story:

Fig. 1. Encapsulated shoot tips of cherry plum (left); bead burst (right)

Fig. 2. Foil strips with explants in droplets of vitrification solution - *Rubus fruticosus, droplet-vitrification*

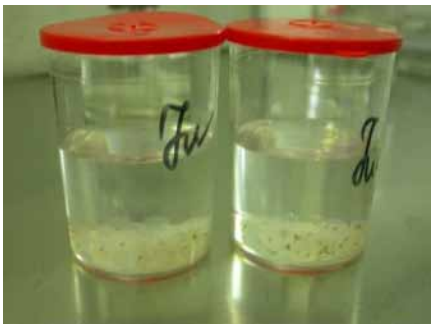

Fig. 3. Encapsulated shoot tips of *Imapatiens* sp.

Fig. 4. Shoot regeneration of *I. walleriana* after vitrification.

Country Report: Slovak Republic

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1. Major achievements in Slovakia related to plant cryopreservation

1.1. Working Group 1

As regards Working Group 1, the emphasis was put especially on some fundamental aspects of cryopreservation comprising complex approach of a study including overall assessment at different levels. Experiments were performed using some medicinal plant species, especially *Hypericum perforatum* L. was used as a model. Study of fundamental aspects brought new knowledge in the field of oxidative stress gene expression at both, transcript and protein levels, on ABA signalling and its regulation of dehydration during pre-cryogenic stages and on physical aspects of cooling regime dependent dehydration and ice growth.

Overall assessment of conifers was focused at structural and maturation capacity of embryogenic cultures and evaluation after long-term storage. Saint John´s wort evaluation comprised (i) genetic variation by cytogenetic and molecular markers, (ii) physiological status expressed by physiological parameters such as H_2O_2 and MDA content along with proline and carotenoid synthesis in order to estimate a balance between production of ROS and effectiveness of antioxidant system and (iii) biosynthetic potential of secondary metabolites with anti-cancer activities after cryostorage expressed as the content of hypericins.

1.2. Work Group 2

As regards Working Group 2, we have focused on the development of procedures using slow cooling and/or vitrification applied to embryogenic cultures of conifers and shoot tip apices of the studied medicinal plant species. The final protocols developed represent a result of testing several variables comprising manipulation of plant material during pre-cryogenic treatments, optimisation of pre-culture and cryoprotection conditions, cooling rate regime and thawing optimisation and post-cryogenic recovery and regrowth assessment parameters.

Participation in the COST action brought essential opportunities for young researchers and doctoral students in the field of sharing experience, use of research facilities available at host institutions, career development, involvement in European initiatives, etc.

2. Collaborations initiated during the 4 years of the COST Action

2.1 Ad hoc collaborations

- Institute of Plant Genetics and Biotechnology, Nitra, Slovak Republic and the Laboratory of Tropical Crop Improvement, KU Leuven, Belgium
  Subject: Embryogenic tissues of several conifer species (*Pinus nigra, hybrids Abies alba x A. cephalonica and Abies alba x A. numidica*).
- P. J. Šafárik University in Košice , Slovakia and Research Crop Institute, Prague, Czech Republic

Subject: DSC of *Hypericum perforatum* shoot tips

- P. J. Šafárik University in Košice, Slovakia and St. Kliment Ohridski University in Sofia, Bulgaria
  Subject: Physiological status and chloroplast ultrastructure of *Hypericum perforatum* shoot tips
- National Forest Centre in Zvolen and University of Oulu, Finland and University of Udine, Italy

Table 1. Summary of research activities performed in Slovakia during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
P. J. Šafárik University, Institute of Biology and Ecology, Košice	Eva Čellárová	*Hypericum* spp. *Orthosiphon stamineus*	Procedure development Overall assessment Gene expression	2007-2008 2008-2010 2009-2010
Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra	Terézia Salaj	*Pinus nigra* Arn. *Abies* spp. and hybrid *Abies* (*Abies alba* x *A. cephalonica*, *Abies alba* x *A. numidica*)	Procedure development Overall assessment	2007-2010
Forest Research Centre, Zvolen	Jana Krajňáková	*Abies* spp.	Procedure development Energy balance	2007-2010

2.2 Regular COST-871 actions

Since the kick off meeting, the representatives of Slovakia took part actively at all regular COST-871 meetings of both WGs.

2.3 Other COST-871 activities

17-21 September 2007, COST 871 Training school on thermal analysis, Crop Research Institute, Prague, Czech Republic

19-30 October 2009, Crop Research Institute, Prague, Czech Republic

Topic: DSC and DTA experiments on *Hypericum perforatum* L. shoot tips

Beneficiary: Matúš Skyba, *Dept. of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Košice, Slovakia*

Host: Dr. Jiří Zámečník, *Dept. of Molecular Biology, Crop Research Institute, Praha, Czech Republic*

Period: from 19/10/2009 to 30/10/2009

COST-STSM-871-05554

Topic: Thermal analysis of *Hypericum perforatum* L. shoot tips prior to and post vitrification

Beneficiary: Dr. Katarína Bruňáková, *Dept. of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Košice, Slovakia*

Host: Dr. Jiří Zámečník, *Dept. of Molecular Biology, Crop Research Institute, Praha, Czech Republic*

Period: from 10/05/2010 to 21/05/2010

2.4 Non-COST-871 activities

8-9 October 2009, International Scientific Conference 5th Biological Days, Nitra, Slovakia

Title: The use of cryopreservation for storage of conifer embryogenic tissues, *T Salaj, I Matušíková, B Piršelová, B Panis, R Swennen and J Salaj*

5-8 April 2009,1st International Symposium Cryopreservation in Horticultural Species. Leuven (Belgium)

Title: Effect of cryoprotectant exposure on post-thaw recovery, growth and genetic stability of *Pinus nigra* embryogenic tissues, *T Salaj, I Matušíková and B Piršelová*

25-26 May 2009 Progress and Perspectives. Ljubljana, Slovenia

Title: Conifer somatic embryogenesis – a biotechnological tool with potential to improve trees quality, *T Salaj, J Moravčíková and J Salaj*

7-9 September :2009 SLTB Meeting, Hannover, Germany

Title: Cryopreservation of conifer embryogenic tissue – an overview. *T Salaj, I Matusikova, B Panis, R Swennen and J Salaj*

26-29 April 2010, Bratislava, Slovakia, 11th Cryogenics 2010, IIR International Conference

Title: Effect of Thermal Gradients in the Specimen on Survival of Cryopreserved Plant Cells Subjected to Cryopreservation by Slow Cooling Aproach, *M Skyba, M Faltus, J Zamecnik and E Cellarova*

Title: Cryopreservation of *Hypericum perforatum* L. shoot tips by vitrification: the role of ABA in dehydration tolerance, *K Brunakova, M Urbanova and E Cellarova*

6-13 June 2010, St. Louis, MO, USA, 12th IAPB Congress

Title: Expression of antioxidative genes in *Hypericum perforatum* L. subjected to cold stress, *M Skyba, J Kosuth and E Cellarova*

14-17 September 2010, Prague, Czech Republic, 12th conference of experimental plant biology

Title: Thermal analysis of *Hypericum perforatum* L. shoot tips after pretreatment and cryoprotection, *K Brunakova, J Zamecnik, E Cellarova*

29 October 2010, Padova, Italy, Annual *Hypericum* meeting

Title: Cryopreservation of *Hypericum* spp.: procedures, mechanisms, assessment, *M Skyba, L Petijova, K Brunakova and E Cellarova*

3. Future of plant cryopreservation in Slovakia

Future plans comprise continuation in cryobiological fundamental research aimed at a study of balance between oxidative stress and effectiveness of antioxidant system at gene, transcript and protein levels, ABA signalling, cytokinin-ABA interaction, energy balance and complex assessment of impact of cryopreservation by multifactorial analysis.

Applied research and practical implications contain adoption of available protocols for other genotypes and species, evaluation of the effect of mother tree genotype and long-lasting field performance of plants regenerated from cryopreserved explants (true-to-typeness); from perspective view point establishment of gene bank of *Hypericum* species with high content of anticancer compounds is expected.

Future plans include also prepared collaboration with world governmental organisation (International Institute for Refrigeration) on biodiversity of plant species.

4. Most important publications

Salaj T, Matusikova I, Panis B, Swennen R, Salaj J (2009) Application of the slow-freezing protocol for the long-term storage of embryogenic tissues of several conifer species. CryoLett 30: 158-159

Skyba M, Urbanova M, Kapchina-Toteva V, Kosuth J, Harding K, Cellarova E (2010) Physiological, biochemical and molecular characteristics of cryopreserved *Hypericum perforatum* L. shoot tips. CryoLett 31: 249-260

Skyba M, Faltus M, Zamecnik J, Cellarova E (2011) Thermal analysis of cryopreserved *Hypericum perforatum* L. shoot tips: cooling regime dependent dehydration and ice growth. Thermochim Acta 514: 22-27

Salaj T, Matušíková I, Piršelová B, Fráterová L, Salaj, J (2011) Regrowth of embryogenic tissues of *Pinus nigra* following cryopreservation. Plant Cell Tiss Org Cult (accepted)

Country Report: Spain

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1. Major achievements in Spain related to plant cryopreservation

1.1. Working Group 1

As regards to Working Group 1 "Fundamental aspects of cryopreservation/cryoprotection and genetic stability", much of the work carried out has been done on the latter subject (Table 1). Already in the first meeting of the working group, four oral communications were presented by Spanish research groups. Dr. Ana Vázquez (Universidad Complutense de Madrid) presented a keynote lecture on "Cryopreservation and genetic instability". Two presentations from University of Oviedo showed the results from the work carried out on transgene stability in cryopreserved cork oak somatic embryos and on cryopreserved and cold stored hop *in vitro* cultures (group of Dr. Revilla). The group from Universidad Politécnica de Madrid (UPM, Dr González-Benito) presented different approaches to study genetic and epigenetic variability in cryopreserved chrysanthemum apices.

The groups of University of Oviedo and UPM worked actively during the four years on genetic and epigenetic stability of hop and chrysanthemum, respectively, and their results have been presented in several meetings of the Working Group 1.

The group of Dr. Revilla used RAPD (Random Amplification of Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) markers to study *in vitro* cold-stored and cryopreserved hop shoot apices and no genetic variation was found. Epigenetic stability was evaluated by means of MSAPs (Methylation Sentitive Amplification Polymorphism) and 36% of the loci resulted polymorphic when stored plants were compared to control ones, and the *in vitro* establishment process seemed to be the main cause of the epigenetic variation (Peredo *et al.* 2008, 2009). Other interests of this group are the use of molecular markers and flow cytometry prior to cryopreservation actions.

The UPM group studied the genetic stability by means of RAPD and AFLP markers of chrysanthemum shoots derived from apices after the different steps of an encapsulation-dehydration cryopreservation protocol. The possible role of osmotic stress on genetic instability has been hypothesized as variable regenerants were found after the sucrose pretreatment step. The sequences of the variable markers were determined and similarities with known genes were found (Martin *et al.* 2009, 2011). The epigenetic stability was studied by CRED-RA technique. A preliminary work on the relationship between (epi-)genetic changes and the morphology of the regenerated plants was presented but with no conclusive results. This group has been recently started working on the genetic stability of mint cultures after cryopreservation and *in vitro* cold storage, and relating those results with the genetic profile of field collections, by means of RAPD markers.

1.1. Work Group 2

Regarding Working Group 2 "Technology, application and validation of plant cryopreservation", several groups carried out experiments to established suitable protocols for different species and types of explants (Table 1). Dr. Sánchez-Romero, at the University of Málaga, studied several aspects of the cryopreservation of embryogenic cultures of avocado and olive, comparing slow cooling and vitrification-based techniques (Sánchez-Romero *et al.*

2009). Through collaborations with different groups, protocols for the cryopreservation of *in vitro* cultures of Spanish endemic species and of grapevine embryogenic cultures were developed at UPM (Gonzalez-Benito *et al.* 2009, Marco-Medina *et al.* 2010). Before this COST Action started, the UPM group worked together with IFAPA-Churriana (Junta Andalucía, Málaga, Spain) developing cryopreservation protocols for several strawberry cultivars. The field performance of plants derived from cryopreserved apices was studied (Medina *et al.* 2007).
Cryopreservation studies have been also carried out by other Spanish groups in a more intermittent basis, with recent publications on the following subjects:

- University of Santiago de Compostela: endangered species (Mallón *et al.* 2010)
- Instituto Investigaciones Agrobiológicas, CSIC: forest species (Vidal *et al.* 2010)
- University of Alicante: endangered species (Marco-Medina *et al.* 2010 a, b).

Table 1. Summary of research activities performed in Spain during the 4 years of COST Action 871.

Institute	Team leaders	Plant species	Thematic area	Period
University of Oviedo	Dr. M.A. Revilla	Hop	WG1 (epi-) genetic stability	2006-2010
University of Málaga	Dr. C. Sánchez-Romero	Embryogenic cultures of avocado and olive	WG2 Method development	2008-2010
UPM	Dr. M.E. González-Benito	Chrysanthemum, mint shoot apices	WG1 (epi-) genetic stability	2006-2010
		Strawberry	WG2 Field performance after cryopreservation	2006
		Grapevine embryogenic cultures	WG2 Method development	2006-2008

2. Collaborations initiated during the 4 years of the COST Action

The 871 COST Action has allowed Spanish groups working on cryopreservation to start collaboration with other European laboratories.
Through the Short Term Scientific Missions scheme several young Spanish scientists have started or strengthened their knowledge and abilities in different cryopreservation techniques:

- Raquel Folgado (University of Oviedo) visited the Catholic University of Leuven (Dr Panis's laboratory) in 2008 to use thermal analysis as a tool for the establishment of cryopreservation protocols.
- Aida Heras Rodríguez (University of Oviedo) carried out studies on the cryopreservation of apple and olive germplasm using the dormant bud method at the University of Copenhagen (Dr. Grout's laboratory) in 2009.
- Carolina Kremer Morales (UPM) visited the laboratory of Dr. Keller (IPK, Germany) in 2009, to compare different procedures for mint cryopreservation.

In addition, there is ongoing collaboration between the University of Málaga with the Catholic University of Leuven and the Millennium Seed Bank (Kew Gardens) on cryopreservation of embryogenic cultures and calorimetry; and between the University of Oviedo and the Julius Kühn Institute (Germany) regarding cryopreservation of dormant buds.
The group of UPM has established relationships with IPK regarding (epi-)genetic stability and

cryopreservation of mint cultures and with the University of Algarve (Portugal) on the cryopreservation of Portuguese endangered species, through a co-supervised PhD thesis.

3. Future of plant cryopreservation in Spain

There are few examples in which cryopreservation is currently used for the long-term conservation of plant genetic resources in Spain. At the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (I.V.I.A, http://www.ivia.es/germo/) there is a collection of embryogenic callus cryopreserved in liquid nitrogen. There are prospects for a future chestnut (shoot apices) and cork oak (somatic embryos) cryopreserved germplasm collection (Vidal *et al.* 2010). Although cryopreservation has not been considered so far as a possible tool in the Spanish strategy for plant germplasm conservation, there are research projects in which the study of basic aspects of cryopreservation are being studied supported by the Spanish Ministry of Science, as for example those of the UPM group (AGL2007-65938-C02-01, AGL2010-21989-C02-01).

In summary, there are already many expertise groups in Spain that are actively working on cryopreservation. However, for a more widely spread application of these technologies for the long-term conservation of plant germplasm, stronger and sustained economic support is necessary.

4. Most important publications

Gonzalez-Benito ME, Martin C, Vidal JR (2009) Cryopreservation of embryogenic cell suspensions of the Spanish grapevine cultivars 'Albarino' and 'Tempranillo'. Vitis 48: 131-136

Marco-Medina A, Casas JL, Gonzalez-Benito ME (2010a) Comparison of vitrification and encapsulation-dehydration for cryopreservation of *Thymus moroderi* shoot tips. CryoLetters 31: 301-309

Marco-Medina A, Casas JL, Swennen R, Panis B (2010b) Cryopreservation of *Thymus moroderi* by droplet-vitrification. CryoLetters 31: 14-23

Martin C, Gonzalez-Benito ME (2009) Cryopreservation and genetic stability of *Dendranthema grandiflora* Tzvelev *in vitro* cultures. Agricultural and Food Science 18: 129-135

Martin C, Cervera MT, Gonzalez-Benito ME (2011) Genetic stability analysis of chrysanthemum (*Chrysanthemum x morifolium* Ramat) after different stages of an encapsulation-dehydration cryopreservation protocol. J Plant Physiol 168:158-66

Medina JJ, Clavero-Ramirez I, Gonzalez-Benito ME, Galvez-Farfan J, Lopez-Aranda JM, Soria C (2007) Field performance characterization of strawberry (*Fragaria x ananassa* Duch.) plants derived from cryopreserved apices. Scientia Horticulturae 113: 28-32

Peredo EL, Arroyo-Garcia R, Reed BM, Revilla MA (2008) Genetic and epigenetic stability of cryopreserved and cold-stored hops (*Humulus lupulus* L.). Cryobiology 57: 234-241

Peredo EL, Arroyo-Garcia R, Reed BM, Revilla MA (2009) Genetic stability of *in vitro* conserved germplasm of *Humulus lupulus* L. Agricultural and Food Science 18: 144-151

Sanchez-Romero C, Swennen R, Panis B (2009) Cryopreservation of olive embryogenic cultures. CryoLetters 30: 359-372

Mallon R, Rodriguez-Oubina J, Gonzalez ML (2010) Vitrification of mosses: a useful method for the cryopreservation of *Splachnum ampullaceum* Hedw. CryoLetters 31:24-28

Vidal N, Vieitez AM, Fernandez MR, Cuenca B, Ballester A (2010) Establishment of cryopreserved gene banks of European chestnut and cork oak. European Journal of Forest Research 129: 635-643

Country Report: United Kingdom

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1. Major achievements in the United Kingdom related to plant cryopreservation

1.1. Working Group 1

The UK has an established international profile in fundamental cryobiology research, enhanced across the national plant cryopreservation community by participation in COST 871 (Table 1). WG1 achievements in fundamental research relate to biophysics, cryoinjury and stability. Attendance of UK WG1 scientists at the COST 871 Differential Scanning Calorimetry (DSC) workshops enabled technology transfers resulting in:

a. Thermal analysis being widely used by the Royal Botanic Gardens Kew, Millennium Seed Bank (RBG-MSB) for research and training purposes.
b. The optimisation of dehydration regimes for dormant apple bud cryopreservation by the University of Reading (UoR).

Collaborations between the University of Derby (UoD), Damar Research Scientists (DRS) and IVALSA, (Firenze) Italy used oxidative stress and antioxidant biomarkers to gain an understanding of stresses incurred during olive cryopreservation. Cryostability research undertaken in the framework of cryobionomics involved collaborations between Keith Harding (DRS) and the Universiteit Göttingen, Sammlung von Algenkulturen, Germany and the University Mánesova, Kosice, Slovakia.

1.2. Working Group 2

Research expertise and knowledge generated within WG1 facilitated technology transfers for cryopreservation applications (WG2), specific UK examples include:

a. Cryostorage: a novel genotype-independent vitrification method for cocoa cryopreservation developed by Ghanaian UoR PhD student Raphael Adu-Gyamfi.
b. Physiology: a collaboration between UoD, DRS and IPK Gattersleben, Germany demonstrated correlations between post-harvest storage duration in *Allium sativum* (garlic) cloves and post-warming survival/regrowth of stem disks.
c. Storage stability: collaboration between the RBG-MSB and University Polytechnic Madrid (UPM), Spain analysed thermal finger prints of seeds of 17 *Brassicaceae* species stored up to 44 years in dry, cold conditions in the UPM Seed Bank.

UK partners are involved with an important collaboration initiated by COST 871 assessing modifications required for dormant bud cryopreservation applied in the less severe climates of European partners as compared to those in continental USA. As a result of COST 871 workshops replicated protocols and equipment are being utilized for the cryopreservation of *Malus* germplasm in the UK, Denmark, Italy and France. This combined with participation in

COST 871 DSC workshops (WG 1) facilitated dormant bud work at UoR resulting in the cryopreservation of >25% of the 2400 *Malus* accessions that form the major part of the UK's National Fruit Collection in the past 2 years. In addition RBG-MSB is using cryopreservation storage as back up for conventionally-stored orthodox seeds.

Table 1. Summary of plant cryopreservation research activities in UK during COST 871.

Institute	Team leaders	Plant species /germplasm	Thematic area	Period
RBG-MSB & UPM	H. Pritchard & E. González Benito	Species in the Brassicaceae	WG2	Oct. 2010
RBG-MSB & Bilbao University, Spain.	I. Kranner & B. Fernandez	*Tortula ruralis*	WG2	June-July 2010
RBG-MSB & Massey University, New Zealand	J.Nadarajan & C. McGill	*Dysoxylum spectabile*	WG1 & 2	2008-on going
RBG-MSB, Universities of Sussex & Bedfordshire (MSc student projects)	H. Pritchard, J. Nadarajan & T. Marks	Seeds of various spp.	WG1 & 2	2007-on going
UoR & UoC	A.Wetten, B.Grout & T. Toldam-Andersen.	*Malus* dormant buds	WG1 & 2	2007-on going
UoR , IICQC	A.Wetten & R. Adu-Gyamfi	*Theobroma cacao*	WG1 & 2	2007-on going
DRS& Universiteit Göttingen, Germany	K. Harding & T. Friedl	Algae sp.	WG1	2005-on going
DRS & University, Mánesova, Slovakia	K. Harding & A. Cellarova	*Hypericum*	WG1	2009-2010
DRS, UoD & IVALSA, (Firenze) Italy	E. Benson, P.T. Lynch C. Benelli, & M. Lambardi	*Olea europaea*	WG1 & 2	2004-on going
UoD, DRS & IPK Gattersleben, Germany	P.T. Lynch, K. Harding & J. Keller	*Allium sativum*	WG2	2002-on going
UoD & IFBD-P, Germany	P.T. Lynch & M. Höefer	*Fragaria x ananassa*	WG2	2007-on going

1.3. Education, Technology Transfer and Networking

During COST 871 all members of the UK group have been involved with the successful supervision of >15 UK and international PhD and MSc projects involving plant cryopreservation. A new taught MSc in Conservation Biology has been launched at the UoD, to which DRS contribute. DRS have collaborated in the development and delivery of a new ISBER-endorsed Certificate in Biobanking with the Integrated Biobank of Luxembourg and the University of Luxembourg. UK networking through conference and meetings is summarised in Table 2.

2. Collaborations initiated during the 4 years of the COST Action

Additional to the continuation of existing collaborations, (Table 1) variously enhanced by COST 871, a number of new collaborations have been initiated:

a. RBG-MSB, UPM and Bilbao University, Spain on seed cryopreservation
b. Joint supervision of a PhD student between UoR and University of Copenhagen (UoC) on cryopreservation of *Malus* dormant buds.
c. UoD and Institute of Fruit Breeding Dresden-Pillnitz (IFBD-P), Germany, as part of a COST 871 STSM, exchanging strawberry cryopreservation technology.
d. DRS international links concerning cryopreservation capacity building including outside the EU include visiting lectureships with: (a) the National Biotechnology Centre of the

Republic of Kazakhstan; (b) UPM, Spain, (c) research fellowships with the University of The Witwatersrand, Johannesburg, University of KwaZulu-Natal, National Zoological Gardens Wildlife Biological Resource Centre, wBioBankSA to progress the strategic development of a Sub-Saharan Centre of Excellence in Cryobiology; (d) cryopreservation of endangered species of the Mata Atlantica with the University of Santa Caterina, Brazil; and (e) UK appointments as (i) associate lecturers and Research Fellows (Cryobiology, Conservation and Molecular Genetics) at UoD and (ii) conferment as Honorary Research Associates at RBG-MSB.

Table 2. UK involvement in the participation and organisation of conferences and production of proceedings outputs during COST 871

Date of event	Event Title	Organisations involved	UK COST 871 members involvement
September 2007	Annual Scientific Meeting, AGM and Symposium	Society for Low Temperature Biology (SLTB). Co-hosted with COST 871 MC meeting	Co-organised by UoD and DRS UoD, RBG-MSB and UoR participants
February 2008	2nd Meeting of WG 1&2 COST 871	COST 871, MTT (Agrifood Research, Finland), Univ. of Oulu,	Keith Harding, key note & proceedings editor. All UK partners participated.
February 2009	Fundamental Aspects of Plant Cryopreservation	Joint meeting between COST 871 and SLTB	Organised by RBG-MSB. Erica Benson, key note. All UK partners participated.
April 2009	First International Symposium on Cryopreservation in Horticultural Species	International Society for Horticultural Science, COST 871, and Katholieke Universiteit Leuven	UoD and RBG-MSB on local and international scientific committees and participants. Paul Lynch co-editor of proceedings.
July 2010	Cryo 2010	Cryobiology Society and STLB	UoD, RBG-MSB and DRS on international scientific committee and participants.
March 2011	SLTB, Spring Meeting	SLTB	Organised by RBG-MSB. Participation by DRS and RBG-MSB.

3. Future of UK plant cryopreservation

Significant future plant cryopreservation initiatives include the commitment by the RBG-MSB to establish a cryo-seed bank. The cryopreservation at UoR of dormant buds of *Malus* accessions that form the major part of the UK's National Fruit Collection to provide a secure back up to field based accessions. The UoR is also implementing a cryopreserved backup collection of *in vitro* clones of *Theobroma cacao* to support the International Intermediate Cocoa Quarantine Centre (IICQC).

4. Most important publications.

During COST 871, the UK group has authored 36 peer reviewed journal articles and review publications, 62 conference proceedings and 9 book chapters, 52 of these are related to COST 871 and 24 include international (non EU) collaborations.
Most significant ones:

Benson EE (2008) Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Crit. Rev. Plant Sci. 27: 141-219

Berjak P, Bartels P, Benson EE, Harding K, Mycock D, Pammenter NW, Sershen, Wesley-Smith J (2011) Cryo-conservation of South African plant genetic diversity. *In Vitro* Cell. Devel. Biol. – Plant 47: 65-81

Daws MI, Pritchard HW (2008) The development and limits of freezing tolerance in *Acer pseudoplatanus* fruits across Europe is dependent on provenance. CryoLett.29 :189-198

Fang J-Y, Sacande M, Pritchard HW, Wetten A (2009) Influence of freezable/non-freezable water and sucrose on the viability of *Theobroma cacao* somatic embryos following desiccation and freezing. Plant Cell Rep 28: 883-889

Fang J-Y, Wetten A, Johnston J (2008) Headspace volatile markers for sensitivity of cocoa (Theobroma cacao L.) somatic embryos to cryopreservation. Pl. Cell Rep. 27: 453-461

Hamilton KN, Ashmore SE, Pritchard HW (2009) Thermal analysis and cryopreservation of seeds of Australian wild Citrus species (Rutaceae): Citrus australasica, C. inodora and C. garrawayi. CryoLett. 30: 268-279

Harding K, Johnston JW, Benson EE (2009) Exploring the physiological basis of cryopreservation success and failure in clonally propagated in vitro crop plant germplasm. Agri. Food Sci. 18: 3-16

Li D-Z, Pritchard HW (2009) The science and economics of *ex situ* plant conservation. Trends Plant Sci. 14: 614-621

Lynch PT, Siddika A, Mehra A, Fabbri A, Benelli C, Lambardi M (2007) The challenge of successful cryopreservation of olive shoot tips. Adv. Hort. Sci. 21:211-214

Lynch PT, Souch G, Al Majathoub M., Keller J, Höefer M and Harding K, 2008. Steps towards the validation of *Allium* and strawberry cryopreservation. CRYOPLANET - COST Action 871. Agrifood Research Working Papers 153. Oulu Finland, 20-23/02/2008. 43–44

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Nadarajan J, Mansor M, Krishnapillay B, Staines HJ, Benson EE, Harding K (2008) Applications of DSC in developing cryopreservation strategies for *Parkia speciosa*, a tropical tree producing recalcitrant seeds. CryoLett.29: 95-110

Pritchard HW, Fuller BJ (2009) Beyond the cell wall: a comparison Between Plant and Animal Cell Cryopreservation. In Vitro Cell. Devel. Biol. – Animal 45**:** S18 - S19

Skyba M, Urbanová M, Kapchina-Toteva V, Košuth J, Harding K, Čellárová E (2010) Physiological, biochemical and molecular characteristics of cryopreserved *Hypericum perforatum* l. Shoots tips. CryoLett. 31: 249-260

Vogiatzi C, Grout BWW, Wetten A, Toldam-Andersen TB (2011) Cryopreservation of Winter-Dormant Apple Buds: I - Variation In Recovery With Cultivar and Winter Conditions. CryoLett. (in press)

Vogiatzi C, Grout BWW, Wetten A, Toldam-Andersen TB (2011) Cryopreservation of Winter-Dormant Apple Buds: II - Tissue Water Status After Desiccation at -4°C and Before Further Cooling. CryoLett. (in press)

Wetten A., Adu-Gyamfi R., Fang J-Y, Rodriguez-Lopez, C (2008) Use of secondary somatic embryos improves genetic fidelity of cocoa (*Theobroma cacao* L.) following cryopreservation. CRYOPLANET – COST Action 871. Agrifood Research Working Papers 153. Oulu Finland 20-23/02/2008. 23-24

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