



Cryopreservation
of Crop Species
in Europe

1st Meeting of Working Group 2

Technology, application and validation
of plant cryopreservation

Florence, May 10-12, 2007

Organization

CNR-IVALSA, Florence

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CryoPlanet
Cost Action 871

COST Action 871

Cryopreservation of Crop Species in Europe

1st Meeting of Working Group 2 (Florence, May 10-12, 2007)

TECHNOLOGY, APPLICATION AND VALIDATION OF PLANT CRYOPRESERVATION

Cryopreservation is the storage of viable plant germplasm in liquid nitrogen (-196°C). At this temperature metabolic and most physical processes are stopped. Hence, plant germplasm preserved under cryogenic storage can be safely maintained for very long term, theoretically ad infinitum. The major purpose of the “CryoPlanet - Cryopreservation of Crop Species in Europe”, COST Action 871, is to create a network that brings together European scientists with an expertise and/or interest in plant cryopreservation, with the main aim of developing and implementing efficient cryogenic procedures. At present, already 16 European countries (i.e., Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Netherlands, Poland, Portugal, Serbia-Montenegro, Slovakia, Spain, United Kingdom) have signed the Memorandum of Understandings, while two other countries (Romania and Turkey) have indicated their intention to sign. Two Working Groups are distinguished within the Action, i.e., WG1/Fundamental aspects of cryopreservation/cryoprotection and genetic stability, and WG2/Technology, application and validation of plant cryopreservation. Dr. Bart Panis is the Chair and Prof. Paul Lynch the Vice-Chair of the Action, which will run until December 2010.

At the 1st Meeting of Working Group 2, organized by the IVALSA Institute at the CNR (National Research Council) Area of Florence on May 10-12, 2007, scientists from 14 European Countries presented 24 lectures, dealing with various aspects of plant cryopreservation, from the development of efficient procedures to their application on germplasm collections in Europe. Keynote lectures on cryotherapy (Qiaochun Wang, China), cryopreservation of invertebrate organisms (Tiziana Irdani, Italy) and germplasm cryopreservation in Argentina (Luis Mroginski, Argentina) completed the programme.

Bart Panis (Chair of the Action)

Maurizio Lambardi, Carla Benelli (Editors)



1st Meeting of the Working Group 2

**TECHNOLOGY, APPLICATION AND VALIDATION
OF PLANT CRYOPRESERVATION**

Florence, May 10-12, 2007



CryoPlanet
Cost Action 871

Programme

Thursday 10th

16.30 Meeting of the Executive Committee

Friday 11th

8.30-9.30 Registration of the participants

9.30-9.45 Welcome of Prof. Ario Ceccotti, Director of IVALSAs

9.45-10.00 Welcome of Dr. Bart Panis, Chair of the COST Action 871

Theme 2.1. Technology aspects of cryopreservation

(Session 1 - Chair: Florent Engelmann)

10.00-10.20 Cryopreservation of *Betula in vitro* shoot tips by vitrification
A. Meier-Dinkel

10.20-10.40 Study of phytohormone composition of growth medium for hop plant recovery improvement after cryopreservation
M. Faltus, A. Bilavcik, J. Zamecnik

- 10.40-11.00 The challenge of successful cryopreservation of olive (*Olea europaea* L.) shoot tips
P. Lynch, A. Siddika, A. Mehera, A. Fabbri, C. Benelli, M. Lambardi
- 11.00-11.20 Cryopreservation of *Drosophyllum lusitanicum* seeds, an endangered insectivorous plant
S. Gonçalves, A. Romano
- 11.20-11.40 The current status of conservation of agricultural and forestry genetic resources in Slovakia and related cryopreservation activities
J. Krajnakova, M. Sušková, R. Longauer, D. Benediková, H. Häggman
- 11.40-12.00 Cryopreservation of dormant buds of apple cultivars in a mild maritime winter climate
T. B. Toldam-Andersen, K. Suszkiewicz, T. B. Nygaard
-

12.00-14.00 Lunch

14.00-14.30 **Keynote lecture**

Elimination of viruses and phytoplasma by cryotherapy of *in vitro*-grown shoot tips

Q. Wang, J. P. T. Valkonen

(Session 2 - Chair: Paul T. Lynch)

14.30-14.50 Cryopreservation of *in vitro* cultures from *Maesa lanceolata*
E. Lambert, D. Geelen

14.50-15.10 Survival of apical meristems of aspen after cryopreservation and slow thawing
P. M. Pukacki, P. Chmielarz, K. Bojarczuk, W. Wesoly

15.10-15.30 Preservation and identification by confocal microscopy of fern spores
H. Fernández, R. A. Arbesú, A. M. Nistal, V. Menéndez, M. A. Revilla

15.30- 15.50 Cryopreservation and vegetative multiplication of *Cyathea australis*
A. Miłkula, J. J. Rybczyński

15.50-16.10 Cryopreservation of yam germplasm in Guadeloupe (FWI)
S. Gallet, F. Gamiette, D. Filloux, F. Engelmann

16.10-16.30 Recent advances in cryopreservation of small fruit germplasm
C. Damiano, M. D. Arias Padró, A. Frattarelli

16.30-17.00 Coffee break

17.00-17.10 *Presentation of the “Azienda Vivai Battistini” of Cesena, Italy*
(*G. Dradi, R. Roncasaglia*)

17.10-17.40 **Keynote lecture**

The challenge of preserving invertebrate’s species by cryopreservation
T. Irdani, E. Cosi, P. F. Roversi

Theme 2.2. Impact and applications of cryopreservation in plants

(Session 1 - Chair: Joachim Keller)

17.40-18.00 Cryopreservation of the banana germplasm collection at the ITC (INIBAP Transit Centre)

B. Panis, I. Van den houwe, B. Piette, R. Swennen

18.00-18.20 Implementing a strawberry cryogenic genebank: field performance of plants derived from apices cryopreserved for one year

C. Soria, J. Gálvez-Farfán, M. T. Ariza, J. J. Medina1, M. E. González-Benito

18.20- 18.40 Definition of a new research program on cryopreservation

A. Grapin, A. Gallard, N. Dorion

18.40- 19.00 Towards a microplate technology platform for plant breeding

E. Anastassopoulos

Saturday 12th

8.30-9.00 **Keynote lecture**

Cryopreservation of plant germplasm in Argentina

L. Mroginski, H. Rey

(Session 2 - Chair: Carmine Damiano)

9.00-9.20 Introducing cryopreservation for plant germplasm preservation in Germany

E. R. J. Keller

9.20-9.40 Cryopreservation in certified plant production

M. Uosukainen, J. Laamanen, A. Nukari

- 9.40-10.00 Preliminary results of *Malus* germplasm cryopreservation from the Institute of Fruit Breeding gene bank collection in Dresden
M. Höfer
- 10.00-10.20 Seed cryopreservation for conservation of ancient *Citrus* germplasm
M. Lambardi, A. Halmagyi, C. Benelli, A. De Carlo, C. Vettori
- 10.20-10.30 *Presentation of “ProClimatic – Climatic Simulation Chambers”, Imola, Italy*
(B. Antignani)
-

10.30-11.00 Coffee break

(Session 3 - Chair: Maurizio Lambardi)

- 11.00-11.20 Cryopreservation in the Finnish national germplasm programme for the horticultural plants
A. Nukari, M. Uosukainen
- 11.20-11.40 Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech Cryobank
J. Zamecnik, M. Faltus, A. Bilavcik
- 11.40-12.00 Physiological and structural characterization of embryogenic tissues of *Pinus nigra* following cryopreservation
T. Salaj, B. Panis, R. Swennen, J. Salaj
- 12.00-12.20 The current status of plant genetic diversity in Turkey and implications for cryopreservation studies
A. E. Ozudogru, Y. Ozden-Tokatli, F. Gumusel, C. Benelli, M. Lambardi
-

12.20-13.00 General discussion and final considerations (B. Panis, P. Lynch)

13.30 Farewell lunch at the Restaurant “La Limonaia”, Sesto Fiorentino (Firenze)

Sunday 13th

- 08.30-13.30 Post-Meeting Tour
Visit to the Chianti Estate “Castello di Nipozzano”
(Marchesi de’ Frescobaldi, Firenze)

Theme 2.1. Technology aspects of cryopreservation

Coordinator: Florent Engelmann (overall WG 2 Leader)

GERMANY

Cryopreservation of *Betula in vitro* shoot tips by vitrification

A. Meier-Dinkel

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Introduction

Elite trees of *Betula pendula* and *Betula pubescens* have been selected in progeny trials for good stem form and growth characteristics. Ramets of these trees were produced by micropropagation and planted in clonal field trials with the aim of approval and certification of the clones as tested basic material according to the council directive on the marketing of forest reproductive material and the German law on forest reproductive material. The aim is to cryopreserve *in vitro* shoot tips of more than 100 birch clones for the duration of the field testing in order to use the material of superior clones for commercial micropropagation as soon as the field trials are evaluated.

Material and methods

Two birch clones (one *B. pendula* and one *B. pubescens*) were used for the adaptation of a PVS2-vitrification protocol used in our lab for the cryopreservation of *in vitro* shoot tips of ash (2) to the requirements of birch. Shoot cultures were propagated on WPM containing 1.0 mg/l BAP and 0.1 mg/l Kinetin and used for the experiment three weeks after the last subculture. The following factors were tested: cold hardening (8 h light, 37 $\mu\text{mol m}^{-2} \text{s}^{-1}$ /16 h darkness at 20°C/3°C) for two and three weeks, respectively, and - after cold hardening - preculture of dissected shoot tips on WPM containing 3% sucrose and 0.8M glycerol at 4°C for one and three days, respectively. Shoot cultures grown in 250 ml glasses were placed in a cooled incubator for cold hardening (2 weeks, 3 weeks). Apical shoot tips of 1 mm length comprising the meristem and a few leaf primordia were dissected using a cannula (syringe needle), placed on the preculture medium in Petri dishes and kept in a refrigerator (1 day, 3 days). Then they were pretreated for 25 min in 2M glycerol and 0.4M sucrose dissolved in liquid WPM. This pretreatment solution was removed from the cryovials and replaced by 1.5 ml PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO [w/v] and 0.4M sucrose in liquid WPM). After 30 min incubation on ice the cryovials were immersed in LN.

For rewarming the cryovials were kept for 1 min in a 42°C water bath and then for 10 s in 20°C water. The PVS2 was immediately drained from the cryotubes and replaced by WPM containing 1.2M sucrose. The shoot tips were rinsed three times more with the 1.2M sucrose solution and kept for 3 min in the last rinse, then drained on sterile filter paper and placed on regeneration medium in Petri dishes (WPM containing 1.0 mg/l BAP and 0.1 mg/l Kinetin). The PVS2 controls (without freezing in LN) were treated in the same way immediately after 30 min PVS2 treatment. The Petri dishes were kept in the dark for the first two days in the plant growth room. The culture conditions were 16 h photoperiod with a light intensity of 60 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a day/night temperature of 24/21°C. Survival and regeneration of the explants was assessed 7, 14, 21 and 28 days after rewarming.

Results

The PVS2 controls showed 100% shoot formation with only one exception with 86%. This shows that the cold hardening, preculture, pretreatment and vitrification treatment methods are well adapted to *in vitro* shoots of birch. The highest shoot formation after freezing in LN with

60% for the *B. pendula* clone and 75% for the *B. pubescens* clone was achieved after three weeks of cold hardening and three days of preculture on WPM with 0.8M glycerol (Table 1). After two weeks of cold hardening shoot formation for both clones and both preculture periods was less (43 to 50%). The result after three weeks hardening and one day of preculture was inconsistent. For the *B. pubescens* clone shoot formation was 67%, which is at the same level as after one day of preculture. However, none of the frozen *B. pendula* explants formed shoots in this treatment combination. The results obtained so far are promising for the aim of cryopreserving a large number of birch clones. The experiment will be repeated with more clones in order to confirm the best treatments. In a study with *in vitro* shoot tips of five *Betula pendula* genotypes using the slow-cooling procedure with polyethylene glycol as cryoprotectant (1) the genotypic differences in re-growth ranged from 10 to 87%. Our results show that similar shoot regeneration rates can be obtained with the described vitrification protocol.

Table 1. Regeneration and shoot formation of *Betula* shoot tips over a four week post-thaw period after different cold hardening and preculture periods (Clone 106-43 = *B. pendula*; clone 1386-10 = *B. pubescens*. b, brown explant; g, green explant; s, explant with shoot formation).

Hardening 2 weeks, preculture 1 day			Regeneration after				Shoot
	Clone	No. explants	7 d	14 d	21 d	28 d	formation %
PVS2 cont.	106-43	6	6 g	6 g	6 s	6 s	100
LN	106-43	6	6 g	4 g, 2 b	5 s, 1 b	3 s, 1 g, 2 b	50
PVS2 cont.	1386-10	10	10 g	10 g	10 s	10 s	100
LN	1386-10	12	12 g	6 g, 6 b	6 s, 6 b	6 s, 6 b	50
Hardening 2 weeks, preculture 3 days							
PVS2 cont.	106-43	7	7 g	7 g	6 s, 1 b	6 s, 1 b	86
LN	106-43	7	7 g	6 g, 1 b	5 s, 2 b	3 s, 3 g, 1 b	43
PVS2 cont.	1386-10	12	12 g	12 g	12 s	12 s	100
LN	1386-10	11	11 g	6 g, 5 b	7 s, 4 b	5 s, 2 g, 4 b	45
Hardening 3 weeks, preculture 1 day							
PVS2 cont.	106-43	6	5 g, 1 b	5 g, 1 b	6 s	6 s	100
LN	106-43	7	2 g, 5 b	2 g, 5 b	1 g, 6 b	7 b	0
PVS2 cont.	1386-10	12	12 g	12 g	12 s	12 s	100
LN	1386-10	12	12 g	9 g, 3 b	9 s, 3 b	8 s, 4 b	67
Hardening 3 weeks, preculture 3 days							
PVS2 cont.	106-43	8	8 g	8 g	8 s	8 s	100
LN	106-43	5	5 g	3 g, 2 b	3 s, 1 g, 1 b	3 s, 2 b	60
PVS2 cont.	1386-10	12	12 g	12 g	12 s	12 s	100
LN	1386-10	12	12 g	10 g, 2 b	9 s, 3 b	9 s, 3 b	75

References

1. Ryyänänen, LA & Häggman, HM (2001) *Plant Cell. Rep.* **20**, 354-360.
2. Schoenweiss, K, Meier-Dinkel, A & Grotha, R (2005) *CryoLetters* **26** (3), 201-21.

CZECH REPUBLIC

Study of phytohormone composition of growth medium for hop plant recovery improvement after cryopreservation

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Introduction

Plant recovery after cryopreservation is determined by explant pretreatment, by ways of freezing or thawing, and by conditions of plant recovery after cryopreservation – culture conditions and regrowth medium. Aim of this study was to analyze the effect of phytohormone composition of regrowth medium on hop plant growth and recovery.

Materials and methods

Effect of phytohormones on hop plant recovery was studied on apical shoot tips and nodal cuttings of cultivar ‘Saazer’ of hop (*Humulus lupulus*, L.). Modified solid media according to MS (1) without casein and myoinositol, with lower amount of nitrogen (25% of NH_4NO_3 and 50% of KNO_3 of the original MS medium), with 40 g l⁻¹ glucose, pH 5.5 and with different phytohormone composition were used:

- 1) Medium without phytohormones – standard multiplication medium
- 2) IBA medium – standard medium + 0.1 mg l⁻¹ indole-3-butyric acid (IBA)
- 3) BAP medium - standard medium + 1 mg l⁻¹ 6-benzylamino-purine (BAP)
- 4) GA₃ medium - standard medium + 0.2 mg l⁻¹ gibberellic acid (GA₃)
- 5) IBA + BAP medium - standard medium + 0.1 mg l⁻¹ IBA + 1 mg l⁻¹ BAP
- 6) IBA + GA₃ medium - standard medium + 0.1 mg l⁻¹ IBA + 0.2 mg l⁻¹ GA₃
- 7) BAP + GA₃ medium - standard medium + 1 mg l⁻¹ BAP + 0.2 mg l⁻¹ GA₃
- 8) IBA + BAP + GA₃ medium - standard medium + 0.1 mg l⁻¹ IBA + 1 mg l⁻¹ BAP + 0.2 mg l⁻¹ GA₃
- 9) 1/10 (IBA + BAP + GA₃) medium - standard medium + 0.01 mg l⁻¹ IBA + 0.1 mg l⁻¹ BAP + 0.02 mg l⁻¹ GA₃

Maternal plants were cultivated on medium without phytohormones at 22±1°C, 80 μmol m⁻² s⁻¹, photoperiod 16/8 h; subculture interval was 8 weeks. Subsequently, the apical shoot tips and nodal cuttings were prepared and put on different regrowth media. Plant regrowth was determined after next 4 weeks. Four characteristics were evaluated: shoot growth, root growth, callus production and plant recovery by 4-step scale (0 - no growth, 1 - weak growth, 2 - medium growth, 3 - strong growth).

Results

The presented study confirmed hypothesis of the decisive effect of phytohormones on recovery of hop plants (Table 1). The differences in plant recovery were also found between apical shoot tips and nodal cuttings. Apical shoot tips stopped their initial growth of shoots after the first week of recovery, and the growth of roots was not observed on medium without phytohormones. No callus production was detected in both, apical shoot tips and nodal cuttings on medium without phytohormones. Slow growth of shoots and roots were observed in nodal cuttings on medium without phytohormones. No callus production was also observed at apical shoot tips and nodal cuttings on medium with IBA. The IBA medium improved particularly root growth in nodal cuttings. Plant recovery of apical shoot tips was better on this medium in

comparison with the medium without phytohormones. Strong or medium callus production was found on the medium with BAP in apical shoot tips or nodal cuttings, respectively. This medium had no effect on root growth in apical shoot tips and very weak effect on root growth in nodal cuttings. The BAP medium had strong effect on shoot growth in apex and medium or weak effect on shoot growth in nodal cuttings. Presence of BAP in regrowth medium had positive effect on plant recovery in apex and nodal cuttings. Presence of GA₃ in medium affected shoot growth, particularly in nodal cuttings. This medium did not affect root growth or callus production in both, apical shoot tips and nodal cuttings. Effect of BAP on callus production was observed in combination with other phytohormones – GA₃ or IBA in both, apical shoot tips and nodal cuttings. Positive effect of IBA on root growth was also found in mixture with GA₃ but not with BAP. However, BAP had always positive affect on shoot growth. The medium with lower content of the all phytohormones had weak effect on callus production and positive effect on plant recovery.

Table 1. Growth and recovery of apical shoot tips and nodal cuttings on different media

Medium - phytohormone composition	Shoot growth		Root growth		Callus production		Plant recovery	
	apex	cutting	apex	cutting	apex	cutting	apex	cutting
Without phytohormones	1	1	0	1-2	0	0	1	2-3
IBA	1-2	1-2	1	2	0	0	2	2-3
BAP	2-3	1-2	0	0-1	2	3	2	2
GA ₃	1	3	0	1	0	0-1	1	2-3
IBA+BAP	3	0-1	0	0	3	3	2	0-1
IBA+GA ₃	1-2	3	1	1-2	0	1	1-2	2-3
BAP+GA ₃	3	2	0	0	3	3	1	1-2
IBA+BAP+GA ₃	2	2	0	0	2	2	1	1
1/10 (IBA+BAP+GA ₃)	2	1-2	0	1-2	0-1	1	2-3	2-3

Growth and recovery were evaluated by 4-step scale (0 – no growth/ recovery, 1 – weak growth/ recovery, 2 – medium growth/ recovery, 3 – strong growth/ recovery).

Conclusions

The presence of phytohormones was necessary for recovery of tiny shoot tips in contrast to nodal cuttings. The highest effect on plant recovery was observed at the medium with BAP. This medium had a positive effect on initial very strong shoot growth and plant recovery. But this medium had very strong effect on callus production that led to the reduction of growth and plant recovery. The IBA medium affected significantly only root growth and the GA₃ medium only shoot growth in nodal cuttings. Any combination of BAP with others phytohormones led usually to callus production; only application of the all three phytohormones (BAP+IBA+GA₃) in 10 times lower concentration led to favorable stimulation of plant recovery with reduced callus production.

Acknowledgement: This study was supported by research projects of Ministry of Agriculture of The Czech Republic QF3039 and 0002700602.

Reference

1. Murashige, T & Skoog, FA (1962) *Physiol. Plant.* **15**, 473 - 497.

UNITED KINGDOM

**The challenge of successful cryopreservation of olive (*Olea europaea* L.)
shoot tips**

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Olive (*Olea europaea* L.) is a commercially very significant vegetatively propagated crop species. Traditionally olive germplasm is conserved in field collections, but remains vulnerable due to factors such as pests, diseases and economic pressures. Therefore *in vitro* approaches, including cryopreservation may provide an important component for a sustainable olive germplasm conservation strategy. Some success has been reported using slow growth approaches for the conservation of olive germplasm (2). However, after up to 8 months storage in the dark at 4-6°C post-storage growth was poor, therefore indicating the need for the development of a protocol for the cryopreservation of olive shoot tips. Successful recovery of *in vitro* derived olive shoot tips after cryopreservation has been reported (1, 3). Although both reports described post-thaw olive shoot survival and Martinez et al., (3) demonstrated post-thaw shoot regrowth; no sustained olive shoot proliferation has been reported after cryopreservation. Hence the aim of this study has been to develop a suitable protocol for the cryopreservation of *in vitro* culture derived olive shoot tips.

In vitro shoot cultures of olive cultivars ‘Frantoio’ and ‘Leccino’ were maintained on OM medium (6) supplemented with 2.0 mg l⁻¹ zeatin. The effectiveness of three protocols for the cryopreservation of shoot tips derived from these cultures was assessed, specifically encapsulation/dehydration, encapsulation-osmoprotection/dehydration and PVS2. No post-thaw shoot regrowth was observed after either encapsulation/dehydration, encapsulation-osmoprotection/dehydration protocols. The two stage addition of PVS2 (incubation in 50% PVS2 for 30 minutes followed by incubation in 100% PVS2 for 1 hour) was shown to significantly (p<0.01) enhance post-thaw shoot regrowth (Table 1). During the first 4 weeks after thawing shoot tip regrowth was observed in terms of the development of new leaves, but over this period the rate of growth slowed, leaves thickened and callus formed over the base of the growing shoots. Within 8-10 weeks after thawing growth stopped and the shoots turned brown and died. Non-frozen control shoots which had been subjected to the PVS2 treatment continued to proliferate.

The similarity of these results with published reports (1, 3) and the DSC study of Martinez *et al.* (4) which indicates that formation of stable vitrified glasses suggest that the lack of sustained post-thaw shoot regrowth may be due to *in vitro* culture shortcomings. Hence the effect of supplementing the post-thaw culture medium with different types and concentrations of growth factors was assessed. Increasing the concentration of zeatin in the post-thaw culture medium up to 10mg l⁻¹ did not result in a significant increase in the frequency of post-thaw regrowth.

However, post-thaw growth in terms of the development of new leaves and shoot elongation was greater following recovery on OM medium containing 8- 10mg^l⁻¹ zeatin. Addition of GA₃ (10 - 20mg^l⁻¹) to post-thaw recovery medium at standard or elevated zeatin concentrations did not affect the frequency of post-thaw regrowth. The elongation of thawed shoots was not enhanced by the presence of GA₃. However, none of the modified post-thaw culture media supported sustained shoot elongation and proliferation.

To determine if the post-thaw growth response of olive shoot tips could be related to tissue/cell damage, histological studies were undertaken using shoot tips of the Italian cultivar ‘Gentile di Larino’ which exhibited 30% post-thaw regrowth after PVS2 cryopreservation (5). Although PVS2 treatment and subsequent plunging into liquid nitrogen did not appear to modify the structure of the meristematic apex, several changes were noted in the sub-apical cells following PVS2 treatment which became more enhanced by immersion in liquid nitrogen. Specifically:

- i. marked cell wall gelification of sub-apical cells
- ii. significant dehydration of external parenchyma cells
- iii. cellular starch accumulation.

This presentation will consider the significance of these results in terms of olive shoot cryopreservation and also the significance of *in vitro* culture to support effective cryopreservation protocols.

Table 1. The effect of PVS2 treatment on the regrowth of non-frozen and frozen shoot tips of olive cv Frantoio, maintained on OM medium for 4 weeks

PVS2 Treatment	Regrowth (%)	
	Non-frozen	Frozen
100% PVS2 1h		
60% PVS2 10min, 100% PVS2 1h	46.6 ± 6.6	6.6 ± 6.6
60% PVS2 20min, 100% PVS2 1h	60.0 ± 11.5	13.6 ± 13.3
60% PVS2 30min, 100% PVS2 1h	46.6 ± 6.6	13.3 ± 6.6
50% PVS2 10min, 100% PVS2 1h	40.0 ± 0.0	6.6 ± 6.6
50% PVS2 20min, 100% PVS2 1h	46.6 ± 6.6	13.3 ± 6.6
50% PVS2 30min, 100% PVS2 1h	53.3 ± 6.6	26.6 ± 6.6

Each value represents mean regrowth of 15 shoot tips ± standard error

Refereces

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PORTUGAL

Cryopreservation of *Drosophyllum lusitanicum* seeds, an endangered insectivorous plant

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Drosophyllum lusitanicum (L.) Link, the portuguese sundew, is an insectivorous plant native to the southern part of the Iberian Peninsula and northern Morocco. Recent surveys have considered *D. lusitanicum* as a severely threatened species with fragmented distribution, and the large disturbances by construction of housing and infrastructure being main agents causing population losses (1, 2). Therefore, it is urgently needed to establish reliable methods for conservation of this species.

Cryopreservation methods have been successfully applied for preservation of seeds of several wild and endangered species. However, to the best of our knowledge, there is no works about storage or cryopreservation of *D. lusitanicum* seeds. In this context, the aim of this study was to develop a simple and reliable method of cryopreservation of *D. lusitanicum* seeds.

Mature seeds of *D. lusitanicum* collected from wild plants were surface sterilized and used in the following assays. For cryopreservation without any pre-treatment, seeds were transferred to 1.5 ml test tubes and directly plunged into liquid nitrogen. Following storage during 30 min, the tubes were removed and were rapidly or slowly warmed. For rapid warming, the tubes were placed in a water bath at $38\pm 2^{\circ}\text{C}$ for 12 min. For slow warming, the tubes were warmed to ambient temperature for 30 min in a laminar airflow cabinet.

To test the effect of an air desiccation treatment before cryopreservation, sterilized seeds were arranged in a single layer in sterile Petri dishes and placed in the air current of laminar airflow cabinet during 4 h at room temperature. After the desiccation period, seeds were placed in sterile 1.5 ml tubes and cooled rapidly by direct immersion in liquid nitrogen, stored for 30 min or 24 h, and than warmed using rapid or slow warming.

Following cryopreservation treatment, seeds were aseptically scarified and inoculated in $\frac{1}{4}$ MS medium (3). Seeds were placed in a growth chamber at $25\pm 2^{\circ}\text{C}$, under cool-white fluorescent lights ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) on a 16/8-h photoperiod. Six weeks after inoculation of the seeds the germination percentages were calculated.

Results showed that rapid warming after 30 min immersion in liquid nitrogen permit to obtain higher germination rates ($P < 0.05$) than slow warming (Table 1). Moreover, the use of rapid warming significantly increased ($P < 0.05$) the germination rate as compared with un-cryopreserved seeds (94 and 82%, respectively).

When the seeds were slowly warmed the duration of the immersion period in liquid nitrogen did not influence the germination rate. On the contrary, when the seeds were subjected to rapid warming the increase of immersion time reduced the germination rate. Yet, this germination rate was not significantly different ($P \geq 0.05$) from that of un-cryopreserved seeds.

Table 1. Germination rates of cryopreserved seeds of *D. lusitanicum*.

Drying	Cooling	Warming	Germination rate (%)
Control	-	-	82 b
No	30 min	Rapid	0 c
No	30 min	Slow	0 c
Yes	30 min	Rapid	94 a
Yes	30 min	Slow	80 b
Yes	24 h	Rapid	80 b
Yes	24 h	Slow	84 b

Values represent means \pm SE of 5 replications with 10 seeds. Values of germination rates followed by the same letter are not significantly different at $P \geq 0.05$ (Duncan's New Multiple Range Test).

This work reports, for the first time, the cryopreservation of *D. lusitanicum* seeds. Results show the feasibility of cryopreserve these seeds with no decrease in germination rate, which offers opportunities for creating a cryobank for this endangered species, without needing the use of expensive facilities.

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SLOVAK REPUBLIC

The current status of conservation of agricultural and forestry genetic resources in Slovakia and related cryopreservation activities

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The presentation has two main objectives: to describe the current status of conservation agricultural and forestry genetic resources in the Slovak Republic, and to present the results of the cryopreservation activities with embryogenic cell masses of coniferous species which were obtained in collaboration with the Department of Biology of the University of Oulu, Finland.

Slovakia has two principal programmes aimed at the conservation of genetic resources launched in 2005: “National Programme for Conservation of Plant Genetic Resources for Food and Agriculture for 2005-2009” based on the Act No.215/2001, and coordinated by the Slovak Agriculture Research Center in Piešťany. The “National Programme for Conservation of Forest Genetic Resources for 2005-2009” is coordinated by the National Forestry Centre in Zvolen.

Bodies implementing the both national programmes for conservation of plant genetic resources are entitled to ask for assistance within the state aid scheme. The conservation is done mainly *in situ*, *ex situ*, *in vitro* and *on farm*. The national programme for plant genetic resources for food and agriculture registered 28,758 genotypes by 31.12.2006. The Gene Bank aimed at the long-term (-17°C) and medium-term (at 0°C) seed storage is the integral part of Programme. The capacity of the Gene bank is 50,000 lots/ samples of seeds. As to their species structure, it contains mainly cereals, legumes, fodder crops, grasses, root, technical, and oil crops, vegetables, medicinal crops, spice plants and flowers. The Gene Bank provides seed samples of plant genetic resources free of charge to breeders, for research and scientific purposes, and to the other gene banks for exchange.

The system for conservation of forest genetic resources includes both *in situ* and *ex situ* methods. For the *in situ* conservation, 95 gene reserve forests with the area of 29,000 hectares have been established and managed using nature-conforming measures. The *ex situ* system consists of 2 clonal archives, 154 generative reproductive plantations and 84 regional seed orchards established for 14 coniferous and broadleaved tree species. The *ex situ* system includes the Forest Seed Bank for long-term storage of forest tree seeds. The bank comprises 300 seed lots of 6 coniferous species. Each lot consists of 40 single tree progenies.

Cryopreservation as a long-term storage technique has not been until now actively implied in the National Programmes. Cryopreservation is part of research activities done at J.P. Šafárik University Košice aiming the cryostorage of *Hypericum perforatum* meristems and *Orthosiphon stamineus*.

With forestry species, cryopreservation activities are mainly focused on the elaboration of the storage method for embryogenic cell masses of coniferous species (*Pinus nigra*, Institute of

Plant Genetics and Biotechnology, Nitra and *Abies alba*, *A. cephalonica*, National Forestry Centre, Zvolen).

In our previous research activities we developed the cryopreservation protocol for embryogenic cell masses of *Pinus sylvestris* (2) and *Abies cephalonica* (1). The best cryopreservation protocol was then used for long-term storage experiment (5-6 years) and the embryogenic cell masses of *A. cephalonica* (2 cell lines), and *A. alba* (12 cell lines) were cryopreserved using the PGD I cryo-preservation treatment (10% polyethyleneglycol 6000, 10% glucose, and 10% DMSO in H₂O). The new proliferation activity was observed with 2 embryogenic cell lines of *Abies cephalonica* and 4 cell lines of *A. alba*. The proliferation ratio for embryogenic cell lines of *A. cephalonica* has reached higher values (1.7 and 3.3, respectively) compared to growth ratio of cell lines of *A. alba* (1.3, 1.2 and 2.0). Later on, the maturation experiments were performed. The results on maturation experiments shown that cryopreserved cell lines have not lost their maturation abilities.

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DENMARK

Cryopreservation of dormant buds of apple cultivars in a mild maritime winter climate

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Since 1956, the genebank collection in Denmark of local and international fruit and berry cultivars has been stored in a field planting at the research station 'Pometet' now belonging to Copenhagen University. No security backup has been established for most of the accessions. Cryopreservation with the 'Dormant bud' method has in 2005 been tested as an opportunity to make a backup of the 750 apple cultivars in the collection in an effective and safe way. The 'Dormant bud' method was developed at the Plant Genetic Resources Unit in Geneva, US, in a cold continental climate (1). Scion wood is to be collected in the field after at least 72 consecutive hours below -5°C to allow the natural cold hardiness of the buds to be fully developed. In the mild maritime Danish climate average temperatures in January and February are about 0°C , and frost is seldom experienced for longer periods than a few days. Thus, the dormant bud method may not be possible to use in such conditions as hardiness can be expected to be relatively low and dehardening may easily occur especially when endodormancy has been broken. To monitor the dormancy level and optimize collection time shoots were collected from 6 apple cultivars from October until late February at 2 week intervals. The water content in the buds was determined in buds taken directly from the field and again after forcing for 6 days at 22°C , 55-60% humidity, and 12 hours light. The water content was quite constant at around 50% humidity (Fresh weight) but in early February, and particularly in late February, an increase in bud humidity up to about 70% occurs after forcing. Buds in the terminal end of the annual shoot react first, basal buds a little later. Collections for cryo tests of the 6 cultivars plus additional 10 cultivars were made in the field in January (27/1) and in late February (24/2). Average temperature the last 72 hours before sampling was -2.7°C and -0.1°C respectively.

To see if artificial acclimatisation had any effect on the survival after cryopreservation some shoot samples were wrapped in plastics to avoid desiccation and given a two week acclimatization pre-treatment at -4°C . Of 'Ildrød Pigeon' some shoots were also given a deacclimatization treatment at $+5^{\circ}\text{C}$. Thereafter the shoots were cut into 35 mm long scion pieces and dehydrated to 28-30% water content in a ventilated cooler at -4°C . This was followed by slow freezing to -30°C over 2 days before the samples were plugged into liquid nitrogen (LN). After one week in LN the samples were thawed for 24 hours and rehydrated in moist peat moss for 16 days. Thereafter the buds were grafted with a chip bud. After 3 months the vitality was examined.

The results from the forcing of the buds showed the tested cultivars had a long dormant period until the beginning of February where more than 2000 Chill Units had been accumulated. The cryopreservation experiment also showed it is possible to cryopreserve apple cultivars using the dormant bud method, in the Danish maritime climate (Table 1). Most cultivars showed high survival rates.

Table 1. Per cent survival rate in grafts of apple cultivars after storage in LN. Based on 10 grafts. Scions sampled 24th February.

Aroma	80 %	Dronning Louise	70 %	Ingrid Marie	80 %	Pigeon	80 %
Belle de Boskoop	60 %	Gloster	80 %	Judar	80 %	Prima	20 %
Bramley	70 %	Gråsten	70 %	Juliane	90 %	Rød Ananas	70 %
Discovery	20 %	Holstein Cox	60 %	Maglemer	80 %	Topaz	30 %

There was also found a difference in when the collection of the different cultivars was most successful. Some were better on the first date others on the last. If many flower buds were present the survival rate was reduced. The acclimatisation treatment had no clear effect on the survival.

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BELGIUM

Cryopreservation of *in vitro* cultures from *Maesa lanceolata*

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BELGIUM

Maesa lanceolata is a medicinal shrub growing in the tropics of Africa and Asia. It produces a number of different saponins for which was shown that they exert antiviral, haemolytic, molluscicidal and antiangiogenic activities (1, 2). As conventional plant growth and production is not very well controlled, *in vitro* propagation was explored as an alternative for rapid multiplication. A fast and reliable reproduction of the species was achieved through the development of an axillary shoot formation protocol. *Maesa lanceolata* seeds were collected in Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). Surface sterilized seeds were germinated on basal medium supplemented with 0.8% (w/v) agar. The basal medium consisted of MS full strength basal salts with MS vitamins and 3% (w/v) sucrose. Cultures were maintained at 26±1°C with a 16h photoperiod.

Three month old seedlings were used as explant source to generate material for subsequent propagation, and for the transformation of leaf discs. For shoot multiplication, the medium was supplemented with 6-benzyladenine (BA) at 3 mg/l concentration. For rooting after multiplication, single shoots of 3 to 5 cm in length excised from multiple shoots were inoculated in hormone-free basal medium gelled with 0.7% (w/v) agar. An average 11.3 shoots per explant were obtained after 50 days of culture (3). *In vitro* root cultures were generated from the micropropagated material and used for cryopreservation tests.

Maesa lanceolata roots tips (2–3 mm in length) excised from 2-week old root cultures were precultured for 1 day at 25°C, in the dark, on SH solid medium containing 0.3M sucrose alone or combined with 0.5M glycerol. The next day samples were transferred to cryovials and incubated at 0°C in PVS2 solution (0.4M glycerol, 15% DMSO, 15% ethylene glycol and 0.4M sucrose). Excess of PVS2 was removed prior to freezing in liquid nitrogen. After storage overnight or longer periods, samples were rewarmed rapidly in a water bath at 40°C and then transferred without washing onto solid MS medium containing 0.09M sucrose, 0.2% (w/v) Gelrite, and 2.7 µM naphthaleneacetic acid (NAA). The surviving root tissues were used to start new cultures and stability and viability analysed.

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POLAND**Survival of apical meristems of aspen after cryopreservation and slow thawing****P.M. Pukacki¹, P. Chmielarz¹, K. Bojarczuk¹ and W. Wesoly²**¹Physiology of Abiotic Stress Laboratory, Institute of Dendrology, Polish Academy of Sciences, PL-62-035 Kórnik, POLAND²Department of Silviculture, August Cieszkowski Agricultural University, PL-60-637 Poznań, POLAND

Winter vegetative buds of aspen (*Populus tremula* L.) were collected in the first week of March from 12-year-old trees growing on the experimental plot of the Institute of Dendrology, Kórnik, Poland. The mean weekly air temperature in February 2006 (before twig collection) was -5.7°C. Collected buds were stored at -10°C for 11 days and next were sealed in aluminium tubes (5.0 × 0.5 cm) and stored at -10°C for 24 h. Then the buds were prefrozen to -40°C at a rate of 5°C h⁻¹, and then immersed in liquid nitrogen (-196°C). After 10 days the samples of *P. tremula* were thawed in a water bath at a slow rate of 6.2°C s⁻¹ or at rapid rates of 9.2, 10.5 and 11.0°C s⁻¹. The decrease in temperature of samples was controlled by 0.25-mm Cu-constantan thermocouples. After thawing, the buds were sterilized with 70% ethanol, sodium hyperchlorite solution, and then the meristems extracted from the buds were cultured on a regeneration medium (WPM = woody plant medium) (1). The resultant rooted shoots were potted in peat:perlite (4:1). The survival of buds after slow thawing and culture for 5 weeks reached 64%, whereas after rapid thawing (11°C s⁻¹) only 21% (Table 1). The fast thawing led to a significant decrease in survival of meristems, as compared to slow thawing. There were no significant differences between the 3 fast-thaw procedures in the first 3 weeks. After slow thawing, the production of meristem callus, regrowth and rooting of shoots were higher than in other treatments. These results show that survival and regrowth of cryostored *P. tremula* buds depend on thawing rate. The storage of living plant tissues in liquid nitrogen may be improved by several techniques, e.g. freezing dehydration before cryopreservation and slow thawing.

Table 1. Effect of thawing rate on the survival (%) of apical meristems of aspen *P. tremula* after storage in liquid nitrogen without cryoprotectants. Values are means ±SD.

Thawing rate (°C s ⁻¹)	Post-thaw apical survival (%) during the culture period (days)				
	7	14	21	28	35
Slow					
6.2	94±9	86±8*	78 ±6*	72±10*	64±5*
Fast					
9.2	88±7	53±7	48±9	37±7	34±10
10.5	63±8	50±4	45±6	34±9	31±4
11.0	77±9	43±10	32±5	29±8	21±6

* significantly different from the fastest thaw procedure ($P \leq 0.05$)

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SPAIN

Preservation and identification by confocal microscopy of fern spores

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Ferns are a diverse, highly-evolved group of plants. Nowadays, there are probably about 12,000 species of ferns in the world, most of these found in the tropics, and it would be interesting to preserve this ancient plant group. Cryopreservation of spores has been previously assayed (1, 2, 3).

In a first approach to this subject in our laboratory, spores of four species of ferns: *Davallia canariensis*, *Ceterach officinarum*, *Dicksonia antarctica* and *Polypodium cambricum* were collected during the spring of 2006 and stored at room temperature until use. Keeping spores in a chamber at 4°C stimulates germination and preserves viability but it could not be a successful method for long time. In this work, we decided to compare the germination capacity of spore after being treated as follows: a) two weeks at 4°C, b) lyophilization for four days and c) immersion for one hour in liquid nitrogen. Data of spore germination were compared among species at 7, 14 and 21 days of culture. The results show that either lyophilization or immersion in liquid nitrogen can be potentially used for the preservation of fern spores (Figure 1). Moreover, germination of spores of *C. officinarum* and *P. cambricum* lyophilized or immersed in liquid nitrogen resulted improved compared to cold treatment at 4°C.

Confocal Laser Scanning microscopy (CLSM) was used to identify the spores by their morphology and the fluorescence emission spectrum. For this purpose, the spores were placed on microscope slides and covered with cover slips. Spore samples were excited with an Argon Laser line of 488 nm. For each spore sample a Lambda Series of the autofluorescence emission spectra was performed. The Lambda Series function records a stack of 20 individual images starting at 495 nm and ending at 695 nm with a detection range of 10 nm for each stack. Each section is taken from top to bottom in the spores. The result is a three dimensional recording that contains the entire volume of the spores. Each species shows a different morphology and fluorescent emission spectrum (Figure 2).

In the life cycle of ferns, spore germinates and forms the gametophyte, which is the phase encharged of reproduction either by sexual or asexual mechanisms. Further studies will be driven in order to preserve gametophyte as a means to avoid start a culture from spore.

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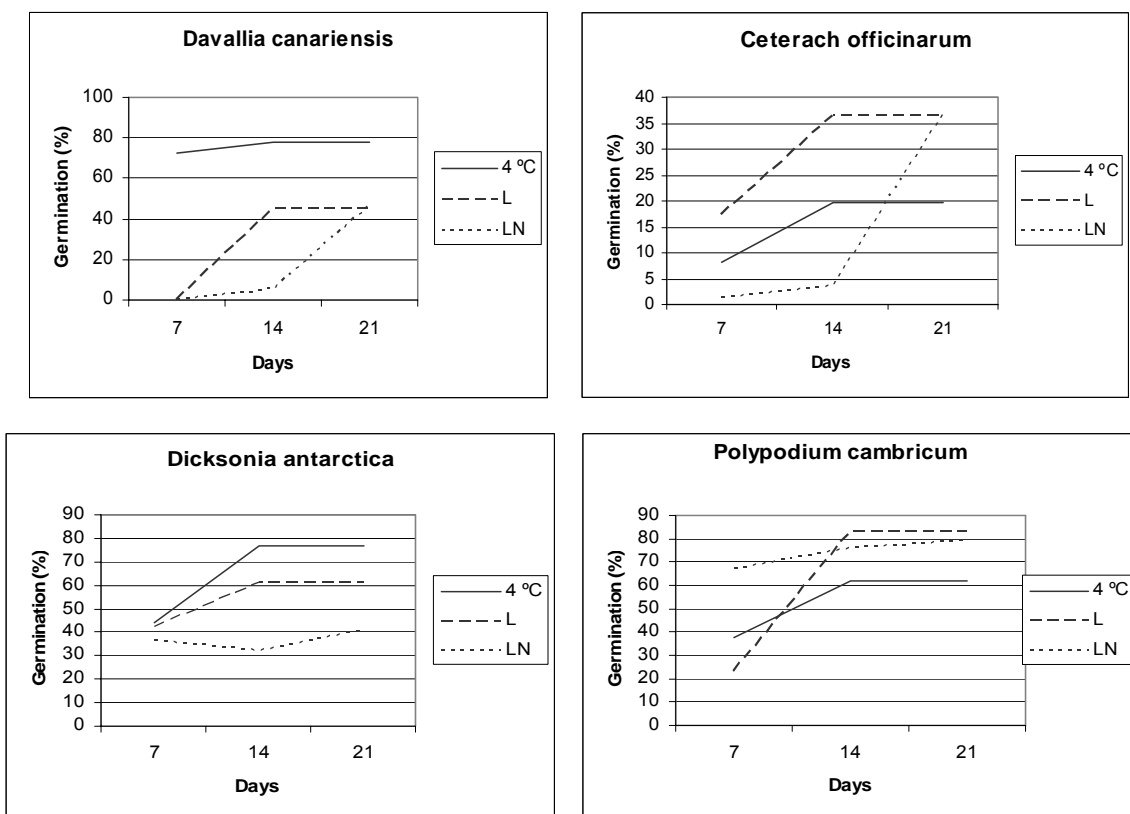


Figure 1. Germination percentages of fern spores of four species after being stored at 4°C, or liophylised (L) or immersed in liquid nitrogen (LN).

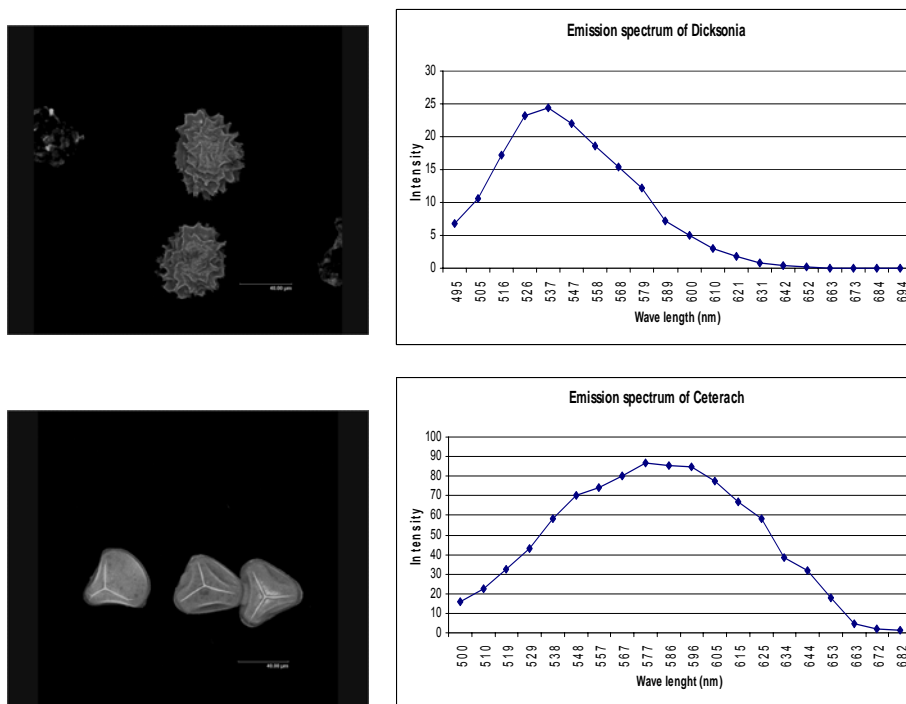


Figure 2. Spore morphology under confocal microscopy and fluorescence emission spectrum of *Dicksonia antarctica* and *Ceterach officinarum*

POLAND

Cryopreservation and vegetative multiplication of *Cyathea australis*

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Plant *in vitro* technologies and cryopreservation are employed to *ex situ* conservation of the genetic diversity. Tissue culture techniques have been allowed to rapid multiplication and storage but only cryopreservation is safe and cost-effective option for long-term conservation of different types of the plant material. Spore storage is the most effective and efficient method for conservation of pteridophytes. Gametophytes (1) and sporophytes (2) have been successfully stored in liquid nitrogen, too. In this paper, we describe the primary studies concerning cryopreservation of *Cyathea australis* spores and gametophytes.

Spores, very young and mature gametophytes of *Cyathea australis* were used in this study. For germination, sterile spores were encapsulated in alginate beads. Beads were put into a liquid 0.5MS medium with 0.088M sucrose. Young gametophytes which have been released from alginate beads to the liquid medium were harvested and transferred on the agar 0.5MS medium with or without 0.058M sucrose, 0.8% (w/v) agar (Difco). Gametophyte clusters were precultured on 0.5MS agar medium supplemented with 0.16M sucrose for 2 days and further 0.25M sucrose for 1-4 weeks. Spores after sterilization (see above) were washed with 0.5M sucrose solution and closed into alginate beads as describe above, but a solution of 0.1M CaCl₂ was supplemented with 0.5M sucrose. Capsules with spores were treatment with 0.5 and/or 0.75 and/or 1.0M sucrose during 1-3 days and after 4 h air – drying were directly subjected into LN.

Intact young gametophytes and 3 mm pieces of heart-shaped one were embedded in 3% (w/v) sodium-alginate. Next, the capsules were incubated with medium containing 0.3M (3 days), 0.5M, 0.75M (for both concentration 48 h) and 1.0M sucrose (24 h). Finally, the beads were harvested and surface-dried by air in laminar-flow chamber at room temperature for 1- 6 h dry. After air-drying, capsules were transferred to cryotubes and directly cooled in LN. Cryotubes removed from LN were re-warmed in water bath at 35°C.

Spores of *Cyathea australis* closed in alginate beads and cultured in liquid medium with sucrose germinated during 10 days in culture. Young prothallia development occurred like in natural conditions. This method spore germination allowed to release juvenile gametophytes individually from alginate beads. Later development of gametophytes which have been harvested from liquid medium was dependent on the sucrose presence in medium. The medium supplemented with sucrose stimulated proliferation of prothallium cells. After 4 months numerous gametophytes and first tiny fronds were visible found. In this culture mass new sporophytes were achieved.

The effect of the time and pretreatment methods on spore survival was observed. Capsulated spores without treatment with sucrose and dried did not survive LN exposure. The viability of cryopreserved spores was the best when capsules were treated with 1M (1 day) or 0.5M and 1.0M sucrose solution (1 day at each concentration) prior to LN exposure. The germination of encapsulated spores immersed in LN was 5 days delayed than non frozen those. However, development of gametophytes after both treatment similarly occurred.

The survival of cryopreserved gametophytes was depended on their age, time of air drying and sucrose concentration during preculture (Table 1).

Table 1. Influence of air drying duration and pretreatment of capsules on survival (%) of mature gametophyte pieces after LN exposure.

Pre-treatment	Air drying duration (h)					
	1	2	3	4	5	6
I*	0	3.7 ±0.69	3.3 ±0.44	3.9 ±0.73	5.3 ±0.62	2.9 ±0.51
II*	0	9.7 ±1.15	22.4 ±2.09	30.6 ±1.86	36.0 ±0,84	17.9 ±0.83

* I: 0.3M(72h), 0.5M(48h), 0.75M(24h)

**II: 0.3M(72h), 0.5M(48h), 0.75M(24h), 1.0M (24h)

Three types of response of the cryopreserved young prothalia were observed: lack of surviving, entirely surviving and partly surviving what consisted on capability to continue cell division and proliferation by one or few cells spread over explant. In post-thawing culture, surviving cells of prothalia resumed growth and formed gametophytes. Entirely survived immature prothalia, reached maturity stages during 5 weeks of culture. About 50% of young prothalia survived cryopreservation. However, in cultures of cryopreserved explants derived from mature gametophytes, the level of viability was lower (5-36%). The optimal dehydration time of encapsulated gametophyte explants for ensuring the best their survival was 5 h long with application of 7-day-long pretreatment with increasing of sucrose concentration from 0.3 to 1.0M. The highest viability of freezed gametophyte was achieved when preculture with 0.25M sucrose was applied. After cryopreservation more cells of gametophyte stay alive and their proliferation was very intensive. After 7 weeks of post-thawing cultures, numerous young gametophytes was received. About 60% of gametophyte explants was survived.

Cryopreservation of *Cyathea australis* spores and gametophytes ensures their long-term storage. As described previously, the gametophyte tissue of some fern species could survive open drying and LN exposure (1). Results of our experiments indicated that the encapsulation/dehydration procedure could be applied to cryopreserve not only gametophytes of *C. australis* but also for other tropical tree ferns.

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FRANCE

Cryopreservation of yam germplasm in Guadeloupe (FWI)

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Introduction

Yam is of great importance in tropical European Ultraperipheral regions and Overseas Countries and Territories, notably in the Caribbean. Cultivated species are propagated vegetatively and yam genetic resources are traditionally conserved *ex situ* in field collections. Conservation in field collections is costly and risky, as the plant material remains exposed to pests and pathogens. Moreover, vegetative propagation leads to the progressive accumulation of viruses, which can cause the loss of sensitive accessions. *In vitro* techniques can be efficiently used to improve the health status, conservation and propagation of plant genetic resources. Meristem culture, associated, or not, with thermotherapy, is employed for virus eradication (4). However, complete virus eradication is not systematically obtained. *In vitro* slow growth is used for medium-term storage of accessions, whereas cryopreservation is still the only technique ensuring the safe and cost-effective long-term storage of plant genetic resources. Today, thanks to the recent development of vitrification-based cryopreservation protocols, cryopreservation has been achieved for over 200 plant species (2, 5, 11).

Recently, cryopreservation has been used for eliminating viruses (cryotherapy) in *Prunus* and grape shoot tips (1, 13) and banana meristematic clumps (6). In all cases, the percentage of virus-free explants was higher after cryopreservation than after classical virus elimination techniques.

As regards virus sensitivity, yam can be infected by several viruses. The most frequent are potyviruses, badnaviruses and potexviruses. Potyviruses are the most aggressive, particularly on *D. trifida*, the culture of which is drastically regressing because of virus attacks. Meristem culture/thermotherapy are employed with yams, but complete virus eradication is not always achieved (3, 10, 12).

In vitro slow growth is routinely employed for medium-term storage of yam germplasm collections. As regards cryopreservation, the preliminary studies performed indicate that yam shoot tips can be frozen using encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet-vitrification (7, 8, 9).

Project objectives

The project aims at developing cryopreservation techniques in order to improve the conservation of yam genetic resources in Guadeloupe. It will focus in particular on optimizing cryopreservation techniques for yam shoot tips and on evaluating the efficiency of shoot tip cryotherapy for eliminating viruses, in comparison with classical virus eradication techniques.

Accessions of the three main cultivated yam species [*D. alata* (Asia), *D. trifida* (Amazon basin and Caribbean) and *D. cayenensis-rotundata* (Africa)] will be employed in the project.

For shoot tip cryopreservation, we will compare the efficiency of three vitrification-based techniques, which have already produced positive results, *i.e.* encapsulation-dehydration, vitrification and droplet-vitrification.

For virus elimination, we will compare the efficiency of cryotherapy with that of meristem culture for eliminating potyviruses, badnaviruses and potexviruses. Molecular detection techniques (PCR and RT-PCR) will be employed to identify the presence of viruses in plants regenerated from control shoot tips, from cultured meristems and from cryopreserved shoot tips of the yam accessions studied in the project.

Results to date

Cryopreservation: droplet-vitrification has been tested with shoot tips of *D. alata*, *D. trifida* and, most recently, *D. cayensis-rotundata*. We observed no survival after freezing for *D. alata* and *D. trifida*. We already noted differences in reactivity of pretreated controls: *D. alata* and *D. trifida* seem much more sensitive to PVS2 treatment than *D. cayensis-rotundata*.

Virus elimination: meristem culture and virus detection methods have been transferred from Cirad Montpellier to INRA Guadeloupe and are now operational in the INRA laboratory. The INRA *in vitro* collection includes about 450 accessions, 300 of which are indexed for the presence of viruses. Thirty of them are currently undergoing thermotherapy before meristem culture is implemented.

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ITALY

Recent advances in cryopreservation of small fruit germplasm

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Cryopreservation techniques have been developed for long-term conservation of more than 100 plant species cultured *in vitro* as protoplasts, cell suspensions, calluses, shoot apices, or somatic and zygotic embryos (3, 5). Cryopreservation is employed for conservation of plant genetic resources, but it is also applied to the conservation of biotechnological products including e.g. metabolite-producing cultures and genetically engineered cell strains.

The ISF (Istituto Sperimentale per la Frutticoltura, Rome) has implemented a research programme on the development of cryopreservation techniques for temperate fruit species.

The objectives of this research were to develop methods for *in vitro* germplasm conservation and cryopreservation of shoot apices of minor fruits. These species are increasingly utilized as dietary complements, in the pharmaceutical industry, in reforestation programs and for ornamental purposes (1, 2). Moreover, due to the fact that cultivation of such species is limited to marginal areas, there is an increasing risk of losing these genetic resources; therefore it is necessary to develop new approaches to preserve their biodiversity.

In vitro apices of mulberry (*Morus alba* L.), blackberry (*Rubus fruticosus* L.) cv “Kotata”, raspberry (*Rubus idaeus* L.) cv “M. Exploit” have been successfully stored using encapsulation-dehydration techniques.

The explants were excised from micropropagated plantlets at the stage of multiplication after 15 days of subculture. The basal media MS (6) and sucrose (3%) were the same for all the species, but hormones were different: BAP 0.5 mg/l for mulberry; BAP 0.4 mg/l for blackberry; BAP 0.5 mg/l, GA₃ 0.03 mg/l, IBA 0.05 mg/l, adenine hemisulfate 3 mg/l for raspberry.

Shoot apices (2-3 mm long) were encapsulated in 3% alginate beads (4), then were pre-grown in liquid MS medium with sucrose concentrations of 0.3M, 0.5M, 0.75M, 1M, 1.25M. Desiccation was performed by placing the beads in a small jar with 8 g silica gel for 0-4-6-8-9-14-20-24 hours. After dehydration, beads were plunged into liquid nitrogen (LN). After freezing, apices were placed, without thawing, in Petri dishes, containing propagation medium. Apices were grown in the dark for one week, then cultured under standard conditions (photoperiod 16 h, light intensity 37.5 $\mu\text{mol m}^{-2}\text{s}^{-1}$).

The highest survival has been obtained using sucrose concentrations of 0.75M or 1M and water content lower than 23% and higher than 17%. The survival rates were 74% for mulberry, 57% for blackberry and 43% for raspberry (Table 1).

These conditions are within the range of pre-growth and desiccation conditions which have proven optimal for the majority of species cryopreserved using encapsulation-dehydration (7). The results obtained in these experiments show that the encapsulation-dehydration is a useful method for the species investigated.

To speed up the routine application of cryopreservation it seems essential to give more attention to fundamental aspects including the physiology of cell and tissue dehydration, genetic stability and to the related molecular markers.

Table 1. Encapsulation-dehydration conditions in apices of mulberry, blackberry and raspberry.

Species	Dehydration (hours)	Water content (%)	Sucrose Treatment (M/days)	Control 24°C (-LN) Re-growth (%)	Cryopres. - 196°C (+LN) Re-growth (%)
mulberry	14	19	1/1	92	74
blackberry	9	20	0.75/3	65	57
raspberry	14	19	1/1	62	43

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Theme 2.2. Impact and applications of cryopreservation in plants

Coordinator: Joachim Keller

BELGIUM

Cryopreservation of the banana germplasm collection at the ITC (INIBAP Transit centre)

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The Laboratory of Tropical Crop Improvement hosts, under the authority of the International Network for the Improvement of Banana and Plantain (INIBAP, Bioversity International), the largest collection of bananas in the world. The accessions are maintained *in vitro* at slow growth conditions (15°C and reduced light). Cryopreservation is a valuable alternative to the *in vitro* collection for conserving biodiversity in banana for the long term. Accessions from the ITC are now routine cryopreserved using the two most successful protocols, i.e. vitrification of proliferating sucrose precultured meristem clumps (1) and vitrification of meristems excised from rooted plants (3). For banana accessions belonging to the ABB group and AAB (non-plantain) bananas, always the first method is applied while the preferred method is the second one for *Musa Acuminata* and most of the East African highland bananas. For all other accessions, the method of choice is based on (i) the quality of the scalps that is obtained after 3 subculture cycles on a medium containing high cytokinin concentrations and (ii) survival of the scalps after a preliminary cryopreservation trial.

For a total of 540 accessions, at least one successful cryopreservation experiment was executed. To decide whether an experiment is successful or not, we used the mode of calculation developed by Dussert and coworkers (2). These calculations were applied to all our data. An experiment is considered as successful provided that the chance to regenerate at least one shoot from the stored material is more than 95%. This probability is depending on (i) number of explants stored in liquid nitrogen (ranging between 30 and 50) (ii) number of representative explants screened (ranging between 16 and 50) and (iii) post-thaw regeneration rate (%) of these representative explants. An experiment leading to a lower probability level will be discarded, irrespective of its regeneration rate. These 540 cryopreserved accessions belong to 22 different genomic banana groups (Figure 1).

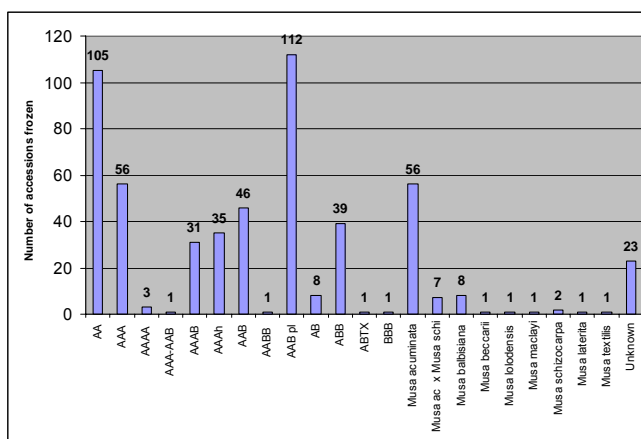


Figure 1. Number of banana accessions cryopreserved per genomic group.

Out of these 540, we can consider now 409 accessions as definitively cryopreserved for the long term. These accessions contain 3 independent repetitions, are derived from rejuvenated and virus free material (if possible) or non-rejuvenated material (if virus eradication and thus rejuvenation is not possible, mainly in case of plantains that contain the integrated BSV sequences).

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SPAIN

Implementing a strawberry cryogenic genebank: field performance of plants derived from apices cryopreserved for one year

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A strawberry germplasm collection was started at IFAPA (Instituto Andaluz de Investigación y Formación Agraria y Pesquera - Institute for Agricultural Research and Training- Churriana-Málaga, Spain) in the late 1980s. It currently includes more than 400 accessions of cultivars, related wild genotypes and selections from breeding programs. Strawberry germplasm is preserved in potted plants under insect-proof screens and *in vitro* stored at refrigerated temperatures (4°C). *In vitro* conservation is used for medium-term storage (active collection). Cryopreservation protocols have been developed for several cultivars to explore the use of these techniques for the long-term conservation of germplasm in IFAPA Centre of Churriana genebank (1). Cryopreservation would reduce the work of maintaining the *in vitro* bank and the risks of losses due to contamination or accidents. Before the cryogenic bank is routinely established, besides testing the chosen protocol with a higher number of genotypes, studies on the field performance of plants derived from cryopreserved apices stored for one year are also being carried out.

Strawberry is an annual crop in the Huelva area (the main strawberry production region in Spain). The plants grown one year are never kept for a second fruiting season. They are uprooted and new plants (multiplied in the high elevation nurseries) are planted. They are never multiplied from the plants that have already been in the production fields (Huelva) to avoid spreading diseases.

In the present study, we have compared the agronomic traits of three strawberry cultivars (Andana, Camarosa and Carisma) of plants derived from apices cryopreserved for one year with plants derived from conventional propagation and micropropagation. The agronomic traits evaluated were fruit production and fruit quality.

The cryopreservation protocol used was based on previous studies (1), and consisted mainly in: 1) *in vitro* shoots cold-hardening (4°C and darkness for 4 weeks), 2) apices preculture on medium with 0.3M sucrose for one day, 3) encapsulation in alginate beads and immersion in a solution of 2M glycerol and 0.4M sucrose for 60 minutes. The beads were then desiccated with silica gel for 4 h and subsequently immersed (inside cryovials) in liquid nitrogen, where they remained for one year. After rewarming, the strawberry beads were cultured on recovery medium (2). From this stage plants were treated like the micropropagated plants and then vegetatively multiplied by runners at elevation nurseries during April 2005 (3).

The agronomic study was carried out in the production fields (Huelva, South Spain). At the end of October 2005, plants from the three different origins were planted in three completely randomized plots (50 plants) on two-row raised beds covered with black plastic.

Accumulative data of fruit production was studied until the end of March (early production) and until the end of May (total production). Marketable (first and second class) fruits were periodically harvested and weighed, starting in early January. Individual fruit weight was calculated by dividing the total yield by the total number of harvested fruits. For the estimation of the fruit quality, three to six fruits per plot were evaluated three times throughout the season for external and internal colour, internal cavity size, shape, firmness, and soluble solids content.

Most of the production traits studied showed no significant differences between the plants of different origin. However, Andana and Camarosa plants of cryopreserved origin showed higher marketable yield in the early production. On the other hand, Carisma showed lower marketable yield of the total production and fruit weight. The fruit quality traits did not show significant differences between groups.

No adverse effect on agronomic traits could be associated with the use of cryopreservation. Plants from *in vitro* and cryopreservation origin were phenotypically similar to the conventional propagated plants in production field.

Further research is being carried out to establish if the changes observed in the present work are maintained in plants obtained by further vegetative propagation of the plants recovered from cryopreservation, and to determine the effect of longer storage periods in liquid nitrogen (two and three years). The establishment of a cryopreserved germplasm bank for strawberry would reduce the requirements for personnel and space, when compared to the currently used *in vivo* or *in vitro* collections.

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FRANCE

Definition of a new research program on cryopreservation

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In order to guarantee safe, long-term conservation of the National Institute of Horticulture (I.N.H.) *Pelargonium* collection, meristem cryopreservation studies have been undertaken since 1999. Survival and regeneration were obtained on *P. x peltatum* 'Balcon Lilas' using an encapsulation-dehydration process involving apex preculture in a liquid medium progressively enriched with sucrose (up to 1M) prior to dehydration with silica gel.

Various steps of this process (such as preculture, dehydration, rewarming, etc.) were studied choosing 'Balcon Lilas' as a model in order to simplify and optimize it. One of the main objective of these studies was to determine if sucrose is specific in conferring dehydration tolerance to *Pelargonium* apices. The newly determined process was tested on 19 genotypes representative of the diversity of our collection. Apex survival was obtained for 18 of them, and variability was observed. Survival rates were between 27.5% (*P. x hortorum* 'Renard Bleu') and 83.9% (*P. x hortorum* 'Bicolor'). Plants were obtained after cryopreservation for various genotypes.

However, some difficulties slowed down the obtaining of an efficient and reliable cryogenic protocol: the repeatability of the survival rates between the experiments remained poor, hyperhydric tissues were often obtained during the apex regeneration through the alginate bead and furthermore this method of encapsulation-dehydration is time consuming.

In 2005, studies were undertaken in order to adapt the droplet-vitrification procedure established by Panis *et al* on *Musaceae* (3). A reproducible process was obtained by optimizing the duration in the loading solution (LS) and in the plant vitrification solution 2 (PVS2). This success permitted us to undertake the studies on the quality of the regenerated plants regarding the conformity and the sanitary status.

In the near future, as research on the *Pelargonium* genus will stop in the UMR GenHort, it will be the opportunity to define a new research project on cryopreservation.

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GREECE

Towards a microplate technology platform for plant breeding

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Microplates have been used in diagnostics for more than 40 years now (4). Their use in plant science has not been limited to phytopathology. In plant breeding laboratories, microplates are used for screening large number of plant samples whether in culture or not. In some applications many plant samples are accommodated in each microplate well, while this is not the case for some others.

In the literature there is a discrepancy regarding whether small samples are suitable for measuring the response of plants to low temperatures. According to Ashworth *et al.* (3), small samples may not provide a reliable estimate of whole plant cold hardiness. In other studies, like the one of Zatylny *et al.*, *in vitro* shoots, callus and cell suspensions have been reported to respond to the cold in a manner similar to that of intact plants (5).

Techniques involving small samples, in general, require less space, are cheaper, faster, amenable to automation and most importantly, facilitate screening of large number of samples.

The use of microplates in plant breeding facilitates large scale screening, under uniform growing/assay conditions. Our laboratory explores the feasibility of using microplates to screen for freezing tolerance. Freezing assays with *in vitro* growing seedlings have been applied successfully to transgenic tobacco (1), as well as to non transgenic Arabidopsis. Results from seedling freezing assays are complimented by results obtained from leaf disc freezing assays. Leaf disc freezing assays are used at a later developmental stage, so that to avoid selecting plants that are resistant only at the seedling stage and not at later developmental stages. Leaf discs freezing assays have been used successfully in transgenic sugarbeet (2) and tobacco (1). In both assays, only one sample (either seedling or leaf disc) is placed in each microplate well. That prerequisite increases the labor required, if samples are placed manually in the wells, but there is the alternative of “loading” the microplate wells automatically.

Work in progress involves screening seedlings of various Greek oregano biotypes for freezing tolerance.

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GERMANY

Introducing cryopreservation for plant germplasm preservation in Germany

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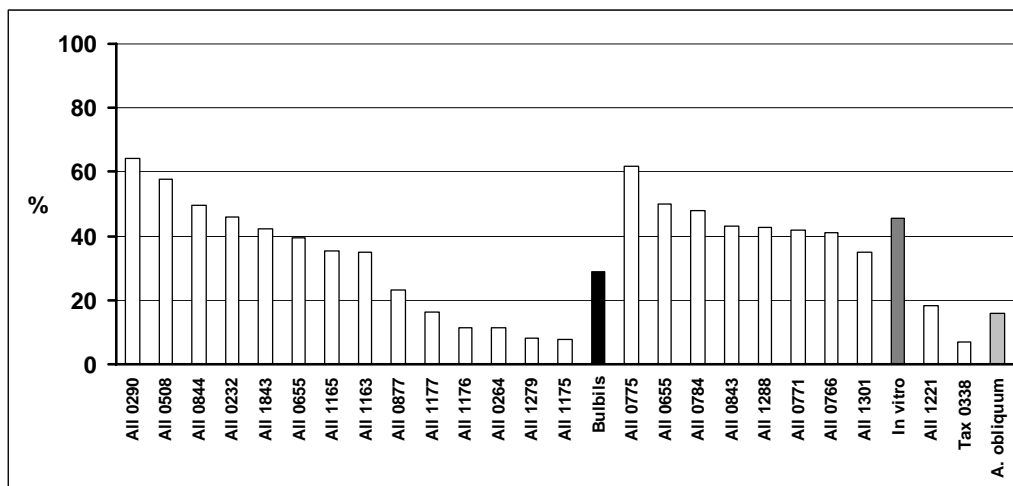
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The implementation of cryopreservation methods reaches more and more the level of their practical applicability. This is true also for plant living collections. In Germany, several groups are interested to collaborate within the workpackage 2 of COST.

Concerning the crops of interest, the progress of the various groups is at different levels. The largest collection is the potato collection of IPK. Other crops, of which initial collections have been established there, are garlic and mint. In Germany, major groups like woody fruit crops and strawberry as well as forest trees got already sufficient consideration to be envisaged for cryopreservation. However, progress is limited there because of insufficient funds. For some potential candidates in the ornamental sector, only limited research activities were endeavoured, so for chrysanthemum and roses. Some other crops, of which foreign publications are known, are still waiting for investigations like Jerusalem artichoke.

Despite the obvious fact that storage in liquid nitrogen has a very high safety potential, which is a sufficiently good justification for endangered germplasm, there is always a discussion about its usability in terms of logistics and costs. The Gatersleben potato cryobank comprising already more than 1000 accessions is a good example to discuss these factors. Due to the special political situation in Germany, its history began in two places, at Braunschweig and at Gatersleben. The entire potato collection is now structured in several parts depending on its origin and use. Correspondingly, the need to store material is different for different parts of the collection. The genebank unification measures in 2002 offered a good chance to re-evaluate the regeneration rates obtained via DMSO droplet freezing which is the basic method for both collection parts. After the unification, several organizational steps were necessary to increase the quality of the collection: re-definition of the number of samples needed for one accession and completion of samples with low numbers of explants. At the same time, some of the steps of the procedure were critically re-evaluated: preculture steps, preconditioning prior to freezing, morphogenesis of regeneration. In course of the development of both German collections, complementary selection of the accessions had been considered. However, some overlapping was found during the re-evaluation. Thus, duplicates were screened and, if necessary, eliminated. Finally a cost comparison was started. Several components were included in this analysis like frequency of requests and regeneration of samples.

The storage strategy may be different depending on whether the action is needed for long term safety storage of a well-funded collection or for emergency rescue. This can be compared in garlic, where cryopreservation activities started already in 1997, and sufficient input could be given to elaborate a safe method. In contrast to this, material of a wild species, *A. obliquum* had to be included at the end of a research project without special preparation in order to rescue at least a part of the material.



Survey on the *Allium* cryobank at Gatersleben; accessions with more than 200 explants each. The marked bars show the averages, left of them the respective special accessions.

During a first meeting of the German users of cryopreservation, a list of the main tasks of interest within WP2 was agreed. It covers four topics which will be discussed in the presentation: interlaboratory tests, comparison of methods, economical analyses, and international integration of cryobanks.

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FINLAND

Cryopreservation in certified plant production

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Introduction

In 1970's, the basic rules for certified plant production were developed in Europe. In Scandinavia, the pathogen and pest free basic material was called nuclear plants, the first generation propagation material was called elite plants, the third generation elite stock plants and after the fourth generation the material was called certified plants in 1-2 generations. Certified production has been legislated, controlled and certified by the state authorities.

The conformed procedures were evolved on the assumption that nuclear stocks would be maintained and the elite plants propagated *in vivo* in the field, where the risk of recontamination of the material was high. Therefore the rules that were set down for control testing and renewal of the basic material were strict. E.g. the strawberries were to be retested and renewed every four year, raspberries and currants every eight year. Consequently the maintenance and the propagation of the first two generations were expensive.

For a long time, certified plant production in Finland covered only the most important berry plants. Only just recently, in 2003, the certified production was extended to cover all berry plants, fruit trees and perennial ornamentals. It was an enormous task to add in a multiple number of new plants and to maintain the old nuclear plants. Due to the high maintenance costs of the material, it became an impossible equation to put into practise. Therefore, the Ministry of Agriculture and Forestry ordered to revise the procedures in the certified production.

Material and methods

Risk assessment was done and a new strategy was designed at MTT (Agrifood Research Finland). A combined greenhouse and *in vitro* storage strategy was used in maintenance of the Finnish nuclear plants. The production of elite plants was mostly based on micropropagation. The experience for over 20 years demonstrated that the applied methods had effectively protected the stock materials against recontamination. Cryopreservation became a research topic in the Finnish national programme for plant genetic resources of horticultural plants. The possibilities to utilize cryotechniques in virus eradication on raspberries and in maintenance of nuclear plants were screened in the research project.

Results

A new strategy was designed at MTT (Agrifood Research Finland) and written in the statute on certified plant production in 2006 (3). According to the new protocol the *in vitro* cultures of a new nuclear stock are placed into cryopreservation after the testing programme is completed, controlled and result is certified by the plant inspection authorities. From the phytosanitary point of view, time stops in cryopreservation. As long as the nuclear material is cryopreserved, it is safe against plant pathogens and pests and it can be propagated as certified material without time limits and without renewal of test programmes. *In vitro* cultures for elite plant production can be taken from cryopreservation instead of excising new meristems from the *in vivo* grown mother stock. Cryotechniques was also included into the Finnish national programme for plant genetic resources of horticultural plants (1, 2, 3). Cryopreservation technique can also be used as

cryo-therapy. It is a rapid and effective method in virus elimination when combined with meristem culture (4).

Discussion

Utilization of cryopreservation technique and combining it with new test methods and DNA fingerprinting allow a higher security in the nuclear plant preservation than methods adapted in 1980's. Clone archives in the field and retesting procedures are no longer necessary. This is a radical change and lowers the maintenance costs of nuclear plants. Cryopreservation enables the maintenance of more extensive selection of certified mother stock material than the traditional methods. It also allows a greater flexibility in establishment and maintenance of the elite plant propagation, because the production timetable is no longer dependent on the season.

Application of cryopreservation technique into the certified plant production simplifies also the corporation between the nuclear plant banks and gene banks. When the cultivar is no longer needed in active production, it already exists as a virus free core collection for germplasm preservation.

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GERMANY

Preliminary results of *Malus* germplasm cryopreservation from the Institute of Fruit Breeding gene bank collection in Dresden

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The maintenance of woody perennials within orchards is very costly for germplasm repositories and germplasm loss may be expected due to disease or inclement environmental conditions.

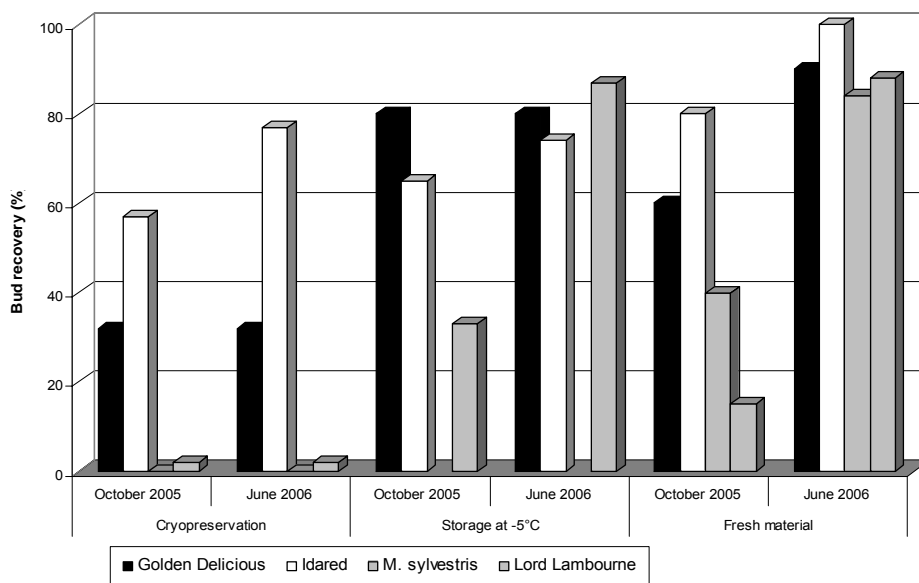
The establishment of reliable, safe cryogenic storage at a back up collection can protect against loss and confer some financial benefits by allowing repositories to hold fewer trees in the field per accession. The possible involvement of cryopreservation in the management of fruit genetic resources will be discussed and preliminary results in *Malus* will be presented.

Malus germplasm may be cryopreserved using buds from either *in vitro* or *ex situ* plants. Cryopreservation using a winter vegetative bud method according to the USDA-ARS National Centre for Genetic Resources Preservation, Fort Collins, will be applied to the *Malus* collection maintained at the Institute of Fruit Breeding in Dresden-Pillnitz.

In the first year three apple cultivars and a mixture of accessions of the European crab apple *Malus sylvestris* were included in the experiment. Scion wood containing the current season's growth was cut from the field trees in Dresden-Pillnitz in December, January or beginning of March. Temperatures below about -5°C for at least 72 h were desired but not always possible. Mild winter temperatures in Central Europe could be the initial problem adapting the method. Scions were cut into 35 mm long single node sections. Moisture content of the sections was determined gravimetrically and expressed on fresh weight basis and water content (g H₂O/ g Dry matter). In the first year experiment the sections were desiccated to 30% moisture by placing them in a -5°C cold chamber. Dehydration time of scion pieces at -5°C reaching about 30% of moisture content (app. 0.4 g H₂O/ g dry matter) varied between 3 and 12 d depending on the genotype. The wild species *Malus sylvestris* required the shortest drying time.

When the sections reached the described moisture level they were placed into cryotubes and cooled at 1°C/ h to -30°C and then held at -30°C for 24 h. The tubes were transferred to the vapor phase over LN in cryo tanks. Ship grafting was performed after a 15 d rehydration period at 4°C in sand. Bud emergence and growth were evaluated in the orchard in autumn and spring time.

Seventy-seven percent of buds survived in 'Idared' and 32% in 'Golden Delicious'. The cultivar 'Lord Lambourne' and *Malus sylvestris* showed a very low percentage or no bud recovery. Grafting of buds which were stored at -5°C and fresh material from the orchard revealed adequate control data for all experiments.



In the second year five apple cultivars and five accessions of *Malus* species originating from different continents were tested. The sections were desiccated using additionally to the -5°C a salt dehydration with NaCl and KNO_3 . Different dehydration levels were tested. Application of salts speeds the dehydration time up and causes browning of the material; NaCl more as compared to KNO_3 . Dehydration time varied between the cutting times within the year; correlating with the moisture content at the beginning of the experiment. Wild species demonstrated the lowest value of moisture content corresponding with the shortest dehydration times. The following steps are still in progress. Recovery results will be presented and future tasks of cryopreservation in apple and strawberry will be discussed.

For investigating intercellular ice formation differential scanning calorimeter measurements were performed in cooperation with Bilavcik from the Research Institute of Crop Production, Prague. Dormant buds were cooled down to -120°C (rate $10^{\circ}\text{C}/\text{min}$). After equilibration at -120°C for few minutes they were heated up to 20°C (rate $10^{\circ}\text{C}/\text{min}$). The data were measured from the heating curve. The extrapolated onset of the melting peak and the amount of crystallized water was evaluated by the TA Universal Analysis software 2000. Preliminary DSC measurement showed that with dehydration to 18% of moisture content/ $0.22 \text{ g H}_2\text{O}/ \text{g Dry matter}$, practically no ice crystallization occurred during the measurement. The amount of crystallized water did not vary among observed cultivars or species at the same dehydration level.

ITALY

Seed cryopreservation for conservation of ancient *Citrus* germplasm

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Aim of this study was the development and optimization of a cryogenic procedure for the preservation of an ancient *Citrus* germplasm, maintained at the botanical garden of a Medicean villa, the “Villa Reale di Castello”, in the outskirts of Florence, Italy. This collection is one of the most important in Europe. It includes ornamental citrus species of high historical value, as well as rare varieties characterized by bizarre or huge fruits. The collection was initiated by Cosimo I de’ Medici in the XVI^o century, and it includes more than 600 accessions, some of them not to be found in other sites or collections. Among the historical germplasm preserved, the following can be mentioned: *Citrus medica* ‘Florentina’, *C. limon* ‘Digitata’ and ‘Peretta’, *C. aurantium* ‘Virgatum’, ‘Bizzarria’ and ‘Foliis variegatis’ (1). This germplasm is of inestimable value, and it is by ancient time preserved mainly in big earthenware basins. Obviously, citrus plants maintained this way are highly vulnerable to damage due to the action of pests and diseases (the control of which requires careful periodical monitoring of plants), and to the effects of late and hard frosts. Recent advances in the cryogenic technology have greatly enhanced the possibility of long-term preservation of seeds and embryonic axes (e.g., 3, 4). Taking advantage from the fact that many *Citrus* species have seeds containing both zygotic and nucellar embryos, promising results have been obtained with the preservation in liquid nitrogen (LN) of entire seeds from different polyembryonic citrus species (2). Hence, in order to develop an effective procedure of seed dehydration and direct immersion in LN, ripe healthy fruits were harvested from four *Citrus* accessions of the collection, i.e., *C. aurantium* ‘Foetifera’, *C. volkameriana*, *C. lumia* ‘Pyriformis’, *C. sinensis* and the hybrid *C. aurantium* x *C. paradisi*. After removal of the mesocarp, the percentage of moisture content (MC, on FW basis) of the seeds was determined by drying a batch of seeds at 60°C for 48 h. In a preliminary trial, the seeds were placed in open Petri dishes under sterile laminar air flow at room temperature, and the MC of seed samples determined every 2 h. Afterwards, for each species, 3 dehydration times were selected, in order to obtain seeds with a MC not over 25%. The dehydrated seeds were then directly plunged into LN. After at least one hour, the seeds were thawed in water bath at 40°C for 2 min and evaluated for germinability. All the *Citrus* germplasm tested in this study showed good adaptability to seed cryopreservation (Table 1). Maximum germinability ranged from 27% (*C. aurantium*) to 100% (*C. aurantium* x *C. paradisi*). For all the species, a reduction of MC around or below 20% was fundamental to achieve a good tolerance of seeds to ultra-rapid freezing. Following cryopreservation, seed germination and seedling development was slower than that of unfrozen seeds. However, the number of seedlings per seed was roughly the same, and no morphological variation was detected between seedlings from unfrozen or cryopreserved seeds. Molecular analyses are in progress to detect the nucellar or the zygotic origin of seedlings from seed conservation in LN.

Table 1. Effects of different dehydration levels, followed or not by cryopreservation, on seed MC, germinability and the number of seedlings per germinated seed.

Species	MC (% FW)	Dehydrat. time (h)	Final seed MC	Germinability (%)		Seedlings	
				Dehydrated (-LN)	Dehydrated (+LN)	Dehyd. (-LN)	Dehyd. (+LN)
<i>C. aurantium</i> 'Foetifera'	48	22	25.2	100	25	2	1
		24	21.2	90	27	1	1
		26	17.5	86	15	1	1
<i>C. aurantium</i> x <i>C. paradisi</i>	55	30	24.3	100	87	1	1
		32	19.2	100	91	1	1
		34	15.6	100	100	1	1
<i>C. volkameriana</i>	34	6	25.6	100	86	2-3	1
		10	21.6	86	87	1-2	1-2
		14	15.3	80	88	2-3	1-2
<i>C. lumia</i> 'Pyriformis'	41	28	24.7	100	50	1	1
		36	18.4	100	37	1	1
		40	15.7	100	38	1	1
<i>C. sinensis</i>	47	18	25.4	86	50	1	1
		22	20.5	100	30	1	1
		24	17.6	86	80	1	1

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FINLAND

Cryopreservation in the Finnish national germplasm programme for the horticultural plants

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Introduction

In Finland, The National Plant Genetic Resources Programme was established in 2003 to facilitate the conservation of national agricultural and forest genetic resources. MTT Agrifood Research Finland is responsible for the coordination of the programme in general and especially for the execution of the programme on field and horticultural crop genetic resources. The following genetic resource working groups at MTT have the organisational responsibility for managing the collections: 1) Landscape gardening; 2) Fruits and berries; 3) Vegetables, herbs and medicinal plants; 4) Field crops.

At the moment the genetic resources in Finland are maintained in field, greenhouse and *in vitro* collections. Vegetatively propagated crop species, such as fruit trees, small fruits and berries, ornamentals and perennials are maintained in national field gene banks and laboratories. MTT with its network of regional stations is the main keeper of vegetative collections. Botanical gardens, arboretums, universities and schools also maintain genetic resources to a significant extent.

At the beginning the guidelines for the preservation of the national genetic resources needed to be drawn. Cryopreservation was considered to be a necessary tool to become a new back-up preservation method, which can be utilized to handle this huge task of maintaining the national collections.

Material and methods

The work was started by inventing and evaluating the collections at MTT. The introduction of cryopreservation was started at MTT in 2004 by studying the modern plant cryopreservation methods. Several different cryopreservation methods were tested like encapsulation-dehydration (4, 8) or vitrification (7) based methods like encapsulation-vitrification (5) and droplet-vitrification (6). Different cultivars of strawberry, raspberry and potato were used as model plants. Both apical shoot tip and axillary bud meristems were used depending on the plant species. As pre-treatment, media with increasing sucrose concentrations were used. Survival and regeneration rates were observed.

Results

The genetic resource working groups drew up and published guidelines for long-term preservation of Finnish plant genetic resources regarding fruits and berries (1), woody ornamentals (2) and vegetables, herbs and medicinal plants (3). The guidelines described the practical implementation of the national gene resource preservation and the present scope of MTT's clone archives, presented the criteria for selecting material to be preserved and listed the necessary measures for the preservation. Instructions were included for the establishment and renewal of clone archives and for selecting alternative preservation methods. Cryopreservation was recommended as the first back-up collection preservation method for most plant species.

Droplet vitrification method was chosen to be modified for different plant species at MTT. Cryopreservation was introduced in long-term preservation at MTT starting with species, for those we already had the cryopreservation protocols ready and experience of using them. The cryopreservation of a collection of 33 raspberry accessions and the cryopreservation of a hops collection including 9 accessions was started.

Discussion

At present, most of the woody ornamental, fruit and berry collections are preserved in clone archives. Vegetables, herbs and medicinal plants are mainly preserved as seeds and in field gene banks. The inventory of nationally valuable genetic materials has not yet been completed. Genetic material representing species-specific diversity and regional diversity is included in long-term storage. Species-specific cryopreservation applications are still under development. For the future, plans call for the most valuable material to be cryopreserved. In some cases, maintaining plant material under slow growth conditions *in vitro* is appropriate. At MTT, the experience of long-term cryopreservation is yet limited to raspberry and only to one year long preservation.

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CZECH REPUBLIC

Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech Cryobank

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Introduction

The collections of vegetatively propagated plants are maintained mostly in field or orchards. Nowadays, such a type of germplasm collection is considered unsafe and not fully reliable. The environmental (climatic conditions, pests, and other diseases) endanger collection. Demand of safety of the collection leads to use cryopreservation techniques. That is why a great effort has made to cryopreserve the most important vegetatively propagated crops in the Czech Republic during last decade till now.

Materials and methods

Cryopreservation methods are explored for conservation of various plant parts. We used shoot tips including meristematic parts tissue, dormant buds (for fruit trees) and pollen. Cryopreservation of hop pollen grain is used mainly for breeding purpose.

One of the cryopreservation methods is vitrification. The vitrification method is based on biological glass forming. There are a lot of modifications of this method in literature. We used some modification and improvement of this method (1). The most used vitrification methods of cryopreservation are based on vitrification procedures (dehydration encapsulation, dehydration and modified method of dehydration based on preconditioning of plant shoot tips on an osmotic solution) and two-step freezing procedures.

Results

The main four crops (*Allium*, potato, apple, pear, and hop) are cryopreserved in the Czech Cryobank. The encapsulation-dehydration method is used for potato, *Allium* and fruit trees. For example the *Allium* shoot tips were dissected from bulblets or from micro bulbs of bolting accession form *in vitro* plants. The shoot tips were encapsulated in alginate beads. Two cryopreservation methods have been used - encapsulation-dehydration and plant vitrification in cryoprotective solutions (PVS2, PVS3). Experiments with dynamic dehydration studies demonstrated the necessity of encapsulation of shoot tips to avoid the high rate of dehydration. The vitrification solution gives up to two times higher regenerating plants then encapsulation-dehydration method.

Two main approaches in cryopreservation of apple germplasm were used, two step cryopreservation procedure and encapsulation-dehydration. The ultra rapid cryopreservation method is used for potato and hop meristems. This method is based on dehydration shoot tips on aluminium foil above silica gel and after subsequent dehydration them the foil with meristems is quenching in to liquid nitrogen. The temperature rate during this procedure is hundreds degrees per minute.

Constraints of the vitrification method are following: There are some problems with vitrification of plant meristem due to high amount of water. It is necessary to avoid the possible ice formation during cooling and recrystallization during heating. The high speed of warming is

achieved by immersion of samples into water 40°C hot. The long-term sample heating can cause damage of plant meristems.

The possible ways how to increase low regeneration after cryopreservation are in improvement by the physical condition of the plants before the freezing by acclimation at low temperatures, by alternating of hardening temperatures, by dehydration to remove the excess of water from plant tissue for increase their resistance to low temperatures. Phytosanitary situation (virus free) of cryopreserved material can influence the results of plant regeneration.

Differential scanning calorimetry was used to determine temperature of glass transition (T_g) of encapsulated dehydrated meristems to different levels. The dehydration increases the temperature of glass transition during sample heating.

Conclusions

The cryobank collaborates with the collection keepers in specialized crop research institutes; Potato Research Institute Ltd. Havlickuv Brod, Hop Research Institute, Zatec, Research and Breeding Institute of Pomology, Holovousy and CRI Research Station in Olomouc concerning *Allium* genera and Viticulture Research Station Karlstejn. The database of cryobank is connected to the national database of genetic resources (EVIGEZ http://genbank.vurv.cz/genetic/resources/asp2/default_c.htm) through the ECN number.

The strategy for choosing the accession to be cryopreserved is based on preferring the Core collection, national old cultivars and accession with problematic propagation. The philosophy of operation of the Czech Cryobank is to be a safe duplicate to repositories of germplasm kept in field or in *in vitro* conditions. Since the end of 2003 the cryobank has started the routine cryopreservation of main vegetatively propagated crops.

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SLOVAK REPUBLIC

Physiological and structural characterization of embryogenic tissues of *Pinus nigra* following cryopreservation

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Introduction

Recently, cryopreservation has been successfully applied to embryogenic cultures of several conifer species, e.g. *Pinus radiata* (6), *Pinus sylvestris* (5), *Pinus patula* (3), *Pinus pinaster* (7, 8), *Picea abies* (9), *Abies nordmanniana* (10), *Picea sitchensis* (2) and others. Different pretreatments as well as cryoprotective solutions have been tested. The tissues survived low-temperature treatments without too severe damage and in most of the cases produced plantlets (2, 7). These results suggest cryopreservation is a convenient method for long-term storage of embryogenic cultures of conifers. During storage in liquid nitrogen, possible genetic changes that can take place during long term maintenance on nutrient media (somaclonal variation), the loss of embryogenic capacity and the risk of contamination are prevented. The aim of the present study is the physiological and structural characterisation of embryogenic cell lines of *Pinus nigra* after cryopreservation.

Material and Methods

Plant material: Embryogenic tissues were initiated from immature zygotic embryos on medium DCR (4) containing PGR 2 mg/l 2,4-D and 0.5 mg/l BA. Six cell lines were selected (E 177, E 182, E 184, E 190, E 193, E 196), representing the diversity regarding somatic embryo structure and maturation capacity.

Cryopreservation: Approximately 300 mg of tissue was pretreated with sucrose (180 g/l in MS proliferation medium). Then, 15% DMSO was added stepwise to reach at final concentration 7.5%. The suspension was pipetted into 1.8 ml cryocanes, placed into a Mr. Frosty container (Nalgene TM) and stored in deep freezer until the temperature reached –40°C. Then the cryovials were plunged into liquid nitrogen for 30 min. Thawing took place in a water bath at 40°C. The thawed suspension was pipetted on three stacked filter paper discs and the cells were cultured on MS proliferation medium.

Growth characteristics: Fresh weight and dry weight were compared in cryopreserved and non cryopreserved cultures (control 2).

Microscopic observation: Pieces of tissues were squashed and stained with 2% acetocarmine and examined under light microscope Axioplan 2 (Carl Zeiss, Jena).

Somatic embryo maturation: The maturation DCR medium contained 94.69 µM ABA and 1% gelrite. Germination occurred on hormone free DCR medium in dark. After hypocotyl and cotyledon elongation the germinating plants were transferred to light.

Results and discussion

Microscopic observations revealed that most meristematic cells located in the embryonal part of somatic embryo survived storage in liquid nitrogen. The tissues started to grow on proliferation

media one week following cryopreservation. Out of 6 cell lines tested, only one (E 190) stopped the growth after two weeks. The tissue recovery was evaluated as a percentage of cultures that resumed growth. Tissues pretreated with sucrose and DMSO but not frozen (control 1) showed 90-100% regrowth. The survival of cryopreserved cell lines reached 62.5% (E 182) to 100% (E 177, E 184). The cell lines surviving cryopreservation showed typical features of conifer embryogenic tissues. The morphology of somatic embryos was not altered due to cryopreservation. The cryopreserved tissues were compared to non-cryopreserved and non-pretreated tissues (control 2). Growth parameters (fresh and dry weights) were similar in both types of tissues. Statistical analysis of the mentioned parameters did not reveal any significant differences at levels P 0.05 and 0.01.

For the maturation experiment, cell lines containing well developed somatic embryos were selected. Cotyledonary somatic embryos appeared around the 7th week of culture on maturation medium. Mature somatic embryos developed in all the three cell lines but in different number reaching 24 (E 193), 50 (E 196), 54 (E 193) cotyledonary embryos calculated per petri plate. Somatic embryos germinated on hormone-free medium and formed plantlets.

In our experiments we have chosen to apply a working protocol initially established for embryogenic banana suspension cultures (11, 12). Variation in the survival rate has been observed in the recovered cell lines. The differences in the cell growth could be attributed to genetic differences among cell lines (9) or to the physiological status of tissue. The survival rate of embryogenic tissues is also affected by different pretreatment and cryoprotective solutions containing maltose (8) or sorbitol (1, 2). In *Pinus nigra*, DMSO had only a limited effect on cell metabolism. The regeneration of newly formed embryos occurred from meristematic cells of the embryonal part. These cells survived storage in liquid nitrogen, suggested by microscopic observations immediately after thawing.

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TURKEY (*)

The current status of plant genetic diversity in Turkey and implications for cryopreservation studies

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Turkey is one of the most significant countries in the world from the point of its unique position and richness in genetic diversity. It covers a total area of about 78.000.000 ha, surrounded by Black Sea in the North, Aegean Sea in the West, Mediterranean Sea in the South and divided by the Dardanelles, the sea of Marmara and the Bosphorus in the West. Being a land bridge between Europe and Asia in a temperate climatic belt, Turkey offers a great diversity in its geomorphology, topography and climate, where ecological factors may change greatly even in a very short distance. The Asian part, ‘Anatolia’, consists of an inner high plateau, extending from West to Aegean coasts, with many river valleys and mountain areas along the North and South coasts. The European part, on the contrary, is a fertile hilly land. West and South Anatolia have typical Mediterranean climate with mild and wet winters, long and hot summers and almost constant drought from May to September. However, North Anatolia exhibits heavy rainfall that continues almost all the year (6).

A consequence of such a vast diversity in the country’s topography and climate is reflected in very rich habitat diversity and plant genetic resources. Besides the two of the Vavilov’s centre of origin, Turkey also includes diversity centres for many wild and cultivated forms of annual and perennial, herbaceous and woody plants, that are of great importance world-wide (e.g., spruce, cedar, wheat, barley, lentil, chickpea, apple, pear, walnut, pistachio) (1). Over 10.000 plant species are present in Turkey, one-third of which are considered endemic. The richness of Turkish flora is essential not only for the continuation of crop improvement in the world temperate belt, but also for the national economical structure, being Turkey still greatly dependent and closely related to the increase in the agricultural income. Several approaches for *in situ* and *ex situ* conservation of plant genetic diversity are available and often interrelated, among which ecosystem reserves (national parks, botanic gardens, etc.), seed and field genebanks.

As regards *in situ* conservation, attempts in Turkey initiated only in 1993 with the first National Project, aimed to the preservation of both wild relatives of cultivated plants and forest tree species with worldwide importance. Among the outcomings of the project, six gene management zones for five wild relatives of wheat were selected, and a national plan for *in situ* conservation of genetic resources was prepared (2). To date, 32 national parks, 11 nature parks, 32 nature reserves and 54 nature manuments are officially declared in Turkey. Differently from *in situ* activities, *ex situ* conservation in Turkey dates back to 1964, and in 1972 the first National Seedbank was established in Izmir. Today, *ex situ* preservation is financed by Turkish Government and is represented by seed genebanks (where “basic collections” are kept in cold rooms at -18/-20°C for long-term storage, while “active collecions” are kept at 0°C for medium-term storage), and by field genebanks (clonal collections). The exploration of biotechnological approaches for the germplasm preservation in Turkey is very recent. In 2005, a collaboration between the Gebze Institute of Technology of Kocaeli and the Italian CNR-IVALSA was established with the aim of developing the

cryopreservation technology in Turkey. As a first result, a TUBITAK-CNR Bilateral Project (2006-2007) was focused on the development of micropropagation and cryopreservation techniques for the medium- and long-term conservation of the plant germplasm which is economically important for both the Countries. Results of the project evidenced the adaptability of *Pistacia* seeds (*P. vera* L., *P. terebinthus* L. and *P. lentiscus* L.) to be stored in liquid nitrogen (LN) after appropriate dehydration (3). In a first trial, two methods of dehydration were compared, i.e., placing the seeds over silica gel or in the sterile air flow of a laminar air-flow hood. A maximum of 90% germinability (corresponding to a final moisture content of 11.7% on FW basis) was obtained after the *P. vera* seeds underwent 8 hours of dehydration over silica gel, prior to being directly immersed in LN. In *P. terebinthus* and *P. lentiscus*, shorter periods of dehydration over silica gel (one hour and 15 min, respectively) were sufficient to reduce their moisture content to about 20%, which was shown to make for best germination percentages (16% and 47%, respectively) of seeds from cryostorage. When used with seeds of *P. vera* and *P. terebinthus*, the 2,3,5-triphenyltetrazolium chloride (TTC) test proved to be a useful marker for an immediate evaluation of seed survival after cryopreservation. As for *Arachis hypogaea* L., effective procedures of both vitrification and dehydration, followed by direct immersion in LN, were developed for embryonic axes of cv. Virginia type. The best vitrification procedure (80% explant survival and regrowth) required loading of embryonic axes with PVS2 for 2 h at 25°C. The dehydration technique involved a 2.5 h desiccation in sterile air flow, after which the initial moisture content of the embryonic axes (25%) dropped to 8.5%. This procedure showed to be very effective, as 100% recovery was achieved. Optimized protocols were then applied to three Turkish cultivars, resulting in best recovery percentages of 87% and 92%, following the vitrification/ and the dehydration/one-step freezing approach, respectively.

In addition, after the development of effective micropropagation procedures for peanut (4) and pistachio (5), cryopreservation studies on vitrification/one-step freezing of shoot tips are currently in progress and under evaluation for their applicability to germplasm preservation.

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(*) At the time of printing the Abstract Book, Turkey was evaluating the subscription of the Memorandum of Understandings of COST Action 871.

Keynote lectures

KEYNOTE LECTURE**Elimination of virus and phytoplasma by cryotherapy of *in vitro*-grown shoot tips****Q. Wang^{1,2} and J.P.T. Valkonen¹**¹Department of Applied Biology, University of Helsinki, P.O. Box 27, 00014 Helsinki, FINLAND²Plant Biotechnology Laboratory, Chengdu Center of Food Test and Analysis, Sichuan Academy of Agricultural Science, Chengdu, Sichuan, P. R. CHINA**Introduction**

Virus and phytoplasma diseases remain a threat to production of agricultural and horticultural crops even though great efforts have been made to control them in last decades (2). In practise, effective means to control these diseases is through cultivation of virus- or phytoplasma-free plants and choosing resistant cultivars if they are available. Recently, cryotherapy has been used for efficient elimination of viruses from *Prunus* (1), banana (3), grapevine (4), and potato (5). The aim of this study was to extent this technique for elimination of virus in raspberry and phytoplasma in sweet potato.

Materials and Methods

Sweet potato shoots (*Ipomoea batatas* L.) infected with sweet potato little leaf (SPLL) phytoplasma, and raspberry shoots (*Rubus idaeus* L.) infected with raspberry bushy dwarf virus (RBDV) were established in *in vitro* culture. For sweet potato, shoot tips (1 mm) from *in vitro* stock shoots were subjected to cryotherapy. For raspberry, *in vitro* stock shoots were heat-treated for 4-5 weeks, followed by cryotherapy of shoot tips (1 mm). All recovered plants were tested for their sanitary status by different methods. For better understandings of the possible mechanism by which virus and phytoplasma were eliminated by cryotherapy, investigations were carried out on localization of virus, phytoplasma and surviving cells, and observation of cell ultrastructure in shoot tips.

Results

Table 1. Effects of cryotherapy of shoot tips on survival, regrowth and elimination of SPLL phytoplasma from sweet potato line 199004.2.

Treatments	Survival (%)	Regrowth (%)	SPLL-free (%)
Shoot tip culture	100	100	10
Dehydrated shoot tips	100	95	7
Cryotherapy	88	85	100

Data were collected from three independent experiments and presented as means

Table 2. Effects of meristem culture, cryotherapy and thermotherapy followed by cryotherapy on survival, regrowth and elimination of RBDV from raspberry genotype Z-13.

Treatments	Survival (%)	Regrowth (%)	RBDV-free (%)
Meristem culture			
0.1 mm	25	40	0
0.2 mm	40	65	0
0.3 mm	95	95	0
Cryotherapy	88	87	0

Data were collected from three independent experiments and presented as means

Table 3. Effects of time duration of thermotherapy applied on stock shoots on survival, regrowth and elimination of RBDV of raspberry genotype Z-13 shoot tips excised from the heat-treated shoots and subjected to cryotherapy.

Duration of thermotherapy (days)	Survival (%)	Regrowth (%)	RBDV-free (%)
0	85	78	0
21	48	60	0
28	36	40	33
35	20	30	35
42	0	0	0

Data were collected from three independent experiments and presented as means

Localization of virus in shoot tips

Cells in the lower part of apical dome and in the first two leaf primordia in non-heat-treated shoot tips were heavily infected with RBDV. After heat treatment, RBDV was still detected in the first two leaf primordia, but not in the area of apical dome.

Localization of phytoplasma in shoot tips

SPLL phytoplasma was observed in sieve elements of vascular tissues in 1.0 and 1.5 mm shoot tips, respectively, but not in 0.5 mm shoot tips.

Distribution of surviving cells in shoot tips after cryotherapy

With sweet potato, most of the cells in upper part of apical dome and in the first two leaf primordia survived, while those in lower part of apical dome and in the third leaf primordium were killed. A similar survival pattern was found for non-heat-treated shoot tips of raspberry. After heat treatment (28 days), only top layers of cells in apical dome and very few cells in the first two leaf primordia survived.

Cell ultrastructure

Size and vacuoles of cells increased, while nucleo-cytoplasm ratio decreased, with an increase in distance from apical dome. Under stress imposed by heat treatment, cells became bigger with larger vacuoles and decreased nucleo-cytoplasm ratio.

Conclusions

Cryotherapy of shoot tips was found to be an efficient method for elimination of SPLL phytoplasma from sweetpotato. Cryotherapy alone failed to eliminate RBDV that can infect meristematic tissues. However, thermotherapy followed by cryotherapy was able to eliminate RBDV. Furthermore, cryotherapy can be used simultaneously for long-term storage of germplasm and production of virus- and phytoplasma-free plants.

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KEYNOTE LECTURE

The challenge of preserving invertebrate's species by cryopreservation

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Cryobiology is the study of the living things at low temperatures. For more than 50 years, science has explored whether cold may preserve life. Today, cryobiology plays an important role in medicine, agriculture, horticulture, forestry and the conservation of endangered or economically important species. When cell or cellular systems are exposed to subzero low temperatures they are subjected to profound changes in the physical and chemical state. All living organisms primarily consist of water where it plays an essential role in the living systems. The most obvious change accompanying freezing is that the amount of this liquid is progressively reduced and eventually vanished and, their response to freezing is necessary dramatic. Nonetheless, we see an enormous number of organisms that can withstand the harshest winter climate. Artic and Antarctic terrestrial ecosystems (algae, higher plants, nematodes, insects and arthropods) provide an excellent opportunity for applied low temperature biologists to explore extreme survival strategies and adaptive responses thus offering insights into how life tolerate and endures the “frozen state” *in vivo*. Ice formation inside the cell is injurious and often lethal (1), so the mechanisms of survival at subfreezing conditions fall into two categories: freeze tolerance and freeze avoidance. In the former case, the organism survives extracellular ice formation; in the latter case, freezing is avoided altogether. During partial freezing of extracellular fluids, the concentration of soluble material remaining in solution increases. This creates an osmotic imbalance that causes water to flow out of the cell. As a result, the concentration of solutes in the cell increases. The alteration of the normal levels of solute concentrations inside the cell is often lethal. In addition, water depletion may cause the collapse of the cell membrane (2). Thus, a large number of living systems cope with prolonged exposure to subfreezing temperatures by avoiding ice formation. Freeze avoidance in animals is achieved by depression of the freezing point and through the action of antifreeze proteins.

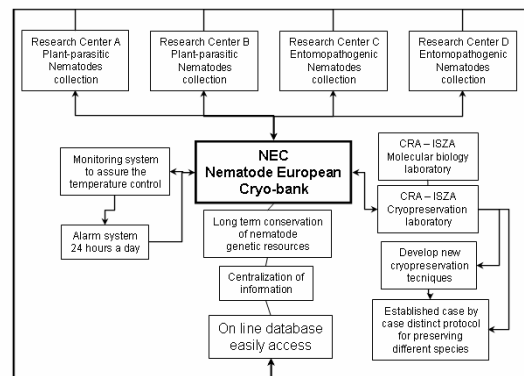
In many terrestrial invertebrate animals, the cold tolerance is achieved by an increase in the supercooling point of the body fluids (haemolymph) by increasing of solutes (such as glycerol and sorbitol) and sugars (such as trehalose and glucose) referred to as cryoprotectant (3, 4). Among land-based animals, antifreeze proteins have been found in beetles, spiders, centipedes and mites but not in flies, mosquitoes, wasps or ants (5). Several insect antifreeze proteins have been identified and their amino acid composition has been determined (5). Nevertheless, a full understanding of the mechanism through which antifreeze proteins inhibit ice formation is not yet available. The most plausible hypothesis is that inhibition occurs by (hydrogen-bond-mediated) adsorption of the proteins on growing ice embryos (6,7).

With the advent of penetrating cryoprotectant, primarily glycerol and DMSO (dimethyl sulfoxide), the cryopreservation has enabled to leap-frog basic research; the intracellular ice formation so lethal to most eukaryotic cells could be prevented by transforming liquids into an amorphous state (vitrification or glassy-state). Vitrification can be considered as the most suitable technique allowing complex tissues and organs cryopreservation. This process requires the use of rapid cooling rates in the presence of highly concentrated cryoprotective solutions:

sugars, alcohols either alone or in combination. Presently, the main challenge in cryobiology is to find the best composition for a cryoprotective solution owing a low biological toxicity while allowing vitrification on cooling. A wide range of animal cells can now be cryopreserved at -196°C for many years in a stable state. However, cooling is only half of the story. To function, a frozen cell or organisms must be returned to normal temperature and must survive the accompanying warming and the thawing of the ice. Generally, with high cooling rates smaller internal ice crystals are formed and, small ice crystals appear to be less damaging than large ones; however, if subsequent warming is slow, those crystals can enlarge to damaging size by the process of re-crystallization.

For multicellular organisms such as nematodes (small-invertebrate worms less than 1 mm long and 20-30 µm width) reviving from a period in liquid nitrogen is a challenge, but it can be done. Nematodes can be parasites (of animals, including humans, and plants) or soil free-living species (i.e. *Caenorhabditis elegans*). Recently, we have described a cryopreservation method employing ethylene glycol (EG) as cryoprotectant, coupled with rapid-cooling in liquid nitrogen but a final storage in a mechanical freezer (at -140 °C). With this procedure the percentage of nematodes able to survive at ultra low temperature is increased (8). The new protocol has been successfully applied to several phytoparasitic nematodes and then extended to other important nematode's species.

The idea of developing a Nematode Cryo-bank in Italy began in 2003 when scientific and technological developments were joined at CRA-ISZA laboratory in Florence (Italy). Since then, nine different species belonging to five genera of nematodes were collected and stored in the cryogenic-freezers. Actually, thousand of different nematode populations and/or the relative genetic material (DNAs) might be potentially stored, almost un-definitely, in whole safety system (9).



For this reason, the existing bio-repositories have been provided of monitoring systems to assure the temperatures control (inside and outside the freezers) and the efficacy of the alarm system 24 hours a day besides an emergency generator for the occurrence of electric black-outs. The creation of a nematodes cryo-bank would allow the centralization of the information that will easily be accessible in an on-line database and it will be possible, for instance, to check the availability of rare biological samples by getting simultaneous information from all the collections inside the bank.

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KEYNOTE LECTURE

Cryopreservation of plant germplasm in Argentina

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Argentina is an agricultural country with different climates. Many of its economically important plant species bear either recalcitrant or suborthodox seeds. Many crop species have also to be maintained by vegetative propagation. Genetic resources of these crops are actually maintained either *in situ* or *ex situ* as plants in field. Losses of plant material may occur owing to diseases or accidents. Additionally, a great deal of labour and time are required for the maintenance of this field collections. Cryopreservation is an alternative strategy for an efficient and economic long-term conservation of these plant species.

Research on cryopreservation of plant species in Argentina was initiated in 2001. At the present, no collection is maintained under cryopreservation, but at least two Institutions (our lab at IBONE, and the Facultad de Ciencias Agrarias, UNMP-INTA) are making efforts to develop protocols, in order to establish banks for long-term conservation of some economical or ecological important species.

Table 1 presents a preliminary summary of results on cryopreservation of plant germplasm in Argentina. Different plant species and various organs and explants were used. Research was focused mainly on cryopreservation of seeds and various explants of *Arachis pintoi* (pinto peanut), a perennial herb which is an important forage legume in tropical and subtropical areas of the world. *Arachis pintoi* has two cytotypes. There is a diploid cytotype, $2n=2x=20$, and a triploid cytotype, $2n=3x=30$. The first one produces seeds, but, in terms of conservation, is recalcitrant. By using vitrification and rapid cooling methods, as much as 90% of the cryopreserved seeds germinated. The triploid does not produce seeds and its propagation is obligatory by vegetative procedures. By using the encapsulation-dehydration method, 57% (diploids) and 60% (triploids) of cryopreserved shoot tips produced plants after reculturing. Hence, these procedures are suitable to cryopreserve valuable germplasm of this species.

Research was also carried out with two important forest trees for Argentina: a) the Paradise tree (*Melia azedarach*), for which two explants were used in cryopreservation, i.e., apical meristems and somatic embryos, with percentages of plant regeneration of 60% and 36%, respectively; b) the Australian cedar (*Toona ciliata*), for which as much as 35% of cryopreserved seeds (by direct immersion in liquid nitrogen, without cryoprotectants) germinated.

With orange (*Citrus sinensis*), var. Valencia late, 65% of the cryopreserved shoots tips regenerated whole plants. Another study was carried out with *Ilex paraguariensis* (mate) and others 7 species of the genus *Ilex*. As regards germplasm conservation possibilities, most of the tropical and subtropical species of *Ilex* present two major constraints: 1) they usually have seeds with rudimentary embryos that remain in the immature heart-shaped stage for a long time after the fruits reach maturity; therefore, the percentage of seed germination is very low. Embryoculture technique has been proved to be a very useful tool to overcome the problem; 2) they have seeds which are highly sensitive to desiccation, as well as cold sensitive; in other words, they are recalcitrant seeds and, therefore, unsuitable for dry and/or cold seed-storage procedures, traditionally employed for germplasm preservation. In our lab, we were able to cryopreserve both immature fruits and embryos, and by embryoculture we got 3-83% (depending upon the species) production of whole plants.

Moreover, a maximum of 11% of the cryopreserved shoot tips of *Solanum tuberosum*, spp. *andigenum*, produced plants after reculturing. Similar results were obtained with the Orchid *Oncidium bifolium*. Also anthers (at the uninucleate stage) of rice (*Oryza sativa*) were successfully cryopreserved and 35% of them formed calli.

Table 1. Summary of results obtained on cryopreservation of plant germplasm in Argentina.

Plant specie	Material cryopreserved	Method	Results (% survival)	Cooling	Ref.
<i>Arachis pintoi</i>	Seeds	vitrification	90	rapid	8
	Apical meristems	encaps-dehyd.	17	rapid	7
	Shoot tips	encaps-dehyd.	57	rapid	7
<i>Citrus sinensis</i>	Shoot tips	encaps-dehyd.	65	slow ^(*)	2
<i>Ilex</i> spp.	Immature fruits	vitrification	3-23	slow	5
	Immature zygotic embryos	encaps-dehyd.	15-83	slow	6
<i>Melia azedarach</i>	Apical meristems	encaps-dehyd.	60	rapid	9,11
	Somatic embryos	encaps-dehyd.	36	slow	12
<i>Oncidium bifolium</i>	Seeds	encaps-dehyd.	5	rapid	3
	Protocorms	encaps-dehyd.	11	rapid	3
<i>Oryza sativa</i>	Anthers	encaps-dehyd.	15	slow	4
<i>Solanum tuberosum</i>	Shoot-tips	vitrification	2-11	rapid	1
<i>Toona ciliata</i>	Seeds	direct	35	rapid	10

(*) slow cooling ($1^{\circ} \text{C m}^{-1}$ to -30°C , followed by immersion in liquid nitrogen)

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