

Abstracts to a Meeting of **Workgroup 2 of the COST Action 871** ("Cryopreservation of Crop Species in Europe"). The title of the meeting was "Integration of Cryopreservation in Genebank Strategies". It was held at IPK Gatersleben, Germany, on September 11 09 2009.

## **HOW CAN ECPGR AND AEGIS CONTRIBUTE TO A MORE EFFICIENT LONG-TERM CONSERVATION IN EUROPE THROUGH A BETTER USE OF CRYOPRESERVATION?**

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The European Cooperative Programme for Plant Genetic Resources (ECPGR) is a collaborative programme among most of the European countries aimed at facilitating the long-term conservation and the increased utilization of plant genetic resources in Europe. Since a few years ECPGR has been in the process of establishing a European genebank integrated system (AEGIS) with the aim of conserving the genetically unique and important accessions for Europe and making them available for breeding and research. Countries are invited to formally accept long-term conservation responsibilities for identified unique and important accessions and to make these readily available to *bona fide* users. Collectively, the selected accessions (i.e. European Accessions) will form the European Collection. Countries are invited to conclude a Memorandum of Understanding with AEGIS to formally agree on responsibilities as a member of AEGIS. Conservation standards will be developed as part of the AEGIS quality management system.

The following responsibilities of AEGIS member countries over registered accessions have been identified:

- Ensure long-term conservation of the accessions according to agreed quality standards;
- Participate in and/or to facilitate supporting conservation activities;
- Provide for safety-duplication of the accessions at another genebank;
- Facilitate the access to and availability of European Accessions according to agreed international instruments;
- Document information on the European Accessions according to agreed standards;
- Keep information updated and make it available to EURISCO.

Cryopreservation is anticipated to play an important role in AEGIS, including:

- a. Possibly the only way that vegetatively propagated germplasm and/or non-orthodox seed producing species can be conserved safely and effectively for the long-term;
- b. It allows to avoid management problems related to maintaining germplasm in an active growth state and might thus complement traditional methods;
- c. It provides for an effective way of safety-duplicating "difficult" germplasm;
- d. It allows integration of long-term conservation and safe movement of germplasm;
- e. It facilitates distribution of disease free or disease indexed germplasm.

## **COORDINATING CRYOPRESERVATION RESEARCH PROJECTS: WAYS TO MINIMIZE LAB TO LAB VARIATION**

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The administration of large scale projects that involve multiple laboratories requires careful coordination in order to produce valid data. Due to the variability of laboratory practices it is necessary to have clear and precise protocols and ensure that they are followed by every participant. Our earlier studies comparing the use of cryopreservation protocols in several laboratories found several critical points that must be controlled so that all laboratories can produce comparable data. Initially the four laboratories following the same protocols had very different results. Examination of each part of the process from plant growth conditions to protocol steps and regrowth parameters was used to determine critical steps that caused most of the variation. Initial planning was vital for successful coordination of the experiments. It was very important for all participants to carefully follow specific written protocols with all details clearly stated. As a result of this experiment we found that our initial protocol was lacking in the detail needed for successful intra-lab coordination. Study of procedures used in each lab allowed us to detail the critical factors that must be addressed in planning a coordinated project. In vitro plant health is a primary factor. This includes the growth conditions, medium used, length of subculture, hyperhydricity, and any pretreatments. Next operator skills or experience can greatly impact all steps of a protocol from explant excision to manipulation of explants during the procedure, so specific training is important. The plant part used and its location on the plant (apical, axillary or basal) also affects recovery after cryopreservation and this must be standardized in any experiments. The physical laboratory conditions, temperature, humidity and type of laminar air flow play a role in several of the techniques as well. Stringent identification and equalization of these critical factors are needed to produce comparable data for the same experiment done in several laboratories.

## **CAN PLANT CRYOPRESERVATION LEARN FROM THE OECD ACTIVITIES TO ESTABLISH A GLOBAL NETWORK OF BIOLOGICAL RESOURCE CENTRES?**

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Although the foundation of biological resource centres for microbial and agronomic biological resources was laid in the late 19<sup>th</sup> century, the development of institutions took different directions.

In the 70<sup>th</sup>, International agreements led to the establishment of rules and institutions to save biological resources for food production as a common global human heritage. In the microbial field, institutions remained in a private or academic environment. This is also reflected by the development of patent protection rights on microbes and related materials under the Budapest treaty.

Recently, the OECD took the initiative to work on international guidelines for maintenance and exchange procedures to be used in “Biological Resource Centres”. In the future, collections should work in compliance with these rules to be recognized as BRC’s (Biological Resource Centres). OECD activities also led to the foundation of GBRCN, the Global Biological Resource Centres Network, so far dealing only with microbial collections. Although these activities have been initialized by an international group of microbial collections, it has been taken up by collections in the medical field. The EU financed the BBMRI project (Biobanking and Biomolecular Resources Research Infrastructure) dealing with all kinds of human resources for medical research. This initiative also refers to a set of OECD guidelines. Thus far, plant genetic resources and even plant cell cultures have not been addressed by the OECD. Nevertheless, the plant field has to be aware of these activities. On the one hand, already existing guidelines for technical procedures in other fields may be helpful to develop similar issues in the plant sector. Anyway spreading of rules should be watched to avoid the establishment of requirements which cannot be fulfilled by plant cryopreservation practise.

### **LONG-TERM STORAGE OF *PINUS NIGRA* Arn. EMBRYOGENIC TISSUES THROUGH CRYOPRESERVATION**

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Cryopreservation of *Pinus nigra* Arn. embryogenic tissues has been studied using the slow freezing method. In the first series of experiments, twenty cell lines were included. The aim of this study was to store the tissues in liquid nitrogen for 1 year and 1 hour and to compare their recovery frequencies after thawing.

Prior to storage in liquid nitrogen, the tissues were pre-treated with sucrose (180 g.l<sup>-1</sup>) and DMSO (7.5%). After short-term storage, 80% of cell lines regenerated with frequencies ranging between 20 to 100% and their growth was not negatively influenced by storage in liquid nitrogen. After the second week of culture massive growth was observed in most of recovered cell lines. After the long-term storage (1 year) a prolonged lag-phase was observed in growth of tissues. It took 9-10 days for cell lines with high regeneration ability and even 8 weeks for cell lines with lower regeneration ability. The long-term storage resulted in recovery of 70% of cell lines with recovery frequencies ranging between 10 and 100%. Growth and molecular analyses are in progress.

In the second series of experiment, the relationship between maturation capacity and recovery after cryopreservation has been studied. Altogether 20 cell lines were included in the experiment. Preliminary results indicate no correlation between maturation capacity of cell lines and their post-thaw recovery.

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## PHYSIOLOGICAL AND GENETIC ASPECTS OF LONG-TERM CRYOPRESERVATION OF *ABIES CEPHALONICA* Loud. EMBRYOGENIC CELL LINES

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*Abies cephalonica* belongs to the Mediterranean fir species, which are important in coniferous forests. However, most stands are rather degraded due to climate change. Interspecific *Abies* hybrids with high vigour and growth have been proposed to survive better than native species. Among *Abies* hybrids, the most promising are those where *A. cephalonica* is the mother tree. Conifer somatic embryogenesis is emerging as a key component of advanced forestry breeding programmes. Cryopreservation should be considered as an integral part of such programmes. We have developed a cryopreservation protocol for eight half-sib embryogenic *A. cephalonica* cell lines and we have also tested genetic fidelity of cryopreserved material using molecular markers. Maturation experiments included studies on the effect of sucrose concentration, polyethylene glycol and activated charcoal on quality and quantity of somatic embryos.

In the present study, recovery of 6-year-old cryostored samples representing two cell lines was assessed by means of proliferation rate and microscopical performance of embryogenic tissue. Both cell lines reached stable proliferation rate 3 months after thawing when the ATP and glucose-6-phosphate cellular level were analysed at different phases of proliferation cycle. Success with multiple maturation experiments depended on the composition of media and the *in vitro* technique used (standard approach or spreading embryogenic cells onto filter paper). We report the first results on long-term cryopreservation of *A. cephalonica* embryogenic cell lines.

## CRYOPRESERVATION OF *IN VITRO* SHOOT TIPS OF WILD CHERRY (*PRUNUS AVIUM*), ASPEN (*POPULUS TREMULA*) AND ASPEN-HYBRIDS (*P. TREMULA* x *P. TREMULOIDES*)

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The deployment of clonal varieties of valuable broadleaved forest species is one of the tasks within the scope of utilization of forest genetic resources at the Northwest German Forest Research Institute. In order to safely maintain reference material of clones currently being on trial and of tested clones, cryopreservation protocols for *in vitro* shoot tips of different species have been developed.

Shoot cultures of wild cherry (*Prunus avium*) were micropropagated on MS medium containing 0.5 mg/l BA, 0.1 mg/l IBA and 0.1 mg/l GA<sub>3</sub>. Shoot cultures of aspen (*Populus*

*tremula*) and aspen hybrids (*P. tremula* x *P. tremuloides*), later shortly called aspen, were cultured on MS medium containing 0.2 mg/l BA. *In vitro* shoot tips of wild cherry and aspen were cryopreserved applying a modified PVS2-vitrification protocol developed *Betula*. Compared to the birch protocol, the incubation times with the PVS2 solution were modified for wild cherry and aspen. The experiments were carried out with four wild cherry clones and 15 aspen clones. Ten shoot tips per treatment variant and clone were frozen in liquid nitrogen (LN), rewarmed after a few days and placed on multiplication medium in order to determine survival and shoot regeneration. Assessments were made 2, 4 and 6 weeks after rewarming.

The best treatment variants for four investigated wild cherry clones resulted in 60 to 80% shoot regeneration. In aspen, shoot regeneration percentages of 15 clones varied from 50 to 100%. Regenerated shoots of both species formed shoot clusters which could be propagated in the same way as unfrozen controls.

### **CRYOPRESERVATION OF SHRUBBY CINQUEFOIL (*DASIPHORA FRUTICOSA*) BY DROPLET VITRIFICATION METHOD**

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Cryopreservation represents an alternative for long-term preservation of shrubby cinquefoil genetic resources no longer used for vegetative nursery propagation. Four cultivars were used to optimize the method. Suitability of apical and lateral meristems was tested. Use of activated charcoal in preculture medium after meristem isolation and effect of different sucrose pretreatment concentrations were studied. Different PVS2 treatment durations were screened. Buds excised from micropropagated plantlets were pretreated with activated charcoal on Murashige and Skoog medium for 0 to 3 days, and with a progressive increase in sucrose concentration (0.25, 0.50 and 0.75 M) for 2-3 days, loaded for 30 min, treated with PVS2 for 35-60 min and frozen on aluminium foil strips. Apical buds of 2-3 mm in length were superior to lateral and smaller apical meristems. Stepwise preculture with increasing sucrose concentrations (0.25-0.50 M) was beneficial, but extending pretreatment with 0.75 M sucrose decreased regrowth and induced some visual damage like vitrification during regrowth. Two-3 mm long apical buds of cultivars ‘Goldteppich’ and ‘Tervola’ showed cryopreservation tolerance with regrowth between 16-90% and 20-60%, respectively but ‘Dart’s Cream’ and ‘Elizabeth’ had lower regrowth with 0-60% and 0-40%, respectively when treated with activated charcoal, stepwise sucrose increase and PVS2 for at least 40 min. Sucrose treatment for one day on 0.25 M and two days on 0.5 M, instead of one day on 0.25 M, 0.50 M and 0.75 M each, combined with PVS2 treatment for 60 min increased regrowth of ‘Dart’s Cream’ up to 70-78%. In conclusion, droplet vitrification combined with activated charcoal and 0.25 M and 0.50 M sucrose pretreatments is suitable for cryopreservation of shrubby cinquefoil.

## **BANANA CONSERVATION ACTIVITIES AT THE BIOVERSITY INTERNATIONAL TRANSIT CENTRE (ITC), BELGIUM**

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The ITC, hosting around 1245 accessions is the world's largest banana germplasm collection. All material is conserved *in vitro* under slow growth conditions at reduced light intensity and low temperature (MTS: Medium Term Storage), while 697 accessions are safely cryopreserved (LTS: Long term storage). Cryopreservation takes place using a droplet vitrification protocol that is applicable to all banana accessions that are present at the ITC and that are representing the whole *Musa* diversity. A safe back up (= black box) of the cryopreserved material containing 480 accessions has been established in IRD, Montpellier.

Besides these "living collections", also a lyophilized leaf tissue collection of some 700 accessions has been established in 2006 from which samples can be requested for molecular studies (DNA) studies.

In this report, also the cost of conservation of MTS was compared to LTS. The calculated time, when the cumulative costs of MTS become equal/higher than for LTS, is 15 years. If stored for a longer time period, LTS becomes the cheaper option for conservation.

## **PRACTICAL IMPLICATIONS TO USE DIFFERENT EXPLANT SOURCES FOR ROUTINE CRYOPRESERVATION**

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After screening various parameters, efficient cryopreservation procedures were established. Biological material differs from technical objects with respect to its high levels of individual variability. Therefore, individual parameters can only solve a part of the methodical development and resulting protocols. Genetic variability and the actual morpho-physiological state of the donor plants influence the result of the procedure to a major extent. Variability is expressed by different reactions of a plant towards given conditions as well as by the variability of morphological characters and morphogenetic pathways.

The main species in the cryobank of IPK, potato, garlic, and mint are very diverse with respect to the parameter donor plant. On the species level major differences exist between monocot bulbous and dicot herbaceous plants. Between potato and mint there are differences in the shoot architecture (sympodium vs. monopodium) making potato a difficult object for morphogenetic analyses. Morphological differences on the infraspecific level are obvious within garlic, where the ability to form bulbils in the inflorescences is limited to some groups and the bulbils moreover differ in size, number and location. Within a species, the availability and quality of the target organs are very much depending on growth cycles and the given

physiological state. The location of a given bud may determine regeneration percentages as has been demonstrated for potato. In garlic, differences in regeneration rates were found between types with small versus large bulbils. As the mean regeneration rates over different garlic accessions between explants from bulbils (mean 32%), cold-precultured in-vitro plantlets (mean 47%) and inflorescence bases (45% in a test case) are in the same range, technological factors will determine what method to use. Sometimes different regeneration frequencies may be influenced by intrinsic genetic factors. Some accessions of garlic turn into dormancy forming in vitro bulblets whereas others go on growing after rewarming. Cold preculture stimulates regeneration in cryopreservation. All these factors play a role in the genebank logistics.

## **INTEGRATION OF CRYOPRESERVATION IN PLANT GENETIC RESOURCE CONSERVATION STRATEGIES IN FRANCE**

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In France, conservation of plant genetic resources (PGRs) is organized in a decentralized way, through the operation of 26 crop-specific networks. The GIS IBiSA (Scientific Interest Group/Infrastructures in the Biology, Health and Agronomy Sectors) established in 2007 to coordinate the national policy for accreditation and support to platforms and infrastructures in life sciences, regularly opens and administrates calls for project proposals aiming at providing equipment to these platforms and infrastructures and/or at developing and implementing new technologies.

Until today, cryopreservation has been little applied to horticultural or other agricultural species. In order to capitalize upon the dramatic progress made in the development of cryopreservation techniques for plant species and to stimulate the integration of this technology in the national PGR conservation strategy, IBiSA has decided to fund in 2009 the CRYOVEG project (Cryopreservation of French collections of PGRs). The project aims at 1) developing or optimizing cryopreservation techniques in a range of selected species; 2) establishing a national scientific and technical network of BRCs using cryopreservation. The project has a network organization, with IRD/INRA Montpellier as the cryopreservation expertise centre and partners in continental France and overseas departments in charge of genetic resource conservation for various species: INRA Guadeloupe (yam); Corsica (*Citrus*); Bordeaux (*Prunus*); Angers (apple and pear); Montpellier (grapevine); Ploudaniel (potato, *Brassica*); IRD La Réunion (coffee); CIRAD Guadeloupe (sugarcane) and La Réunion (vanilla, garlic). The group will work as a think-tank on the integration of cryopreservation in the national PGR conservation strategy and an action plan for its implementation will be collectively designed. The project will work towards establishing a French research network on PGR cryopreservation, open to all partners interested in cryopreservation. The group will prepare research and development projects whose results will be disseminated through European PGR conservation networks *e.g.* ECPGR. Finally, network members will train French and foreign (from North and South countries) researchers and technicians in cryopreservation techniques.

## PLANT CRYOPRESERVATION ACTIVITIES IN FINLAND – TOWARDS CRYOBANKING

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Plant germplasm conservation relies on *in situ* and *ex situ* strategies. Among *ex situ* conservation methods, attention has recently focused on cryopreservation. Cryopreservation offers a safe and cheap alternative for maintaining genetic fidelity and protecting germplasm from external threats. The possibility of large scale cryobanking has thus been evaluated.

In Finland, the responsibility for conservation of germplasm of horticultural plants lies with MTT Agrifood Research Centre and with Metla (Finnish Forest Research Institute) for forest tree species. For horticultural plants and forest trees, both *in situ* and *ex situ* strategies are employed and cryopreservation is in active use in both institutes. In addition to these governmental institutes, Helsinki and Oulu Universities teach cryopreservation and perform cryopreservation research. In Oulu, cryopreservation protocols are actively researched and there is increasing interest for using cryopreservation as one method for conserving wild plant species. A large liquid nitrogen container has recently been purchased for this purpose.

Integrating cryopreservation within Nordic genebank (NordGen) activities is envisaged in Nordic countries. However, integration has been concretely initiated only in Finland. In Finland, the national raspberry and hop collections are cryobanked, strawberry collection is partly cryobanked, and cryobanking *Ribes*, *Malus*, *Prunus* and *Syringa* has been initiated. NordGen has made a tentative plan for using the facilities of MTT Agrifood Research (Laukaa) for establishing a backup long-term collection of vegetatively propagated horticultural plants. The apple germplasm collection will most probably be established in Denmark where most Nordic apple accessions are conserved in the field and already partly cryopreserved. We are thus moving towards cryobanking. Integrating laboratories already engaged in cryopreservation as satellites of the existing genebanks seems the most efficient and cost-effective strategy to make the best use of resources and know-how.

## CRYOCONSERVATION STRATEGY OF FRUIT GENETIC RESOURCES IN GERMANY

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The ‘National Program for Genetic Resources of Agricultural and Horticultural Plants’ in Germany is designed to provide long-term preservation, utilization, research and development for these species. In the Institute for Breeding Research on Horticultural and Fruit Crops in Dresden-Pillnitz the Fruit Gene bank with 2600 accessions is integrated. The conservation strategy requires the application of different methods for safety duplication.

One way is the German Fruit Genebank established as a network of collections held at several locations. Based on an assessment performed in different institutions, 950 apple cultivars and 369 strawberry cultivars were selected to be included into the National German

Apple respectively Strawberry Genebank. High operating expenses and budget limitations for field collections do not allow further duplication. The cryopreservation should be established as another safety duplication method.

Cryopreservation using winter vegetative buds was successfully applied to the *Malus* collection maintained at the Institute in Dresden-Pillnitz. Following a two step freezing (prefreezing at 1°C/h to -30°C and maintenance at -30°C for 24 h) and cryopreservation for five months, chip-budding was performed after a 15 days rehydration period at 4°C. Successful recovery was only observed for scion pieces collected in January. Rates between 0 and 77% were reached. Further specifications of the cryopreservation protocol are necessary to adapt the method to mild winter conditions in Central Europe.

For strawberry, first *in vitro* cold storage was elaborated and adapted as a safety duplication method. The average storage duration at 4°C for a range of strawberry cultivars was 22 months and for wild species accessions 9 months. Preliminary experiments with PVS2 vitrification using 22 cultivars and 7 wild species accessions demonstrated recovery data varied from 37 to 86% for the cultivars and from 0 to 60% for the wild species. These experiments indicate that it is likely to be a successful protocol for strawberry, but additional modifications may be needed for species material.

## **HOW TO START A PROGRAMME ON ROSA CRYOPRESERVATION?**

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GenHort research activities are dealing with genetics of horticultural species and this unit is in charge of several collections of plant genetic resources. Some works are focused in increasing knowledge of our collections, studying the impact of domestication and selection on the genetic variability, defining strategies for an optimal management of these genetic resources. In order to secure the vegetatively propagated collections, studies on cryopreservation have been undertaken on *Pelargonium* since 1999. The main objective was to determine if shoot tip cryopreservation could play a role in the long term maintenance of healthy collections of vegetatively propagated plants. An efficient and reproducible protocol of cryopreservation with a droplet-vitrification procedure allowed obtaining good survival and regeneration rates. Soon, new activities will take place in the cryopreservation studies developed at GenHort. For an applied aim, cryopreservation of *Malus* and *Pyrus*, using a dormant bud protocol will be undertaken. A new program focused on the genus *Rosa* is just about to be initiated. The sub-genus *Rosa* comprises over 180 wild species, assigned in 10 sections, and plentiful of cultivars within 2 clusters: the old garden roses (20 groups) and the modern roses (13 groups). It presents a huge diversity regarding geographic origins, plant architecture, flowering physiology, environmental adaptation, morphological characters etc. Knowing the few reports previously published on *Rosa* cryopreservation, the present purpose is to define the scientific questions which can emerge and the best way to approach their solution. It is the aim to set up three kinds of cryopreservation protocols (encapsulation-dehydration, droplet vitrification and dormant buds). The emerging questions would be: 1) is the tolerance to cryopreservation influenced by the genotype, 2) could the level of tolerance be related to the infrageneric diversity classification? The diversity within a collection of genetic resources could then be affected by the chosen conservation method.

## **EPIGENETIC ANALYSIS OF CHRYSANTHEMUM SHOOTS REGENERATED FROM CRYOPRESERVED APICES: EMPLOYMENT OF CRED-RA TECHNIQUE**

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The analysis of epigenetic changes that can appear during tissue culture and cryopreservation is taking an important role in the studies of genetic stability of germplasm during these processes.

The aim of this study has been to apply and to assess the capacity of the CRED-RA technique (“Coupled restriction enzyme digestion and random amplification”) (1) to obtain the methylation patterns of chrysanthemum cryopreserved shoots, and by comparing methylation patterns to analyse the epigenetic stability of the cryopreserved material in relation to control non-treated tissues. The methodology of this technique is based on the capacity of certain restriction enzymes (Hpa II and Msp I) to recognize the same cut sequence, but with different restriction capacity depending on the methylation status of the cytosine residues. Subsequently, PCR amplifications, using arbitrary primers, are produced with the digested DNA. These amplifications could show different bands patterns according to the DNA methylation status of the original sample.

Analyses were carried out with chrysanthemum regenerated shoots derived from two different cryopreservation protocols: encapsulation-dehydration and vitrification. Controls (unfrozen samples) for each tested samples were included. Besides, the genetic stability of the samples was assessed using RAPD markers.

The results obtained in this work showed a high genetic and epigenetic stability of the chrysanthemum samples studied. Nevertheless, two out of six samples, derived from the encapsulation-dehydration treatment, presented different band-patterns compared to their controls when the CRED-RA technique was applied. These differences cannot be correlated to a possible somaclonal variation since the RAPD data did not show any genetic change. No variation was detected in the four samples derived from the vitrification method, although to conclude that this protocol implies a higher stability, compared to encapsulation-dehydration method, further analyses would be necessary.

CRED-RA technique has proven to be a useful tool in the assessment of stability in DNA-methylation patterns of regenerated plants of chrysanthemum after cryopreservation.

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## **GENOMIC DNA METHYLATION PATTERNS AND SURVIVAL OF APPLE BUDS TO CRYOPRESERVATION**

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Cryopreservation using the winter vegetative bud method (1) is being implemented in Asturias (Spain), a region with a mild oceanic climate. Two apple cultivars were assayed. Twigs with buds were collected from the field in February and March. Cultivar 'De la Riega' taken on February gave high survival after cryopreservation but very low survival was obtained with the cultivar Raxao and no survival was achieved in samples of both cultivars when collected on March. So although it is feasible to apply this method to apples growing in mild temperatures, results differ between cultivars and the dormant bud stage seems to have a capital importance.

Experiments made with chestnut buds have shown a relationship between DNA cytosine methylation and bud dormancy (2). Hypermethylated DNA was found in dormant buds coinciding with unfavourable conditions for active growth, and DNA became increasingly hypomethylated during bud burst, when conditions are favourable for growth and development. Opposite patterns were found with the levels of acetylated histone H4 which were higher during bud burst than during bud dormancy. A similar work on global DNA methylation has been carried out in samples of these two apple cultivars in order to find out a correlation between responses of cultivars concerning survival after cryopreservation and the collection time. DNA was extracted from lyophilised buds excised from only desiccated scion woods (approx. 30% moisture content) and from cryopreserved scion woods and global methylation is quantified by High Performance Capillary Electrophoresis (3). The determination of global methylation from isolated DNAs takes less than 10 minutes per sample, so it can be a good tool for screening in case there is a correlation between methylation level and survival percentage to cryopreservation. Experiments need to be repeated as controversial results have been obtained.

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### **CRYOPRESERVATION OF DATE PALM 'BARHEE' PROEMBRYOGENIC MASSES USING THE ULTRA-RAPID DROPLET FREEZING TECHNIQUE: EXPRESSION OF THE OSMOTIC STRESS GENES AND GENETIC STABILITY**

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Our study is focused on the morphogenetic capacity of date palm cultivar 'Barhee' proembryogenic masses (PEMs) after their cryogenic treatment. Proembryos of this genetically instable cultivar have been induced using small pieces of juvenile leaves as primary explants placed on MS medium supplemented with 0.3 mg/l 2,4-D. These regeneration conditions lead to true-to-type plants as it is observed after plant fructification.

Compared to the standard vitrification protocol, the ultra-rapid droplet freezing technique proved its high efficiency for the cryopreservation of the date palm proembryos of the cultivar 'Barhee'. Sucrose preculture, which can induce activation of genes coding for resistance towards osmotic stress, increases considerably post thaw recovery rates after vitrification. The highest survival rate (60%) after cryogenic exposure was obtained when PEMs were treated with PVS2 solution for 30 min at 0°C and 15 min at 25°C. Regrowth of the cryopreserved PEMs requires from 2 to 3 weeks. We showed that cryopreservation does not affect the morphogenetic capacities of this plant material, and a highly proliferating suspension culture was established using the protocol that we previously established. The overall production of somatic embryos reached 9,000 units per litre per month. Morphological and molecular studies show the genetic stability of clonal material following cryopreservation.

## **ASSESSMENT OF AN *IN VITRO* SCREENING ASSAY FOR THE IDENTIFICATION OF CHICKPEA PLANTS TOLERANT TO FREEZING**

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Chickpea (*Cicer arietinum* L.), a traditional crop in Greece cultivated in dry fields, plays a significant role in rotation programs. Chickpea is essential for the Mediterranean diet and it is also used as fodder crop. In the past two decades, new varieties have been produced at the Fodder Crops and Pastures Institute, which can be sown in late autumn as opposed to mid February, in order to exploit the humid period of late winter and early spring. These varieties are also characterized by high yields.

In search of a fast, efficient and reliable screening technique for identifying chickpea plants resistant to low temperatures *in vitro*, we used these new varieties to test the efficacy of a microplate freezing assay.

In Greece, chickpea is susceptible to frost at its early vegetative stages, therefore, chickpea embryos were used as experimental plant material in our microplate freezing assay. Six varieties with various degrees of freezing resistance, based on field data, were used. Twenty-four embryos from each variety were included in each experiment. Each chickpea embryo was cut in three segments namely root, hypocotyl and epicotyl (with foliage leaves). Each embryo segment was placed in a microplate well, prefilled with water, following a Latin square experimental design, in order to control the environmental variability. Samples in microplates were precooled in a water/alcohol bath to -1°C for 5 min and subsequently gradually cooled to -20°C. Each experiment was repeated three times. Evaluation of results will determine the usefulness of the microplate freezing assay in breeding programmes aiming at producing chickpea plants tolerant to freezing.

## **CRYOPRESERVATION OF *CITRUS* GERMPLASM: CONSIDERATIONS ON A CASE-STUDY**

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As vegetatively-propagated plants, the germplasm of citrus is mainly conserved either in field orchards, or in gardens where ancient collections are often maintained in big earthenware containers. Germplasm preserved this way is costly and vulnerable to damage and losses due to pests, diseases and extreme environmental conditions. However, numerous reports have shown the possibility to successfully cryopreserve various explants (somatic embryos, embryogenic callus, nucellar cells, ovules, seeds and embryonic axes) from different *Citrus* species and hybrids by both slow-cooling and one-step freezing procedures. In 2006, a study was started at the CNR-IVALSA of Sesto Fiorentino, Italy, with the aim to develop cryogenic procedures which could allow for the duplication of the ancient citrus collection at the botanical garden of “Villa Medicea di Castello” in Florence. The collection was initiated by Cosimo I de’ Medici in the 16th century, and it includes more than 600 accessions, some of them unique. Up to now, a protocol of seed dehydration and direct immersion in liquid nitrogen (LN) has been developed for 14 ancient accessions from 9 *Citrus* species and one hybrid, achieving, for 11 of them, over 75% germinability in post-cryopreservation. As these accessions are polyembryonic (i.e. seeds contain zygotic and nucellar embryos, the latter being genetically identical to the maternal parent), their seeds are a good material for clonal cryostoring. The project is now dealing with the development of effective encapsulation-based procedures. As a preliminary step, the effect of different pre-freezing treatments on moisture content (MC) and survival of encapsulated (in 3% alginate) buds has been evaluated. The exposure of synseeds to increasing sucrose concentrations (0.1 M, 0.3 M and 0.7 M, each one for 24 h), followed by 5 hours of desiccation in silica gel, produced a reduction of MC from 81% (on FW basis) to 23%, with 50% of bud survival. However, osmoprotection plus desiccation was not able to induce tolerance to freezing in LN. Hence, other cryopreservation techniques (encapsulation-vitrification, droplet freezing) are now considered.

## **CRYOPRESERVATION OF MULBERRY (*MORUS ALBA* L.) APICES: APPLICATION OF THE MOST USED METHODS TO AN ITALIAN CULTIVAR**

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White mulberry (*Morus alba* L.), native of East Asia, naturalized in North America and Europe, is the food source of silkworm. It is also cultivated for ornamental, pharmaceutical and commercial uses, such as for wood and cellulose production. In Italy, it was introduced around the tenth century, and now there are either plants derived from spontaneous

hybridisation or selected varieties, reproduced by vegetative propagation. Mulberry is a rustic plant, able to grow in calcareous, dry or saline soils, in hot or cold areas. Therefore, it can be planted for reforestation of marginal zones. In 2000, the Italian Ministry for Agriculture financed a project for conservation of Italian vegetal biodiversity, including mulberry germplasm. Cryopreservation procedures have been developed for the most diffused Japanese and Chinese cultivars. In this work, cryopreservation is applied to *in vitro* shoot tips with the aim to evaluate applicability of cryopreservation to Italian germplasm. Apices of the cultivar 'Florio', excised from *in vitro* grown shoots, were cryopreserved using encapsulation-dehydration (ED) or various vitrification protocols. For ED, shoot tips were pre-cultured for 1, 3, 5 or 7 days in liquid medium supplied with 0.5, 0.75, 1.0 or 1.25 M sucrose. Desiccation was performed with silica gel for 0, 4, 6, 8, 9 or 14 hours. For vitrification (standard PVS2, droplet vitrification, pretreatment with 5% DMSO, three-step and encapsulation-vitrification), tips were immersed in PVS solution for 5, 15, 30 or 60 minutes. Only for droplet vitrification (DV) tips were treated with 10% DMSO for 1, 2, 3 or 4 hours, transferred into droplets of PVS2 on aluminium strips and quickly immersed in liquid nitrogen. The highest regrowths were obtained using the ED with 0.75 M sucrose pre-treatment of beads for 3 days and 9 hours of silica gel desiccation and DV with explant pre-culture of 2 h in 10% DMSO (67 and 61%, respectively).

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## **PRESERVATION OF POTATO (*SOLANUM TUBEROSUM*) GERMPLASM BY MEANS OF CRYOPRESERVATION**

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Potato is the fourth main food crop after rice, wheat and maize in the world. The consumption of potato has been assumed to increase strongly in the future because of its good yield on a relatively small land area and high nutrition values. The varieties of potato are propagated only vegetatively because of the high heterozygosity of the seeds. Numerous potato varieties have been preserved in the genebank of Estonian Research Institute of Agriculture (ERIA) for already more than 30 years. Their morphological properties have been characterised both in laboratory and field conditions. Currently 810 clones from 410 potato varieties are preserved and maintained by micropropagation in slow growth conditions. Maintenance of the plant material by micropropagation is time and labor consuming and, moreover, the influence of the long term *in vitro* preservation on the genetic stability is not known. Therefore, safe storage methods and studies on the fidelity of the preserved material are needed. The general aim of the present studies will be to characterise the genotype of the most important varieties in ERIA, and to develop cryopreservation protocols for their safe storage. In addition, the genetic fidelity during cryostorage will be evaluated. There are several molecular markers available for potato genotype and genetic fidelity analyses. Markers, such as simple sequence repeats (SSR) will be used, and depending on the number of selected varieties and clones, diversity arrays technology (DaRT) will be tested. The work will be done in collaboration between ERIA (Estonia) and the University of Oulu (Finland).

So far two cryopreservation protocols were tested: the droplet vitrification and the DMSO droplet method. In preliminary tests performed at Agrifood Research Laukaa (Finland) both protocols were successful on shoot tips of variety 'Bintje'. During autumn 2009, the methods will be applied to several Estonian potato varieties.

## **METHODS FOR EVALUATION OF SHOOT TIP DEHYDRATION FOR PLANT CRYOPRESERVATION**

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Low water content is a pre-requisite condition for plant cryopreservation. Low water content results in a low amount of freezable water. Two dehydration methods for shoot tips were tested. In the first method the potato shoot tips were dehydrated by sterile air flow in laminar flow bench in open Petri dishes. The dehydration curves were notable to evaluate according to the known water relation. In the second method the dehydration to the equilibrium water content was employed. The constant equilibrium water vapour concentration was achieved by placing the material over different saturated salt solutions. The equilibrium was possible to evaluate according to the first order kinetics. The dehydration curves of dormant apple buds and *Allium* shoot tips were separated in two parts according to the dehydration rate. The first part of dehydration was dependent on relative humidity and was significantly faster, in contrast to the second part of dehydration. The second part of dehydration was not dependent on relative humidity. The controlled dehydration over saturated salt solution can be described by the first order reaction which can be used for the prediction of the critical dehydration rate and final water content of shoot tips for cryopreservation.

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## **GENETIC STABILITY ANALYSIS OF MINT CULTURES (FIELD, *IN VITRO* AND CRYOPRESERVATION) FROM A GENE BANK**

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Plant germplasm is conventionally preserved as seeds in an efficient and economic way. However, this procedure is not available for some species (those vegetatively propagated, or with non-orthodox seeds). Field collections have been the traditional germplasm conservation method for them. In the last decades *in vitro* conservation techniques have also been used to avoid field risks, and, more recently, cryopreservation protocols have been developed for several plant species. Although cryopreservation is usually claimed as a guarantee for genetic

stability, in comparison with other *in vitro* long term storage procedures, genetic variation in cryopreserved material has been reported (1).

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

Apex cryopreservation is an alternative procedure to the classical methods for medium- and long-term conservation of mint genotypes. Optimization of this preservation system implies the development of protocols that can guarantee the regeneration of the preserved material together with maintenance of the characteristics of the preserved genotypes through the whole process.

The aim of this work was to study in detail the droplet vitrification protocol developed for cryopreservation in the genebank of IPK at Gatersleben. Plants from the field collection, *in vitro* plants and regenerated plants from cryopreservation have been studied in order to evaluate their genetic stability as a fundamental aspect in the adequacy for long term germplasm conservation of the different procedures. The genetic stability analysis has been carried out with RAPD markers. No variation was found among the studied material.

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## **HISTORY AND DEVELOPMENT OF THE POTATO CRYOPRESERVATION METHOD AND THE CRYOPRESERVED COLLECTION IN THE IPK GATERSLEBEN**

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The German collection of cryopreserved potato shoot tips was started 1991 during the project 'Refinement of cryopreservation techniques for potato' performed at the German Collection of Microorganisms and Cell Cultures (DSMZ) in cooperation with Gunda Mix-Wagner of the Institute of Crop Science of the Federal Agricultural Research Center (FAL) in Braunschweig, Germany, by Angelika Schäfer-Menuhr. It was founded by the International Plant Genetic Resources Institute (IPGRI, today Bioversity International). During the following five years Angelika Schäfer-Menuhr developed and optimized the DMSO droplet method for potato shoot tips. In 2002, a total collection of about 550 accessions have been stored at the Institute of Crop Science of the Federal Agricultural Research Center (FAL) in Braunschweig, Germany. In 1997, cryopreservation of potato was also started with the same method in the research group *In Vitro* Storage and Cryopreservation at IPK Gatersleben. In the year 2002, both collections were unified at IPK Gatersleben. All samples from Braunschweig and a major part of the IPK collection were rewarmed and regeneration was documented. Problems during the unification were 1) different numbers of explants stored per accessions, 2) accessions with no or insufficient regeneration rate and 3) accessions without

virus test results within the Braunschweig collection. To overcome these problems, all samples were restocked to at least 100 explants per accession. If possible, explants of the respective corresponding accessions, which were tested virus free, were used to fill up the collection. Furthermore, some methodical improvements were introduced, such as solid regeneration medium. The actual number of safely (at least 5 plants can be obtained) cryopreserved accessions is 1119 (July 2009). Therefore, IPK is hosting the largest cryopreserved collection of potato worldwide. In the future, predominantly such accessions will be cryostored, where the frequency of request is low.

### **PRELIMINARY RESULTS OF ANALYSES ON GENETIC STABILITY AND PHYTOCHEMICAL PROFILE OF PLANTLETS REGENERATED FROM CRYOPRESERVED *THYMUS MORODERI* SHOOT TIPS**

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As a final step in the *in vitro* conservation of *Thymus moroderi* Pau ex Martínez (Labiatae), a species endemic to southeastern Spain, genetic stability and phytochemical profiles of plantlets cryopreserved by the vitrification method were checked. The genetic fidelity was assessed by RAPD analysis conducted on six cryopreserved plantlets and comparing the results with those made on *in vitro* grown plantlets. Plant material dehydrated with PVS2 but not cryopreserved was also included as control. A total number of nine primers yielding 10-13 scorable bands per primer were evaluated. The total number of bands was 1339 with a band size ranging from 125 to 2000 bp. The first results showed that storage in liquid nitrogen seemed not to cause genetic changes. For phytochemical comparisons, dichloromethane extracts from micropropagated and cryopreserved plantlets were used, focusing the analysis to the terpene fraction. The study of the composition of the extracts was carried out by gas chromatography/mass spectrometry (GC-MS). The identification of the components was assigned by comparison of their retention indices relative to (C8-C20) *n*-alkanes with those in the literature. Further identification was made by matching the recorded mass spectrum to those stored in the Wiley mass spectra library of the GC-MS data system. The results showed a similar profile between cryopreserved and non-cryopreserved samples although differences regarding the relative abundance of certain compounds were obtained. In all cases, 1,8-cineole was the most abundant component.

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### **PRELIMINARY STUDIES OF THE APPLICABILITY OF CRYOPRESERVATION METHODS IN ANCIENT APPLE VARIETIES IN SWITZERLAND**

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The Horticulture Centre of Zurich University of Applied Sciences (ZHAW) preserves many different ancient plant varieties. This collection contains more than 500 apple, 130 vegetable and 180 grape cultivars and is supported by the National Action Plan (NAP) for Conservation of Ancient and Endangered Species of the Swiss Federal Office of Agriculture. At the moment the collection of apple cultivars is maintained in the field. But the risk of losing parts of the collection by attacks of fire blight, other diseases or unfavourable weather conditions, such as heavy hail showers, is increasing. These facts point up the need for alternative preservation methods to insure the survival of the collection in the long term. Institutions like CPC, FRUCTUS and other owners of big collections (approximately 2000 apple varieties) are beginning to back up their collections as nuclear stocks in the greenhouse, a method which is labour-, and cost-intensive. Cryopreservation as cost-efficient and space-saving method could be an alternative for conserving these collections. Therefore, the aim of this work is to test the usability of cryopreservation, with emphasis on the dormant-bud method, for the maintenance of ZHAW's collection of ancient apple cultivars. In collaboration with M. Höfer of the Julius Kühn Institute in Germany, in a first set of experiments the dormant-bud method for cryopreservation was tested on two varieties. The success rates of bud growth after chip-budding and the dormant-bud method were evaluated. Preliminary results indicate that regrowth rates after chip-budding of cryopreserved buds are not yet on a satisfying level. Further research is needed to improve the chip-budding method and to build up satisfying back up collections.

## **WOODY FERNS GAMETOPHYTE CRYOPRESERVATION OF THREE DIFFERENT TAXA**

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The aim of the presentation is to show the possibility to employ an earlier developed cryopreservation protocol of woody fern *Cyathea australis* to other species belonging to three different taxa. Ferns present unique experimental material due to alteration of haploid and diploid generations. Their haploid generation includes two different propagules: 1) easy to collect single cell - spores and 2) in majority one cell layer composed of mitotically and photosynthetically active cells - gametophytes, which are successfully *in vitro* propagated. Diploid generation sporophytes in very early stage of development only could be the donor of explants for cryopreservation experiments. Due to woody ferns phylogenesis, they are unique experimental material for cryopreservation, since the global areas of their origin did not meet glacial era and desiccation stress.

Experiments have been carried out on vegetatively multiplied gametophytes of the following woody fern species: *Cibotium glaucum*, *Cibotium schedii*, *Cyathea australis*, *Cyathea dealbata*, *Cyathea schan-chin*, *Cyathea smithii*, *Dicksonia fibrosa*. The culture of woody fern gametophytes followed an earlier developed protocol in our laboratory.

In present study, encapsulation-dehydration was employed with a preculture in liquid or agar solidified 1/2 MS medium supplemented with or without ABA at the presence of sucrose concentrations increasing from 0.25 M to 1.0 M. Explants included only vegetative parts of gametophyte body.

Encapsulated and dehydrated tissue of gametophyte explants of *Cyathea schan-chin* and *Dicksonia fibrosa* fully survived the protocol, irrespective of time and type of used subculture. The cells of gametophytes of another five studied species were able to survive only at rates of 60 to 80%. The highest level of survival was achieved in the presence of ABA during two weeks long preculture. All studied species were able to regenerate rich gametophyte cultures in postfreezing time.

In conclusion: of the existing gametophyte culture system and cryopreservation protocol (using encapsulation-dehydration) seems to be promising for seven different species belonging to three various taxa when attempting the cryopreservation of endangered tropical fern species.

### **CRYOPRESERVATION OF *ORTHOSIPHON ARISTATUS* – AN ATTEMPT TO TRANSFER A DROPLET-VITRIFICATION METHOD USING PVS2 FROM MINT TO *ORTHOSIPHON***

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*Orthosiphon aristatus*, a member of the family Labiatae, has poor seed set under central-European conditions. Therefore, vegetative propagation is necessary for germplasm preservation. Cryopreservation is the only efficient long-term storage method for clonal material. Related publications are still limited for labiates, being mainly restricted on *Mentha*. No publication exists so far about *Orthosiphon* species.

The PVS2 droplet-vitrification protocol developed for mint was modified and adopted for *Orthosiphon* shoot tips. Regrowth was 82% in controls compared to 4% after cryopreservation. Thermal analysis of shoot tips was performed using Differential Scanning Calorimetry after different cryoprotectant treatments.

After incubation in loading solution, DSC showed ice crystallisation in all conditions. Onset temperature of melting peaks during warming was -17.8°C independent of incubation time. Glass transitions could not be detected. All loaded shoot tips regrew into normal plants. Incubation of shoot tips in PVS2 for 20 min reduced or eliminated ice formation. Glass transition was recorded between -108°C and -114°C with heat capacities between 0.2 and 0.4 J/W. Highest regrowth (100%) was noted after 60 min loading and 20 min PVS2.

When extending PVS2 incubation to 40 or 60 min, regrowth of normal plants decreased to 60%. Thermal analysis showed that T<sub>g</sub> and heat capacity of glass transitions were similar in all treatment combinations. No crystallisation was recorded during cooling, but recrystallisation and melting during warming phases were sometimes detected. Regrowth of plants after treatment with cryoprotectants but without cooling was optimal after 60 min loading and 20 or 40 min PVS2 treatment.