60 years of plant cryopreservation: from freezing hardy mulberry twigs to establishing reference crop collections for future generations

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In this presentation, 60 years of plant cryopreservation research will be highlighted. It is generally accepted that plant cryopreservation really started in 1956 when Prof. Akira Sakai (Sakai, 1956) reported the survival of cold hardened and prefrozen mulberry twigs that were exposed to liquid nitrogen. He already found out that hardening on the one hand and dehydration on the other were essential for survival. In a next phase, plant in vitro culture were tested for their resistance towards cryopreservation. This posed an extra challenge since it is difficult to cool down freeze such fully hydrated tissues to the temperature of liquid nitrogen with the formation of lethal intracellular ice–crystals. Therefore slow freezing protocols, often in the presence of DMSO were developed. These proved to be very efficient system for non-organized tissues like calli and cell suspensions but organized tissues remained problematic. But it was only with the development of fast freezing protocols, such as droplet freezing, encapsulation dehydration and droplet vitrification that the large scale application of cryopreservation to reference crop germplasm collections became possible.

Currently, over 10,000 accessions starting from in vitro cultures are safely preserved for the long term through cryopreservation. More than 80 % of these belong to 5 crops; potato, cassava, bananas, mulberry and garlic. Other important cryopreservation collections representing thousands of accessions are those of dormant apple buds.


Keywords: Genetic resources, cryopreservation, vitrification
Development of V and D cryo-plate methods as effective protocols for cryobanking

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Two efficient and simple cryopreservation methods using aluminium cryo-plates have been developed. In the V cryo-plate method, dehydration is performed using the vitrification solution PVS2 while in the D cryo-plate method, dehydration is achieved using the air current of the laminar air-flow cabinet or silica gel. Both methods can be adapted to different species after marginal changes in the procedures. Until now, more than 20 papers have been published related to both methods. The main advantages of V and D cryo-plate are the following: Handling of specimens throughout the procedure is very easy and quick because only the cryo-plates are manipulated. The specimens attached on cryo-plates can be efficiently treated with loading solution (LS) and PVS2/air flow. Cooling and warming are performed very easily by immersing the cryo-plates in LN and 1.0 M sucrose solution, respectively, resulting in ultra-rapid cooling and warming rates. High regeneration can be obtained using both methods. For species, which are sensitive to PVS2, the D cryo-plate method can be used. Both methods include preparation of material to be cryopreserved, preconditioning, excision, preculture, mounting of explants on cryo-plates, osmoprotection, dehydration by PVS or air flow, liquid nitrogen storage, rewarming and regeneration. Both protocols appear promising for cryopreservation of both herbaceous and woody plants including tropical plants after marginal modifications of the procedures. Optimization of the dehydration time, preconditioning of materials and post-cryopreservation regrowth conditions are crucial to achieve high regrowth. These new cryopreservation methods will facilitate the efficient implementation of cryo-storage and long-term maintenance of plant genetic resources in genebanks.

Keywords: cryo-plate, cryobank, V cryo-plate, D cryo-plate.
1.3
Seed thermal analysis - applications in cryopreservation and ecology

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Physical transformations in the extracellular and intracellular space contribute to stress and survival of plant cells in cold environments; and controlling the extent and location of such changes contributes to successful cryobanking (conventional and cryopreservation). Over the last few decades research in seed thermal analysis has developed into a powerful tool for understanding the importance of water (high moisture freezing limit, the glassy state) and lipid (crystallisation, melting) transformations to the storage stability of seeds, and other tissues, and survival in nature.

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Keywords: differential scanning calorimetry, dynamic mechanical analysis, water freezing, lipid crystallization, seed longevity, cryopreservation
Oaks (genus *Quercus*) are dominant and iconic trees in most European, American, Asian and North African forests, from cool temperate to tropical environments. There are at least 600 species of *Quercus* globally with high ecological and economical importance. However, 45% of the species evaluated by IUCN are considered threatened, being affected by habitat destruction or diseases and pests, such as acute oak decline or chronic oak dieback in the UK. As a result, conservation of oaks is becoming increasingly important for many countries. Oak’s seeds are desiccation sensitive (i.e. recalcitrant), so conventional seed bank strategies are not suitable for the long-term conservation of these species. Tissue culture is a useful tool for *Quercus* species conservation, but cryopreservation is identified as the most favourable and cost-effective option for their long-term conservation. Numerous tissues could be used as germplasm to preserve the genetic diversity of the different species, including winter dormant buds, shoot tips, plumules, embryogenic calli, somatic embryos and embryonic axes. Pollen cryopreservation could also be used as a complementary technology to support conservation and breeding programmes. Interestingly, whilst the seeds of oak are recalcitrant, it seems that the pollen has much greater desiccation tolerance (from the same genome). Seed embryonic axes (with shoot and root meristems) are the preferred explants for oak ex situ conservation, as they can capture high genetic diversity and be grown into full plants with relatively simple micropropagation procedures. However, their cryopreservation can be limited by many challenges, including physical damage to the embryo during excision, oxidative stress associated to excision and cryopreservation procedures, differential response of shoot and root meristems (within a species and among species) to the stresses imposed by partial desiccation and cooling, and the need to improve *in vitro* growth and acclimation procedures. Current cryobiotechnological options and successes for diverse species of *Quercus* are reviewed in this paper and suggestions are made for future studies on these species.

Keywords: cryopreservation, in vitro culture, seed, pollen, shoot tips, embryos, dormant buds, conservation
Cryopreservation of tropical recalcitrant-seeded species - Perspectives

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Cryopreservation is an alternative strategy for long-term conservation of tropical recalcitrant-seeded species. Embryonic axes/embryos or shoot tips are possible explants for this purpose as the seeds are sensitive to cold temperature and they are not tolerant to desiccation to a low moisture content required for seed banking. Shoot tips are used for species with large axes or those without clear differentiated embryonic axes in their seeds. Though not strictly, desiccation and vitrification are usually used for embryonic axes and shoot tips, respectively. Several species under study provide examples, including the *Garcinia* species. Cryopreservation of shoot tips of tropical recalcitrant species faces many challenges. Amongst these are the availability of experimental materials throughout the year; osmotic and chemical (toxicity to chemicals used) stresses and lack of tolerance to ultra-low temperature. With a systematic approach in addressing each problem/challenge, a successful cryopreservation protocol could be obtained. Ultrastructural changes observed during each step of the protocol give useful information in understanding success or failures of the cryopreservation technique used.

Keywords: tropical recalcitrant-seeded species, challenges, ultrastructural changes

Improvement of regeneration after cryopreservation of vegetatively propagated plants determined by differential thermal analysis

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Three critical key parameters for cryoprotocol improvement resulting in increasing regeneration rate after cryopreservation can be determined by thermal analysis. (1) The volume of frozen water. The boundary volume of frozen water affected by dehydration over desiccants or mixture of cryoprotectants can be determined by the differential scanning calorimeter. For example, the cryopreservation of garlic shoot tips by different concentrations of Plant Vitrification Solution No 3 (PVS3) for dehydration. Particular concentration of cryoprotectants can regulate the
final frozen water volume. Although the regeneration of garlic shoot tips after their exposure to PVS3 of lower concentration of sucrose/glycerol components 45/45 was similar to PVS3 of higher concentration 50/50. The use of less concentrated PVS3 results in less possible dehydration damage before ultra-low temperature application. (2) The volume of unfreezable water. The intersection of the volume of frozen water and the absolute volume of water determined gravimetrically at differently dehydrated shoot tips can be derived as volume of unfreezable water. The unfreezable water volume can be the limit of the minimum water volume below which the shoot tips cannot be dehydrated. (3) Glass transition temperature. Determination of glass transition temperature is important for long-term cryopreservation without undesirable alterations. A pure cryoprotectant usually added in an excessive amount has a constant Tg; whereas, the Tg of shoot tips increases with decreasing volume of water taken up by the cryoprotective solution. The highest recovery value after cryopreservation treatment should be achieved at the obtained highest sample glass transition temperature. The three above mentioned parameters define the important threshold dehydration of shoot tips for high regeneration rate after cryopreservation.

Keywords: Differential scanning calorimeter, frozen water volume, unfreezable water volume, glass transition temperature, plant vitrification solution no.3

O_4

Differential expression protein `MDH and CAT` increase the germination of cryopreserved Paeonia pollen by regulating the ROS and apoptosis event

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Previous work in our group has shown that malic dehydrogenase (MDH) and catalase (CAT) expressed differentially in peony cryopreservation. To study the relationships between differential expression proteins, reactive oxygen species (ROS) and apoptosis in pollen cryopreservation, we determined changes in pollen germination, ROS level, caspase-3-like activity and apoptosis rate during pollen cryopreservation of Paeonia lactiflora ‘Hong Pan Tuo Jin’ and ‘Fen Yu Nu’ added with or without MDH or CAT. Pollen from ‘Hong Pan Tuo Jin’ showed a great loss of viability, but marked increase of ROS level and caspase-3-like activity after cryopreservation. Caspase-3-like / protein and apoptosis rate in them remained unchanged. MDH
addition improved the post-LN pollen viability significantly, decreased the ROS level, caspase-3-like activity and caspase-3-like / protein, but had no effect on apoptosis rate. Pollen from ‘Fen Yu Nu’ showed marked increase in ROS level and apoptosis rate, unchanging in pollen viability, caspase-3-like activity and caspase-3-like / protein. CAT addition significantly increased the post-LN pollen viability, greatly decreased the apoptosis rate. ROS level in them also declined, but caspase-3-like activity and caspase-3-like / protein didn’t change. In conclusion, MDH and CAT may increase the post-LN pollen germination by regulating the ROS and apoptosis event, but through different manners. By reducing ROS and caspase-3-like activity, MDH made apoptosis in a stable state. CAT may increase the cryopreservation pollen germination by directly reducing apoptosis rate and ROS.

Keywords: pollen cryopreservation, malic dehydrogenase, catalase, apoptosis, ROS

O_5
Expression analysis of Trx and Grx genes in Dendrobium nobile protocorm-like body response to cryopreservation

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Reactive oxygen species (ROS)-induced oxidative stress is a vital factor that reduces cell viability during cryopreservation. Thioredoxin (Trx) and glutaredoxin (Grx) participate in ROS scavenging and manage redox homeostasis in cells. Our previous proteomic study found proteins Trx M1 and monothiol-Grx (M-Grx) in protocorm-like bodies (PLBs) of Dendrobium noile Lindl. ‘Hamana Lake Dream’ were differentially expressed during cryopreservation. Therefore, this study was to clarify the molecular regulation of Trx M1 and M-Grx, and their relationship with oxidative stress during cryopreservation, the mRNA expression of Trx M1and M-Grx in Dendrobium noile PLBs was analyzed using real-time quantitative PCR method in both the control vitrification procedure and an improved vitrification procedure by adding ascorbic acid (AsA) in unloading solution. The results showed that both of genes Trx M1 and M-Grx were up-regulated after loading, PVS2-treated and unloading, however significantly down-regulated after preculture and cooling-rewarming. Notably, our previous study also found the obvious decrease of survival, along with the occurrence of oxidative damage in PLBs after preculture and cooling-
rewarming, which was consistent with the change of \textit{Trx M1} and \textit{M-Grx} gene expression. The addition of AsA significantly increased the survival and \textit{M-Grx} expression in cryopreserved PLBs. In conclusion, genes \textit{Trx M1} and \textit{M-Grx} play important roles in \textit{Dendrobium nobile} PLB tolerance to ROS-induced oxidative stress during cryopreservation. Adding AsA in unloading solution might relieve oxidative damage by enhancing \textit{M-Grx} gene expression.

Keywords: Dendrobium; vitrification; thioredoxin; glutaredoxin; gene expression; oxidative damage

\textbf{O_6}
\textbf{Comparing mRNA expression levels to protein abundance of \textit{Magnolia denudata} pollen following cryopreservation}

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Although genetic properties of a cell are determined by gene, genetic information is ultimately embodied by function of protein. It has been known that there are four levels of regulation from gene to protein: transcriptional control, post-transcriptional control, translational control and post-translational control. In our previous study, eight differentially expressed proteins from \textit{Magnolia denudata} pollen cryopreservation have been identified. In order to further complete the molecular mechanism of cryopreservation, this study compared mRNA expression levels to protein abundance of \textit{enoyl-ACP reductase} and \textit{putative dehydrogenase} in \textit{M. denudata} pollen at pre-, during- and post- liquid nitrogen (LN) stages of cryopreservation, using quantitative real-time polymerase chain reaction. The results indicate that mRNA expression levels of 2 genes were similar following cryopreservation, with during- and post-LN showing almost equal expression level, but decreasing by half compared to pre-LN. These changes were both inconsistent with the protein abundance changes of these 2 genes, which suggest that the changes at protein abundance probably come from translational control or
post-translational control, rather than transcriptional control or post-transcriptional control. It was revealed how the pollen cell response to cryopreservation to some extent.

Keywords: cryopreservation, pollen, transcription and translation, mRNA, protein

O_7
KRYOBEG-Elimination of endophytes and cryopreservation of begonia

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Begonia is an economically important perennial bedding plant belonging to the family Begoniaceae. Endophytes in the tissue culture give contamination and cause severe problems for commercial tissue culture production worldwide. The prerequisite for a constant production and free exchange is to ensure that plant materials are free of endophytes and plant pathogens. Cryopreservation of begonias will provide major savings and reduce risks for the complete breeding program, and secure the breeding lines by giving a safety backup and minimizing space, keep the material free of diseases and pests, and maintain genetic stability of the selections. Diagnosis and elimination of endophytes from begonia tissue cultures, and cryopreservation of valuable begonia new breeding cultivars are the main goals of this study.

Thirty-two begonia in vitro cultures have been screened for bacteria with 16S sequencing: 18 cultivars were tested to be infected with at least one bacteria, including Curtobacterium flaccumfaciens, Agrobacterium radiobacter, Cellulomonas flavigena and etc., all together 10 bacteria genera were found. Effect of different antibiotics to eradicate bacterial infection has been tested and four antibiotics (chloramphenicol, Rifampicin, Cefotaxime and Streptomycin Sulfate salt) have been selected, in which Rifampicin (200mg/L) showed the most effective to eliminate
bacteria from begonia in vitro cultures. Droplet-vitrification procedures with both PVS2 and PVS3 were applied for cryopreservation of in vitro cultured begonia cv. Sky White and cv. Sun Red. The highest regrowth rate (40%) were achieved using droplet-vitrification with PVS3 for 90min.

Keywords: cryopreservation, begonia, endophytes, antibiotics
**Session II: Cryobanking**

**O_8**

**National genebank of Thailand at its dawn**

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Genebank facilitating storing and caring of plant genetic resources has been established for the first time in Thailand in 1984. This “National Genebank of Thailand (NGT)” was constructed in the compound of the Thailand Institute of Science and Technological Research (TISTR) in Bangkok with funding supports of the International Board for Plant Genetic Resources (IBPGR); the latter had worked in accordance to the Food and Agriculture Organization (FAO) of the United Nations (UN) and subsequently reorganized and changed their operating name to Bioversity International. The NGT housed five parts, i.e. cold rooms (medium term storage at 0 to 10°C and long term storage at -20 to -10°C), a preparatory room (20°C), a seed lab, a processing room and a control room. Main aims of the NGT at the beginning emphasized on collecting and storing crop germplasm in the form of seeds to serve as resources for national and international breeders. Moreover, full services of documentation, exhibition, exchange and distribution of seeds and information were planned. Projects were done on storing of edible legumes including winged bean, pigeon pea, yardlong bean, glutinous maize, popcorn, Job’s tear, *Momordica*, *Solanum*, *Amaranthus* and indigenous vegetables. Due to lack of funding and personnel, the former NGT was unfortunately demised. It is still hopeful, however, that the NGT, though requiring large amount of budgets, knowledge management, workforces, and proper long-term administration, could one day be revived. As other germplasm collections elsewhere in the world, the national seed bank is the promise for our food security assurance and sustainable future.

Keywords: plant genetic resource conservation, plant germplasm bank, plant germplasm preservation, seed bank, seed vault
Towards securing diversity of clonally propagated crops: results and recommendations of the feasibility study for a safety back-up cryopreservation facility

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Crop diversity is critical to global food security and sustainable agriculture. Genetic diversity of most staple crops can be conserved ex situ through their seed. Many countries have equipment and trained personnel to maintain seed collections; and Svalbard Global Seed Vault provides a secure back-up for crops with orthodox seeds. This level of security does not exist for clonally propagated crops, such as banana, sweet potato, cassava, yams, and others, although their global annual production exceeds one billion tonnes and millions of people depend on them for food, nutrition and livelihoods. Securing diversity of these crops at -196°C in liquid nitrogen offers a solution. We present the results of the study commissioned by Bioversity International, International Potato Center and Global Crop Diversity Trust with support of governments of Australia, Germany and Switzerland to investigate the status of cryobanking of clonally propagated crops and access the feasibility of establishing a safety back-up cryo-facility for their long-term conservation. The Study conducted by the group of independent experts is based on data received from 26 world institutions holding or ready-to-hold cryopreserved crop collections. It also collates information on clonal crops maintained in field and in vitro genebanks around the world. The Study recommends a collaborative effort among researchers and genebanks to facilitate wide-scale implementation of cryopreservation to >100,000 unique accessions of Annex 1 and Article 15 clonal crops currently held in costly and vulnerable field and in vitro genebanks. It also
recommends ensuring safety back-up of 5,000-10,000 accessions arising from current cryopreservation activities.

Keywords: Cryopreservation, clonal crops, backup, genetic diversity

**O_10**

Developing coconut cryopreservation protocol and establishing cryo-genebank at RDA with the Bioversity International

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Coconut (*Cocos nucifera* L.) is one of the most important palm crops in the world. Coconut palms produce highly recalcitrant seeds that are big, sensitive to desiccation and show no dormancy. Maintenance of coconut collection in the field genebank is risky since it is prone to exposure to unknown climatic factors and diseases. Cryopreservation of plant tissue in liquid nitrogen (-196°C) is currently the only method allowing safe and cost effective long-term conservation of recalcitrant seed species, such as coconut. The project between RDA of Korea and Bioversity International aims at developing and validating robust cryopreservation protocols, starting with coconut and then exploring the potential for cryopreserving other priority species. In the framework of this project, three LOAs were finalised, related to establishing cryopreservation protocols for coconut genetic resources; (i) The Suncheon National University (SNU, Korea) is responsible for developing cryopreservation protocol of intact zygotic embryos, (ii) The University Leuven (KU Leuven, Belgium) will develop cryopreservation protocol for plumules and meristems, (iii) the Philippine coconut authority (PCA, the Philippines) is supplying on a regular basis KU Leuven and Suncheon University with good quality zygotic embryos. In the collaborative project between SNU and the PCA, the two protocols were proposed as a candidate for routine implementation of cryobanking for coconut collections; preculture-desiccation of intact embryos
and vitrification of plumular cube. At KU Leuven, work is concentrated on the establishment of proliferating shoot cultures of coconuts. From these, meristems will be excised to be cryopreserved through droplet-vitrification. We present an overview of existing cryopreservation work as preliminary results.

Keywords: coconut, cryopreservation, protocol, zygotic embryo

**O_11**

**Implementation of citrus shoot tip cryopreservation in the USDA-ARS National Plant Germplasm System**

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The USDA-ARS National Plant Germplasm System (NPGS) maintains 540 *Citrus* cultivars and crop wild relatives as duplicate clones in a screenhouse at the National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) in Riverside, California. These 540 accessions are pathogen-tested and apparently free of all known graft-transmissible pathogens. An additional 815 citrus and citrus relative accessions are maintained by the NCGRCD and the University of California, Riverside, in greenhouses until sanitation for pathogens can be
completed. Between 2012 and 2017, a total of 451 of the pathogen-tested citrus accessions were cryoprocessed at the NCGRCD and the National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, Colorado. Vegetative budwood was collected and sent to NLGRP. A minimum of 170 shoot tips from each accession were excised from surface-sterilized budwood, which were then cryopreserved using a PVS2 droplet-vitrification technique. Viability was assessed by micrografting 10 liquid nitrogen-exposed shoot tips onto in vitro grown ‘Carrizo’ seedling rootstock. The remaining shoot tips were placed into long-term liquid nitrogen storage. Of the 451 Citrus and crop wild relative accessions cryoprocessed, 354 accessions had regrowth levels of 40% or greater, 17 had 0% regrowth, 47 had viability levels between 10 and 30%, and 33 had endogenous contaminants. This large-scale effort has revealed that shoot tip cryopreservation can be successfully scaled-up to secure the NPGS Citrus collection.

Keywords: Citrus, cryopreservation, shoot tips, vitrification, implementation, micrografting

O_12
Cryobanking of garlic (Allium sativum L.) germplasm at ICAR-NBPGR in India: Achievements, Constraints and Prospects

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Allium sativum L. (garlic) represents one of the most widely cultivated species from amongst 35-40 species of Allium occurring in India. A crop of wide commercial importance, garlic owes its importance to its culinary and medicinal properties. Being a major foreign exchange earner for India, garlic is an integral constituent of large number of recipes and preparations in India.

The National Bureau of Plant Genetic Resources (ICAR-NBPGR), has been leading the Allium genetic resources conservation programme in India since over last three decades in the form of seed, field repositories and as in vitro collections. Maintenance of garlic collections in the field is labour intensive, requires annual regeneration and suffer from viral infestation; whereas, in vitro conservation is fraught with the problem of bacterial contamination. Reports exist for successful cryopreservation of garlic employing new vitrification-based protocols, there is none for Indian genotypes. Shoot tips excised from cloves of more than 100 accessions were cryopreserved using vitrification (V) or droplet-vitrification (DV) technique. Both PVS2 and
PVS3 were used as cryoprotectants with latter proving to be more effective as a vitrification solution for garlic shoot tips. Although explants survived PVS2 dehydration, shoots were healthier with PVS3. Exposure of explants to low temperature (10°C) and high sucrose (10%) was beneficial for successful cryopreservation of garlic. Depending on the genotype, post-thaw survival of shoot tips varied from 10-100% with shoot regrowth varying from 0-90%. Cryobanking has been initiated in more than 60 accessions. Present findings will focus on achievements and constraints with respect to cryopreservation of Indian garlic genotypes at ICAR-NBPGR in India.

Keywords: garlic, Allium sativum, cryopreservation, vitrification, PVS2, PVS3

O_13
IITA clonal crop cryopreservation started: result of many years and multi-aspect setting up

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Ex situ long-term conservation of crop diversity is critical for global food security and sustainable agriculture, especially in this era of global warming and climate changes. Clonally propagated crops (i.e. non-orthodox and/or recalcitrant seeds), such as cassava and yam, are mainly conserved as living plants in field genebanks or as tissue cultures in vitro slow growth genebanks. These conservation systems do not ensure long-term preservation due to several limitations. Cryopreservation, which refers to the maintenance of plant tissue, vegetative parts or embryos at ultra-low temperature (e.g. -196 °C in liquid nitrogen) offers a reliable and ultimately most cost-effective solution for long-term conservation of clonally propagated crops.

IITA holds international collections of clonally propagated crops, such as cassava (Manihot esculenta Crantz), yam (Dioscorea spp.), banana and plantain (Musa spp.), maintained on field and vitro in medium-term storage genebanks. To ensure long-term conservation for those important collections of around 11,000 accessions, cryopreservation was emphasized on. However, cryopreservation entails many prerequisites including setting a suitable conservation strategy, well-trained staff, quality/risk management system and most importantly facilities, consumables and equipment. IITA cryobank setting entails an in-house fit-on-purpose LN generator and cryo-storage tanks to support the adapted procedure. This later is backed up with by
a main tank for the long-term conservation, another one for the safety duplication and a last one for the temporary conservation and viability validation of any accessions to be cryopreserved. The implementation of IITA cryobanking have been started with cassava. Any accession to be cryopreserved will first be tested clean (from virus and endophytes), amenable to cryo-method and most importantly confirmed unique and true-to-type as quality insurance for sustainable and trustworthy system. This latter validation is done using SNP markers, generated from DArT sequencing, for fingerprinting. Considering all these criteria, the IITA cryobank is aiming to have in the cryobank up to 1,000 cassava and 500 yam accessions in 2022.

Keywords: Cryobank, clonal crops, Plant genetic resources, Quality management system

O_14
Cryopreservation of in vitro shoot tips of cassava using cryo-plate methods

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Cassava (Manihot esculenta Crantz) is one of the most important tuber crop in the tropics and simple reliable cryopreservation method is indispensable for stable long-term storage of their genetic resources. Then, we tried to apply the cryopreservation methods using aluminum cryo-plates to in vitro-grown cassava shoot tips. In vitro shoots of a cassava line ‘M VEN 297-A’ were used for optimization of cryo-plate procedure. At first, dehydration time was tested for both V and D cryo-plate methods. Both same 45 min of dehydration by PVS2 for V cryo-plate method and by air desiccation in Petri dish containing 35 g silica gel for D cryo-plate method gave the best
regrowth after liquid nitrogen (LN) storage. Because the growth after cryopreservation was more vigorous by air desiccation, duration after last subculture, preculture conditions and sucrose concentration of loading solution (LS) for D cryo-plate method were tested. The tentative optimal procedure is as follows. Shoot tips were dissected from the shoots 4 weeks after the last subculture and precultured 1 d on solidified MS medium containing 0.3 M sucrose. They were attached on cryo-plates with circular wells in 1.5 mm diameter with alginate gel, osmoprotected for 30 min in LS with 0.8 M sucrose and dehydrated by silica gel for 45 min. All treatments were done at 25°C. Then, the cryo-plates were stored in LN and rewarmed by 1 M sucrose solution. Using this procedure, the regrowth rate reached 76.7%. This procedure will contribute to facilitate the cryostorage of cassava germplasms in genebanks.

Keywords: cassava, cryo-plate, cryopreservation, D cryo-plate method

O_15
The development of a droplet-vitrification method to conserve Vitis collections in the USDA-ARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil

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Both the United States and Brazil maintain vast collections of grape genetic resources. We share a common interest in using cryopreservation methods for the secure, long-term back-up of accessions within these field collections of the USDA-ARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil. We have developed a Vitis droplet-vitrification method that results in high levels of shoot tip regrowth after liquid nitrogen (LN) exposure. Uniform shoot tips were obtained from nodal sections of either in vitro or growth chamber stock plants. Pretreatments included 0.3 M sucrose, salicylic acid, ascorbic acid and glutathione. Half-strength PVS2 was applied for 30 min at 25°C, prior to full-strength PVS2 treatment at 0°C. The optimum PVS2 exposure duration was 30 min for growth chamber-derived
shoot tips and 90 min for in vitro derived shoot tips. Shoot tips were then placed onto foil strips and plunged into LN. Regrowth levels ranged from 45 to 72 % for the seven *Vitis* species tested from in vitro plants, and from 35 to 65 % for three *Vitis vinifera* cultivars and one *Vitis* hybrid tested from growth chamber plants. The high levels of regrowth suggest that *Vitis* cryopreservation may soon be ready for implementation within genebanks.

Keywords: *Vitis*, plant vitrification, genetic resources, cryopreservation, shoot tips

**O_16**

**In vitro cryopreservation of medicinally important RET plants of India: Success and limitations**

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Indian Gene Centre is immensely rich in diversity of medicinal plants, which are still to a large extent gathered from the wild. The unprecedented loss of this valuable germplasm due mainly ruthless and unscientific/destructive collection of plants has resulted in many plants becoming threatened with extinction. According to the IUCN designed Conservation Assessment and Management Plan (CAMP) methodology, about 112 species from southern India, 74 species from Northern and Central India and 42 species from the Himalayas are threatened in the wild. Current rates of species extinction have made conservation of threatened plants, an urgent activity. Significant advances have been made in the in vitro propagation and medium-term conservation of ~25 RET medicinal plants at the In Vitro Genebank at ICAR-NBPGR, India. Presumably the only available option for long term conservation, cryopreservation was attempted in five threatened genera *Bacopa monnieri*, *Dioscorea deltoidea*, *Gentiana kurroo*, *Picrorhiza kurroa* and *Rauvolfia serpentina*, using various vitrification based techniques (vitrification, encapsulation-dehydration and droplet freezing). Depending on species and accession, varying degree of post-thaw success (0-60% survival and regrowth) have been achieved using shoot tip explants. In *B. monnieri* after cryopreservation, based on random amplified polymorphic DNA (RAPD) and bacoside A analyses, the regenerated plants exhibited morphological, molecular and biochemical genetic stability. The protocol, once standardized, will have the potential application in cryobanking the germplasm to ensure material availability for pharmaceutical purposes for future. The paper mainly outlines strategies adopted, success achieved and limitations experienced. Impact of various parameters on successful cryopreservation will be the focus of discussion.

Keywords: rare, endangered, threatened, medicinal plants, India, cryopreservation
Survey of literature on in vitro conservation of plants shows that successful cryopreservation has been developed for many economically important crops using mostly droplet-vitrification and V-cryo-plate methods. For a few crops that were “left behind”, the process of optimization of culture conditions is in progress, and for those species which regenerate, cryopreservation has been attempted although with limited success. Yet, the sensitivity or tolerance of many wild species to cryopreservation assessed using existing methods remains unknown, and the conditions successfully used for their cultivated relatives may not work with the wild germplasm. We present a systematic approach that helps to i) quickly assess the osmotic and chemical tolerance of the new plant material and ii) achieve relatively high levels of plant regeneration after cryopreservation using very limited number of treatments and explants irrespective of species or the origin of material. This systematic approach implies variation of preculture, loading, and dehydration steps based on the material sensitivity to osmotic and chemical stress. It was first tested for cryopreservation of cultivated plants (allium, garlic and potato) at the National Agrobiodiversity Center, RDA, Republic of Korea, and later adapted for isolated root cultures, somatic embryos and shoot tips of medicinal and wild species, such as *Betula lenta*, *Hypericum perforatum*, *Panax ginseng*, *Castilleja levisecta* and several others at the University of Guelph, Canada, with 60-100% post-cryopreservation regrowth. The Differential Scanning Calorimetry analysis confirmed the effectiveness of the newly developed protocols. For some species, plants developed from the cryopreserved shoot tips were acclimated ex situ and reintroduced in their natural habitats. It is likely that this approach may lead to effective conservation of many wild and cultivated plant species.

Keywords: Cryobank, droplet-vitrification, protocol development, systematic approach
Session III: Methods of Plant Cryopreservation

O_18
Optimization of cryopreservation protocols for zygotic embryos of *Citrus reticulata* ‘Mandarin’

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*Citrus* seeds are known for their short life span in a conventional seed bank environment. They show a typical intermediate seed storage behaviour where they survive desiccation to a considerably low level ca. 7-10%. However, their longevity is evidently compromised at this low moisture content at sub-zero temperatures. Cryopreservation of zygotic embryos is a potential option for long-term storage of this species: we investigated its feasibility for *Citrus reticulata* ‘Mandarin’ in this study. The experimental strategies evaluated included seed and embryo desiccation sensitivity assessment, naked embryo cryopreservation, encapsulation-dehydration cryopreservation and cryopreservation of embryos following PVS2 vitrification technique. Differential Scanning Calorimetry was used at every desiccation step to determine the critical water content for cryopreservation. Desiccated and cryopreserved naked embryos with a moisture content of 7% (fresh weight basis) showed the highest regrowth (60%). Maximum regrowth for encapsulated embryos with a moisture content of 24–27% was around 30–40%. When a vitrification technique was used, the highest regrowth (60%) was recorded following 120 minutes of PVS2 treatment. Although vitrification and naked embryo cryopreservation had similar regrowth percentages, the seedlings obtained from naked embryo cryopreservation appeared more vigorous, probably due to the lack of exposure to cryo-protectant, a known plant stressor, compared to PVS2-exposed embryos. From our results, we concluded that naked embryo desiccation was the best technique for cryopreservation of *C. reticulata* zygotic embryos.

Keywords: seed storage, intermediate seed, desiccation, encapsulation-dehydration, vitrification, differential scanning calorimetry

O_19
Current perspectives on pollen cryopreservation in horticultural species

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Pollen with viability retained through cryopreservation is very important input for pollination, breeding, biodiversity conservation, biotechnology research in general for conservation and other biological and non-biological studies. In general, cryopreserved pollen collections provide long-term security for wild flora, ornamentals, fruit and vegetable crops, medicinal herbs and endangered species. Technology has been developed for long-term cryopreservation of Nuclear Genetic diversity through long-term cryopreserved pollen in the form of nuclear genetic diversity (NGD) of mango, amla, pomegranate, jackfruit, guava, passionfruit, jamun (different species and varieties citrus, papaya (different species), grape, tomato, eggplant (*Solanum* gene pool), onion, capsicum, rose, gladiolus, gerbera, carnation and RET species of medicinal plants are maintained in liquid nitrogen. Pollen collecting is usually made on a bright sunny day between 10-11 AM. Pollen collections are subjected to viability indexing by germination in vitro by the hanging drop or cellophane techniques. Pollen samples are packed either in gelatin capsules or butter paper packets, sealed airtight in polyethylene aluminium laminated pouches and lowered into a canister of a cryoflask. The fertility of stored pollen is tested under field conditions through controlled pollination. The pollen cryobank is managed by periodic replenishment of the cryogen, for maintaining a constant cryogenic temperature throughout the storage duration. For pollen cryopreservation, there are many promising applications which has come to focus on the recent advances in allied bio-scientific areas. Pollen cryopreservation protocols developed for different horticultural species will be discussed in detail.

Keywords: pollen cryopreservation, pollen cryobank, horticultural species protocols

**O_20**

**Cryopreservation and cryotherapy research in horticultural crops in New Zealand**

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The New Zealand Institute for Plant & Food Research Limited holds tens of thousands of accessions from over 100 cultivated plant species and their wild relatives. Many of these accessions are clonally propagated and held in the field, exposed to the vagaries of climate and biotic threats. The incursion of a highly virulent strain of *Pseudomonas syringae* pv. *actinidiae* in 2010 reduced the genetic diversity of kiwifruit, one of the most commercially important horticultural crops in New Zealand. This accelerated research into implementation of cryopreservation technologies. We have successfully developed protocols for cryopreservation of grapevine, gentian, potato, raspberry, apple, kiwifruit and dragon fruit (Pitaya). Conservation of our potato germplasm collection (more than 700 accessions) using droplet vitrification is under way. Prospects for conserving kiwifruit germplasm using droplet vitrification are also good. Dormant apple bud cryopreservation experiments resulted in at least 50% of genotypes having >40% viability after cryopreservation. In addition to long-term storage of crop germplasm, cryo-technologies are also being used to eradicate pathogenic microorganisms from infected clonal material. Using droplet vitrification, we have eradicated *Cucumber mosaic virus* from gentian, three leafroll viruses from four clones of grape, and two species of *Pseudomonas* bacteria from kiwifruit. Although cryotherapy alone was not sufficient to eradicate *Raspberry bushy dwarf virus* from infected raspberry, a combination of chemotherapy, thermotherapy and cryotherapy was successful, with all regenerated plants testing negative for the virus by qRT-PCR assay. Method development and its application to conserve high-health clonal germplasm will be discussed.

Keywords: Droplet vitrification, Virus eradication, Pseudomonas, Potato, Kiwifruit, Raspberry, Grapevine, Gentian, Apple, Pitaya

**O_21**

**Effect of immersion time in PVS2 and pericarp removal on cryopreservation of papaya (*Carica papaya* L. ‘Sukma’) seeds**

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Cryopreservation of papaya seeds is very important because some of the papaya seeds are
intermediate seed type which cannot be stored for long periods because this process can reduce seed viability. Cryopreservation is a long-term conservation method of germplasm using liquid nitrogen. This study aimed to compare the viability of seeds after being cryopreserved with and without pericarp and difference Plant Vitrification Solution 2 (PVS2) immersion times. This research had been conducted at the Seed Storage Laboratory of Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, from August to December, 2015. The experiment was designed as Factorial in Completely Randomized Design with two factors. The first factor was seed condition either with pericarp or without pericarp. The second factor was the immersion duration in PVS2 with 5 incubation times, i.e. 0, 15, 30, 45, and 60 minutes. The results showed that seeds without pericarp gave the best germination rate and the best immersion duration in cryoprotectans were at 30 minutes, with the results of the germination rate, growth rate and growth maximum potential were 48.0%, 2.9%, 48.0 %, respectively.

Keywords: Vitrification solution, germination rate, growth rate, cryoprotectant, liquid nitrogen, intermediate seed type.

O_22
Just a spoonful of sugar' myth or fiction in rewarming solution (RS)?

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Cryopreservation protocols have been successfully developed for hundreds of species and thousands of genotypes in laboratories around the world. In many of them, the thawing process is performed in Rewarming Solution (RS) with a high concentration of sucrose (0.8-1.2M). As a baseline, 50 publications were reviewed and classified by RS sucrose concentration, species and regrowth rate. The need for high sucrose concentrations in the RS post-thawing regrowth rate (+LN) was assessed in the range of 0.0M, 0.3M, 0.6M, 0.9M and 1.2M with a set of 15 potato landraces, cryopreserved with a PVS2-droplet vitrification method. The results showed no significant difference for the average regrowth rate (81.1-86.8%) between sucrose concentrations from 0.3M to 1.2M. However, complete removal of sucrose (0.0M) from RS resulted in a significantly lower average regrowth rate of 66.5%, yet the assessed accessions still showed good regrowth, ranging from 46.7-86.7% regrowth of complete plantlets. This led to the assumption that potato shoot tips are relatively robust with fast rehydration during thawing, causing minor damages to the cells’ water channel system. Fourteen of 15 accessions showed its
highest regrowth rate with sucrose concentrations of 0.3-0.9M. The results are currently being screened at the International Potato Center (CIP) on a group of ~ 100 diverse accessions to determine if RS sucrose concentration can be reduced for large-scale routine cryopreservation.

Keywords: Potato, cryopreservation, RS, regrowth, sucrose

O_23
The role of hydrogen peroxide in cryopreservation of cockscomb (Celosia plumosa) seedlings

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Recently, many research showed that oxidative stress is a potential cause of damage in plant cells during cryopreservation, and H₂O₂ may be the leading ROS components. However, H₂O₂ is not only being a toxic by product causing oxidative damage to membrane lipids, protein and DNA at high concentrations, but also involving in signal transduction pathways leading to activate the plant defense against biotic and abiotic stresses at low concentrations. The present study investigated the effects of H₂O₂ in cryopreservation by adding H₂O₂ or CAT in each step of cryopreservation, detected the dynamic changes in antioxidant systems during cryopreservation of cockscomb seedlings.

The results showed that: (1) The process of cryopreservation resulted in the rise of H₂O₂, and it reached the peak after unloading. (2) Addition either low concentration of H₂O₂ or CAT can improve the regeneration rate of cryopreserved cockscomb seedlings, while the addition stage were different. Adding 2 mmol/L H₂O₂ in loading stage got 73.33 ± 3.52% regeneration rate, and adding 400 u/mL CAT during the unloading process result in 76.67 ± 4.17% regeneration rate. (3) The addition of H₂O₂ in loading phase increased the activity of CAT in the seedlings, resulting a sharp drop of H₂O₂ content in PVS2 stage and the continuous decline of MDA content after frozen. Therefore, we concluded that adding low concentration of H₂O₂ at early cryopreservation steps improved cockscomb seedlings cryo-tolerance by enhancing the antioxidant capacity, which prevented ROS accumulation.

Keywords: cryopreservation, hydrogen peroxide, antioxidant defense system, Celosia
Cryopreservation and micropropagation of banana (*Musa paradisiaca* ‘Robusta’) with reference to droplet-vitrification and cytokinin application

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Shoot tips of banana (*Musa paradisiaca* ‘Robusta’) taken for effective micropropagation were cryopreserved through droplet-vitrification using PVS2 solution. Sucrose-precultured shoot tips were isolated and kept for 20 min in a filter-sterilized loading solution containing 2M glycerol and 0.4 M sucrose dissolved in MS medium at room temperature. Later, the loading solution was replaced by ice-cooled and filter-sterilized PVS2 solution consisting of 30% (3.26M) glycerol, 15% (2.42M) ethylene glycerol, 15% (1.9M) dimethylsulfoxide and 0.4 M sucrose dissolved in MS medium. This was followed by rapid thawing. The PVS2 solution was replaced by filter-sterilized solution (1.2 M sucrose dissolved in MS medium). Plantlets regenerated from this technique were high quality, genetically similar, pathogen-free ones. In the present study, banana, cultivar 'Robusta' was subjected to different cytokinins amended in MS basal medium. Cytokinins showed differential effects in the micropropagation of Robusta cultivar. The multiplying cultures of banana in medium supplemented with BAP showed the highest multiplication ratio (3), when compared to kinetin (2.5) and zeatin (2.6). Among the different cytokinins tested, the BAP was found to be effective one at optimum concentration of 4 mg/L in the micropropagation of banana. All plantlets regenerated from shoot tips that had cryopreserved and treated with cytokinins were high quality and disease-free ones.

Keywords: Banana cultivar 'Robusta', cryopreservation, in vitro multiplication, cytokinins, BAP

Does cryopreservation stress impact genotype integrity? A case study with germplasm of *Musa* spp.

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The new-age vitrification-based methods of cryopreserving meristems have been amply demonstrated in many plant species, including crops. However, use of penetrating and non-penetrating cryoprotectant agents (CPAs) carries an inherent toxicity risk, affecting structural integrity, physiological functionality, osmotic and biochemical stress and induction of mutational changes. In terms of genebanking of germplasm, strong curiosity among curators remains about the cryogenic effects (e.g. CPAs, freezing injury) and non-cryogenic factors (e.g. in vitro culture, genotype), on the exposure of explants to physical, chemical and physiological stresses, which need investigation on the potential impacts on the genome, transcriptome, proteome and metabolome, especially events causing undesirable instability.

Genetic integrity was studied in cryopreserved meristems of 24 accessions of *Musa* by morphological (11 descriptors) and molecular traits (21 simple sequence repeats markers). The germplasm comprised accessions belonging to ABB, AAB, AAA, AB and AA genomic groups, besides four wild species. Use of vitrification method resulted in 20-67% regrowth after liquid nitrogen (LN), whereas when droplet-vitrification method was applied, a higher regrowth of 25-92% was achieved. Morphological data (ANOVA) revealed that most of the traits of plants derived from frozen meristems were non-significantly different from non-frozen controls and in vitro conserved material. Using SSR primers, UPGMA (Nei and Li/Dice) analysis showed no significant differences among the control and cryopreserved samples in eight accessions tested, with 93-97% coefficient of similarity, demonstrating no genetic drift or mutations at these loci. Only one accession (Borjahaji, AAA) gave 76.6% similarity coefficient, which did have any phenotypic expression. Overall, it is concluded that vitrification-based cryo-procedures can be safely used for long-term preservation of *Musa* genetic resources, without any severe impediment of somaclonal variation or genetic instability.

Keywords: Cryoprotectant agents, banana, cryopreservation, genetic stability
Cryopreservation of in vitro shoot tips of chayote (Sechium spp.) by D cryo-plate method

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Chayote (Sechium spp.) is a valuable fruit and a main source of nutrition to the people of Mexico. The Banco Nacional de Germoplasma deSechium edule (BANGESe), located at the Centro Regional Universitario Oriente of Universidad Autonoma Chapingo in Huatusco, Veracruz, Mexico, was founded in 2005. BANGESe maintains over 245 accessions of S. edule as a field genebank. However, maintenance of field collections of chayote is cumbersome and costly. An alternative method for safe and cost-effective long-term preservation of chayote is required. Adapted version of the D cryo-plate procedure for long-term preservation of chayote was developed. Variants of the D cryo-plate procedure were optimized for post-liquid nitrogen (LN) regrowth in chayote shoot tips (accession “H635-12” (S. edule: nigrum spinosum × S. compositum). The procedures tested included variation in preculture duration, loading solutions (LSs), duration of LS treatment, air dehydration time, and high temperature exposure. The most effective procedure for high post-LN regrowth consisted of: exposure of shoots to 29°C for 5 days just before excision, 2 days preculture on 0.3 M sucrose medium, osmoprotection with LS containing 0.4 M sucrose and 2.0 M glycerol for 45 min, and dehydration for 45 min in Petri dish containing 35 g silica gel. The optimized procedure resulted in high rate of regrowth (83.3%) in post-LN regrowth. Now this optimized procedure is going to be applied to 10 additional chayote accessions. D cryo-plate has shown to be a practical and simple procedure for cryo-storage of i vitro grown chayote shoot tips; and can be rapidly integrated in the Genebank everyday conservation system.
O_27

Cryopreservation of alginate-coated in vitro-grown apices of apple, pear and sweet cherry

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In vitro shoot tips of 3 temperate fruit crops (apple, pear and sweet cherry) encapsulated in sodium alginate were successfully cryopreserved using 2 different methods based on vitrification and two-step freezing. Initial shoot tip cultures were cold-hardened for 4 weeks in 4°C. Explants (3 mm) aseptically dissected from cold hardened in vitro cultures were cold treated in MS medium supplemented with 2.0 M sucrose and 100 mg/L vitamin C for 48 h at 4°C. After encapsulation in alginate gel, embedded shoot tips were dehydrated by exposure to a sterile air flow for a period of 8 h. Two cooling procedures were used: rapid cooling by direct immersion of cryotube with embedded shoot tips in liquid nitrogen (LN) (vitrification) and two-step freezing by progressive cooling (0.5 to 10°C/min) from +20°C to -150°C before immersion in LN. Thawing was performed by placing the cryovials in a 40°C water bath for approximately 1 min. Survival was defined as the percentage of the total number of shoot tips that showed a green colour after post culture according to evaluation scale. Average survival rate amounted to 91% in the case of two-step freezing by progressive cooling. Encapsulated shoot tips immersed directly to liquid nitrogen were also cryopreserved successfully (72–77% survival). Both cryopreservation procedures were successfully applied for 8 cultivars of apple, pear and sweet cherry. Encapsulation-dehydration method using alginate beads appears promising for the cryopreservation of rosaceous fruit species.

Keywords: fruit species, cryopreservation, encapsulation, vitrification, two-step freezing

O_28

Cryopreservation of Ashe magnolia shoot-tips by droplet-vitrification
The Magnoliaceae are one of the earliest angiosperms. Although they are mainly known for their ornamental and horticultural uses, they also have medicinal properties. Half of the Magnolia species are considered to be endangered on a global scale, and an efficient cryopreservation method for the specimens of interest that are maintained in living collections or collected in the wild would, therefore, help to ensure their long-term conservation. A study was performed comparing three different preculture conditions prior to cryopreservation. In vitro shoots from Magnolia macrophylla var. ashei were cultivated onto a basal medium (Ctrl) or sucrose supplemented medium (Suc) at 25°C, and onto the basal medium at 13°C during one week (Cld). Shoot-tips from in vitro donor plants were dissected and cryopreserved using the droplet-vitrification technique. Survival, shoot-tip regrowth, callus formation, oxidation and hyperhydric shoot-tips were recorded four weeks after rewarming from liquid nitrogen. Shoot recovery was recorded twelve weeks after the cryoprocess. Survival and shoot-tip regrowth after cryoprocessing were improved by treating the donor plants with sucrose and cold (Survival: Ctrl 60%, Suc 93%, and Cld 100%; Shoot-tip regrowth: Ctrl 30%, Suc 50%, and Cld 93%). Shoot recovery was only obtained when shoot tips were dissected from donor plants that were cold treated (30%). These results show that cold acclimation is essential for the plant regeneration from shoot tips of Ashe magnolia that were submitted to droplet-vitrification. Further studies are ongoing to gain more insight into the regeneration process and improve the recovery rates.

Keywords: cryopreservation, magnolia, PVS2, droplet-vitrification, shoot-tips, abiotic stress, cold acclimation, sucrose

O_29

Cryopreservation of apple (Malus domestica ‘Benoni’) dormant buds using two-step freezing method

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Apple is the most important temperate fruit in India. The top apple producing states are Jammu and Kashmir (J&K), Himachal Pradesh (HP) and Uttarakhand (UK). The apple germplasm is mainly conserved in the field genebanks at the ICAR-National Bureau of Plant Genetic
Resource’s Regional Station, Shimla (HP) and Bhowali (UK); ICAR-Central Institute of Temperate Horticulture, Srinagar (J&K); ICAR-Indian Agricultural Research Institute’s Regional Station, Shimla (HP); Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (HP); Govind Ballabh Pant University of Agriculture and Technology, Pantnagar (UK); Sher-e-Kashmir University of Agricultural Science and Technology, Srinagar (J&K). To have a backup, some accessions are being maintained under in vitro genebank at New Delhi. For long-term conservation of apple accessions cryopreservation is the only method. Dormant buds of apple (Malus domestica ‘Benoni’) were cryopreserved by two-step freezing method. Apple twigs were collected from Srinagar at the sub-freezing temperature. Twigs were stored at 5-6°C until use. The buds were scooped from the twigs and desiccated on silica gel. Then the buds were subjected to gradual low temperature treatments up to -30°C before submerging into liquid nitrogen. Programmable freezer was not used for freezing treatments. Post-thaw recovery (in vitro) was about 20%. The average rate of shoot formation was about 80%. This protocol of two-step freezing method was successfully applied to other three apple cultivars with the post-thaw in vitro recovery about 11-40%. This method appears to be a promising technique for cryopreserving dormant buds from field grown apple trees.

Keywords: Ex situ conservation, Malus spp., cryopreservation, winter bud, germplasm

O_30
Cryopreservation of embryogenic callus of Colchicum atticum and Colchicum szovitsii by vitrification

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Colchicium is one of the 19 genera belonging to Colchicaceae family and an important geofit in Turkey. Colchicium species grown naturally in Turkey are under extinction due to destruction of natural habitats, unconcious usage of agricultural areas and taking out the corms from nature for export. In this study, embryogenic callus of Colchicum atticum and C. szovitsii Fisch. & C.A. Mey. subsp. szovitsii were successfully cryopreserved by vitrification. In this method, callus structures were sufficiently dehydrated with cryoprotective solution (PVS2) prior to direct plunge in LN (-196°C). The PVS2 contains (w/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in ½ Murashige & Skoog medium (MS) containing 0.5 M sucrose. Callus were treated with PVS2 at 0°C for 30, 60 or 90 min. After rapid warming, the callus stucture was expelled in 2 ml of MS medium containing 1.2 M sucrose. The average rate of survival was about 60%. The vitrified calli regenerated plantlets.

This research was supported by TUBITAK (The Scientific and Technical Research Council of Turkey) (Project No. TOVAG 1160215) project.

Keywords: Colchicium, ornamentals, preservation, corm, PVS2

O_31
Cryopreservation of some cyclamen species embryogenic callus by vitrification and genetic stability

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The origin of 20 Cyclamen taxons, belonging to Myrsinaceae and growing under trees and bushes, is in Mediterranean region. In this study, in vitro preservation possibilities of cyclamens (*C. coum*, *C. persicum* and Melody F1 Red cultivar) which are important genetic resources in Turkey were investigated. Embryogenic callus obtained from ovule and petiole were successfully cryopreserved in liquid nitrogen (LN) (-196 °C) by vitrification. In cryopreservation period, different concentration of osmotic reagents (2% mannitol, 2% sorbitol and 0.5 M sucrose) and different temperatures (0 and 25°C) were treated with PVS2. The best cryopreservation result was obtained from the treatment: 0.5 M sucrose + Callus induction medium (CIM), 48 h, +4°C, darkness and PVS2 solution at 0°C, 60 min for all species. This study showed that cryopreservation protocol worked effectively because the plantlets from cyclamen species had same genetic structure after cryopreservation. It was determined that there was no somaclonal variation or genetic alteration between the plantlets obtained after cryopreservation and control plants using RAPD markers.

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Keywords: Cyclamen, ornamental, callus, tuber, PVS2

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**O_32**

*Cryopreservation of Arabidopsis - A model system for shoot tip maintenance of plant genetic resources?*

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The federal ex situ collection of agricultural and horticultural plants at IPK Gatersleben houses more than 1,800 cryopreserved accessions of plant genetic resources, predominantly shoot tips of potato, mint and *Allium*. Though, recent advances have increased our understanding of the genetics of many crops, Arabidopsis is still a valuable tool and holds a plethora on genetic resources to investigate specific questions.

During cryopreservation, meristematic tissues have to cope with different stresses including desiccation, cold stress and rehydration. To elucidate molecular mechanisms during cryopreservation, the aims of the study were to investigate the stress response mechanisms at
physiological and transcriptomic levels. Using Arabidopsis shoot tips as a model tissue, gene expression analyses after cryopreservation revealed a range of induced genes, which are involved in several biotic and abiotic stress mechanisms. First results indicate that \textit{Pathogenesis Related gene 5 (PR5)} interacts in mechanical-induced stresses; whereas, \textit{WRKY22} transcription factor (\textit{WRKY22}) has regulatory functions. Thus, a transcriptomic approach was pursued to gain new insights into the potential signal cascades in which \textit{WRKY22} participates. Thereby, \textit{WRKY22} seems to modulate \textit{PR5} expression in response to shoot tip preparation. In conclusions, this study will increase our understanding of fundamental process underlying Arabidopsis cryopreservation and may improve cryopreservation protocols of some crops.

Keywords: Arabidopsis, shoot tips, PVS2 vitrification, transcriptome analysis, gene bank
Cryopreservation of non-precultured protocorms of *Acampe rigida* (Buch.-Ham. ex Sm.) P. F. Hunt using V-cryo-plate and D-cryo-plate methods

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Non-precultured protocorms of *Acampe rigida* (Buch.-Ham. ex Sm.) P. F. Hunt were used to find suitable duration time for dehydration on V-cryo-plate and D-cryo-plate methods. For V-cryo-plate method, non-precultured protocorms were dehydrated with PVS2 solution for 0, 20, 40, 60 and 120 min. The results showed that protocorms dehydrated with PVS2 solution for 40 min gave the highest survival score at 0.08 and gave the highest percentage of survival at 16.67% which higher than control (+LN; 0 min), but most of protocorms showed dark green color and developed slower than control (-LN; 0 min). The suitable treatment for V-cryo-plate in this study is exposing to PVS2 solution for 40 min. For D-cryo-plate method, non-precultured protocorms were dehydrated with silica gel for 0, 0.5, 1, 1.5, 2, 2.5 and 3 h. The results showed that protocorms dehydrated with silica gel for 1.0 h gave the highest survival score at 0.56 and gave the highest percentage of survival up to 74.19%. Most protocorms with dark green developed slower than control (-LN; 0 min). The suitable treatment for D-cryo-plate method in this study is dehydrating with silica gel for 1.0 h. An unpaired t-test was used to compare the data of survival score between suitable treatments from V-cryo-plate and D-cryo-plate methods. The suitable treatment for cryopreservation of protocorms of *A. rigida* is cryopreserving with D-cryo-plate method dehydrated with silica gel for 1 h.

Keywords: Plant cryopreservation, V-cryo-plate, D-cryo-plate, Orchidaceae
**P_2**

**Morphology characterization of papaya (Carica papaya L. ‘Sukma’) derived from cryopreserved-seed**

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The cryopreservation techniques allow reducing of tissues or organs metabolism rate and the cell division are able to continue right after released from desiccation and freezing storage. This method is prospected to conserve plant material for long time without any morphological and genetic change. Seeds of papaya var. Sukma that have been cryopreserved are planted in the experimental field of Bogor Agricultural University at Pasir Kuda, Ciomas, from June to October 2017 to prove that there is no morphological changes occur. The objective of this study was to characterize morphologically papaya plants derived from seeds that have been stored by cryopreservation and compare them with papaya plants whose seeds are not stored by cryopreservation. The overall results showed there are no differences and morphological changes occur in papaya plants whose seeds have been preserved by cryopreservation, where the plant height is obtained equally 90-100 cm, stem diameter 4-5 cm, color of stems light green, color of petioles are light green, shape of petiole sinus are strongly closed, and shape of mature leaf are straight.

Keywords: Cryopreserved seed, conservation, color of stem, petioles, mature leaf, IPB

**P_3**

**Cryopreservation of Habenaria radiata and Habenaria rhodocheila seeds by aluminum cryo-plate vitrification method**

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Genus *Habenaria*, including 600 species, are terrestrial orchids distributed in Asia, America and Africa. *Habenaria rhodocheila* Hance is found in Thailand, Myanmar, Cambodia and Laos. The main characteristics of *H. rhodocheila* flowers is the large lip with various colors including orange, pink, red and yellow. *Habenaria radiata* (Thunb.) K. Spreng is native to Japan. Main allure of this plant is white egret shaped flower. The number of both species decreased in their habitat because of over collecting and environmental changes in the habitat. Conservation of the surviving plants is an urgent issue to keep the genetic diversity of the species. Cryopreservation is a reliable way to keep plant germplasm for long term. Pods of *H. radiata* and *H. rhodocheila* were harvested and kept at room temperature until use. Seeds were surface sterilized, desiccated in a laminar air-flow cabinet, and then used in the experiment. Cryo-plate vitrification method was performed according to Yamamoto et al. (2012). Viability of the seeds with or without immersion in liquid nitrogen (LN) was estimated by seed germination on Malmgren Modified Terrestrial Orchid Medium supplemented with 3% sugar, 0.5% active charcoal and 0.25% gellan gum. Cultures were kept at 23°C with 16 h, 31.5 µmol m⁻² s⁻¹ of inflorescence lamp. The plates were exposure to PVS2 for 0 to 120 min. Seeds of *H. radiata* showed high viability after immersion in LN without PVS2 treatment and the viability decreased according to increase of exposure time to PVS2 after immersion in LN. On the other hand, viability of *H. rhodocheila* seeds after immersion in LN increased by PVS2 treatment for 10 to 30 min, kept high levels by 40 to 60 min PVS2 treatment and then decreased by more than 90 min PVS2 treatment. The *H. rhodocheila* seeds treated with PVS2 for 40 to 60 min without LN treatment showed lower viability than cryopreserved seeds.

Keywords: Liquid Nitrogen, PVS2, Terrestrial orchid

**P_4**

Vitrification-based cryopreservation of *Dendrobium cruentum* Rchb. f. seeds

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Dendrobium cruentum Rchb. f. is one of epiphytic orchids originated from southwestern of Thailand. It faces extinction. The cultivation of this species was successful but there is a problem for long-term storage. Meanwhile, cryopreservation is the potential technique for long-term conservation of several biological germplasm based on plant tissue at ultra-low temperature of liquid nitrogen (LN, −196°C). Vitrification technique is the use of plant vitrification solution especially PVS2 to provide a glassy state of plant materials. Under the ultra-low temperature, T_g, T_m and T_c were observed. From the experiment, encapsulation-vitrification and V-cryo-plate methods were used by using 2% sodium alginate with various exposure times (0, 30, 60, 90 and 120 minutes). The maximum germination percentage (68.1%) was observed in V-cryo-plate method with 60 minute exposure time of PVS2 solution, while the encapsulation-vitrification method showed lower germination percentage of 21.6% comparing with V-cryo-plate on the same conditions.

Keywords: Cryopreservation, encapsulation-vitrification, V-cryo-plate Dendrobium cruentum Rchb. f., orchid seed

P_5
Compatible solutes on cryopreserved Hancornia speciosa Gomes lateral buds

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Hancornia speciosa is a native fruit species from Brazil with important economic applications. However, the use of this species has been challenged by difficulties in its storage. Cryopreservation is a safe and efficient tool for long-term storage. Nevertheless, cryopreservation may promote reactive oxygen species (ROS) formation with subsequent lipid peroxidation, which causes membrane damage. The use of in vitro antioxidant compounds could help eliminate ROS and consequently improve explant survival. In this study, the lateral buds of H. speciosa were precultured in proline or glycine betaine (GB) - containing solutions, and the effect of these compounds on the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), and on lipid peroxidation were measured after sample warming. The present findings indicated that oxidative stress induced by cryopreservation was significantly reduced by the preculture of lateral buds with proline and GB. SOD activity increased and
subsequently hydrogen peroxide (H₂O₂) production increased, followed by the immediate activation of CAT and APX. The enzymatic activity increased for 7 d following rewarming, indicating that preculture with 0.1 M proline was fundamental to achieve high levels of shoot regeneration from *H. speciosa* lateral buds. Acknowledgements: CNPq, CAPES and FAPEMIG.

Keywords: Recalcitrant species, Long-term storage, Proline, Glycine betaine, Oxidative stress

**P_6**

Droplet vitrification as a viable alternative cryopreservation to the dormant bud technique for recalcitrant pears

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The French INRA Pip Fruit Biological Resource Center is in charge of the maintenance, management, characterization and promotion of the traditional and scientific genetic resources of apple, pear, quince and related species. In 2010, the first cryopreservation trials were performed on apple and pear using the dormant bud technique with the main aim of cryopreserving a major part of the INRA *Malus* and *Pyrus* collections. However, some accessions, mainly from the genus *Pyrus*, sometimes responded badly to the dormant bud technique. To optimize the ability to cryopreserve the largest number of accessions in our collections, the droplet vitrification technique was tested on 2 cultivars of *Pyrus*: Williams and Conférence, used as controls since 2010. Our objective was to evaluate this method under our experimental conditions and to compare it with the dormant bud technique. Experiments were carried out using samples from a batch of budsticks collected in January 2015, in order to test the impact of several critical steps in the process. With both techniques, the immersion in liquid nitrogen step appeared to have the most important impact on the final results, much more than dehydration and slow-freezing for dormant bud technique or LS and PVS2 steps for droplet vitrification. Regenerated plants were obtained after using droplet vitrification (Williams: 40%, Conférence: 10%). With some improvements, this method could be a viable alternative cryopreservation to the dormant bud technique and opens up the possibility of new development paths for the optimization of long-term preservation systems of our genetic resources collections.
Keywords: cryopreservation, droplet vitrification, dormant bud technique, pear.

P_7

Genipa americana seed cryopreservation

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The jenipapeiro (Genipa americana) is a forest species of high socioeconomic and ornamental potential. However, predatory collectivism actions threaten its existence, being necessary the use of conservationist practices. The seeds of this species have sensitivity to desiccation and cooling, making it unfeasible for conservation by conventional methods. Cryopreservation is the most promising alternative for the long-term conservation of species that produce unorthodox seeds, such as the genipap. The objective was to cryopreserve seeds of Genipa americana and evaluate the effects of desiccation and freezing on germination and establishment of seedlings. Initially, the seeds were dehydrated in silica gel (0, 16, 18, 20, 22 and 24 h), and then were cryopreserved in liquid nitrogen (LN) (-196°C). After thawing, the viability and germination were analyzed. Dehydrated and non-cryopreserved seeds were also analyzed. The silica gel desiccation period promoted a significant reduction in viability and germination. However, initial seed water content was so high (47%) that storage in LN without prior dehydration treatment resulted in seed mortality. It was verified that, the removal of up to 14% seed water content induced to a higher freezing tolerance, allowing the successful cryopreservation. Silica gel dehydration followed by immersion in LN was shown to be highly efficient for cryopreservation of seeds of Genipa americana, with a germination rate of 35% after thawing, and a rate of 100% survival, with growth and normal establishment of the seedlings after acclimatization. The authors are thankful for FAPEMIG, CNPq and CAPES for the financial support and scholarships.
Keywords: Jenipapeiro; Rubiaceae; long-term storage

P_8
Cryopreservation of Vanda denisoniana Bens. & Rchb.f

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Vanda denisoniana Bens. & Rchb.f., a wild Thai orchid, is a valuable species for both research and economics. Presently, this species has been threatened due to climate change and habitat lost. Cryopreservation is a powerful tool for long-term plant genetic resources conservation. This research has been conducted to optimize the important conditions for cryopreservation of protocorm-like bodies (PLBs). The parameters used in this study were types of Plant Vitrification Solution (PVSs), sucrose concentration, preculture duration, dehydration duration, and unloading duration. The viability and recovery of the PLBs were recorded after 8 weeks on regeneration medium (consisted of New Dogashima Media (NDM) containing 5 µM 6-benzylaminopurine (BAP), 1 µM naphthaleneacetic acid (NAA) and 3% (w/v) maltose). The use of PVS3 displayed higher survival percentage of PLBs compared to those using PVS2. The optimum conditions for cryopreservation of PLBs were precultured with semi-solid NDM media supplemented with 0.5 M sucrose for 48 h, followed by treatment with 0.8 M sucrose for another 24 h. The PLBs were immersed into loading solution (0.8 M sucrose and 2 M glycerol) at 25°C for 30 min and then dehydrated with PVS3 at 0°C for 20-40 min prior to immersion into liquid nitrogen. For unloading step, the PLBs were immersed into NDM liquid media supplemented with 1.2 M sucrose for 20 min. The PLBs were able to regenerate and develop into normal plantlets.

Keywords: New Dogashima Media, NDM, Plant Vitrification Solutions, PVS3, protocorm-like bodies, Thai wild orchid

P_9
Development of effective cryopreservation protocols using aluminium cryo-plates for mulberry
The adaptation of D cryo-plate method to in vitro mulberry shoot tips from the tropics and subtropics was tested. The optimal D cryo-plate procedure is the following: Precultured shoot tips (1.5 mm) are attached on the cryo-plate by calcium alginate gel. Then, osmoprotection is performed by immersing the cryo-plates in LS (2.0 M glycerol and 0.6 M sucrose) for 30 min at 25°C. The shoot tips on cryo-plate are dehydrated under the air current of a laminar air-flow cabinet for 2.5 h at 25°C, 40-50% RH. This optimized procedure was applied to shoot tips of 10 mulberry lines, resulting regrowth ranged from 73.3 to 90.0%, with an average of 78.7%. Also, the possibility of using mulberry shoot tips dissected from the axillary buds of current shoots after sterilization was tested in V cryo-plate procedure. The optimal dehydration time of shoot tips excised from current shoots by PVS2 was 50 min at 25°C. This optimized procedure was applied to shoot tips from current shoots of 10 mulberry lines. Regrowth ranged from 70.0 to 96.7%, with an average of 80.3%. The protocols developed for mulberry are useful techniques for cryopreservation of other woody plant species after marginal modifications.

Keywords: cryo-plate, current shoot, D cryo-plate method, mulberry, V cryo-plate method
The V cryo-plate method was successfully developed using *in vitro* Rakkyo plants (*Allium chinense* G. Don ‘Shima-rakkyo’). Rakkyo is a cash crop of west part of Japan. The optimal V cryo-plate method was the following: Shoot tips were collected from in vitro plants and precultured 1-3 d at 25°C on solidified MS medium containing 0.3 M sucrose. Those precultured shoot tips were placed in elliptical wells on an aluminium cryo-plate and embedded in calcium alginate gel. Osmoprotection was performed by immersing the cryo-plates for 30 min at 25°C in LS (2.0 M glycerol and 1.0 M sucrose). The optimal dehydration time of shoot tips excised from *in vitro* shoot tips by PVS2 is 30 min at 25°C. In this method, cooling was performed by transferring in uncapped 2 ml cryotubes held on a cryo-cane which was directly plunged into liquid nitrogen for at least 1 h. For setting off regrowth, the cryopreserved shoot tips attached to the cryo-plates were rewarmed by immersion in petridish containing 1.0 M sucrose solution at 25°C. giving the survival and regrowth rate of cryopreserved shoot tips 100%. This protocol appears to be a promising technique for cryopreservation of *Allium* genetic resources.

Keywords: Allium, Rakkyo, cryo-plate, vitrification, cryopreservation

**P_11**

**Efficiency of aluminium cryo-plates for cryopreservation of Dendrobium signatum Rchb. f. pollinia**

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The efficiency of V cryo-plate and D cryo-plate methods for cryopreservation of *Dendrobium signatum* Rchb. f. pollinia, a Thai orchid was investigated. Pollinia were collected from flowers and then placed on the aluminium cryo-plate containing 12 wells embedded with 3% sodium alginate gel. In V cryo-plate method, cryo-plates with pollinia were immersed in 0.6 M sucrose (loading solution) for 15 min, then dehydrated with PVS2 solution for 40 min at room
In the D cryo-plate method, cryo-plates with pollinia were treated with 0.6 M sucrose for 15 min, then dehydrated in a laminar air-flow cabinet for 3 h at room temperature (29±2°C). In both methods, cryo-plates were directly plunged into liquid nitrogen for 40 min, and rapidly warmed in 1.2 M sucrose (unloading solution) for 15 min. The cryopreserved and non-cryopreserved pollinia were used to pollinate flowers of the same species. The results showed that cryopreserved pollinia retained fertilizing ability, and had similar pod formation as those pollinated with non-cryopreserved pollinia. The pod formation from cryopreserved pollinia in V cryo-plate and D cryo-plate methods were 55.6% and 50%, respectively. Seeds from non-cryopreserved and cryopreserved pollinia were successfully produced and germinated into plantlets with well-formed leaves and roots. Multiple stems (4.8 stems/plant) produced from one plantlet when cultured on modified VW agar medium (1949) supplemented with 100 g/L banana, 150 ml coconut water, 50 g/L potato, and 10 g/L sucrose for 120 d at 25±2°C. Cryopreservation of pollen using aluminium cryo-plate can be used successfully for pollination.

Keywords: Orchid, Dendrobium signatum, V cryo-plate, D cryo-plate, Vaccin and Went medium, pollination

P_12
Cryopreservation of an endangered pharmaceutically important orchid, *Cymbidium finlaysonianum* Lindl. using vitrification technique

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Cryopreservation is an *in vitro* conservation method which has become an important tool for a long-term storage of plant genetic resources. New protocorm-like bodies (PLBs) (about 4-5
mm in diameter) of *Cymbidium finlaysonianum* Lindl. were isolated individually from 2-month-old proliferating PLB clusters which had been cultured in VW liquid medium (VW; Vacin and Went, 1949) supplemented with 8.84 µM 6-benzylaminopurine were successfully cryopreserved using a vitrification method. In this cryogenic procedure, PLBs were precultured in MS liquid medium (MS; Murashige and Skoog, 1962) supplemented with 0.5 M sucrose at 25±2°C for 2 d on an orbital shaker at 110 rpm. The PLBs were treated with loading solution (2 M glycerol plus 0.4 M sucrose) for 20 min at 25±2°C to make the precultured PLBs tolerant to plant vitrification solution 2 (PVS2), consisting of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide and 0.4 M sucrose in MS medium, pH 5.8. Subsequently, the selected PLBs were subject to PVS2 treatment at various exposure times (0-120 min) at 0°C and plunged into liquid nitrogen (LN) for 1 d. After storage in LN, the PLBs were rewarmed and washed by MS liquid medium containing 0.5 ml of 1.2 M sucrose for 20 min. One week after rewarmed PLBs, viability was determined by TTC reduction and regrowth assessed. The results showed that the PLBs precultured with 0.5 M sucrose for 2 d, followed by dehydrated with vitrification solution for 60 min had the highest post rewarmed viability in terms of TTC reduction (40 %) and regrowth (33.5 %). No survival rate of PLBs was found without vitrification treatment. Regenerated plants showed the same morphological characters as control.

Keywords: *Cymbidium finlaysonianum*, plant vitrification solution 2, cryopreservation, TTC, protocorm-like bodies (PLBs), 6-benzylaminopurine

**P_13**

*Cryopreservation of Arundina graminifolia* seeds using D cryo-plate method

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In this study, encapsulation-vitrification, encapsulation-dehydration, V cryo-plate and D cryo-plate methods for cryopreservation of *Arundina graminifolia* seeds were investigated. The results showed that the D cryo-plate method gave the highest regrowth of 82%, followed by encapsulation-dehydration (74%), V cryo-plate (71%) and encapsulation-vitrification (39%). The D cryo-plate protocol is as follows: Pour the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½ MS basal medium with 0.4 M sucrose in the wells of the cryo-plate. Place 20 seeds from 3 month selfing in each well. Pour calcium chloride solution containing 0.1 M calcium chloride in ½ MS basal medium with 0.4 M sucrose. Expose to 0.4 M sucrose + 2 M glycerol (loading solution) for 30 min and then dehydrated under a laminar air-flow cabinet for 3 h. Put each cryo-plate in a 2 ml cryotube and plunge 2 ml cryotubes into liquid nitrogen for 1 d. Warming in 1.2 M sucrose solution (unloading solution) for 15 min then removed the solution and cultured cryopreserved beads on ½ MS agar medium.

Keywords: Cryopreservation, encapsulation-vitrification, encapsulation-dehydration, V cryo-plate, D cryo-plate, Arundina graminifolia

**P_14**

**Cryopreservation of excised embryonic axes of *Quercus serrata* using desiccation method**

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*Quercus serrata* is a major tree species of forest areas in Korea, China and Japan. Oaks are susceptible to a large number of diseases. However, ex situ conservation of *Q. serrata* for cryogenic storage has not been established. Therefore, the sensitivity of desiccation and liquid nitrogen (-196°C) were investigated to develop a cryopreservation method for *Q. serrata*. Excised
Embryonic axes were dried rapidly over silica gel for 0-20 h, which produced 1.33-0.02 g H2O g DW-1. To assess tolerance of desiccation and cryo-exposure, they were immersed in LN [LN(+)] for 3 weeks prior to viability assessment. Axes were immediately exposed in 42°C with 0.5 M sucrose solution for 5 min for rewarming rapidly. Then, they were slowly thawed on ice for 15 min. Air-drying to 1.33-0.02 H2O g DW-1 showed axes survival of 70 - 100% in LN (-) and 10-75% in LN (+). Both root and shoot regeneration was 5-65% to 1.33-0.13 H2O g DW-1 in LN (-) but not observed to all desiccation condition in LN (+). Root regeneration presented positive correlation with desiccation levels and 75% survival and 65% germination were obtained to 0.02 H2O g DW-1 in LN (+). Shoot germination was 40% to 0.30 H2O g DW-1 in LN (-) and 10% to 0.04 H2O g DW-1 in LN (+). Desiccation of embryonic axes improved root regeneration after cryo-exposure; whereas, additional protective treatments were needed for higher regeneration of root and shoot growth.

Keywords: Desiccation, cryopreservation, Quercus serrata, regeneration, viability

P_15
Dehydration effects on cryopreservation of Cymbidium finlaysonianum protocorms

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Protocorms of Cymbidium finlaysonianum cultured on MS agar medium without plant growth regulator were dehydrated with PVS2, PVS3 and glycerol for 0, 30, 60, 90 and 120 min prior to plunge into liquid nitrogen (LN) for 1 h. The results showed that treated controls (PVS2, PVS3 and glycerol without LN) gave lower survival when the exposure times were longer. The PVS2 and PVS3 exposure time for 60 min prior to plunge into LN gave the highest survival of 40% and 10%, respectively. There is no survival after cryopreservation when glycerol was used for dehydration. After that, cryopreserved protocorms developed into plantlets within 3 months of culture.

Keywords: Cymbidium finlaysonianum, protocorms, cryopreservation
Some special features of the valuable essential oil rose cultivars conservation: in vitro deposition and cryopreservation

In order to solve global problems of plant genetic resources conservation and taking into account the essential oil, aromatic, medicinal and decorative properties of essential oil rose different cultivars grown in the south of Russia, the aim of our studies was to optimize the conditions for long-term deposition and cryopreservation of valuable cultivars. The regenerants of essential oil rose were cultured on MS medium with 0.1 mg/L NAA, 0.5 mg/L GA3, 0.5-1.5 mg/L BAP, 30 g/L sucrose and 8 g/L agar at 25±1°C under 16-h photoperiod with light intensity 37.5 μmol m⁻² s⁻¹. Explants for deposition were placed on ½ and ¼ MS media with 0.2 g/L and 0.4 g/L CCC and 60 g/L sucrose. Preservation was performed at 4±1°C under 16-h photoperiods supplied by cool-white fluorescent lamps giving 1.25-3.75 μmol m⁻² s⁻¹. Microshoots for meristem isolation were hardened off for 2 weeks before cryopreservation. Encapsulation-dehydration method (application of 3% alginate solution in MS medium with 0.75 M sucrose, polymerized in calcium chloride solution, dehydrated in MS medium with 0.75 M sucrose and dried in a laminar air-flow cabinet to 20% moisture content) was used for cryopreservation. Regeneration viability and frequency were assessed on the 30th day after thawing and cultured on the regeneration medium. Multiplication index in the long-term collection in vitro was 1:3-1:6, the maximum in the cvs. Raduga and Festivalnaya. Microshoot height and leaf amount were less under the deposition, leaf blade morphology differed with those under the standard in vitro culture. After the cryopreservation, viability of the studied samples varied from 42 to 81%. The efficiency of regeneration did not differ significantly from the control. In some cultivars we observed an increase in leaf number, adventitious microshoots appearance and active rhizogenesis. This study was funded by a research grant № 14-50-00079 of the Russian Science Foundation.

Keywords: Rosa damascene, R. gallica, R. alba, meristem, in vitro deposition, cryopreservation, viability, regeneration rate
Challenges in the development of a widely applicable method for sugarcane (Saccharum spp.) shoot tip cryopreservation

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The USDA-ARS National Plant Germplasm System (NPGS) maintains 946 accessions of sugarcane (Saccharum spp.) in the field at the Subtropical Horticulture Research Station in Miami, Florida. These accessions are particularly vulnerable to hurricanes, diseases and other threats. We sought to identify a method whereby clonally propagated sugarcane accessions could be successfully introduced into tissue culture, multiplied, and then cryopreserved as shoot tips for long-term preservation at the National Laboratory for Genetic Resources Preservation in Fort Collins, Colorado. For many accessions, 70% isopropyl alcohol and 20% commercial bleach treatments, followed by three rinses of sterile water were sufficient to remove microbial contaminants during the introduction process. However, in some cases, cefotaxime was particularly effective for removing bacterial contamination. Published methods for encapsulation-dehydration and V-plate vitrification cryopreservation procedures were tested to determine if acceptable results could be obtained. Droplet-vitrification methods were tested and then modified to seek improved results. We found that antioxidant treatments of glutathione, glycine betaine, and ascorbic acid did not improve regrowth after liquid nitrogen exposure, using either PVS2 or PVS3 as cryoprotectants. Exposure durations of PVS2 and PVS3 were optimized, with and without exposure to liquid nitrogen (LN), and shoot tip regrowth levels ranged from 0 to 37% after LN exposure. We concluded that the sugarcane cryopreservation methods that we tested are not yet ready for implementation in the NPGS.
Keywords: sugarcane, Saccharum, cryopreservation, shoot tips

P_18
Slow growth in vitro conservation of fruit crops

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Research and Breeding Institute of Pomology (RBIP) Holovousy Ltd. holds a fruit germplasm of about 2,300 accessions in field collections. Unfortunately, trees in these collections are at permanent risk from environmental conditions, pests and diseases. Germplasm preservation of vegetatively propagated fruit species in field collections may be supplemented using biotechnological methods. In vitro storage under slow-growth conditions is one of these techniques. The experiment with medium term storage of in vitro cultures of strawberry, apple, pear and sweet cherry in low above zero temperature at 4°C was used in RBIP Holovousy Ltd. as a duplicate method to field collections. In vitro cultures were established on MS medium supplemented with 1 mg/L 6-benzylaminopurine (BAP) in aluminium-capped 100 ml Erlenmeyer flasks with 5 explants/25 ml of medium. The experiment was successful and viable shoot tips were obtained at the end of storage period. The best overall results were obtained with sweet cherry genotype P-HL-A (rootstock), where 92.0% explants survived without an intervening subculture after seven months. In contrary, the lowest survival was observed with sweet cherry P-HL-C (rootstock), where only 2.0% of shoots survived 7 months from the beginning of experiment. Remaining genotypes of fruit species used in the experiment had intermediate results. The storage of in vitro cultures at 4°C appears to be promising technique as a duplicate medium-term conservation method for fruit germplasm, which can considerably reduce labor and material costs. Moreover, in vitro collections are year-long accessible form of germplasm for distribution among germplasm curators and community users.

Keywords: cold storage, fruit species, in vitro, germplasm, genotype

P_19
Cryopreservation of Chinese cherry (Prunus pseudocerasus Lindl.) shoot tips by vitrification
Chinese cherry (*Prunus pseudocerasus* Lindl.) belongs to the family Rosaceae and genus *Prunus*, originated from the Yangtze river basin in China and has been cultivated for more than three thousand years. Chinese cherry produces not only delicious fruits, but also the excellent rootstocks and breeding resources for sweet cherry. It is widely distributed in China and its characteristics are outstanding. Especially, wild cherry germplasm resources, with unique genetic structure of wild population and special genes with great potential in sweet cherry breeding. It is a valuable gene pool for resistance breeding and functional component breeding, which is the basis for improving the genetic germplasm of cherry. But in recent years, intensified human activities, deterioration of environment and vigorous promotion of commercial varieties make the genetic diversity of Chinese cherry germplasm resources rapidly losing. Simple, efficient, economical and long-term cryopreservation was chosen to preserve these resources. To optimize the protocols of the conservation for Chinese Cherry, in vitro-grown shoot tips of ‘Taishan GanYing’ used as the material, orthogonal test and single factor test were designed. A simple and effective vitrification method for Chinese cherry was successfully established. It is recommended for the long-term conservation of diverse Chinese cherry germplasm.

Keywords: Chinese cherry; cryopreservation; vitrification; shoot tip; germplasm resource

**P_20**

**Germination responses of Dyera costulata seeds to cryopreservation**
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A study on the possibility of seed cryopreservation of *Dyera costulata*, one of the important commercial timber species, was set up at the Seed Technology Laboratory, FRIM. Matured pods were collected from tree stand in FRIM's area. Upon receiving at the laboratory, the seeds were removed manually from the pods. Fresh seeds were immediately subjected to liquid nitrogen storage and another set of control seeds were kept at 20°C cold room. These two sets of seeds were kept up to six weeks and seed samples were consequently germinated after 1, 2, 4 and 6 weeks. Prior to the storage and after each storage period, moisture content and germination of the seeds were checked. Fresh seeds measured 8.31% of moisture showed 94% germination. Seeds kept under liquid nitrogen temperature (-196°C) showed moisture content ranging from 5.47% to 6.26%. However, germination percentage reduced from 90% to 55% throughout the storage time. Meanwhile, control seeds showed increased in moisture content during the storage; from 7.15% to 8.28%. Reduction in the germination percentage of the control seeds was noticed from 85% after one week in storage to 65% after six weeks. These findings showed that *D. costulata* seeds tolerated sub-zero storage temperature in the liquid nitrogen up to six weeks although germination percentage was quite low. Survived germinated seeds were transferred to vermiculite for growth evaluation. Further study needs to be carried out to determine the most suitable *D. costulata* seed moisture content prior to cryopreservation.

Keywords: Dyera costulata, seed storage, moisture content, germination, cryopreservation

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**P_21**

**Modified droplet-vitrification cryopreservation of arctic bramble (*Rubus arcticus*) and hybrid arctic bramble**

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The goal of this study was to find a cryopreservation method for arctic bramble (*Rubus arcticus* ssp. *arcticus*) and hybrid arctic bramble (*Rubus arcticus* ssp. *arcticus* × *Rubus arcticus* ssp. *stellatus*) in order to continue the long-term cryopreservation of the mandate variety collections with arctic brambles at Natural Resources Institute Finland (Luke). The droplet-
vitrification cryopreservation method has been successfully modified and applied at Luke for long-term cryopreservation of *in vitro* grown shoot tips of black currants and lateral buds of raspberries. Pretreatments and modifications were tested with the in vitro cultures, established in 2016, of the Finnish arctic brambles ‘Pima’ and ‘Susanna’ and the Swedish hybrid arctic brambles ‘Beata’ and ‘Sofia’. Cold acclimatization pretreatment was tested, after which shoot tips were excised for long-term cryopreservation using modified droplet-vitrification cryopreservation method. The durations of the cold pretreatment and the plant vitrification solution 2 pretreatment, the sucrose pretreatment before freezing and the in vitro growth media prior to freezing and during regrowth were tested. The highest regeneration rates were obtained by one week cold pretreatment for *in vitro* cultures and by increasing sucrose concentrations from 0.25 to 0.5M for meristems before freezing. As a result, the cultured buds from unfrozen and cryopreserved treatments showed regeneration into well dividing *in vitro* cultures. In conclusion, this cryopreservation method was shown applicable for cryopreservation of arctic brambles and long-term cryopreservation for two clonal lines for each cultivar with three repetitions was performed.

Keywords: Cryotechnology, Rubus, micropropagation, plant genetic resources

**P._22**

**The production of virus-free material of raspberry ‘Ninni’ by combined thermo- and cryotherapy**

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In Norway, raspberry is an economically important crop of increasing importance for the last 10 years. Raspberry is vegetatively propagated, and is often infected with several viruses that cause significant economic losses. A prerequisite for long-term development and production of this crop is virus diagnosis, virus elimination and preservation of healthy mother stock of important cultivars. Raspberry ‘Ninni’, a newly bred cultivar, was found to be infected by raspberry bushy dwarf virus (RBDV) by indicator plants and next generation sequencing of small RNAs. A combination of thermo- and droplet-vitrification cryotherapy has been developed in order to eradicate RBDV from ‘Ninni’. After 30 days of thermotherapy at 38°C, shoot tips (1mm) were excised from 6-week-old stock plants and stepwise precultured with increased sucrose concentrations from 0.25, 0.5 to 0.75 M. each for 24h. Shoot tips were loaded with a loading solution composed of 2 M glycerol and 0.5 M sucrose for 20 min, before exposure to PVS2 for 20
min at room temperature. Dehydrated shoot tips were transferred to aluminum foils (2 × 0.8 cm), prior to a direct immersion into liquid nitrogen (LN) for 1h. Thawing was done by immediately transferring from LN into 1.2 M sucrose for 20min. With this procedure, 44 of 120 shoot tips survived and regenerated into new plantlets, 13 (30%) of them were found to be RBDV-free.

Keywords: raspberry, RBDV, virus elimination, thermotherapy, cryotherapy

P_23
Cryopreservation of vanilla (Vanilla planifolia) root-tips: A new alternative for in vitro long-term storage of its germplasm

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Vanilla planifolia (Orchidaceae) is the natural source of vanillin, which is the most widely appreciated flavor compound in the world. Although vanilla is cultivated throughout the tropics, their natural distribution areas are being severely reduced due to the anthropogenic impact. Therefore, ex situ preservation actions are necessary to safeguard the threatened diversity of this species. Cryopreservation of vanilla shoot-tips has been previously reported using droplet-vitrification technique. However, survival and plant regeneration were low and little reproducible. In this study, we developed a successful protocol for cryopreservation of root-tips, as an alternative for long-term storage of vanilla germplasm. Maximum survival (~60%) and further regeneration (43%) were obtained by preconditioning root-tips from in vitro propagated plants on semisolid MS with 0.3 M sucrose (1 d), exposing to loading solution containing 0.4 M sucrose plus 2 M glycerol (30 min), and then, to glycerol-sucrose plant vitrification solution PVS3 (60 min in ice) prior to direct immersion of samples into liquid nitrogen in droplets of PVS3 solution placed on aluminum foil strips. Tissues were rewarmed by plunging the aluminum foils directly in liquid MS medium with 1.2 M sucrose (15 min) at room temperature. Growth recovery and bud induction after cryopreservation were efficiently achieved by culturing root-tips on semisolid MS medium with 3 mg L⁻¹ KIN and 1 mg L⁻¹ IBA. Plant regeneration was developed by transferring the induced buds to MS media. This protocol has great potential for long-term conservation of V. planifolia germplasm and of other vanilla relatives.

Keywords: Vanilla, root-tips, cryopreservation
Cryopreservation of *Paphiopedilum javanicum* seeds by encapsulation-dehydration for conservation

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*Paphiopedilum javanicum* is one of five slipper orchids that can be found in the area around Mount Kinabalu in Malaysian Borneo. A cryopreservation method based on encapsulation-dehydration was established for the storage of *P. javanicum* seeds. To evaluate the effect of sucrose treatment, immature seeds encapsulated in 3% (w/v) sodium alginate beads (4–5 mm) were incubated in sucrose solutions with different concentrations (0.0, 0.2, 0.4, 0.6 and 0.8 M); for optimization of the duration of sucrose treatment, the encapsulated seeds were incubated in sucrose solution for different duration (1, 2, 3 and 4 days); and to test the effect of moisture content, the seed-containing beads were dehydrated to obtain different moisture contents (25%, 30%, 40%, 50%, 60%, 70% and 80%). Cryopreserved seeds stored in liquid nitrogen consistently showed higher germination percentage as compared to those that were not placed in liquid nitrogen. The highest seed germination (49.4%) was observed when the beads were treated with 0.6 M sucrose while the best incubation period in sucrose solution was 3 days with 47.1% seeds germinated and 25% moisture content of beads gave the highest seed germination (52.5%). This study proved that the species is responding positively to encapsulation-dehydration.

Keywords: *Paphiopedilum javanicum*, cryopreservation, encapsulation-dehydration, germination, immature seed, conservation

Conservation of oil palm pollen via cryopreservation

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Oil palm pollen, a valuable genetic resource and used for research, has been successfully developed for conservation. Some advantages of the use of pollen are pollen serves as a source of genetic diversity in collections where it is hard to maintain its diversity with seeds. Seeds of some species have low fertility, large in size and require an investment of labour to store. Oil palm pollen was shown to be tolerant to desiccation and low temperature. Methods for pollen collection, desiccation, viability testing and longevity assessment have been developed for oil palm. Pollen viability can be measured by vital staining pollen grains, germinating pollen grains in vitro or by demonstrating successful fertilization and seed development. Oil palm pollen of many germplasms can be kept by maintaining pollen at liquid nitrogen (LN) temperatures (−196°C) for long-term storage. Meanwhile, short-term viability can be managed by storing pollen at temperatures between 4°C and −20°C. Based on current results evaluated on pollen of various oil palm germplasms, no significant difference was observed between storage at (-5°C) freezer and storage in LN. Therefore, long-term storage of oil palm pollen via cryopreservation has potentially been applied for conservation of oil palm genetic resources in MPOB.

Keywords: oil palm, pollen, germplasm

**P_26**
An improved droplet-vitrification cryopreservation method for taro shoot tips

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Taro, *Colocasia esculenta* (L.) Schott cultivars, as vegetatively propagated collections, are conserved in genebanks. In the current study, *Colocasia esculenta* cultivar “Ya Nuo” was used to improve droplet-vitrification cryopreservation method for taro shoot tips. Shoot tips (2 mm) were dissected from *in vitro* plantlets, and precultured in liquid MS medium supplemented with 0.3 M sucrose and 0.2M reduced glutathione (GSH) for 2 days, incubated with 2.0 M glycerol + 0.4 M sucrose solution for 20 min at 25°C, cryoprotected with plant vitrification solution 2 (PVS2, 30% glycerol, 15% dimethyl sulfoxide, 15% ethylene glycol, 0.4 M sucrose) for 40 min at 0°C and rapidly immersed in liquid nitrogen (LN). The cryopreserved shoot tips were rapidly rewarmed at
25°C in MS liquid medium containing 1.2 M sucrose for 20 min and then recovered on MS medium. Survival and regrowth levels were as high as 68% and 66%, respectively, and there is no change detected during propagation, cryopreservation and recovery.

Keywords: Droplet Vitrification, Reduced glutathione, Taro, Shoot tips

**P_27**

*Long-term storage of chickpea (Cicer arietinum) germplasm by cryopreservation*

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Chickpea (*Cicer arietinum* L.) is one of the most important pulse worldwide. This crop plays a significant role to maintain soil fertility through symbiotic nitrogen fixation, as well as in the Mediterranean diet for its important content of noble proteins. In South Italy, particularly in the Apulia region, many traditional landraces are still cultivated in marginal areas, becoming therefore at strong risk of genetic erosion or even extinction. In vitro culture is a useful and innovative approach for the collection and the long-term preservation of threatened germplasm by means of the cryopreservation technology. The aim of this study was to develop an efficient protocol for the multiplication and cryopreservation of two Apulian black chickpea landraces. Seeds of Apulian black chickpea were inoculated on agarized sucrose-free nutrient medium. The cotyledonary node and axillary buds were excised from the seedlings and then cultured on the same medium, supplemented with 6-benzylaminopurine and sucrose. After three subculture cycles, shoot tips from in vitro proliferated shoots were used for germplasm conservation by comparing three cryopreservation techniques, i.e. PVS2-vitrification, droplet-vitrification and V-cryoplate. Preliminary results are promising in terms of explant survival, although the subsequent development of cryopreserved and thawed shoot tips is still difficult. Hence, the study of the recovery medium composition for excised shoot tips is currently in progress, in order to improve shoot regrowth rates.

Keywords: landraces, genetic erosion, extinction, PVS2-vitrification, droplet-vitrification, V-cryoplate
Bacterial contamination of coconut zygotic embryo cryopreservation

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This experiment has been carried out as an international cooperative project between RDA and Bioversity International through Korea's RDA fund for sustainable use of coconut genetic resources and long-term safety preservation. In order to implement a strategic plan for the conservation of coconut genetic resources, the preparation of microbial-free embryos is essential for embryo culture, material transfer, as well as cryobanking. During the first phase of cryopreservation experiments, bacterial contamination of in vitro embryos was one of the main problems to be solved. Hence, it was recommended to develop a system to clean in vitro embryos and/or to control the contamination during the experiments. The application of nutrient agar prior to preculture was efficient for the detection of bacterial contamination. The contaminant colonies were transferred to R2A, tryptic soy agar and nutrient agar medium and 20 strains were isolated. The 16S ribosomal RNA gene was used for the identification of the bacterial strains. The universal primer 8F and 1512R primer were used for the polymerase chain reaction (PCR) amplification of 16S rRNA gene. The 16S rRNA sequences were run through the EzBioCloud BLAST (http://eztaxon-e.ezbiocloud.net/) program and the species of the highest homology was investigated. Among the contaminants, 20 strains were classified in 15 bacterial species. There were two strains closely related to Sphingomonas echinoides and Bacillus safensis, respectively, and four strains showing the highest sequence similarity to Sphingomonas panni. Most of the bacteria identified were considered as non-pathogenic. Endogenously-borne contamination from
coconut embryos poses the highest risk during the experiments. Some of the bacteria did not initially appear on nutrient agar upon receipt of embryos but could breakout, spread and contaminate clean embryos both in preculture and post-culture. A combination of antibiotics, cefotaxime and rifampicine, proved to reduce the bacterial growth but also suppressed germination of the embryos. Surface sterilization of embryos combined with the application of a vacuum was successful to decrease the bacterial contamination. Two protocols are now proposed as a candidate for routine implementation of cryobanking for coconut collections: 1) preculture-desiccation of intact embryos; 2) vitrification of plumular cubes.

Keywords: coconut, preservation, bacterial, contamination, vitrification

P_29
Cryopreservation and synthetic seed production in ornamental flower bulbs (geophytes)

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Ornamental flower bulbs called geophytes are divided into five groups, (i) bulbs, (ii) corms, (iii) tubers, (iv) tuberous root and (v) rhizomes. Ornamental geophytes are important floricultural crops in the worldwide. Geophytes grown naturally in many places are under extinction due to destruction of natural habitats, unconcious usage of agricultural areas and taking out the tubers, corms, bulbs and rhizomes from nature for export. For this reason, in vitro conservation studies of geophytes are significant to improve new varieties and produce new products in the future. In addition, representing genetic resources of the world, preservation of these resources and gaining to floriculture sector are also very important.

In this review, basic and the newest cryopreservation techniques and synthetic seed production for ornamental geophytes are discussed: (i) traditional methods, (ii) vitrification-based methods (droplet, dessication, vitrification, encapsulation-vitrification and their combinations), (iii) synthetic seed production and (iv) V-Cryo-plate and D-Cryo-plate techniques.

Keywords: Geophytes, preservation, vitrification, encapsulation, genetic resources
Cryopreservation of embryogenic callus of *Crocus ancyrensis* (Herbert) Maw via encapsulation-vitrification

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Alginate coated embryogenic callus from in vitro-grown corm explants of *Crocus ancyrensis* endemic to Turkey were successfully cryopreserved by vitrification. Embryogenic callus was precultured on sucrose-enriched MS medium including 1 mg/l NAA and then encapsulated. To induce dehydration tolerance (osmotolerance), encapsulated embryogenic calli were treated with a 2% Sorbitol, 2% Mannitol, 2% Sorbitol+ 2% Mannitol and 0.5 M sucrose at 4°C during 48 h for pretreatment. These synthetic seeds were dehydrated with a vitrification solution (PVS2 solution) for 0, 30, 60 and 90 min at 4°C prior to a plunge into liquid nitrogen. Afterwards, synthetic beads were thawed in a waterbath (38°C). After PVS2 solution removed from the cryotubes, washing solution was added in cryotubes and synthetic beads incubated during 4 d in fresh MS medium at 25°C dark conditions. At the end of this stage, beads were transferred to regeneration medium. Regeneration was observed from the beads incubated in MS medium containing %4 Mannitol and immersed PVS2 during 1 hour as 8%. This protocol was examined in *C. ancyrensis* for the first time but it should be developed by different applications.

This research was supported by TUBITAK (The Scientific and Technical Research Council of Turkey) (Project No. TOVAG 113O795) project.

Keywords: Corm, genetic resources, endemic, preservation, ultra low temperature

Cryopreservation of chilli seeds by vitrification
Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) has collaborated with DOA Genebank (Genebank Research and Development Group) Biotechnology Research and Development Office, Department of Agriculture to study cryopreservation technique of chilli seeds (Huareo no.13) by vitrification. The cryoprotectant immersion durations were experimented in order to study the most suitable time for this method. Chilli seeds were immersed in loading solution containing 2M glycerol and 0.4M sucrose for 0, 10 and 20 min. They were then soaked in PVS2 solution containing 30% glycerol, 15% ethylene glycol 15% DMSO for 0, 30 and 60 min. After that, chilli seeds were plunged into liquid nitrogen. After thawing and reculturing on MS medium + 0.5M sucrose for 1 d, chilli seeds were taken out from the medium and transferred onto MS medium. They were then incubated for 14 d. It was found that the survival rate of chilli seeds before liquid nitrogen immersion was 100% and after liquid nitrogen immersion was also 100%. They were able to grow in outside conditions. These in vitro seedlings were successfully transplanted to field conditions.

Keywords: chilli seeds, cryopreservation, vitrification

**P_32**

**Cryopreservation of lettuce, Chinese cabbage, white-flowered cabbage, Chinese kale and pak choi seeds by encapsulation-dehydration**

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Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) has collaborated with DOA Genebank (Genebank Research and Development Group, Biotechnology Research and Development Office, Department of Agriculture), to study cryopreservation techniques of lettuce, Chinese cabbage, white-flowered cabbage, Chinese kale and pak choi seeds by encapsulation-dehydration. It was found that the seeds which were not plunged into liquid nitrogen (LN) had 100% viability in all experiments. Seeds germinated to be normal seedlings on MS medium for 14 d. It was shown that duration time in soaking loading solution at 10 and 20 min and duration time for silica gel at 14 and 21 h did not damage the seeds. Cryopreserved seeds showed normal seedlings on MS medium for 14 d, It is found that lettuce, Chinese kale and pak choi had 100% survival. Chinese cabbage and white-flowered cabbage had 60-90.9% and 25-91.60% survival, respectively and were able to grow in outside conditions. Therefore, conservation of these vegetable seeds in LN or the cryopreservation technique by encapsulation-dehydration is suitable.

Keywords: cryopreservation, lettuce, Chinese cabbage, white-flowered cabbage, Chinese kale, pak choi, seeds, encapsulation-dehydration

**P_33**

**Cryopreservation and virus eradication of shallot (**Allium cepa** ‘Aggregatum’) by droplet-vitrification**

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Shallot (Allium cepa ‘Aggregatum’) is one of the important vegetable crops. The present study attempted to develop an efficient droplet-vitrification cryopreservation method for shallot ‘10603’ shoot tips. In vitro stock shoots were maintained on medium consisting of Murashige and Skoog (1962) medium (MS) supplemented with 30 g/L sucrose, 0.5 mg/L BAP, 0.1 mg/L NAA and 8 g/L agar (pH=5.8). Shoot tips (2.0 mm in length) were excised from 4-week-old stock shoots and stepwise precultured with increased sucrose concentrations from 0.3 to 0.5 M, each concentration for 1 d. The precultured shoot tips were then loaded with a loading solution composed of 2 M glycerol and 0.5 M sucrose for 20 min before exposure to PVS3 for 3 h at room temperature. Dehydrated shoot tips were transferred on to aluminum foils (2 × 0.8 cm) prior to a direct immersion into liquid nitrogen (LN) for cryostorage. For thawing, frozen aluminum foils were immediately transferred from LN into unloading solution composed of liquid MS containing 1.2 M sucrose. After incubation at room temperature for 20 min, shoot tips were post-cultured on solidified MS medium containing 0.5 M sucrose for 1 d and 0.3 M sucrose for 1 d before finally post-cultured on SMM for recovery. With this procedure, 94% and 58% of shoot tips survived and regenerated into shoots following cryopreservation. Onion yellow dwarf virus (OYDV) and shallot latent virus (SLV) have been detected and immunohistologically localized from shallot plants and cryotherapy is involved in the attempt of obtaining virus-free plants.

Keywords: Shallot. In vitro culture. Cryopreservation. OYDV. SLV

P_34
Plant cryopreservation in Norway

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Plant genetic resources, including cultivated varieties and wild species, are a prerequisite for breeding in both conventional and genetic engineering programs. Germplasm conservation has an important role to play in the maintenance of biodiversity and in avoidance of genetic erosion. Therefore, conservation of plant germplasm is important and has attracted great attentions internationally. Cryopreservation is considered an ideal means of the long-term conservation of plant genetic resources. Moreover, such storage requires a small volume, demands very limited maintenance area, and thus lowers the cost. Nuclear stock collections represent plants with an added value as free of relevant pathogens. The safeguarding of such collections is an important aspect of cryopreservation.

In Norway, we have been working with cryopreservation of vegetative propagated crops since 2010. We have been focusing on cryopreservation of *Argyranthemum*, strawberry, potato, begonia, raspberry, blackberry and shallot onions. Five research projects regarding cryopreservation have been funded by and involved the following institutions and companies: NIBIO, Norwegian Research Council, NMBU, Sagaplant AS, Piql, Grønn Næringskompetanse, Graminor AS, Norwegian Genetic Resource Center, NorGen, Norsk Gartnerforbund, Gartnerhallen and Tiboplant. Combination of virus diagnosis, virus-free plant material production, cryopreservation of healthy nuclear stocks and relevant plant information, a cryobank of important germplasm and healthy plant material are under development in Norway.

In two of the projects, they have been developed technology, methods and equipment for co-cryo-preservation of plant germplasm and their related digital data and visual information. This guarantees that the relevant data and information will be "brought back to life" at the same time as the plant is.
“Together with relevant companies and international partners, we want to investigate the concept ‘Svalbard Green Vault’, for cryopreservation of vegetative propagated germplasm resources.

Keywords: cryopreservation, genetic resources, Norway

**P_35**

**How applicable are dormant buds in cryopreservation of horticultural woody plant crops?**

**The Malus case**

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The method of using dormant buds (DB) for germplasm cryopreservation was published a few decades ago and since then, it was used in preserving genetic resources of selected horticultural woody-plant species. The advantages of employing DB in preservation are widely known; the most relevant are no requirements of aseptic cultures, high processing throughput and involvement of a relatively low skilled technical support; but the method has also shortcomings as seasonality of processing and lack of procedural modifications that might support preservation of all accessions in a collection. The U.S., NLGRP cryopreserved DB of 2,115 unique Malus accessions (in 51 species) with a \( \geq 40\% \) post cryo viability (lab standard). The method worked for all accessions in 20 species, for \( \geq 90\% \) of accessions in six species and at a various percent (0-89\%) in the remaining 25 species. For species with the largest number of processed accessions, as *M. domestica*, *M. hybr.* and *M. siviersii* (1,334, 326 and 88 cryopreserved accessions respectively), the percent of accessions responding favorable to the DB method was high (97, 96 and 82\%). The results indicated variability in *Malus* DB response to LN exposure; similar results were recorded in the *Pyrus* and *Ribes* collections. Procedural refinements of the DB cryopreservation method might support the method applicability to a much higher number of accessions and its use in other collections of horticultural woody plants. Our study on *Vaccinium* cryopreservation showed twig pre-harvest temperature was critical for a successful cryopreservation of DB, as well as application of cryoprotectants and antioxidants in cryopreservation processing of *Prunus* DBs.

Keywords: modifications, processing throughput, variability, viability