

**COST Action FA1104 'Sustainable production of high-quality cherries for the European market'**

**Workshop**

***Long Term Preservation of Woody Species  
by Cryo-Techniques***

26 & 27 March, 2015

CNR Research Area of Florence, Italy

**Book of Abstracts**



**Workshop “Long Term Preservation of Woody Species by  
Cryo-Techniques”. Florence, Italy, 26-27 March 2015**

# Workshop

## *Long Term Preservation of Woody Species by Cryo-Techniques*

26 & 27 March, 2015

CNR Research Area of Florence, Italy

### Organizers

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### Acknowledgements

This workshop has been organized within WG1 ‘Genetic resources and breeding, Genetics and Genomics’ established in the COST Action FA1104.

COST Action FA1104 ‘Sustainable production of high-quality cherries for the European market’ (<https://www.bordeaux.inra.fr/cherry/>) is aimed to develop innovative strategies to safeguard European cherry production through active networking.



Members of ECPGR (European Cooperative Programme for Plant Genetic Resources) Working Group on *Prunus* (<http://www.ecpgr.cgiar.org/working-groups/prunus/>)

participated and supported the organization of the workshop, whose aims are in line with the scope and objectives of ECPGR. The outputs of the event will be disseminated in

collaboration with the ECPGR *Prunus* WG.



**Società di Ortoflorofrutticoltura Italiana**

**Working Group**

**‘Micropropagation and *In Vitro* Techniques’**

**Workshop “Long Term Preservation of Woody Species by  
Cryo-Techniques”. Florence, Italy, 26-27 March 2015**

## PROGRAMME

March 26 <sup>th</sup> , Thursday	
9.00 – 9.45	<i>Registration of participants</i>
9.45 – 10.15	<p><b>Welcome greetings</b>  Daniela Giovannini, <i>Management Committee of COST Action FA1104</i>  Mauro Centritto, <i>Director of IVALSA</i>  Maurizio Lambardi, <i>SOI-Italian Society of Horticulture</i></p>
10.15 – 11.00	<p>Applied plant cryobanking at CGIAR plant collections: from protocol development to genebank management  <b>Bart Panis</b>  <i>Biodiversity International, Leuven, Belgium</i></p>
11.00 – 11.30	<i>Coffee break</i>
11.30 – 12.00	<p>Cryopreservation of woody species and cryotherapy  <b>Maurizio Lambardi</b>  <i>CNR-IVALSA, Trees and Timber Institute, Florence, Italy</i></p>
12.00 – 12.30	<p>Cryopreservation of <i>Malus</i> and <i>Fragaria</i>  <b>Monika Höfer</b>  <i>Julius Kuhn Institute, Dresden, Germany</i></p>
12.30 – 13.00	<p>Cryopreservation of Ornamentals  <b>Adela Halmagyi</b>  <i>Institute of Biological Research, Cluj-Napoca, Romania</i></p>
13:00– 13:30	<p>Cryopreservation of conifers: challenges and bottlenecks  <b>Jana Krajnakova</b>  <i>University of Udine, Italy</i></p>
13.30 – 15.00	<i>Lunch</i>
15.00 – 15.45	<b>Short presentations of video-posters (5-10 minutes each)</b>
15.45 – 16.15	<i>Coffee break</i>
16.15 – 17.00	<b>Visit to the Laboratory of Cryopreservation and In Vitro Techniques of IVALSA</b>
17.00	<i>End of first day</i>
20.00	<i>Social Dinner (only for invited speakers and COST FA1104 members)</i>

**Workshop “Long Term Preservation of Woody Species by  
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<b>March 27<sup>th</sup>, Friday</b>	
<b>9.00 – 9.30</b>	<p>Cryopreservation of synthetic seeds  <b>Aylin Ozudogru</b>  <i>CNR-IVALSA, Trees and Timber Institute, Florence, Italy</i></p>
<b>9.30 – 10.00</b>	<p>Overview on <i>Prunus</i> cryopreservation with focus on cherry  <b>Emilia Caboni</b>  <i>CRA-FRU, Fruit Tree Research Center, Rome, Italy</i></p>
<b>10.00 – 10.30</b>	<i>Coffee break</i>
<b>10.30 – 11.00</b>	<p>Genetic and epigenetic stability assessment of plants regenerated from cryopreservation  <b>Anna De Carlo</b></p>
<b>11.00 – 11.15</b>	<p>Pollen cryopreservation  <b>Carla Benelli</b>  <i>CNR-IVALSA, Trees and Timber Institute, Florence, Italy</i></p>
<b>11.15 – 11.30</b>	<p>Some experiences in the field of <i>in vitro</i> cryopreservation of woody species (cherry species included) in the Fruit Research Institute of Čačak (Serbia)  <b>Tatjana Vujović</b>  <i>Fruit Research Institute Kralja Petra, Čačak, Serbia</i></p>
<b>11.30 – 12.00</b>	<b>Final discussion and general considerations</b>
<b>12.00 – 13.00</b>	<i>Lunch</i>
<b>13.00 – 13.30</b>	<b>Conclusion of the Workshop</b>
<b>14.00</b>	<p>Departure to <b>Oscar Tintori Nursery</b>  <i>(only for invited speakers and COST FA1104 members)</i></p>
<b>15.00 – 17.00</b>	<p><b>Visit to the Oscar Tintori Nursery, Pescia, Pistoia.</b>          Guided visit to the "Hesperidarium", a reach and unique collection of ornamental citrus plants. Tasting of own-produced olive oil and citrus jam.</p>
<b>17.00 – 18.00</b>	Return to Florence

## Video-Posters

### ***In vitro* preservation of patrimonial fruit varieties of Emilia-Romagna region, Italy**

Irene Quacquarelli, Nives Gimelli, Daniela Giovannini  
*CRA-FRF, Fruit Tree Research Unit, Forlì, Italy*

### **Preliminary results of cryopreservation experiments aimed to long-term maintenance of Hungarian sour cherry germplasm**

Tamás Lakatos, Tímea Tóth  
*Research Institute for Fruitgrowing and Ornamentals, Budapest, Hungary*

### **Droplet vitrification cryopreservation of *Prunus mahaleb* using shoot tips from *in situ* plants**

Heider Saleh, Sezai Ercisli  
*Department of Horticulture, Ataturk University Agricultural Faculty, Erzurum*

### ***In vitro* methods for increasing genetic variability and preserving woody fruit and ornamental species biodiversity applied in Fruitgrowing Institute Plovdiv, Bulgaria**

Lilyana Nacheva, Petya Gercheva  
*Fruitgrowing Institute, Plovdiv, Bulgaria*

### **Application of the droplet vitrification method for cryopreservation of temperate fruit species**

Alessandra Sgueglia, Federica Piombino, Diletta Dietrich, Maria Antonietta Germanà, Cinzia Forni, Adele Gentile, Andrea Frattarelli, Simona Monticelli, Emiliano Condello, Emilia Caboni

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# ***ABSTRACTS***





## Applied plant cryobanking at CGIAR plant collections: from protocol development to genebank management

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Key words: banana, cryopreservation, droplet vitrification, *Musa*

Research at CGIAR (the Consultative Group on International Agricultural Research) is dedicated to reducing rural poverty, increasing food security, improving human health and nutrition, and ensuring more sustainable management of natural resources. This research is carried out by 15 Centers, in close collaboration with hundreds of partner organizations. The CGIAR collections encompass seed propagated as well as vegetatively propagated crops. Seed is classically stored at -20°C (and sometimes cryopreserved) while vegetatively propagated crops are maintained in the field, stored as in vitro collection under reduced growth conditions or through cryopreservation. Both crop types, seed and vegetatively propagated crops, are preserved in 11 CGIAR centers and it is estimated that by 2015 the number of stored accessions will increase to 750 000.

Until recent, banana collections existed mainly as field and in vitro collections. However, in the year 2003 a cryobank was established at the Bioversity International Transit Centre (ITC), Leuven Belgium. First experiments on banana cryopreservation already started back in 1985 but it was only after the optimization and application of the droplet vitrification protocol to banana that cryobanking could practically start. This method can be applied to meristems excised from rooted in vitro plants as well as to “cauliflower-like” meristem clumps. Which tissue to use depends on the genome constitution of the banana cultivar.

A banana accession is considered as safely stored provide three independent and successful experiments are executed. For extra safety reasons, one repetition per accession is safely backed up at IRD (Institut de recherche pour le développement) in Montpellier, about 1000 km from Leuven. One skilled technician can cryopreserve on average 40 accessions per year, medium preparation, plant multiplication, post-thaw testing included. Thanks to funds received from donors such as the World bank, The Gatsby Charitable Foundation, Genebank Coordinated Research Programme managed by the Crop Trust we have cryopreserved 902 accessions to date belonging to all *Musa* groups. This represents 64 % of the accessions stored in vitro and is thus one of the world’s largest cryopreserved plant collections percentage wise.

More and more plant genebanks start now to cryopreserve their collections as a safety backup. Genebank curators, however, are often confronted with the fact that their cryopreservation protocol is not applicable to all accessions within a given species or that the preserved material (shoots, seed, embryos, callus,...) does not grow out into normal plants at desired frequencies. In this presentation, issues dealing with safety (of material as well as of staff), management, the importance of testing regeneration towards a normal rooted plants versus survival will be discussed.



## Cryopreservation of woody species and cryotherapy

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Key words: cryopreservation, shoot tips, embryogenic callus, dormant buds, seeds

First attempt to cryopreserve woody plant material dates back to 1960, when Akira Sakai showed that one-year-old twigs of poplar and willow species were not injured when cooled to  $-196^{\circ}\text{C}$ , if they were first held at low temperatures for some hours. Thirty years later, Sakai and co-workers developed the Plant Vitrification Solution n° 2 (PVS2) which showed to be very effective for the induction of vitrification in nucellar cells of *Citrus sinensis* during ultra-rapid freezing in liquid nitrogen. Since then, the number of PVS2-based protocols, developed for the cryopreservation of woody plant shoot tips, increased yearly, while at the same time new and effective encapsulation- and droplet-based methods were also proposed. A range of different cryo-techniques is now available for the cryostorage of woody species germplasm, allowing the safe long-term conservation in liquid nitrogen of different organs and tissues, coming from tissue culture or directly collected in the field, such as:

- shoot tips and nodal segments, obtained *in vitro* by axillary or apical buds and used naked or encapsulated in Ca-alginate beads. They are the most used explants with broad-leaf trees, provided that optimized protocols of micropropagation have been achieved;
- embryogenic callus samples, largely used with conifer species for which efficient micropropagation procedures from mature stock plants are rarely available. Also organogenic callus can be used with broad-leaf species;
- dormant buds, directly collected from the field, which are cryo-stored after the application of a simple protocol, based on bud desiccation and slow cooling. The technique allows for a marked reduction of time and labour, in comparison with the cryopreservation of germplasm using tissue culture-based methods, but it can be developed only with species that can be chip-grafted, such as apple, pear and cherry;
- seeds and embryonic axes, useful material for the long-term preservation of seed-propagated species. In polyembryonic species (such as citrus), they allow also the conservation of clonal germplasm;
- pollen, a particularly precious material in fruit plant breeding.

Recently, cryotherapy (i.e., the use of liquid nitrogen to recover pathogen-free plants) showed to allow eradication of viruses, phytoplasma and bacteria from infected material. The examples of efficient procedures for woody species, although still limited, are promising.



## Cryopreservation of *Malus* and *Fragaria*

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**Key words:** *Malus*, *Fragaria*, cryopreservation, dormant buds, PVS2 vitrification

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

The aim was the elaboration and optimization of the cryopreservation in *Malus* and *Fragaria* to establish a basis for a duplicate collection and to perform one demand in the National Program of Plant Genetic Resources. At present the Fruit Genebank in Dresden-Pillnitz comprises 281 strawberry cultivars, 270 *Fragaria* wild species accessions and 507 *Malus* wild species accessions.

The method of the cryopreservation elaborated within the EU-Project ‘European Small Berries Genetic Resources’ GENBERRY (AGRI GEN RES 036) in strawberry (PVS2 vitrification protocol with cold acclimation; Höfer and Reed 2012) was adapted and 84 strawberry cultivars (*Fragaria* × *ananassa* Duch.) and 50 *Fragaria* wild species accessions were tested. The average of recovery was 86 %; all tested accessions passed the aspired baseline for storage minimum recovery of 40 %. Currently, the institute has 174 different *Fragaria* accessions in the permanent cryopreservation.

Based on the preliminary results of the cryopreservation of dormant *Malus* buds under the conditions of the Fruit Genebank in Dresden-Pillnitz (Höfer, 2007; Höfer, 2010), the results of the further optimization and standardization were summarized in the article ‘Cryopreservation of winter-dormant apple buds – Establishment of a duplicate collection of *Malus* germplasm’ (Höfer 2015; Journal Plant Cell, Tissue and Organ Culture DOI 10.1007/s11240-015-0735-1). Of the 73 *Malus* wild species accessions tested, 60 had living buds, and recovery was > 40 % for 34 accessions and these averaged 71 % recovery.

The results of the project demonstrated that the cryopreservation of *Malus* and *Fragaria* accessions could be an effective method of duplicate collection. A concept including the implementation for the further establishment of the cryopreservation of the genetic resources in apple and strawberry (cultivars and wild species accessions) will be elaborated.



## Cryopreservation of ornamentals

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Key words: *Chrysanthemum*, *Dianthus*, *Rosa*, long-term storage, shoot apices

The commercial production of ornamental species and varieties is constantly growing and becoming an important part of the economy. New cultivars with horticultural and economic value are produced every year replacing the existing assortments. Over the years several cryopreservation methods have been performed as the only viable procedures for the long-term conservation of vegetatively propagated plant genetic resources. Despite of the growing number of ornamental species cryopreserved in various countries, a large-scale routine application of cryopreservation for long-term germplasm storage in genebanks is still limited. Different cryopreservation methods have been implemented for some vegetatively propagated ornamental species like chrysanthemum (*Chrysanthemum morifolium* L.), carnation (*Dianthus caryophyllus* L.) and roses (*Rosa x hybrida*). Chrysanthemum shoot tips were cryopreserved by controlled-rate freezing (highest regrowth after preculture in 0.5 M sucrose and 0.25°C/min cooling rate), encapsulation-dehydration (regeneration after preculture in 0.5 M or 0.75 M sucrose and 4-5 h dehydration in laminar flow cabinet), DMSO-droplet freezing (maximum shoot regrowth after 2 h incubation in 7% DMSO) and droplet-vitrification (highest regrowth after 5 min dehydration in PVS2 100% or 15 min dehydration in PVS2 60%). Droplet-vitrification of carnation and rose cultivars lead to high regeneration after 20-25 min PVS2 dehydration (according to cultivar) while cryopreservation by encapsulation-vitrification of carnation shoot tips after 24 h sucrose (0.5 M) preculture and dehydration at 0°C was the most effective in achieving high rates of regeneration. Although many cryopreservation studies have been developed to minimize freezing damage and to enhance regrowth, the genetic stability of conserved material still remains a major concern.





## Cryopreservation of conifers: challenges and bottlenecks

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Key words: somatic embryogenesis, cryopreservation, slow-cooling method, clonal forestry, genetic fidelity

In the present moment, forests cover more than 4 billion hectares of the Earth's surface and their importance as a carbon sink is enormous. In Europe, forests represent almost half of the land surface (102 million ha, which amount to 25 % of the world total), of which 65 % are conifers. Conifers are characterized, as many forest tree species, by long rotation times and regeneration intervals and by large size. Traditionally, the conservation strategies for forest trees are based on *in situ* and *ex situ* conservation. Somatic embryogenesis (SE) of conifers became an important propagation technique and together with cryopreservation has been employed into tree breeding programs of several coniferous species. For clonal forestry, cryopreservation facilitates the recovery of elite genotypes following long-term selection trails and the subsequent establishment of production clone banks for commercial application. In conifer SE, cryopreservation is applied exclusively to the juvenile cultures, embryogenic cell masses, although there is potential for cryogenic storage of somatic and zygotic embryos. To date, cryopreservation of embryogenic cultures has been successful for more than 30 species and their hybrids, represented by the genera *Abies* (3), *Larix* (6), *Picea* (9), *Pinus* (11) and *Pseudotsuga* (1). The most frequently applied cryopreservation technique is slow-cooling and fast-thawing; however, vitrification-based method has been successfully used as well. Because zygotic embryos of many conifers develop desiccation tolerance during maturation, methods for developing desiccation tolerance in SE in preparation for cryostorage have also been successful. Although, the first reports on the cryopreservation of conifers were published more 25 years ago, the experience and reports on the effects of prolonged storage in liquid nitrogen are still limited, and the genetic fidelity at DNA level of the cryopreserved material has rarely been considered. On the other hand, cryopreservation as a cost-effective, low labor- and space-demanding alternative will have an important role for conservation of coniferous tree species.



## Cryopreservation of synthetic seeds

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Key words: alginate encapsulation, encapsulation-dehydration, encapsulation-vitrification, gelation method, cryo-plate method

Synthetic seed, also referred as synseed, artificial seed, manufactured seed, bead or capsule, is, by definition accepted today, an encapsulated somatic embryo, or any other tissue (shoot bud, seed, zygotic embryo, callus tissue, etc.) which mimics the real seed for sowing and possesses the ability to convert into a plantlet under *in vitro* or *ex vitro* conditions. It serves as an analytical tool in zygotic embryogeny for determination of the roles of endosperm and seed coat for embryo development and germination, enables the safe, easy and cost-effective transportation of the disease-free germplasm, even between the countries, without obligations from quarantine department, and is frequently used for large-scale propagation of especially rare, endangered, genetically-engineered elite genotypes. As synthetic seed can retain its ability to convert into a plantlet even after storage, it is also used effectively for *ex situ* conservation, both for medium- and long-term. Its long-term conservation by cryopreservation was first proposed by Dereuddre *et al.* (1990), where *in vitro* axillary shoot tips of pear tree (*Pyrus communis*) were alginate-coated and dehydrated prior to their immersion in liquid nitrogen. This very first application of cryopreservation by encapsulation-dehydration was followed by many others, using several different explant types (i.e., seeds, zygotic embryos or embryonic axes, somatic embryos, embryogenic callus cultures, cell suspensions, shoot tips, protocorms, hairy root cultures, ovules and gametofits) of numerous plant species. A year later, encapsulation-vitrification approach, where encapsulated explants were dehydrated chemically through the use of highly-concentrated vitrification solutions, was developed by Tannoury *et al* (1991), using *in vitro* shoot tips of carnation (*Dianthus caryophyllus*). The method was then applied successfully to zygotic or somatic embryos, embryogenic callus cultures, shoot tips, protocorms/protocorm-like bodies, and hairy root cultures of numerous species. Today, also cryo-plate method, a novel approach that combines the advantages of encapsulation and droplet vitrification is also available, which already produced very promising results with *in vitro* shoot tips of potato (*Solanum tuberosum*), mint (*Mentha* spp.) and Dalmatian chrysanthemum (*Tanacetum cinerariifolium*). In the present paper, different types and preparation methods of a synthetic seed, its advantages, as well as its application in cryopreservation will be summarized.



## **An overview on *Prunus* spp. cryopreservation with focus on cherry**

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Key words: *Prunus avium*, *Prunus cerasus*, encapsulation-dehydration, vitrification, droplet-vitrification

*Prunus* is a genus of the *Rosaceae* family which includes a large number of ornamentals and fruit trees such as peach, apricot, plum, cherry and almond. Many of these species are of great economic importance in temperate regions. Due to their high heterozygosity, these species are vegetatively propagated and are traditionally conserved in *ex situ in vivo* collections. However, in field conservation presents some limits related to the costs of orchard management and to the climatic adversity and pathogen attacks. Thus, cryopreservation of apices of lateral buds of *in vitro* growing shoots represents a suitable tool for a safe and low-cost long-term conservation of these fruit species. During the last years several approaches for cryopreservation of these species have been proposed, based, according with the species, on encapsulation-dehydration, vitrification and droplet-vitrification methods. An overview on the principal studies realised will be presented and discussed, trying to highlight the critical factors to obtain efficient protocols in cherry and the other *Prunus* species.



## Genetic and epigenetic stability assessment of plants regenerated from cryopreservation

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Key words: genetic stability, DNA methylation, cryopreservation, *in vitro* culture

The aim of successful cryopreservation is to maintain genetically stable plant material stored in liquid nitrogen although some questions often arise on the genetic and epigenetic stability. The presentation has main objective to focus attention on this fundamental aspect of cryopreservation in order to respond properly to the strategies of germplasm preservation. Some papers will be presented on possible effects of cryopreservation on genetic and epigenetic stability.

In theory, cell division and all metabolic activities should be stopped when plants are exposed to ultra-low temperatures, so after rewarming from cryopreservation true-to-type plants are to be expected. Genetic changes are characterized by changes in DNA nucleotide sequences. In contrast, epigenetic modifications do not change the original DNA sequence, but may, nevertheless, result in heritable changes of gene expression patterns. Processes that can be epigenetically altered include regulation of gene expression, activity of transposable elements, defence against foreign DNA, and inheritance of specific gene expression patterns. Modifications of these processes can be caused by DNA methylation, modifications to particular histones and alterations in chromatin structure. DNA methylation is required for regular development of plants and can have an impact on vigour and morphogenesis, so modification of epigenetic features could result in altered phenotypes in the recovered plants. Different molecular markers (RAPD, AFLP, RFLP) have proven to be key tools for the detection of variations in primary DNA structure. There are several publications reporting the use of these markers to assess stability after cryopreservation; in most of these studies, no differences in DNA markers were found before and after cryopreservation. To analyse potential epigenetic changes, the DNA methylation status was assayed by methylation-sensitive amplified polymorphism (MSAP) analysis.

Modifications in DNA methylation have been found in same tissue culture and cryopreserved plants. They might be caused by stressful *in vitro* conditions, osmotic dehydration and the application of cryoprotective agents.

It is, therefore, of high practical and scientific interest to assess how genetic and epigenetic stability of *in vitro* propagated and cryopreserved plants is affected by parameters of *in vitro* culture procedures or by cryo-injury caused by physical, chemical and physiological stresses during cryopreservation.





## Pollen cryopreservation

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Key words: long-term preservation, low temperatures, liquid nitrogen, kaki, olive

Pollen is used to a limited extent in ex situ conservation, mainly for forest/tree genetic resources although several reasons can validate an its much wider use in conservation, moreover the importance of pollen conservation in horticultural species is well documented. Collection and storage of pollen grains can be a tool to obtain a wide range of genetic diversity in a population and it represents an effective propagule for genebanks. The possibility to store pollen for long-term in cryobank and prolong its longevity increases the opportunity for researchers in biotechnological and basic studies and in particular in breeding programs overcoming seasonal and geographical restrictions. Pollen is manageable for cryopreservation and this preservation method has been applied in different plant species. Few information are available on cryopreservation of *Prunus* pollen, the first investigation has been reported by Parfitt and Almedhi in 1984.

The present research shows our experience on ultra-low temperatures,  $-80^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$  (liquid nitrogen), applied to pollen grains of three accessions of *Diospyros kaki*, one of *D. lotus* one of *D. virginiana* and eight cultivars of *Olea europaea* for various time periods (15, 30, 90, 180 and 360 days). After each storage period the viability was assessed on different sets of pollen grains by fluorochromatic reaction with the double coloration with fluorescein diacetate/ propidium iodide and *in vitro* germination rate was observed. Ultra-low temperatures exerted a significant positive effect on viability and *in vitro* germinability of pollen grains, in particular, preservation in liquid nitrogen resulted the most reliable method of storage for all tested genotypes.



## Some experiences in the field of *in vitro* cryopreservation of woody species (cherry species included) in the Fruit Research Institute Čačak (Serbia)

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**Key words:** encapsulation-dehydration, vitrification, droplet-vitrification, V-cryo-plate, D-cryo-plate

Research on the application of different cryopreservation techniques for the long-term conservation of temperate fruit species has been initiated within the Department of Fruit Physiology of FRI, Čačak at the beginning of 2008. Namely, COST Action 871 CryoPlaNet had a great impact in plant cryopreservation activities within this Department and enabled collaboration with institutions and researchers that are great experts in this field. Encapsulation-dehydration technique has been successfully applied for cryopreservation of genotypes belonging to *Prunus* (cherry plum, *P. cerasifera* Ehrh.) and *Rubus* (*R. fruticosus* L. ‘Čačanska Bestrna’) genera. The possibility of cryopreservation of raspberry (*R. idaeus* L. ‘Latham’) using droplet-vitrification, encapsulation-dehydration and vitrification techniques was examined in collaboration with research group from CRA-FRU in Rome, Italy. Also, optimization of the droplet-vitrification protocol for cryopreservation of *in vitro* grown shoot tips of blackberry ‘Čačanska Bestrna’ and cherry plum was performed by evaluating the effect of different vitrification solutions, treatment durations, temperature of dehydration, as well as duration of unloading on recovery of explants after rewarming. This reserach was performed in collaboration with IRD, Montpellier, France. The droplet-vitrification technique has been successfully applied to other representatives of *Prunus* genus – autochthonous plums ‘Crvena Ranka’ (*P. insititia*) and ‘Sitnica’ (*P. domestica*), plum cultivars ‘Požegača’ and ‘Krina’ (*P. domestica*) and cherry rootstock Gisela 5 (*P. cerasus* × *P. canescens*) and *Malus* genus – apple cultivar ‘Gala Must’ (*Malus* × *domestica* Borkh.). Research on application of vitrification technique for cryopreservation of *Prunus* and *Malus* genetic resources has also been performed and results obtained have shown that significant increase in regrowth of cryopreserved shoot tips of cherry rootstock Gisela 5 and apple ‘Gala Must’ can be achieved by varying duration of dehydration and/or type of vitrification solution employed. Recently, the research on application of newly developed cryopreservation techniques using aluminium cryo-plates (V-Cryo-plate and D-Cryo-plate methods) has also been initiated in the framework of bilateral cooperation with IRD, Montpellier. The shoot tips of plum ‘Požegača’, cherry plum and cherry rootstock Gisela 5 were successfully cryopreserved using these methods which allow overcoming such disadvantages as mistiming dehydration, damage and loss of material, and manipulative problems of different vitrification procedures.



## ***In vitro* preservation of patrimonial fruit varieties of Emilia-Romagna region, Italy**

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Key words: *Prunus avium*, genetic resources, micropropagation, cold storage

In the recent years, the Fruit Research Unit of Forlì (CRA-FRF) has embarked on a programme aimed to recover the patrimonial fruit varieties of Emilia-Romagna region (Southern Po Valley, Italy), to preserve from erosion their valuable historical, agronomic and adaptive traits. Accessions recovered at local farms have been included in the CRA-FRF fruit tree Heritage collection where they are now undergoing phenological, morphological and genetic characterization. In parallel, as a further guarantee against accidental losses or damage from abiotic and biotic agents, CRA-FRF has also started a plan of *in vitro* preservation.

The most valuable and genetically unique accessions of the Heritage collections are being replicated *in vitro* and protocols of establishment and *in vitro* proliferation are being optimized for each variety. Moreover, the application potentials of the slow growth preservation technique are being evaluated, with the aim to slow down *in vitro* shoot growth and limit demands and risks of subcultures. For sweet cherry, in particular, protocols of micropropagation and slow growth conservation have been optimized for each of the patrimonial varieties recovered, and trials of cryopreservation have been recently initiated.



## **Preliminary results of cryopreservation experiments aimed to long-term maintenance of Hungarian sour cherry germplasm**

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Key words: sour cherry, controlled rate freezing, PVS2

The horticultural germplasm collection of the Research Station at Újfehértó contains more than 2 000 accessions, mainly apple, pear and sour cherry varieties. The series of local sour cherry clones is the most valuable part of the collection, because a successful breeding work based on it. The whole germplasm is located in a 20 ha orchard. The management cost of the collection is significant and increasing. The aim of this present cryopreservation work is to find safe and labour effective alternative of the classical field germplasm collection.

According to Zhao et al. (2008) five weeks old micropropagated sour cherry plants cv. ‘Csegöldi’ were cold adapted by hold them in an incubator programmed to 16/8 h day/night cycle with 23/4 °C. Dissected CA shoot tips were precultured for 48 h in MS medium containing DMSO (5%) and proline (2%). In 2 ml cryotubes shoot tips were equilibrate with PVS2 or modified PVS2 (containing 3% polyethylene glycol) solution for 30 minutes, and cooled at 1 °C/min rate to -40 °C and submerged into LN2.

The recovery rate after six weeks period was relatively low, 27% and 38% treated with PVS2 or modified PVS2, respectively. However, this result suggests that cryopreservation would be a useful technique to maintain our local sour cherry clones.





## **Cryopreservation of *Prunus mahaleb* using shoot tips from *in situ* plants**

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Key words: vitrification, *Prunus mahaleb*, micropropagation

The present study aims to use vitrification method for long-term storage of shoot tips in *Prunus mahaleb*. For micropropagation, maximum number of shoots per explant was obtained on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L BA (benzyl amino purine). Several pretreatments prior to cryogenic storage were conducted to enhance shoot tips recovery after cryostorage. Using vitrification method, two different preculture treatments were tested to enhance regrowth. Further accumulation of sucrose and glycerol was tested. The highest regrowth using this method was (up to 70.4%) when shoot tips were exposed to 60 min PVS2 at 0°C. Recovery of whole plantlets from cryopreserved shoot tips took place directly without transitory callus formation. Vitrification method is promising technique for *in vitro* propagation and germplasm preservation of *P. mahaleb* shoot tips.



***In vitro* methods for increasing genetic variability and preserving woody fruit and ornamental species biodiversity applied in Fruitgrowing Institute Plovdiv, Bulgaria**

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Key words: micropropagation, embryo rescue, somatic regeneration, somaclonal variation

This paper presents the major achievements in the field of biotechnological approaches for woody trees improvement in Fruitgrowing Institute in Plovdiv, Bulgaria. Our research is focused on *in vitro* embryo rescue of sweet cherry and apricot, development of efficient systems for somatic regeneration of pome and stone fruit species (*Malus*, *Pyrus*, *Prunus*), somaclonal variation, virus elimination through *in vitro* techniques, optimization of micropropagation protocols of woody fruit (*Malus*, *Pyrus*, *Prunus*, *Juglans*, *Pistacia*) and ornamentals (*Magnolia*, *Taxus baccata*, *Ginkgo biloba*) and physiological studies of *in vitro* cultivated plants.



## Application of the droplet vitrification method for cryopreservation of temperate fruit species

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Key words: droplet vitrification, temperate fruit species, vitrification solutions

In recent years several new techniques have been developed for the cryopreservation of shoot tips of temperate fruit tree species (Sakai and Engelmann, 2007) and, among those, a modification of the vitrification method, the droplet-vitrification technique, has been also proposed (Panis *et al.*, 2005). In this method the excised explants are frozen in individual micro-droplets of vitrification solution allowing to reach cooling rates considerably higher compared to other vitrification methods (Panis *et al.*, 2005). In our laboratory this method was applied to different fruit species, such as apple (Condello *et al.*, 2011a), raspberry (Condello *et al.*, 2011b) and, recently, hazelnut, pear and pomegranate. To develop the protocols for each species some factors were compared such as preconditioning of the donor explants, type and time of application of the plant vitrification solutions (PVS2 and 3), growth regulation combination of the recovery medium. A description of the results so far obtained will be presented.

**Workshop “Long Term Preservation of Woody Species by  
Cryo-Techniques”. Florence, Italy, 26-27 March 2015**