STATUS OF CRYOPRESERVATION TECHNOLOGIES IN PLANTS (CROPS AND FOREST TREES)

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Summary
Over the past decades, plant cryopreservation technologies have been evolving rapidly, opening the door to the possibility of long-term storage of valuable genetic resources of many crop and forest species. From the original slow-cooling approach, research has moved to easier and more reproducible techniques which allow a complete vitrification of extra- and intra-cellular liquids through the direct immersion of explants in liquid nitrogen. This report describes concisely most of the procedures which have been proposed in time for the cryopreservation of a wide range of tissues and organs, such as cell suspensions, embryogenic callus, pollen, meristematic tissues, seeds and embryo axes. Also the most important achievements in the cryopreservation of herbaceous, hardwood and softwood species are discussed.

Keywords
Cryopreservation, encapsulation/dehydration, plant genetic resources, slow cooling, vitrification

Introduction

It is estimated that up to 100,000 plants, representing more than one third of all the world's plant species, are currently threatened or face extinction in the wild [3]. Preservation of the plant biodiversity is essential for classical and modern (genetic engineering) plant breeding programmes. Moreover, this biodiversity provides a source of compounds to the pharmaceutical, food and crop protection industries. Since the 1970s, large numbers of landraces and wild relatives of cultivated crops have been sampled and stored in ex situ gene banks. It is estimated that 6 million samples of plant genetic resources are held in national, regional, international and private gene bank collections around the world [26]. Storage of desiccated seeds at low temperature, the most convenient method to preserve plant germplasm, is not applicable to crops that do not produce seed (e.g., bananas) or with recalcitrant seed (i.e., non-orthodox seed that can not be dried to moisture contents that are low enough for storage, as for instance many tropical trees), as well as to plant species that are propagated vegetatively to preserve the unique genomic constitution of cultivars (such as fruit and several timber and ornamental trees). Preservation only in field collections is risky, as valuable germplasm can be lost (genetic erosion) because of pests, diseases and adverse weather conditions. Moreover, the maintenance of clonal orchards is labour-intensive and expensive. The maintenance of *in vitro* collections (established for some vegetatively-propagated species) is labour-intensive as well, and there is always the risk of losing accessions due to contamination, human error or somaclonal variation (i.e., mutations that

occur spontaneously in tissue culture, with a frequency that increases with repeated subculturing).
Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid nitrogen) is a sound alternative for the long-term conservation of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested. As such, plant material can be stored for unlimited periods. Moreover, besides its use for the conservation of genetic resources, cryopreservation proved to be extremely useful for the safe long-term storage of plant tissues with specific characteristics, such as medicinal- and alkaloid-producing cell lines, hairy root cultures, genetically transformed [14] and transformation-competent culture lines [20]. Recently, it was also proven that cryotherapy can be successfully applied to eradicate viruses from plum, banana and grape [4, 23, 66]. However, despite the fact that cryogenic procedures are now being developed for an increasing number of recalcitrant seeds and *in vitro* tissues/organs, the routine utilization of cryopreservation for the preservation of plant biodiversity is still limited.

1. Theoretical basis of plant cryopreservation

Cryopreservation of biological tissues can be successful only if intra-cellular ice crystal formation is avoided, since this causes irreversible damage to cell membranes thus destroying their semi-permeability. In nature, some plant species adopted systems where ice crystal formation at sub-zero temperatures can be avoided through the synthesis of specific substances (such as sugars, proline and proteins) that lower the freezing-point in the living plant cells, resulting in ”supercooling”. Such ‘avoidance’ of crystallization, while still maintaining a minimal moisture level needed to maintain viability, it is not possible when dealing with ultra-low temperatures of cryopreservation (-196°C). Crystal formation, without an extreme reduction of cellular water, can only be prevented through “vitrification”. Vitrification refers to the physical process of transition of an aqueous solution into an amorphous and glassy (i.e., non-crystalline) state [51]. Two requirements must be met for a cell to vitrify: (i) rapid freezing rates, and (ii) a concentrated cellular solution. Rapid freezing rates (6°C/sec) are normally obtained by plunging explants enclosed in a cryovial into liquid nitrogen. Higher cooling rates can be obtained by enclosing the meristems in semen straws, resulting in cooling rates of about 60°C/sec, or using a “droplet freezing protocol” where the material is placed on aluminium foil strips that are plunged directly into liquid nitrogen, giving rise to cooling rates of 130°C/sec [44, 53].
The cell cytosol can be concentrated through air drying, freeze dehydration, application of penetrating or non-penetrating substances (cryoprotectants), or adaptive metabolism (hardening). For a solution to be vitrified at high cooling rates, a reduction in water content to at least 20-30% is required. For dehydration, the following techniques are applied:

1.1. Air drying

Usually, samples are dried by the sterile airflow of a laminar airflow cabinet. Doing so, there is not any control of temperature and air humidity, both influencing strongly the evaporation rate. More reproducible is the air-drying method that uses closed vials containing a fixed amount of silica gel [63].

1.2. Freeze dehydration

Because plant cells rarely contain ice-nucleating agents, during slow cooling crystallization is initiated in the extra-cellular spaces. Since only a proportion of the water that contributes to the extra-cellular solution undergoes transition into ice, the remaining solution becomes more and more concentrated and thus hypertonic to the cell. To restore the osmotic equilibrium, cellular water will leave the protoplast, resulting in cell dehydration. Generally, freezing rates

of 0.5 to 2°C/min, depending on the type and physiological state of the plant material, are applied. These slow-cooling rates are generally obtained using computer-driven cooling devices, stirred methanol baths, and propanol containers held at –70°C.

1.3. Non-penetrating cryoprotective substances
Osmotic dehydration can be obtained through the application of non-penetrating cryoprotective substances, such as sugars, sugar alcohols and high molecular weight additives like polyethylene glycol (PEG).

1.4. Penetrating cryoprotective substances
Commonly used penetrating cryoprotective agents are dimethyl sulphoxide (DMSO) and glycerol. For many applications, DMSO is preferred because of its extreme rapid penetration into the cells. Where DMSO toxicity is a problem, glycerol or amino acids (e.g., proline) are often applied.

1.5. Adaptive metabolism (hardening)
Hardening is a process that increases plants ability to survive the impact of unfavorable environmental stress. This is triggered by environmental parameters, like reduction in temperature and shortening of daylength. Also osmotic changes and abscisic acid (ABA) treatments can have similar effects. Hardening can result in a considerable increase of, for instance, proteins, sugars, glycerol, proline and glycine betaine which will all participate in the increase of osmotic value of the cell solutes.

Most hydrated tissues, however, do not withstand dehydration to moisture contents needed for vitrification (20-30%) due to solution and mechanical effects. Exceptions are pollen, seeds and somatic embryos of most orthodox seed species. The key for successful cryopreservation is thus shifted from freezing tolerance to dehydration tolerance. This tolerance can be induced by chemical cryoprotection with substances like sugars, amino acids, DMSO, glycerol, etc. The mode of action of most of these substances is, however, still far from being understood. Alternatively, tolerance to dehydration can also be induced by adaptive metabolism. For example, it has been observed that cold acclimation in nature often leads to the accumulation of specific proteins, sugars, polyamines and other compounds that can protect cell components during drying. Also alterations in membrane composition are reported, influencing both their flexibility and permeability [47].

2. Available plant cryopreservation protocols

All cryopreservation protocols described in literature use the above-mentioned techniques or combinations. The most commonly applied protocols are:

2.1. Air drying (flash drying, normal drying)
This method is directly applicable to orthodox seed, zygotic embryos and pollen of many common agricultural and horticultural species. Some of these orthodox seeds can even withstand drying below 3% moisture content, without any damage and reduction of viability. Flash (or ultra-rapid) drying proved to be beneficial for recalcitrant zygotic embryos of some plant species [2].

2.2. Classical slow-cooling (or slow-freezing) protocol
This was the first ‘standard’ protocol that was developed for hydrated plant tissues [67]. It is based on slow cooling of specimens (at a rate of 0.5-2°C/min) in the presence of a cryoprotectant solution, generally containing DMSO at a 5-15% concentration. When during the slow-cooling process a temperature of about –40°C is reached, the intra-cellular solution

is considered to be concentrated enough to vitrify upon a subsequent liquid nitrogen plunging. Now, this method is mainly used for cryopreservation of non-organized tissues, like cell suspensions and calli.

2.3. Encapsulation/dehydration

In this method, developed by Fabre and Dereuddre [16], explants (usually meristems or embryos) are firstly encapsulated in alginate beads (which can contain also mineral salts and organics), thus forming "synthetic seeds" ("artificial seeds" or "synseeds"). Then, the synseeds are treated with a high sucrose concentration, dried down to a moisture content of 20-30% (under airflow or using silica gel) and subsequently rapidly frozen in liquid nitrogen. Although the procedure can be considered rather lengthy and labour-intensive, it is observed that the presence of a nutritive matrix (the bead) surrounding the explant can promote its regrowth after thawing.

2.4. Vitrification

First reports on the use of a vitrification solution with plant tissues appeared in 1989 [34, 62]. The technique relies on treatment of explants with a concentrated vitrification solution for variable periods of time (from 15 minutes up to 2 hours), followed by a direct plunge into liquid nitrogen ("vitrification/one-step freezing"). This results in both intra- and extra-cellular vitrification. The vitrification solution consists of a concentrated mixture of penetrating and non-penetrating cryoprotectant substances. The most commonly applied solution, named "PVS2" (Plant Vitrification Solution n° 2), consists of 30% glycerol, 15% ethylene glycol, 15% DMSO (all v/v) and 0.4 M sucrose [52]. Fifteen years after its first report, vitrification is today by far the most widely used cryopreservation protocol. The success of the procedure can be attributed to its easiness, high reproducibility and to the fact that it can successfully be applied to a wide range of tissues and plant species.

2.5. Other protocols

Other available methods are the "droplet freezing" [53], the "preculture method" [42] and the "preculture/dehydration" [12]. These techniques have been up to now applied to only a limited number of plant species and are not described in detail in this report. A recent and promising technique, the "encapsulation/ vitrification" [51], is described in *4.2*.

3. Application of cryopreservation to herbaceous species

3.1. Cell suspensions and callus cultures

Cell suspension and callus cultures are often cryopreserved using the classical slow-cooling protocol. Generally, the main aim of cryopreserving these non-organized tissues is not the long-term storage of the genetic diversity, but the conservation of specific features of tissues that can be lost during normal *in vitro* maintainance. It has been repeatedly reported that the morphogenetic potential of embryogenic callus lines is not affected by their storage in liquid nitrogen (see also *4.1*). Moreover, it was proven that cryopreservation did not affect the expression of a foreign *sam* gene in transgenic *Papaver somniferum* cells [14], or it was even beneficial for pyrethrin biosynthesis by *Chrysanthemum cinerariaefolium* cell cultures [25]. Also the production of regenerable protoplasts is not influenced by cryopreservation, as shown for rice [27, 37], *Festuca* and *Lolium* species [65]. Moreover, cryopreserved rice [7] and maize calli [20] proved to be a constant source of regenerable cell cultures for the production of transgenic plants. Moukadiri and coworkers [39] showed that rice calli, which were stored into liquid nitrogen, had a higher competence for transformation, as indicated by transient gene expression levels. In banana and grape, comparable levels of transient expression, as well as stable transformation, were obtained from cryopreserved and non-

cryopreserved suspension cells [43, 65]. Large scale cryopreservation of non-organized cultures is reported for coffee [17], oil palm [13] and banana [43].

3.2. Cryopreservation of pollen

Pollen is stored for facilitating crosses in breeding programmes, distributing and exchanging germplasm among locations and preserving nuclear genes of germplasm, as well as for studies in basic physiology, biochemistry, fertility and biotechnology, involving gene expression, transformation and *in vitro* fertilization [58]. For example, preservation of pollen can be very useful for cross-pollination of cultivars differing in flowering period. Methods exist for the cryopreservation of pollen from many crops [57], but its application is still rather low and limited to a few research centers [15].

3.3. Cryopreservation of meristematic tissues

Meristematic tissues are the most common explants for the cryopreservation of vegetatively-propagated species, such as fruit trees, and many root and tuber crops. Also, in view of a lower chance for somaclonal variation, organized tissues, like meristems, are often preferred over non-organized tissues, like calli and cell suspensions. Currently, the majority of the reports deal with the encapsulation/dehydration or the vitrification methods. It is presumed that, in view of its rather severe freeze-dehydration, the classical slow-cooling method is good in retaining the integrity of individual cells, but less efficient in retaining the tissue integrity necessary for meristem survival. Many scientific reports exist on the cryopreservation of meristems ("shoot tips"), but its large-scale application is mainly found in fruit crop germplasm collections (see *4.2.*). In case of herbaceous species, the number of accessions stored in liquid nitrogen is significantly smaller, but continuously growing.

At the German Collection of Micro-Organisms and Cell Cultures (DSMZ, Braunschweig, German), meristems of 519 old potato varieties are cryopreserved using the droplet freezing method [38], while at the International Potato Centre (CIP, Lima, Peru), 345 potato accessions are preserved using the vitrification protocol (Panta, *personal communication*). At K.U. Leuven, Belgium, 306 banana accessions are currently safely stored using the droplet vitrification method [44], representing more than 1/4 of the world banana collection (*unpublished*). Significant efforts are also made for cassava [49], garlic [28], mint [24, 59] and Australian endangered species [61].

3.4. Cryopreservation of seeds

Seeds of most common agricultural and horticultural species are tolerant to desiccation and exposure to liquid nitrogen [54]. Cryogenic storage of orthodox seeds can be considered as an alternative to the traditional storage at –20°C. For some plants species, seed longevity at –20°C is only a few years and can thus considerably be increased through storage in the vapour phase of liquid nitrogen. In most cases, simple drying to lower the moisture content to 5-10% is sufficient to resist ultra-low temperatures. Celery is an example of orthodox seed cryopreservation [19].

4. Application of cryopreservation to woody species

Up to now seed and field collections have been the only reliable option for the long-term germplasm preservation of woody species. However, numerous forest Angiosperms (e.g., *Acer* spp., *Quercus* spp., chestnut, horsechestnut, many tropical species) have non-orthodox seeds with a very limited period of conservability. Fruit trees, which are mainly vegetatively propagated, require the conservation of huge numbers of accessions in clonal orchards, including old and newly selected cultivars, local varieties and wild material. The preservation of the *Prunus* European germplasm, for instance, requires the maintenance of over 30,000

accessions in the field repositories of 21 countries [18]. Although clonal orchards play a pre-eminent role in assisting woody plant conservation programmes, their maintenance requires large areas of land, high running costs (mainly for pruning operations, weed and pest management, irrigation, etc.), they are prone to environmental stresses (e.g., heavy frosts, flooding) and to the hazards of pests, diseases and genetic alterations. Hence, a periodic and careful monitoring of the preserved trees is essential.
Nowadays, biotechnology offers a broad range of techniques for the collection, molecular characterization, disease indexing, pathogen elimination, propagation, documentation, preservation and exchanging of disease-free plant genetic resources [1]. In this context, cryopreservation is a sound alternative to seed- and in-field banks for the long-term storage of woody plant germplasm. It is important to emphasize that the storage of specimens at –196°C does not aim to replace the traditional *in situ* and *ex situ* approaches to tree germplasm preservation. Rather, it should be regarded as complementary, in order to develop a multi-option *modus operandi* for the conservation and use of genebanks, to provide a real guarantee against accidental loss of plant genetic resources.

4.1. Organ and tissues from in vitro *culture used for cryopreservation*
Recent advances in tissue culture technology have greatly increased the variety of organs and tissues that have been tested for storage in liquid nitrogen. Among the explants above described for herbaceous species, three categories are mainly used for woody plant cryopreservation, i.e., (i) the shoot tips, differentiated organs which are used for the preservation of vegetatively-propagated plants, such as many fruit and timber tree cultivars for which the maintenance of genetic fidelity is fundamental. Shoot tips (1-2 mm, on average) are obtained from apical or axillary buds, excised from *in vitro*-grown shoot cultures. Under sterile conditions, the bud is handled in order to obtain the apical meristem, surrounded by some of the original leaf primordia and leaflets [40]; (ii) the seeds or the isolated embryo axes, for the preservation of species that are mainly reproduced by seeds. For those species characterized by non- or sub-orthodox seeds, which cannot be stored in traditional seed-banks, cryopreservation can be regarded as an important option for the long-term conservation of genetic resources [46]; (iii) the embryogenic callus, i.e., the most powerful *in vitro* morphogenetic system. Cryopreservation of embryogenic cultures has to be regarded as an important way for long-term preservation of valuable germplasm of woody plants. In seed-propagated species, cryopreservation of embryogenic cultures allows the conservation of plants with non-orthodox seeds. As regards vegetatively-propagated species, embryogenic cultures are not recommended for germplasm preservation, since the appearance of somaclonal variation cannot be excluded. Nevertheless, cryopreservation allows the maintenance of valuable embryogenic lines utilized in bioengineering, e.g., avoiding the loss of embryogenic potential due to repeated subculturing or allowing the storage of transgenic material while field trials are ongoing.

4.2. Cryopreservation of hardwood trees
In recent years, the “vitrification/one-step freezing” technique has been continuously improved and applied to an increasing number of hardwood species. The application of this cryogenic technique to woody species became very popular after the PVS2 solution was introduced for the cryopreservation of *Citrus sinensis* nucellar cells [52]. To date, the PVS2 solution has been successfully used for the cryopreservation of shoot tips from several economically-important hardwood species (e.g., *Malus*, *Pyrus*, *Prunus*, and *Populus* spp., *Vitis vinifera*). In time, the PVS2 solution has repeatedly shown its effectiveness in protecting hardwood-tree shoot tips from damage caused by ultra-rapid freezing, as survival rates higher

than 50% have been generally achieved [32]. Highest percentages of shoot-tip survival (over 90%) are reported for *Prunus jamasakura* [41] and *Populus alba* [30]. When explants are loaded with the PVS2 solution, post-thaw survival is to a great extent influenced by the duration of treatment, which must be long enough to ensure sufficient cell dehydration, without cytotoxic effects. When the solution is applied at 25°C, exposure time ranges from 20 [36] to 120 min [41]. Alternatively, a chilled solution (0°C) can be used to minimize the risk of toxicity [5]. Following the storage in liquid nitrogen, rapid warming in a waterbath is required. This will avoid recrystallization and ensures a proper recovery of the vitrified material. Thawing temperatures ranging from 20°C to 40°C have been proposed for woody species [32]. In *Populus* spp., for instance, after cryopreservation shoot tips were thawed at different temperatures (30 to 50°C). While *P. alba* shoot tips showed best survival when warmed at 40°C, negligible differences were recorded with *P. canescens*. However, for both species, a majority of healthy, well-developing shoots originated, in time, from shoot tips rewarmed at 40°C [29].

The "encapsulation/dehydration" technique has been used for the cryopreservation of shoot tips from 9 different Genera of hardwood species, among which *Malus*, *Pyrus* and *Prunus*. It is noteworthy that, for 50% of the species cryopreserved following an encapsulation-dehydration procedure, a shoot-tip survival of 80% or more has been reported [32]. Recently, a new technique has been proposed, named "encapsulation/vitrification" [51]. The procedure combines the encapsulation of explants with the application of a vitrification mixture. This technique has been already successfully applied to the shoot-tip cryopreservation of apple [45] and plum [11].

Compared to shoot tips, reports dealing with the cryopreservation of embryogenic calli and somatic embryos from hardwood trees are limited. The number of temperate hardwood species for which a "vitrification/one-step freezing" procedure has been developed increased in recent years, including *Castanea sativa* [8], *Fraxinus angustifolia* [56], *Quercus suber* [64], *Olea europaea* [31] and *Aesculus hippocastanum* [33]. Also embryogenic calli of some forest and fruit tropical species have been successfully cryopreserved [e.g., 55, 68]. In the above-mentioned reports, isolated somatic embryos or samples of embryogenic callus are used as explants and treated with the PVS2 or, alternatively, encapsulated and dehydrated, prior to a direct plunge into liquid nitrogen.

For hardwood species, in addition to the "vitrification/one-step freezing" method, the use of the slow-cooling technique is still sporadically reported. However, compared to the vitrification method, slow cooling results in a higher variability of shoot tip survival, which ranges from a minimum of 34% (*Juglans regia*; [10]) up to a maximum of 92% (*Malus* spp.; [69]). For walnut somatic embryos, an encapsulation/dehydration protocol was proposed, followed by the slow cooling and the immersion in liquid nitrogen of the beads [9].

4.3. Cryopreservation of softwood trees

Cryopreservation of embryogenic cultures of conifers is an advanced technology, already successfully applied to numerous species of *Picea*, *Pinus*, *Larix*, *Abies* and *Pseudotsuga*. Over 5000 genotypes of 14 conifer species, for instance, are cryostored in one facility in British Columbia alone [6]. Here, cryopreservation protocols are mainly based upon the slow-cooling technology. At present, no report deals with the cryopreservation of conifer shoot tips.

4.4. Other cryopreservation procedures

Recently, a cryopreservation method for dormant-vegetative buds, based on the original procedure described by Sakai [50], has been applied to 1,915 accessions of apple [60]. For this, winter-collected scions were desiccated in a cold room at –5°C to 30% moisture and very slowly cooled (1°C/h) to –30°C, where they remained for 24 hours prior to being transferred

to the vapour phase of liquid nitrogen (-160°C). After retrieval from storage and grafting onto rootstocks, over 90% of the accessions showed a survival higher than 30%. A more classic "vitrification/one-step freezing" approach has been proposed for the cryopreservation of persimmon (*Diospyros kaki*) winter-dormant axillary buds, directly collected from the field [36].

Finally, as for seed cryopreservation, dehydration/one-step freezing procedures have been successfully applied to intact seeds or embryonic axes of several woody species (see [35, 46]).

4.5. Cryostored woody plant germplasm

The first examples of woody plant repositories using the cryopreservation technology are now available. In addition to the collection of conifer embryogenic lines in British Columbia (see *4.3.*), the following cryobanks can be mentioned [48]:

- the National Seed Storage Laboratory (NSSL) of Fort Collins (USA), with about 2,100 accessions of apple (dormant buds);
- the National Clonal Germplasm Repository (NCGR) of Corvallis (USA), with over 100 accessions of pear (shoot tips);
- the AFOCEL of France, with over 100 accessions of elm (dormant buds);
- the National Institute of Agrobiological Resources (NIAR) of Japan, with about 50 accessions of mulberry.

But also some tropical and sub-tropical woody species are presently cryopreserved, e.g., at ORSTOM (now IRD), France (80 accessions of oil palm [15]), and at the National Bureau of Plant Genetic Resources (NBPGR), India (numerous accessions of citrus, jackfruit, almond, litchi and tea [48]).

5. Genetic integrity of plants from cryopreservation

In the past, numerous reports on somaclonal variation were described as a result of the introduction, manipulation and regeneration of plants *in vitro*. Obviously, this problem can concern the conservation of germplasm at ultra-low temperatures, too. Some peculiarities of this technology (e.g., the blocked metabolism of cells and the absence of subcultures) reduce the risks of genetic and epigenetic alterations to a minimum. On the other hand, cryopreservation results in the exposure of tissues to physical, chemical and physiological stresses which all can cause cryoinjury. Moreover, some threats to genetic stability arise from particular reactions (free radical formation, molecular damage due to ionising radiation) that can still occur at the temperature of –196°C [21], as well as from the common practice of using DMSO as cryoprotectant at concentrations up to 10%. Although the number of reports studying these aspects in detail are still limited, the fact that, up to now, no clear evidence of morphological, cytological or genetic alterations due to cryopreservation has been produced is promising [22].

6. Conclusions

Although the slow-cooling approach was already introduced in the '70s, for a long time cryopreservation of plant tissues was not studied on a wide scale. This was mainly due to the complexity of procedures and the high cost of cryo-freezers. With the development in the early '90s of "new" and simplified cryopreservation protocols, based on the prevention of intra- and extra-cellular ice crystals by means of cell vitrification and direct immersion of explants in liquid nitrogen, the cryostorage of genetic resources has become a realistic target for most plant species. Nowadays, although the vitrification protocol can almost be

considered as a standardized protocol, a large amount of the work is still performed in the framework of academic studies and involves only one or a few accessions per plant species.
Hence, the main drawback for a wider application of plant cryopreservation is the unavailability of efficient cryopreservation protocols for many plant species. The two most important parameters that need to be optimized for each species and tissue are the preparation phase of tissues towards dehydration (most important are sugar and/or cold treatments) and the length of explant treatment with the vitrification solution. Research should move in the direction of simplifying and standardizing the procedures as much as possible, in order to make the technology available to a wide range of public institutions and private companies. Moreover, to facilitate the development of even more efficient cryopreservation protocols, a better knowledge of the physico-chemical background of cryopreservation is needed. This can only be unraveled through fundamental studies that involve both thermal analysis and a thorough examination of the different parameters that can influence the cryo-behaviour, like endogenous sugars, membrane composition, oxidative stress and cryoprotective proteins. In the framework of a European project (CRYMCEPT, see *http://www.agr.kuleuven.ac.be/dtp/tro/CRYMCEPT/*) these parameters are now investigated for different plant species.

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