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Review. The use of cryopreservation for germplasm conservation of vegetatively propagated crops†

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Abstract

Cryopreservation is the conservation at very low temperature (≤ 150ºC) of living propagules. Cryopreservation offers a viable and economical method for the long-term conservation of genetic resources of vegetatively propagated plants. Various techniques have been developed to minimize desiccation and freezing damage, thus ensuring high propagule recovery. In most cases, shoot apices obtained from *in vitro*-grown shoots are the plant material used for cryopreservation of vegetatively propagated plants. Cryopreservation techniques are based on either freeze-induced dehydration («classical methods»), or vitrification of internal solutions («new methods»). Various considerations should be taken into account when cryopreservation techniques are actually used for germplasm conservation.

Key words: *in vitro* culture, genebank, liquid nitrogen, plant genetic resources.

Resumen

Revisión. La utilización de la crioconservación para la conservación de germoplasma de plantas cultivadas propagadas vegetativamente

La crioconservación consiste en la conservación de progágulos vivos a temperaturas muy bajas (≤ 150ºC). La crioconservación es un método viable y económico para la conservación a largo plazo de recursos genéticos de plantas propagadas vegetativamente. Se han desarrollado diversas técnicas para minimizar los daños producidos por desecación o congelación, asegurando así un alto porcentaje de recuperación de propágulos. En la mayoría de los casos se han utilizado ápices obtenidos de vástagos cultivados *in vitro* como material a conservar en la crioconservación de plantas propagadas vegetativamente. Las técnicas de crioconservación se basan bien en una desecación inducida por el descenso controlado de la temperatura («métodos clásicos»), o en la vitrificación de las soluciones de los tejidos («nuevos métodos»). Se debe tener en cuenta una serie de consideraciones cuando se desean utilizar las técnicas de crioconservación para la conservación a largo plazo de recursos fitogenéticos.

Palabras clave: banco de genes, crioconservación, cultivo i*n vitro,* nitrógeno líquido, recursos fitogenéticos.

Introduction

Conservation of plant genetic resources ensures maintenance of agrobiodiversity. National and International Institutes have been created with that aim. The Programme for the Conservation and Use of Plant Genetic Resources (*Programa de Conservación y Utilización de Recursos Fitogenéticos*) was established in Spain in 1993 (BOE, 1993). This Programme has been implemented through several strategic actions. At the international level, the International Plant Genetic Resources Institute (IPGRI) is a «research institute with a mandate to advance the conservation and use of genetic diversity for the well-being of present and future generations» (http://www.ipgri.cgiar.org/ institute/about).

Plant genetic resources conservation can be carried out either in the natural habitats of species' populations (*in situ*) or outside them (*ex situ*) (for a general review on plant germplasm conservation see Frankel and Hawkes, 1975; Iriondo, 2001). *Ex situ* plant genetic resources conservation can be carried out in an

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efficient and economical way by seed conservation, in seedbanks (Anon., 1994). Seeds of most crops can be stored for long periods under low relative humidity and low temperature conditions. However, seed storage is not feasible in some cases. Seeds of some species are recalcitrant or intermediate, i.e. they cannot stand desiccation below a relatively high critical water content value (10-12% or 20% fresh weight basis, respectively, Hong *et al.*, 1996) and cold storage without losing viability (see reviews of King and Roberts, 1980; Berjak and Pammenter, 1997). Species with recalcitrant or intermediate seeds are usually native from tropical or aquatic habitats, some of them with agricultural importance such as *Hevea brasiliensis* (rubber tree), and *Elaeis guineensis* (oil palm). Some forest tree species from temperate climates have also recalcitrant seeds (*Juglans* spp., *Quercus* spp.). Intermediate seeds can be stored by partial drying, although for shorter periods than those of orthodox seeds. Conservation of recalcitrant seeds under humid conditions can be carried out only for short periods, due to germination onset, fungal attack or viability loss.

A second group of species for which seedbanking is not feasible are those vegetatively propagated. They are usually highly heterozygous and, in some cases, do not produce seeds. Some important crops belong to this group, including *Dioscorea* spp. (yam), *Solanum* spp (potato), *Musa* spp. (banana), *Manihot* spp. (cassava), *Colocasia esculentum* (taro), and *Ipomoea batatas* (sweet potato). The genetic conservation of these species must be carried out in field collections. These are exposed to losses due to plague attacks or natural disasters. Besides, field collections are labour intensive. In the mid seventies, the use of *in vitro* culture was pointed out as a feasible alternative for genetic conservation of plants for which seedbanking was not possible (Henshaw, 1975). *In vitro* culture does not only provide a method for clonal propagation and safe exchange of plant material but it can also be used for medium-term germplasm conservation. This is achieved by slowing growth, thereby extending the intervals between subcultures (Dodds, 1991). Most procedures are based on the incubation of plant material at lower temperature and irradiance (or darkness) than those used for micropropagation. In some cases, culture medium has lower mineral or sucrose concentration. These procedures are currently used for genetic conservation of some vegetatively propagated species (Ashmore, 1997). For example, cassava accessions (over 5,000) are kept by this procedure at the *Centro Internacional de Agricultura Tropical* (International Centre for Tropical Agriculture, CIAT, Colombia), and cultures are subcultured every 10-18 months, depending on genotype. At the *Institut für Pflanzenbau der Bundesforschungsanstalt für Landwirtschaft* (Institute of Crop Science of the Federal Agricultural Research Centre, FAL, Germany), potato plantlets are stored on MS liquid medium with 2% sucrose at 10ºC and subcultures are carried out every 2-3 years.

In vitro storage, based on slow growth techniques, is routinely applied to a range of crops as a medium-term storage (Ashmore, 1997). However, concern can be risen on the genetic stability of cultures in the long-term (somaclonal variation, Larkin and Scowcroft, 1981) and on the possible losses due to contamination (see Ashmore, 1997).

Cryopreservation is, so far, the only viable procedure for long-term germplasm conservation of vegetatively propagated species and, therefore, can be used for base collections. Cryopreservation implies the conservation of plant propagules at very low temperatures (below –150ºC), thus ensuring that all metabolic processes stop. In this way, subcultures are not required and the threat of somaclonal variation is reduced. Liquid nitrogen (–196ºC) is usually used as refrigerant, although freezers with working temperatures of –150ºC are now available. Cryopreserved material requires a limited space. However, power or liquid nitrogen supply must be guaranteed.

Different types of plant cell, tissues and organs can be cryopreserved, including cell suspensions, pollen, embryogenic cultures, somatic and zygotic embryos, shoot apices or meristems. For vegetatively propagated species, the most widely used organs are shoot apices excised from *in vitro* plants. In some woody species, cryopreservation of dormant buds has also been developed, and recovery was achieved by grafting (Towill and Forsline, 1999). Cryopreservation of *in vitro* cultured apices has certain advantages, among them that cryoprotective treatments can be applied to the shoots or apices while cultured *in vitro*. Besides, *in vitro* culture can be used for clonal propagation of the starting plant material and for virus-free plant production. Plant germplasm distribution in the form of *in vitro* cultures is usually less voluminous and improves health status. Furthermore, somaclonal variation is less probable to occur when recovery of plants is carried out directly from apices compared to other methods, for example by direct or indirect organogenesis (Scowcroft, 1984). In several cases,

embryogenic cell lines have shown to be highly stable, although that stability may differ among species and could decrease with time in culture (Brar and Jain, 1998). Besides, propagation procedures through somatic embryogenesis are not fully developed in many species.

The aims of this review were to provide a summary of the cryopreservation techniques available for vegetatively propagated plants, and to discuss issues involved in the application of those techniques for plant germplasm conservation.

Cryopreservation techniques

Most plant living cells have high quantities of water and they are extremely sensitive to temperatures below 0ºC. Therefore, cells should be dehydrated to avoid ice crystal formation (Mazur, 1984). However, extreme desiccation also produces damages (on cell membrane, due to high concentration of internal solutes, protein denaturation). Cryopreservation techniques have been developed to minimize both types of damages.

Classical methods

Plant cryopreservation procedures were firstly developed following the success of animal cell cryopreservation (Grout and Morris, 1987). They were based on chemical cryoprotection and slow cooling, followed by rapid immersion in liquid nitrogen. It is named controlled freezing, slow freezing or two step-freezing method. By decreasing temperature at a relatively slow rate, ice crystals are formed in the extracellular solution and water is removed from the intracellular one, leading to cellular dehydration and therefore avoiding intracellular ice formation (Meryman and Williams, 1985). Such «classical» cryopreservation procedures have been highly successful with callus and cell cultures, consisting of rather uniform and small units (Schrijnemakers and Van Iren, 1995; Lynch, 2000). For example, sugarcane and banana embryonic calli were successfully cryopreserved with this method and the field performance of the recovered plants studied. In both cases, no differences between cryopreserved and control plants, on agronomic traits and/or growth descriptors, were found after a period (two culture cycles or 12 months) of growth in the field (Martínez-Montero *et al.*, 1998; Côte *et al.*, 2000; Martínez-Montero *et al.*, 2002). This technique is usually not very effective to cryopreserve larger structures which comprise different cell types, such as shoot apices. However, there are some successful examples of two-step shoot-tip cryopreservation, some of them with vegetatively propagated species such as potato or chrysanthemum (Benson *et al.*, 1989; Fukai *et al.*, 1991; Engelmann, 1997a). In this method, shoot apices are treated with cryoprotective substances such as dimethyl sulfoxide (DMSO), ethylene glycol, polyethylene glycol, mannitol, sorbitol or sucrose, either alone or in mixtures. Subsequently, they are slowly cooled, usually at rates of 0.5-2ºC min^{-1}, to –40ºC and then rapidly immersed in liquid nitrogen. Rapid rewarming is usually required to avoid recrystallisation phenomena. Transfer to fresh medium after one day in culture is recommended due to the toxic effect of cryoprotective mixtures (Engelmann, 1997a). Controlled cooling rate can be achieved with the use of (expensive) programmable freezers or by more simple devices. A cooling rate of 0.4-0.6ºC min^{-1} was obtained with an ethanol bath and a –40ºC freezer for cryopreservation of sugarcane cultures (Martínez-Montero *et al.*, 1998).

New methods

For the last ten years, new plant cryopreservation procedures have been developed, which are based on vitrification. Vitrification can be defined as «the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling» (Fahy *et al.*, 1984). During vitrification, the solution is said to become an amorphous glassy solid, or glass. Vitrification of solutions is achieved by reducing intra- and extracellular freezable water, either by exposure of plant tissues to highly concentrated cryoprotective mixtures or by physical desiccation, and subsequent very rapid cooling, generally by direct immersion in liquid nitrogen. Cryopreservation techniques based on vitrification are usually simple to carry out and applicable to complex structures such as embryos and shoot apices (Withers and Engelmann, 1997).

There are two main types of new cryopreservation techniques, although combinations of them have also been used. The first one is actually termed vitrification (*sensu stricto*) and the second one encapsulation-dehydration (Table 1).

Table 1. List of vegetatively propagated plant species for which shoot-apex cryopreservation has been developed using different techniques (see text)

Species	Technique	Reference
Actinidia sinensis	Encap.-dehydration	Wu *et al.*, 2001
Allium sativum	Vitrification	Makowska *et al.,* 1999
Allium wakegi	Vitrification	Kohmura *et al.,* 1994
Asparagus officinalis	Pregrowth-desiccation[1]	Uragami *et al.*, 1990
Camellia sinensis	Vitrification	Kuranuki, 1995
Cichorium intybus	Encap.-dehydration	Vandenbussche *et al.,* 1993
	Vitrification	Demeulemeester *et al.,* 1992, 1993
Citrus spp.	Encap.-dehydration	González-Arnao *et al.,* 1998
Chrysanthemum morifolium	Encap.-dehydration	Sakai *et al.,* 2000
	Vitrification	
Dioscorea spp.	Encap.-dehydration	Mandal *et al.,* 1996
		Malaurie *et al.*, 1998
Fragaria spp.	Encap.-dehydration	Navatel and Capron, 1997
	Encap.- vitrification	Hirai *et al.,* 1998
		Hao *et al.*, 2002
	Slow freezing	Reed and Hummer, 1995
	Vitrification	Kartha *et al.,* 1980
Humulus lupulus	Encap.-dehydration	Martínez and Revilla, 1999
Ipomoea batatas	Vitrification	Towill and Jarret, 1992
Eucalyptus spp.	Encap.-dehydration	Poissonnier *et al.,* 1992
Lilium spp.	Vitrification	Matsumoto *et al.*, 1995b
Malus spp.	Encap.-dehydration	Wu *et al.,* 1999; Zhao *et al.,* 1999b
	Vitrification	Niino *et al.*, 1992c
	Droplet	Zhao *et al.,* 1999a
Manihot spp.	Vitrification	Charoensub *et al.,* 1999
Morus spp.	Encap.-dehydration	Niino *et al.,* 1992b
	Vitrification	Niino *et al.,* 1992a
Musa spp.	Vitrification	Thinh *et al.,* 1999
Olea europaea	Pregrowth[2]	Pannis *et al.,* 1996
Phoenix daylifera	Encap.-dehydration	Martínez *et al.,* 1999
Prunus domestica	Vitrification	Bagniol and Engelmann, 1991
Prunus dulcis	Vitrification	Brison *et al.,* 1995
		De Carlo *et al.,* 2000
Pyrus spp.	Encap.-dehydration	Shatnawi *et al.,* 1999
	Encap.-dehydration	Scottez *et al.,* 1992
	Vitrification	Niino *et al.,* 1992a
	Slow freezing	Reed, 1990
Ribes spp.	Encap.-dehydration	Reed and Yu, 1995
Rubus spp.	Slow freezing	Chang and Reed, 1999
Saccharum spp.	Encap.-dehydration	González-Arnao *et al.,* 1999
Solanum spp.	Encap.-dehydration	Fabre and Dereuddre, 1990
	Vitrification	Lu and Steponkus, 1994
	Droplet	Schäfer-Menuhr *et al.,* 1994
Vitis vinifera	Encap.-dehydration	Plessis *et al.,* 1993
Wasabia japonica	Encap.- vitrification	Matsumoto *et al.,* 1995a

[1] Nodal segments. [2] Meristematic clumps.

In the vitrification technique, the plant material is exposed to highly concentrated cryoprotectant solutions for short periods. Previously, to induce desiccation tolerance, tissues are cultured on medium with high sucrose (e.g. 0.3 M) or sorbitol (e.g. 1.4 M) concentration and subsequently transferred to a glycerol-sucrose solution, called loading solution (e.g. 2 M glycerol + 0.4 M sucrose) (Sakai, 2000). A widely used vitrification solution is that developed by Sakai *et al*. (1990) and named PVS2, which consists of 30%

(w v^{-1}) glycerol, 15% (w v^{-1}) ethylene glycol and 15% (w v^{-1}) DMSO in liquid medium with 0.4 M sucrose.

The droplet (see Table 1) technique can be considered a modification of the previous one. It was developed for potato germplasm cryopreservation at FAL (Germany) and consists on treating shoot apices in drops of a 10% DMSO solution placed on aluminium foil strips, which are rapidly immersed in liquid nitrogen (Mix-Wagner *et al.*, 2003).

The encapsulation-dehydration technique is based on the artificial seed technology. This technique was developed by Fabre and Dereuddre (1990), and consists on the inclusion of apices in alginate beads and their subsequent culture in a highly concentrated (0.7-1.5 M) sucrose solution followed by physical dehydration, and direct immersion in liquid nitrogen. Culture of apices on sucrose enriched medium (0.3-0.7 M), prior to encapsulation, usually improves survival after desiccation and freezing. Physical desiccation is carried out either with silica gel or in the air flow of the laminar flow cabinet (Paulet *et al.*, 1993). Water contents of around 20% (fresh weight basis) have resulted appropriate for high survival after freezing of vegetative explants in several species (Scottez *et al.*, 1992; Niino *et al.*, 1992b; González-Arnao *et al.*, 1996; Engelmann, 1997a; González-Benito *et al.*, 1998).

More recently, protocols combining the above techniques have been developed and named encapsulation-vitrification techniques (Matsumoto *et al.*, 1995a; Hirai *et al.*, 1998; Sakai *et al.*, 2000). Apices are firstly encapsulated and then submitted to vitrification, with no requirement for physical desiccation.

In many temperate species, incubation of the *in vitro*-shoots, nodal segments or apices at low temperature (generally 4º to 10ºC), for periods ranging from days to weeks, increases survival after freezing for both types of techniques, classical and new (Reed, 1990; Scottez *et al.*, 1992; Niino and Sakai, 1992; Wu *et al.*, 1999; Martínez and Revilla, 1999). During cold acclimation cellular changes (numerous smaller vacuoles, more abundant mitochondria and rough endoplasmic reticulum) and accumulation of certain proteins occur, as it has been shown in cryopreservation studies of peach cell suspensions (Arora and Wisniewski, 1995). During shoot tip cryopreservation of non cold-hardy tropical species, preculture on sucrose enriched medium (0.3-0.8 M) improved survival after cooling either by encapsulation-dehydration or by vitrification (Takagi, 2000).

In some cases, apices pregrowth on sucrose rich medium has proven sufficient for survival after cooling in liquid nitrogen. That is the case of banana meristem clumps cultured on 0.4 M sucrose for 2 weeks (Panis *et al.*, 1996). Uragami *et al.* (1990) obtained 63% survival, after cooling in liquid nitrogen, in asparagus nodal explants that had been previously precultured for 2 days on 0.7 M sucrose and subsequently desiccated to 20% water content with silica gel. These two methods, named «pregrowth» and «pregrowth and desiccation», have been especially successful to cryopreserve zygotic and somatic embryos of several species (Engelmann, 1997a).

Conservation and recovery

Long-term conservation can take place in liquid nitrogen (–196ºC) or in the vapour phase. However, storage temperature should not be higher than –130ºC to prevent solution devitrification and large ice crystal formation (Towill, 1991). Those temperatures can be also achieved by deep freezers running at –150ºC.

At the Federal Centre for Breeding Research on Cultivated Plants (BAZ, Germany) the viability of potato apices after long-term storage in liquid nitrogen has been studied (Mix-Wagner *et al.*, 2003). The cryopreservation technique employed was the droplet method and storage was carried out in liquid nitrogen. Plant regeneration from long-term (3 to 8 years) *versus* short-term stored apices was studied in 51 cultivars. Only in three of them recovery showed a significant decrease in the long-term stored apices.

For the recovery of apices after cryostorage, rapid rewarming is usually required to avoid recrystallisation (Towill, 1991). Vials containing the apices are usually immersed in a water bath at 35-40ºC. When apices are not included in vials (e.g. the droplet technique, Mix-Wagner *et al.*, 2003) rewarming usually takes place in liquid medium at room temperature.

In many species, recovery of apices cryopreserved with the new techniques is direct, without callus formation. By contrast with the classic techniques, the structural integrity of most cells is well preserved (Engelmann, 1997a). Some studies have shown the importance of the appropriate post-thawing culture conditions to enhance organised growth. For example, potato cv. Desirée shoot tips showed higher recovery when incubation during the first week after thawing was performed under low light intensity (Benson *et*

al., 1989). In many cases, selection of a suitable growth medium for apex recovery may be necessary. Adjustment of growth regulator concentration (Withers *et al.*, 1988) or even medium salt formulation (Pennycooke and Towill, 2001; Decruse and Seeni, 2002) could be required for the normal development of frozen shoot apices. Some media supplements such as iron chelating agents or surfactants have been shown to improve recovery (Benson *et al.*, 1995; Pennycooke and Towill, 2001).

Application of cryopreservation techniques for plant genetic resources conservation and management

Work is being carried out in several research institutes and universities to develop appropriate cryopreservation techniques for vegetatively propagated species (Ashmore, 1997; Engelmann, 1997b). However, the number of plant genetic resources centers where cryopreservation is used is still low and usually concerns few accessions only (Ashmore, 1997; Reed, 2001; Reed, pers. com.). Potato is the crop in which the number of accessions is the highest. At BAZ (Germany) a total of 519 varieties are stored under cryopreservation (Mix-Wagner *et al.*, 2003). Apices have been cryopreserved using the droplet method. At the *Centro Internacional de la Papa* (International Potato Centre, CIP, Perú) 197 accessions are cryopserved by the vitrification method (Reed, 2001). Lower numbers, but still important collections, are those of cassava (95 accessions, CIAT, Colombia) and pear (106 accessions, National Clonal Germplasm Repository, Corvallis, USA) (Reed, 1990, 2001; Roca *et al.,* 2000).

Certain aspects, for which there is still not too much experience, should be considered before the establishment and management of a cryogenic germplasm bank. Probably, the two first questions to answer are the technique to use and the number of replicates required. The new cryopreservation techniques do not require expensive equipment (programmable freezer). In all cases, personnel should be properly trained in *in vitro* and cryopreservation techniques, otherwise results may vary greatly (Mix-Wagner *et al.*, 2003). Some preliminary testing should be carried out with a few cultivars. Vitrification usually requires less handling than encapsulation-dehydration, but solutions may be toxic. Encapsulation-dehydration protocols are usually simple, but more handling of alginate beads (although easier due to their size) is required and some species do not tolerate the high sucrose concentrations employed. The advantages and disadvantages of each technique should be considered. However, other factors such as personnel, available facilities, and type of plant could influence the selection of the technique (Reed, 2001).

Those first tests with few cultivars could give indications on how the protocol should be improved to obtain high percentage recovery. But, which recovery percentage should be aimed and in how many cultivars? For genebank management, recovery as normal apex regrowth giving healthy *in vitro* plants should be the goal. Care should be taken when interpreting some published cryopreservation works where survival (any kind of growth) is reported. In some germplasm conservation centers, 20% recovery is considered enough for long-term preservation (Golmirzaie and Panta, 2000). Other authors consider that survival should be higher than 40% (Reed *et al.*, 2000; Reed, 2001). It is important that those percentages be reproducible. These survival percentages may not seem high but we should take into account that we are dealing with clonally propagated plants, where homogeneity should be expected. The case is different when cryopreservation of heterogeneous material is considered (e.g. zygotic embryos from species with recalcitrant seeds). As genotypes usually respond differently to the same protocol (Golmirzaie and Panta, 2000; Mix-Wagner *et al.*, 2003), curators should make the decision of: 1) either modify the protocol for each genotype or group of genotypes to obtain high recovery in most of them, or 2) store more propagules (to compensate for low recovery) with a protocol that may be not optimal for all genotypes. More recently, Dussert *et al*. (2003) developed a probabilistic method to help curators to take certain decisions, for example, on the number of cryopreserved propagules to be tested in order to get a precise estimation of the expected recovery percentage, or on the minimum number of propagules which should be rewarmed per accession to obtain a fixed number of viable plants.

Three examples on the number of replicates conserved (number of vials and propagules in each one) are those used for potato and *Pyrus*. Twelve potato apices are kept per cryovial and around 300 apices per variety at BAZ (Mix-Wagner *et al.*, 2003). At CIP, 250 apices are stored per genotype (five cryovials containing 50 shoot tips each) (Golmirzaie and Panta,

2000). In the National Seed Storage Laboratory (Fort Collins, USA) 100 *Pyrus* meristems (25 per cryovial) of each genotype are stored as base collection (Reed *et al*., 2000).

The number of replicates conserved should take into account the recovery percentage expected and the planned periodical monitoring. Storage should be carried out under optimal conditions (no danger of temperature rising and therefore of solution recrystallisation), and therefore periodical monitoring would be necessary with very low frequency. It would be advisable that the container used for long-term conservation is employed only for that purpose. Appropriate handling procedure for inserting or removing accessions should be carefully developed and carried out to avoid accidental temperature increase.

It would be desirable that the liquid nitrogen container or freezer (–150ºC) is in a different site to the field collection (Reed, 2001). It is very important that liquid nitrogen or power supply are secured. Automatic-filling liquid nitrogen containers are available. Alarms for temperature decrease detection and constant personnel availability are required. For further security, a duplicate of the cryopreserved collection could be established in a different site.

As in all types of *ex situ* conservation banks, information recording is very important. A good recording method (accession numbering, data bases) should be established to collect information about the mother plant (passport data; Anon., 1996), the *in vitro* culture phase and the cryopreservation protocol used, including all phases: apex excision, pretreatment, cryopreservation method, thawing method, and recovery medium and incubation conditions. The last two phases are very important to secure adequate recovery and reproducibility in the future (probably after many years) and as much detail as possible should be included (Reed, 2001). It is important to record as well the recovery percentage after a short conservation period (days).

A major concern of curators is the genetic stability of conserved material. *In vitro* culture has been reported to induce genetic changes (somaclonal variation) in some cases (Scowcroft, 1984). Care should be taken to test that the *in vitro* culture phase does not produce genetic instability (Harding, 1999). Although not many studies have been performed yet, cryopreservation protocols seem to ensure genetic stability of the plant material. Sugarcane plants derived from cryopreserved embryonic clumps did not show field performance differences when compared with non-cryopreserved ones (González-Arnao *et al.*, 1999; Martínez-Montero *et al.*, 2002). Genetic stability of cryopreserved potato shoot tips has been studied through ploidy status or ribosomal genes stability (Harding, 1991; Benson *et al.*, 1996; Harding, 1997). Study of the DNA variation through amplified fragment length polymorphism assays have been carried out in strawberry, apple, grape, and kiwi (Hao *et al.*, 2001, 2002; Zhai *et al.*, 2003). To date, there is no substantial evidence to suggest that plants regenerated from cryopreserved shoot apices are genetically changed. In potato, DNA polymorphism observed was not induced by cryopreservation but by the whole process employed, including the tissue culture phase (Harding, 1997). In other works where variants were regenerated from cryopreserved apices, those were due to their chimeric structure (Fukai *et al.*, 1994).

Conclusions

Cryopreservation has proven to be an efficient long-term conservation method for genetic resources of a range of vegetatively propagated crops. Protocols have been or are being developed for many others at an experimental level. Adjustments of those protocols to the genebank level would be necessary to actually exploit all the advantages of cryopreservation.

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