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Securing Plant Genetic Resources for Perpetuity through Cryopreservation

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As the expanding world population increasingly depends on a dwindling number of cultivars and of crop species, so the concomitant loss of crop diversity increasingly threatens global food security. Therefore crop diversity and associated wild relatives urgently need to be conserved for future generations.

Seed conservation is the most used *ex situ* method to conserve crop germplasm. However, it is not an option for those crops that are sterile (do not produce viable seeds, like banana), or produce only recalcitrant (non-storable) seeds (like cocoa and coconut). Nor is it an option for species where specific gene combinations need to be maintained during propagation (many fruit species such as apple and potato). In such cases, vegetative material needs to be maintained in the field or in *in vitro* collections (micro plants grown in test tubes). Even under reduced-growth conditions, maintaining *in vitro* collections is still labour intensive, and there is always the risk of losing accessions due to contamination or human error. Moreover, *in vitro* material of some species is subject to somaclonal variation (spontaneous mutations whose frequency is generally increased during *in vitro* culture). Cryopreservation, or storage of biological material at ultra-low temperatures, is therefore preferred for the long-term conservation of plant genetic resources of vegetatively propagated crops.

Cryopreservation

The main issue when exposing biological tissues to low temperatures is the formation of lethal ice crystals during cooling or thawing. Crystals thus formed penetrate membranous cell structures like nuclear and plasma membranes, causing irreversible damage, and a loss of their semi-permeability. The only way to avoid the formation of ice crystals in a solution is vitrification. Vitrification or glass transition refers to the transformation of a glass-forming liquid into a glass. Two requirements must be met for a liquid to vitrify. The solution must be both: i) rapidly cooled, and ii) "glass forming" or highly concentrated. These two requirements form the basis for developing efficient cryopreservation techniques (Panis *et al*., 2001).

Roughly, three main cryopreservation protocols can be distinguished for hydrated tissues: i) the "classical" slow freezing protocol (currently mainly applied to non-organized tissues such as cell suspensions and calluses) (Withers and King, 1980); ii) the encapsulation/dehydration method that relies on synthetic seeds (Fabre and Dereuddre, 1990), and iii) the methods relying on the application of highly concentrated vitrification solutions such as PVS2 (Plant Vitrification Solution 2) (Sakai *et al*., 1990). The latter two methods are more suitable for organised plant tissues such as shoot cultures.

The droplet vitrification protocol was established because it combines the application of highly concentrated cryoprotective vitrification solutions (often PVS2) with ultra-fast freezing and thawing. Such high freezing and thawing rates are obtained by transferring the biological material (often meristems) to small strips of aluminium foil and exposing them directly to liquid nitrogen (Panis *et al*., 2005) (Fig. 1).

The droplet vitrification protocol can now be considered as the first "generic" cryopreservation method for plant tissues, as it has now been successfully applied to different tissues (shoot cultures/embryos) and a wide variety of plant species from different climatic environments. Moreover, the technique is less cumbersome in its application compared with other cryopreservation methods and no sophisticated equipment is needed (Panis *et al*., 2011).

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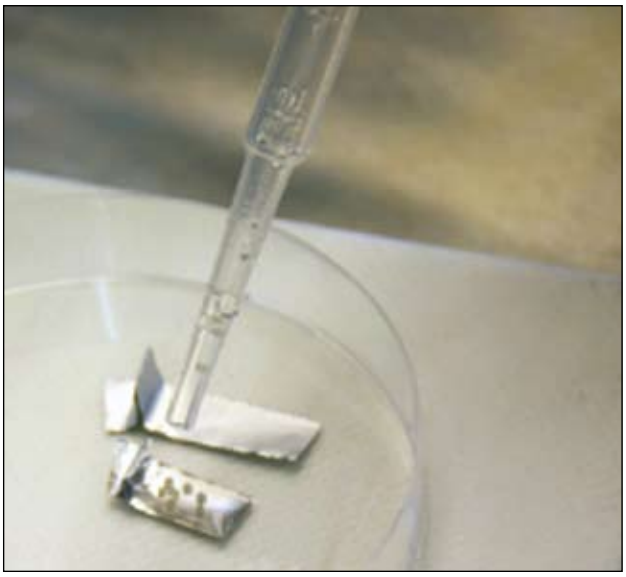

Fig. 1. Excised banana meristems (each 1 mm²) are transferred inside a droplet of vitrification solution onto a strip of aluminium foil for droplet vitrification (Photo credit: Bioversity International/N. Capozio)

Applications of Cryopreservation in Crop Germplasm Collections

Currently, many of the institutes that maintain large collections of vegetatively-propagated crops species use droplet vitrification to store their valuable germplasm for the long-term (Table 1).

The largest collection of cryopreserved, vegetatively-propagated plant material is kept at the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO. Since 1993, dormant apple buds of 2,302 accessions have been cryopreserved (Volk *et al*., 2015). The largest cryopreserved collection of *in vitro* shoot-tips comprises currently about 1,454 potato accessions and is stored at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Keller *et al*., 2016). Other large collections of *in vitro* shoot tips are Bioversity International's International Transit Centre (ITC), in Leuven, Belgium (950 banana accessions) (Fig. 2) and the Centre for International potato (CIP) 's genebank, in Lima, Peru (1450 potato accessions) (CIP, 2016).

Safety Back-up of Cryopreserved Collection

Until now each institute that stores cryopreserved crop germplasm has applied its own conservation standards with respect to 'what is a good regeneration rate to consider the germplasm safely stored'. Some of them consider a crop accession as safely cryopreserved for the long-term provided the regeneration rate of the

Table 1. Cryopreservation methods used in world's largest crop genebanks for storing their vegetatively-propagated germplasm

Institute	Country	Crop	Cryopreservation Method
Bioversity International, Leuven	Belgium	Banana	Droplet vitrification
Crop Research Institute, Prague	Czech Republic	Potato, garlic, hops	Droplet vitrification
International Center for Tropical Agriculture (CIAT), Cali	Colombia	cassava	Droplet vitrification Encapsulation/dehydration
International Institute of Tropical Agriculture (IITA), Ibadan	Nigeria	Yam, banana, cassava	Droplet vitrification
International Potato Center (CIP), Lima	Peru	Potato	Straw vitrification Droplet vitrification
Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden	Germany	Strawberry/ Fruit trees	Vitrification Dormant bud freezing
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Genebank Department, Gatersleben	Germany	Potato, garlic, mint	Droplet freezing Droplet vitrification
National Agrobiodiversity Center (NAAS), RDA, Suwon	South Korea	Garlic	Droplet vitrification
Tissue Culture and Cryopreservation Unit, NBPGR, Delhi	India	Banana, chives, medicinal plants, berries, fruit trees.	Vitrification Droplet vitrification Slow freezing Dormant bud freezing
National Institute of Agrobiological Sciences (NIAS), Tsukuba	Japan	Mulberry	Dormant bud freezing
USDA-ARS, Fort Collins and Corvallis	USA	Citrus species, grape, garlic, mint, fruit trees.	Vitrification Droplet vitrification Slow freezing Dormant bud freezing

representative sample is at least 30 %. Others use 40%, 60% or even 80% as cut-off.

Bioversity International accepts a banana accession as safely stored provided: i) Three successful independent repetitions are executed, and ii) for each successful repetition stored in the cryotank, the ITC [International Transit Centre (previously called INIBAP)] is 95% confident that at least one plant can be regenerated (Dussert *et al*., 2003). As an additional "security" measure, one replicate set of all accessions has been transferred under frozen conditions to the Institut de Recherche pour le Development (IRD), Montpellier, France using a dry shipper. This 'black-box' back-up at IRD is located 1000 km from Leuven thus reducing the risk of germplasm loss due to political and/or environmental hazards.

Bioversity International proposes creating a Global CryoVault for a range of vegetatively propagated crops. The proposed location will be in Leuven, Belgium, although other locations are being considered. This Global CryoVault, will ensure that a copy of cryopreserved crop samples conserved elsewhere in the world will be safely stored. It will act as a complementary facility to the Svalbard Global Seed Vault, in the Arctic tundra, which conserves crops that reproduce through 'storable' seeds. With these two facilities, the majority of existing crop diversity – tens of thousands of species and varieties of all food crops and wild relatives – will be preserved for present and future generations.

Fig. 2. A box of Cryotubes, each tube containing 10 cryo-preserved banana meristems retrieved from liquid nitrogen storage at Bioversity International Transit Centre ITC (Photo credit: Bioversity International/B. Panis)

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