

# Cryobiotechnology of tropical seeds – scale, scope and hope

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## **Abstract**

Plants provide essential ecosystem services that benefit humankind, yet their existence is subject to an increasing number of threats, including global environmental change. Anticipated impacts on future access to medicines, nutritious foods, particularly indigenous fruits, and sustainable biofuels, demands that action is taken to protect and conserve such valuable, neglected and underutilized species (NUS), especially in the tropics where the use of non-timber forest products (NTFP) is considerable. Primarily, the protection of such plant genetic resources is achieved through the complementary approaches of in situ and ex situ conservation; for the latter, generally as dry seeds stored at -20°C. However, tropical forest species – which are estimated to constitute about 50% of the world's plants – tend not to be so readily amenable to such an approach, and attention is now focusing increasingly on cryopreservation. This review considers the following issues: how plant cryopreservation studies have evolved over time and the extent of application of cryobiotechnology (scale); the policy current drivers and biodiversity needs that provide a framework for current and future plant cryopreservation studies (scope); and which technological innovations, knowledge and understanding might fast-track the science and accelerate the mainstreaming of plant cryopreservation particularly for tropical seeds (hope). Whilst significant progress in plant/seed cryobiotechnology has been made in the last decades, a co-ordinated global research and training programme aimed at accelerating the cryobanking of tropical plants of value to humankind is now urgently needed.

**Keywords:** plant cryopreservation, ex situ conservation, recalcitrant seeds, orchids

## **SCALE – THE EVOLUTION OF CRYOPRESERVATION STUDIES**

Cryopreservation as a science accelerated after the 1960s, when the discipline of cryobiology was established. However, significant developments in plant cryopreservation as a science date from the 19<sup>th</sup> century. Table 1 provides an indication of the early, considerable interest in the exposure of plant genetic resources, in this instance seeds, to cryogenic gases. The late 19<sup>th</sup> century was a period of innovations in chemistry resulting in the ability to produce liquid gases, including the discovery by James Dewar in 1898 of the means of making hydrogen. As the preface to this volume explains, in 1899 Dewar then collaborated with William Turner Thiselton-Dyer, the director of the Royal Botanic Gardens Kew, UK, to assess the ability of crop seeds to tolerate exposure to this liquefied gas.

During the 60-year period prior to the work of Dewar and Thiselton-Dyer, and the following 40 years, plant scientists contributed to the first golden era of cryobiology; providing evidence of dry seed tolerance to temperatures of close to zero Kelvin (e.g., -272°C; Lipman, 1936) and many months storage (e.g., 176 d in liquid air at c. -190°C; Busse, 1930).

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Table 1. A short history of early successes in air-dry seed cryopreservation (modified from Pritchard and Nadarajan, 2008).

Seeds of	Treatment	Survival	Reference
Barley, broad bean, rye and wheat	Exposed to cryogen which 'froze mercury' for 15 min	"Their power of germination was unchanged"	Edwards and Colin (1834)
<i>Avena sativa</i> , <i>Clarkia elegans</i> , <i>Eschscholtzia californica</i> , <i>Hordeum vulgare</i> , <i>Lepidium sativum</i> , <i>Linaria bipartita</i> , <i>Nemophila insignis</i> , <i>Portulaca oleracea</i> , <i>Triticum sativum</i>	20-30 min exposure to -110°C	Germination was "unimpaired"	Wartman (1860)
12 species: <i>Hordeum distichon</i> , <i>Avena sativa</i> , <i>Cucurbita pepo</i> , <i>Cyclanthera exfolens</i> , <i>Trigonella foenumgraecum</i> , <i>Impatiens halsamina</i> , <i>Helianthus annuus</i> , <i>Heracleum villosum</i> , <i>Lotus tetragonolobus</i> , <i>Convolvulus tricolor</i> , <i>Pisum elatius</i> , <i>Funkia sieboldiana</i> . Seeds were at 10 to 12% of natural moisture	liquid air (-183 to -192°C), 110 h. Slowly and carefully thawed, a process which occupied about 50 h	No ill effects	Brown and Escombe (1897-1898)
<i>Chenopodium album</i> , <i>Cucumis sativus</i> , <i>Helianthus annuus</i> , <i>Linum usitatissimum</i> , <i>Lupinus luteus</i> , <i>Mimosa pudica</i> , <i>Onobrychis sativa</i> , <i>Pinus sylvestris</i> , <i>Ricinus communis</i> , <i>Secale cereale</i> , <i>Triticum sativum</i> and <i>Zea mays</i>	Exposure to liquid air	"It is not apparent that any marked unfavourable effect on germinable seeds may be traced to the immersion"	Selby (1901)
Sweet clover	Liquid air, 176 d	74% germination, compared to 64% for the unfrozen control	Busse (1930)
(A) Sugarcane, spinach, cucumber, sugar beet, buckwheat, barley, purple vetch, oat, onion, mustard and <i>Melilotus</i> ; (B) Pea, corn, squash, alfalfa and sunflower	-196°C for (A) 30 d; (B) 60 d	No detrimental effects with respect to laboratory/greenhouse germination or vigour	Lipman and Lewis (1934)
Vetch, wheat, barley, tobacco, flax, buckwheat, spinach, milo, maize and <i>Melilotus</i>	Exposure to -272°C	No ill effects when germinated and grown in a greenhouse	Lipman (1936)

In the Busse (1930) study, germination of the stored seeds was not checked until 6 months after thawing, at which point the controls had only about 50% germination; consequently the author felt that "storing in liquid air...may be beneficial in retarding normal aging of ... seeds". Some awareness of the need to control rewarming rates from ultra-low temperature can be assumed from the approach taken by Brown and Escombe (1897-1898), such that seeds were "slowly and carefully thawed, a process which occupied about 50 h". Similarly, the possible need for some control of cooling rate was also evident around this time as Selby (1901) applied a "gradual transition" from vapour to immersion as opposed a "sudden transition" by immersion to liquid air. Thereby, introducing two-step freezing.

Compared to the preservation of dry propagules, the preservation of hydrated tissues provided a more difficult challenge, requiring the balancing of dehydration with avoidance of solution effects and ice crystal nucleation and growth. Following the discovery of 15% glycerol as a vitrification agent for the preservation of fowl spermatozoa at -79°C (solid

carbon dioxide) or -192°C (liquid air) by Polge et al. (1949), investigations into animal cell cryopreservation advanced rapidly. However, plant cryopreservation studies took another 20 years to become established as a field of investigation. A search of Web of Science™ (Thompson Reuters) reveals little published output in plant cryopreservation until the mid-1970s (Table 2). Thereafter, published output in this selection of peer-reviewed journals increased to nearly 300 in the period 1995-2004 and double this value in the following decade.

Table 2. Number of papers on ‘plants AND cryopreservation’ by decade in Web of Science (search: March 2015).

Decade	No. of ‘plant cryopreservation’ papers	Popular topics of papers
1955-1964	0	-
1965-1974	0	-
1975-1984	8	<b>Cultured plant cells, protoplasts, meristems</b>
1985-1994	95	<b>cultured plant cells &gt; meristems &gt; zygotic embryos &gt; somatic embryos &gt; winter buds = pollen</b> ; thermal analysis; lipid phase transitions; vitrification; molecular / genetic stability; NMR; cytoskeleton; simple freezing method; cryoinjury; mitochondrion / chloroplast; ice nucleation
1995-2004	269	<b>Cultured plant cells &gt; meristems &gt; zygotic embryos &gt; somatic embryos &gt; winter buds</b> ; encapsulation-dehydration; molecular genetic stability; genetic resources; cold acclimation; tools and protocols; encapsulation-vitrification; phenotype; ABA; cryoprotection; hairy root culture; protocorms; cryotherapy; ice inhibition; membranes; ultrastructure; oxidative stress
2005-2014	603	<b>Meristems &gt; zygotic embryos (including seeds and axes) &gt; somatic embryos &gt; cultured plant cells = protocorms &gt; winter buds = pollen</b> ; encapsulation-dehydration; molecular, genetic and metabolic stability; methylation; genetic resources (threatened, indigenous or medicinal species; core collection); cold and sugar-mediated acclimation; tools and protocols and models; encapsulation-vitrification; droplet-vitrification; phenotype; ABA; cryoprotection; hairy root culture; cryotherapy / pathogen eradication; ice inhibition; membranes; ultrastructure; oxidative stress / anti-oxidants; volatile markers; recovery and light; field performance; imaging; nodal segment; proteome analysis; hydration window; cytoskeleton

The popular topics described in the titles to these publications suggest biotechnology to be an early driving force for the development of plant preservation as the frequency of plant material worked on tended to be: cultured plant cells > meristems > zygotic embryos > somatic embryos > winter buds ≈ pollen. Only in the last decade has the cryopreservation of plant genetic resources become a ‘hot’ topic. Over the whole period, key topics that emerge show a focus on methodology (e.g., vitrification, encapsulation-dehydration, droplet-vitrification), mechanisms (e.g., ultrastructure, dehydration and oxidative stress/tolerance), stability (e.g., genetic and phenotypic) and wider application (e.g., reference to genetic resources, rare species, etc.).

A more inclusive assessment of the scientific contributions in ‘plant cryopreservation’ activity over the last few decades can be judged from the number of records in Google

Scholar (scholar.google.co.uk), as it includes book chapters and, in some cases, abstracts as well as papers (Figure 1). An interest in plant cryopreservation clearly took off four decades ago and in the last decade there were more than 10,000 references to plant cryopreservation, i.e., about 1,000 contributions per year. This increase in activity in plant cryopreservation science has been accompanied by a greater confidence in the wide-scale application of the technology to core genetic resources collections, usually aimed at contributing to the prevention of extinction (genetic erosion) of both cultivated plants and their related wild species. Some examples follow, as an indication of the efforts across the world.

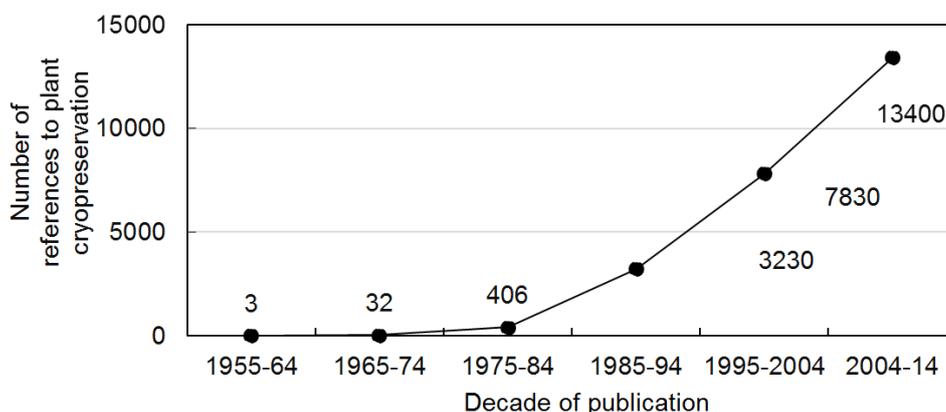


Figure 1. Number of references to ‘plants AND cryopreservation’ by decade in Google Scholar, as an indication of interest in the topic. Papers and book chapters are included, but not patents. NB. Earlier citations may include some references to fungi and plant products.

At the USDA National Centre for Genetic Resources Preservation at Fort Collins, USA, there are about 600,000 samples in the base collection, representing about 9,000 species. About 5,000 are cryopreserved vegetative samples, mostly dormant buds, and 48,872 seed samples are cryogenically stored in vapour above liquid nitrogen (Christina Walters, pers. commun.). This means that about 9% of the total samples at the NCGRP are cryopreserved.

Also in the USA, Cincinnati Zoo and Botanical Garden created in the late 1980s a frozen garden within its CryoBioBank® at the Center for Research on Endangered Wildlife (CREW) (<http://cincinnati-zoo.org/conservation/crew/crew-animal-research/cryobiobank/>). The objective remains the long-term preservation of native and endangered plants of the country. The bank now includes >3,000 samples from about 180 wild plant species. The samples being preserved in LN are not limited to dry seeds and embryonic axes of recalcitrant seeds, but include shoot tips, pollen, callus, dry fern spores, and the gametophytes of ferns and bryophytes. The size, age, and diversity of this collection make it exceptional, allowing both the empirical evaluation of the long-term effects of LN specimen stability and viability and illustration of some of the challenges of dealing with wild seed collections.

At the INIBAP International Transit Center, Leuven, Belgium, there are 1,479 banana accessions in the in vitro cold room and 910 (61%) cryopreserved (Bart Panis, pers. commun.). Cryopreserved means three independent experiments per accession, with the probability of regenerating at least one plant being 95%. As extra security, one repetition per accession is stored in IRD, France. Other cryopreserved clonal crop collections of significance include >500 cassava accessions at the International Center for Tropical Agriculture (CIAT), Colombia (Gonzalez-Arnao et al., 2008) and the collections at IPK, Germany. The mandate of the IPK Genebank comprises the collection, conservation, and distribution of plant genetic resources (PGR). It has a total inventory of 151,379 accessions from 3,220 plant species and 768 genera; of these there are 298 accessions in vitro and 1813 accessions in cryo, i.e., 1,637 as shoot tips and 176 as pollen samples (Joachim Keller, pers. commun.).

In India, the ICAR's National Bureau of Plant Genetic Resources at New Delhi has cryopreserved 11, 770 accessions. These are mainly of non-orthodox seeds (6,120 acc., usually as embryos/embryonic axes), selected orthodox seeded species (3,250 acc.), e.g., of medicinal plants, pollen (460 acc. of highly recalcitrant species), dormant buds (387 acc.), released varieties/registered germplasm/genetic stocks of crops, and some threatened and endangered plant species. Genomic resources (779 acc.) have also been successfully cryobanked (Rekha Chaudhury, pers. commun.).

At RDA in South Korea, 432 accessions of undehisced seeds of ginseng (*Panax ginseng*) have been cryopreserved (Haeng-Hoon Kim, pers. commun.), as well as 221 accessions of five clonal *Allium* species, including garlic, in long term conservation following the droplet vitrification of unripe inflorescences, cloves or bulbils (Kim et al., 2007).

Most of the examples above have a strong focus on the main crop species. In addition, there has been cryo-banking of genetic resources for future in the ornamental, commodity and fruit industries. For example, coffee germplasm at CATIE (Costa Rica) and in IRD (France) (Dussert and Engelmann, 2006) and citrus at NBPGR (New Delhi, India) (Malik et al., 2012). There are many more examples of cryopreserved plant genetic resources, for which details can be found in a number of excellent reviews (Reed, 2008; Engelmann, 2011), including on fruit trees (Benelli et al., 2013) and >200 ornamental species (Kulus and Zalewska, 2014) focus as an indication of interest in the topic. Papers and book chapters are included, but not patents. NB, earlier citations may include some references to fungi and plant products.

#### **SCOPE – INTERVENTION ON BEHALF OF BIODIVERSITY**

There are a number of policy developments in the last decade that provide a framework for plant cryopreservation into the future. These include the Aichi Biodiversity Targets. These targets are wider ranging, as they include plant and animal genetic resources (<https://www.cbd.int/sp/targets/>). There are five strategic goals (A-E), with C aiming to 'Improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity'. Targets 12 and 13 deal with ex situ conservation:

- Target 12: By 2020, the extinction of known threatened species has been prevented and their conservation status, particularly of those most in decline, has been improved and sustained.
- Target 13: By 2020, the genetic diversity of cultivated plants and farmed and domesticated animals and of wild relatives, including other socio-economically as well as culturally valuable species, is maintained, and strategies have been developed and implemented for minimising genetic erosion and safeguarding their genetic diversity.

Following the tenth meeting of the Conference of the Parties to the Convention on Biological Diversity, the second decadal format (2011-2020) of Global Strategy for Plant Conservation was launched. The vision is 'of a positive, sustainable future where human activities support the diversity of plant life (including the endurance of plant genetic diversity, survival of plant species and communities and their associated habitats and ecological associations), and where in turn the diversity of plants support and improve our livelihoods and well-being' (<https://www.cbd.int/gspc/strategy.shtml>). The Strategy consists of the following five objectives: I – Plant diversity is well understood, documented and recognized; II – Plant diversity is urgently and effectively conserved; III – Plant diversity is used in a sustainable and equitable manner; IV – Education and awareness about plant diversity, its role in sustainable livelihoods and importance to all life on Earth is promoted; V – The capacities and public engagement necessary to implement the Strategy have been developed. Objective II, dealing with the conservation of plant diversity, includes two targets relevant to ex situ conservation through cryopreservation:

- Target 8: At least 75% of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes.
- Target 9: 70% of the genetic diversity of crops including their wild relatives and

other socio-economically valuable plant species conserved, while respecting, preserving and maintaining associated indigenous and local knowledge.

The terms and technical rationale for Target 8 state that the 'ex-situ collections should be accessible, backed up, genetically representative and should preferably be in the country of origin. However, this could also include conservation measures undertaken in another country on behalf of the relevant authorities (e.g., seed banks)'.

Finally, the State of the World's Forest Genetic Resources, based on 86 country reports received by June 2013 (Beardmore et al., 2014). The accompanying Global Plan of Action for the Conservation, Sustainable Use and Development of Forest Genetic Resources identifies 27 strategic priorities grouped into four areas: 1) improving the availability of, and access to, information on FGR; 2) conservation of FGR (in situ and ex situ); 3) sustainable use, development and management of FGR; 4) policies, institutions and capacity-building. Strategic Priority 6 aims to 'Promote the establishment and development of efficient and sustainable ex situ conservation programmes, including in vivo collections and genebanks'. Actions envisaged include: promote the use of post-harvesting procedures that maintain the quality of the seed before and after ex situ conservation; foster studies on seed collection, quality, conservation and reproduction; and promote and encourage research on the conservation of recalcitrant-seed species.

The question that needs to be addressed regarding the implementation of banking for the preservation of plant species is: how many species should be cryopreserved and as what type of material? For elite genotypes and vegetatively propagated species maintaining clonal material is critical to retaining genetic homogeneity. Thus for the major clonal crops (potato, yam, banana, cassava, fruit trees, etc.), shoot-tip or somatic embryo cryopreservation is the preferred option. This would also be the case for species of biotechnological value for their secondary products, e.g., medicinal species, of which there are 5,000-7,000 in both India and China. For these species, the preferred material for cryopreservation is somatic embryos, cultured cells and shoot tips; all of which have been worked on extensively regarding methodological development. Some clonal material can, however, be stored as seed. Cleistogamous species have non-opening flowers, are self-pollinating and produce seeds. This reproductive trait is found in about 10-15% of species, particularly grasses and legumes. A similar situation occurs for apomictic species in which asexual reproduction occurs through seeds. This trait is present in about 40 families and about 1% of higher plant species.

Undoubtedly, the three most important groups of species for seed cryopreservation are: 1) otherwise inherently short-lived orthodox seeds (Pritchard, 1995); 2) endangered exceptional species that produce few seeds (Pence, 2014); and 3) recalcitrant seeds (Li and Pritchard, 2009; Walters et al., 2013).

Little is known about the lifespan of the vast majority of the world's seeds when stored under dry cold conditions. In a study on dry seeds of 276 crop species held in the USDA Fort Collins seed bank (initially at 5°C and then at -18°C), a variation in estimated P50s of at least two orders of magnitude was evident (Walters et al., 2005). On this basis, about half the species might be considered to be relatively short-lived. As noted by Walters et al. (2005), 'some plant families had characteristically short-lived (e.g., *Apiaceae* and *Brassicaceae*) or long-lived (e.g., *Malvaceae* and *Chenopodiaceae*) seeds' and 'seeds from species that originated from particular localities had characteristically short (e.g., Europe) or long (e.g., South Asia and Australia)' lifespans.

Sensitivity to conventional banking dry, cold (-20°C) storage has been known since the early 1990s, particularly for tropical and sub-tropical oilseeds, e.g., papaya, oil palm, coffee, citrus, and orchids (see Dussert and Engelmann, 2006; Malik et al., 2012; Pritchard, 2004). But its widespread occurrence has been only more recently realised. Orchids are a particularly challenging group, often requiring an in vitro environment for germination to mimic the nutrient providing role of a symbiotic fungus in nature. As the largest plant family, with 27,000 species, interest in how to maximise seed lifespan remains high. However, there are many examples of poor storage in these minute, oil-rich, mainly tropical species (Table 3). In the example shown here, just one of the species in two genera stored well under

conventional conditions.

Table 3. Dry storage at c. -20°C of seed of seven orchid species. (Modified from Seaton et al., 2013).

Species	Initial germination (%)	Germination after 9 or 12 months storage (%)
<i>Coelogyne asperata</i>	35±2	2±1
<i>Coelogyne foerstermannii</i>	82±3	5±1
<i>Coelogyne pandurata</i>	99±0	7±2
<i>Coelogyne rumphii</i>	65±4	1±1
<i>Dendrobium macrophyllum</i>	70±4	53±1
<i>Dendrobium mirbelianum</i>	96±1	0
<i>Dendrobium stratiotes</i>	96±1	1±0

The long-term survival of plant tissues in cryo is known, with a report on pea and strawberry meristems after 28 years of cryobanking (Caswell and Kartha, 2009). Walters et al. (2004) have shown the benefits of applying cryogenic temperature to slow down the ageing rate in lettuce seeds stored for >10 years. Based on the ageing time courses for these seeds stored at temperatures between 50 and -196°C, a reduction in the temperature dependency on aging rate occurred at about -15°C and coincidental with triacylglycerol phase changes (-40 to -7°C). Nonetheless, cryogenic storage prolongs lifespan, with projected half-lives of c 500 and 3400 years for fresh lettuce seeds stored in the vapour and liquid phases of liquid nitrogen, respectively. However, cryogenic temperatures were not sufficient to stop deterioration completely (Walters et al., 2004; Ballesteros and Pence, 2014). In an attempt to reduce the rapid rate of deterioration of dry seeds of the orchid *Gavilea littoralis* we compared storability at temperatures from 15°C to that of liquid nitrogen (-196°C). Germination was retained best when ultra-cold temperature was used (Figure 2). Further progress in this area can be expected as mature seeds of 57 orchid species are known to tolerate cryopreservation (Popova et al., 2016).

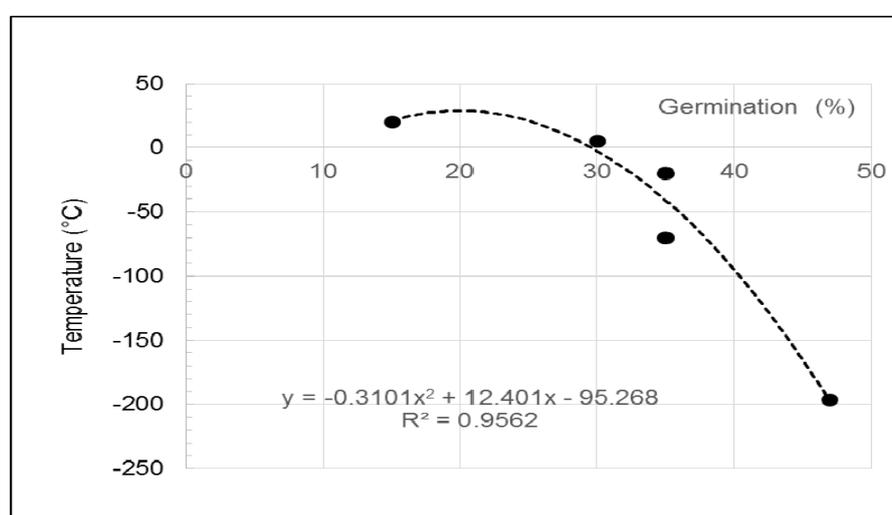


Figure 2. Effect of storage temperature for one year on seed germination of the orchid *Gavilea littoralis*. The initial in vitro germination was 41±5% (modified from Seaton et al., 2013).

Concerning so-called 'exceptional species or 'plant species with extremely small populations (PSESP)', i.e., those for which valuable germplasm in short supply due to extreme rarity or very low seed set, cryo-banking should be considered an essential option

(Pence, 2014; Merrett Wade et al., 2016).

Finally, as recalcitrant (desiccation sensitive) seeds are dispersed at high moisture content (Berjak and Pammenter, 2008) it is not possible to store fresh seeds at sub-zero temperatures due to the induction of ice formation. High desiccation sensitivity is most common in species of moist, relatively aseasonal vegetation zones, and reaches about 47% in evergreen tropical moist forest (Tweddle et al., 2003). As roughly half the world's higher plants inhabit such environments, recalcitrant seeded species could represent close to one quarter of the world's higher plants. Developing cryopreservation protocols for such material is thus an urgent imperative (Li and Pritchard, 2009; Walters et al., 2013).

Overall, there are perhaps 10,000 species of particular interest in cryopreserving clonal material. In addition, there might be about half the world's bankable seeds with quite short-lived seeds, compared with the longest-lived. Then, approaching half of the world's species in the tropical wet forests could have recalcitrant seeds. In all, as many as two-thirds the world's plants might need cryo-banking for long term storage. For orthodox seed, including those with rapid viability loss at -20°C, (the so-called intermediate (Walters, 2015) or Type II [Pritchard, 2004] seeds), cryopreservation should delay significantly the interval to seed regeneration to replenish seed after viability has dropped below an established minimum threshold (i.e., below 85% of initial germinability of the stored seeds) (FAO, 2013). For recalcitrant seeded species, cryopreservation is the *ex situ* conservation method of choice.

### **HOPE – TECHNOLOGICAL INNOVATIONS, KNOWLEDGE AND UNDERSTANDING**

During the last quarter of a century plant cryopreservation has advanced at a rapid rate, mainly due to technological (and biotechnological) developments. Of particular significance has been the steady flow of methodological improvements, including encapsulation-dehydration, encapsulation-vitrification, vitrification and droplet-vitrification, combined with the design of V- and D-plates to maximise cooling rates (see reviews by Benelli et al., 2013; Engelmann, 2011; Sakai and Engelmann, 2007). These approaches have been extremely effective at enabling cryopreservation of specimens about 2 mm across.

For larger samples, such as the isolated axes of recalcitrant seeds, which are c. 5 mm long and composed of heterogeneous tissues, control of dehydration has been often controlled through rapid artificial drying in a flow of air. Results have been variable, with the root generally more tolerant than the shoot; and reflecting differential desiccation rates and thus stresses across the various tissues of an explant or among explants in a batch (Pritchard et al., 1995; Ballesteros et al., 2014; Xia et al., 2014). Insufficient drying of axes increases the risk of intracellular ice formation. Although the formation of small intracellular ice crystals might not cause immediate physical damage, autophagic degradation may follow (Wesley-Smith et al., 2015), inducing programmed cell death, which is also known to contribute to orthodox (desiccation tolerant) seed viability loss (Wang et al., 2015).

Given the challenge of precisely controlling drying and cooling in complex tissues – to avoid desiccation stress (osmotic effects) and ice formation, respectively – new ways of creating an equilibrium state are required. In addition, chemical stress from plant vitrification solutions also needs to be limited. Two developments appear to have started to address these concerns. Firstly, a set of treatments have been developed for droplet-vitrification that uses a standard procedure, associated with additional treatments and alternative vitrification solutions. In this way it has been possible to identify whether the material is tolerant or sensitive to chemical toxicity and to the osmotic stress of dehydration with vitrification solutions, thus revealing which is the main barrier in solution-based vitrification methods (Kim and Lee, 2012). Secondly, vacuum infiltration vitrification (VIV) has been used rapidly infuse PVS into complex embryo tissues, thereby improving cryopreservation success and significantly reducing exposure time to the cryoprotectant (Nadarajan and Pritchard, 2014).

## CONCLUSIONS

The developments in cryobiotechnology over the last few decades provide a solid foundation for the application of cryopreservation for the conservation of any plant species, i.e., the mainstreaming of cryopreservation in the near future. But advances will be made much faster if the knowledge being developed is shared rapidly and widely. The attempts to do this are greatly helped through continent-wide research programmes, e.g., COST Action 871 on the 'Cryopreservation of Crop Species in Europe' (Grapin et al., 2011), and ambitious training programmes, e.g., NBPGR's 'In Vitro and Cryopreservation for Conservation of Plant Genetic Resources: Current Methods and Techniques', which has trained >100 young scientists in plant cryopreservation over the last decade (<http://www.nbpgr.ernet.in/>). Nonetheless, a co-ordinated global research and training programme aimed at accelerating the cryobanking of tropical plants of value to humankind is now urgently needed.

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