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Cryopreservation of Fruit Genetic Resources-A Review

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A B S T R A C T

Conserving the plant genetic resources through any means is of utmost importance to prevent the loss of plant biodiversity. Long-term storage using liquid nitrogen (LN) is usually adopted for developing long-term base collections of crops for use in the distant future. The fruit crops are commonly propagated through vegetative means and are majorly conserved as *ex situ* field gene banks, which require large areas and demand intensive field management. The need for repeated reculturing and chances of contamination during subcultures in *in vitro* banking necessitate a long-term contamination free conservation through cryobanking. Improved cryopreservation techniques will provide added security in the preservation of important woody plant germplasm formerly preserved only as field collections.

Introduction

Conserving the plant genetic resources through any means is of utmost importance to prevent the loss of plant biodiversity. Conservation can be achieved through two broad methods, *i.e.*, *in situ* and *ex situ*. Conservation of germplasm under natural conditions is referred to as *in situ* conservation. The *ex situ* conservation refers to preservation of germplasm in gene banks. Through *ex situ* conservation techniques, it is possible to preserve entire genetic diversity of a crop species at one place, with comparatively lesser expense and less intensive management. The *ex situ* conservation methods comprise of seed gene banks, field gene banks, botanic gardens, *in vitro* banks, cryogene bank and DNA banks. Long-term storage using liquid nitrogen (LN) is usually adopted for developing long-term base collections of crops for use in the distant future, functioning as a safety backup for similar collections held in clonal repositories, field gene banks (FGB) or *in situ* conserved genetic material is subject to threats from natural vagaries.

The cryogene banks (preserving the genetic resources in ultra-low) temperatures helps in long-term conservation (for indefinite periods theoretically) of plant genetic resources which is very easier and require limited space and management.

Cryopreservation

The term cryopreservation is used for the process of storage of living material at ultralow temperatures in liquid nitrogen (−196 °C) or its vapour phase (−150 °C). At this temperature, all cellular, metabolic and biochemical events stop, and the plant material can be stored without any changes or deterioration for extended time periods (Reed, 2017). The principle of cryopreservation is to bring plants cells or tissues to a zero metabolism and non-dividing state by reducing the temperature in presence of cryoprotectant.

The ultra-low temperature storage of plant genetic resources can be achieved by keeping them over solid carbon dioxide (-79℃), in low temperature deep freezers (-80℃), in vapour phase nitrogen (-150℃) or in liquid nitrogen (-196℃). However, liquid nitrogen is most widely used in long term storages owing to its chemically inert and nontoxic nature, low cost, easy availability and due to non-inflammable properties.

Relevance of cryopreservation in conserving fruit genetic resources

The fruit crops are commonly propagated through vegetative means and are majorly conserved as *ex situ* field gene banks. These field gene banks require large areas to conserve the genetically variable germplasm and demand intensive field management. Being highly heterogeneous and recalcitrant in nature, the fruit crops could not be conserved through seed banking. In addition, the field gene banks are always in the threat from pest and disease outbreak, changing climate and natural calamities. The short- and medium-term conservation through *in vitro* banking back up the clonal repositories, the need for repeated reculturing and chances of contamination during subcultures necessitate a long-term contamination free conservation through cryobanking.

Selection of plant material

Cryopreservation involves storage of plant material such as intermediate or recalcitrant seeds (which are difficult to conserve through other techniques), dormant buds from temperate and subtropical fruit crops which undergo winter dormancy, shoot tips or meristems, zygotic or somatic embryos and pollen at ultra-low temperatures. The ideal plant material for cryopreservation should have attributes such as smaller size, less in moisture content, highly vacuolated cells and should be collected from healthy younger parts. Meristems are proven to be ideal explant for cryopreservation (Kalaselvi *et al.,* 2017)

Mechanism of cryopreservation

Cryopreservation of biological tissues can be successful only when intracellular ice crystals do not form, since they cause irreversible damage to cell membranes and thus destroy their semipermeability. Crystal formation without an extreme reduction of cellular water can be prevented by vitrification, i.e., the non crystalline solidification of water. Vitrification is a physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and on further cooling solidifies into a metastable glass, without undergoing crystallization.

As glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to metabolic inactivity and stability over time. To achieve a state of vitrification, there are two conditions required: (1) rapid freezing rates and (2) a concentrated cellular solution. The latter is obtained through air drying, freeze dehydration, application of penetrating or

non-penetrating substances (cryoprotectants) or acclimation (Agrawal *et al.,* 2019)

Techniques of cryopreservation

From the original slow cooling approach, research has moved to easier and more reproducible techniques such as desiccation, pregrowth, pregrowth-desiccation, vitrification (Sakai *et al.,* 1990), encapsulation-dehydration (Fabre and Dereuddre, 1990), encapsulation-vitrification (Matsumoto *et al.,* 1994), droplet-freezing (Schäfer-Menuhr *et al.,* 1994) and ultrarapid freezing or droplet-vitrification (Agrawal *et al.,* 2004 and Panis *et al.,* 2005). Most recent protocols are ones using an aluminium cryoplate or stainless steel cryo-meshes, which combines the methods of encapsulation-vitrification and droplet-vitrification (Funnekotter *et al.,* 2017 and Matsumoto, 2017).

Slow Freezing or Controlled Freezing (Classical Method)

Slow cooling comprises steps of tissue dehydration followed by slow cooling (at rates of 0.1 to 4°C/min) of the explant, down to a defined prefreezing temperature (usually of −30 to −40 °C) and rapid immersion in LN (Engelmann, 2004). As such, cells and the external medium initially supercool, followed by extracellular ice formation and avoidance of intracellular ice formation (Mazur, 1984). Slow freezing requires the use of costly programmable freezing apparatus (with greater temperature control). This method combines the application of penetrating cryoprotective substances such as dimethyl sulfoxide (DMSO) and controlled freeze dehydration. A modification of this method is known as the two-step cooling, where no special programmable freezer is required. Plant tissues are treated with cryoprotectants, normally at room temperature (25 °C), and are kept at −30 °C for 30–120 min. They are then immersed in LN thereafter. Cryopreserved tubes are warmed using hot water (40 °C) for 1~2 min, and cryoprotectants are removed from a tube. After rewarming, samples are moved from the cryotube and recultured (Agrawal *et al.,* 2019).

New Vitrification-Based Techniques

In these techniques, vitrification is achieved by direct immersion in LN without the freeze concentration step (as in slow cooling), by exposing the cells to extremely concentrated (7 to 8 M) cryoprotectant solutions (Sakai and Engelmann, 2004).

Dehydration freezing

Cryopreservation of seeds of Citrus species after desiccation was attempted by Kaya *et al.,* (2016) in Turkey, wherein the seeds of six citrus species viz., *Citrus limon* (L.) Burm, *C. limonia* Osbeck, *C. jambhiri* Lush., *C. aurantium* L., *Poncirus trifoliata* Raf. × *C. sinensis* Osb. (Troyer citrange) and *Fortunella margarita* (Lour.) Swingle were tested for their initial moisture content, viability and germination. These seeds were subjected to air desiccation for different durations (depending on their initial moisture contents) so that the seed moisture content reached to 10-20 per cent. The dehydrated seeds were directly immersed into liquid nitrogen. The fresh seed moisture content was higher in Troyer citrange (56.3%), *C. limon* (51.1%) and *C. jambhiri* (49.7%), while lowest was in *Fortunella margarita* (25.1%). Maximum tolerance to cryopreservation was observed in *C. jambhiri* with 93.3 per cent germination after 12 hours of dehydration to moisture content of 21.1 per cent. Lowest seed germination (43.3%) was observed in *Fortunella margarita* when dehydrated for 3 hours to a moisture content of 24.2 per cent,

while the species had a better germination when desiccated for six hours to moisture content of 19.0 per cent. In other species also, the optimum seed moisture content and dehydration duration were optimized for successful cryopreservation. Thus in Citrus species, which produce polyembryonic non-orthodox oily seeds, air desiccation of seeds to moisture levels of around 20 per cent proved better for successful cryopreservation.

In *Passiflora edulis*, the seeds with initial moisture content of 25 per cent were desiccated to various moisture levels (15, 10 and 5 %) by thin layer drying at 40 ℃ for different durations (42, 70 and 100 minutes respectively and exposed to liquid nitrogen treatment (Generoso *et al.,* 2019). The cryopreserved seeds with water content of 10% produced all normal seedlings under *in vitro* while non-desiccated seeds did not produce normal seedlings. The non-desiccated seeds produced either abnormal seedling (7.5%) or remained as ungerminated embryos (92.5%).

Encapsulation-Dehydration

The technique involves immersion of explants in alginate solution (usually 1–5%) and their subsequent release into a complexing agent (50–100 mM CaCl2 solution) where bead hardening occurs in 20–30 min. These synseeds are next cultured in a highly concentrated sucrose solution (0.7–1.5 M), followed by physical dehydration to a moisture content of 20–30%, using air of laminar flow or silica gel (Engelmann, 2004). The dehydrated beads are then cryopreserved. Culture of explants on sucrose-enriched medium (0.3–0.7%) prior to encapsulation usually improves survival after desiccation and freezing. This method is simple, but somewhat labour intensive, and some species do not tolerate the high sucrose concentrations employed. The use of anti-oxidant and anti-stress compounds (e.g. lipoic acid, glutathione, glycine betaine, polyvinyl pyrrolidone, melatonin) has been shown to increase post-thaw recovery in explants cryopreserved by this method (Uchendu *et al.,* 2010).

In rough lemon (*Citrus jambhiri*), the embryonic axes from three genotypes IPS-7, IPS-85 and IPS-120 were subjected to encapsulation. The fresh beads were precultured in various concentrations of sucrose (0.3, 0.5 and 0.7M) for 40 hours and moisture contents were recorded. Further the embryonic axes were air desiccated for 6 hours so that the moisture contents were reduced to less than 22 per cent in all the accessions. Preculturing beads on 0.5 M sucrose followed by 6 h of air desiccation gave maximum survival of axes before and after cryopreservation in IPS-7 and IPS- 85, but for accession IPS-120 successful cryopreservation with a highest viability of 66.66 and 50 per cent respectively, was achieved for axes precultured for 40 h in 0.3 M sucrose preculture media, followed by 6 h of desiccation. More than 100 accessions of *C. jambhiri* have been cryostored in the national cryobank at NBPGR, New Deli (Rohini *et al.,* 2016).

Vitrification

In this technique, most or all freezable water is removed by physical or osmotic dehydration of explants (thus increasing cellular viscosity), followed by ultrarapid freezing which results in vitrification of intracellular solutes. A number of steps are involved – preculture of explants on medium fortified with cryoprotective substances, treatment with a loading solution (e.g. a mixture of 2 M glycerol and 0.4 M sucrose), dehydration with a highly concentrated vitrification solution (from 15 min up to 2 h), rapid freezing and thawing, removal of

cryoprotectants and recovery. A widely used vitrification solution (mixture of penetrating and non-penetrating cryoprotectant substances) developed by Sakai *et al.,* (1990) is named plant vitrification solution 2 (PVS2). It consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in liquid medium with 0.4 M sucrose and a total molarity of 7.8 M. Cells are osmotically dehydrated by PVS2 at a nonfreezing temperature, ranging from 0 to 25 °C (Sakai *et al.,* 1990).

Vitrification of shoot apices of pineapple *in vitro* plantlets was attempted by Gonzalez-Arnao *et al.,* (1998) wherein the apices were precultured in medium containing 0.3 M sucrose and loaded in medium with 0.75M sucrose + lM glycerol for 25 minutes. When the apices were dehydrated in PVS2 solution under different exposure periods at 25°C, the survival after cryopreservation was very less.

When the apices were dehydrated in PVS2 at 0°C, the apices survived better. The highest survival of apices after cryopreservation (65%) was observed with PVS2 vitrification solution at 0°C for 7 hours before rapid freezing in liquid nitrogen. The study has emphasized on the relevance vitrification temperature on successful cryopreservation of pineapple shoot apices.

In *Passiflora pohlii*, a wild species of Passiflora resistant to soil borne pathogens, studies to cryopreserve the nodal segments of 0.5 cm through vitrification were taken up. The explants after preculture in half strength MS media containing 0.7 M sucrose, were exposed to two vitrification solutions namely PVS2 (30% glycerol, 15% dimethyl sulfoxide (DMSO), 15% ethylene glycol (EG) and 13.7% sucrose, w/v) and PVS3 (50% glycerol and 50% sucrose, w/v) for different exposure timings before immersing to liquid nitrogen. The highest recovery (65%) was obtained with the vitrification technique using treatment with the PVS3 solution for 30 to 120 min. Also, 40 per cent recovery was observed in segments treated with PVS2 for 60 minutes when incubated in dark conditions while post-warming incubation in light did not recover any cryopreserved nodal segments (Merhy *et al.,* 2014).

Encapsulation-vitrification

In this technique, the explants are encapsulated and subjected to vitrification, without any further physical desiccation. It combines the advantages of vitrification and of encapsulation-dehydration (ease of manipulation of encapsulated explants) and applied to apices of an increasing number of species (Matsumoto *et al.,* 1994 and Sakai and Engelmann, 2007)

In pineapple varieties MD-2 and Peurto Rico, efficiency of encapsulation-vitrification of shoot apices was compared with vitrification by Gamez-Pastrana *et al.,* (2004). To improve the survival of apices on cryostorage, the pretreatment conditions were modified by supplementing Proline (3 M) along with Sucrose. Optimal conditions involved the encapsulation of pineapple apices in calcium alginate (3 %) followed by two-stage preculture in liquid medium with 0.16 M sucrose + 0.3 M proline for 24 h and then transfer to 0.3 M sucrose + 0.3 M proline for an additional 24 hours.

Encapsulation-vitrification was more effective for cryopreserving apices of both varieties when using PVS3 for dehydration. These conditions gave the highest survival rates after cryopreservation (54 % with apices of MD-2 and 83 % with apices of Puerto Rico) while dehydration with PVS2 resulted in lesser survival of encapsulated beads as compared to vitrification

Droplet-Freezing/Vitrification

Droplet-freezing method (also referred as ultra-freezing or fast-freezing method in literature) was initially established successfully for potato (Schäfer-Menuhr *et al.,* 1994). The method refers to refers to droplets of cryoprotectant on an aluminium foil, into which explants are placed for cooling and rewarming. In this method, a drop of PVS2 is placed on aluminium foil strips, onto which explants are placed and plunged in LN. The application of droplet-freezing allows higher cooling and thawing rates compared to normal vitrification (of about 130℃/min), and the chance to obtain a vitrified state during freezing and to avoid devitrification during thawing increases. Varied explants such as shoot tips, meristems, embryogenic callus, hairy roots and embryonic axes have been used.

In banana, explants used for cryopreservation of banana (*Musa* L. spp.) are mainly sourced from tissue culture. The regenerative potential of cryopreserved sucker derived meristem (SM) was compared with two types of routinely employed explants viz., *in vitro*-raised single-shoot meristems (IVM) and proliferating meristems (PM). The regeneration frequency of SM was high (60.0±11.5%) and statistically comparable to PM (68.3±4.4%) and IVM (55.6±11.1%) after using the droplet vitrification cryopreservation technique.

The total time required for cryopreserving plants from SM (2 months) was substantially less than that for PM (14 months) and IVM (8 months). In addition, the study revealed that cryopreserved plants were statistically comparable to the mother plants raised from suckers for all important growth and yield parameters. Thus Musa germplasm could be effectively cryopreserved using droplet vitrification method with a new type of explant, the sucker derived meristem (Agrawal *et al.,* 2014).

In *Citrus limon*, successful cryopreservation of *in vitro* grown shoot tips through droplet vitrification was reported in two cultivars 'Frost Eureka limon' and 'Cook Eureka limon' by Yi *et al.,* (2018), wherein preculturing shoot tips in MS + 0.3 M sucrose for 48 hours and then treated in MS + 0.5 M sucrose for 16 hours was optimized, followed by loading and dehydration. The shoot tips were treated with two vitrification solutions PVS2 and PVS3 for varied exposure times (30, 60, 90 and 120 minutes) at 0 ℃ and transferred to aluminium foil strips containing PVS2 or PVS3 and directly plunged into liquid nitrogen. In both the cultivars, higher regrowth percentages of shoot apices on micrografting on Troyer citrange seedlings were observed for PVS2 treatment for 60 minutes or PVS3 for 90 minutes.

Cryoplates method

Recent developments in cryopreservation of plants have been steps that have replaced use of aluminium foil with aluminium cryo-plates (V cryo-plate and D cryo-plate, Matsumoto, 2017). In these methods, shoot tips are stuck on special aluminium plates with uniform wells using alginate, and subjected to vitrification procedures. These techniques have two main advantages; (1) they are user-friendly because samples are easy to handle, with reduced risk of damage or loss of explants, and (2) they have a high rate of regrowth owing to the very high cooling and warming rates (Niino *et al.,* 2013; Yamamoto *et al.,* 2015 and Funnekotter *et al.,* 2017). The V cryo-plate method (Yamamoto *et al.,* 2011b) is based on PVS2-vitrification-dehydration of explants on a cryo-plate, and the D cryo-plate method (Niino *et al.,* 2013) is based on air dehydration. Precultured (0.3 M sucrose) shoot tips are attached to small

wells of a cryo-plate with alginate beads and treated with LS solution (2 M glycerol + 0.6–1 M sucrose) for 15–30 min and then dehydrated with PVS2 solution (V cryo-plate method) or in a laminar flow cabinet (D cryo-pate method) for a suitable duration. Then the cryoprotected and dehydrated shoot tips are immersed in LN directly. For regrowth, shoot tips attached to the cryo-plate are transferred to a 1 M sucrose solution for rapid warming and unloading at 25 °C for 15 min and plated on a culture medium. Higher rates of regrowth have been reported using the cryo-plate methods due to the very high cooling and warming rates.

For preserving the shoot tips of *in vitro* and field grown blueberries, Dhungana *et al.*, (2017) have attempted D- cryoplate method. The ideal explant, preculture treatments, loading and desiccation period were investigated and found that shoot tips from *in vitro* grown plants had better survival (86.7%) than shoot tips from field grown current shoots (59.7%). To induce osmo-tolerence, the shoot tips were precultured in MS media containing 0.3,0.5 and 0.7 M sucrose at 25 ℃ for varying durations of 1, 2 and 3 days. Preculturing in MS media with 0.3 M sucrose for one day was found better for higher regrowth (79.5%) after cryopreservation.

The loading solution treatment (2M glycerol and 0.6 M sucrose) of precultured shoot tips for 30 minutes followed by desiccation for one hour resulted in highest recovery of cryopreserved shoot tips in blueberry. Thus *in vitro* grown shoot tips precultured in 0.3M sucrose for one day, followed by loading with 2M glycerol and 0.6 M glucose for 30 minutes at 25 ℃ and desiccation for one hour was optimized for cryopreservation. With this protocol, cryopreservation of six blue berry cultivars using shoot tips from *in vitro* and current shoots was attempted. The percentage regrowth of *in vitro* shoot tips ranged from 46.7 to 100 per cent whereas in current shoot tips, recovery ranged from 17.2 to 71.5 percent. Highest regrowth was recorded in the cultivar Blugold (100%) using *in vitro* grown shoot tips which was significantly higher than current shoot tips (17.2%), followed by Festival (96.9% in *in vitro* shoot tips). In cultivars viz., climax, Magnolia and Weymouth no significant difference was observed in regrowth percentage of *in vitro* and current shoot tips showing the influence of varietal response in cryopreservation (Dhungana *et al.*, 2017).

Successful Cryopreservation of *in vitro*-grown shoot tips of strawberry by the vitrification method using aluminium cryo-plates is reported by Yamamoto *et al.*, (2012). In the present study, the shoot tips (1.5–2.0mm £ 0.5–1.0mm) were dissected from the shoot and pre-cultured at 58C for 2 d on Murashige and Skoog medium containing 2M glycerol and 0.3M sucrose. The pre-cultured shoot tips were placed on the aluminium cryo-plate containing ten wells embedded in alginate gel. Osmoprotection was performed by immersing the cryo-plates in a loading solution (2M glycerol and 0.8M sucrose) for 30 min at 25℃. Dehydration was performed by immersing the cryo-plates in plant vitrification solution 2 for 50 min at 25℃. Then, the cryo-plate with shoot tips was transferred into an uncapped cryotube that was held on a cryo-cane and directly immersed into liquid nitrogen (LN). After storage in LN, shoot tips attached to the cryo-plate were directly immersed into 2ml of a 1M sucrose solution for regeneration. With this protocol, the regrowth levels of shoots of 15 strawberry cultivars were observed. Regrowth was very high for all cultivars, ranging from 70 to 97 per cent, with an average of 81 per cent for the 15 cultivars. Highest regrowth was recorded in Hatsukuni (97%) followed by Cavalier (93%).

Comparison of cryopreservation techniques

There are advantages as well as disadvantages associated with each method. Information on efficacy of protocols is required before selecting the method of choice for long-term conservation of germplasm in gene banks. A study was conducted to compare the efficacy of existing methods namely simple freezing and vitrification with a fast freeze/fast-thaw (droplet freezing) method for meristems in banana by Agrawal *et al.,* (2004). The average post-thaw shoot regeneration of the fast freeze/fast thaw protocol was 51.8 per cent and was superior to the existing protocols (12.5%, 39.2% and 42.8%, respectively). In droplet freezing method, in addition to higher post-thaw regeneration, frequency of callus formation was lowest (3.7%). It was found that the fast freeze/fast thaw technique was the best amongst all the four methods tested for the cryopreservation of *in vitro* banana meristems in the genotype Robusta.

Stability of cryopreserved material

Genetic stability of any plant material after *ex situ* conservation, especially in case of *in vitro* and cryo gene banks are of important concern. Assessment of genetic stability in terms of morphological, histological and molecular integrity has earned greater attention since variations can occur due to various reasons such as tissue culture, toxicity due to cryoprotectants and regeneration process. Hence, before routinely establishing the cryobank any species, studies on the field performance and genetic fidelity of plants derived from cryopreserved plant material need to be carried out.

Agrawal *et al.,* (2009) compared the performance of cryopreserved banana germplasm of cultivar 'Sommarani Monthan' (AAB, Monthan subgroup) for agronomic and molecular traits (using simple sequence repeats; SSR) with that of its parental type. Experiment included cryopreserved germplasm, cryopreservation control, *in vitro*-conserved plants, and natural sucker raised plants.

Genetic stability of original mother plants maintained in the field genebank, *in vitro*-conserved plants, PVS2-treated plants and cryopreserved plants was assessed using 12 agronomic traits and nine SSR primers. Results indicated that no significant differences existed for the major growth and yield parameters analyzed, except in one plant. Even the *in vitro*-conserved plants (conserved for 6 years) were statistically comparable to the original mother plants raised from natural suckers in all the important growth and yield parameters. In one of the plants raised from cryopreserved meristems, the fruit colour was found to be green instead of ash green. Among the nine primer pairs tested, seven primers pairs namely AGMI-33,34, AGMI-35,36, AGMI-67,68, AGMI-93,94, AGMI-95,96, AGMI-129,130 and MbSSR 1-149 amplified products resulting in discrete repeatable amplicons. All the alleles were found to be monomorphic. The SSR primer pairs tested also did not show any variation for the green fruited variant as compared with control plants

Choudhary *et al.,* (2013) examined the genetic stability of cryopreserved dormant buds of *Morus* germplasm that were stored in liquid nitrogen using two-step freezing, then rewarmed and regrown. The plants were regenerated directly from dormant buds (before and after cryopreservation) without intermediary callus phase. Fifteen primers were selected for the RAPD analysis based on the reproducibility and banding patterns. All the primers have shown monomorphic banding patterns and did not reveal any polymorphism among the mother plant and *in*

vitro regenerants before and after cryopreservation, suggesting that cryopreservation, using two-step freezing, does not affect genetic stability of mulberry germplasm.

Status of cryopreservation in fruit crops

Globally, successful cryopreservation protocols have been optimized for Banana, Pine apple, Citrus, Papaya, Strawberry, Japanese Persimmon, Olive, Date palm, Bluberry and Grapes using various explants adopting different cryogenic storage techniques. At the international, National Institute of Agro biological Resources (NIAR) of Japan holds about 50 accessions of mulberry and INIBAP, Laboratory of Tropical Crop Improvement, Belgium has 440 banana accessions in cryogenebank. At present, the institutes such as National Bureau of Plant Genetic Resources (NBPGR, New Delhi) and Indian Institute of Horticultural Research (IIHR, Bengaluru) are involved large-scale cryobanking of horticultural crops in India. The NBPGR holds 13363 accessions of various agri-horticultural species in the form of seeds/ embryo/ embryonic axes/ dormant buds/ meristems or pollen, of which 3465 accessions of fruits and nuts are cryopreserved as seeds, while IIHR has 675 accessions of pollen of various horticultural species (Agrawal *et al.,* 2019).

In conclusion the cryopreservation protocols developed in various fruit crops can aid in long term conservation of genetic resources with several advantages of saving land, time, labour and energy without losing the maternal genetic fidelity. But the technique has its own demerits of using toxic chemicals, posing oxidative stress to the tissues, which needs to be investigated thoroughly. Though with manifold benefits, the cryogenic storage cannot be a standalone approach for conservation of fruit genetic resources, yet it can complement other conservation methods. Thus, it is expected that improved cryopreservation techniques will provide added security in the preservation of important woody plant germplasm formerly preserved only as field collections.

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