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Cryopreservation of Forest Tree Seeds: A Mini-Review

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Abstract

Since forest trees form the basis of forest ecosystem, their prolong subsistence is crucial for various flora and fauna. The foremost challenges to sustain the forest ecosystem comprise of the declining forest tree population accompanied with structural changes due to afforestation and exploitation of forest area, environment changes, pests, pollution, and introgressive hybridization. For *ex situ* conservation approach, *in vitro* techniques encompass basic role for conserving tree genetic resources, predominantly where natural propagules like recalcitrant seed might not be appropriate for long-term conservation. The practice includes restricted growth techniques, conventional micropropagation, production and storage of synthetic seeds, and cryopreservation. Even though these practices have been applied chiefly to herbaceous species, but recently, woody species were also focused upon. Key conceptions, challenges and techniques for forest tree seed conservation are discussed briefly in this review with special emphasis on some successful cryopreservation approaches for long-term storage.

Key Words: conservation, desiccation, liquid nitrogen, orthodox seed, recalcitrant seed

Introduction

Forests, one of the major bio-resources, are economically, ecologically, environmentally, and aesthetically important. The forestland area is consistently shrinking to meet the intensifying world demand for food, timber and other products and thus enhances the importance of conservation of the forest resources (Ledig 1988; Rajora and Mosseler 2001). Forest trees are less domesticated as compared with agricultural crop plants and even the seeds formed through breeding program are heterogeneous. Due to this reason, there is rising interest for creating germplasm banks of seed, meristem, pollen, and cell cultures as the only probable means for the long-term storage of genomes and conservation of the genetic standards of cultivated and wild-growing plants (Pence 1991a; Rao 2004; Panis and Lambardi 2005).

All of the forestlands have a specific function in maintaining the ecological balance of biodiversity. To protect indigenous species, exotic rapid-growing trees such as *Eucalyptus* could be transplanted for fuel-wood production (Merkle and Nairn 2005). Generally, *in situ* and *ex situ*

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management strategies are applied in case of forest trees (Ledig 1986, 1988; Finkeldey and Gregorius 1994). *In situ* conservation signifies a more evolutionarily approach in comparison to the stationary *ex situ* conservation method (Finkeldey and Gregorius 1994). For certain tree species where seed storage is always challenging, conservation through tissue culture might be the lone choice since *in vitro* cultures of tissue and cells and/or cryopreservation might offer exceptional opportunities for the conservation of tree genetic resources (Suranthran et al. 2012; Palanyandy et al. 2013; Sinniah and Gantait 2013; Gantait et al. 2015; Palanyandy et al. 2015).

Seed storage through cryopreservation is the most proficient technique since it provides an organized and ancillary way to maintain the forest germplasm (Linington and Pritchard 2001). Multiple attempts have been successfully made to conserve tree genetic resources through subjecting the seeds or parts of it to ultra-low temperature counting from mid twentieth century (Engstrom 1966) on *Pinus* sp. Later on, oil palm (*Elaesis guineensis*) came into area of interest (Grout et al. 1983) and that is still under advancement towards its improvement of cryoconservation strategies (Suranthran et al. 2012; Palanyandy et al. 2013; Gantait et al. 2015; Palanyandy et al. 2015). Establishment of cryo-storage protocols was successfully reported on several forest tree seeds such as *Astronium urundeuva* (Medeiros and Cavallari 1992), *Bletilla striata* (Hirano et al. 2005), *Fraxinus excelsior* (Chmielarz 2009a), *Prunus avium* L. (Chmielarz 2009b), *Betula pendula* (Chmielarz 2010), *Salix* sp. (Popova et al. 2013), *Mimusops* sp. (Wen et al. 2013), *Parkia* sp. (Sinniah and Gantait 2013), and most recently in *Populus nigra* (Michalak et al. 2015).

In the present review an illustration on outlook will be drawn based on the issues and concerns of forest seed conservation and successful reports developed to combat with those limitations.

Cryopreservation: Principles and Techniques

Cryopreservation is the lone procedure accessible to date to conserve the germplasm of problem species safely and cost-efficiently for long period of time. Cryopreservation is based upon the storage of plant materials in liquid nitrogen (LN) (-196^{o}C). The main principle is conserving the plant material in a viable but dormant stage. At this ultra-low temperature, cell division as well as metabolism remains stagnant without undergoing any kind of physical changes. Several resources such as dormant seeds or orthodox seeds can be cryopreserved without any pretreatment and demonstrates natural dehydration processes. Mazur (1984) focused on the need to dehydrate the tissue prior to cryopreservation since the intracellular water forms ice crystals, raptures cells and leads to non-viability. In the classical protocols, the desiccation of explants took place both prior to and right through cryopreservation (also called freeze induced desiccation), while the more updated techniques employ desiccation prior to cryopreservation. In the classical controlled rate of cooling, (also called two-step freezing or slow controlled-freezing) technique, hydrated tissues are generally preserved. The samples are cooled to about -40^{o}C in gradual rate (frequently 0.1-4^{o}C per min) followed by quick dipping in LN (Gonzalez-Arnao et al. 2008). The plasma-membrane offers a considerable obstruction and evaluates the transfer of ice crystals into the intracellular components of the cells so as to prevent the cell from any kind of freezing injuries inspite of its exposure to such lower temperature conditions. The excess water present inside a cell is oozed out to the peripheral medium as the temperature decreases further, so naturally the intracellular solutes become more concentrated. Controlled cooling practices are commonly complicated as they may require programmable freezers. The technique is mainly employed for dormant buds (Sakai and Nishiyama 1978; Towill and Ellis 2008), apical parts of the species that are cold sensitive (Reed and Uchendu 2008) and undifferentiated cultures (Kartha and Engelmann 1994; Withers and Engelmann 1998).

In the modern techniques, cellular dehydration is carried out just before cryopreservation by introducing the explants to a strenuous cryoprotectant solution and/or air desiccation, till the majority of freezable cell sap has been pulled out from the cells, which results in vitrification of the aqueous compartment and thus intracellular ice formation is bypassed. Generally, dehydration step is carried out by directly immersing the explants in LN. This technique is more appropriate for composite organs such as shoot tips, embryos or embryonic axes. This is particularly significant

for cryopreservation of tropical forest tree germplasms, which can now be attempted in tropical countries, provided that basic tissue culture facilities along with a dependable supply of LN. Generally five specific cryopreservation methods are practiced namely desiccation, encapsulation-desiccation, vitrification, encapsulation-vitrification and droplet-vitrification.

Desiccation method principally utilizes seeds, embryonic axes or zygotic embryos. In case of plant species where seed can be used as a source of germplasms, desiccation is generally carried out by moderate drying in a relatively humid environment by soaking them in saturated salt solutions where as in case of zygotic embryos or embryonic axes desiccation through the wind action of laminar air flow is much more prevalent and much more effective as it is attained by arid sterilized condensed air, silica gel or saturated salt solutions. Another work reported by Berjak et al. (1989) suggested that for the prevention of desiccation injuries, ultra-rapid drying could be an effective choice for cryopreserving samples with comparatively higher moisture content (MC). Encapsulation-dehydration is a technique based on the production of artificial seeds or synthetic seeds that brings about effective germplasm storage as well as its transport to various other countries. In this practice encapsulation of plant propagules in calcium alginate, regenerated in a liquid medium supplemented with sucrose for the period of 1-7 days and then dehydrating up to roughly 20% on the basis of its fresh weight by means of silica gel method followed by rapid cooling. Bonnart and Volk (2010) suggested a modification of this technique by the usage of calcium alginate along with 2M glycerol and 0.5M sucrose as the encapsulating agent and dehydrating it by air-drying instantly. Vitrification is the conversion of excess cell sap present in the cells of the plant material into an amorphous fluid thus avoiding the formation of ice crystals (Fahy et al. 1984). Plant samples are exposed to an intermediate concentrated loading solution that comprises of 2M glycerol and 0.4M sucrose (Matsumoto et al. 1994). Kim et al. (2009) have formulated a series of additional loading solutions, which are found compatible with several plant species like *Chrysanthemum*. The Plant Vitrification Solutions (PVS) that are generally used includes PVS2 (Sakai et al. 1990) and PVS3 (Nishizawa et al. 1993) which consists of (w/v) 15% ethylene glycol + 30% glycerol + 15% DMSO + 13.7% sucrose and 50% glycerol + 50% sucrose, respectively. Combining it with encapsulation, the vitrification technique has further been modified. Explants are encapsulated in a calcium alginate matrix and cryopreserved by a standard vitrification protocol that termed as 'encapsulation-vitrification'. In spite of several vitrification techniques, droplet vitrification is a much more advanced approach and most commonly used. In droplet vitrification the explants are wrapped up in an aluminum foil and immersed freely in LN. The main credit of this technique is that the explant remains in contact with LN throughout freezing and then thawing in presence of the unloading solution helps in maintaining a suitable temperature rhythm.

Forest Seeds: Concerns

According to Chin (1988) tree species of tropic and sub-tropic territories for instance cacao, coconut, coffee, citrus, rubber, oil palm and several other forest species, develop seeds that are not dehydrated, sheds at elevated levels of MC and become highly sensitive towards freezing thus, cannot be preserved by traditional methods such as low temperature. There are only few species (around 2% of the world flora) that produce 'intermediate' seeds (Tweddle et al. 2003), so, conservation of germplasm of these tree species is of prime significance as even though these seeds are stored using a standard protocol, their viability is compromised. Intermediate seeds typically comprises of a limited MC regime that limits its tolerance to LN (Dussert et al. 2001). To regulate the pre-cryopreservation treatment to maintain seed MC, use of saturated saline solutions was successful for intermediate seed forming tree species for instance coffee (Dussert and Engelmann 2006) and *Citrus* species (Hor et al. 2005). Nonetheless, species-specific protocol needs to be established for effective cryopreservation of intermediate seeds (Malik et al. 2010).

Plentiful research has been carried out to augment the competence of conservation procedures such as introducing several field genebanks and also to consider the preservation of the recalcitrant seeds. However, several *in vitro* and tissue culture techniques have been known for its conservation properties by maintaining the genetic homogeneity of the regenerated plantlets from the stored germplasms (Engelmann 2012) as these techniques confirmed

the mid-term storage of plant materials ensuring higher germination rates (Bunn et al. 2007). But on the other side for long-term storage of plant germplasms, cryopreservation is the lone technique. A wide range of explants such as seeds, latent buds (*in vivo*) and cell suspensions, calli, shoot tips, somatic as well as zygotic embryos and embryonic axes (*in vitro*) have been exploited for cryopreservation. Quite a few factors propose that the immature embryos can be more flexible to work with than the mature embryos because of two significant factors. Firstly, they are of much minute structure than mature embryos and tenders homogeneous cryoprotection, cooling, and thawing in hydrated freezing methods, and further consistent dehydration, which improves endurance of the plant cells from freezing injuries (Withers 1979). Secondly, immature embryos are more embryogenic in nature than the aged embryos (Pence et al. 1980). In case any freezing injury is caused to the zygotic embryos they can revive themselves instantly due to their high regenerating capabilities. As evidence to this above statement, Pence (1991b) reported that the zygotic embryos of *Theobroma cacao* L. (Cacao) had the capability to form callus instantly even after undergoing a fast freezing method of preservation.

Issues Regarding Cryopreservation of Forest Tree Seeds

Cryopreservation has been proven effective in conservation of embryos or embryonic axes (Kartha and Engelmann 1994; Engelmann et al. 1995; Pence 1995; Engelmann 1997). As a matter of fact freezing of embryos is an effective approach for any species in case of recalcitrant seeds (Engelmann 1999). Difference between moisture retention and stage of maturity are the two major factors that make the cryopreservation of recalcitrant embryos challenging. Embryos are widely occupied for cryopreservation since seeds of numerous species are too robust to be iced up directly. Nevertheless, embryos are comprised of multifaceted tissue compositions that demonstrate susceptible to dehydration and freezing, mostly the radicle portion seeming difficult than the plumule portion. Chandel et al. (1995) experimented with *Cacao* and its various species and concluded that embryos are exceedingly susceptible towards desiccation and decrease in their moisture content levels leads to severe internal injury to the embryo structure. Lastly, a number of species forming embryos are very rare that makes cryopreservation more complicated. Selection of embryos at the accurate stage is intricate for any experiment as it may determine the formation of ice crystals in an embryo (Engelmann et al. 1995; Engelmann 1999).

An Account on Forest Tree Seed Cryopreservation

Cryopreservation has been recognized as a prospective tool for forest tree seeds for the long-term storage. There are ample numbers of investigations that have been carried out on *in vitro* conservation of forest trees using whole seeds, their components, and embryonic axes via several protocols as a move concerning development of appropriate systems for cryopreservation. In the present review, we investigated the responses of the whole seeds to multiple number of cryopreservation protocols. The cryopreservation techniques that have been majorly employed for forest seed conservation are either desiccation or direct exposure to LN and the conclusions are demonstrated in Table 1 and 2. A schematic illustration has also been presented in Fig. 1. It is observed from the information of the tables that the tree species belonging to Fabaceae family is the most common as they respond actively towards desiccation or to direct LN.

Engstrom (1966) and Stanwood and Bass (1978) found more than 99% germination rate in case of *Pinus echinata*, *Ulmus pumila*, *Abies concolor* and *Pinus ponderosa*. Following long time presentation, Styles et al. (1982) demonstrated cryopreservation for 24 species and calculated their germination percentages after storage of 600 days. Commonly, no remarkable changes in germination rate took place after 600-day storage in LN. The MC of the seeds taken was less than 10%. Grout et al. (1983) found that in *Elaesis guineensis* there occurs a significant difference in MC between the whole seed and the embryo and thus consequences in the distress of sub-zero conservation of whole seeds. They concluded that embryos could offer a practical technique for the long-term storage of the germplasm of the species. On the other hand, Ahuja (1986) took six species (*Abies alba*, *Fagus sylvatica*, *Larix deciduas*, *Picea abies*, *Pinus sylvestris*, and *Populus tremula*×*P. tremuloides*) and found >90% germination except *Fagus*

Table 1. Examples on Success of Desiccation for Cryopreservation of Forest Seeds (In Chronological Order)

Botanical name	Family	Seed class	Regrowth (%)	Reference
Ulmus pumila	Ulmaceae	Orthodox	100	Engstrom (1966)
Pinus echinata	Pinaceae	Orthodox	100	
Abies concolor, Pinus ponderosa	Pinaceae	Intermediate Orthodox	100	Stanwood and Bass (1978)
Elaesis guineensis	Arecaceae	Intermediate	0	Grout et al. (1983)
Abies alba, Larix decidua	Pinaceae	Recalcitrant Intermediate	95	Ahuja (1986)
Fagus sylvatica	Fagaceae	Intermediate	0	
Picea abies	Pinaceae	Orthodox	99	
Pinus sylvestris	Pinaceae	Orthodox	100	
Populus tremula×*P. tremuloides*	Salicaceae	Recalcitrant	95	
Astronium urundeuva	Anacardiaceae	Orthodox	-	Medeiros and Cavallari (1992)
Bursaria occidentalis	Pittosporaceae	-	0	Touchell and Dixon (1993)
Acacia acuminata	Mimosaceae	Recalcitrant	33	
Cassia venusta	Caesalpiniaceae	-	84	
Petalostylis millefolium		-	30	
Bossiaea ornata	Papilionaceae	Orthodox	16	
Jacksonia floribunda		-	94	
Templetonia retusa		-	62	
Viminaria juncea		-	79	
Zygophyllum aurantiacum	Zygophyllaceae	-	20	
Agonis flexuosa	Myrtaceae	-	0	
Eucalyptus burracoppinensis		Orthodox	4	
Eucalyptus lanepoolei		Orthodox	2	
Eucalyptus Ioxophleba v. gratiae		Orthodox	12	
Melaleuca cuticularis		-	3	
Melaleuca huegelii		-	1	
Melaleuca uncinata		-	82	
Acer saccharinum	Aceraceae	Recalcitrant	21	Connor and Bonner (2001)
Aesculus pavia	Hippocastanaceae	Recalcitrant	24	
Chorisia speciosa	Bombacaceae	Orthodox	-	Wetzel et al. (2003)
Aegiphila lhotzkiana	Verbenaceae	Orthodox	-	
Qualea parviflora	Vochysiaceae	Orthodox	-	
Tabebuia umbellata	Bignoniaceae	Orthodox	-	
Anadenanthera macrocarpa, Albizia lebbeck, Bauhinia sp., Cassia ferruginea, Hymenaea stigonocarpa, Mimosa setosa, Platypodium elegans, Sclerolobium aureum	Fabaceae	Orthodox	-	
Caesalpiniae chinata	Fabaceae	Orthodox	-	Zanotti et al. (2007)
Fraxinus excelsior	Oleaceae	Orthodox	62-74	Chmielarz (2009a)
Betula pendula	Betulaceae	Orthodox	77	Chmielarz (2010)
Salix hallaisanensis, S. gracilistyla, P. alba×*P. Glandulosa, Populus*	Saliaceae	Recalcitrant	>80	Popova et al. (2013)
Mimusops elengi, Manilkara zapota	Sapotaceae	Recalcitrant	0	Wen et al. (2013)
Populus nigra	Salicaceae	Recalcitrant	86	Suszka et al. (2014)
Citrus maxima cv. Feizhouyou	Rutaceae	Recalcitrant	77.5	Yan et al. (2014)
Citrus maxima cv. Mansailong	Rutaceae	Recalcitrant	63.3	
Populus nigra L.	Salicaceae	Recalcitrant	>90	Michalak et al. (2015)
Jatropha curcas L.	Euphorbiaceae	Recalcitrant	100	Prada et al. (2015)

Blank spaces (-) denote the unavailability of information.

Table 2. Examples on Success of Direct Plunging in Liquid Nitrogen (LN) for Cryopreservation of Forest Seeds (In Chronological Order)

Botanical name	Family	Seed class	Regrowth (%)	Reference
Pinus canariensis	Pinaceae	Orthodox	91	Pita et al. (1998)
P. halepensis		Orthodox	84	
P. nigra		Orthodox	88	
P. pinaster		Orthodox	61	
P. pinea		Orthodox	96	
P. sylvestris		Orthodox	71	
P. uncinata		Orthodox	97	
Astronium fraxinifolium	Anacardiaceae	Orthodox	95	Salomao (2002)
Schinopsis brasiliensis		Orthodox	86	
Spondias mombin		Orthodox	53	
Aspidosperma discolor	Apocynaceae	Orthodox	95	
Aspidosperma parvifolium		Orthodox	93	
Aspidosperma pyrifolium		Orthodox	91	
Anemopaegma arvense,	Bignoniaceae	Orthodox	84	
Jacaranda cuspidifolium		Orthodox	89	
Jacaranda decurrens		Orthodox	89	
Tabebuia aurea		Orthodox	71	
Tabebuia impetiginosa		Orthodox	89	
Tabebuia serratifolia		Orthodox	94	
Tabebuia roseo-alba		Orthodox	93	
Zeyheria montana		Orthodox	88	
Chorisia pubiflora	Bombacaceae	Orthodox	53	
Eriotheca gracilipis		Orthodox	100	
Pseudobombax cf. tomentosum		Orthodox	63	
Buchenavia tomentosa	Combretaceae	Orthodox	59	
Dioscorea sp.	Dioscoreaceae	Orthodox	61	
Amburana cearensis	Fabaceae	Orthodox	94	
Anadenanthera colubrina		Orthodox	93	
Bowdichia virgilioides		Orthodox	85	
Crotalaria cf. *spectabilis*		Orthodox	94	
Dalbergia miscolobium		Orthodox	53	
Machaerium aculeatum		Orthodox	28	
Machaerium cf. *acutifolium*		Orthodox	68	
Machaerium brasiliensis		Orthodox	100	
Ormosia fastigiata		Orthodox	77	
Platypodium elegans		Orthodox	83	
Pterodon emarginatus		Orthodox	80	
Kielmeyera coriacea	Guttiferae	Orthodox	58	
Cariniana estrellensis	Lecythidaceae	Orthodox	53	
Cariniana legalis		Orthodox	74	
Lafoensia pacari	Lythraceae	Orthodox	90	
Byrsonima basiloba	Malpighiaceae	Orthodox	30	
Cedrela fissilis	Meliaceae	Orthodox	70	
Pinus echinata	Pinaceae	Orthodox	100	
Guettarda pohliana	Rubiaceae	Orthodox	78	
Tocoyena formosa		Orthodox	83	
Magonia pubescens	Sapindaceae	Orthodox	100	
Sterculia striata	Sterculiaceae	Orthodox	85	
Styrax camporum	Styracaceae	Orthodox	36	
Apeiba tibourbou	Tiliaceae	Orthodox	54	
Luehea sp.		Orthodox	46	

Table 2. Continued

Botanical name	Family	Seed class	Regrowth (%)	Reference
Roupala montana	Proteaceae	-	-	Wetzel et al. (2003)
Bletilla striata	Orchidaceae	-	23	Hirano et al. (2005)
Astronium fraxinifolium, Myracrodruon urundeuva, Schinopsis brasiliensis	Anacardiaceae	Orthodox	-	Lima et al. (2008)
Aspidosperm apyrifolium	Apocynaceae	Orthodox	43	
Cavanillesia arborea	Bombacaceae	Orthodox	-	
Jacaranda brasiliana, Tabebuia aurea, Cordia trichotoma	Bignoniaceae	Orthodox	-	
Tabebuia impetiginosa		Orthodox	70	
Acacia polyphylla, Amburana cearensis, Anadenanthera colubrine, Copaifera langsdorffii, Enterolobium contortisiliquum, Hymenaea courbaril var. stilbocarpa, Lonchocarpus montanus, Machaerium scleroxylon	Fabaceae	Orthodox	-	
Cedrela fissilis	Meliaceae	Orthodox	-	

Blank spaces (-) denote the unavailability of information.

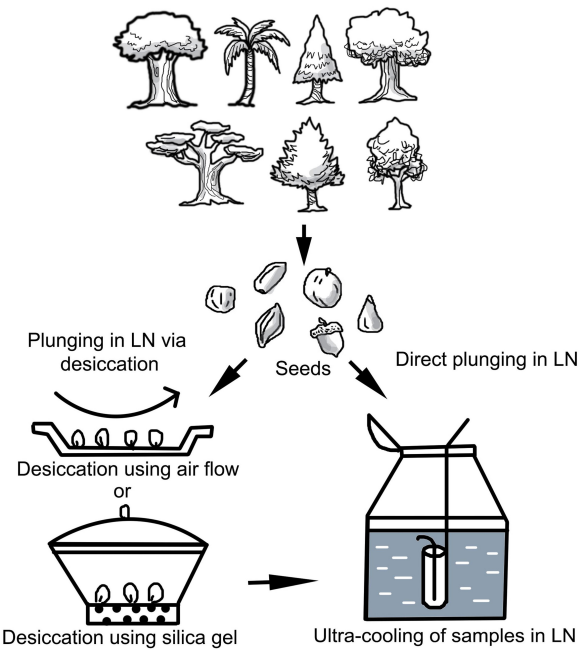

Fig. 1. A schematic illustration on two major cryopreservation methods embracing ultra-cooling in liquid nitrogen (LN) either through direct exposure or via desiccation of forest tree seeds.

sylvatica which lost its viability in LN. Medeiros and Cavallari (1992) in experimentation on *Astronium urundeuva*, which is a priceless timber and curative tree, the orthodox seeds were subjected to drying for 24, 48, 72, 96, and 120 h at 25°C and 10-15% relative humidity prior to immersion in LN; thawing was done for 30 min at room temperature. Seeds even without desiccation (8.01% MC) survived cryogenic storage, but Medeiros et al. (1992) obtained exceptional germination even after 24, 72, and 96 h drying, subsequent to which, seeds accomplished individual humidity contents of 7.13%, 5.89%, and 5.96%. Touchell and Dixon (1993) in their study specified that in dominant families of native Australian species conservation using LN is very effective and this was proved by an experiment conducted on 90 native western Australian species that comprised of 84 genera as well as 33 families respectively. Amongst the 90 species, 68 of them germinated after being stored in liquid nitrogen for two weeks either by direct immersion into liquid nitrogen or slow cooling at 0.4°C min^{-1} at 15% or 35% dimethyl sulphoxide (DMSO). In other case, few other species in Papilionaceae and Myrtaceae family displayed alike germination rates on treatment with 35% DMSO as well as directly soaked into LN but when treated with 15% DMSO showed lower germination rates. Connor and Bonner (2001) trailed that the initial germination rate in both the silver maple and red buckeye seeds was high, 89% and 94% correspondingly after nine days of desiccation, but after 11 days, silver maple had reduced to 21% and red buckeye to 24%. Another experiment conducted by Salomao on the year 2002 did not involve dehydration of seeds before drenching them in liquid nitrogen for three consecutive days and then thawed at a room temperature

(25°C) for another three more days. From this it was concluded that all the species that were tested were cryostorage tolerant but many species were found highly sensitive towards LN. Although all the seeds of the several tree species studied by Wetzel et al. (2003) were found tolerant to the injuries caused by direct immersion in LN, but in certain exceptional cases of *Cassia ferruginea, Platypodium elegans, Sclerobium aureum*, and *Roupala montana* the germination rates were lower than the controls.

Influence of seed age

It has been established from the earlier reports that the life of the forest tree seeds significantly depends on the preliminary attribute of seed. According to Simpson et al. (2004), longevity of forest seeds under storage has a proportional relationship with the quality of seeds. To ensure the highest genetic and physiological attributes, seeds should be collected at its mature phase for its prior treatment as well as long-term storage. This is evident especially in case of those forest tree seeds that require post-harvest ripening (such as *Picea glauca*) or stimulated desiccation (such as, *Shorea roxburghii, S. siamensis*) for the completion of their maturation (Panochit et al. 1984; Caron et al. 1992). According to Hirano et al. (2005), when the immature seeds of *Bletilla striata* were directly soaked into LN devoid of pre-treatment, the survival rate was directly proportional to the seed age as 0%, 9%, and 23% in the seeds of 2, 3 and 4 months after pollination (MAP), respectively. But when juvenile seeds of distinctive growth phases (2-4 MAP) were cryopreserved through vitrification, the maximum resurgence rate (82%) was attained in the seeds of 3 MAP trailed by those of 4 MAP (72%) and 2 MAP (44%). As high as 92% resurgence rate was obtained when under developed seeds of 3 MAP were exposed to vitrification solution subsequent a three-day pre-culture in 0.3 M sucrose.

Influence of moisture content

Tarre et al. (2007) established a skilled procedure of cryopreservation while working with eight Bromeliaceae species that are native to the Atlantic forest. In that very experiment the moisture content of the seeds ranged between 11.2% to 28.2% and required desiccation before immersing them directly into LN, except for *Encholirium pedicellatum* (where, seeds were dehydrated to 2.5% of MC). After recovering the seeds from LN, thawing was carried out at a room temperature (25°C) and the regeneration rates were duly recorded. Another work by Lima et al. (2008) on 19 different species of deciduous Parana River valley in the forest area of Goias (Brazil) demonstrated that without desiccation, the seeds (MC ranged between 5.7% to 12%) were directly plunged into LN for three days, followed by thawing at room temperatures (25°C). All the species survived in LN but an exception was noticed as regeneration was declined in seeds of *H. courbaril* having 5.8% MC and *Aspidosperma pyrifolium* (6.8% MC). Chmielarz (2009a) accounted that storing the dormant seeds of European ash for two years in LN after desiccation ensuring the water content (WC) of 0.06-0.24 g g^{-1} showed no variation in germination rates. In another study by the same researcher (Chmielarz 2009b) on mazzard cherry seeds (*Prunus avium* L.) it was revealed that desiccation up to an MC of 9.0-16.9% proved to be better for post-freezing regeneration (maximum of > 60%) instead of deep desiccation (1.6-7.3% MC). Chmielarz (2010) reported that *Betula pendula* seeds could tolerate desiccation over silica gel till the level of 0.02 g g^{-1} (WC) and sustained germination rate of 77% after 14 days. According to that report, the optimum range of seed WC, within which the seeds tolerate the ultra-low temperature of LN was 0.02-0.23 g g^{-1} and further confirmed that the seeds if dehydrated properly can be stored in LN for about two years with proper after-storage germination rates. Research work carried out on the seeds (10% WC) of both *Salix hallaisanensis* and *S. gracilistyla* regenerated with 80% germination rates but were sensitive when their WC was reduced. Salix seeds were observed to be effectively cryopreserved with WC of 0.25 g g^{-1} without any significant change in after storage regeneration frequencies. Wen et al. (2013) carried out a detailed comparative study between the embryos of *M. zapota* and *M. elengi* and it was observed that *M. zapota* displayed better tolerance towards dehydration and exposure to LN. Survival and regeneration was better noticed either on the embryonic axes that were chopped off and dehydrated prior cryopreservation treatment or from the embryonic axes extracted from already cryopreserved seeds. Suszka et al. (2014) reported that both fresh and desiccated seeds of *Populus nigra* could be preserved using standard cryopreservation techniques for about two years.

In that study the fresh seeds exhibited higher germination rates (90%) than desiccated seeds (86% germination rate). Michalak et al. (2015) also demonstrated that MC within 0.11-0.17 g g^{-1} plays an important role in the cryopreservation of black poplar seeds irrespective of its origin, time of harvesting and the seed quality.

Post-Cryopreservation Regrowth

Growth recovery is a fundamental step for the endurance of the seeds following their exposure to ultra-low temperature for a specific period (longest possible). However, post-freezing viability and subsequent germination of seeds chiefly reliant on genotype and age of the seeds, cryoprotectants employed, degree of freezing and thawing etc. (Tsukara and Hirosawa 1992). To assess the viability of the germplasms several tests are there. Yet, most commonly used tests are triphenyltetrazolium chloride and flourescein diacetate assays. The consequent step following viability assessment is regrowth of germplasm. The researchers followed different protocols for the post-cryopreservation regrowth and maintenance of the cryopreserved seeds. Touchell and Dixon (1993) after storing the seed in LN thawed quickly by quick dipping into a 40°C water bath and kept at room temperature for 20 min. To break the dormancy of legume tree seeds, they were soaked in boiling water and kept overnight to cool. The seeds were then transferred to the ground in a soil comprising composted hardwood saw dust, hardwood fines, and quartz sand in a proportion of 3:1:2 (*v*/*v*) and put a thin layer of gravel on top to attain successful germination. Muthusamy et al. (2004) propagated the post-freezing re-warmed seeds in a typical germination container having garden soil substrate for *in vivo* germination following which the seeds were incubated in a growth chamber at 25°C with a 16 h photoperiod. Contrastingly, for *in vitro* germination, Murashige and Skoog (1962) (MS) basal medium fortified with 30 g l^{-1} sucrose was used. The temperature was controlled at 26°C with a 12 h photoperiod. However, Hirano et al. (2005) cultured the cryopreserved immature seeds on 0.2% gellan gum-solidified New Dogashima medium (Tokuhara and Mii 1993) supplemented with 0.3 M sucrose at 25°C for as long as 3 days under continuous illumination. Pritchard and Nadarajan (2008) accounted that the dried seeds are prone to damage at some stage in rehydration during the germination test. Damage was often noticed if the seeds are directly dipped in water. According to them moistening the seeds on wet filter paper overcome the risk to damage. As a better alternative, Halmagyi et al. (2010) thawed the samples quickly in liquid MS basal medium (with 30 g l^{-1} sucrose) (pH 5.7) and shaked the medium for about 10 sec at room temperature. The emerged shoot tips were transferred on a modified semisolid MS medium supplemented with plant growth regulators. Gantait et al. (2015), on the other hand, unloaded the sample in 1.2 M sucrose after quick thawing and then relocated onto double layered sterile filter paper kept on Petri dishes enclosing semi-solid MS medium with 3% (*w*/*v*) sucrose and 100 mg l^{-1} myoinositol devoid of any plant growth regulators. The samples were given dark phase for consecutive 7 days before disclosure to light with a 16 h photoperiod at 26±2°C. Suszka et al. (2014) germinated the desiccated seeds in a Jacobsen germinator. Moist filter paper was used to rehydrate the seeds and cool white light was supplied on a 12 h cycle. The temperature was sustained at 23°C and 27°C for 22 h and 2 h per day, respectively. The use of Jacobsen germinator was also described in the reports of Chmielarz (2010) and Michalak et al. (2015). As per their description, the seeds were thawed by keeping in a water bath at 40°C for 5 min following LN exposure before transferring them in Jacobsen germinator. They maintained the temperature at 23°C for 22 h and 27°C for 2 h per day and light given in a 12 h cycle ensuring successful regrowth and maintenance.

Conclusion and Prospect

There are several cryopreservation practices established for the protection of forest trees as its conservation is an imperative issue for the reason since forest trees are less cultivated than domestic crops, and incorporates genetic variation. Cryopreservation being a lone technique cannot completely provide an efficient solution to this problem but yet can reduce it a certain extent. It serves as an active technique for the conservation mainly in case of tree species involving the use of whole seeds or its components as explants for germplasm storage. The technique preserves the reservoirs of germplasm and the escapes the threat to extinction. It also maintains the biosynthetic characteristics of the

plant. The corresponding tissue features can be conserved in this method that might has been lost during regular *in vitro* maintenance protocols. This technique also helps to easy collect and manages all groups of genetic material around the world. It aids exchange of germplasm internationally since the size of sample material is small and could be travelled anywhere in the world. Moreover it reduces the chance of contamination, disease and mutation since the whole procedure is performed aseptically. Overcoming the two critical factors that is seed desiccation and seed moisture content and manipulating their parameters to standardize effective cryopreservation procedures are much focused in today's research world. A minimum level of damage mechanisms involved during dehydration of desiccation-sensitive tissue is that tolerant tissue can survive easily but sensitive tissues fail. The occurrence of desiccation-sensitivity or recalcitrance is not associated with phylogeny since, few families comprised of species that produce seeds with this trait and few other families don't. In general, perennial, aquatic or rainforest trees hold recalcitrant character. Hence, future methods should address to resolve this setback involving reduction of desiccation-derived oxidative stress.

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Conflict of Interest

The authors of this article declare that there is no conflict of interest and no financial gain from it.

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