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REVIEW PAPER

Cryobiotechnology of forest trees: recent advances and future prospects

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Abstract Globally, forests are of great economic importance and play a vital role in maintaining friendly ecological environments, sustainability of eco-systems, and biodiversity. Harsh environments, human activities and climate warming have long threatened the diversity of forest genetic resources. Among all conservation strategies, cryopreservation is at present time considered an ideal means for long-term conservation of plant genetic resources. To date, studies on cryopreservation of forest trees have been far behind

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agricultural and horticultural crops. The present review provides a comprehensive and update information on recent advances in cryopreservation of shoot tips, somatic embryogenic callus and seeds of forest trees. Assessments of genetic stability in the regenerants following cryopreservation were also analyzed and addressed. Further studies on cryopreservation of forest trees are proposed and needed. By doing so, we expect to re-evoke research interests and promote further developments in forest tree cryobiotechnology, thus assisting to ensure maintenance of biodiversity of genetic resources of forest trees.

Keywords Cryopreservation · Embryogenic callus · Forest · Genetic resource · Genetic stability · Seeds · Shoot tips

Importance of forest trees

Forest is referred as to an area with a high density of plants. By 2015, forests covered about 31% of the world's total land area, accounting for approximately 4 billion ha (The World Bank 1990–2015). About 30% of the global forests are used for wood and non-wood products, accounting for about 1% of world gross domestic products (Rittenhouse and Rissman 2012). Besides its economic importance, the forest plays a vital role in maintaining friendly ecological environments, sustainability of eco-system and biodiversity (Ciccarese et al. 2012; Rittenhouse and Rissman 2012). Many of the major food and medicinal crops that are cultivated today originated from the forest (Ciccarese et al. 2012). The forest is among the most important in vivo genebanks which possess valuable genes that can be exploited for breeding of novel cultivars.

According to botanical classification, forest plants are divided into two main groups: gymnospermae and angiospermae (Häggman et al. 2008). The gymnosperms are exclusively composed of trees and woody shrubs, while the angiosperms are a diverse group of plants including trees, shrubs, grasses and herbaceous plants. The present review focuses on forest trees, which dominate both the structure and the function of major temperate and tropical forests.

Needs for cryopreservation of genetic resources of forest trees

In the last two decades, although increased forest areas occurred in many countries and regions, including the United States, Europe, China, Latin America and the Caribbean such as Chile, Uruguay, Cuba and Costa Rica, serious deforestation still continued in some countries of Africa and Asia, as well as large Pacific and tropical areas of the Latin America (The World Bank 1990–2015). Globally, more than 1/3 of the forest is the primary forest, which is composed of native species. However, the primary forest has decreased, at a rate of 0.4% annually over the last ten years, by more than 50 million since 1990, mainly due to excessive exploitation for different purposes (The World Bank 1990–2015). The world lost nearly 10 hectares of forest per minute and the world deforestation was estimated to be 14.5 million ha per year between 1990 and 2005 (http://www.fao.org/news/story/en/item/95180/icode). Most of deforestation took place in the countries located in the tropical region. A recent report warns that about 21% of the total plant species

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(approximately 390,900 plants) on the earth are at risk of extinction, mainly due to industrialization, urbanization, soil salinity, and climate warming (Royal Botanic Gardens Kew 2016).

Obviously, there is a global need to conserve biodiversity of forest species (Tsai and Hubscher 2004; Panis and Lambardi 2006; Häggman et al. 2008; Lambardi et al. 2008). Traditionally, seed-propagated plant species can be conserved in seed banks (Solberg et al. 2017), while those that are vegetatively propagated can be preserved in field collections or in in vitro cultures (Barazani et al. 2017).

Seed banks are relatively easier and low cost (Pritchard 1995). According to storage behavior, seeds can be grouped into three catalogues: orthodox, intermediate and recalcitrant (Pritchard 1995; Gantait et al. 2016). Orthodox seeds are tolerant to desiccation and can be dried to relatively lower moisture contents (3–7%, Gantait et al. 2016). Their storage duration depends on moisture content of seeds and temperature of the storage (Marzalina and Krishnapillay 1999). Intermediate seeds can be desiccated to a moisture content similar to that of orthodox seeds, however are sensitive to low temperatures (Gantait et al. 2016). Therefore, they can be stored under similar conditions applied to orthodox seeds, but for relatively shorter time period (Marzalina and Krishnapillay 1999). A great number of forest trees, especially broad-leaf tropical species, belong to recalcitrant group, which is not tolerant to desiccation and storage at low temperature (below 16 °C) and can be conserved only for weeks (Pritchard 1995; Gantait et al. 2016).

Field collections are another important approach to conservation of forest species. However, such conservation system has several limitations, such as adverse climatic conditions, attacks by pests and pathogens, needs of large land areas and great labors for maintenance (Engelmann 1997; Panis and Lambardi 2006; Häggman et al. 2008). In vitro storage at low temperatures (4–10 °C) can be considered for medium-term conservation of genetic resources, generally up to 1 year (Bamberg et al. 2016). However, this strategy is labor-intensive, and has risks of losing stored material, due to contamination and human errors (Bamberg et al. 2016).

Cryopreservation, i.e., the storage of plant material such as cells, tissues or organs at extra-low temperatures, usually that of liquid nitrogen (LN, − 196 °C), has been considered an ideal means for long-term conservation of germplasm (Tsai and Hubscher 2004; Panis and Lambardi 2006; Benson 2008a; Wang et al. 2014a). At such a temperature, almost all the cellular processes are arrested, and thus the stored material can theoretically be conserved for an unlimited period of time (Engelmann 1997; Benson 2008a). Compared to the traditional methods, cryopreservation requires minimal maintenance of the stored material, and reduces to the greatest degree the risk of genetic and epigenetic variations (Harding 2004; Panis and Lambardi 2006; Benson 2008a; Wang et al. 2014a).

Updated information on technical development, progress and application of cryopreservation are available in agricultural and horticultural plants (Feng et al. 2011; Kaczmarczyk et al. 2011; Benelli et al. 2013; Kulus and Zalewska 2014; Wang et al. 2009, 2014a; Popova et al. 2016), but quite limited in forest trees (Harvengt et al. 2004; Tsai and Hubscher 2004; Panis and Lambardi 2006; Häggman et al. 2008; Lambardi et al. 2008; Gantait et al. 2016; Ozudogru and Lambardi 2016). The present review provides comprehensive and updated information on cryopreservation of forest trees and proposes the further prospective research, in order to re-evoke interests in research on the said subject.

Major steps of cryopreservation of forest trees

As with other crops, earlier studies used two-step cooling for cryopreservation of forest trees (Panis and Lambardi 2006; Lambardi et al. 2008; Ozudogru and Lambardi 2016). As advances in cryobiology, novel cryoprocedures were established since the early 1990s. Now, various cryoprocedures have been developed for cryopreservation of forest trees, including encapsulation-dehydration, encapsulation-vitrification, vitrification, droplet-vitrification and pregrowth-desiccation. In general, major steps involved in these cryoprocedures include: (I) Pre-conditioning of stock cultures. Cold-hardening is frequently used for pre-conditioning of stock cultures to induce their tolerances to dehydration and subsequent freezing in LN. Cold-hardening is usually performed at 0–4 °C in either darkness or light for days to weeks; (II) Encapsulation, in which samples are encapsulated into 3% (w/v) calcium alginate beads in encapsulation-based protocols, in order to protect the samples from mechanical damage and easy operation; (III) Preculture, in which samples are usually precultured on a medium containing high concentrations of sugars or sugar alcohols to enhance their tolerances to dehydration and subsequent freezing in LN; (IV) Cryoprotection, in which samples are cryoprotected by cryoprotectants to avoid cryo-injury caused by freezing in LN, particularly in two-step freezing protocols; (V) Loading, in which samples are treated with loading solution (LS) to alleviate osmotic pressures imposed by PVS (Plant Vitrification Solution) in PVS-based vitrification protocols. LS contains 2 M glycerol and 0.4 M sucrose (Matsumoto et al. 1994); (VI) Dehydration, in which samples (either encapsulated or naked) are dehydrated by physical drying or exposure to PVS in vitrification-based protocols. Among several PVSs, PVS2 is the most frequently used, which contains 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) dimethyl sulphoxide (DMSO) and 15% (w/v) ethylene glycol in a basic medium (Sakai et al. 1990); (VII) Pre-cooling, in which samples are usually pre-cooled to − 35 or − 40 °C before immersion in LN in two-step cooling; (VIII) Transfer of single PVS-treated samples onto 2.5–3.5 μL PVS carried on aluminum foil strips, which is a necessary step in droplet-vitrification; (IX) Direct immersion in LN for cryostorage in vitrification-based protocols; (X) Thawing, in which samples cryostored in LN are re-warmed; (XI) Unloading, in which thawed samples are treated with an unloading solution, which is usually composed of 1.2 M sucrose in a liquid medium, to remove toxic chemicals contained in PVS in PVS-based vitrification method; and (XII) Post-thaw culture for recovery. Each of these steps may be modified according to a specific cryoprocedure, plant species or genotypes and types of samples.

Cryopreservation of forest trees

In vitro shoot tips or buds and in vivo dormant buds

Shoot tip, also called shoot meristem, usually refers to 0.5–2.0 mm (in length) explants that comprises an apical dome and 5–6 leaf primordia (LPs) (Wang et al. 2014a), while buds refer to explants containing shoot tips and older tissues, which are larger in size (2.0–10.0 mm) than shoot tips. Dormant buds refer to winter buds from in field trees. Shoot tips or buds and dormant buds are preferred for use in the conservation of genetic resources of vegetatively-propagated plants, because they are genetically more stable than

other explants such as cells and embryogenic tissues (Tsai and Hubscher 2004; Wang et al. 2014a).

Gymnospermae

Up to date, shoot tip has been successfully cryopreserved in only a few gymnospermae species including *Pinus kesiya* (Kalita et al. 2012), *Sequoia sempervirens* (Halmagyi 2008; Halmagyi and Deliu 2011; Ozudogru et al. 2011) and *Tetraclinis articulata* (Serrano-Martinez and Casas 2011). In the study of Kalita et al. (2012), shoot tips of *Pinus kesiya* were first-step precultured with 0.1 M sucrose for 11 days, followed by second-step preculture with 0.4 and 0.7 M sucrose, each for one day. Precultured shoot tips were then exposed to a modified plant vitrification solution L (PVSL, Suzuki et al. 2008) for 10 min at 25 °C, prior to a direct immersion into LN for cryostorage. PVSL contains (w/v) 20% glycerol, 5% sucrose, 25% ethylene glycol and 10% DMSO. Following cryopreservation, thawed shoot tips were post-cultured on a medium containing 4 mg l^{-1} benzyladenine (BA) for recovery. This cryoprocedure resulted in 76% of shoot regrowth in cryopreserved shoot tips (Kalita et al. 2012). Cryopreservation of *Sequoia sempervirens* was achieved by vitrification-based methods (Halmagyi and Deliu 2011; Ozudogru et al. 2011). In the study of Ozudogru et al. (2011), stock cultures were cold-hardened at 4 °C in darkness for 4 weeks. Apical and axillary buds excised from the cold-hardened stock cultures were precultured with 0.12 M for 48 h and 0.25 M sucrose for 72 h, respectively. Droplet-vitrification resulted in the highest shoot regrowth (22%) following cryostorage among the three cryogenic procedures tested. In the study of Halmagyi and Deliu (2011), apical shoot tips (2–3 mm) were encapsulated in 3% sodium alginate beads and then precultured at 24 °C in liquid MS medium containing 0.5 M sucrose for 24 h on a rotary shaker (98 rpm). Precultured shoot tips were dehydrated by air drying in a laminar flow for 3 h to reduce the water content of the beads to approximately 27%, prior to direct immersion in LN. About 67% of shoot regrowth rate was obtained in cryopreserved shoot tips that had been precultured with 0.5 M sucrose, while preculture with sucrose concentrations higher than 0.5 M caused excessive cell dehydration (Halmagyi and Deliu (2011). Using a modified PVS2-based vitrification protocol, Serrano-Martinez and Casas (2011) reported successful cryopreservation of *Tetraclinis articulata*. The authors demonstrated that high concentrations of sucrose in loading solution (0.4 M), PVS2 (0.4 M) and unloading solutions (1.2 M) were toxic to the explants, while the replacement of sucrose by sorbitol in equal molar concentration in the corresponding solutions eliminated the toxicity, thus beneficial for recovery of shoot tips.

Angiospermae

Both in vitro shoot tips and in vivo dormant buds were used in cryopreservation of Angiospermae species. Ryynänen's group in Finland conducted the systematic studies on cryopreservation of silver birch (*Betula pendula*). In vivo dormant shoots were collected from field-grown mature trees from March to April, during which the average temperature was about − 2.5 to − 8 °C (Ryynänen 1996a). The collected material was stored at − 5 °C for 1–4 weeks. Then, buds (1 cm in length) excised from the stored shoots were kept at approximately 0 °C for 36 h before cryopreservation. In fast cooling, buds were directly immerged in LN for cryostorage. In two-step cooling, samples were pre-cooled at 10 °C/h to − 38 °C for 24 h and then plunged into LN for cryostorage. Cryopreserved buds were removed from LN and thawed in a water bath at 37 °C for 5 min. Then, thawed

buds were surface-sterilized and cultured in in vitro conditions for shoot recovery, according to Ryynänen and Ryynänen (1986). With the fast cooling, only about 6% survived freezing in LN in one of the three experiments (Ryynänen 1996a). The best results (100% survival and 55–88% regrowth) were obtained by two-step cooling. With in vitro shoot tips, both two-step cooling and vitrification were tested, but only the former was successful (Ryynänen 1996b). In two-step cooling, in vitro stock cultures were cold-hardened at 5 °C for 3 weeks. Shoot tips (0.3–0.4 mm in size) containing 4–5 LPs were excised and precultured with 0.5% DMSO for 72 h. Precultured shoot tips were incubated in PGD solution on ice for 30 min, followed by two-step cooling procedures, as reported by Ryynänen (1996a). PGD solution contains 10% polythylene glycol (PEG), 10% glucose and 10% DMSO in sterile water (Ryynänen 1996b). Shoot regrowth rate of cryopreserved shoot tips varied from genotypes, with the highest and lowest rates of 58 and 11% obtained among the genotypes tested. Key factors affecting shoot tip cryopreservation of silver birch were studied, including sampling time (Aronen and Ryynänen 2014), bud type (with or without a female catkin) (Ryynänen 1999), age of the stock shoots (Ryynänen and Häggman 2001), cold-hardening of the stock cultures (Ryynänen and Aronen 2005a; Ryynänen 2011), compositions of preculture medium (Ryynänen 1998, Ryynänen and Häggman 1999, 2001) and post-thaw culture medium (Ryynänen and Häggman 1999, 2001), light conditions during preculture (Ryynänen 1998; Ryynänen and Aronen 2005a) and cryostorage time durations (Ryynänen 1999). These studies allowed establishment of efficient cryoprocedures for in vitro shoot tips and in vivo dormant buds of *Betula pendula* (Ryynänen et al. 2002; Ryynänen 2011; Aronen and Ryynänen 2014).

Several key factors affecting successful cryopreservation of Angiospermae species have been studied. Cold-hardening of the stock cultures was repeatedly found to improve recovery of cryopreserved shoot tips or dormant buds in various plant species including forest trees such as *Betula pendula* (Ryynänen 1996b, 1998, 1999; Ryynänen and Häggman 1999, 2001), *Fraxinus* spp. (Volk et al. 2009), *Cedrela odorata* (Maruyama et al. 1996), *Guazuma crinita* (Maruyama et al. 1996), *Jacaranda mimosaefolia* (Maruyama et al. 1996), *Populus alba* (Lambardi et al. 2000) and *Castanea sativa* (Vidal et al. 2005, 2010). Use of ammonium-free preculture medium improved recovery of cryopreserved shoot tips of *Betula pendula* (Ryynänen and Häggman 1999, 2001). Addition of abscisic acid (ABA) at 10 mM into the preculture medium was beneficial to recovery of cryopreserved shoot tips of *Betula pendula* (Ryynänen 1998). Inclusion of melatonin (0.1–0.5 μM) in preculture, as well as in regrowth medium, was for the first time found to significantly improve the recovery of cryopreserved shoot tips of in vitro-grown plantlets and dormant winter buds of American elm (*Ulmus americana*) (Uchendu et al. 2013). Nearly 100% of cryopreserved shoot tips resumed shoot growth in the two cryogenic procedures. Melatonin (*N*-acetyl-5-methoxytryptamine), a small molecule that is widely present in animals and plants, has abilities to fortify plants against abiotic stress by scavenging reactive oxygen species (ROS) (Shi et al. 2016) and to regulate plant growth, development and regeneration including in vitro cultures (Sarropoulou et al. 2012). Although the mechanism as to why exogenous application of melatonin increases recovery of cryopreserved explants remains unknown, the antioxidant potential of melatonin and its metabolites may contribute to these positive effects (Uchendu et al. 2013).

Addition of Supercool© X-1000 solution (20% w/w) to PVS2 improved survival of cryopreserved shoot tips in the 8 *Castanea sativa* genotypes tested, and increased shoot regrowth in 5 out of 8 (Vidal et al. 2010). Use of ammonium-free medium in post-thaw culture was found to be beneficial to recovery of cryopreserved shoot tips (Ryynänen and Häggman 1999, 2001). Working on *Populus alba*, Lambardi et al. (2000) observed lower

survival rate and no shoot regrowth in cryopreserved shoot tips when post-cultured on PGRs-free. Post-culture medium containing 1.5 μM BA and 0.5 μM gibberellic acid (GA_3) produced significantly higher shoot regrowth rate in cryopreserved shoot tips and did not produce any callus, compared to the media containing 3.0 μM BA or zeatin (ZT) or thidiazuron (TDZ) in combination with 1.0 μM GA_3 (Lambardi et al. 2000).

For recovery of cryopreserved in vivo dormant buds, surviving buds can be either in vitro micrografted upon seed-derived rootstocks as in *Ulmus glabra* (Harvengt et al. 2004), *Fraxinus* spp. (Volk et al. 2009) and *Populus trichocarpa* (Bonnart et al. 2014) or directly rooted in a rooting medium as in *Salix* (Bonnart et al. 2014), to regenerate whole plants.

A cryobank of 444 European elm clones (*Ulmus* spp.) has been set up, using in vivo dormant buds (Harvengt et al. 2004), which contained three European elm species and their hybrids collected from 9 European countries, and represented the genetic diversity of European elms. Cryopreserved buds of 26 clones that were randomly selected from the cryobank could regenerate into whole plantlets and be established in field conditions. A list of successful cryopreservation of angiospermae forest trees is shown in Table 1.

Somatic embryogenic callus (SEC)

Somatic embryogenesis of forest trees has great potential applications to micropropagation, genetic transformation and production of artificial seeds (Lambardi et al. 2008; Yang and Zhang 2010). However, establishment of efficient somatic embryogenesis requires skills, takes long time and is even difficult in many plants (Lambardi et al. 2008; Yang and Zhang 2010; Ozudogru and Lambardi 2016). Once established, SEC needs repeated subcultures to maintain its embryogenic potential. However, regenerative potential of SEC decreases with increased subculture times, and repeated subculture increases risks of losing the cultures, due to contamination or human error, and induces genetic variations (Lambardi et al. 2008; Ozudogru and Lambardi 2016). For these reasons, it is necessary to establish efficient cryopreservation protocols for long-term maintenance of SEC. Advances in cryopreservation of SEC in forest trees can be found in several reviews (Lambardi et al. 2008; Vieitez et al. 2010; Ozudogru and Lambardi 2016).

Gymnospermae

Embryogenic callus clumps or suspension cultures were most frequently used for cryopreservation of gymnospermae species. Although SEC has been successfully cryopreserved using various cryogenic procedures, two-step cooling was the most often applied cryoprocedure (Table 2). In this strategy, a solution containing sucrose (0.3–0.5 M) or sorbitol (0.3–0.8 M) and DMSO (5.0–7.5%) was frequently used to treat samples (Salaj et al. 2007, 2010, 2011; Álvarez et al. 2012). Use of maltose (0.4–0.5 M) (Marum et al. 2004; Salaj et al. 2011; Latutrie and Aronen 2013), or 0.4–0.8 M sorbitol (Touchell et al. 2002; Gale et al. 2008) or polyethyleneglycol (PEG, 10–20%) (Marum et al. 2004; Álvarez et al. 2012) or 10 μM ABA (Hazubska-Przybył et al. 2013) was found to improve recovery rates of SEC (Latutrie and Aronen 2013). Cryoprotected samples were usually pre-frozen to − 30 to − 40 °C, followed by immersion in LN for cryostorage (Maruyama et al. 2000; Marum et al. 2004; Gale et al. 2007, 2008; Salaj et al. 2007; Latutrie and Aronen 2013). In encapsulation-dehydration cryopreservation, embryogenic masses were encapsulated into calcium alginate beads (4–5 mm in diameter), followed by preculture in sucrose-enriched medium (0.4–0.75 M) for hours (18 h) to days (3 days) and subsequent desiccation to

Table 1 Examples of successful cryopreservation of in vitro shoot tips or in vivo dormant buds of angiospermae forest trees

Plant species	Cryogenic method	Survival or regrowth (%)	References
Betula pendula	Two-step cooling	75–95(survival), 55–88(regrowth)[a]	Ryynänen (1996a)
	Fast cooling	8.7 (survival), 1.7 (regrowth)	
	Two-step cooling	23–59 (regrowth)	Ryynänen (1996b)
	Vitri	0.7 (regrowth)	
	Two-step cooling	>40 (regrowth)	Ryynänen (1998)
		24–67 (regrowth)[a]	Ryynänen (1999)
		70 (regrowth)	Ryynänen and Häggman (1999)
		22–74 (regrowth)	Ryynänen and Häggman (2001)
	Vitri	71 (regrowth)[a]	Ryynänen and Aronen (2005a)
	Two-step cooling	70 (regrowth)[a]	Ryynänen and Aronen (2005b)
Castanea sativa	Vitri	38–54 (regrowth)	Vidal et al. (2005)
		63 (regrowth)	Vidal et al. (2010)
Cedrela odorata	Vitri	60 (survival), 10 (regrowth)	Maruyama et al. (1996)
	Two-step cooling	50 (survival), 20 (regrowth)	
Eucalyptus spp.	Drop-vitri	38–85 (survival)	Kaya et al. (2013)
Eucalyptus grandis × *E. camaldulensis*	Encap + two-step cooling	49 (survival)	Blakesley and Kiernan (2001)
Fraxinus spp.	Two-step cooling	34–100 (regrowth)[a]	Volk et al. (2009)
Guazuma crinita	Two-step cooling	50 (survival), 0 (regrowth)	Maruyama et al. (1996)
Jacaranda mimosaefolia	Two-step cooling	20 (survival), 0 (regrowth)	
Populus alba	Vitri	90 (survival)	Lambardi et al. (2000)
Populus tremula *Populus tremula* × *tremuloides*	Two-step cooling	37–100 (regrowth)[a]	Aronen and Ryynänen (2014)
Populus trichocarpa	Two-step cooling	42–100 (regrowth)[a]	Bonnart et al. (2014)
Robinia pseudoacacia	Vitri	70–78 (survival)	Verleysen et al. (2005)
	Encap-dehy	80 (survival)	
Salix spp.	Two-step cooling	40 (regrowth)[a]	Bonnart et al. (2014)
Salix hybrid	Encap-dehy	8–83 (regrowth)	Blakesley et al. (1996)
Trichilia emetica	Vitri	38 (regrowth)	Varghese et al. (2009)
	Two-step cooling	71 (regrowth)	
	Vitri + ultra-rapid cooling	55 (regrowth)	

Table 1 continued

Plant species	Cryogenic method	Survival or regrowth (%)	References
Ulmus americana	Vitri	80–100 (regrowth)	Uchendu et al. (2013)
Ulmus spp.	Two-step cooling	72–75 (regrowth)[a]	Harvengt et al. (2004)

Drop-vitri droplet-vitrification, *Encap* encapsulation, *Encap-dehy* encapsulation-dehydration, *Encap-vitri* encapsulation-vitrification, *Vitri* vitrification

[a]In vivo dormant buds

17–35% water content, prior to a direct immersion in LN for cryostorage (Fernandes et al. 2008; Gale et al. 2008). In PVS-based vitrification protocol, precultured samples without or with loading treatment were exposed to PVS2 at 0 °C for 30 min, prior to direct immersion in LN for cryostorage (Touchell et al. 2002). Following rapid thawing at 40 °C for 2–3 min, cryopreserved samples were unloaded with an unloading solution containing 2.0 M sucrose at room temperature for 20 min and post-cultured for recovery. Hargreaves et al. (2002) reported that, although all of the 60 *Pinus radiata* cell lines tested could survive following two-step cooling, use of nurse culture largely improved post-thaw regrowth in the majority of the 60 cell lines. Of the 23 lines crystored for 4 years, five recovered only when the nurse culture was used. The nurse tissue consisted of a vigorously growing cell line of *Pinus radiata* that was genetically distinct from all of the thawed lines.

Genotypes (Gale et al. 2008; Salaj et al. 2007, 2010, 2011; Álvarez et al. 2012; Latutrie and Aronen 2013), developmental stages (Valladares et al. 2004; Gale et al. 2008), culture age (Find et al. 1998; Gale et al. 2008; Latutrie and Aronen 2013) and cell density (Find et al. 1998; Marum et al. 2004) of SEC were found to affect success of cryopreservation. A comprehensive study was conducted by Gale et al. (2008), who tested encapsulation-dehydration and vitrification for cryopreservation of four genotypes (A1–A5, C1–C5, D1 and D5) of somatic embryos at the globular and torpedo stages of *Picea sitchensis*. They found that, although all the four lines tested survived, their survival rates ranged from 20 to 100% in different lines in the two methods. In vitrification, higher survival rates were observed in C1 and D5 than in A1–A5 and D1–D5, but surviving somatic embryos proliferated only non-SEC in all four genotypes tested (Gale et al. 2008). In encapsulation-dehydration, somatic embryos at torpedo stage were more tolerant to desiccation than those at globular stage, and the optimal water content for survival was about 20% (fresh water basis, FWB) (Gale et al. 2008). Somatic embryos of the genotypes A1, A5 and C5 survived following cryopreservation, among which genotypes A1 and C5 proliferated only non-SEC, and genotype A5 proliferated both non-SEC and SEC. Marum et al. (2004) reported that survival rates were much higher in cryopreserved embryogenic cell suspensions of *Pinus pinaster* when the initial density of the suspension of 250 mg l^{-1} was used than 50 mg l^{-1}. In addition, Maruyama et al. (2000), Gale et al. (2007), and Álvarez et al. (2012) reported length of cryostorage did not significantly affect the proliferation rate and somatic embryo development of the cryopreserved SEC, up to 10 years of cryostorage. Latutrie and Aronen (2013) reported that recovery rates (80–93%) were higher in embryogenic cultures of *Pinus sylvestris* cryostored for 10 years than those (59%) for 12 years, but their proliferation rates were similar. Plants regenerated from cryopreserved

Table 2 Cryopreservation of suspension cells and somatic embryogenic callus of gymnospermae forest trees

Plant species	No. of genotypes or lines tested	Type of explant	Cryogenic method	Recovery (%)	References
Abies cephalonica	1	Embryogenic callus	Two-step cooling	75	Aronen et al. (1999)
Abies hybrids	1	Embryogenic callus	Two-step cooling	> 37	Salaj et al. (2010)
Cryptomenia japonica	1	Embryogenic cells	Two-step cooling	Not specified	Maruyama et al. (2000)
Picea abies	72	Suspension cells	Two-step cooling	Not specified	Nørgaard et al. (1993)
	1	Suspension cells	Two-step cooling	Not specified	Find et al. (1998)
Picea abies	137	Embryogenic callus	Two-step cooling	Not specified	Högberg et al. (1998)
Picea glauca × *engelmanni* complex	357	Suspension cells	Two-step cooling	73–100	Cyr et al. (1994)
Picea glauca	1	Suspension cells	Two-step cooling	94	Kartha et al. (1988)
Picea mariana	9	Embryogenic callus	Vitri	3.3–66.7	Touchell et al. (2002)
Picea omorika	1	Embryogenic callus	Pre-des	Not specified	Hazubska-Przybył et al. (2010)
Picea sitchensis	1	Somatic embryos	Two-step cooling	Not specified	Kristensen et al. (1994)
Picea sitchensis	1	Somatic embryos	Encap-dehy	66	Gale et al. (2013)
Pinus caribaea hondurensis	1	Suspension cells	Two-step cooling	100	Lainé et al. (1992)
Pinus nigra	7	Embryogenic callus	Two-step cooling	87	Salaj et al. (2011)
Pinus patula	1	Embryogenic callus	Two-step cooling	60	Ford et al. (2000)
Pinus pinaster	3	Suspension cells	Two-step cooling	20–100	Marum et al. (2004)
	137	Embryogenic callus	Two-step cooling	100	Álvarez et al. (2012)
Pinus radiata	60	Suspension cells	Two-step cooling	78–100	Hargreaves et al. (2002)
Pinus roxburghii	1	Suspension cells	Two-step cooling	69.9	Mathur et al. (2003)
Pinus sylvestris	9	Embryogenic callus	Two-step cooling	78	Häggman et al. (1998)
	108	Embryogenic callus	Two-step cooling	80–93	Latutrie and Aronen (2013)

Encap-dehy encapsulation-dehydration, *Pre-des* pregrowth-desiccation, *Vitri* vitrification

SEC by PVS-based vitrification were identical to those derived from non-cryopreserved control (Latutrie and Aronen 2013).

Angiospermae

To date, although various cryopreservation protocols have been described for SEC of angiospermae forest trees, fast cooling methods were most frequently in this type of forest trees (Table 3). In some cases, cold hardening of the stock cultures at 4–5 °C in the dark or light for days to weeks was required to improve recovery of cryopreserved SEC of *Aesculus hippocastanum* (Lambardi et al. 2005) and *Quercus robur* (Sánchez et al. 2008). In PVS-based vitrification methods, PVS2 was frequently used and the treatment time durations ranged from 60 to 90 min at room temperature (Martínez et al. 2003; Corredoira et al. 2004; Valladares et al. 2004; Lambardi et al. 2005; Sánchez et al. 2008; Adu-Gyambi and Wetten 2012). In dehydration protocols, precultured SEC without encapsulation was dehydrated for about 1 h and with encapsulation for about 5 h over silica gel or in laminar

Table 3 Cryopreservation of somatic embryos and embryogenic callus of angiospermae forest trees

Plant species	No. of genotypes or lines tested	Types of explant	Cryogenic method	Recovery (%)	References
Aesculus hippocastanum	1	Somatic embryos	Vitri	94	Lambardi et al. (2005)
Castanea sativa	1	Somatic embryos	Preg-des	33	Corredoira et al. (2004)
Fraxinus angustifolia	1	Somatic embryos	Encap-dehy	31	Tonon et al. (2001)
Fraxinus excelsior	1	Embryogenic callus	Two-step freezing	100	Ozudogru et al. (2010)
			Encap-dehy	0	
			Encap-vitri	0	
Liquidambar spp	7	Embryogenic callus	Two-step freezing	100	Vendrame et al. (2001)
Liriodendron tulipifera	5	Embryogenic callus	Two-step freezing	75–100	
Quercus ilex	3	Embryogenic callus	Vitri	80	Barra-Jiménezet al. (2015)
Quercus robur	2	Somatic embryos	Vitri	70	Martínez et al. (2003)
			Preg-des	31.5–56.7	
	1	Embryogenic callus	Preg-des	Not specified	Chmielarz et al. (2005)
	1	Somatic embryos	Vitri	88–93	Valladares et al. (2004)
	1	Somatic embryos	Preg-des	90	Fernandes et al. (2008)
	51	Somatic embryos	Vitri	18–100	Vidal et al. (2010)

Encap-dehy encapsulation-dehydration, *Preg-des* pregrowth-desiccation, *Vitri* vitrification

flow to reduce water contents of the samples to 17–35% FWB (Chmielarz et al. 2005; Grenier-de March et al. 2005; Fernandes et al. 2008). Following direct immersion in LN for cryostorage, cryopreserved samples were rapidly thawed in a water bath set at 38–40 °C for 1–2 min (Lambardi et al. 2008). In same cases, thawing at a higher temperature (45 °C) was found to increase recovery rates of cryopreserved SEC, for example in *Aesculus hippocastanum* (Lambardi et al. 2005).

Several factors affecting the success of SEC cryopreservation have been studied. Using vitrification, Barra-Jiménez et al. (2015) found that although survival rates were similarly high in cryopreserved embryogenic callus of three *Quercus ilex* lines, surviving samples of the two lines completely lost their regenerative ability and only one was able to differentiate into somatic embryos. Working on PVS vitrification, Lambardi et al. (2008) found that a higher recovery rate (75%) was obtained in cryopreserved somatic embryos at torpedo stage than those at globular stage (32.5%) in *Aesculus hippocastanum*. Chmielarz et al. (2005) showed that higher regrowth rate was obtained in cryopreserved SEC of *Quercus robur* when precultured on sucrose-enriched solid medium than in liquid medium. Corredoira et al. (2004) compared effects of pregrowth-desiccation and vitrification on recovery of cryopreserved somatic embryos of *Castanea sativa*, and found that the latter produced 68% of recovery rate, much higher than the former (33%). Applying three cryopreservation protocols to SEC of *Fraxinus excelsior*, Ozudogru et al. (2010) found that neither encapsulation-dehydration nor encapsulation-vitrification gave survival, while only two-step cooling achieved recovery following cryopreservation.

Vidal et al. (2010) tested vitrification for somatic embryos of 51 *Quercus suber* genotypes and found that all genotypes tested survived following cryopreservation and the surviving samples were able to produce new somatic embryos. These somatic embryos were able to germinate and convert into plantlets, and their morphologies were identical to those derived from non-cryopreserved SE.

Seeds

Up to date, seeds of a number of forest trees have been successfully cryopreserved. Recently, a review paper provided detailed information on advances in cryopreservation of forest tree seeds (Gantait et al. 2016). Hence, the present article addresses only some important issues relevant to the topic.

Orthodox seeds can be cryopreserved without any pretreatment. Medeiros et al. (1992) found that *Astronium urundeuva* seeds, which contained 8.01% water content at harvest, were able to survive cryopreservation without desiccation. But, germination rates were improved when the seeds were dehydrated by air drying for 24–96 h to reduce seed water content to 5.96–7.13%. Working with 90 forest species native to Australia, Touchell and Dixon (1993) achieved seed germination in 68 using direct immersion into LN or slow cooling. Chmielarz (2009) reported that seed water contents of 0.04 and 0.06 g H_2O dry mass (g g^{-1}) were optimal for germination and seedling emergence in cryopreserved seeds of *Fraxinus excelsior.* Germination rate was lower in cryopreserved seeds that had broken their dormancy than those at dormancy stage. Furthermore, germination rates of the seeds cryopreserved for 2 years were similar to those stored with traditional conservation method at − 3 °C.

Compared to orthodox seeds, intermediate seeds require considerably more attentions to maturity degree and moisture content at which seeds are collected for cryopreservation (Touchell and Walters 2000; Gantait et al. 2016). Nadarajan et al. (2006) reported that viability of cryopreserved seeds of *Sterculia cordata* increased with seed maturity and the

greatest germination rates obtained in fully-matured seeds. Lombardo et al. (2013) found that *Fraxinus angustifolia* seeds collected at early stage of maturation exhibited physiological dormancy, while those collected at the final stage of maturation did not. Germination rates of cryopreserved seeds were lower in the former than in the latter. In this case, the viability was associated with the dry weight of seeds at harvest time: the more mature the seeds were, the higher the dry weight was. Rapid desiccation was frequently used to achieve an optimal moisture content of seeds, thus avoiding or minimizing desiccation injury (Touchell and Walters 2000). In general, sufficient recovery was obtained when the seeds were desiccated to 7–20% moisture content (i.e., 0.08–0.25 g H_2O g^{-1}) (Normah and Makeen 2008; Gantait et al. 2016). Rapid thawing at 37–41 °C for 1–20 min was most often used for cryopreserved seeds, with quite a few cases in which slow thawing at the ambient temperature was used (Touchell and Dixon 1993; Dumet and Berjak 2002; Pukacki and Juszczyk 2015; Gantait et al. 2016).

Cryopreservation of recalcitrant seeds has been quite limited and still remains unfeasible. Wen (2009) found that seeds of Chinese fan palm (*Livistona chinensis*) were not able to survive cryopreservation, following desiccation to different levels of water contents (14.5–32%). However, zygotic embryos excised from the seeds could be successfully cryostored only when they were desiccated to about 20% of water content, and no significant differences were detected in the viability of the zygotic embryos cryopreserved for 2 years. Cryopreserved seeds of *Mimusops elengi* and *Manilkara zapota* failed to germinate, while 27 and 94% of recovery rates were obtained in cryopreserved embryonic axes for the former and the latter, respectively (Wen et al. 2013). Also, high recovery rates, up to 76% for *M. elengi* and 98% for *M. zapota*, were achieved in cryopreserved hypocotyls and radicles (Wen et al. 2013). Working on *Castanea sativa*, Corredoira et al. (2004) obtained high survival rates (93–100%) of cryopreserved embryogenic axes, following desiccation to 20–24% of moisture contents in a laminar flow cabinet. Zygotic embryogenic organs or tissues have been used for successful cryopreservation of recalcitrant seed forest species, such as in *Quercus faginea* (González-Benito and Perez-Ruiz 1992), *Quercus suber* and *Quercus ilex* (González-Benito et al. 2002), *Quercus robur* (Plitta et al. 2014) and *Sterculia cordata* (Nadarajan et al. 2006), thus providing an alternative for efficient cryopreservation of recalcitrant seeds (Normah and Makeen 2008).

Genetic stability in cryo-derived regenerants

For conservation of plant genetic resources, a major concern is the genetic integrity in regenerants recovered after cryopreservation (Harding 2004; Benson 2008b; Wang et al. 2014a, b). Cryogenic procedures involve not only freezing in LN but also other steps such as preculture and dehydration. All these steps cause severe stresses to the samples and may result in genetic variations in the regenerants recovered from cryopreservation (Harding 2004; Benson 2008a, b; Wang et al. 2014b). Therefore, assessment of genetic stability in cryo-derived regenerants is necessary (Harding 2004; Benson 2008a, b; Wang et al. 2014a, b). Some examples of genetic stability assessments in regenerants recovered from cryopreserved forest trees are given in Table 4.

Flow cytometry (FCM) and molecular markers such as amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and inter-simple sequence repeat (ISSR) are frequently used for assessments of genetic stability. Earlier studies mainly used single methods, while more recent studies preferred a combination of two methods for this purpose, because different DNA markers

Table 4 Some examples of assessment of genetic integrity assessments in regenerants recovered from cryopreserved forest trees

Plant species	Type of explants	Assessment method	Polymorphism (%)	Causes	References
Abies	Embryogenic callus	RAPD	16.8	DMSO	Aronen et al. (1999)
	Embryogenic callus	RAPD	0	–	Salaj et al. (2010)
	Embryogenic callus	RAPD	Not specified	Freezing in LN and in vitro culture	Krajňáková et al. (2011)
Melia azedarach	Shoot tips	RAPD	0	–	Scocchi et al. (2004)
Picea glauca × *engelmanni* complex		RAPD	0	–	Cyr et al. et. (1994)
Picea glauca	Embryogenic callus	RAPD	0	–	De Verno et al. (1999)
Pinus nigra	Embryogenic callus	RAPD	–	–	Salaj et al. (2011)
Pinus sylvestris	Embryogenic callus	RAPD	0	–	Häggman et al. (1998)
Quercus	Embryogenic callus	SSR	Not specified	Cell lines	Barra-Jimenez et al. (2015)
	Somatic embryos	RAPD	Not specified	Cell lines	Sánchez et al. (2008)
	Somatic embryos	FCM	Minor difference	Cell lines	Fernandes et al. (2008)
		SSR	0	–	
		AFLP	2.5	Dehydration	
	Plumules	AMP	10.39–11.00	Desiccation, cryoprotectant and freezing in LN	Plitta et al. (2014)

AFLP amplified fragment length polymorphism, *AMP* amplified DNA methylation polymorphism, *FCM* flow cytometry; *RAPD* randomly amplified polymorphic DNA, *RFLP* restriction fragment length polymorphism, *SSR* single sequence repeats

cover wider genomic regions and use of combined markers gives more reliable results of genetic integrity analyses (Harding 2004; Benson 2008a; Wang et al. 2014a, b).

Using RAPD, Häggman et al. (1998) did not detect any reproducible variations in cryopreserved SEC of *Pinus sylvestris*. Maintenance of genetic fidelity assessed by RAPD has been reported in various forest trees such as *Melia azedarach* (Scocchi et al. 2004), *Populus tremula* × *P. tremuloides* (Jokipii et al. 2004), *Quercus robur* (Sánchez et al. 2008), *Eucalyptus* (Padayachee et al. 2009), *Abies* hybrids (Salaj et al. 2010) and *Pinus nigra* (Salaj et al. 2011). Assessments of genetic stability by SSR in *Picea abies* plants recovered from PVS-based vitrification did not show any polymorphic bands, compared to the non-cryopreserved control (Hazubska-Przybył et al. 2013). Fernandes et al. (2008) reported no polymorphic bands were detected by SSR in the regenerants derived from cryopreserved somatic embryos by encapsulation-dehydration in *Quercus suber*, but some minor variations in genetic stability were detected by FCM and AFLP. These variations were caused by dehydration degree and could be avoided when samples were dehydrated to 35% water content, but not 25% (Fernandes et al. 2008). Although about 16.8% of genetic variations revealed by RAPD were found in cryopreserved SEC of *Abies cephalonica*, DMSO was identified to be the major causal agent, but not freezing in LN (Aronen et al. 1999). Fingerprints analyzed by RAPD were found similar in the regenerants recovered from cryopreserved SEC and the control in 6 out of 8 lines of *Pinus nigra* (Salaj et al. 2011). The polymorphic bands found in the other 2 lines were caused by pretreatments with sorbitol, while not with sucrose or maltose. A relationship between the genetic stability and the genotype in cryopreserved embryogenic tissues was also found in *Quercus ilex* (Sánchez et al. 2008) and *Quercus suber* (Fernandes et al. 2008). More recently, using DNA methylation evaluation, Plitta et al. (2014) found that cryopreservation had negligible influence on DNA methylation levels in cryopreserved plumules (shoot meristems of embryonic axes) of *Q. robur*, and therefore suggested cryopreservation of plumules as an appropriate method for long term conservation of *Q. robur* germplasm.

Taking together all data addressed above, it can be seen that results obtained so far are promising. Use of vegetative tissues such as shoot tips for cryopreservation can to great content maintain genetic stability in the regenerants following cryopreservation, while careful manipulations of SEC to be cryopreserved can help maintenance their genetic stability following cryopreservation in forest trees (Harding 2004; Tsai and Hubscher 2004; Benson 2008a, b; Padayachee et al. 2009; Wang et al. 2014a).

Conclusions and further prospects

Cryopreservation is at present time considered as an ideal means for the long-term storage of biodiversity of plants. Although progresses have been made in developments of cryogenic procedures applicable to shoot tips, SEC, seeds and zygotic embrogenic tissues of forest trees, studies on forest trees have been far less advanced than on agricultural and horticultural crops. Therefore, more efforts should be exerted to study cryobiology of forest trees. Research should be strengthened to help our better understanding of the mechanisms of dehydration and freezing tolerance of forest species. Wide-spectrum cryopreservation protocols applicable to a wide range of genotypes in a given species, which is still a bottleneck for setting-up cryobanks of forest trees, should be further developed. Like *Malus* and *Pyrus* species, cryopreservation of in vivo dormant buds of forest tree species grown in temperate regions should be further developed. Use of in vivo dormant buds can

avoid the establishment and maintenance of in vitro stock cultures, which is difficult in some forest tree species, and is labor-expensive and time-consuming. Assessments of genetic stability in the regenerants recovered from cryopreservation should be strengthened by use of combined molecular markers or more sensitive methods like DNA methylation and gene sequencing. Speeding up of establishment of cryo-banks of forest tree species would certainly help to avoid dangers of genetic erosion facing the forest trees.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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