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Synthetic Seeds

Germplasm Regeneration, Preservation and Prospects

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In Vitro Conservation Through Slow-Growth Storage

Ravishankar Chauhan, Vikram Singh, and Afaque Quraishi

Abstract In vitro approaches are valuable for the conservation of plant biodiversity that includes the preservation of genetic resources of vegetatively propagated species, threatened plant species, taxa with recalcitrant seed, elite genotypes, and genetically modified/engineered material. The mid-term conservation is usually achieved by reducing the growth of in vitro cultures through the application of minimal media and growth retardant or storage at low temperatures resulting in prolonged intervals between the subcultures. Moreover, the combinations of all these factors are also employed for slow-growth storage. The medium-term conservation strategies are consistently employed for a large number of plant species, including various threatened species, from tropical as well as temperate origin. For long-term conservation of plant species, cryopreservation (storage in liquid nitrogen at −196 °C) is commonly employed. However, the main difficulties associated with cryopreservation are the maintenance of in vitro cultures as the procedure is highly technical and expensive since it involves a huge amount of resources and labor. In vitro slow-growth storage, therefore, enables a possible solution for mid- to long-term conservation of plant materials in limited space and at reduced costs too. Slow-growth procedures allow clonal plant conservation for several months to years (depending upon the species) under aseptic conditions, requiring the infrequent successive transfers of the cultures.

Keywords Cold storage · Endangered species · Germplasm storage · Growth retardant · Minimal medium

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1 Introduction

The conventional method of plant germplasm conservation includes their maintenance as the whole plant in the field (Pathirana et al. 2016). Field maintenance of plant materials not only carries the risks of infections of viral, fungal, and bacterial diseases and insect pests but also includes losses due to the environmental disasters such as flood, earthquake, drought, fire, volcanic eruptions, etc., which has led to the erosion of valuable germplasm resources (Barba et al. 2008; Carimi et al. 2011). However, duplication of materials in different fields is an option but is a quite expensive approach. The major obstacles in in situ conservation practices are the requirement of larger space, the high cost of operation, complicated management, and risk of damage by both biotic and abiotic factors of the environment (Rao 2004). Therefore, risks involved in field maintenance have led us to search for secure, cost-effective, and efficient protocols for effective conservation of plant diversity. The plant genetic resources are conserved in the forms of seeds, bulbs, or tissue culture-derived propagules in various gene banks and termed as ex situ conservation (Paunescu 2009). Ex situ conservation is the maintenance of plant genetic resources under controlled conditions, i.e., away from their native habitats and cultivation in botanic gardens and nurseries and by seed storage or in gene banks through in vitro conservation (Dhillon and Saxena 2003; Paunescu 2009). In vitro approaches have two kinds of storage strategies: (1) active strategy which refers to short- to mid-term storage of samples and (2) base strategy referring to long-term preservation of materials (Linington 2003; Engelmann 2011). In gene banks, both the strategies are complementary to each other in which germplasms are stored in an environment free from vulnerable depletion by nature and by arthropods (Linington 2003; Li and Pritchard 2009). Both the strategies distribute disease-free plants, thus minimizing the cost of disease indexing (Lynch et al. 2007). Among these, the most suitable method suggested for long-term ex situ conservation of any species is storage of their seeds.

The species having orthodox seed form can be stored at a low temperature for extended periods by dehydrating down their moisture level (Roberts 1973). However, the conservation of other species and seed form is little problematic (Engelmann 2011), for example, the vegetatively propagated species that do not produce seeds. Similarly, recalcitrant seeds can't be dried sufficiently at the low moisture level with viability to let their storage at low temperatures (Roberts 1973). Moreover, the seeds of few species are generally highly heterozygous in nature and, therefore, unsuitable for the conservation purpose. Such species are thus chiefly maintained as clones (Engelmann 2011). Until now, most of the activities on ex situ conservation of plants have focused particularly on crops. However, conservation of wild and threatened plant species has also become an issue of concern. The statistics of the International Union for Conservation of Nature (IUCN) revealed that out of over 12,000 plant species, approx. 70% are in the threatened category and 19% are critically endangered (Trejgell et al. 2015). In addition, 28 species are extinct in the wild. In situ conservation strategy alone may not be sufficient to rescue the

threatened species (Sarasan 2010). In line, advancements in the biotechnology lead to the introduction of few novel categories of germplasm that includes clones obtained from elite genotypes, the cell lines with special attributes, and genetically transformed clones (Engelmann 1992). This new category is often of high added value and a bit problematic to produce (Engelmann 2011). The development of efficient techniques to ensure its safe preservation is, therefore, of paramount importance. A lot of efforts have been made to improve the quality and conservation methodologies by field gene banks and botanic gardens. However, clearly alternative approaches to plant genetic conservation are needed, and since the early 1970s, attention has turned to the possibilities offered by biotechnology, specifically in vitro culture system. Besides the conventional forms of protection of economically important and threatened species in the past decades, advancements in biotechnology and especially in the area of in vitro culture techniques led to the development of procedures that can be used as an excellent tool in plant conservation (Maryam et al. 2014). Plant tissue culture systems allow propagating plant material in an aseptic environment with high multiplication rates (Sharma et al. 2018). Disease-free clones can be obtained through meristem culture in combination with different therapies such as chemo-, thermo-, and electrotherapy, thus ensuring the production of disease-free stock materials and simplifying procedures for the germplasm exchange throughout the world (Singh et al. 2018). The miniaturization of explants allows reducing space requirements and, consequently, labor costs for the maintenance of plant germplasm. In vitro conservation protocols have been established for ample plant species, including a number of endangered species (Chauhan et al. 2016; Kamińska et al. 2016, 2018). In addition, another importance of ex situ conservation is that it is an internationally accepted strategy, as stated in the Global Strategy for Plant Conservation (UNEP 2002), and is frequently employed by a number of organizations known for biodiversity conservation (Sarasan et al. 2006). Plant tissue culture technique has been reported as an effective tool to conserve many plant species, especially of tropical origin (Engelmann 1991). For the short- and mid-term conservations, various techniques have been developed, which not only results in slow growth of the cultures but also prolongs the time interval between two subcultures (Cha-um and Kirdmanee 2007; Cordeiro et al. 2014).

2 Germplasm Storage Strategies

The maintenance of plant stocks or material under aseptic and adequate environmental conditions can be conducted using the two main approaches. The first one of these approaches is based on conserving material without disturbing its growth, i.e., successive transfer in a fresh medium, while the second one is based on conservation under slow-growth condition (Withers 1980; Engelmann 1991; Sarasan et al. 2006; Novikova et al. 2008). The shortcomings of a successive transfer are an increase in work expenses and the consumption of basic materials and nutrients (Cordeiro et al. 2014). It should also be taken into consideration that long-term subculture can be

followed by a decrease and/or the loss of the morphogenetic potential of the culture as well as by an increase in the probability of genetic changes during long-term subculturing (Joy et al. 1991; Bessembinder et al. 1993; Hao and Deng 2003). Furthermore, there is a risk of losing propagating material as a result of a human errors or microbial contamination in the process of subculture (Grout 1990); therefore, it is advisable to reduce frequent interventions during conservation.

With due regard for all these factors, in vitro culture under slow-growth conditions is supposed to be the most effective method of plant germplasm conservation. The use of this approach is aimed at slowing down the growth of cultures and prolonging the interval between two successive transfers (Cordeiro et al. 2014), as well as raising the degree of safety during the conservation of cultures as a result of a decrease in interferences in a culture system and the minimization of the risk of contamination during subculture (Grout 1990; Engelmann 2011). The success of the use of certain approach depends on numerous factors, such as the possibility of extending the time period between two successive transfer, how long the influence of a limiting factor lasts until the moment when that factor begins to negatively affect the culture, and how fast the regular developmental functions could be restored after reverting to standard culture conditions (Grout 1990). The essential condition for using slow-growth procedures is the study of vital capacities of various kinds of cultures and the stability/instability of the preserved material (Shibli et al. 2006; Rai et al. 2009).

3 Slow-Growth Storage Technique

Slow-growth storage (also known as mid-term conservation) is based on the reduction of the metabolic activity, i.e., the growth rate of in vitro cultures by maintaining them on modified growth medium or in altered culture conditions (Lambardi and Ozudogru 2013). The motto is to prolong the duration between two subcultures (depending on the species) of in vitro cultures without negatively affecting their regrowth potential. Reduction in the growth of in vitro cultures is generally achieved by modifying the culture medium and/or the culture conditions (Engelmann 1998, 2004). Among these approaches, the most widely applied practice is temperature reduction, which can popularly be coupled with a decrease in light intensity or incubation of culture in the dark condition (Engelmann 2011). A number of tropical species often show their susceptibility to low-temperature damage and hence can be stored at a comparatively higher temperature, which further depends on the cold sensitivity of the species (Engelmann 2011). And, to maintain in vitro culture, it should be subsequently subcultured under standard culture conditions to avoid contamination and/or deterioration of stock materials (Niino and Arizaga 2015). Manipulations of the culture medium may include dilution of mineral elements, reduction/enhancement of sugar concentration, changes in nature and/or concentration of plant growth regulators, and an addition of osmotically active compounds. Moreover, in a few cases, plant growth retardants were also applied (Acedo and

Arradaza 2012; Trejgell et al. 2015). Various parameters influence the efficiency of in vitro slow-growth storage procedures that includes the selection of explants, its chemical/physiological state during storage, the type of culture vessel, its volume, as well as the volume of a culture medium used for storage (Niino and Arizaga 2015, Engelmann 1991).

4 Low-Temperature Storage

The most extensively applied technique is temperature reduction, which can be pooled with a decrease in the light intensity or by maintaining the cultures in the dark conditions. Tropical and sub-tropical plant species are often cold-sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the particular species. Potato in vitro plants can be stored at 7 °C without transfer for up to 18 months (Gopal and Chauhan 2010). Other species such as *Ananas* are much more cold-sensitive since the 66 accessions of *Ananas* shoot cultures have to be conserved at temperatures higher than 20 °C (Souza et al. 2004; Silva et al. 2016).

In vitro slow-growth storage procedures are being frequently used for medium-term conservation of a number of species, both from tropical and temperate origins, including crop and medicinal plants, e.g., *Coffea*, *Vitis*, *Musa*, and *Acorus* (Nassar 2003; Sajid et al. 2006; Kulkarni and Ganapathi 2009; Quraishi et al. 2017), and rare and endangered species (Thakur et al. 2015; Chauhan et al. 2016). However, if in vitro conservation appears as a simple and practical option for long-term conservation of various species and has extensive medium-term applications, its implementation still requires customization for any new species; continuous inputs are mandatory, and a question remains in regard to the clonal fidelity of the stored species. Moreover, it is not always possible to apply a single protocol for preserving genetically diverse species. For example, slow-growth storage experimentation performed with an in vitro collection of *Ananas* germplasm including 66 accessions revealed a huge variability in the response of the accessions to the storage conditions (Silva et al. 2016). Some of them showed somaclonal variation during storage, while others did not show any erosion.

Plant species storage at non-freezing low temperatures has been very successful (Koc et al. 2014). At lower temperature regime, the aging of the plant cells/tissues is slowed down but not completely stopped. Consequently, successive transfer of the plant material is necessary although very infrequently. Some examples where shoots/plants have been stored with different strategies of slow growth for various durations are listed in Table 1.

Preil and Hoffmann (1985) stored approx. 700 breeding lines of *Chrysanthemum* at 2–3 °C in the diffused light of 10–15 lux. At this condition, few of the lines survived up to 5 years, and the authors noticed that aeration of the cultures played a crucial role in storage. In the poor gas exchange conditions, the shoots became vitrified. Cold storage at a temperature of 10 °C in the diffuse light also induces vitrification of *Cheiranthera volubilis* shoots and, thus, reduces survival too (Williams and Taji 1987).

Table 1 In vitro slow-growth storage of plant species by employing three important factors, i.e., minimal medium, cold storage, and growth retardant either singly or in combination for mid-term conservation

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Taraxacum pieninicum	Shoot tip	Minimal medium + cold storage + growth retardant	½ MS + sucrose (3%) + ABA (5 μM) at 10 °C	9 months	90%	Kamińska et al. (2016)
Chlorophytum borivilianum	Shoot	Minimal medium	MS + sucrose (6%) + BA (2 mg l^{-1}) + NAA (0.2 mg l^{-1})	4 months	100%	Chauhan et al. (2016)
Indigofera tinctoria	Nodal explants	Minimal medium	MS + IBA (0.5 mg l^{-1}) + mannitol (10 g l^{-1})	4 months	100%	Nair et al. (2016)
Senecio macrophyllus	Shoot tip	Minimal medium + growth retardant	½ MS + ABA (3.8 μM) + sucrose (3%) at 10 °C	9 months	100%	Trejgell et al. (2015)
Tetrastigma hemsleyanum	Shoot tip	Minimal medium + cold storage + growth retardant	½ MS + BA (0.3 mg l^{-1}) + sucrose (10 g l^{-1}) + maleic hydrazide (0.2 mg l^{-1}) at 8 °C	10 months	96%	Peng et al. (2015)
Vitis heyneana	Axillary buds	Minimal medium + growth retardant	MS + IBA (0.05 mg·l^{-1}) + IAA (0.1 mg l^{-1}) + ABA (0.5 mg l^{-1}) + mannitol (10 g l^{-1})	12 months	48%	Pan et al. (2014)
Pistacia lentiscus	Shoot tip	Cold storage	Basal MS in the dark condition at 4 °C	12 months	100%	Koc et al. (2014)
Castanea sativa	Shoot tip	Minimal medium + cold storage	WPM + BA (0.44 μM) at 8 °C	48 months	82%	Capuana and Lonardo (2013)
Glycyrrhiza glabra	Shoot apices	Minimal medium + cold storage + growth retardant	MS + BA (0.25 mg l^{-1}) + PEG (1 mg l^{-1}) + Ancymidol (5 mg l^{-1}) + ABA (0.1 mg l^{-1}) + mannitol (20 g l^{-1}) at 10 °C	6 months	100%	Srivastava et al. (2013)
Dioscorea alata	Node	Minimal medium	MS + mannitol (4%)	13 months	–	Acedo and Arradaza (2012)
Turbinicarpus	Shoot apices	Minimal medium + cold storage	MS + mannitol (30 g l^{-1}) + sorbitol (30 g l^{-1}) at 4 °C	12 months	–	Pérez-Molphe-Balch et al. (2012)

Solanum tuberosum	Node	Minimal medium	MS + sucrose (20 g l^{-1}) + sorbitol (40 g l^{-1})	18 months	58%	Gopal and Chauhan (2010)
Elettaria cardamomum	Node	Minimal medium	½ MS + BA (5 μM)	18 months	70%	Tyagi et al. (2009)
Saccharum spp.	Globular embryo	Minimal medium + cold storage	½ MS + sucrose (10 g l^{-1}) at 18 °C	8 months	–	Watt et al. (2009)
Dendranthema grandiflora	Node	Minimal medium	Modified hyponex medium + ½ MS + DMSO (2.5%) + sucrose (4%)	12 months	80%	Budiarto (2009)
Malus domestica and *Malus sieversii*	Shoot	Minimal medium + cold storage	MS + sucrose (3%) at 4 °C	21 months	–	Kovalchuk et al. (2009)
Fragaria x *ananassa*	Shoot	Minimal medium + cold storage	Knop's medium + sorbitol (0.2 M) at 4 °C	15 months	76%	Hassan and Bekheet (2008)
Veronica multifida ssp. *Capsellicarpa*	Node	Minimal medium	MS + mannitol (3 or 6%)	3 months	–	Holobiuc et al. (2008)
Drosophyllum lusitanicum	Node	Minimal medium + cold storage	MS + mannitol (2%) + sucrose (6%) + zeatin (0.91 μM) at 5 °C	8 months	100%	Gonçalves and Romano (2007)
Curcuma longa	Shoot	Minimal medium	MS + BA (2.5 mg l^{-1}) + isabgol (3.5%)	12 months	56%	Tyagi et al. (2007)
Allium sativum	Bulblet	Minimal medium + cold storage	MS + sorbitol (0.2 M) at 4 °C in dark	18 months	100%	Hasan et al. (2007)
Cedrus atlantica and *Cedrus libani*	Micro-cuttings	Minimal medium + growth retardant	MS + ABA (10 mg l^{-1})	6 months	83%	Renau-Morata et al. (2006)
Olea europaea	Shoot	Minimal medium + cold storage	OM medium at 4 °C in the dark condition	8 months	–	Rugini and Pesce (2006)
Vanilla spp.	Shoot buds	Minimal medium	½ MS + sucrose (1.5%) + mannitol (1.5%)	12 months	90%	Divakaran et al. (2006)
Dierama luteoalbidum	Corm	Minimal medium + growth retardant	MS + paclobutrazol (5–10 mg l^{-1})	6 months	100%	Madubanya et al. (2006)

(continued)

Table 1 (continued)

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Vriesea reitzii	Shoot	Minimal medium	MS + NAA (2 µM) + BA (4 µM)	4 months	100%	Filho et al. (2005)
Garcinia indica	Shoot	Minimal medium	½ MS + BA (5 µM) + sucrose (3%)	11 month	95%	Malik et al. (2005)
Plumbago indica	Nodal segments	Minimal medium	MS + mannitol (20 g l^{-1})	8 months	100%	Charoensub and Phansiri (2004)
Melia azedarach	Apical mer-istem-tip	Minimal medium + cold storage	¼ MS + BA (0.5 µM) at 4 °C in dark	12 months	67%	Scocchi and Mroginski (2004)
Ananas comosus	Shoot	Minimal medium + growth retardant	MS + paclobutrazol (0.5 mg l^{-1})	3 months	100%	Canto et al. (2004)
Ipsea malabarica	Shoot	Minimal medium	½ MS only	27 months	100%	Martin and Pradeep (2003)
Mentha spp.	Apical and nodal explants	Cold storage	MS only at 2 °C	6 months	–	Islam et al. (2003)
Malus pumila cv Gala	Shoot tip	Minimal medium + cold storage	MT + BA (0.5 mg l^{-1}) + NAA (0.05 mg l^{-1}) + sucrose (2%) + mannitol (2%) at 4 °C	12 months	100%	Hao and Deng (2003)
Phoenix dactylifera	Shoot bud	Cold storage	MS only in darkness at 5 °C	12 months	70%	Bekheet et al. (2002)
Solanum tuberosum	Node	Minimal medium + cold storage + growth retardant	MS + sucrose (6%) + Ancymidol (25 µM) at 6 °C	16 months	92%	Sarkar et al. (2001)
Ensete ventricosum	Shoot	Minimal medium + cold storage	MS + BA (10 µM) + mannitol (1%) at 15 °C	12 months	86%	Negash et al. (2001)
Malus pumila cv Moscatella	Apical/node	Cold storage	½ MS in darkness at 4 °C	12 months	100%	Negri et al. 2000

Malus pumila cv Starkspur red	Apical/ node	Cold storage	½ MS in darkness at 4 °C	18 months	90%	Negri et al. (2000)
Musa spp.	Shoot tips	Cold storage	MS only at 17 °C	15 months	66%	Pedroso de Oliveira et al. (2000)
Solanum tuberosum	Shoot	Cold storage	MS only at 4 °C in red light illumination	3 months	–	Pruski et al. (2000)
Prunus virginiana	Shoot	Cold storage	MS only at 4 °C in red light illumination	3 months	–	Pruski et al. (2000)
Quercus suber	Shoot	Cold storage	GD medium at 5 °C in the dark	24 months	50%	Romano and Martins-Loução (1999)
Coffea arabica	Zygotic embryo	Minimal medium + growth retardant	MS + ABA (18.9 or 37.8 μM)	24 months	74%	Naidu and Sreenath (1999)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + mannitol (2%) at 6 °C	16 month	88–100%	Sarkar et al. (1999)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + sucrose (40 g l^{-1}) + mannitol (20 g l^{-1}) at 6 °C	30 months	83%	Sarkar and Naik (1998)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + mannitol (4%) + acetylsalicylic acid (100 μM) at 8 °C	12 months	90%	Lopez-Delgado et al. (1998)
Lilium hybrid	Scale bulblet	Minimal medium + cold storage	¼ MS + sucrose (9%) at 2 °C	28 months	73–90%	Bonnier and van Tuyl (1997)
Miscanthus x *ogiformis*	Shoot	Minimal medium + cold storage	½ SH medium + NAA (0.1 μM) at 8–16 °C	6 months	95–100%	Hansen and Kristiansen (1997)
Coffea spp.	Shoot tip	Minimal medium	MS + BA (1.3 μM)	36 months	–	Dussert et al. (1997)
Xanthosoma spp.	Shoot	Minimal medium + cold storage	MS + mannitol (3%) at 13 °C	24 months	–	Zandvoort et al. (1994)

(continued)

Table 1 (continued)

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Colocasia esculenta	Single shoot	Minimal medium + cold storage	MS + BA (10 µM) + mannitol (1–2%) at 9 °C	96 months	90%	Bessembinder et al. (1993)
Saccharum spp.	Apical meristem	Minimal medium + cold storage	½ MS + sucrose (1%) at18°C	12 months	–	Taylor and Dukic (1993)
Cocos nucifera	Zygotic embryo	Minimal medium	MS + sodium ascorbate (100 mg l^{-1}) + sucrose (1.5%)	12 months	51%	Assy-Bah and Engelmann (1993)
Populus alba x *Populus grandidentata*	Shoot	Minimal medium + cold storage	MS + BA (1.33 µM) at 4 °C	60 months	25%	Son et al. (1991)
Musa acuminata	Meristem tip	Minimal medium + cold storage	SM + ribose (3%) at 17 °C	24 months	67%	Ko et al. (1991)
Ipomoea batatas	Axillary buds	Minimal medium + growth retardant	MS + ABA 10 (mg l^{-1})	12 months	20–80%	Jarret and Gawel (1991)

ABA abscisic acid, *BA* 6-benzylaminopurine, *DMSO* dimethyl sulfoxide, *GD* Gresshoff and Doy (1972) medium, *IBA* indole-3-butyric acid, *MS* Murashige and Skoog (1962), *MT* Murashige and Tucker (1969) medium, *NAA* naphthalene acetic acid, *OM* Rugini (1984) medium, *SH* Schenk and Hildebrandt (1972) medium, *SM* Smith and Murashige (1970) medium, *WPM* woody plant media (McCown and Lloyd 1981)

The double-node cuttings of *Drosophyllum lusitanicum* could be kept alive for 8 months at 5 °C in growth-limiting condition (Gonçalves and Romano 2007). Low-temperature storage has been applied with most promising results to in vitro shoot/plantlet cultures and less successfully to undifferentiated cell cultures. Slow-growth can retard the loss of totipotency of cultured cells/tissue and the ability to synthesize secondary metabolites too in callus cultures stored for relatively short periods (Seitz 1987). The storage temperature usually depends on the sensitivity of the species. Whereas for temperate species it ranges from 5 to 9 °C, for tropical species, it is often much higher (Kulkarni and Ganapathi 2009). Shoot tips of *Actinidia* spp. could be maintained at 8 °C for 52 weeks with 100% survival (Monette 1987). The shoots retrieved after a storage period appeared normal in respect to growth and proliferation rates. Similarly, the cultures of *Colocasia esculenta*, another tropical species, conserved for 3 years at 9 °C (Zandvoort and Staritsky 1986). On the contrary, *Musa* cell suspension cultures were not able to tolerate temperatures lower than 15 °C (Kulkarni and Ganapathi 2009). Few banana cultivars that were stored below 15 °C suffered damage within 3 months (Withers and Williams 1986). At 15 °C some of the accessions of banana survived up to 17 months with a viability of 92%, but in others, viability was sharply reduced to 50% within 13 months (Withers and Williams 1986). According to Watt et al. (2009), the best condition for the storage of globular somatic embryos of *Saccharum* spp. is to place them on ½-strength MS medium (Murashige and Skoog 1962) supplemented with only 10 g L^{-1} of sucrose and incubate at 18 or 24 °C. *Saccharum* spp. stored under these conditions for 8 months showed approx. 80% survival, and most of the plants appeared normal. Similarly, cassava plantlets must be stored at temperatures higher than 20 °C (Roca et al. 1984). The low-temperature storage protocols of maintaining in vitro cultures hold great promise in the nursery industries (Preil and Hoffmann 1985). During the periods of low demand for a particular species or variety for which potential markets exist in the future, the in vitro cultures may be efficiently shelved in normal refrigerators and the time and, consequently, money required to maintain them by successive transfer or restarting fresh cultures saved. This methodology may also apply to research stocks for further experimentation. However, one of the expected limitations in low-temperature storage of plant germplasm may be the gradual habituation of some materials to slow-growth conditions (Withers 1991).

5 Minimal Medium

By modifying the medium composition usually by reducing the sugar content, minerals, growth regulators, or osmotic agents such as sorbitol and mannitol, inhibition of cell division can be achieved, which significantly limits both callus formation and shoot development (Shibli et al. 2006; Lambardi and Ozudogru 2013). In this context, the in vitro slow-growth storage of *Elettaria cardamomum* was achieved on the half-strength MS, fortified with 30 g L^{-1} of sucrose, of which

about 70% of the cultures survived up to 18 months at 25 °C (Tyagi et al. 2009). A regrowth potential of 96% was obtained by culturing nodal explants excised from 18-month-old conserved cultures of *E. cardamomum*.

Photinia sp. micro-shoots were able to store at 4 °C and up to 15 months in a combination of sucrose and mannitol 15 g L^{-1} each containing QL medium (Quoirin and Lepoivre 1977) with more than 90% of survival rate (Akdemir et al. 2010). The stored materials were further recovered and found to proliferate normally in 1 mg L^{-1} BA supplemented QL medium. The micro-plants of six genotypes of *Solanum tuberosum* could be preserved for up to 12 months, without any phenotypic abnormalities, and had enough nodes for further subculturing. The conservation was conducted at 24 °C in the MS medium supplemented with 20 g L^{-1} sucrose along with 40 g L^{-1} sorbitol at a photoperiod of 16/8 h, in which the survival rate was 77.8% (Gopal et al. 2002). This approach was an effective alternative to low-temperature (6–8 °C) storage, especially for the species of tropical and sub-tropical origins, where summer temperature may reach up to 45–50 °C (Gopal et al. 2002).

The effects of osmotic doses along with different temperature regimes were found efficient for tuber- or bulb-producing species. MS medium, comprising 3% (w/v) sucrose, 4% (w/v) sorbitol, and 1 mg L^{-1} ancymidol, was seen to be the best suited for slow-growth storage of in vitro cultured crowns of *Asparagus officinalis* (Fletcher 1994), in which crowns were stored at 6 °C for 16 months and were regrown with 100% survival. Similarly, Bonnier and van Tuyl (1997) successfully stored the in vitro bulblet of *Lilium* spp. for a period of 28 months at 25 °C on ¼-strength MS medium supplemented with 9% (w/v) sucrose. Afterward, these cultures were successfully regenerated with a survival rate of 92%.

Further, the combined effects of sucrose, mannitol, and photoperiod were assessed at 6 °C of temperature for the conservation of micro-shoots of *S. tuberosum* by Sarkar and Naik (1998). Their slow-growth media were comprised of 30, 40, 50, 60, 70, or 80 g L^{-1} sucrose along with 20, 40, or 60 g L^{-1} of mannitol. Over 30 months of storage, sucrose alone did not improve the viability of these cultures. However, the addition of 20 g L^{-1} of mannitol in the storage medium increased the survival rate (83%) of micro-shoots. Further, in order to in vitro conserve the *Saccharum officinarum* germplasms, lateral buds onto the MS medium with an osmoticum were screened (Sarwar and Siddiqui 2004). In the 2% mannitol (w/v)-supplemented MS medium, the lateral buds were healthy up to 165 days and with 75% survival rate, while the cultures with 3% (w/v) mannitol showed 100% survival up to 105 days only, at 17 °C of temperature; conducted study also suggested that low temperature (10 °C) was unfavorable for in vitro storage of *S. officinarum* under both light and dark conditions.

The in vitro shoot tips of *Vanilla planifolia* could successfully be maintained for more than 1 year, without subculturing, on the MS medium supplemented with 15 g L^{-1} each of sucrose and mannitol, at 22 °C and with 90% recovery (Divakaran et al. 2006). After few years of this report, the in vitro shoots of *Saccharum* sp. were successfully stored in the ½-strength MS medium amended with 30 g L^{-1} sorbitol, at both 18 and 24 °C for a duration of 8 months (Watt et al. 2009). The highest survival percentage and shoot regrowth (90%) were observed in cultures stored at 18 °C.

Studies pertaining to in vitro preservations of *Dianthus spiculifolius* and *D. tenuifolius* were performed (Mitoi et al. 2009), in which addition of 0.16 and 0.32 M mannitol in the MS medium, in combination with vitamin of B5 medium (Gamborg et al. 1968), was found to be most suitable for their conservation and regeneration after 6 months of storage at 25 °C. The addition of mannitol (58.4 mM) in the MS medium was found best for 7 months' storage at 5 °C of *Prunus* sp. with a survival rate of 100% (Marino et al. 2010). In the case of *Podophyllum peltatum*, storage of micro-shoots at 10 and 25 °C of temperatures revealed 100% survival after the addition of mannitol (2%, w/v) or sorbitol (2%, w/v) to the MS medium (Lata et al. 2010). However, a negative impact of both the osmoticum, in terms of shoot proliferation rate, was there when stored micro-shoots were cultured on a recovery medium (Lata et al. 2010). Likewise, the shoot tips of *Pyrus* sp. could be stored successfully on the MS medium containing 2.5% (w/v) mannitol, with highest survival (63.41%) and regeneration (58.81%) potentials at 25 °C (Ahmed and Anjum 2010). The efficiencies of sucrose, mannitol, or sorbitol, in a series of concentrations (3%, 6%, 9%, or 12%, w/v), were assessed for storage of *Stevia rebaudiana* micro-shoots (Shatnawi et al. 2011). Afterward, a dose of 3–9% (w/v) sucrose was found to favor higher survival (94.6%) of micro-shoots even after their storage for 32 weeks. However, under dark storage, the survival rate of these cultures was reduced significantly. The micro-shoots of two *Pistacia vera* cultivars were maintained at 4 °C in the dark conditions for 12 months in the MS medium containing 2% (w/v) of mannitol (Akdemir et al. 2013). More than 90% of proliferation in the micro-shoots of both the cultivars was observed after 12 months of storage.

6 Application of Growth Retardant

Growth retardants are natural/synthetic chemical compounds that can be applied in the culture medium to change vital processes by modifying hormonal balance in a plant in vitro (Espindula et al. 2009). Growth retardants act as signalling compounds in the regulation of plant growth and development. They typically bind to receivers in the plant and induce a series of cell changes that can affect the initiation/modification of tissue development (Espindula et al. 2009). The augmentation of plant growth inhibitors in the culture medium is also a significant measure to retard cell growth; such inhibitors include abscisic acid (ABA), maleic hydrazide, paclobutrazol, and few others (Renau-Morata et al. 2006; Sharma et al. 2012; Trejgell et al. 2015).

Kovalchuk et al. (2009) successfully conserved the micro-shoots of *Malus domestica* for 21 months in the MS medium augmented with 1 mg L^{-1} of ABA at 4 °C, while *Glycyrrhiza glabra* shoot apices cultures responded best for storage up to 6 months, when incubated at 10 °C under a dark condition in 5 mg L^{-1} ancymidol, 0.1 mg L^{-1} ABA, and 1 mg L^{-1} polyethylene glycol (Srivastava et al. 2013). Addition of 9.5 μM ABA and 1.5% (w/v) each of sucrose and sorbitol could enhance survival and proliferation of 9-month-old micro-shoots of *Senecio macrophyllus*

during their re-culture in optimal conditions, compared to those cultures stored on the MS medium lacking ABA (Trejgell et al. 2015).

Recently, the subculture duration of *Tetrastigma hemsleyanum* micro-shoots was significantly prolonged up to 10 months, by using 0.2 mg L^{-1} of maleic hydrazide 8 °C with 8/16 h photoperiod (Peng et al. 2015). In this study, the addition of growth retardants such as ABA, chlormequat, and paclobutrazol did not improve *T. hemsleyanum* micro-shoot survival at both 8 and 25 °C of temperatures.

7 Slow-Growth Storage of Elite Tree Species

In vitro propagation technique plays a key role in increasing the production of woody plants and the re-establishment of threatened plant germplasms (Quraishi 2013). However, those techniques involve periodic subculturing of cultures to change the gaseous state of the vessels and to refresh the components of a medium (Ozden-Tokatli et al. 2010). Plant biodiversity comprises ample old mature tree diversity having various featuring characteristics such as *Ginkgo biloba*: the living fossil or the *Taxus* trees famous for their anti-cancer bioactive compound. For the exploitation and conservation of such elite clones, slow-growth storage can be useful as few hard-wood species efficiently conserved through these techniques.

Pistacia lentiscus can be efficiently stored in 6 months at 4 °C in the dark (Koc et al. 2014). Further, the response of mannitol was examined for mid-term conservation of *Eucalyptus ventricosum* (Negash et al. 2001). *E. ventricosum* could be effectively conserved up to 6 months' duration at 15 °C. Similarly, in vitro shoot tips of *Malus pumila* were stored at 4 °C on the Murashige and Tucker (1969) medium fortified with 2% (w/v) each of sucrose and mannitol, in which all the shoot tips were able to survive up to 12 months of storage, revealing 100% recovery (Hao and Deng 2003). The mid-term storage of *Eucalyptus grandis* shoot cultures was achieved for up to 10 months by the addition of 10 mg L^{-1} of ABA at 25 °C of temperature (Watt et al. 2000). In this approach, reductions in the light intensity and addition of mannitol to the MS medium were found to be less effective for the conservation of *E. grandis*. Very recently, 45 or 60 g L^{-1} of sucrose fortified DKW medium (Driver and Kuniyuki 1984) was found to be suitable for in vitro conservation of *Prunus avium* shoots (Ozudogru et al. 2017). At 4 °C and under dark condition, these cultures survived up to 16 months of time. However, the inclusion of mannitol in the storage medium did not reveal any significant impact on shoot quality of *P. avium*.

8 Conclusion

For long-term storage, cryopreservation, i.e., storage at an ultra-low temperature, usually that of liquid nitrogen (−196 °C), is the only method. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus

be stored without alteration or modification for a theoretically unlimited period of time (Barraco et al. 2013). However, the main difficulties associated with long-term maintenance of in vitro cultures are that the procedure is a bit problematic, highly technical, and expensive as it involves the huge amount of resources and labor (Rao 2004; Capuana and Lonardo 2013). In vitro slow growth, therefore, represents a possible solution for mid- to long-term storage of plant materials in limited space and at reduced costs. Furthermore, slow-growth procedures allow clonal plant conservation for several months to years (depending upon the species) under aseptic conditions, requiring infrequent subculturing (Cha-um and Kirdmanee 2007). Of the numerous methodologies tried for short-/medium-term conservation of germplasms, lowering the temperature regime of culture has been most accepted so far and is being used for routine maintenance of germplasm of a range of plant species (Withers 1991).

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