Chapter 23

Rubber, Tea, Coffee, Oil Palm and Arecanut

☆ *Rekha Chaudhury and S.K. Malik*

1. Introduction

The conservation of plant genetic resources (PGR) is essentially to protect and safeguard invaluable genetic resources that are fast depleting due to various biotic and abiotic stresses and have large potential future use. Utilization of these PGRs is linked to food security and agrobiodiversity. Conservation strategies are dependent on plant species and explants to be conserved, availability of best techniques and resources and feasibility. *In situ* and *ex situ* conservation methods that can complement one another based on specific needs are essential for holistic efficient and cost-effective conservation.

Over the past six decades, biotechnological research has focused on developing and improving protocols for (i) embryo culture; (ii) clonal propagation *via* somatic embryogenesis; (iii) homozygote production *via* anther culture; (iv) germplasm conservation *via* cryopreservation; and (v) genetic transformation. Cryopreservation, in particular, has shown enormous potentiality in effecting safe long-term conservation of PGRs over the past 40 years in view of simplicity and the applicability to a wide range of genotypes and explants like seeds, embryos, embryonic axes, meristems, pollen, dormant buds and genomic resources (Chaudhury *et al.*, 2015).

Plantation crops need to be propagated and maintained clonally in field, however with several limitations. Field maintenance proves most expensive due to (1) high labour costs (2) vulnerability to environmental hazards and pest and pathogens and (3) large space requirement. This can lead to sudden loss of valuable germplasm or accumulation of systemic pathogens, especially viruses. It can be overcome using *in vitro* repositories or seed genebanks or cryobanks for

short-, medium- and long-term conservation. *In vitro* conservation using mainly normal and slow growth conditions, induction of storage organs *etc.* have been reported for several plantation crops (Mandal, 1999; Mandal *et al.*, 2000; Manivel, 2000; Sreenath, 2000; Thulaseedharan *et al.*, 2000). Arecanut is the least worked out species in this regard.

Cryopreservation technology, the *ex situ* storage of biological materials at ultra-low temperatures, usually between -150 to -196°C using liquid nitrogen (LN) as a safe and efficient long-term technology, has remained an important biotechnological tool for storing wide range of plant germplasm (Chaudhury *et al.*, 2015) and for pathogen-free status of germplasm (Wang *et al.*, 2014). Cryopreservation is the only technique available presently, for long-term storage of vegetatively propagated species (in the form of shoot tips, meristems, somatic embryos and cells), difficult-to-store non-orthodox seed species (comprising intermediate and recalcitrant seeds), to which several plantation crops belong, for pollen to meet breeders need. For plantation crops, cryopreservation of vegetative tissues such as shoot apices and somatic embryos and zygotic tissues like seeds, embryos and embryonic axes and pollen have been reported from several laboratories.

Seeds are the most preferred explants for storage due to ease of handling. However, many plantation crops, which are indigenous to the tropics belong mainly to the category of recalcitrant seeds (Chaudhury and Malik, 2004). These cannot be desiccated to low moisture content without substantial loss of viability. In addition, seeds with intermediate seed storage behavior can withstand desiccation up to 10-14 per cent, however with viability decline. Careful controlled processing of such seeds result in extension of their storage life at low and ultra-low temperatures.

Storage of pollen is another option which is comparable to seed storage. Pollen of several species is desiccation-tolerant (*i.e.* can be dried to less than 5 per cent moisture content on a dry weight basis) when shed at binucleate state and can be stored below 0°C in desiccated state. In contrast, trinucleate pollen produced from species like arecanut and oil palm, have high moisture content at anthesis, survive only limited desiccation and have short life span ranging from a few hours to days (Barnabas and Kovacs, 1997). Pollen storability trends are not correlated with seed storability trends and hence pollen storage of recalcitrant seed species can be undertaken to complement their conservation efforts.

The chapter deals with current status of use of various cryotechniques to achieve *ex situ* conservation of some important plantation crops, *Camellia sinensis* L. (O.) Kuntze (tea), *Elaeis guineensis* Jacq. (oil palm), *Areca catechu* L. (arecanut), *Hevea brasiliensis* Muell.-Arg. (rubber) and several spp. of *Coffea* (coffee) using explants like seeds, zygotic embryos, somatic embryos, embryonic axes, shoot apices and pollen.

2. Cryopreservation Methods

Cryopreservation techniques commonly used for various species are as follows:

2.1. Air Desiccation-Freezing

In this technique, the seeds are desiccated to different target moisture contents by maintaining over charged silica gel in a desiccator for 4-48 hrs before freezing in LN. Aseptically excised embryos and embryonic axes are desiccated in sterile

air under laminar airflow cabinet up to 5 hours, to around 11-16 per cent moisture level depending on critical moisture content of each, and then frozen by rapid plunging in liquid nitrogen.

2.2. Pregrowth-Desiccation

Explants like zygotic embryos, somatic embryos, shoot apices and embryonic axes are pregrown on media containing different cryoprotectants in order to impart greater desiccation tolerance and homogeneity in the behaviour. The explants are then dehydrated under the laminar airflow cabinet or over silica gel or processed by encapsulation-dehydration or vitrification.

2.3. Encapsulation-Dehydration

Explants are suspended in 3-5 per cent sodium alginate solution and picked up to dispense individually into 100 mM $CaCl_2$ followed by shaking to obtain beads. The beads are then dehydrated in sucrose-enriched media for a minimum period of 17 hours followed by desiccation and freezing.

2.4. Vitrification

It involves the treatment of tissues with cryoprotectants in vitrification solutions followed by fast freezing. Most commonly used is Plant Vitrification Solution 2 (PVS2). Recovery growth of explants is tested after thawing and removal of cryoprotectants. Initially developed for shoot apices, cell suspensions and somatic embryos, it has been lately applied to zygotic embryos and embryonic axes.

2.5. Droplet Vitrification

Developed by Panis *et al.* (2005), this technique follows a normal vitrification protocol except during immersion in LN, droplets of vitrification solution enclosing the explants are placed on sterile aluminum strips. A very high cooling and rewarming rates are ensured as explants are in direct contact with LN and with unloading solution.

2.6. Pollen Storage

Pollen samples are desiccated using various desiccants for few hours before sealing in cryovials and storing at temperatures at or below -150°C.

2.7. Thawing

For thawing of frozen samples, polypropylene cryovials containing the explants are dipped into a water bath (37-40°C) to warm the specimen rapidly.

3. Success using Cryotechniques in different Genera of Plantation Crops

3.1. Rubber (*Hevea brasiliensis* L.)

3.1.1. Cryostorage of Zygotic Embryos and Embryonic Axes

Cryopreservation of rubber was reported to be successful by Normah *et al.* (1986) using air desiccation-freezing method for embryonic axes with 50 per cent

survival after cryoexposure. In later studies (Normah, 1987), higher survival rates (69-71 per cent) were achieved when axes were desiccated for 2 and 3 hours, cooled by stepwise method or by direct immersion and thawed rapidly. A detailed review on storage attempts on rubber seeds and embryonic axes was provided by Normah and Chin (1995). It was concluded that best cryoprotocol was desiccation of rubber axes to 19 per cent followed by stepwise cooling and rapid thawing.

In later studies by Sam and Hor (1999), zygotic embryos were first precultured for 16 hours on 0.3 M sucrose medium before subjecting to desiccation by PVS2 treatment for 70 to 120 min which led to 57 per cent survival after rapid freezing and thawing. In another study, using encapsulation-dehydration technique, rubber embryos were encapsulated in beads and then precultured on medium enriched with sucrose (0.3, 0.5, 0.7, 0.9 M) for 24 hours followed by desiccation in laminar flow to obtain 15 per cent moisture in the explant (Yap *et al.*, 1999). Preculture in 0.3 M sucrose, after LN exposure, led to 45-70 per cent viability, and 30-60 per cent survival.

3.1.2. Cryostorage of Pollen

Pollen of rubber has been reported to be successfully cryostored for 3-7 weeks as viability was confirmed by seed set resulting from field pollination (Hamzah *et al.*, 1999). It demonstrated the full functionality of cryostored pollen when used for hand-pollination.

3.2. Tea (*Camellia sinensis* L. (O.) Kuntze)

3.2.1. Cryostorage of Seeds and Embryonic Axes

Fully mature tea seeds were reported to be highly desiccation sensitive (Kim *et al.*, 2005) and could not survive LN exposure (Chaudhury *et al.*, 1990) with success later achieved with embryonic axes excised from seeds (Chaudhury *et al.*, 1991). In later studies, seeds were partially desiccated up to 14 per cent (Hu *et al.*, 1994) and 8-10 per cent (Wang *et al.*, 1999) and could be successfully cryostored using simple protocol. No loss of germinability of tea seeds, cryostored for two months, was recorded (Wang *et al.*, 1999). Simultaneously, tea axes could be successfully cryostored with high survival values of up to 95 per cent (Chaudhury *et al.*, 1991) following desiccation-fast freezing. Kim *et al.* (2002) used cotyledonary embryonic axes at moisture contents between 21.5 and 15.0 per cent for cryopreservation and highest percentages of plantlet production from cryopreserved explants ranged between 75.1 and 80.4 per cent. In studies by Kaviani (2010), success in cryopreservation was achieved with alginate encapsulated embryonic axes of *C. sinensis* which were kept in MS liquid medium with 0.75 M sucrose for 2 h followed by air dehydration with moisture contents of about 15-20 per cent.

3.2.2. Cryostorage of Shoot Tips

Cryostorage of shoot tips have been successful in tea. In *Camellia sinensis* cv. Yabukita, two month old *in vitro* grown plantlets were cold-hardened for five weeks. Vitrification of shoot tips, precultured for two days on sucrose-enriched media, was then carried out. The normal shoot formation rate of cryopreserved apices was 60 per

cent. Using the encapsulation technique, 40 per cent shoot formation was reported (Kuranuki and Sakai, 1995). In both cases, apices developed shoots directly within 2 or 3 weeks without intermediary callus formation producing true-to-type plants (Kuranuki and Sakai, 1995).

3.3. Coffee (*Coffea* spp.)

Different *Coffea* species exhibit non-orthodox (intermediate or recalcitrant) seed storage behaviour (Dussert *et al.*, 2002; 2007). Extensive cryopreservation studies have been carried out on different *Coffea* species with diverse explants (Dussert *et al.*, 2002).

3.3.1. Cryostorage of Seeds and Zygotic Embryos

In *Coffea arabica* seeds, optimal water content for cryopreservation was found to be 0.2 g H_2O g^{-1}dry weight. Seedlings after cryopreservation could be successfully recovered if seeds were rehydrated above water immediately after warming (Dussert *et al.*, 1997). Extraction of zygotic embryos from whole seeds after cryopreservation led to higher (80-90 per cent) survival values (Dussert *et al.*, 1998). In subsequent studies, four coffee species, desiccated to different moisture contents followed by fast and slow freezing, showed variable success rates (Dussert *et al.*, 1998). Using air desiccation–freezing method, seeds of *C. liberica* showed about 53 per cent survival after cryopreservation (Normah and Vengadasalam, 1992). Zygotic embryos of *C. canephora*, *C. arabica*, hybrid *arabusta* (*C. arabica x C. canephora*) and *C. liberica* have been able to withstand freezing after partial desiccation (survival, 41-95 per cent) (Abdelnour-Esquivel *et al.*, 1992). At IRD-Montpellier (France) since 1997, cryopreservation efforts have been made to investigate basic principles in desiccation and freezing sensitivity in seeds of different *Coffea* spp. to enable cryobanking (Dussert et al., 2001). Continued efforts led to establishment of world's first coffee cryobank (Dussert *et al.*, 2007). Optimization of rewarming and rehydration protocols led to 100 per cent survival of seeds when recovered after cryostorage.

3.3.2. Cryostorage of Somatic Embryos

Somatic embryos have also been attempted for cryostorage. In coffee (*C. arabica* and *C. canephora*), using conventional methods, slow freezing globular embryos were cultivated on a medium enriched with sucrose, pretreated later with sucrose and DMSO before slow freezing (Bertrand-Desbrunais *et al.*, 1988). A 50 per cent recovery of cryopreserved samples was achieved through secondary embryogenesis. Higher recovery (100 per cent through adventitious embryos) was achieved using simplified freezing of heart shaped somatic embryos at –20°C (Tessereau, 1993). Heart and torpedo shaped embryos, processed through encapsulation-dehydration technique, were later recovered directly (Hatanaka *et al.*, 1994). After cryopreservation, 63 per cent of embryos were viable and half of them developed into whole plantlets. Using desiccation method, *C. canephora* somatic embryos were subjected to 12-week freeze-hardening on high sucrose and abscisic acid (ABA) before seven day desiccation and fast freezing (Tessereau *et al.*, 1994). Approximately 64 per cent cryopreserved embryos developed directly into plantlets. Using a similar methodology, 70 per cent recovery of plantlets was reported in *C. arabica* (Mycock *et al.*, 1995).

Abdelnour-Esquivel (2000) used method of pregrowth-desiccation followed by cryoprotectant treatment and slow freezing to somatic embryos. Embryos were pretreated with increasing concentrations of sucrose up to 0.75 M followed by incubation with 5 per cent DMSO and slow freezing at 0.5°C min^{-1} to -40°C before plunging in to LN. Variable success (9-61 per cent) was observed in *C. arabica* and *C. canephora*.

3.3.3 Cryostorage of Shoot Tips

In *Coffea sessiliflora* and *C. racemosa*, shoot apices excised from three week old nodal cuttings were cultured overnight on standard medium and then processed through the standard encapsulation technique. In the former species, a 3-10 day treatment of beads in 0.75 M sucrose before cryopreservation led to 38 per cent survival and in the later, treatment of beads in increasing sucrose concentration from 0.5 to 1 M led to 27 per cent survival (Mari *et al.*, 1995).

3.3.4. Cryostorage of Pollen

No efforts have been reported for long term coffee pollen cryostorage however, 1-2 months longevity by storing at low humidity and temperature (Carvalho and Monaco, 1969; Ferwerda, 1969) and by more than two years by storing under vacuum at -18°C (Walyaro and van der Vossen, 1977) have been reported.

3.4. Oil Palm (*Elaeis guineensis* Jacq.)

3.4.1. Cryostorage of Zygotic Embryos

Oil palm seeds, earlier classified as orthodox, were later defined as intermediate in seed storage behaviour. In studies by Grout *et al.* (1983), oil palm embryos desiccated to 10.4 per cent moisture content were frozen in LN with recovery rate of 75 per cent and there was no loss in viability even after 8 months storage. Before cryopreservation, partial rehydration of oil palm embryos was reported useful (Engelmann *et al.*, 1995b). Zygotic embryos were extracted from dehydrated or rehydrated seeds and desiccated to 0.12-0.3 g H_2O g^{-1} dry weight before freezing in LN rapidly. When extracted from rehydrated kernels, 65 per cent of the embryos desiccated to around 0.3 g H_2O g^{-1} dry weight developed into plantlets after cryopreservation. In contrast, only 25 per cent of embryos at 0.12 g H_2O g^{-1} dry weight extracted from cryopreserved dry kernels developed into plantlets indicating a role of rehydration of kernels before embryo extraction. Rajanaidu and Ainul (2013) reported cryobanking of 33,250 diverse accessions of oil palm as zygotic embryos by the Malaysia Palm Oil Board, Malaysia.

3.4.2. Cryostorage of Somatic Embryos

In oil palm, finger-shaped somatic embryos, pregrown with 0.75 M sucrose followed by rapid freezing and rapid thawing (Engelmann and Dereuddre, 1988), led to 20 per cent success. Later, pregrown somatic embryos subjected to 16 hours desiccation in silica gel before fast freezing led to 80-90 per cent survival (Dumet *et al.*, 1993a, b). Inclusion of the desiccation step allowed the use of standard somatic embryos. Later Dumet *et al.* (1993c) applied this procedure to diverse clones (39) of

somatic embryos resulting in highest survival of 31 per cent for clones in optimal physiological state and lowest survival of 12 per cent for those in a poor state.

For the first time, droplet vitrification method was applied to polyembryoids of oil palm which led to 68 per cent survival after cryopreservation (Gantait *et al.*, 2015). Polyembryoids (3-5 mm diameter) were pre-cultured before processing using PVS2 and were frozen on aluminium strips. It proved to be a better method in terms of higher regeneration in oil palm in comparison to the earlier reported conventional vitrification method. Polyembryoids with haustoria bearing a number of embryoids were the best stage of explants for cryopreservation as judged by morphological examination. These explants exhibited maximum morphogenetic competency in terms of developing plantlets after treatments.

3.4.3. Cryostorage of Meristem

There is one report on success in cryostorage of apical meristems of oil palm (Ainul *et al.*, 2009) resulting in 45 per cent recovery growth when tested after 24 hours.

3.4.4. Cryostorage of Pollen

Oil palm pollen could be cryogenically stored for periods beyond eight years without any significant loss in their viability and germinability and hence can be used effectively for pollinations (Tandon *et al.*, 2007). Oil palm pollen cryostored for up to eight years retained as high as 54 per cent viability as judged by fluorescein diacetate reaction (FCR) test and 49 per cent by *in vitro* germinability. This was comparable to 52 per cent germination before storage (Tandon *et al.*, 2007).

3.5. Arecanut

3.5.1. Cryostorage of Zygotic Embryos

Arecanut is propagated through seeds which are recalcitrant (Raja *et al.*, 2002), and desiccation sensitive, with critical moisture content of 32.8 per cent and hence, Raja *et al.* (2014) attempted to cryopreserve zygotic embryos. The fully developed zygotic embryos (4.0-4.5 mm L X 3.0-3.5 dia) were desiccated to different moisture levels for 1-5 hours under laminar air flow. Desiccated embryos were subsequently fast frozen to -196°C. Recovery, after an eight hour cryoexposure, was 85 per cent under *in vitro* when moisture content of the axes was 21.8 per cent on four hours of drying. After cryostorage at this moisture, maximum survival (70 per cent) was observed after 30 days of culture. Recently pollen collection and *in vitro* germination method has been standardized in arecanut (Anonymous, 2014, www. cpcri.gov.in).

4. Factors Determining Successful Cryopreservation

There are several pre- and post cryoexposure factors determining the success and recovery of plantlets which fall under following themes:

4.1. Collection and Handling of Fruits and Seeds

Seeds of non-orthodox species possess high moisture contents and are short-lived necessitating collection, handling and processing for storage readily. Fresh

harvests are required to be transported in shortest possible time through courier. For retaining healthy viable condition for subsequent cryopreservation experiments, freshly harvested fruits, seeds and vegetative materials are to be processed for storage within a few days or weeks of harvest.

4.2. Developmental Stage

The maturity level of explants is of immense importance during cryopreservation. Recalcitrant embryos and embryonic axes acquire some degree of desiccation tolerance at a particular physiological maturity stage, which is variable for each species. As the seeds develop towards maturity, a gradual decrease in desiccation tolerance has been observed in non-orthodox species which necessitates selection of the right maturity stage of explant for cryopreservation.

4.3. Desiccation and Freezing Rates

For most of the species, fast drying (3-5 hours in laminar air flow) followed by fast freezing has shown to cause minimum damage to cells and hence is widely adopted. Flash drying, using forced drying rapidly in a stream of dry air flowing at rates of approximately 10 $lmin^{-1}$, have proved advantageous in retention of viability (Wesley-Smith *et al.*, 1992).

The direct plunging of small explants into LN, causing freezing rates of 200°C min^{-1}, has been the best. Keeping the explant size and cryo-containers to a minimum and sub-cooling of LN under vacuum to cause quenching increases the rate of freezing.

4.4. Recovery Growth

The suitability of a protocol for cryopreservation can be assessed only in terms of good recovery growth of cryopreserved explants. Whole seeds are germinated in Petri plates in controlled lab conditions. In case of embryos, embryonic axes, somatic embryos and shoot apices recovery growth is to be assessed under *in vitro* conditions. Optimised culture media and culture conditions are essential for achieving normal recovery growth from cryopreserved tissues, especially during the first weeks of culture. In coffee, coconut and oil palm, the use of the optimal recovery medium enhances not only the survival percentage of embryos but increases normal growth without callusing after cryopreservation (Engelmann *et al.*, 1995a). Addition of 2,4-D (0.2 mgl^{-1}) for short period increased the recovery growth after cryopreservation in oil palm (Engelmann *et al.*, 1985). IBA (0.1 mgl^{-1}) and BA (0.5 mgl^{-1}) improved the growth of cryopreserved embryonic axes of *Coffea liberica* (Normah and Vengadasalam, 1992). Improved growth of immature embryonic axes of *C. arabica* was observed after culturing them on a recovery media supplemented with GA_3 (Abdelnour Esquivel *et al.*, 1992). Cryopreservation in some cases affected normal growth of embryonic axes as in *Theobroma cacao* where only regenerative callus could be obtained after cryopreservation (Pence, 1991).

5. Cryobanking

Large-scale testing experiments for practical and feasible establishment of cryo-banking has been successful for major important crops and are being continued

(N'Nan *et al.*, 2012; Benelli *et al.*, 2013). Establishment of cryobanks ensure availability of diverse germplasm, thus consistently providing valuable genes for breeding more productive elite cultivars by traditional breeding and genetic transformation. Reviews enlisting operational risk and safety issues in cryobanking have been published (Benson, 2008; Keller *et al.*, 2005; Benelli *et al.*, 2013). Cryo-banking cannot replace conventional conservation methods, but does serve as a complementary conservation strategy to field gene banking and *in vitro* conservation, *i.e.*, a backup and safety storage technique (Benson, 2008).

6. Prospects and Future Strategies

Variable success in plantation crops, after cryoexposure of explants using different cryotechniques, has been reported. Limitations such as low survival percentage and extent and mode of regrowth in few of the species have led to non-uniform applicability of these to diverse genotypes. In addition, insufficient research efforts for understanding of mechanisms underlying the desiccation and freezing sensitivity have been undertaken.

In vitro culture methods especially using zygotic embryos has been standardized for several plantation crops. This aids in conservation due to ease in handling seeds and embryos for various manipulations. The varying results obtained after seed/embryo/plumule cryopreservation can be attributed to heterogeneity of the explants, availability of limited quantity of explants resulting in less replication, non-optimization of recovery conditions, suitable *in vitro* recovery media, strong link between amenability of a genera/accession to in vitro conditions and their recovery post-cryoexposure. However, there is still scope for various technical improvements in the current cryopreservation protocols as percentage survival in several cases is less than 50 per cent.

Overall cryopreservation, where biological materials are conserved for long-term when stored below the glass transition temperatures, has proven to be the exclusive cost-effective method for non-orthodox species to which most plantation crops belong. In fact cryobanking has proven its worth for germplasm belonging to plants, animals, fishes and microbes (Chaudhury *et al.*, 2015). In several cryolabs around the world, cryopreservation costs have been worked out to be much cheaper than that of *in vitro* slow growth and field genebanks. Cost of coffee field collection was compared with coffee cryo-selection at CATIE in Costa Rica (Dulloo *et al.*, 2009) and found it to be the cheapest method.

Except studies on oil palm and coffee, where large number of diverse germplasm have been cryobanked, there is no other large-scale experimentation on range of genotypes of other species of plantation crops.

At present, pollen storage is not often used in genebanks and the technique needs to be thoroughly assessed based on needs for a species. It is essential to undertake this methodology to supplement the seed or clonal genebanks. Since the technique is simple, effective and relatively inexpensive, it should be widely adopted for plantation crop species. This would have selective advantage in these species since several of these are not amenable to *in vitro* conditions and also

produce difficult-to-store intermediate and recalcitrant seeds. During storage the viability and fertilizing ability of pollen should be maintained. Pollen used for germplasm conservation should remain viable for many years and this is achievable only through cryogenic storage. Herrera *et al.* (2002) has reported protocol for the induction of androgenensis and plant regeneration in *Coffea arabica* from isolated microspores *in vitro* using colchicine pretreatment. With the possibility of haploid and diplodized plant regeneration directly from pollen assuring pure gametophytic origin their storage assumes a new significance. Hopefully, in near future, it would be feasible to raise whole plants from cryostored pollen.

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