Chapter 20

Coconut

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1. Introduction

The coconut palm, *Cocos nucifera* L. (2n=32), is a woody perennial monocotyledonous tree that belongs to the family Arecaceae that includes 27 genera and 600 species. Gunn *et al.* (2011) proposed the South East Asian Island and Southern margins of Indian subcontinent as the ancestral homes of coconut. Coconut is distributed mainly in coastal regions between 20° N and 20° S from sea level to 1000 m above sea level in more than 86 countries which can be grouped into eight distinct coastal/oceanic regions on four continents (Harris, 2001). The ideal growing temperature for coconut ranges between 24 and 30°C (Woodroof, 1979). It is grown on more than 12.19 million ha, 94 per cent of which are in Asia and the South Pacific (APCC, 2014).

The genus *Cocos* is monotypic and has no known truly wild forms. The varieties in coconut fall into two main groups: the tall *typica,* which is commonly cultivated for commercial production and the dwarf *nana,* grown for ornamental and breeding purpose (Narayana and John, 1949). The palm grows from a single terminal vegetative bud with a juvenile period of 3-4 years for dwarfs and 8-10 years for talls. The palm is monoecious with large number of male flowers and few female flowers. The seed is a large one seeded drupe. The economically productive period for talls is 80-90 years and that of dwarfs are 30-40 years (Menon and Pandalai, 1958).

While considering the conservation objectives for coconut genetic diversity, *ex situ* conservation in field gene bank becomes the choice for conserving and utilizing maximum genetic diversity in coconut. The field gene banks provide easy and ready access to conserved material for research as well as for use. But its major drawbacks are threat from pests, diseases, natural calamities, availability of land resources, urbanization that results in loss of valuable collections and high maintenance costs (Withers and Engels, 1990). Moreover, diseases such as root wilt and incidence of insects and pest attacks necessitates the conservation of target genes present in the existing varieties that becomes inevitable for future use in breeding programmes. This envisages the development of a complementary conservation strategy integrating *in situ* and *ex situ* approaches (Maxted *et al.*, 1997) and also utilization of alternate techniques of conservation such as *in vitro* conservation.

2. Coconut Genetic Resources

Plant genetic resources are the most valuable and essential basic raw material of any crop improvement programme and its conservation is highly essential to safeguard against from genetic erosion, genetic vulnerability and genetic wipeout for a sustainable agricultural production. Being a monotypic genus, the genetic diversity in coconut is mainly found in different ecotypes/landraces. The genetic diversity in coconut is gradually getting reduced due to various reasons. The high production cost, market fluctuations in price, labour requirement, incidence of pests and diseases compel coconut farmers to shift the land use for more profitable crops. Consequently, some of the traditional, locally adapted populations are gradually getting diminished or lost. Environmental disasters as well as developmental activities also pose risk and cause the loss of diversity in coconut. Hence there is urgent need to collect and conserve coconut genetic resources for its efficient utilization as unexplored genes or alleles may have better use in future breeding programmes. The large stature and recalcitrant (Chin and Roberts, 1980) or homoiohydrous behaviour of coconut seeds makes it impossible for seed storage by conventional methods. At present, *ex situ* conservation of coconut genetic diversity is through field gene banks whereas *in situ* conservation is mainly on farm or in home gardens or on remote islands and atolls (Sthapit *et al.*, 2005). The ICAR-Central Plantation Crops Research Institute (CPCRI) located at Kasaragod in India maintains the world's largest collection of coconut germplasm constituting a total of 455 accessions (ICAR-CPCRI, 2015-16). The National and multi-site International Gene Banks for the conservation of coconut genetic diversity have been developed by the coconut growing countries world over under ADB-funded project of COGENT (Coconut Genetic Resources Network) of the Bioversity International (Batugal and Jayashree, 2005). The highest priority is to duplicate field collections *in vitro* as embryos and pollen (Ramanatha Rao and Batugal, 1998) and to facilitate international exchange of germplasm. In India the Central Plantation Crops Research Institute, Kasaragod hosts the International Coconut Genebank for South Asia (ICG-SA) that is located at Kidu, Karnataka. In ICG-SA India has conserved 91 accessions of indigenous and exotic coconut germplasm. The other multi-site gene banks are located in Indonesia (for Southeast and East Asia), Papua New Guinea (for the South Pacific), Cote d' Ivorie (for Africa and the Indian Ocean) and Brazil (for Latin America Caribbean).

3. Conservation Strategies

The concept of germplasm conservation demands that collection methods initially capture maximum variation and subsequently, conservation and regeneration techniques minimize losses through time (Astley, 1992). The two approaches for conservation of plant genetic resources are *in situ* and *ex situ*. In the case of *in situ* conservation, the genetic resources are maintained in the natural habitat in which the diversity has evolved *e.g.* natural reserves and on-farm conservation. The advantage of in *situ* conservation is that it facilitates the ongoing processes of natural evolution (Krogstrup *et al.*, 1992). It is probably the best way to preserve biodiversity. Nevertheless, it is expensive in terms of the area required and in the cost of establishment and maintenance. Conserving plant genetic resources outside their original habitat is termed as *ex situ* conservation *e.g.* botanic gardens, seed banks, field gene banks, *in vitro* collections, pollen and DNA storage. It is generally used to protect populations in danger of destruction, replacement or deterioration. For any given gene pool, a complementary conservation strategy involving *in situ* and *ex situ* approaches and methods are necessary for efficient and cost effective conservation (Maxted *et al.*, 1997).

4. Conservation of Coconut Genetic Resources

Coconut is a monotypic genus without any known wild forms. Consequently, the conservation of coconut diversity is within a single species that includes many ecotypes. As seeds are large in size and highly recalcitrant (Chin and Roberts, 1980), *ex situ* conservation of seeds under conventional dry and low temperature conditions is not feasible in coconut. In coconut, the *in situ* conservation method presently being utilized is on farm conservation in the farmer's fields as components of the traditional agricultural systems and in remote islands and atolls. *Ex situ* conservation is mainly in field gene banks. Field gene banks provide easy access to conserved material for use. But they confer the risk of destruction by natural calamities, pests, diseases and are costly for maintenance. Hence there is urgent need to develop safety duplicates of the living collections using alternate strategies of conservation such as *in vitro* conservation.

In vitro culture techniques provide some important tools for improved conservation and management of plant genetic resources (Ramanatha and Riley, 1994). In view of high maintenance cost of field gene banks and loss of important genetic material due to diseases and pest attacks, conservation of coconut genetic diversity can be complemented utilizing other systems like *in vitro* conservation. The advantages of *in vitro* conservation are maintenance of material in pathogen-free condition that facilitates safer distribution, conservation of vegetatively propagated plants, somaclonal and gametoclonal variants, rare germplasm arising through somatic hybridization and genetic engineering, storage of pollen enhancing longevity, meristem culture, conservation of plants from endangered species, storage of cell cultures for industrial applications, and those that produces recalcitrant seeds. Further, the cultures are not subjected to environmental disturbances (Withers and Engelmann, 1997). *In vitro* options provide short to medium (slow growth) and long-term (cryopreservation) conservation of coconut genetic resources.

For short and medium term storage, the objective is to increase the intervals between subcultures by reducing growth. This is achieved by modifying the environmental conditions and/or the culture medium. The advantages of this method are saving space and other resources, cost effective exchange and distribution of material at any times across the world and rescue of germplasm in the event of outbreak of diseases. In coconut, it is especially beneficial for germplasm collection, exchange and surveillance of diseases before the introduction of collected material (embryo or pollen) for further evaluation (Assy-Bah and Engelmann, 1993). The various methods for limiting *in vitro* growth are addition of osmotic agent like sucrose (Sarkar and Naik, 1998), use of growth retardants (Tahtamouni *et al.*, 2001), storage at low temperature (Bertrand-Desbrunais *et al.*, 1992), and encapsulation in alginate beads (Karun *et al.*, 1996). Short to medium term *in vitro* conservation of coconut has been mainly utilized for collection and international exchange of germplasm. Karun and Sajini (1994).) reported short-term storage of coconut zygotic embryos in sterile water without losing its viability for two months. This technique has been utilized in one indigenous and five international expeditions conducted by ICAR-CPCRI for the collection of coconut genetic diversity (Karun *et al.*, 2002). Mature zygotic embryos encapsulated in alginate beads and stored in sterile water at room temperature for two months showed normal germination upon transfer to the retrieval medium. But none germinated after storage in a refrigerator at 10° C for the same period (Karun *et al.*, 1996). Assy-Bah and Engelmann (1993) reported medium term storage (6 to 12 months) of coconut zygotic embryos. They identified the balance of sucrose and activated charcoal for the slow growth action.

Cryopreservation is the *ex situ* storage of biological materials at ultra low temperature (-196 °C) using liquid nitrogen (Withers and Engelmann, 1997) at which all metabolic process within them are completely arrested allowing conservation for a theoretically unlimited period of time (Engelmann, 2004). Even though the initial inputs for cryogene banking are high, its maintenance requires minimal resources. Therefore, it is cost effective (Reed *et al.*, 2004) and requires limited space (De-Zu and Prichard, 2009). Additional advantages are maintenance of cultures in disease-free state without genetic alteration or modification.

It has emerged as an important biotechnological tool for storing wide range of plant germplasm especially those which are difficult to store due to large size, high moisture content, high desiccation/freezing sensitivity (recalcitrant) and those propagated vegetatively and endangered plant species (Nikishina *et al.*, 2007). This technique ensures safe and efficient long term conservation of different types of seeds (Chaudhury and Chandel, 1994), zygotic embryos (Engelmann, 2000), somatic embryos (Gonzalez-Arnao *et al.*, 2003), embryonic axes (Laura *et al.*, 1996), shoots tips (Escobar and Roca, 1997) and pollen (Zhang *et al.*, 2009). Cryopreservation is also employed in *in vitro* propagation systems based on somatic embryogenesis, to avoid somaclonal variation and loss of embryogenic potential that can occur during long-term maintenance of actively growing embryogenic cultures (Silvia *et al.*, 2004). Cryopreservation is also be utilized for virus eradication (cryotherapy) (Brison *et al.*, 1997; Helliot *et al.*, 2002).

The standard techniques of cryopreservation are the classical controlled rate cooling and new vitrification based ones. Classical and new cryopreservation approaches are different based on the techniques employed and the physical mechanisms upon which they are attained (Withers and Engelman, 1997). The classical cryopreservation techniques involve slow cooling in a programmable freezer to a defined pre-freezing temperature (- 40° C) followed by plunging in liquid nitrogen. The material, after treatment with cryoprotectant mixture to a certain period of time, was slowly cooled (from 0.1 to 0.5 °C/min) so that ice is formed in the extracellular spaces. Depending upon the rate of cooling and prefreezing temperature, most or all intracellular water is removed that will avoid the detrimental intracellular ice formation upon subsequent immersion of specimen in liquid nitrogen. As freezable water is reduced the cytoplasm becomes concentrated and vitrifies upon exposure to liquid nitrogen. In order to avoid recrystallization in which ice melts and reforms larger and more damaging crystal size, rapid rewarming was performed (Mazur, 1984). The classical approach to cryopreservation was most successful in preserving protoplast culture, cell suspensions and callus cultures. It was not much valuable in shoot tips and mature zygotic and somatic embryo cultures that comprise mixture of cell sizes and types (Withers and Engelmann, 1997; Fukai, 1995).

The new cryopreservation techniques are based on vitrification. In vitrification based procedure, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation followed by rapid cooling. Vitrification is defined as the transition of the liquid phase to an amorphous glassy solid at the glass transition (T_g) temperature (Fahy *et al.*, 1984). The biological material stored in this stable condition may be maintained for a long time without alteration or modification (Burke, 1986). Vitrification-based procedures offer practical advantages in comparison with classical freezing techniques (Steponkus *et al.*, 1992). This procedure is simple than classical ones, and requires minor modifications for different cell types. It is more suitable for complex organs like shoot tips and embryos. Here the critical step to achieve survival is the dehydration step and not the freezing step as in classical protocols. Therefore if samples to be frozen are amenable to desiccation to critical water content (which vary depending on the procedure employed and type and characteristics of the propagule to be frozen) then further decline in survival was not observed after cryopreservation (Engelmann, 1997).

The development of an effective cryopreservation protocol requires the optimization of numerous variables such as type of explants, size, water content, and concentration of cryoprotectant (if utilized) and freezing behavior of the species. Before cryopreservation, the cells have to be dehydrated artificially to protect them from damage caused by the crystallization of intracellular water into ice (Mazur, 1984). As membranes are the primary site of injury (Fujikawa *et al.*, 1999), tolerance mechanisms for desiccation and freezing mainly depends on membrane stabilization through changes in lipid composition, accumulation of sugars and production of membrane protecting polypeptides, induction of anti-oxidative mechanisms, and the transcription of genes coding molecular chaperones (Thomashow, 1999). Engelmann (2000) described seven vitrification-based procedures for cryopreservation those were air desiccation, pre-growth, pre-growth desiccation, encapsulation dehydration, encapsulation vitrification, vitrification and droplet freezing.

5. Steps Involved in Cryopreservation

The successive steps involved in cryopreservation are selection of material, pretreatment, freezing, storage, thawing, and post retrieval treatments.

5.1. Selection of Material

It is preferable to select young and meristematic tissues for cryopreservation studies. The cells of these tissues are small and are characterized by low water contents, few vacuoles, dense cytoplasm, high nucleo-cytoplasmic ratio that enables them to withstand freezing (Engelmann, 1991). The physiological stage of the material is also vital for the successful recovery after cryopreservation (Berjak *et al.*, 1993).

5.2. Pre-treatment

The material is to be pretreated for a specific period for preparing it for the freezing process. The samples are cultured in a medium enriched with various cryoprotective substances like monosaccharides, oligosaccharides, polysaccharides, mannitol, sorbitol, DMSO etc (Benson and Withers, 1988) for different durations. Cryoprotective substances like sucrose act as an osmotic agent in dehydrating the samples and also protect the membrane (Fujikawa *et al.*, 1999). The nature of cryoprotectants, their concentration and duration will have to be determined on each species basis.

5.3. Freezing

The two types of freezing processes followed are slow and ultra rapid freezing. For slow freezing, a programmable freezer is required to obtain precise and reproducible freezing condition. For rapid freezing, the specimens have been enclosed in cryovials and rapidly immersed in liquid nitrogen as done in case of coconut (Assy-Bah and Engelmann, 1992b) or oil palm (Engelmann *et al.*, 1995).

5.4. Storage

The samples have to be kept at the temperature of liquid nitrogen for storage for theoretically unlimited period of time (Engelmann, 2004).

5.5. Thawing

Immersing the cryotubes containing the samples in a water bath set at 37-40°C carries out rapid thawing. It reduces the chance of ice micro crystals formed during freezing to larger crystals of a size, which would cause damage to cellular integrity (Engelmann, 1991). Fast rewarming of coconut zygotic embryos at 40±1°C for 3 min produced regrowth after cryopreservation (Sisunandar *et al.*, 2010).

5.6. Viability Assessment

Viability tests are conducted for quick and accurate prognosis of the efficiency of cryoprotection and to forecast the chances for recovery of explants after cryopreservation. Viability tests are usually correlated with the survival and regrowth of the plant material subsequent to cryopreservation (Verleysen *et al.*, 2004). Different techniques for viability testing include staining and meristem electrolyte leakage (MEL) tests. The commonly used staining tests are with FDA (flurescein diacetate) and TTC (triphenyl tetrazolium chloride). FDA is absorbed by the living cells and transformed into flurescein, and measured in UV (Windholm 1972). In TTC test, the tetrazolium salt solution is reduced to red colored formazan by the activity of dehydrogenase ions released by respiration of the viable plant cells in the mitochondria (Stenponkus and Lanpher, 1967). In non-optimized cryopreservation protocols, the primary cause of cell death is due to damaged cell membranes (Senaratna and McKersie, 1986). This led to cell lysis and leakage of electrolytes that can be quantified in terms of cell critical to survival and also injured cells (Ketchie *et al.*, 1972; Becwar *et al.*, 1982; Kioko *et al.*, 2006; Pammenter. *et al.*, 1991; Malik and Chaudhury, 2006; Savage, 1992). But the actual assessement of viability is regrowth upon transfer into the medium after cryostorage. Gomes-Copeland *et al.* (2012) used electrolytic conductivity and potassium leaching tests to assess the viability of cryopreserved embryos of coconut cultivar 'Anão Verde do Brasil de Jiqui' (AVeJBr).

5.7. Post-retrieval Treatment

The samples after cryopreservation are to be set for recovery in best conditions (Benson and Withers, 1988). To eliminate the toxic effect of cryoprotective substances, the samples are rinsed and diluted in the culture medium. By successive transfer to progressively less concentrated media avoided osmotic shock for the samples (Benson and Withers, 1988). For better regrowth the nature of the medium (solid/ liquid) can also be changed. To avoid photo oxidation, the samples are placed initially in dark condition for better regrowth. Growth hormones may also be supplemented to the medium for the stimulation of growth (Sajini, 2011). Addition of non vitamin antioxidant and anti-stress compounds (LA: lipoic acid, GSH: glutathione, GB: glycine betaine) during the PVS 2 vitrification protocol was found to be effective for the improvement of regrowth (Esther *et al.*, 2010).

6. Cryopreservation of Coconut Zygotic Embryos

Bajaj (1984) suggested the possibility of long term conservation of coconut zygotic embryos when the embryos resumed growth after freezing at -196°C. Here, the immature embryos (1-1.5 cms) of West Coast Tall cultivar of coconut were partially dehydrated and cut into transverse halves. They were treated with a cryoprotectant solution [7 per cent dimethyl sulfoxide (DMSO) and 7 per cent sucrose in MS liquid medium] blotted dry and wrapped in a single layer of sterile aluminum foil. It was then frozen by gradually lowering into liquid nitrogen and kept for five minutes. The frozen samples were thawed in warm water (35 to 40° C), washed and cultured on MS medium containing 2, 4-D (0.2 mg L^{-1}) NAA (0.5mg L^{-1}) and kinetin (0.1 mg L^{-1}). The retrieved embryos and their segments in cultures showed a lag period of up to 4 months without any sign of growth. In some cultures, the embryo subsequently showed an overall swelling and elongation. Chin *et al.* (1989). reported the survival of one single coconut embryo, 15 months after freezing using a classical protocol (cryoprotection with DMSO and slow freezing).

Using immature embryos of coconut (7-8 months after pollination), Assy-Bah and Engelmann (1992a) could successfully establish rooted plantlets from embryos after freezing in liquid nitrogen. The immature embryos from the coconut hybrid PB 121 were placed for 4 hours in Petri dishes on standard medium containing 600 g L^{-1} glucose. Pregrowth on this medium was compared with pregrowth on medium supplemented with the cryoprotectants glycerol, sorbitol or polythyleneglycol

(PEG) 6000 at 5, 10 or 15 per cent. Thereafter, the embryos were immersed rapidly in liquid nitrogen. Thawing was carried out by immersion of the cryotubes for 30 sec in a water bath at 40 °C. After freezing in liquid nitrogen, survival was obtained in three conditions only: pregrowth with 10 and 15 per cent glycerol (25 and 10 per cent survival respectively) and 10 per cent sorbitol (43 per cent survival). PEG showed no cryoprotective effect at the concentrations tried. But only one rooted plantlet could be obtained from embryos pre-treated with 15 per cent glycerol after 2.5 months (Assy-Bah and Engelmann, 1992a). Embryo pre-treatment with cryoprotectant solution containing, 1.75 mol L^{-1} sucrose + 15 per cent glycerol for 12 and 16 hours presented lower moisture content and larger viability by tetrazolium test in coconut cultivar Brazil Green Dwarf (BGD) (Gomes-Copeland *et al.*, 2015).

Assy-Bah and Engelmann (1992b) reported cryopreservation of mature embryos of four varieties of coconut (hybrid PB 121, Cameroon Red Dwarf, Indian Tall, Renell tall). The embryos were pretreated in the laminar air current for 4 hours and subsequently incubated in medium containing 600 g L^{-1} glucose and 15 per cent glycerol for 11-20 hours. After rapid freezing and thawing a recovery rate of 33 and 93 per cent was observed depending on the variety. Karun *et al.* (2005) reported cryopreservation of mature embryos of West Coast Tall variety of coconut after desiccation pretreatments. Maximum retrieval of healthy plantlets was obtained from the embryos subjected to 18 h silica gel or 24 h laminar air flow desiccation treatment. Irreversible damage of shoot meristem was observed when the moisture content of the embryo was reduced below 20 per cent.

Sisunandar *et al.* (2010a) reported an improved cryopreservation protocol for a wide range of coconut cultivars from Indonesia. The method included four optimized steps *viz*: rapid dehydration, rapid cooling, rapid warming and recovery *in vitro* and acclimatization and soil supported growth. For rapid dehydration, the embryos were placed in a glass jar equipped with a stainless steel mesh (1-2 mm) platform and an autoclavable fan placed below the platform and housed in a polycarbonate tube. Activated silica gel (680 g) was placed into the lower portion of the glass jar in two separate zones. Following this protocol, 20 per cent (when cryopreserved 12 days after harvesting) and 40 per cent (when cryopreserved at the time of harvest) of all Malayan Yellow Dwarf embryos cryopreserved could be retrieved to normal seedlings. Differential scanning calorimetric studies showed that this protocol induced a drop in embryo fresh weight to 19 per cent and significantly reduced the amount of water remaining that could produce ice crystals (0.1 per cent). Of the 20 cultivars tested, 16 were found to produce between 10 per cent and 40 per cent normal seedlings, while four cultivars generated between 0 per cent and 10 per cent normal seedlings after cryopreservation.

Sisunandar *et al.* (2010b) conducted morphological, cytological or molecular studies in coconut plantlets recovered after cryopreservation. The embryos from four different cultivars were subjected to rapid dehydration in a drying chamber containing activated silica gel for 8 h to decrease the moisture content from 78-80 per cent to 19-20 per cent. The plants recovered from cryopreservation showed no morphological variation through measurement of shoot elongation rates, production of opened leaves and number and total length of primary roots. There was no variation in chromosome number (2n=32), type of chromosomes, the length of the long and short arms, the arm length ratio, and centromeric index in all studied cultivars independently of cryopreservation as revealed by karyotype analysis. Genetic and epigenetic fidelity of coconut plants recovered from cryopreservation was assessed through microsatellite (SSR) analysis and global DNA methylation rates. There were no significant genetic or epigenetic differences between the seedlings originating from cryopreserved embryos and respective control.

Sajini *et al.* (2011) studied the effect of preculture conditions, vitrification and unloading solutions on survival and regeneration of coconut zygotic embryos after cryopreservation. Among the seven plant vitrification solutions tested, PVS3 was found to be the most effective for regeneration of cryopreserved embryos (Figure 20.1). The optimal protocol involved preculture of embryos for 3 days on medium with 0.6 M sucrose, PVS3 treatment for 16 h, rapid cooling and rewarming and unloading in 1.2 M sucrose liquid medium for 1.5 h. Under these conditions, 70-80 per cent survival (corresponding to size enlargement and weight gain) was observed with cryopreserved embryos and 20-25 per cent of the plants regenerated (showing normal shoot and root growth) from cryopreserved embryos were successfully established in pots.

Figure 20.1: Plantlet Formation from Coconut Zygotic Embryo following Cryopreservation in PVS3.

7. Cryopreservation of Coconut Plumular Tissues

Hornung *et al.* (2001) followed encapsulation dehydration protocol for the cryopreservation of plumular tissues of coconut. The encapsulated plumules were precultured for 72-96 h in medium with 0.75 M sucrose and desiccated with silica gel to around 30 per cent moisture content. Callus growth was observed from the plumule after freezing in liquid nitrogen. Bandupriya *et al.* (2007) studied the effect of abscisic acid in the encapsulation-dehydration method for cryopreservation of coconut plumules. The survival and recovery rate of frozen plumules was significantly increased after the addition of ABA (40 µM) to the sucrose pretreatment medium. Post cryopreservation, 84 per cent of plumules showed survival 39 per cent of which had been recovered.

Coconut plumule (apical dome with 3-4 leaf primordial) extracted from mature embryos (11-12 months after pollination) was also utilized for cryopreservation studies by Nan *et al.* (2008). Plumules excised from Malayan Yellow Dwarf embryos were first precultured on standard medium with 0.12 M sucrose for three days. Thereafter, for encapsulation, the plumular tissues were suspended in standard medium containing 3 per cent (v/v) sodium alginate and 0.15 M sucrose. After making beads in 0.1 M $CaCl_2$ containing 0.15 M sucrose, it was pretreated for 2-3 days sequentially in standard medium containing various sucrose concentrations (0.5 M, 0.75 M and 1 M). Thereafter, the beads were dried for 6 to 24 h on sterile filter paper over 40 g silica gel in 125 ml air tight boxes. After freezing, regrowth of plumules was obtained for plumules dehydrated for 14 h (21 per cent) and 16 h (20 per cent). Pregrowth of encapsulated plumule beads in 1 M sucrose for 16 hours resulted in 20 per cent leafy shoot production from the cryopreserved samples. The histological studies in recovered plumules after cryopreservation showed structural similarity with control cells.

Bandupriya *et al.* (2010) investigated the most suitable method to transport/store mature zygotic embryos of coconut (for excision of plumules) for cryopreservation work. Three different conditions tested were transportation as solid endosperm cores containing embryos (refrigerated for 10 days), embryos in solidified agar or KCl solution (stored at 27°C in dark for 10days). Following encapsulation-dehydration technique, plumular tissues, excised from embryos stored in KCl and solidified agar showed significantly higher rate of recovery than embryos in albumen cores in unfrozen samples. In frozen plumules, there was no significant difference in recovery under three conditions tested. In frozen plumules pretreated with 1.0 M sucrose, the rate of recovery (40 per cent) was significantly higher in ones excised from embryos stored in solidified agar when compared to other two conditions.

8. Conservation of Coconut Pollen

Pollen represents the haploid phase in the life cycle of plants. At maturity, the pollen grains are dispersed either in two celled (vegetative and generative cell) or three celled stage (vegetative and two sperm cells). Under natural conditions the two-celled pollen has much longer life span because of their protective structure, low water content, and reduced metabolic activity. The trinucleate pollen is short lived due to its wall characteristics, high moisture content, and high rate of metabolism. The life span of pollen is primarily determined by the plant genome and also influenced by environmental conditions (Kozaki, 1975).

Based on pollen longevity, the plant taxa are classified into three main groups (Harrington, 1970) such as, long-lived pollen (6 months to 1 year), pollen with a medium life span (approximately 1-3 months) and short-lived pollen (few minutes to few days). Depending on water content, pollen having more than 30 per cent water is classified as partially hydrated like recalcitrant seeds and if it is less than 30 per cent as partially dehydrated like orthodox seeds (Kermode and Finch-Savage, 2002). Jain and Shivanna (1989) reported a positive correlation between the loss of viability and a reduction in the amount of membrane phospholipids irrespective of pollen storage conditions. The desiccated pollen is to be rehydrated in humid air for restoration of membrane integrity and viability of pollen (Shivanna and Heslop-Harrison, 1981; Bernard, 1973; Karipidis *et al.*, 2007; Hoekstra, 1984; Hoekstra and Van der Wal, 1988; Crowe *et al.*, 1989 a, b).

Maintenance of pollen viability for breeding and research purposes necessitate its storage (Nath and Anderson, 1975). Apart from the use of stored pollen in breeding programmes like storage of multiple genotypes in small space, its transportation, use of individual genotype over a long period of time, pollination in asynchronously flowering plants, it also offers successful exchange and conservation of plant genetic resources (Ganeshan *et al.*, 2008; Panella *et al.*, 2009; Tandon *et al.*, 2007). Pollen conservation provides a viable backup method for conserving genes (Towil and Walters, 2000).

The viability of pollen is to be ensured for effective use in hybridization as well as conservation studies. The pollen viability can be assessed by utilizing staining methods (Panella *et al.*, 2009) or by germination on artificial media (Verdeil and Pannetier 1990; Muccifora *et al.*, 2003; Nandakumar *et al.*, 1988) and was found to correlate with its fertilizing ability (Visser, 1955; Aloni *et al.*, 2001). Temperature is one of the most important environmental factors affecting pollen germination, pollen tube growth and fruit set (Kakani *et al.*, 2002; Burke *et al.*, 2004; Lee *et al.*, 1985). Karim *et al.* (2011) reported variation for cardinal temperatures (T_{min}, T_{opt} and T_{max}) of pollen germination percentage and pollen tube growth. The principal component analysis (PCA) identified maximum percentage pollen germination and pollen tube length of the species, and T_{max} for the two processes as the most important pollen parameters in describing a species tolerance to high temperature.

The percentage of *in vitro* germination of stored pollen has been correlated with its fertilizing ability (Visser, 1955). But the ultimate test for viability is the ability for fruit and seed set after pollination. There are reports that storage and other stresses affect vigour (vigorous pollen tubes) before affecting viability of pollen (Shivanna *et al.*, 1991). So in addition to pollen viability, the study of pollen vigour (pollen tube growth) is also of much importance for pollen storage studies (Page *et al.*, 2006; Harding and Tucker, 1969; David, 1971, 1974; Ottaviano *et al.*, 1980, 1982,).

In the case of coconut, the microspores inside microsporangia undergo nuclear division to give rise to a lenticular shaped generative cell and large tube nucleus (Menon and Pandalai, 1958). When the anthers become fully mature, the pollen sacs burst and shed their pollen before the opening of the male flower. The mature coconut pollen grain contains three nuclei (Patel, 1938). The male flowers on the upper spikes of the inflorescence and those situated on the sides of the female flower open first and commence opening from the apex of the spike and extends downwards. Even though the male flowers open throughout the day, most of them open from 8 A.M. to 10 A.M. Each male flower carries about 272 million pollen grains (Aldaba, 1921). Fresh pollen grains are spherical and smooth while within the anther, but after few seconds of exposure to dryness, they turn ellipsoidal with a longitudinal groove in the middle and measure about 0.063 mm in length and 0.020 mm in breadth (Menon and Pandalai, 1958). Pollen production in individual male flowers in coconut was significantly higher in summer and cold seasons than in the rainy season (Gangolly *et al.*, 1961). Under natural condition, the life span of fresh coconut pollen is only few days (Patel, 1938). The pollen must be conditioned to prolong and maintain high viability. Coconut pollen is desiccation tolerant and can be dried to 5 per cent moisture content without any lozss in viability (Whitehead, 1963). Franchi *et al.* (2011) classified coconut seed and pollen as recalcitrant seed and orthodox pollen (RSOP). Under Indian conditions, Patel (1938) observed 25 per cent of infertile pollen grains in coconut. Aldaba (1921) observed 3 to 33 per cent of infertile pollen grains in Philippines. Nair and Sharma (1963) reported the occurrence of coconut pollen variations comprising the trichotomocolpate, porate, operculate and I-furrowed forms in coconut. Several workers have reported *in vitro* pollen germination in coconut (Aldaba, 1921; Marechal, 1928; Patel, 1938; Nambiar, 1960; Nampoothiri, 1970). Several workers have noticed the importance of boron for germination of pollen in the stigmatic secretions (Gaugh and Ouggar, 1953) or in the germination medium (Johri and Vasil, 1961; Richards, 1986; Shivanna and Johri, 1985; Asif *et al.*, 1983; Leduc *et al.*, 1990; Muccifora *et al.*, 2003; Mortazavi *et al.*, 2010).

8.1. Short Term Storage of Coconut Pollen

The pollen after dehiscence from the anther can be stored through the manipulation of moisture content, storage temperature and humidity. Patel (1938) observed that fresh coconut pollen wrapped in bloting paper become nonviable after ninth day at room temperature. The longevity of pollen was prolonged for 16 days by storage over 35 per cent sulphuric acid (Marechal, 1928) wherein the viability estimates was 35-60 per cent. Desiccation to 5 per cent moisture content by oven drying at 40°C and storage over damp calcium chloride (RH 40 per cent at 5°C) resulted in considerable reduction of viability during the first month of storage, but there was very little further reduction after seven months after collection (Whitehead, 1963). By freeze-drying and storage at room temperature, coconut pollen could be conserved for short periods (Whitehead, 1965).

8.2. Coconut Pollen Cryopreservation

Even though partial dehydration can prolong coconut pollen viability for short periods, cryopreservation is the only available option for its long term storage (Engelmann, 1997; Towill and Walters, 2000). Coconut pollen cryopreservation could be utilized for long-term storage of pollen from palms of distinguishable characteristics that can be utilized for future breeding programmes, facilitating crosses for the production of hybrids and also for the conservation of diverse genotypes. It can also be advantageous in distributing and exchanging germplasm and also for the continuous availability for pollination over extended periods of time. The viability of pollen was studied by *in vitro* germination on media supplemented with sucrose (Kakani *et al.*, 2005).

Karun *et al.* (2006) reported pollen cryopreservation in West Coast Tall (WCT) accession of coconut. The authors accounted medium comprising 8 per cent sucrose, 1 per cent gelatin, 1 per cent agar and 0.01 per cent boric acid for best pollen germination. The variation observed for pollen germination among WCT genotypes was reduced after cryopreservation. The overall pollen tube growth was significantly more when pollen was incubation dried or cryopreserved. Karun *et al.* (2014). studied the effect of cryopreservation and cryostorage on pollen germination and fertility of coconut cultivars, WCT and Chowghat Orange Dwarf (COD). Germination and vigour of cryopreserved pollen were generally higher compared to that of pollen dried in oven and non-cryopreserved. After 4 years and 6 years of cryopreservation, 29 per cent and 32 per cent germination was reported in COD and WCT cultivars (Figure 20.2). Cryopreserved pollen of five Tall and five Dwarf accessions also displayed 24-31 per cent and 25-49 per cent germination, respectively (Karun *et al.*, 2014). Normal nut set was also observed when WCT pollen, cryostored for six years, was used for hybridization in COD palms (Figure 20.3).

9. Light and Ultrastructural Studies Conducted in Cryopreserved Materials

In coconut, Nan *et al.* (2008) reported cellular changes with respect to dehydration duration and pregrowth treatment during cryopreservation of plumular tissues of coconut. For shorter dehydration of plumules (6 hours), the cells in the first level of meristematic zone recovered from its shorted size whereas underlying layers showed high plasmolysis with significant periplasmic space. For intermediate dehydration, apart from this, the occurrences of starch grains were also

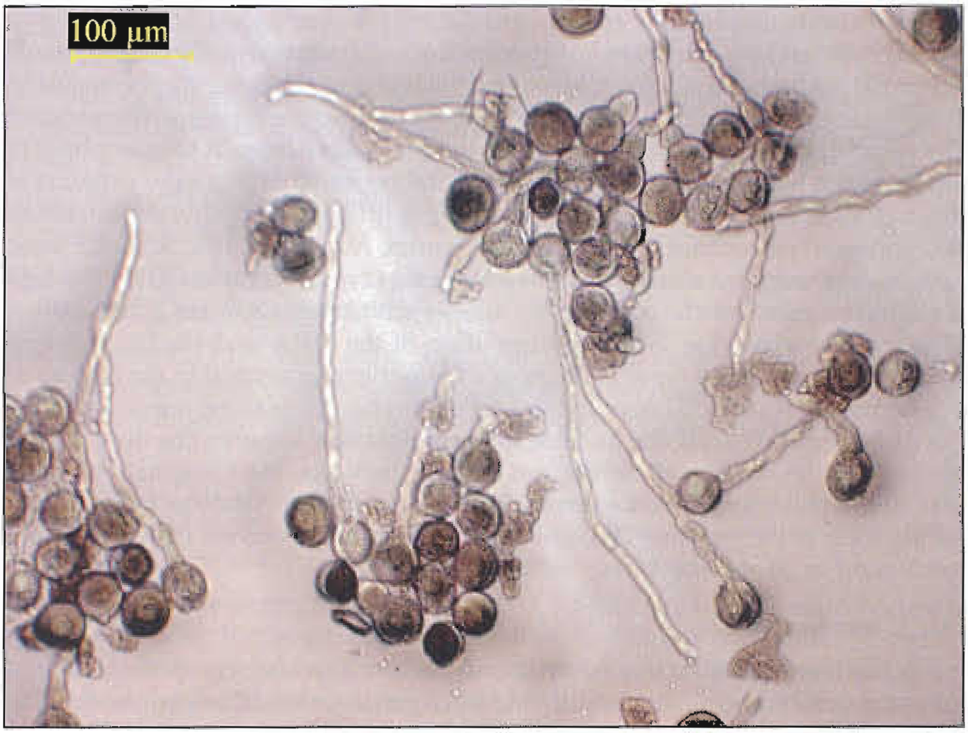

Figure 20.2: Germination in WCT Pollen Cryostored for Four Years.

Figure 20.3: Nut Set in COD Palms Hybridized with WCT Pollen Cryostored for Six Years.

observed. When long dehydration durations (16 hours) were used, the cells appeared of normal isodiametric shape, spherical nuclei with high nucleo-cytoplasmic ratio. There was increase in starch grains from 0.5 M to 1 M sucrose in pregrowth medium. The occurrence of active dividing cells in the plumular tissues at the periphery of the meristem was in accordance with the actual regrowth of plumular explants of coconut after cryopreservation. After studying different dehydration durations of cryopreserved coconut embryos and plumules, Nan *et al.* (2014) reported three categories of cell ultra structural damages during cryopreservation. The first stage of changes concerned the plasmolysis of cells with small vacuoles, condensation of chromatin, changing in the conformation of the DNA and the nucleus and arrest of mitosis. These types of changes are described in general in the context of a desiccation tolerance. The second degree of the changes was the retraction of the cytoplasm inside the cell, the increase in the periplasmic volume. The third degree of modification concerned the deformation of the walls, the invagination or the lysis of the plasma membrane resulting in the observation of distorted cells and the bursting of the nucleus. These two types of modifications are irreversible and correspond to an absence of regrowth of the samples.

10. Conclusion

It has been observed that survival and germination after cryopreservation of coconut zygotic embryos is mainly dependent on genotype as well as cryopreservation methods. Hence it is necessary to develop an efficient cryopreservation protocol independent of genotypic effect so that maximum diversity can be conserved with ease. The studies presented in this chapter elucidate the effectiveness of various cryopreservation methods in coconut zygotic embryo cryopreservation and efficacy of pollen cryostorage on viability and fertility of stored pollen. Though cryopreservation of coconut zygotic embryos, plumule and pollen is feasible, post-retrieval percentage cryopreserved material is still reported as low. Perfect protocols for different cryopreservation methods need to be standardized for different explant material including embryogenic calli, somatic embryos in addition to embryos, plumule and pollen for long term maintenance coconut germplasm.

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