Chapter 6

Biotechnological Approaches

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1. Introduction

Coconut (*Cocos nucifera* L.) is an important perennial palm crop cultivated mainly for the edible oil from dried kernel, but virtually every part of the palm possesses some human use and therefore, the palm is designated as 'tree of life'. It is a monotypic species in the genus *Cocos* and belongs to the Arecaceae family. Coconut palm products possess high nutritional and medicinal value (Foale, 2003). Coconut is cultivated in more than 93 countries, mainly between 26°N and 26°S (Harries, 2001) in about 12 million hectares of land in tropical and subtropical coastal lowlands (FAOSTAT, 2013). About 10 million farmers and their families depend exclusively on coconut; many others in rural and semi-urban locations depend on the crop marginally for their livelihood (Rethinam, 2006).

Coconut consists mainly of two types, talls and dwarfs. Since coconut is heterozygous in nature with a long juvenile period, genetic improvement becomes difficult and is prolonged. Supply of high-yielding, disease-resistant seedlings is essential for stabilized yield in the traditional coconut-based farming system. Availability of quality planting materials has been a major constraint in coconut productivity. Several factors are known to influence coconut production which has declined slightly in last two years in India (http://www.coconutboard.nic.in/stat.htm). In India, the annual demand for 10 million coconut seedlings cannot be met by conventional method, which could produce only about 35 per cent of the demand (Karun *et al.*, 2015). Since the palm has a single apical meristem, without branches or suckers, its propagation through vegetative means is restricted with dependence exclusively on seed propagation. With no other known method of propagating the coconut palm by vegetative methods, tissue culture is only the option for rapid

multiplication of coconut palms. Several factors pose challenges in coconut farming which warrants an improvement in existing coconut varieties. In this context, germplasm collection and their conservation assume significance (Arunachalam and Rajesh, 2008). Crop improvement research in coconut would result in significant improvement in yield potential and higher profits. Biotechnological tools such as embryo culture, cryopreservation, tissue culture, molecular markers and marker-assisted selection (MAS) and transcriptomics could play crucial roles in coconut improvement programmes.

2. Embryo Culture

2.1. Germplasm Collection

Production of planting material for propagation in coconut is solely through seed nuts since other vegetative means are not available. Bulkiness of the seed, short dormancy period, nut water, stringent phytosanitary requirements and high cost for transportation are the major difficulties encountered during germplasm collection and storage. Field gene banks host genetic resources in coconut. The collection and transportation of coconut for the safe movement of coconut germplasm through embryo cultures, instead of seed nuts, is recommended by the technical guidelines finalized by FAO/IPGRI (currently Bioversity International) (Diekmann, 1997). The success of *in vitro* germination of coconut zygotic embryos provides an alternative way of transportation of coconut germplasm in the form of embryo cultures. This method also avoids the formalities of quarantine regulations, which include treatments of the nuts with insecticide, fungicide and fumigants. Further, collection of embryos *in vitro* also greatly reduces the cost of transport. Thus coconut zygotic embryo culture has practical value in collection and exchange of germplasm overseas and therefore, simple and efficient *in vitro* field collecting techniques have great significance. Many attempts in zygotic embryo culture have been reported in the past (Cutter and Wilson, 1954; Abraham and Thomas, 1962; Ventura *et al.*, 1966). ICAR-CPCRI has developed a successful protocol for culturing coconut zygotic embryos from 8-11 months old nut, which was successfully utilized in the germplasm expeditions of the institute (Karun *et al.*, 1992, 1999, 2004). This technique makes use of effective artificial media with available macro and micronutrients and microclimatic conditions which support the embryo to grow and to form entire plantlets. The field collection technique involves inoculating the sterilized zygotic embryos excised from the nuts on to the nutrient medium *in vitro* (Assy-Bah *et al.*, 1987; Sossou *et al.*, 1987; Karunaratne, 1988; Rillo and Paloma, 1991; Karun *et al.*, 1993; Ashburner *et al.*, 1995). Storage of zygotic embryos is necessary when the collection sites are far off and exotic. It has been reported that endosperm plugs scooped from mature nuts can be stored in KCl solution for two weeks (Assy-Bah *et al.*, 1987). Sterile water could also be used as medium for storing zygotic embryos for two months and this method has been employed in germplasm collection efforts carried out by ICAR-CPCRI (Karun and Sajini, 1994). Germination of embryos was inhibited in sucrose and activated charcoal free medium (De Guzman *et al.*, 1971; Karunaratne *et al.*, 1985; Assy Bah *et al.*, 1987; Rillo and Paloma, 1990). Assy-Bah and

Engelmann (1993). stored dwarf coconut embryos for upto six months in a medium containing activated charcoal without sucrose.

The embryo culture protocol developed by ICAR-CPCRI (Karun *et al.*, 1993) (Figure 6.1) was first implemented during 1994 for six Pacific Ocean accessions maintained at the World Coconut Germplasm Center, Andaman Islands wherein 87 embryos were brought to main land. Out of 83 plantlets retrieved, 25 plantlets were field planted at International Coconut Gene Bank for South Asia (ICG-SA) Kidu, Karnataka during 1996. Later five international expeditions were conducted by ICAR-CPCRI during the period 1997- 2001 for the collection of coconut genetic diversity (Karun *et al.*, 2002). A total of 4182 embryos of 45 accessions were collected from eight countries, *viz.*, Mauritius, Madagascar, Seychelles, Maldives, Comoros, Reunion, Sri Lanka and Bangladesh. The per cent retrieval of embryos varied among the locations and among accessions. The germination percentage varied between 54 (Sri Lanka) to 82.2 per cent (Bangladesh) among expeditions. All the exotic accessions collected through embryo culture protocol developed by ICAR-CPCRI have been planted in International Coconut Gene Bank (ICG- SA) and have started flowering since 2004 from the earlier observations on *in vitro* retrieval of embryos and their *ex vitro* establishment, it is suggested that about 300 to 400 embryos are needed to be collected for field establishment of 100 palms in a gene bank. The diverse collections, after evaluation, are being utilized for breeding new varieties in coconut. Thus *in vitro* germplasm collection as well as *in vitro* and *ex vitro* establishment of plantlets in turn may contribute in adding new varieties as well as hybrids that has better adaptation to biotic as well as abiotic factors, which could be utilized in coconut breeding programmes.

2.2. Embryo Rescue

Embryo culture technique can effectively be used for obtaining plantlets from embryos resulting from incompatible crosses which naturally fail to germinate (De Guzman *et al.*, 1971; Gosal and Bajaj, 1983; Thomas and Pratt, 1981). Special types of coconuts such as 'Makapuno' (homozygous recessive embryo) can be germinated *in vitro* using these techniques, as reported by De Guzman *et al.* (1971). A commercial application of embryo culture is the rescue and culture of Makapuno variety in Philippines (De Guzman, 1970; Del Rosario and De Guzman, 1976). Embryo rescue in Dikiri coconut has been carried out at Coconut Research Institute, Sri Lanka (Vidhanaarachchi *et al.*, 1998). Embryos from sweet kernelled nuts collected from Konkan region of Maharashtra were rescued and plantlets were regenerated successfully through embryo culture protocol (CPCRI, 2011). The Horned Coconut produces multiple ovaries resulting in formation of horn like structures over the mature fruits, which delays germination from the Horned Coconut accessions collected from Andaman Islands in 1999, embryos were cultured *in vitro* using embryo culture technique. Field evaluation of these plantlets in the National Coconut Gene Bank at ICAR-CPCRI has revealed that the trait is inherited to the next generation. Moreover the field planted embryo cultured palms commenced flowering in about six to eight years (CPCRI, 2012). Embryo culture technique thus helps in both germplasm collection and to rescue embryos from special coconut varieties which fail to germinate naturally.

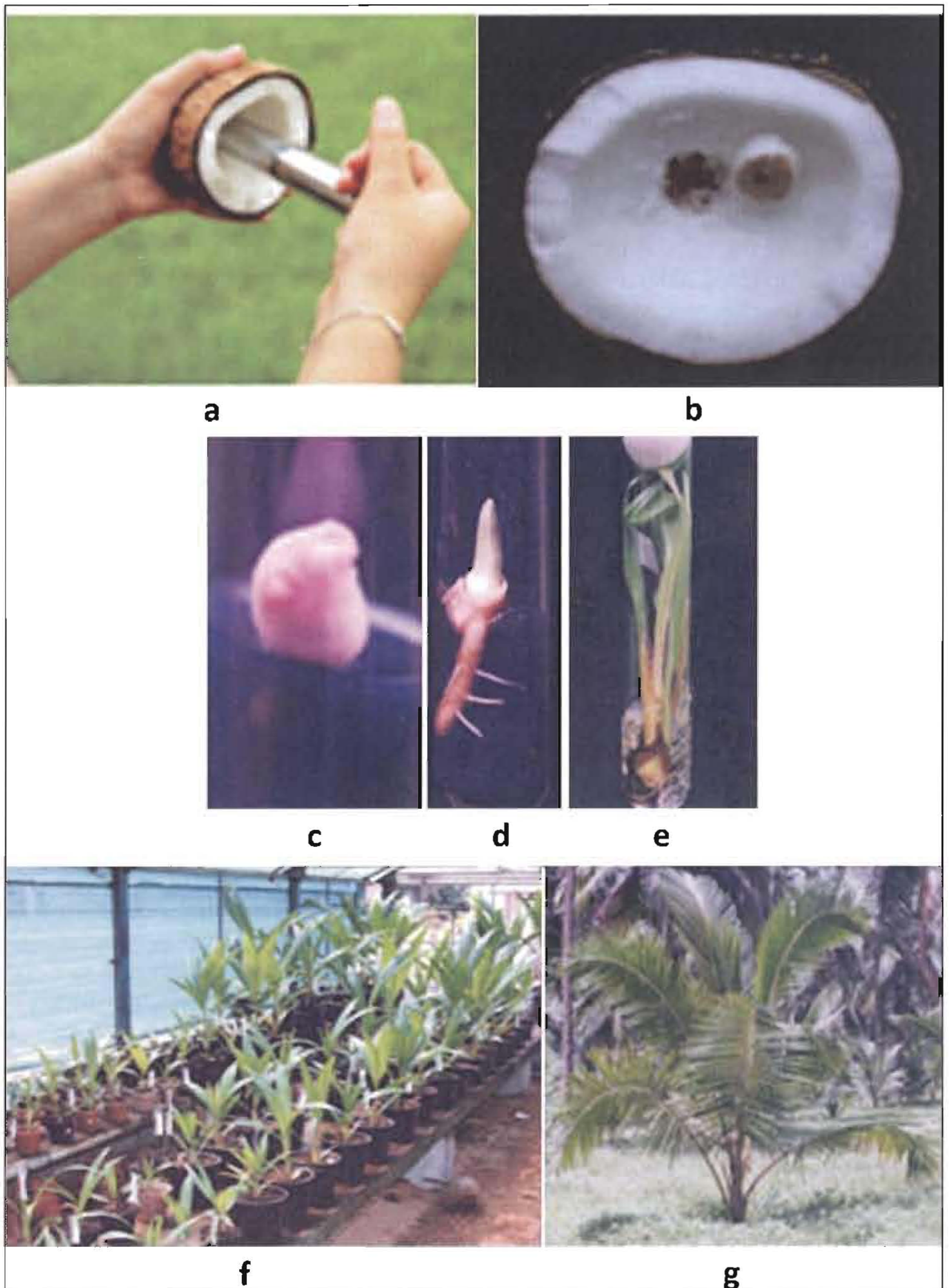

Figure 6.1: Embryo Culture.
a, b) Embryo scoping, b) Embryo, c,d,e) Embryo culture,
f) Hardening of plantlets, g) Successful establishment of plants.

3. Cryopreservation

Cryopreservation technique is widely used for long-term conservation as it is safe and enables conservation of large genetic variability. Coconut pollen and embryo cryopreservation are an excellent option for long-term conservation of genetic resources, which can provide a viable backup to field gene banks. In case of embryos, pre treatment is very much essential before conserving in liquid nitrogen at –196 °C.

Palm genetic resources are traditionally conserved *ex situ* as whole plants in field gene banks and require large area. Being a recalcitrant crop, it is sensitive to desiccation and can thus be conserved for short periods only even in the optimal moisture condition (Assy-Bah and Engelmann, 1992b). The pre-treatments include simple desiccation (using laminar air current and use of silica gel), the use of high concentration of sucrose, various cryoprotectants in various combinations (glycerol, propylene glycol, DMSO, sorbitol, formamide) and encapsulation with 3 per cent sodium alginate and dehydration techniques. These techniques are described in detail below:

3.1. Embryo Cryopreservation

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 cm) 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 DMSO and 7 per cent sucrose in MS liquid medium) blotted dry and wrapped in a single layer of sterile aluminium foil. It was then frozen by gradually lowering into liquid nitrogen and kept for five minutes. The frozen samples was 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 upto four 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). Hornung *et al.* (2001). followed encapsulation dehydration protocol for the cryopreservation of plumular tissues of coconut. The encapsulated plumules were precultured for 72-96 hours 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.

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 four 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 polyethylene glycol (PEG) 6000 at 5, 10 or 15 per cent. Thereafter the embryos were immersed

rapidly in liquid nitrogen. Thawing has been carried out by immersion of the cryotubes for 30 seconds 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 (1992b) reported cryopreservation of mature embryos of four varieties of coconut (Hybrid PB 121, Cameroon Red Dwarf, Indian Tall, Rennell Island Tall). The embryos were pretreated in the laminar air current for four 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 has been 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 hours silica gel or 24 hours 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.

Coconut plumule (apical dome with 3-4 leaf primordial) extracted from mature embryos (11-12 months after pollination) was also utilized for cryopreservation studies (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 plumules were suspended in standard medium containing 3 per cent (v/v) Na -alginate and 0.15 M sucrose. After making beads in 0.1 M calcium chloride 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, 1 M). Thereafter, the beads were dried for 6 to 24 hours 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 hours (21 per cent) and 16 hours (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. This observation corroborates with the histological data showing similarity with control cells without any treatments (Nan *et al.*, 2008). Plumule with a portion of radicle was selected as explant because of its small size and its efficiency for complete plantlet development. Experiments conducted using PVS3 for droplet vitrification for coconut plumule cryopreservation showed 20 per cent shoot growth in plumule pre-cultured for 3 days in medium containing 0.4M sucrose and subsequent dehydration for 20 min to 100 min. with PVS3 (CPCRI, 2013; Figures 6.2d, e f, g).

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 embryos cryopreserved could be returned to normal seedlings. Differential Scanning Calorimetry (DSC) 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 and 10 per cent normal seedlings after cryopreservation.

Sisunandar *et al.* (2010b) conducted morphological, cytological or molecular studies in recovered coconut plantlets after cryopreservation. The embryos from four different cultivars were subjected to rapid dehydration in a drying chamber containing activated silica gel for eight hours 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 differences between genomic DNA isolated from seedlings originating from cryopreserved embryos and respective controls. Bandupriya *et al.* (2007) studied the effect of abscisic acid (ABA) 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. In cryopreserved plumules, 84 per cent showed survival from which 39 per cent had recovered. In a later study, 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 10 days). Following encapsulation dehydration technique, the plumule 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.

Cryopreservation of mature coconut embryos through vitrification was attempted by Sajini *et al.* (2011) (Figures 6.2a,b,c). In this study, authors have investigated the effect of preculture conditions, vitrification and unloading solutions on survival and regeneration of coconut zygotic embryos after cryopreservation. The PVS 3 solution, which consists of equal proportion of glycerol and sucrose, was most effective for regeneration of cryopreserved embryos among the seven

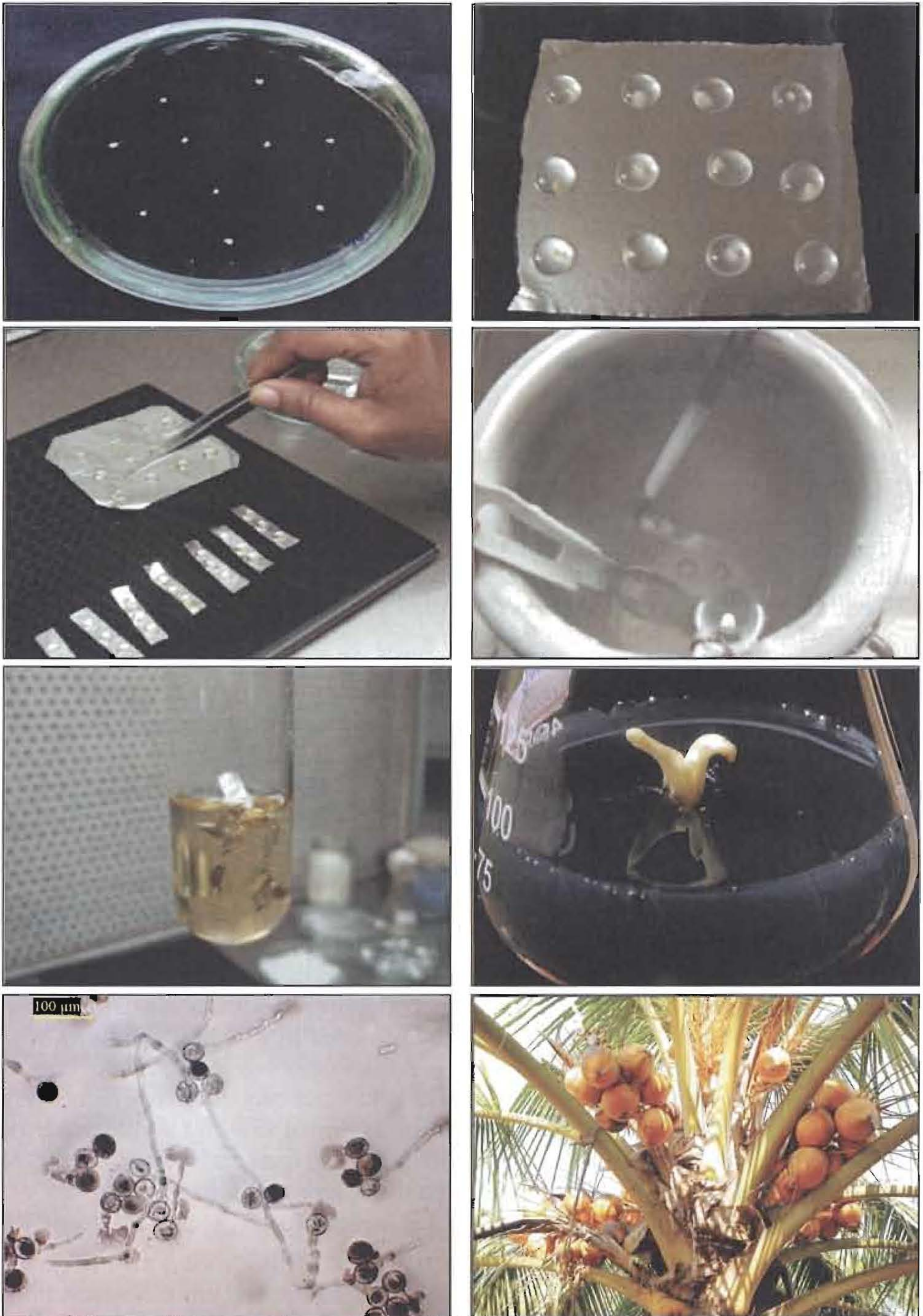

Figure 6.2: Cryopreservation of Coconut Embryo, Plumule and Pollen.
a, b c: Stages of coconut embryo cryopreservation; d, e, f, g: Droplet vitrification method for coconut plumule cryopreservation; h: Germination in cryopreserved pollen; i: Normal nut set in COD palm with WCT pollen cryopreserved for 7 years.

plant vitrification solutions tested. Most effective protocol involved preculture of embryos for three days on medium with 0.6 M sucrose, followed with PVS3 treatment for 16 h thereafter cooling rapidly in liquid nitrogen and rewarming and unloading in 1.2 M sucrose liquid medium for 1.5 h. The survival rates of 70-80 per cent (corresponding to size enlargement and weight gain) was achieved with this protocol and 20-25 per cent of the plants regenerated (showing normal shoot and root growth) from cryopreserved embryos could be established in pots.

Sisunandar *et al.* (2014) investigated the effect of maturity on the outcome of cryopreservation in four cultivars *viz.*, Nias Yellow Dwarf', 'Tebing Tinggi Dwarf', 'Takome Tall' and 'Bali Tall'. The four step cryopreservation protocol included rapid desiccation, rapid freezing, rapid thawing, and recovery and acclimatization for four months in the glasshouse. About 28 per cent of plantlet recovery was obtained with 11 month old nuts which was significantly higher when compared to younger fruits. Authors also suggested that nuts could be stored for a period of three weeks after the harvest for isolating embryos and further process.

3.2. Pollen Cryopreservation

Coconut pollen is another excellent source of diverse alleles within a gene pool. Pollen cryopreservation has been successfully used in variety of plant species (Towill and Walters, 2000). Life span of most coconut pollen is for few days (Patel, 1938). Alternative methods such as freeze drying were also reported for short term storage in coconut (Rognon and Nucé de Lamothe, 1978; Whitehead, 1963). Storage of coconut pollen in liquid nitrogen for 24 hours did not affect *in vitro* germination (Karun and Sajini, 2010). Ultra low temperature storage retards most of the molecular reactions in the cells and effectively extends the viability to hundreds of years. Karun *et al.* (2014) (Figures 6.2h, i) reported long term cryopreservation of coconut pollen. Pollen of both West Coast Tall and Chowghat Orange Dwarf cultivars retained their viability and fertility even after a storage period of four years in liquid nitrogen. The methodology of pollen cryopreservation is briefly as follows: Extraction of pollen was done by sieving (mesh size - 0.2 mm) the male flowers which were incubated at 40°C for 24 h in an oven. Pollen were wrapped in aluminium foil strips and inserted into cryovials and plunged into the liquid nitrogen. Viability of the pollen is tested both *in vitro* and *in vivo*. *In vitro* viability test include growing of pollen in artificial media and *in vivo* by hand pollination in the field. Seed set after hand pollination using cryopreserved pollen for a period of four years was found to be normal one hundred percent germination was observed in embryos extracted from hybrid nuts produced with cryostored pollen and plantlet development was normal. The study confirmed the feasibility of setting pollen cryobank in coconut.

Even though field gene bank is the preferred mode of conservation in coconut, embryo and pollen cryopreservation may also be taken up as complementary conservation strategy since under ultra low temperature, all the metabolic functions are arrested and material is in a state of suspended animation. So even though precious materials are lost, there is always chance for retrieval from cryopreserved germplasm and this may be utilized for breeding of new varieties in coconut.

4. Tissue culture

4.1. Explants and Clonal Propagation

Mass multiplication of elite coconut palms earmarked for high yield, resistance to biotic and abiotic stresses, is of huge significance in coconut farming. Farmers are in need of huge number of quality seedlings either for replanting or to expand coconut growing area. Rapid multiplication of coconut through *in vitro* techniques, therefore, is of vital importance. Progress in clonal propagation efforts in coconut is slow. Repeatable commercial level protocols have been reported in some of the palms species such as arecanut (Karun *et al.*, 2004) and oil palm (Rabechault *et al.*, 1970), utilizing explants such as young leaf, immature inflorescence and immature zygotic embryo mainly through direct and indirect somatic embryogenesis. Somatic embryogenesis is a process where somatic cells differentiate into somatic embryos which share morphological similarity with zygotic embryo. Highly recalcitrant nature of coconut to *in vitro* culture has resulted in poor *in vitro* responses irrespective of explants, cultivars or conditions. Several factors such as influence of genotype and explant maturity, adsorption of nutrients and hormones by activated charcoal making culture conditions undefined, production of compact calli, less percentage of plantlet regeneration, underperformance of regenerated plantlets and very slow rate of growth during *in vitro* culturing have cumulative effects in imparting poor in vitro responses (Fernando *et al.*, 2010). Optimization of culture media, type of explant, plant growth regulators and their concentrations, subculturing periods, other additives, have paramount significance in developing repeatable tissue culture protocol.

Prolonged incubation of immature inflorescence in auxin-cytokinin combination media resulted in conversion of floral primordial to vegetative primordial at a very low frequency (Raju, 2006). Multi cellular embryoids were produced from coconut anthers (Iyer, 1981). The major bottleneck is the development of abnormal tissues and lack of friable callus. In spite of embryogenic nature of the callus obtained from plumular tissues, formation of somatic embryos is limited. Many tissues such as leaves, inflorescence, ovary, anthers and zygotic embryos have been utilized for coconut tissue culture. Since success of *in vitro* culturing depends on the type of explant, its selection is considered as a key element for a successful outcome. Coconut leaf explants from juvenile palms were used in some studies with successful induction of callus (Pannetier and Buffard-Morel, 1982; Raju *et al.*, 1984). However the embryogenic capacity of leaf explants lasts for short dura-tion which limits its use as explant in clonal propagation studies (Karunaratne *et al.*, 1991).

Immature inflorescence is a potential source of explant to clonally propagate important crop plants. These are considered as promising explant as it contains numerous meristematic points. The success depends on the selection of inflorescence of correct maturity stage. Branton and Blake (1983) used immature inflorescence of coconut as a source of explant and observed that sections of inflorescence rachillae proliferated in medium to form callus termed as colloids. Using immature inflorescence as explants, Verdeil *et al.* (1994) achieved somatic embryo formation with a functional bipolar organization and completely differentiated shoot meristem.

Direct development of coconut shoots were reported from the rachilla explants of immature inflorescence (Raju, 2006). Basal media composition influenced the responses of immature inflorescence explants apart from its size (Vidhanaarchchi and Weerakoon, 1997).

Unfertilized ovary is another source of explants. In coconut, its efficiency for callogenesis was increased by culturing them in CRI72 medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). However plantlet regeneration was limited with a total 83 plantlets from 32 cultured ovaries (Perera *et al.*, 2007). Anthers and microspores are used as basal explants to produce haploid plantlets or double haploids. In coconut, stages of coconut inflorescence at which anthers and microspore are to be sampled have been standardized and defined. Ploidy analysis of anther-derived plantlets indicated that half of the regenerated plantlets were haploid and rest was diploid (Perera *et al.*, 2008).

In coconut, immature embryos were found to be more responsive and the response from mature embryos was improved by slicing the embryos (Adkins *et al.*, 1998; Samosir, 1999) or by selective excision of plumular tissue from zygotic embryo (Chan *et al.*, 1998; Lopez-Villalobos, 2002; Perez-Nunez *et al.*, 2006).

As far as coconut is considered, most of the recent studies on clonal propagations have used plumular tissues as source of the explants (Figure 6.3). The results from these explants are promising and more consistent. Immature embryos of coconut (6-7 months post anthesis) cultured in medium supplemented with 2,4-D produced callus with 50 per cent of them turning in to globular embryos. Around 22 per cent of germination was observed in these cultures. Age of the embryo was considered as an important factor determining callus proliferation and subsequent embryogenesis (Karunaratne and Periyapperuma, 1989). More consistent results were obtained with the use of plumules as source of explants (Chan *et al.*, 1998; Lopez-Villalobos, 2002; Fernando *et al.*, 2003; Saenz *et al.*, 2006; Pe´rez- Nu´n˜ez *et al.*, 2006; Rajesh *et al.*, 2005, 2014a). Multiplication of the embryogenic callus is important in scaling up protocols, which was achieved by Perez-Nunez *et al.* (2006)., which could be accelerated by subdividing callus and repeated subculturing. Rajesh *et al.* (2005). have outlined a procedure for regeneration of complete plantlets via organogenesis and embryogenesis from plumular tissues of West Coast Tall cultivar of coconut. Callus was induced from plumular tissues in Y3 media supplemented with either 2, 4-D (74.6 μM) alone or 2,4-D (74.6 μM) in combination with TDZ (4.54 μM). The

Figure 6.3: Plumular Culture.

frequency of callus induction increased and the browning of explants was reduced when a cytokinin (TDZ) was added along with the auxin (2,4-D) in the callus induction medium. The calli developed were subcultured at monthly intervals to media containing lower levels of 2,4-D and a constant level of either cytokinins (BA and TDZ) or polyamines (spermine and putrescine). Higher percentages of embryogenic calli, somatic embryoids and meristemoids were obtained in Y3 media supplemented with either spermine or BA. Plantlets with balanced shoot and roots were transferred to pots and established in the greenhouse. Histological studies of the differentiated tissues confirmed the development of shoot buds (organogenesis) and typical bipolar embryoids (somatic embryogenesis). Even though plantlets have been regenerated and successfully established in the field, a commercial scale protocol has not been achieved and conversion of somatic embryos into plantlets has remained one of the major bottlenecks. Various efforts were made in coconut tissue culture to refine the protocol such as use of polyamines (Rajesh *et al.*, 2014a), and media combinations. Apart from multiplication, maintenance of embryogenic callus for long time could help in providing year round embryogenic calli for further conversion. Bhavyashree *et al.* (2015) reported that coconut embryogenic callus obtained from plumular tissues could be maintained for 21 weeks without compromising on the embryogenic potential by subculturing the calli from lower 2,4-D concentration (74.6 μM/l) to higher levels (90.4 μM/l). However further refinement in the protocol is needed to increase the maintenance period.

4.2. Gene Expression Studies

Regulation of somatic embryogenesis is governed by a number of genes. Knowledge on their expression pattern would help in better understanding the complexity of somatic embryogenesis (Perez-Nunez *et al.*, 2009). Several transcription factors control the transition from vegetative to embryogenic growth. Studies indicate that members of APETALA2/Ethylene-responsive element binding protein domain family play an important role in promoting embryo development. Identification of AINTEGUMENTA like gene (*CnANT*) which codes for two AP2 domains in coconut, is considered to be significant and would be beneficial for further transformation studies to increase plantlet regeneration (Bandupriya *et al.*, 2013). Maintaining embryogenic calli for long time is challenging and is important as it can provide year round embryogenic calli for further conversion. Embryogenic potential of long term maintained calli were confirmed through gene expression studies (Bhavyashree *et al.*, 2015).

4.3. Transformation Studies

Microprojectile bombardment method was used for inserting GUS gene in to embryogeneic calli and young leaf tissues of coconut (Samosir, 1999) which was the first reported genetic transformation effort in coconut. More recently *Agrobacterium*-mediated genetic transformation studies were reported in various tissue of coconut such as immature anthers, excised zygotic embryos, plumule-derived embryogenic calli, and somatic embryogenesis-derived roots and leaves (Andrade-Torres *et al.*, 2011).

For developing a commercial level protocol with increased callogenesis and clonal propagation frequency in coconut, a multiple approach involving optimized media combination with Plant Growth Regulator (PGR), additives and systems such as temporary immersion system and bioreactors, is essential.

5. Molecular Interventions

5.1. Molecular Markers

Strength of the species to adapt to changing environments and to cope up with new pests and climatic conditions depends on the genetic diversity. Knowledge on genetic diversity of a crop is a key element in its breeding programme. Use of molecular markers has proved to be an extremely efficient method in estimation of genetic diversity of germplasm collections and population structure of various crops. In coconut, efficient use of molecular markers has helped in characterization and management of germplasm, genetic diversity studies, linkage mapping and identification of QTLs for marker-assisted selection (MAS). It was also possible to organize accessions into genetic groups and to identify redundant collections. Molecular markers utilized in coconut include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inverse Sequence-Tagged Repeat (ISTR), simple sequence repeats (SSR) and inter simple sequence repeats (ISSR). The use of these molecular methods has opened up new avenues for phylogenetic analysis and provides new tools for the efficient conservation and use of coconut genetic resources.

5.1.1. Restriction Fragment Length Polymorphism (RFLP)

Lebrun *et al.* (1998). employed RFLP technique for the first time in coconut to study the genetic diversity in 100 palms from diverse geographical locations, representing 10 Tall and seven dwarf coconut populations. Results revealed two genetically distinct groups- one included ecotypes from the Far East and from the South Pacific, and the other group comprised the ecotypes from India, Sri Lanka and Western Africa. The results were in accordance with the historical dispersion of coconut. Far East and Pacific regions, which are considered as putative area of origin of the coconut palm which was well supported by the substantial diversity obtained through RFLP studies. Moreover, tall ecotypes exhibited higher polymorphism as compared to dwarfs.

5.1.2. Randomly Amplified Polymorphic DNA (RAPD)

Variations both in alleles and locus could be detected in single assay using RAPD technique. Polymorphism could be detected using this method as it deals with deletions, insertions and point mutations which influence the base sequence of primer binding sites (Williams *et al.*, 1990; Nirmal Babu *et al.*, 2014). Seventeen distinct South Pacific populations exhibited a moderate level of genetic diversity when analyzed by means of RAPD technique using 14 primers (Ashburner *et al.*, 1997). Variability within the populations was over 60 per cent. The low inter-population diversity observed in coconut populations obtained in the study was

attributed to genetic drift and a possible bottleneck in the past of the species A few RAPD mark-ers unique to specific populations were also identified in that study. RAPD technique was utilized to access the genetic diversity of coconut populations from Sri Lanka, the Philippines and Brazil as reported by Everard *et al.* (1996)., Rodriguez *et al.* (1997). and Wadt *et al.* (1999)., respectively. Genetic diversity of four dwarf populations from East Java was analyzed using RAPD (Hayati *et al.*, 2000). They found that variability of coconut population grown outside East Java was higher than that at East Java since those coconut population collected from seeds of open pollinated plants.

Studies were carried to identify RAPD markers associated with resistance to specific diseases. Markers potentially linked to lethal yellowing disease were detected through RAPD technique using three coconut populations *viz.*, susceptible West African Tall, resistant Malayan Yellow Dwarf and a resistant population of Atlantic Tall palms (Cardena *et al.*, 2003). Genetic diversity of 15 Indian and five exotic accessions of coconut were tested using eight polymorphic primers by RAPD method. Results revealed that tall accessions were more heterozygous than dwarf accessions with higher proportions of polymorphic bands and higher genetic diversity. Likewise, exotic accessions displayed higher variation. Dwarf accessions from geographically distant regions clustered together (Upadhyay *et al.*, 2004).

Genetic diversity studies with in yellow dwarf populations *viz.*, Malayan Yellow Dwarf (MYD), Kulashekaram Yellow Dwarf (KYD) and Andaman Yellow Dwarf (AYD) using 16 highly polymorphic RAPD primers indicated the presence of greater diversity within accessions (Ritto *et al.*, 2008). RAPD technique was also employed to differentiate tall, dwarf and dwarf x tall hybrids. The RAPD primer OPBA3 clearly differentiated both the tall and dwarf bulks. This was validated in individual tall and dwarf coconut palms representing different geographic regions. This RAPD primer was also used to screen the parents and validate hybrids of Dwarf x Tall crosses (Rajesh *et al.*, 2014b). Purity of the dwarf x tall hybrids were tested using RAPD-derived sequence characterized amplified region (SCAR) markers (Rajesh *et al.*, 2013).

Figure 6.4: Hybrid Authentication using SSR Markers
M: 100 bp ladder; D: CGD parent; T: WCT parent; H: Hybrid; O: Off-type.
Arrowhead indicates off-types. (Rajesh *et al.*, 2014).

5.1.3. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a high-multiplex PCR-based method for DNA profiling (Vos *et al.*, 1995) with the potential to generate a

large number of polymorphic genetic loci and involved both restriction digestion and PCR. In detection of polymorphism, AFLP technique was found to be more efficient as compared to DAF. Out of 300 primers screened, 28 (9.33 per cent) detected polymorphism in DAF whereas in AFLP 55 (86 per cent) primer combinations generated polymorphic bands (6.42). Cluster analysis revealed clustering of dwarf ecotypes into a single group and the tall ecotypes into three groups (Nagaraju *et al.*, 2003).

AFLP analysis of 42 genotypes indigenous to Sri Lanka was carried out using eight primer pairs. Results revealed that more variation was detected in tall forms (*typica*), rather than intermediate (*aurantica*) and dwarf (*nana*) forms. *Aurantiaca* group was more similar to the dwarf rather than the tall group. In addition, putative duplicate accessions were identified in the *Aurantiaca* group (Perera *et al.*, 1998). Teulat *et al.* (2000) used AFLP markers in combination with SSR markers to analyze genetic diversity of 31 palms from 14 coconut populations from different ecological regions.

5.1.4. Inverse Sequence-Tagged Repeat (ISTR)

Inverse sequence-tagged repeat (ISTR) analysis is a PCR based approach for the detection of DNA polymorphisms. The primers complementary to repetitive, copia-like sequences in the coconut genome (Rohde *et al.*, 1992) were used to amplify a large number of genetic loci with an abundance of polymorphisms occurring among a set of selected coconut genotypes from various regions of the world. Duran *et al.* (1997) extended this technique to analyze East African Tall coconut populations. It was concluded that these molecular marker types represent powerful tool for genotype identification, analysis of germplasm variability and breeding purposes in coconut. These studies also provided evidence for the existence of truncated, copia-like repetitive sequences in the coconut genome indicat-ing that retro-elements may have played a role in the genera-tion of genetic diversity in coconut.

5.1.5. Inter Simple Sequence Repeats (ISSR)

This technique make use of microsatellites, generally 15-25 bp long, to amplify inter SSR sequences of different sizes in multiple genomic loci (Zietkiewicz *et al.*, 1994). Thirty-three coconut accessions representing different geographical regions of the world, maintained at the International Gene Bank in India, were analyzed using 19 ISSR primers (Manimekalai and Nagarajan, 2006). A total of 199 ISSR markers were generated, out of which 154 were polymorphic. Least similarity was found between Nicobar Tall and Chowghat Orange Dwarf, both accessions from India. Coconut accessions from Southeast Asia, South Asia and South Pacific formed separate groups, which was generally in accordance with their origin and dispersal. The use of polymorphic microsatellites for assessing genetic diversity in coconut has been gaining popularity because of their high information content and co-dominant nature.

5.1.6. Simple Sequence Repeats (SSR)

Allelic variations could be detected effectively using SSR method one pair of SSR primer deals with one locus and this technique make use of repeat numbers with

in locus (Weising *et al.*, 1992). Rivera *et al.* (1999). characterized 40 coconut samples from the Philippines using eight SSR primer pairs. Dwarfs grouped separately from talls and showed less genetic diversity. Using a pre-cloning enrichment procedure, Perera *et al.* (1999). isolated eight coconut microsatellites, which were used to study the levels and patterns of genetic diversity of Sri Lankan coconut populations. The results showed that the Sri Lankan tall coconuts exhibit higher levels of diversity than the dwarfs and intermediates, and the intermediate coconuts are more similar to dwarfs than talls. This was in agreement with the results obtained using AFLPs in the same set of genotypes in an earlier study (Perera *et al.*, 1998). Perera *et al.* (2000) used eight pairs of SSR primers to analyze the genetic diversity in 130 individuals of coconut comprising 75 tall and 55 dwarf individuals representing 94 ecotypes from different geographical regions. A phenetic tree based on genetic distance clustered individuals into five groups, each mainly composed of either talls or dwarfs. Thirty-three tall coconut populations from Sri Lanka were subjected to microsatellite assay with eight SSR primer pairs in order to study the levels and distribution of genetic variation for formulating future collection strategies and selecting parents for breeding programmes (Perera *et al.*, 2001). A high level of genetic diversity was detected in all the populations. A coconut microsatellite kit was developed by CIRAD in collaboration with COGENT and it consists of 14 microsatellite markers with sufficient discriminating power for practical identification of coconut cultivars (Baudouin and Lebrun, 2002). Standard protocols, without the use of radioactive probes, as well as dedicated statistical software, Gene Class 2, were developed which could be adapted to use in developing countries. Merrow *et al.* (2003). utilized 15 simple sequence repeat (SSR) microsatellite DNA loci to analyze genetic variation within coconut germplasm collections maintained at two locations in South Florida, representing eight cultivars. Parentage analysis of 'Fiji Dwarf' cultivar was also carried out using these loci. The Red Malayan Dwarfs were found to be genetically distinct from Green and Yellow ones also, genetic identity of 'Red Spicata' was found to be more to "Fiji Dwarf'.

Devakumar *et al.* (2006). carried out an assessment of genetic diversity of 21 Indian and 24 exotic coconut accessions using eight SSR primers (Figure 6.5). The eight coconut microsatellite loci distinguished a total of 48 alleles, with an average of 6 alleles per locus. Genetic diversity values were low for most dwarfs and high for the tall accessions, which is in accordance with their breeding behaviour. However, an indigenous dwarf, Kulasekharam Orange Dwarf, showed genetic diversity higher than many talls. Within population variation (58 per cent) was found to be higher than among population variation (42 per cent). Microsatellite analysis of lethal yellowing disease tolerant genotypes (Vanuatu Tall and Sri Lankan Green Dwarf) and susceptible genotype (West African Tall) was carried out by Konan *et al.* (2007). A total of 58 alleles were detected by the 12 microsatellite loci analyzed. Genotypes of susceptible West African Tall cultivar were found to be less genetically clustered to the genotypes of the two tolerant cultivars. The fingerprinting based on microsatellites aided in identification of suitable parents to be used in crossing programmes for developing a segregating mapping population for marker-assisted selection of lethal yellowing resistant genes. The Maypan, a hybrid of Malayan Yellow Dwarf (MYD) and Panama Tall coconut, earlier considered highly resistant,

is presently being devastated by an outbreak of lethal yellowing disease in Jamaica. Lebrun *et al.* (2007) used 34 SSR markers to compare the MYD sampled from four locations in Jamaica along with a reference DNA of MYD collected from five different countries *viz.*, Ghana, Malaysia, Philippines, Mexico and India to check whether the affected planting material in Jamaica was genetically similar to the material earlier shown to be resistant to lethal yellowing disease. The results revealed more variation at 34 simple sequence repeat loci in MYD samples from Jamaica than from the rest of the world. About 16 per cent of alleles in Jamaican MYD samples did not match the usual typical MYD genotypes showing that the Jamaican MYD palms were only partially true to type and this heterogeneity may have some undesirable effect on its degree of resistance to lethal yellowing disease.

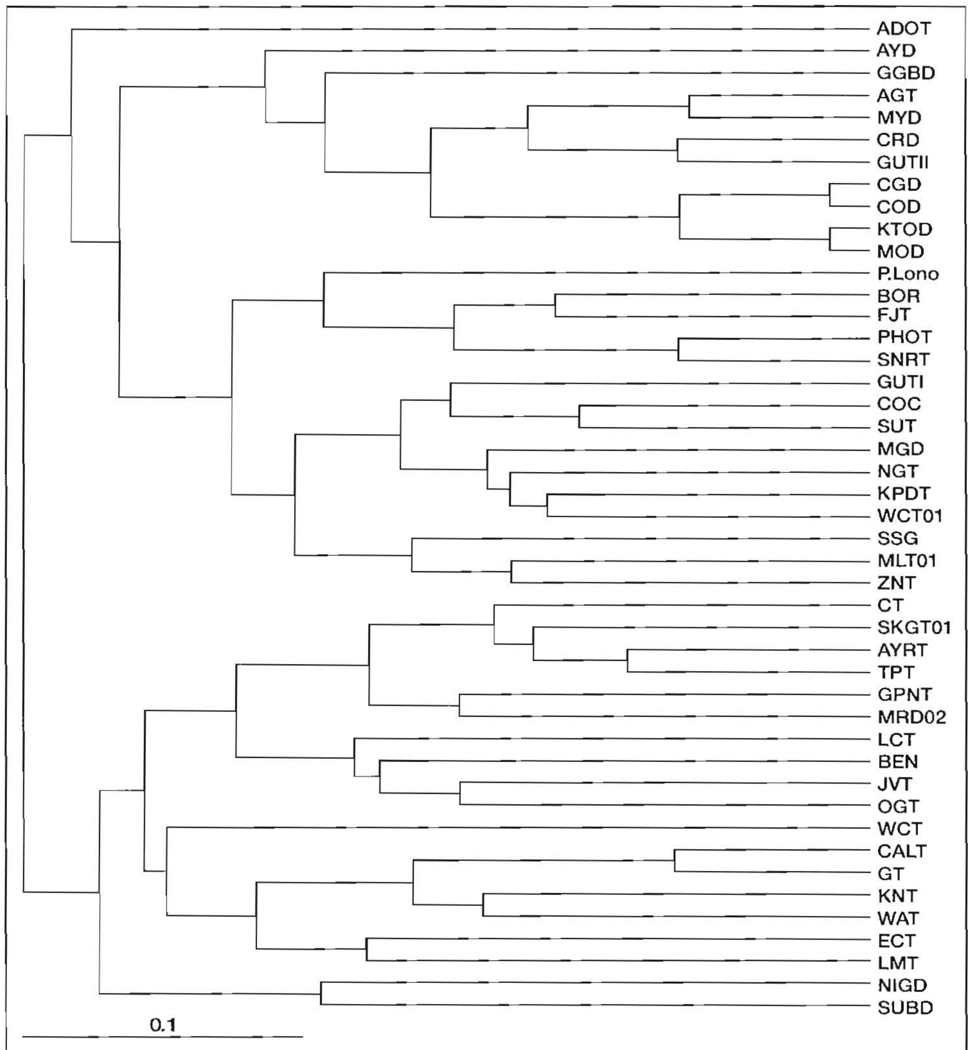

Figure 6.5: Diversity in Coconut Accessions using Microsatellite Markers.

The extent of genetic diversity in 26 coconut accessions from the Andaman and Nicobar Islands, India was determined using 14 microsatellite markers by Rajesh *et al.* (2008a).. A total of 103 alleles were detected by the microsatellite markers with an average of 7.35 alleles per locus. The average observed and expected heterozygosity was 0.29 and 0.66 respectively. A mean fixation index (*F*ST) of 0.49 was observed, indicating a high level of population differentiation among the coconut accessions. Majority of rare alleles were observed in tall accessions from the Nicobar Islands. This study using microsatellites confirms the rich genetic diversity of coconut accessions from these Islands. An important step in genetic analysis is to produce genetic linkage maps. Such maps represent the relative order of genetic markers, and their relative genetic distances from one another, along each chromosome of an organism. Rajesh *et al.* (2008b). using 14 simple sequence repeat (SSR) markers studied the pattern of diversity in 102 coconut palms representing 10 landraces from 3 coconut-growing communities of India. A total of 90 alleles were identified with an average of 6.42 alleles per locus and an average polymorphism information content of 0.61. The heterozygosity was highest for the two tall landraces, while it was lowest for the dwarf coconut landraces. SSR markers were used to characterize two ecotypes of coconut from Kerala, India namely Annur and Bedakam. Clustering analysis shows their distinct nature as compared to local West Coast Tall (WCT) populations. However Annur ecotype was comparatively closer to WCT (Rajesh *et al.*, 2014c).

Rajesh *et al.* (2014d). studied the genetic and phylogenetic relationships of coconut populations selecting Laccadive Ordinary Tall (LCT) and Laccadive Micro Tall (LMT) from Amini and Kadamat Islands from Lakshadweep, India using 20 highly polymorphic SSR markers. The variation was observed to be large among the distinct types of these cultivars. For example, the elliptical type from Amini emerged as distinct type and was found to be related to round type from Amini. The pear shaped nuts from both islands showed affinity and seems to have formed as a result of introgression between elliptical and round types. The round form of LMT from Kadamat Island was found to be distinct according to SSR studies. SSR markers have also been utilized for confirming the hybridity of D × T hybrids which will ensure supply of genuine hybrids to farmers (Rajesh *et al.*, 2012; Figure 6.4).

5.1.7. Start Codon Targeted Polymorphism (SCoT)

This method is based on detection of short conserved region flanking the ATG translation start codon in plant genes. This novel marker system was described first by Collard and Mackill (2009). Genetic diversity in 23 coconut accessions (10 talls and 13 dwarfs), representing different geographical regions were tested using SCoT marker technique. Results indicate the potentiality of SCoT markers in detection of DNA polymorphism in coconut accessions (Rajesh *et al.*, 2015a)

5.2. Linkage Map Construction

The AFLP, ISSR, ISTR and RAPD marker techniques were employed to construct linkage map in coconut for the two parents of the cross Malayan Yellow Dwarf x Laguna Tall. A total of 382 markers generated 16 linkage groups for each parent. QTLs for early germination were identified. These QTLs correlate with

early germination and yield, representing characters, which are important in coconut breeding (Herran *et al.*, 2000). Lebrun *et al.* (2001). made use of AFLP and SSR markers to construct a linkage map in the coconut type Rennell Island Tall. A total of 227 markers were arranged into 16 linkage groups. QTLs were detected for yield characters *viz.*, number of bunches and number of nuts. Baudouin *et al.* (2006). investigated the genetic factors, which controlled fruit components in coconut. QTL analyses were performed for fruit component weights and ratios in a segregating progeny of a Rennell Island Tall genotype. Out of the 52 putative QTLs identified for the 11 traits studied, 34 grouped in six small clusters. Interestingly, the QTLs for fruit component weight, endosperm humidity and fruit production were found at different locations in the genome, which suggested the need for selection of QTLs for individual traits for efficient marker-assisted selection for yield.

5.3. Transcriptome Analysis

Advancement in biotechnology front made available transcriptomic analysis technique to researchers. The RNA-sequencing (RNA-seq) technologies, makes use of Next Generation Sequencing platforms, were first used to study plants only a few years ago (Weber *et al.*, 2007). Fan *et al.* (2013) carried out RNA-Seq analysis to gain global overview of transcriptome of coconut using various tissues. Comparative analysis of sequences between assembled unigenes and released cDNA sequences of coconut and oil palm shows that assembled sequences were of high quality. As compared to the released EST sequences of coconut, overall 99.9 per cent of unigenes were found to be novel. Through transcriptome analysis large amount of genetic information of coconut could be gained which is considered as a valuable resource for further molecular genetic studies and breeding in coconut. Rajesh *et al.* (2015b) reported transcriptome analysis (RNA-Seq) on an Illumina HiSeq 2000 platform of coconut plumule derived embryogenic calli. A total of 40,367 transcripts were obtained after *de novo* transcriptome assembly and functional annotation which showed significant BLASTx matches with similarity greater than 40 per cent and E value of $\leq 10^{-5}$. Fourteen genes involved in coconut somatic embryogenesis were identified. Gene expression of these genes during several stages of embryogenesis was studied using quantitative real time PCR technique which gives insight to gene expression pattern. Transcriptome analysis of coconut leaf tissue was undertaken by Rajesh *et al.* (2015c)., for identifying resistant gene analogues (RGA). Leaf transcriptome was generated using RNA sequence obtained from Chowghat Green Dwarf cultivar which is resistant to root (wilt) disease. A total of 243 RGA classified under six classes were identified using comprehensive bioinformatics analysis. In the past owing to lack of sequence information molecular biology techniques have been used scarcely in assessment of genetic resources and for improvement of important agronomic and quality traits in coconut. Transcriptome based study in coconut was carried out by Nejat *et al.* (2015) in relation to phytoplasma infection. The analysis indicates that due to phytoplasma infection the number of down-regulated genes exceeded the number of genes up-regulated. Following infection, out of 39,873 differentially expressed unigenes, 21,860 unigenes were suppressed whereas 18,013 were induced. As compared to healthy leaves, in phytoplasma infected leaves, significant over expression of genes associated with plant defense

in response to biotic stimuli were noticed. Bandupriya and Dunwell (2015) carried out transcriptome analysis through 454 pyrosequencing for discovering candidate genes involved in embryogenesis in coconut. The EST data generated from different tissue types could be used as a resource bank for genome wide studies and gene discovery in coconut. In coconut transcriptome analysis, which make use of latest technologies, will result in huge genetic information which can be utilized in breeding resistant varieties to various stresses.

6. Conclusions

New avenues have opened up with the advent of biotechnology with their possible use in crop improvement programmes. Embryo culture, embryo rescue and cryopreservation techniques have helped in collection and preservation of germplasm. Scaling up of quality planting material production through clonal propagation method seems to be the solution to fulfill growing demand. However a commercial level protocol with increased callogenesis and clonal propagation frequency in coconut needs a multiple approach involving optimized media combination with plant growth regulators, additives and systems such as temporary immersion system. The important advances in biotechnological techniques, which have been made particularly during the last decade, such as identification of molecular markers associated with polymorphism and potentially linked to specific traits could immensely benefit the coconut breeder in practice and in turn in crop improvement.

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