

Coconut Tissue Culture: The Indian Initiatives, Experiences and Achievements

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Abstract

Coconut is one of the principal crops of India cultivated in over 35 districts mainly in the southern states. The productivity of the crop is declining in many of the traditionally cultivated regions owing to ageing plantations as well as biotic and abiotic stresses. These plantations are to be replanted with high yielding varieties/hybrids for which adequate quantity of quality planting material is not available. Even though tissue culture research was initiated in many laboratories in the country, the work was eventually phased out in most of the laboratories for want of a repeatable protocol. At ICAR-CPCRI, coconut tissue culture programs have been continuing for the past three decades. The attempts made include experimentation with different explants *viz.*, immature inflorescence, plumular tissues, mature palm shoot meristem, ovary and anthers and different culture media supplemented with varying levels and types of hormones. Some of the successful protocols developed at the Institute include coconut zygotic embryo culture for collection and exchange of germplasm, cryopreservation and retrieval of zygotic embryos and pollen and plantlet regeneration from plumular tissues. Even though ICAR-CPCRI has succeeded in obtaining plantlets *via* direct organogenesis from inflorescence explants, the absence of friable calli formation from explants, the low rate of somatic embryo formation, large number of cultures turning to abnormal shoot development, non conversion of somatic embryos into plantlets, and formation of abnormal somatic embryos remain the major bottlenecks. Gene expression studies are being currently undertaken to decipher the molecular basis of *in vitro* recalcitrance.

Keywords: Coconut, cryopreservation, embryo rescue, immature inflorescence, plumule

Introduction

India is one of the leading countries in the world in terms of coconut production and productivity. The annual coconut production in India is 22167 million from 20.88 lakh hectare area and the productivity per hectare is 10614 nuts (<http://coconutboard.nic.in/>). Lives of millions of Indians are woven around coconut palm, which is revered as ‘*Kalpa Vriksha*’, which in Sanskrit language denotes a ‘tree which provides everything to humanity’. The requirement for quality coconut seedlings to replace diseased and senile palms in India is unable to meet the annual demand (Karun *et al.*, 2015). The total production of coconut seedlings from governmental sector works out to around 6 million seedlings where as the demand is for around 24 million seedlings; the production from governmental sector accounts for only 25 % of the total requirement Therefore, there is an urgent need to bridge this demand-supply gap.

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Collection and conservation of plant genetic resources play a pivotal role in the improvement of crop genetic diversity especially for development of high yielding varieties resistant to diseases and pests. In the case of coconut, collection and exchange of germplasm as seed nuts is difficult and uneconomical due to short dormancy (recalcitrant) and bulkiness of the seed. Moreover phytosanitary restrictions too severely limit the germplasm introduction. Embryo culture technique in coconut provides an easy and safe alternative for the movement of coconut germplasm and is emphasized in the technical guidelines of FAO/IPGRI (Diekmann, 1997). Embryo culture protocol in coconut can benefit in collection and exchange of coconut germplasm and embryo rescue in cases where natural germination is inhibited or delayed.

Botanically, coconut palms possess a single apical meristem and since the palm has no branches or suckering habit, propagation is restricted and only through seed nuts. The only alternative for rapid multiplication of coconut palms is via tissue culture. Unfortunately, advancement in coconut tissue culture has been rather sluggish due to its recalcitrant nature, which is apparent from our decade's long experiences in this area. Some of the indispensable pre-requisites to accomplish a commercially viable protocol in coconut are (i) optimization of the most responsive explants (both type and stage), (ii) composition of the tissue culture media and (iii) supplementation with plant growth regulators. Much of the leads in coconut tissue culture have been obtained using plumular tissues as explants. Though promising, there is a need for focused research towards developing a repeatable and commercial-scale protocol from the plumular explants.

Coconut genetic resources are traditionally conserved *ex situ* as whole plants in field gene banks and require large area. The field gene banks are always at the threat of natural calamities, diseases and pests. Coconut, being a recalcitrant crop, is sensitive to desiccation and can thus be conserved for short periods only in the optimal moisture condition. But coconut embryos and pollen are desiccation and freezing

tolerant to a certain extent, which can be exploited for its utility in long term conservation of coconut genetic resources through cryopreservation. Research carried out on all these aspects at ICAR-CPCRI are briefly highlighted below.

Embryo culture

Germplasm collection

An efficient and successful procedure has been worked out and validated at ICAR-CPCRI for culturing coconut zygotic embryos; the procedure has been effectively employed in numerous germplasm expeditions (Karun *et al.*, 1992, 1999, 2004). The modus operandi utilizes artificial media and microclimatic conditions which sustain the growth of embryo and their development into complete plantlets. A field collection technique for zygotic embryos has been outlined by Karun *et al.* (1993) to aid collecting of germplasm during expeditions from regions with no access to laboratories. A simple technique utilizing sterile water as a medium for storing zygotic embryos for up to two months has been developed and successfully employed in germplasm collection initiatives by researchers at ICAR-CPCRI (Karun and Sajini, 1994).

The ICAR-CPCRI embryo culture protocol (Karun *et al.*, 1993) was first implemented in 1994 for collecting six accessions from Pacific Ocean from the World Coconut Germplasm Center, Andaman and Nicobar Islands, India. From 87 embryos collected and brought to the main land, 83 plantlets could be retrieved out of which 25 plantlets were field planted at International Coconut Gene Bank for South Asia (ICG-SA), Kidu, Karnataka State, India, in 1996. Later on, the protocol was successfully utilized in five international germplasm collecting missions conducted by ICAR-CPCRI during the period 1997-2001, mainly for enhancing coconut genetic diversity in India (Karun *et al.*, 2002). A total of 4182 embryos, comprising 45 accessions, were collected from eight countries, *viz.*, Mauritius, Madagascar, Seychelles, Maldives, Comoros, Reunion, Sri Lanka and Bangladesh. Variations were recorded in

frequency of embryos retrieval among the islands from where the germplasm collections were made and also, among different accessions with the germination percentage varying between 54 (Sri Lanka) to 82.2 % (Bangladesh) among collections. The exotic collections, retrieved through the ICAR-CPCRI embryo culture protocol, have been planted in ICG- SA, with the first flowering of palms recorded in 2004. About 300 to 400 embryos are needed to be collected for field establishment of 100 palms in a field gene bank based on the observations recoded from the above studies, especially on the *in vitro* retrieval of embryos and their *ex vitro* establishment. Some of the promising exotic collections have been incorporated into coconut breeding programs, after performance evaluation.

Embryo rescue

Embryo rescue is an effective technique for obtaining plantlets *in vitro* from embryos which either fail to germinate in nature or exhibit delayed germination. Mohachao Narel is a coconut variant reported from Guhagar taluk of Ratnagiri district of Maharashtra State in India, which is characterized by sweet and soft kernel and less fibre content. Embryo weight of sweet endosperm nuts was recorded to be significantly lower than nuts possessing normal endosperm (Samsudeen *et al.*, 2013), which hinders its germination under natural conditions. Embryos from sweet kernelled nuts were ‘rescued’ via embryo rescue and plantlets could be regenerated successfully through ICAR-CPCRI embryo culture protocol (CPCRI, 2011; **Plate 1 a, b**) and field planted at AICRP Centre, Ratnagiri, Maharashtra, India.

Another variant, Horned Coconut, observed from Andaman Islands, is characterized by multiple ovaries which results in the formation of horn like structures over the mature nuts; this arrangement delays natural germination of nuts. From the Horned Coconut nuts collected from Andaman Islands, embryos were cultured *in vitro* using ICAR-CPCRI embryo culture technique. The embryo cultured palms, planted in the field, commenced flowering in about six to eight years (CPCRI,

2012). Thus embryo culture technique has been successfully utilized in India in both germplasm collecting expeditions and to rescue embryos from variants which do not possess the capacity or exhibit delay to germinate in nature.

Cryopreservation

Coconut genetic resources are conserved conventionally *ex situ* as whole palms in field gene banks, which requires large area and is expensive. Coconut, being recalcitrant, is sensitive to desiccation which hinders its conservation for longer periods; even under optimal moisture condition. Cryopreservation is a long-term conservation strategy which facilitates safe and efficient conservation of large genetic variability and which can overcome the limitations of conventional conservation. Cryopreservation of coconut zygotic embryos and pollen serves as excellent alternatives for long-term conservation of valuable coconut genetic resources. In addition, the technique can provide a viable safety backup to coconut genetic resources in field gene banks.

Cryopreservation of zygotic embryos

With respect to cryopreservation of mature zygotic embryos, pre treatment of the embryos was found to be essential before conserving in liquid nitrogen at -196°C . 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% sodium alginate and dehydration techniques. The achievements in cryopreservation are described below:

Karun *et al.* (2005) reported cryopreservation of mature embryos of West Coast Tall cultivar after desiccation pretreatments. Maximum retrieval of healthy plantlets was achieved from zygotic embryos which were subjected to either 18 hours silica gel or 24 hours laminar air flow desiccation treatment (**Plate 1 c, d**). When the moisture content of the embryo was reduced to below 20%, irreversible damage of shoot meristem was noticed.

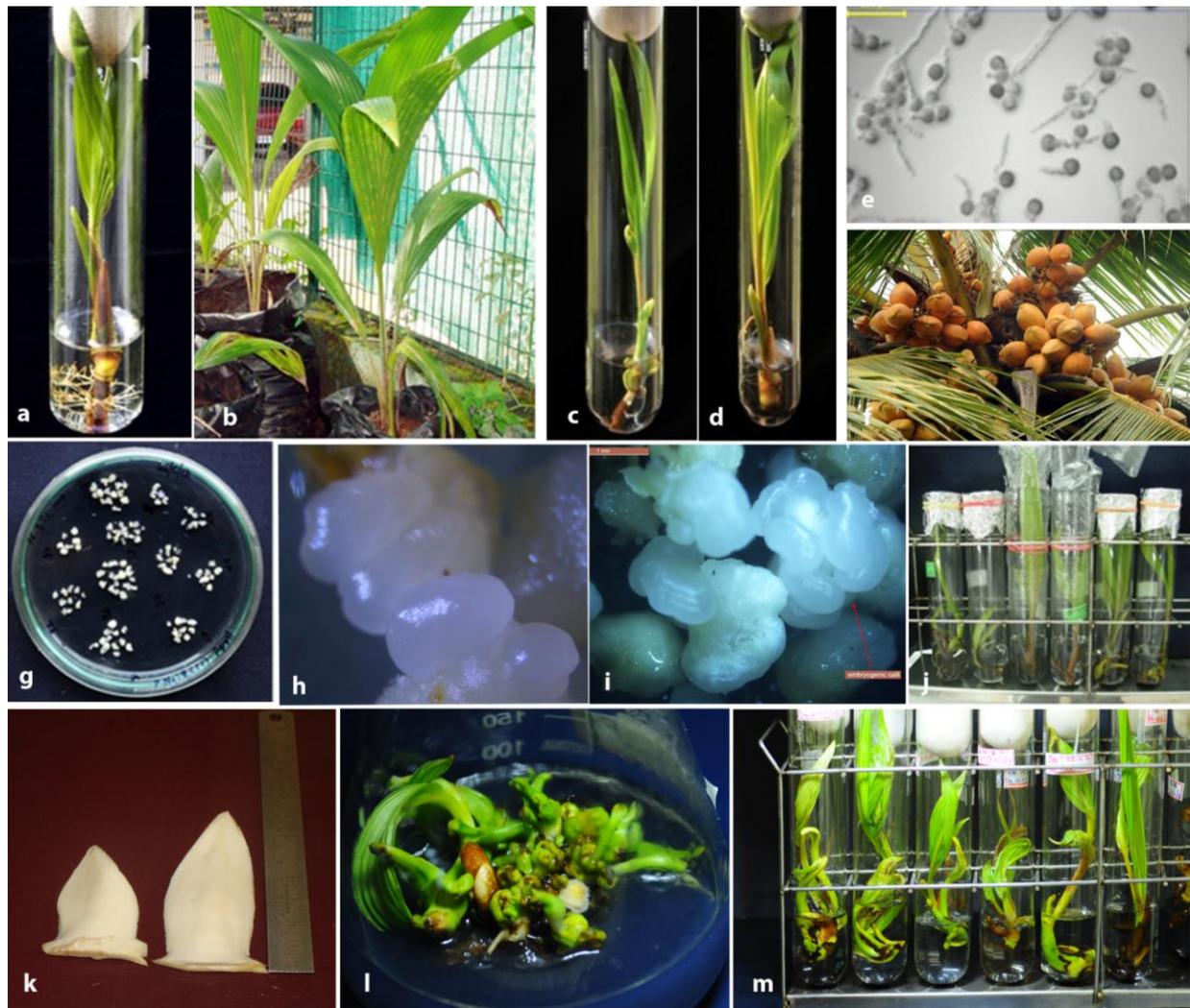


Plate 1. Embryo rescue, cryopreservation of zygotic embryo and pollen and clonal propagation using plumule and immature inflorescence as explants in coconut.

a & b: sweet coconut plantlets raised *in vitro* after rescuing zygotic embryo;

c & d: regenerated plantlets from cryopreserved zygotic embryos of Benaulim Tall and Chowghat Orange Dwarf cultivars;

e: *in vitro* germination of cryopreserved pollen in artificial media;

f: normal nut set obtained using six years old cryopreserved pollen;

g, h, i & j: callus, embryogenic callus, somatic embryo and plantlet regeneration in coconut plumule culture;

k, l & m: immature inflorescence used for culturing, formation of shoots and plantlet derived from immature inflorescence culture in liquid medium.

Pre growth desiccation method using 2 M and 3 M sucrose the moisture content of the embryos was reduced to 30% and 27% with a corresponding final recovery of plantlets after cryopreservation of 20.8% and 29% respectively (Sajini *et al.*, 2006).

Sajini *et al.* (2011) carried out detailed studies on cryopreservation of mature coconut zygotic embryos using vitrification technique. The effect of preculture conditions, vitrification and unloading solutions on both the survival and regeneration of the embryos after cryopreservation were critically examined in the study. The results of the studies revealed that among the seven plant vitrification solutions tested, PVS3 solution, consisting of equal proportions of glycerol and sucrose, was most effective for regeneration of cryopreserved embryos. The best protocol standardized consisted of preculture of embryos for three days on medium with 0.6 M sucrose, followed by PVS3 treatment for 16 hours, cooling rapidly in liquid nitrogen and rewarming and finally unloading in 1.2 M sucrose liquid medium for an hour and a half. The survival rates of 70-80% (corresponding to size enlargement and weight gain) could be achieved with this protocol and 20-25% of the plants regenerated (showing normal shoot and root growth) from cryopreserved embryos could be successfully established in pots.

Cryopreservation of pollen

Studies at ICAR-CPCRI have shown that storage of coconut pollen in liquid nitrogen for 24 hours did not affect *in vitro* germination (Karun *et al.*, 2006; Karun and Sajini, 2010). In a later study, Karun *et al.* (2014) reported long term cryopreservation of coconut pollen of West Coast Tall and Chowghat Orange Dwarf cultivars. Viability and fertility of the pollen of both cultivars were retained even after storage for six years in liquid nitrogen (Plate 1 e, f).

The methodology of pollen cryopreservation consists of the following steps: (i) extraction of coconut pollen by sieving (sieve of mesh size - 0.2 mm) male flowers incubated at 40°C for 24 hours in an oven, (ii) wrapping pollen in strips of aluminium foil, (iii) inserting

these into cryovials and (iv) plunging into the liquid nitrogen. Viability of the pollen is tested both *in vitro* (observing growth of pollen in artificial media) and *in vivo* (hand pollination in the field). Normal nut set was recorded after hand pollination using pollen which had been cryopreserved for a period of six years. One hundred percent germination was observed in embryos extracted from hybrid nuts produced with cryostored pollen and normal plantlet development was also recorded. The study confirmed the feasibility of setting pollen cryobank in coconut. Programs have already been initiated at ICAR-CPCRI to conserve coconut gene pool in the form of pollen at National Cryogene Bank at ICAR-NBPGR, New Delhi, India.

Utilization of cryostored pollen for resistance breeding program

Farmers in the coconut root (wilt) disease affected tract of Kerala State, India prefer dwarfs/ hybrids for replanting due to their precocity, short stature and a higher level of resistance/tolerance to the disease. ICAR-CPCRI has evaluated 36 hybrid combinations for resistance to root (wilt) disease during the past 40 years. CGD X WCT hybrid produced by hybridizing Chowghat Green Dwarf with pollen collected from disease-free West Coast Tall palms located in the 'disease hotspots' has performed excellently at ICAR-CPCRI, Kayamkulam and in demonstration trials carried out in farmers' plots. CGD X WCT hybrid was notified and released as 'Kalpa Sankara', which is tolerant to root (wilt) disease, during March 2012 by the Central Varietal Release Committee. Kalpa Sankara is the first coconut hybrid released for cultivation in the root (wilt) disease prevalent tract.

The demand for seedlings of Kalpa Sankara (CGD X WCT) hybrid is very high. The two key components required for production of this hybrid is the availability of Chowghat Green Dwarf mother palms and disease-free West Coast Tall male parental palms. Chowghat Green Dwarf mother palms are available in farmer's plots and such palms can be used for production of D X T hybrids. Since these palms are located

in far off places in the root (wilt) disease affected tracts, large- scale production of D X T hybrids cannot be achieved through a centralized system. Hence a decentralized method of D X T hybrid production is adopted at ICAR-CPCRI, Regional Station, Kayamkulam, India wherein 25-30 CGD mother palms (identified in one *Panchayat/ Krishi Bhavan*) is artificially pollinated under the supervision of a progressive farmer. ICAR-CPCRI will identify the male WCT parental palms and ascertain their disease-free status through ELISA test. Male flower processing is being done at ICAR-CPCRI and the pollen collected is transferred to cryo vials and stored in deep freezer/ liquid nitrogen. The stored WCT pollen will be transported to each *Panchayat/ Krishi Bhavan* and kept in desiccators. Through this centralized mechanism of pollen processing, disease-free status of the male parental palms and quality of the pollen will be assured. Otherwise the pollen used will not be of uniform quality.

Presently 60 high yielding and disease-free (confirmed using ELISA test), WCT male parental palms are used for collecting male flowers. From one inflorescence approximately 6-8 grams of pollen is obtained and it is transferred to 2.0 ml cryo vials (8-10 nos.). Cryo vials are stored in liquid nitrogen cans; a can of 121 litre capacity can store upto 6000 samples. Pollen vials are taken out from the cans as per requirement and one vial (filled with 1.5 gram pollen) can be used for pollinating six CGD palms for two days. For completing the pollination on all female flowers (25-35 nos.) in all the six palms, approximately 3-4 pollen vials are needed. If this pilot programme is a success, there is a plan to extend this decentralized hybridization technique throughout Kerala State.

Tissue culture

Mass multiplication of elite coconut palms, providing high yield and possessing resistance to biotic and abiotic stresses, is significant for obvious reasons. Since planting materials obtained through conventional methods are unable to meet the demand for seedlings, a technique for large scale multiplication of coconut through *in vitro* techniques holds

paramount importance. Unfortunately, progress in clonal propagation of in coconut has been rather sluggish, with the high recalcitrance of coconut to *in vitro* culture being the main impediment to development of a commercial scale protocol for *in vitro* multiplication. Poor responses have been recorded to *in vitro* interventions irrespective of type of explants, cultivars or culture conditions. Several reasons have been attributed for the *in vitro* recalcitrance: genotype effect and maturity levels of explants, adsorption of nutrients and hormones by activated charcoal which makes the culture conditions undefined, production of calli which is compact, meager percentage of somatic embryogenesis and plantlet regeneration and slow growth of regenerated plantlets; all these factors could have possible cumulative effects in imparting poor *in vitro* responses (Fernando *et al.*, 2010). Selection of explants is considered as a key element for a successful outcome. Numerous tissues *viz.*, leaves, inflorescence, plumular tissues, ovaries, anthers, roots and zygotic embryos have been utilized as explants for coconut tissue culture. The major bottlenecks observed at ICAR-CPCRI include the formation of abnormal tissues and lack of production of friable calli, irrespective of explants utilized or media and culture conditions.

Leaf culture

Coconut leaf explants from juvenile palms were used in some studies with successful induction of callus and somatic embryos (Raju *et al.*, 1984). However the embryogenic capacity of leaf explants lasts for short duration, limiting its use as explant in clonal propagation studies.

Immature inflorescence culture

Immature inflorescence contains numerous meristematic points and therefore is considered a potential source of explant to clonally propagate important crop plants. The success depends on the selection of inflorescence of correct maturity stage. Prolonged incubation of immature inflorescence explants in auxin-cytokinin combination media resulted in conversion of floral primordial to vegetative primordial at a very low frequency (Raju, 2006).

Shareefa *et al.* (2016) have outlined a procedure for obtaining plantlets through immature inflorescence cultures. Inflorescence explants, with spathe lengths of 2-12 cm were collected from 25-30 year old West Coast Tall palms (Plate 1k). The outer and inner spathes were removed under aseptic conditions and rachillae were sliced into 1-1.5 mm long bits and inoculated in test tubes containing 20 ml Y3 media with low concentration of 2,4-D. The cultures were incubated in dark for a period of 16 weeks. After 1 or 2 weeks in culture, the rachillae bits produced tiny swellings, which gradually developed into white transparent calluses within 2-4 weeks. At the end of eight months incubation in the dark, with regular subculturing, maximum shoot like structures and minimum abnormal growth was observed in Y3 medium with low concentration of 2,4-D and picloram. The shoot like structures were transferred to ½ MS media with NAA and BAP and kept under diffused light for a period of one month followed by incubation in light condition. When the cultures were transferred from diffused light to light condition, elongation and formation of vegetative shoots were observed (Plate 1l). At the end of this cycle of incubation, the shoots were quite distinct and multiple shoots were formed. From the multiple shoots, the individual shoots could be separated easily and the individual shoots were cultured in Y3 medium supplemented with NAA and BAP. The number of shoots developed from rachillae explant varied from 1 to 17 (Plate 1m). The sizes of individual shoots were not uniform. It was observed that immature inflorescence with outer spathe lengths of 4-7 cm resulted in maximum (62.5 %) shoot regeneration. However, majority of the cultures did not develop roots at its base.

Plumular explants

The use of plumular tissues as source of explants has provided some of the consistent results among the various explants subjected to *in vitro* culture (Rajesh *et al.*, 2005, 2014). A procedure for regeneration of complete plantlets via organogenesis and embryogenesis from plumular tissues of West Coast Tall cultivar of coconut has been outlined by Rajesh *et al.* (2005). Callus could be induced in Y3 media

supplemented with either an auxin alone (2,4-D; 74.6 µM) alone or an auxin (2,4-D; 74.6 µM) in combination with a cytokinin (TDZ; 4.54 µM) (Plate 1g). Enhancement in the callus induction frequency and reduction in the browning of explants was observed when a cytokinin (TDZ) was supplemented along with an auxin (2,4-D) in the callus induction medium. Subculturing of calli at monthly intervals in to media containing lower levels of 2,4-D and a constant level of either cytokinins (BA and TDZ) or polyamines (spermine and putrescine) was undertaken. Enhanced frequencies of embryogenic calli (Plate 1h), somatic embryoids (Plate 1i) and meristemoids were obtained in Y3 media supplemented with either spermine or BA. Plantlets with balanced shoot and roots (Plate 1j) 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. Later, efforts were made in coconut tissue culture to refine the protocol such as use of polyamines (Rajesh *et al.*, 2014), and media combinations. Apart from multiplication, maintenance of embryogenic callus for long time could help in providing year round embryogenic calli for further conversion. Experiments have also indicated that coconut embryogenic calli, obtained from plumular tissues, could be maintained for 21 weeks by subculturing the calli from lower 2,4-D concentration (74.6 µM) to higher levels (90.4 µM) without compromising on the embryogenic potential (Bhavyashree *et al.*, 2015). Further refinement in the protocol is required to increase the period for which the embryogenic calli could be maintained.

Gene expression studies

A number of genes have been implicated in regulation of somatic embryogenesis in model crops and an understanding of their expression patterns would definitely help in better

deciphering the complexities underlying coconut somatic embryogenesis. Bhavyashree *et al.* (2015) carried out studies to understand the embryogenic potential of long term maintained calli in conjunction with gene expression studies. The results of the study revealed that embryogenic nature of calli could be maintained to a maximum of 21 weeks in medium supplemented with 2,4-D (74.6 μM) and culturing into higher concentration of 2,4-D (90.4 μM) subsequently. Gene expression studies were also carried out using qRT-PCR. Genes such as *ECP*, *GST*, *LEAFY* and *WUS* were highly expressed in long term embryogenic calli (21 week old); on the other hand, genes such as *SERK*, *GLP*, *WRKY* and *PKL* were upregulated in initial embryogenic calli (21 days old).

Bhavyashree *et al.* (2016) have also undertaken studies to compare the response of cultures from shoot meristem explants from two coconut cultivars *viz.*, a tall (West Coast Tall, WCT) and a dwarf (Chowghat Orange Dwarf, COD). Gene expression patterns of eight genes at different development stages of the two cultivars were also analyzed using qRT-PCR. Significant differences in regeneration potential and gene expression patterns in the two coconut cultivars have been reported suggestive of genotypic differences of cultivars to *in vitro* interventions. Expression of *PKL*, *SERK* and *WUS* was upregulated in embryogenic calli in comparison to non-embryogenic calli. Upregulation of *GLP*, *ECP* and *GST* was noticed in normal somatic embryos compared to abnormal ones. Upregulation of *ECP*, *LEAFY*, *GLP* and *WRKY* was recorded in normal meristemoids compared to aberrant ones. Higher frequency of somatic embryogenesis was observed in WCT compared to COD. Three genes *viz.*, *SERK*, *PKL* and *WUS* were upregulated in embryogenic calli of WCT compared to COD. Enhanced expression of *GLP* and *GST* was observed in somatic embryos of COD; in contrast, higher expression of *ECP* was observed in WCT. Enhanced expression levels of *WRKY* and *LEC* were observed in meristemoids of WCT compared to COD. Based on the results obtained in the study, it was suggested that these

genes could serve as markers for different developmental stages in coconut.

Rajesh *et al.* (2016) have carried out transcriptome analysis (RNA-Seq) of coconut plumule derived embryogenic calli on an Illumina HiSeq 2000 platform. After *de novo* transcriptome assembly and functional annotation, a total of 40,367 transcripts were obtained which showed significant BLASTx matches with similarity greater than 40% and E value of $\leq 10^{-5}$. Fourteen genes, with significant roles in somatic embryogenesis, were documented. 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.

Bioinformatics initiatives

Somatic embryogenesis receptor-like kinases (SERKs), belonging to leucine-rich repeat receptor-like kinase super family, have been reported to possess vital roles in the process of somatic embryogenesis. Rajesh *et al.* (2015) carried out homology based modeling and molecular dynamics (MD) simulation of a coconut SERK protein (CnSERK) for examining its structural features, functional characterization of its active sites and binding mechanisms of selected plant hormones and growth regulators by docking studies. A 3-D model was generated for coconut SERK. Fifteen plant growth regulators were docked with the target SERK protein. To validate the results of the *in silico* study, an *in vitro* study was carried out to compare the efficiency of three selected chemicals [adenine sulphate, glutathione and 22(S), 23(S)-homobrassinolide] in enhancing frequency of somatic embryogenesis from plumular explants of coconut. Plumular explants were from West Coast Tall cultivar of coconut and were inoculated into Y3 media supplemented with various concentrations of each of the three growth regulators. Glutathione (100 μM) gave the best response for induction of both embryogenic calli and somatic embryogenesis. The results of this study might aid in the development of regeneration protocols for *in vitro* regeneration in coconut.

Conclusions

New vistas have emerged with the introduction of biotechnological tools in coconut crop improvement programmes. Embryo culture, embryo rescue and cryopreservation techniques have helped in collection and preservation of valuable coconut germplasm. 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. Scaling up of quality planting material production through clonal propagation method seems to be the solution to fulfill the ever growing demand for quality planting materials. Plumular explants have so far proven to be the most responsive explant for somatic embryogenesis compared to other explants. Higher yields of calli and calli bearing somatic embryos could be obtained from plumular explants compared to inflorescence explants. Achievements made in this area of plumule culture include perfecting a procedure for excision of shoot meristem directly from zygotic embryo's, hastening in vitro culture period and identification of best performing palms and season for collection of explants. Presence of genotypic differences in response to in vitro culture, low rate of somatic embryo formation, conversion of somatic embryos into plantlets, and formation of abnormal somatic embryos are the major constraints which still need to be overcome. Efforts are on to decipher the molecular aspects of in vitro recalcitrance in coconut. A commercial level protocol with increased callogenesis and clonal propagation frequency in coconut needs a multiple faceted approach involving optimized media combination with plant growth regulators, additives and bioreactor systems.

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References

- Bhavyashree, U., Rachana, K.E., Lakshmi Jayaraj, K., Rajesh, M.K. and Karun, A. 2016. Comparative gene expression profiling during *in vitro* regeneration in two coconut cultivars. *Emirates Journal of Food and Agriculture* **28**(8): 572-580.
- Bhavyashree, U., Lakshmi Jayaraj, K., Rachana, K.E., Muralikrishna, K.S., Sajini, K.K., Rajesh, M.K. and Karun, A. 2015. Maintenance of embryogenic potential of calli derived from shoot meristem of West Coast Tall cv. of coconut (*Cocos nucifera* L.). *Journal of Plantation Crops* **43**(2): 105-116.
- CPCRI. 2011. Research Highlights 2010-11. Central Plantation Crops Research Institute Kasaragod, Kerala, India. pp. 36.
- CPCRI. 2012. Annual Report 2011-12, Central Plantation Crops Research Institute Kasaragod -671124, Kerala, India, pp. 128.
- Diekmann, M. 1997. The use of biotechnology for the safe movement of coconut germplasms. Paper presented at International Symposium on Coconut Biotechnology Merida, December 1-5.
- Fernando, S.C., Vidhanaarachchi, V.R.M., Weerakoon, L.K. and Santha, E.S. 2010. What makes clonal propagation of coconut difficult? *Asia-Pacific Journal of Molecular Biology and Biotechnology* **18**: 163-165.

- Karun, A., Shivasankar, S., Sajini, K.K., and Saji, K.V. 1992. Field collection and *in vitro* germination of coconut embryos. *Journal of Plantation Crops* **21**: 291-294.
- Karun, A. and Sajini, K.K. 1994. Short-term storage of coconut embryos in sterile water. *Current Science* **67 (2)**: 118-120.
- Karun, A. and Sajini, K.K. 2010. Cryopreservation of coconut zygotic embryos and pollen. Technical Bulletin No. 63, ICAR-CPCRI, Kasaragod, India.
- Karun, A., Rajesh, M.K. and Muralikrishna, K.S. 2015. Somatic embryogenesis and bioreactors: Meeting report. *Current Science* **108(10)**: 1782-1783.
- Karun, A., Sajini, K.K. and Parthasarathy, V.A. 2002. Increasing the efficiency of embryo culture to promote germplasm collecting in India. In: (Eds) Engelmann, F., Batugal, P. and Oliver, J. *Coconut embryo in vitro culture: Part II*. pp. 7-26.
- Karun, A., Sajini, K.K. and Parthasarathy, V.A. 2005. Cryopreservation of mature zygotic embryos of coconut by desiccation methods. *Cord* **21(1)**: 13-19.
- Karun, A., Sajini, K.K. and Shivashankar, S. 1999. Embryo culture of coconut: the CPCRI protocol. *Indian Journal of Horticulture* **56(4)**: 348-353.
- Karun, A., Sajini, K.K., Kumaran, P.M. and Samsudheen, K. 2006. Cryopreservation of coconut (*Cocos nucifera* L.) pollen. *Journal of Plantation Crops* **34**: 568-571.
- Karun, A., Sajini, K.K., Niral, V., Amarnath, C.H., Remya, P., Rajesh, M.K., Samsudeen, K., Jerard, B.A. and Engelmann, F. 2014. Coconut (*Cocos nucifera* L.) pollen cryopreservation. *Cryo-Letters* **35(5)**: 407-417.
- Karun, A., Sajini, K.K., Radha, E. and Parthasarathy, V.A. 2004. Efficacy of CPCRI embryo culture protocol in germplasm expedition. *Journal of Plantation crops* **32**: 139-143.
- Karun, A., Shivasankar, S., Sajini, K.K. and Saji, K.V. 1993. Field collection and *in vitro* germination of coconut embryos. *Journal of Plantation Crops* **21**: 291-294.
- Rajesh, M.K., Fayas, T.P., Naganeeswaran, S., Rachana, K.E., Bhavyashree, U., Sajini, K.K. and Karun, A. 2016. *De novo* assembly and characterization of global transcriptome of coconut palm (*Cocos nucifera* L.) embryogenic calli using Illumina paired-end sequencing. *Protoplasma* **253(3)**: 913-928.
- Rajesh, M.K., Gangaraj, K.P., Jamshinath, T.P., Fayas, T.P., Naganeeswaran, S., Sajini, K.K. and Karun, A. 2015. Virtual screening and *in vitro* evaluation of potential growth regulators against somatic embryogenesis receptor-like kinase (SERK) in *Cocos nucifera* L. *Indian Journal of Biochemistry and Biophysics* **52(5&6)**: 328-340.
- Rajesh, M.K., Radha, E., Sajini, K.K. and Karun, A. 2014. Polyamine-induced somatic embryogenesis and plantlet regeneration *in vitro* from plumular explants of dwarf cultivars of coconut (*Cocos nucifera*). *Indian Journal of Agricultural Sciences* **84 (4)**: 527-530.
- Rajesh, M.K., Radha, E., Sajini, K.K., Karun, A. and Parthasarathy, V.A. 2005. Plant regeneration through organogenesis and somatic embryogenesis from plumular explant of coconut. *Journal of Plantation Crops* **33(1)**: 9-17.
- Raju, C.R. 2006. Direct *in vitro* shoot development from immature rachilla explants of coconut (*Cocos nucifera* L.). *Cord* **22(1)**: 51-58.
- Raju, C.R., Kumar, P.P., Chandramohan, M. and Iyer, R.D. 1984. Coconut plantlets from leaf tissue cultures. *Journal of Plantation Crops* **12**: 75-78.
- Sajini, K.K., Karun, A. and Kumaran, P.M. 2006. Cryopreservation of coconut zygotic embryos by pre growth desiccation. *Journal of Plantation Crops* **34**: 576-581.

- Sajini, K.K., Karun, A., Amarnath, C.H. and Engelmann, F. 2011. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos by vitrification. *Cryo-Letters* **32** (4): 317-328.
- Samsudeen, K., Rajesh, M.K., Nagwaker, D.D., Raghavan Reshmi, Ajith Kumar, P., Devadas, K. and Karun, A. 2013. Diversity in Mohachao Narel, a sweet endosperm coconut (*Cocos nucifera* L.) population from Maharashtra, India. *National Academy of Science Letters* **36** (3): 319-330.
- Shareefa, M., Thomas, R.J., Sreelekshmi, J.S., Raju, C.R and Karun, A. 2016. *In vitro* shoot regeneration from immature inflorescence of coconut. In: *Book of Abstracts, 3rd International Symposium on Coconut Research and Development (ISOCRAD-3)*. (Eds.) Chowdappa, P., Muralidharan, K., Samsudeen, K. and Rajesh, M.K. 10-12th December 2016, ICAR-CPCRI, Kasaragod, Kerala. pp.32.