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Biotechnological advancements in the improvement of Coconut and Arecanut

Coconut and arecanut are principal plantation crops cultivated in the tropics for their multitude of economic uses. These two palms are particularly susceptible to a number of biotic and abiotic stressors that significantly limit their productivity potential. The demand for improved, high-yielding varieties of coconut and arecanut, along with the need for enhanced resistance to biotic and abiotic stresses, has been rising. Further, it is anticipated that the palms must be adapted to climate-change-induced vagaries. Conventional breeding-based crop improvement programs for coconut and arecanut have largely been ineffective for various reasons, including perennial nature, long juvenile period and genetic heterozygosity of palms, among others. Advancements in the field of biotechnology have enabled palm researchers to adopt and apply novel techniques for the improvement of perennial crops. This article provides an overview of the progress made in using tissue culture and cryopreservation, application of molecular marker techniques, and next-generation sequencing to improve coconut and arecanut.

COCONUT embryo culture has been a boon for unhindered international germplasm collection and exchange. Coconut accessions that do not germinate naturally and sterile inter-specific hybrids of arecanut have been regenerated by applying relatively easy embryo rescue techniques. *In vitro* culture-based multiplication of elite accessions is a major goal in palm tissue culture. Despite the rapid strides in tissue culture of other palms, success in direct or callus-mediated regeneration in coconut has remained elusive. The protocol for direct regeneration from coconut immature inflorescence explants, standardized recently, looks propitious.

Conversely, arecanut has witnessed large-scale regeneration of elite genotypes using immature inflorescence tissues as explants. Standardization and application of efficient cryopreservation techniques for zygotic embryos and pollen of coconut and arecanut have made safe and long-term conservation of genetic resources a practically viable endeavour. Diverse molecular markers technologies have been utilized to decipher the genetic diversity of coconut and arecanut accessions. Further, the application of molecular markers has greatly aided in developing linkage maps and identifying QTLs governing yield and yield-attributing traits. Recent advancements in genomics and the availability of whole-genome sequence information are anticipated to further unlock the genomic potential of these crops, especially in the field of breeding and development of economically important, climate-smart and even speciality cultivars. This article briefly discusses significant achievements in the use of biotechnology for improvement of coconut and arecanut.

Embryo culture and embryo rescue

Coconut zygotic embryo culture to support international germplasm exchange: At ICAR-CPCRI, an efficient protocol for culturing coconut zygotic embryos *in vitro* has been developed and validated (Fig. 1) as it has been successfully used in numerous germplasm expeditions. The method relies on artificial media and micro-climatic conditions *in vitro* to support embryo growth and development into complete plantlets. A field collection technique for zygotic embryos has also been devised to assist germplasm collection during expeditions to areas devoid of access to laboratories. Researchers at ICAR-CPCRI have also developed and successfully utilized a simple technique for storing zygotic embryos for up to two months using sterile water as a medium in germplasm collection initiatives. The coconut embryo culture protocol developed at ICAR-CPCRI is an internationally accepted procedure that could be employed as a means for germplasm collection and exchange.

In 1994, the ICAR-CPCRI embryo culture protocol was first utilized to collect six accessions from the Pacific Ocean collections from the World Coconut Germplasm Center (WCGC) in the Andaman and Nicobar Islands, India. Mature zygotic embryos were collected and transported to the mainland, retrieved *in vitro* and acclimatized plantlets were field planted in 1996 at the International Coconut Gene Bank for South Asia and

Fig. 1. Embryo culture of coconut: (A) germinating embryo *in vitro*; (B) plantlet development; (C) hardened plantlet derived from zygotic embryo culture; (D) Embryo culture plantlet in the field; and (E) bearing palm

the Middle East (ICG-SAME), Kidu, Karnataka, India. Later, the protocol was successfully implemented in five international germplasm collecting missions conducted by ICAR-CPCRI between 1997 and 2001, with the primary goal of increasing coconut genetic diversity in India. Zygotic embryos were collected from 45 accessions from eight countries, viz. Bangladesh, Comoros, Madagascar, the Maldives, Mauritius, Réunion, Seychelles and Sri Lanka and retrieved *in vitro*. The exotic collections, obtained using the ICAR-CPCRI embryo culture protocol, were planted in ICG-SAME, India. After performance evaluation, some promising exotic collections have also been incorporated into coconut breeding programmes. The insights gained from the above studies suggest that about 300 to 400 embryos are indispensable for the field establishment of 100 palms in a field gene bank.

Coconut embryo rescue: *In vitro* embryo rescue involves obtaining plantlets from embryos that either do not germinate or germinate very slowly in nature. Mohachao Narel is a coconut variant found in the Guhaghar taluk of Maharashtra's Ratnagiri district. The kernels of this accession are sweet and soft, and the fibre content is low. Owing to sweet endosperm nuts and relatively low embryo weight, these accessions fail to germinate under natural conditions. Using the ICAR-CPCRI embryo culture protocol, plantlets were successfully regenerated following the embryo rescue of sweet endosperm nuts (Fig. 2).

Horned Coconuts are unique to the Andaman Islands- their multiple ovaries result in horn-like structures covering the mature nuts, delaying the natural germination process. The ICAR-CPCRI embryo culture technique was used to culture Horned Coconut embryos *in vitro*. Field planting of the embryo cultured palms derived from horned coconuts resulted in flowering in six to eight years.

Cryopreservation

Genetic resources of coconut and arecanut are traditionally conserved *ex situ* as whole palms in field gene banks, which takes up a lot of space and is expensive to maintain. Cryopreservation is a long-term conservation strategy that allows for the safe and efficient conservation of genetic diversity while also overcoming the limitations of traditional conservation. Cryopreservation of coconut zygotic embryos and coconut and arecanut pollen are excellent strategies for the long-term preservation of valuable genetic resources. Furthermore, the technique can serve as a viable backup for the genetic resources of these crops stored in field gene banks.

Cryopreservation of coconut zygotic embryos: A pre-requisite for successful cryopreservation of mature zygotic embryos is pre-treating the embryos before freezing them in liquid nitrogen at −196°C. Simple desiccation (using a laminar air current and silica gel), high sucrose concentrations, encapsulation with 3% sodium alginate, and cryo-protectants (plant vitrification

Fig. 2. Embryo rescue in coconut: (A) rescued embryo from sweet endosperm coconut in retrieval media; (B) germination of the embryo; (C) plantlet development; (D) hardened plantlets in green house; and (E) field planting of embryo-rescued plantlet

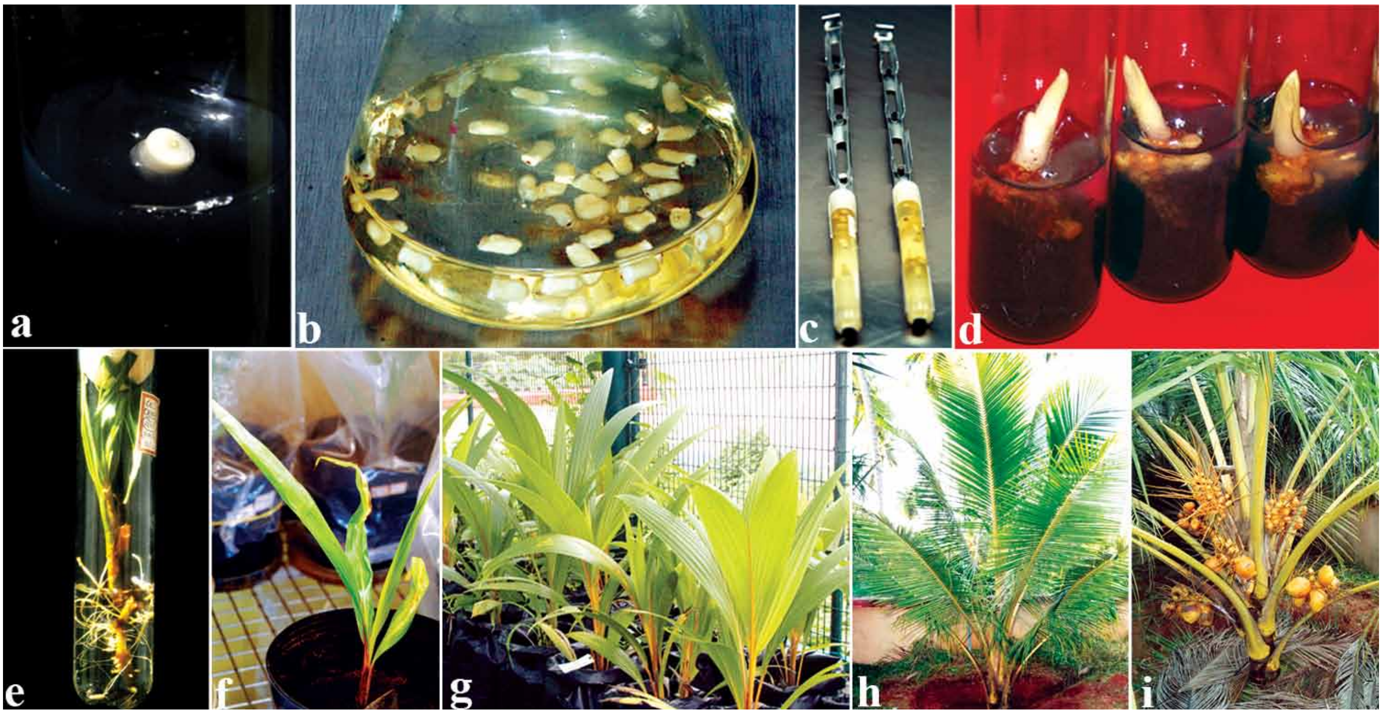

Fig. 3. Vitrification-based cryopreservation of zygotic embryos of coconut: (a) pre-growing of the embryo in sucrose medium; (b) treatment with PVS_3; (c) dehydrated embryos in cryo-vials affixed to cryo-sticks; (d) post-cryopreservation regeneration of embryos in retrieval medium; (e) rooted plantlet; (f) *in vitro* hardened plantlet; (g) *ex vitro* hardened plantlets; (h) planted in field; and (i) bearing palm

solution, PVS) are among the pre-treatments. In 2005, cryopreservation of mature embryos of the West Coast Tall cultivar was standardized using desiccation pre-treatments. Normal plantlets could be derived from zygotic embryos desiccated for 18 h with silica gel or for 24 h with laminar airflow and then cryopreserved. Irreversible damage to the shoot meristem was observed when the moisture content of the embryo was reduced to less than 20%. Following a pre-growth desiccation method, using high sucrose concentration, the moisture content of the embryos could be reduced to 27-30%, respectively, with a final recovery of 20.8-29% after cryopreservation.

Further extensive research on the vitrification technique for cryopreservation of mature coconut zygotic embryos has been undertaken. The PVS_3 (plant vitrification solution) solution, which contains equal amounts of glycerol and sucrose, was the most effective for regenerating the cryopreserved embryo. Pre-culture of embryos for three days on a medium containing 0.6 M sucrose, PVS_3 treatment for 16 h, rapid cooling in liquid nitrogen, rewarming, and finally unloading in 1.2 M sucrose liquid medium for 90 minutes was the best protocol standardized. This protocol resulted in 70-80% survival rates (corresponding to size enlargement and weight gain) (Fig. 3), and 20-25% of the plants regenerated (showing normal shoot and root growth) from cryopreserved embryos could be successfully established in pots.

Cryopreservation of coconut pollen: Since pollen is a useful source of diverse alleles within a gene pool, pollen storage is highly essential for germplasm conservation, hybrid seed production, and assisted pollination. Storage of coconut pollen in liquid nitrogen for 24 h did not affect *in vitro* germination. The following are the steps involved in pollen cryopreservation: (a) extraction of coconut pollen by sieving (mesh size 0.2 mm) male flowers in an oven at 40°C for 24 h; (b) wrapping pollen in strips of aluminium foil; (c) inserting these into cryo-vials; and (d) plunging into liquid nitrogen. Pollen viability is assessed both *in vitro* (by observing pollen growth in artificial media) and *in vivo* (hand pollination in the field) (Fig. 4). Even after seven years of storage in liquid nitrogen, pollen retained its viability and fertility. After hand pollination, utilizing pollen that had been cryopreserved for seven years, normal nut set was recorded. Embryos, extracted from hybrid nuts produced with cryostored pollen, displayed

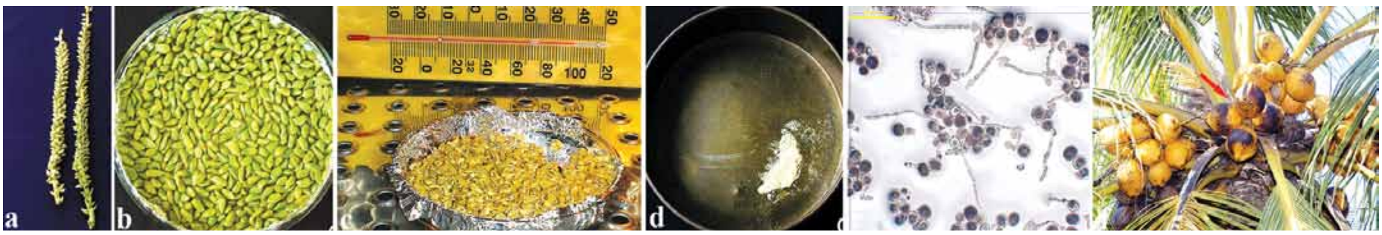

Fig. 4. Pollen cryopreservation in coconut through simple desiccation: (a) spikelets with mature male flowers; (b) & (c) isolated male flowers and desiccation; (d) pollen grains extracted from desiccated flowers; (e) *in vitro* germination assay; and (f) field fertility of cryopreserved pollen

100% germination and normal plantlet development. This study thus confirmed the feasibility of establishing a pollen cryobank in coconut.

Cryopreservation of arecanut pollen: Various aspects of arecanut pollen cryopreservation, viz. desiccation and collection of pollen, *in vitro* germination, viability and fecundity studies have been standardized. *In vitro* viability tests were conducted using fresh and desiccated pollen of arecanut genotypes. The desiccated pollen was cryopreserved by direct immersion in liquid nitrogen for different durations (24 h to two years). Viability and fertility studies were conducted using cryopreserved pollen (Fig. 5). Normal nut set was observed with the use of cryopreserved pollen.

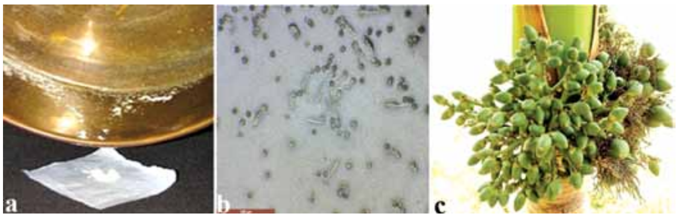

Fig. 5. Pollen cryopreservation in arecanut through simple desiccation: (a) extracted pollen from desiccated male flowers; (b) *in vitro* germination assay; and (c) field fertility of cryopreserved pollen

Cryopreservation of arecanut embryogenic calli: The embryogenic calli were pre-grown in Y3 medium supplemented with sucrose for three days and later desiccated using PVS_3 (Fig. 6). The results showed 8-10% recovery of embryogenic calli that resulted in normal plantlet production. The clonal fidelity studies, using start codon targeted (SCoT) markers, showed no genetic variation of cryopreserved calli compared to the original calli. This preliminary study demonstrated the successful use of the V-cryoplate technique in long-term cryopreservation of embryogenic calli of arecanut.

Tissue culture

Multiplication of elite coconut palms, characterized by high yield and resistance to biotic and abiotic stresses, is important for obvious reasons. A technique for large-scale coconut multiplication *in vitro* is essential since conventional planting materials cannot meet the demand for seedlings, which, unfortunately, is hampered by the high recalcitrance of coconut to *in vitro* culture. Several factors have been attributed to *in vitro* recalcitrance, viz. genotype effect, explant maturity levels, activated charcoal absorption of nutrients and hormones. Among the coconut tissue culture explants are leaves, inflorescence, plumular tissues, ovaries, anthers, roots, and embryos. No matter the type of explants, media and culture conditions used, abnormal tissues and the lack of friable calli remain major bottlenecks in coconut.

Coconut immature inflorescence culture: Recently, immature inflorescence culture has shown promise as a technique for mass-multiplication of elite coconut genotypes. Immature inflorescences possess many meristematic points, making them a good source of explants; also, the selection of immature inflorescences at the appropriate maturity stage determines the success of regeneration.

Immature inflorescence explants of 2-12 cm spathe lengths are collected, and the outer and inner spathes are removed under aseptic conditions. The rachillae are sliced into 1-1.5 mm long bits, inoculated in Y3 media with a low concentration of 2,4-D, and incubated in the dark for 16 weeks. Within one or two weeks of incubation, the rachillae bits produce tiny swellings that soon develop into vegetative buds. Following eight months of incubation in the dark and regular sub-culturing in Y3 medium with low concentrations of 2,4-D, shoot-like structures were observed. These shoot-like structures were transferred to 1/2 MS media with NAA and BAP and incubated for one month under diffused light. Following the switching from diffused to direct light, the cultures exhibited elongation and formation of vegetative shoots. By the end of this incubation cycle, the shoots were quite distinct, and multiple shoots were formed. It was possible to separate individual shoots from multiple shoots and transfer them to media containing NAA and 2iP for shoot elongation. The individual shoots were cultured in Y3 media with NAA and BAP to induce rooting and promote normal shoot growth. Immature inflorescence with an outer spathe length of 4-7 cm showed maximum shoot regeneration. Plantlets developed through this technique could be hardened (Fig. 7).

Coconut plumule culture: The plumular tissues, as explants, have produced some of the most consistent results among the various explants cultured *in vitro*. Plumular tissues provided higher frequencies of embryogenic calli, somatic embryoids, and meristemoids. In Y3 media supplemented with an auxin (2, 4-D) in combination with a cytokinin (TDZ), calli could be induced from plumular tissues. The calli were sub-cultured in media with lower

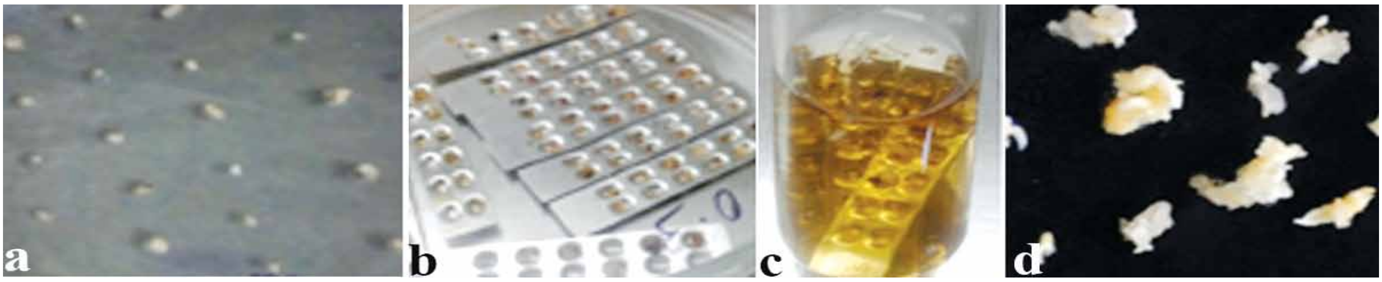

Fig. 6. Cryopreservation of immature inflorescence culture derived embryogenic calli in arecanut through V-cryoplate technique: (a) pre-growth of embryogenic calli, (b) loading of calli onto V-cryoplate; (c) dehydration in PVS_3; and (d) survival and multiplication of embryogenic calli post-cryopreservation in retrieval medium

Fig. 7. Plantlet regeneration from immature inflorescence culture in coconut: (a) immature inflorescence explants of different size and maturity; (b) rachillae bits in Y3-based nutrient medium; (c) & (d) different stages of multiple shoot initiation; (e) completely developed plantlet; (f) hardened plantlet; and (g) field planting

levels of 2,4-D and a constant level of either cytokinins (BA and TDZ) or polyamines (spermine and putrescine) at regular intervals to obtain plantlets (Fig. 8). Plantlets with balanced shoots and roots could be potted and transplanted to the greenhouse. The development of shoot buds (organogenesis) and typical embryoids (bipolar) was confirmed by histological studies. Although plantlets have been successfully regenerated and established in the field, a commercial-scale protocol has yet to be developed. The formation of friable calli and conversion of somatic embryos into plantlets remains a major bottleneck, and the protocol needs to be refined further. Recent studies have shown that a maximum of seven plantlets could be obtained from a single plumular explant by supplementing a novel aromatic cytokinin (meta-topolin) in the tissue culture media.

Arecanut immature inflorescence culture: A reproducible protocol for arecanut somatic embryogenesis and plantlet regeneration from immature inflorescence explants has been developed at ICAR-CPCRI for rapid multiplication of elite genotypes such as Yellow Leaf Disease (YLD) resistant palms, Hirehalli Dwarf (HD), a natural mutant with short stature and dwarf hybrids, VTLAH–1 and VTLAH–2. For mass multiplication, immature inflorescence rachillae are chopped into 1-2 cm sized explants and inoculated in the Y3 basal medium for somatic embryogenesis. Calli were induced on subsequent sub-cultures from higher to lower auxin concentrations. Somatic embryo formation was achieved in a hormone-free Y3 medium, and germination was achieved when supplemented with cytokinins. Subsequent plantlet development was achieved in a regeneration medium under light room conditions with a photoperiod of 16 h (Fig. 9).

Molecular markers

Knowledge of the genetic diversity of a crop is an important cornerstone of a successful breeding programme. The use of molecular markers has proved to be an extremely efficient method in estimating the genetic diversity of germplasm collections and the population structure of various crops. Efficient use of molecular markers will enable characterization and management of germplasm, genetic diversity studies, linkage mapping and identification of quantitative trait loci (QTLs) for marker-assisted selection (MAS).

Molecular markers for genetic diversity studies and marker-assisted selection in coconut: Breeding efficiency can be enhanced by assessing genetic diversity, germplasm characterization and management, linkage mapping, and identifying QTLs, which would enable MAS. Various molecular markers used in coconut for assessment of genetic diversity and population structure include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inverse sequence-tagged repeats (ISTR), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), start codon targeted (SCoT) markers and more recently, single nucleotide polymorphisms (SNPs). Based on these studies, accessions were organized into genetic groups, and redundant collections were identified.

Various molecular marker studies have established that tall and dwarf coconut accessions differ genetically, which can be attributed to their breeding behaviours: self-pollinating dwarf accessions have low genetic diversity and high homozygosity compared to tall accessions, which are cross-pollinated. Through molecular methods, novel avenues have been opened up for phylogenetic analysis, and new marker-based tools have been developed for the efficient conservation and use of coconut genetic resources.

In perennial crops such as coconut, markers associated with important traits can increase breeding efficiency and reduce breeding cycles. Molecular markers capable of

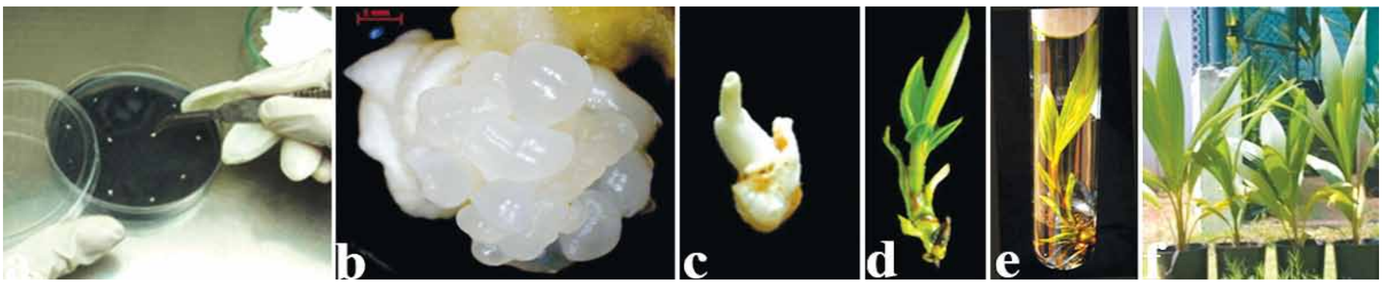

Fig. 8. Plantlet regeneration from plumular explants in coconut: (a) plumule in callusing medium; (b) formation of embryogenic calli; (c) development of somatic embryo; (d) & (e) plantlet regeneration; and (f) hardened plantlets

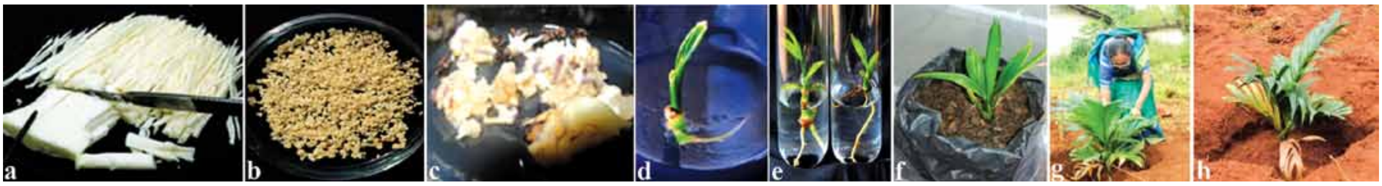

Fig. 9. Plantlet regeneration from immature inflorescence culture in arecanut: (a) immature inflorescence explant; (b) rachillae bits in Y3 based nutrient medium; (c) callogenesis and somatic embryogenesis; (d) & (e) plantlet regeneration; (f) hardened plantlet; and (g) & (h) field planting

distinguishing tall (T) and dwarf (D) coconut cultivars have been identified. Also, markers have been used to confirm the authenticity of D × T hybrids in the early seedling stage, ensuring that farmers are given genuine hybrids.

Molecular markers for genetic diversity and assessment of clonal fidelity in arecanut: Arecanut germplasm has been traditionally characterized and evaluated based on morphological and yield parameters. However, the genetic diversity information provided by morphological characters is limited, and these parameters can be influenced by environmental, genetic and physiological factors. Genetic relationships among arecanut germplasm, consisting of indigenous and exotic accessions, have been assessed using RAPD and SSR primers. Clustering of the accessions correspond to their geographic origins, in general. Molecular markers have also been successfully utilized to confirm the genetic fidelity of tissue-cultured derived plantlets. The low level of variability observed among tissue culture-derived plantlets augurs well for exploiting this technique for the large-scale multiplication of elite arecanut palms.

Molecular markers for detection of inter-specific hybrids in arecanut: *Areca catechu* L. and its wild relatives, viz. *Areca concinna* and *Areca triandra,* were characterized using SCoT markers, leading to the identification of markers unique to *A. catechu* and *A. triandra.* Later, these SCoT markers were converted into *A. catechu* and *A. triandra* specific sequence characterized amplification markers (SCAR). These SCAR markers have been effectively used to authenticate inter-specific hybrids between *A. catechu* and *A. triandra.*

Integrating multi-omics data for coconut improvement: Combining genomics, transcriptomics, proteomics, and metabolomics approaches can enable a comprehensive understanding of the mechanisms underlying the complex architecture of traits of agricultural importance. Several initiatives have contributed to the development of coconut genomic resources, which are briefly highlighted.

RNA-sequencing (RNA-seq) permits the characterization of genes, including those which are differentially expressed, and could be employed for functional genomic studies, especially when there is limited genome information. Transcriptomic studies in coconut have deciphered the molecular basis of somatic embryogenesis and differential response of coconut genotypes to root (wilt) and bud rot diseases and water deficit stress. These resources have yielded valuable insights into the spatio-temporal expression of key genes, their regulatory mechanisms and involvement in a repertoire of biological processes. In addition to aiding annotation of the nuclear genome, RNA-seq data has also unearthed a plethora of genic molecular markers for genetic research and breeding applications in coconut.

The nuclear and organellar genomes of the dwarf cultivar, Chowghat Green Dwarf (CGD), have been made available by utilizing a hybrid sequencing strategy. Availability of CGD genome, possessing enhanced response to root (wilt) disease, is a valuable genetic resource for accelerating genomics-assisted breeding in coconut.

Way forward

Biotechnological tools have opened up new vistas for coconut and arecanut crop improvement. Cryopreservation, embryo culture, and embryo rescue are all techniques that have assisted in collecting and preserving valuable germplasm in these crops. To satisfy the ever-growing demand for quality planting materials, clonal propagation seems like an ideal solution to scale up production. While success has been achieved in arecanut, genotypic differences in response to *in vitro* culture, low rates of somatic embryo formation, conversion of somatic embryos into plantlets, and abnormal formation of somatic embryos remain major challenges in coconut. Optimum media combination with plant growth regulators, additives, and bioreactor systems is necessary to standardize a commercial-level protocol. Also, there is a need for augmentation of omics strategies to uncover the molecular basis of *in vitro* recalcitrance in coconut.

Despite the success of conventional breeding techniques in improving coconut and arecanut, the application of multiple-omics technologies is still in its infancy in both crops. The genome assembly of a dwarf coconut cultivar has been made available; efforts have to be made to re-sequence elite or important genotypes further, to strengthen genomics-assisted breeding in identification and characterization of SNPs, quantitative trait loci (QTLs) linked to traits of economic importance, and tread the path of genotyping by sequencing (GBS) to perform genome-wide association analysis (GWAS) analysis. Embracing new breeding tools like genomic selection (GS) will likely improve genetic gains. These initiatives would pave the way for large-scale adoption of biotechnology programmes worldwide to develop climate-smart and disease-resistant phenotypes.

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