Embryo culture to rescue rare coconut types

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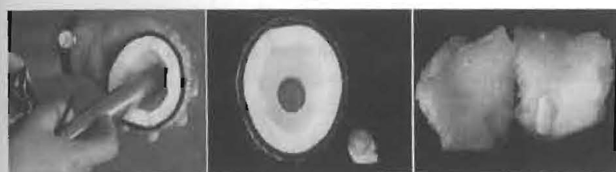
Introduction

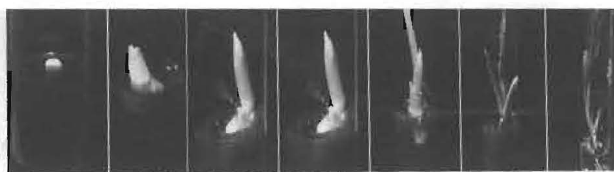
Crop improvement in coconut is complex owing to its extended juvenile phase, perennial nature and inherently high heterozygosity. Biotechnological interventions such as tissue culture, therefore hold immense significance in augmenting traditional breeding approaches to make coconut crop improvement programmes more effective. ICAR-CPCRI has successfully developed protocols in coconut embryo culture- applications of in vitro culturing of zygotic embryos include collection and safe exchange of germplasm between countries and to raise plantlets from coconut accessions special traits of coconut through embryo rescue. Though progress in clonal propagation has been slow, a successful protocol would reduce the demand-supply gap in quality planting material. Issues and strategies in tissue culture of coconut are briefly highlighted below:

Embryo culture: Give and take germplasm safely

Seed is the propagule generally used for as planting material in coconut. The collection of coconut germplasm as seed nuts from far off places is hampered by its recalcitrant nature, lack of dormancy or short dormancy and bulkiness of nuts, which make this method of propagation expensive and cumbersome. Phytosanitory restrictions also limit collection of seed nuts as source material for germplasm. In coconut germplasm collecting, conservation and exchange, the embryo culture technique could ease up and facilitate the limited exchange of germplasm between countries because instead of the bulky seednuts, zygotic embryos could be transported, thus eliminating the encumbrance of phytosanitary restrictions. Consequently, germplasm could be collected and conserved in a limited space free from quarantine problems. Short to medium term *in vitro* conservation is mainly utilized for collection and international exchange of germplasm wherein embryos can be stored in sterile water without losing its viability for around two months. This technique has been utilized in one indigenous and five international expeditions conducted by ICAR-CPCRI during the period 1997-2001 for the collection of coconut genetic diversity. 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 % (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), Kidu, Karnataka and have started yielding. From the earlier observations on in vitro retrieval of embryos and their ex vitro establishment, it has been suggested that about 300 to 400 embryos are required to be collected for field establishment of 100 palms in a gene bank. The diverse collections after evaluation are utilized for 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 development of hybrids that has better adaptation to biotic as well as abiotic factors and which could be utilized in breeding programmes.



Scooping endosperm plug along with the embryo



Different growth stages in coconut embryo culture

Embryo culture: Experimental models

In vitro raised plantlets, via embryo culture could be used for screening variety for important traits such as drought tolerance, salt tolerance, high temperature tolerance and biotic stress resistance. Experiments such as investigations on trace element deficiencies, pathological studies, germination studies and transformation studies could also be undertaken using *in vitro* raised plantlets.

Embryo cultures turning non viable to viable

Embryo culture protocols are widely used for obtaining plantlets from embryos which fail to germinate under natural conditions or take a long time to germinate. Special types of coconuts such as `Makapuno' (homozygous recessive embryo), sweet endosperm coconut and horned coconut have been successfully retrieved *in vitro* using embryo rescue technique and established in the field.

ICAR-CPCRI protocol for coconut zygotic embryo culture procedure can be extended for collecting and exchanging germplasm, rescuing special traits of coconut and also to raise *in vitro* plantlets for experimental purpose. Protocol is viable and has considerable commercial value. The Makapuno embryo does not develop normally because the endosperm, which supports the germination of the embryo is abnormal and rots when the nut matures. The embryo culture technique is the only means known to germinate the Makapuno embryo to produce a pure bearing Makapuno palm. Successfully grown Makapuno palms produce about 75-100% Makapuno nuts if planted together and/or isolated from other coconut palms by a pollen barrier.

Clonal propagation: an enigma

The paramount goal of tissue culture is to clonally propagate selected parental lines and hybrids so as to produce uniform elite planting materials. Research on somatic embryogenesis in coconut was initiated four decades ago at Wye College, UK, and later at ORSTOM, France. These experiments made use of plant somatic tissues such as young leaves, meristem region of young seedlings, sections from rachillae of young inflorescences, as initial explants to generate embryogenic calli. Recent studies related to somatic embryogenesis have utilized zygotic tissues apart from somatic tissues such as

Scooping endosperm plug along with the embryo

Surface sterilization of excised embryo in 20% sodium hypochlorite solution for 20 minutes followed by washing in sterile water for five times

Inoculation of the embryos in full strength Y3 medium supplemented with 40g/l sucrose (60 g/l for dwarf) and 1 g/l activated charcoal in dark till the emergence of first leaf.

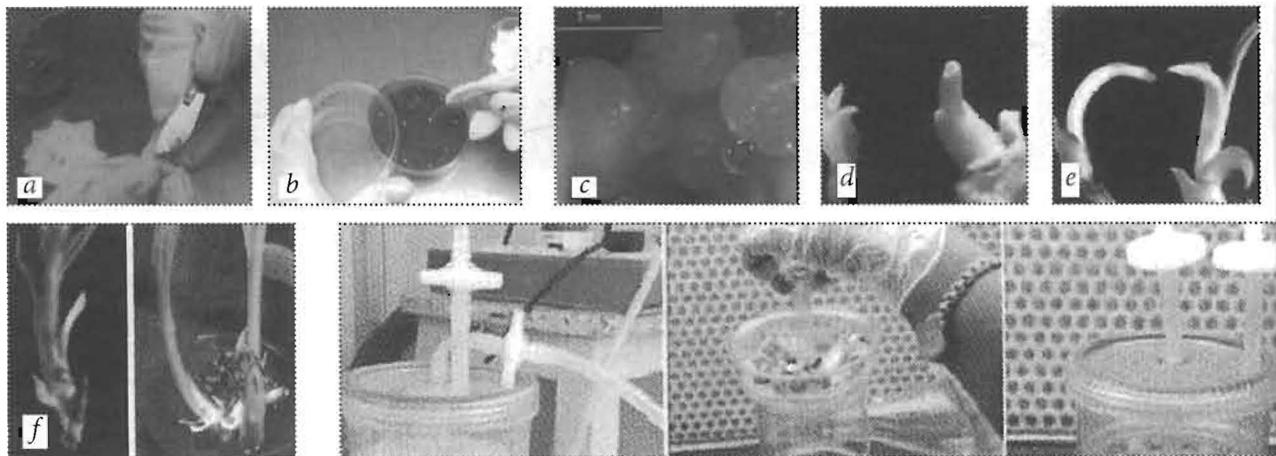
Different growth stages in coconut embryo culture

Sub culturing of the embryos at monthly intervals into full strength solid Y3 media supplemented with 4% sucrose, 0.5 mg/l BAP and 0.5 mg/l NAA. Additional amount of BAP or NAA are added (2 mg/l BAP, 5 mg/l IBA) in order to promote either shoot or root growth, if they are not balanced. Sucrose concentration is reduced to 3% after 3-4 subcultures

In vitro and *ex vitro* hardening of embryo cultured plantlets immature inflorescences and ovaries. Zygotic tissues viz., immature or mature embryos and embryo derived plumular tissues, were found to be easier to manipulate for achieving somatic embryogenesis. In spite of several concerted efforts, reproducible protocol for clonal propagation in coconut has not been achieved. Coconut is considered as one of the most recalcitrant species for *in vitro* culturing. *In vitro* recalcitrance in coconut has been attributed to many factors which include 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, under performance of regenerated plantlets and very slow rate of growth during *in vitro* culturing.

In vitro and *ex vitro* hardening of embryo cultured plantlets

Efforts for developing a protocol for *in vitro* regeneration of coconut using plumular explants was initiated at ICAR-CPCRI in the year 2000. Even though plantlets have been regenerated



Clonal propagation includes optimization of culture media, type of explants and plant growth regulators. (a-f) Plumular explants have shown positive results for callogenesis, somatic embryogenesis and in vitro plantlet regeneration.

and successfully established in the field, a commercial scale protocol has not been achieved and conversion of somatic embryos into plantlets has remained as one of the major bottlenecks. Various efforts were made in coconut tissue culture to refine the protocol such as use of novel plant growth regulators and media combinations. 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 has been limited. Thus refinement in protocol using other viable explants, along with plumule, as well as use of other alternative techniques such as bioreactors and cell suspension cultures assumes importance. Cell suspensions in specific medium would be ideal for producing large number of somatic embryos and to extract commercially important plant metabolites. However, several factors such as aeration, agitation, light and temperature would- influence the process as suspensions are maintained in flask culture. The embryogenic cells produced in cell suspension culture could be used in bioreactors to enhance somatic embryogenesis. Highly recalcitrant nature of coconut to in vitro culture necessitates alteration in conventional tissue culture approaches. Some of the factors such as pH, temperature, dissolved oxygen; CO2 concentrations could play a major role in somatic embryogenesis. Standardizing several of these factors constant could lead to an effective protocol with enhanced somatic embryogenesis.

The adoption of bioreactors in plant tissue culture is considered a major milestone since they offer several advantages viz., time saving, labour-saving, relatively easy to scale-up, allow enhanced growth and multiplication and improved nutrient availability due to the use of liquid medium over traditional tissue culture techniques. Many crop plants have been mass multiplied using bioreactors ever since its inception into plant tissue culture practices. As a plant production technique, bioreactors are far superior to traditional in vitro methods for all the species thus far tested. It is worth noting that with bioreactors, even the difficult-to-propagate woody and tree species can be produced relatively easily at high frequency. A hybrid reactor would be ideal to reduce the in vitro culture duration in coconut and also to enhance the rate of somatic embryogenesis and conversion of somatic embryos into plantlets. A system wherein explants flooded with nutrient medium containing growth regulators at regular time intervals has been successfully used in scaling up of somatic embryogenesis. Temporary immersion systems (TIS) offer the possibility of automating some culture stages and work has been initiated from this angle at ICAR-CPCRI.

Genetic transformation studies could be a tool in developing a viable clonal propagation protocol in coconut. Micro-projectile bombardment method was used initially for inserting GUS gene in to embryogenic calli and young leaf tissues of coconut. Genetic modification in coconut is still a long way away from becoming a reality. This could be useful for the improvement of coconut somatic embryogenesis by introducing genes which are known to regulate somatic embryogenesis in other plant species or by over expressing these genes in coconut in vitro cultures. Optimization of culture media, type of explant, plant growth regulators and their concentrations, sub culturing periods and other additives have paramount significance in developing repeatable tissue culture protocol. ■

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