Coconut Tissue culture

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Coconut is a major crop in tropical areas. It is mainly grown around sea area where temperature remains around 25° C. Its every part is useful in one way or the other. There is high demand of coconut seedlings in rainy season and even in winter season also. There are only a few private nurseries and state government nurseries which are not having the capacity to fulfill the demand of the farmers in the country. Therefore to fulfill the demand Coconut Development Board has established Demonstration cum Seed Production Farms in different states of India. Palms are propagated by seed, and despite continuing selection there is considerable variation between seedlings. Still there is some gap between demand and supply. To fulfill this need, a new technique i.e. Coconut Tissue Culture can be adopted in India to produce large number of quality planting material at a time during planting season. Large scale production of high yielding disease resistant coconut seedlings is possible only through modern rapid multiplication technique (tissue culture). Coconut planting material can be developed by tissue culture. Practically any plant in the world can be tissue cultured or propagated by micro propagation. Introduction of new varieties and hybrids with superior qualities is essential for coconut crop improvement. To achieve this goal by conventional means is very slow. However, the technique of producing the plant by tissue culture is vastly different for each plant and the cost of production varies with the kind of plant as well. One company in Philippines currently uses embryo culture. Plant tissue culture is the culture and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions (*in vitro*). Plant cell and tissue culture include the cultural techniques for regeneration of functional plants from embryonic tissues, tissue fragments, calli, isolated cells, or protoplasts. In commercial settings, tissue culture is often referred to as micro-propagation, which is in fact one of the techniques in tissue culture. Micro-propagation refers to the production of whole plants from cell cultures derived from explants (the initial piece of tissue put into culture) or meristem cells. Various Tissue Culture Types are pollen culture, callus tip culture, stem tip culture, meristem tip culture and protoplast fusion.

Haberlandt was the first scientist to produce whole plants from plant tissues and so he is popularly called as the "Father of Tissue Culture'. Plant tissue culture and molecular biology form the basis for genetic engineering. The history of coconut tissue culture can be traced back to the 1960s when Dr. De Guzman started her work on embryo culture. Since then, there have been review papers published which give more detail than is possible in this paper. They include general reviews emphasizing vegetative propagation by Pannetier and Buffard-Morel and Blake , as well as one another culture by Thanh-Tuyen . There are two further reviews on somatic embryogenesis, by Verdeil and Buffard-Morel and by Blake and Hornung. Coconut growing countries involved in tissue culture are the beneficiaries and mainly the Philippines (the first coconut oil producer) and Mexico. Coconut palm, the "Tree of Life", is one of the most important oil crops in the tropics. Coconut production over the years shows a gradual decline due to various reasons. However, it is still economically important for countries like Sri Lanka where there is still a high industrial demand for lauric oil. Therefore, introduction of new varieties and hybrids with superior qualities is essential for the survival of the industry. Conventional breeding programmes are the basis for the continuous genetic improvement in coconut. The success of the breeding programmes is slow due to limitations attributed to its biology, size and perennial nature. Thus the potential of a clonal propagation method of coconut to assist breeders is exciting. Coconut tissue culture research was initiated worldwide in early 1980s'. However, an economically viable or a practically sound protocol is yet to be developed.

The elaborate work on coconut cloning was done during 1981 to 87, along with other plants such as cashew and few woody trees to extend to certain innovative concepts and ideas. Clonal propagation through somatic embryogenesis will not give the desired results but the work has other potential for application. This article is an attempt to share in brief,

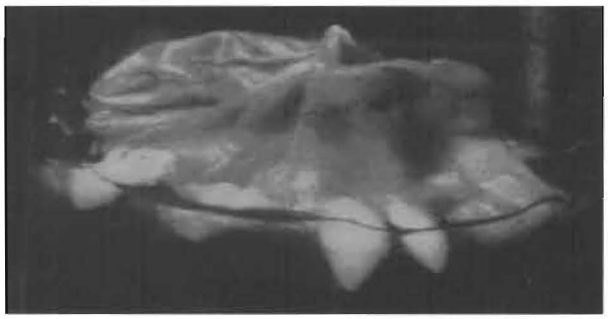


Fig -1

Fig -2

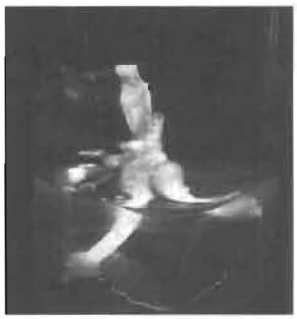


Fig -3

Fig -4

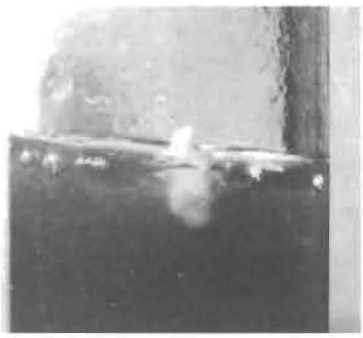


Fig -5

Fig - 6

Fig 1 to 6 shows coconut leaf giving rise to embryoids in high NAA and Sugar containing media and attempts to isolate and grow them resulting root formation.

the tendencies observed during dealing with the system by different research workers. In the random experimentation process those experiments where the NAA concentrations increased to abnormally high levels 60-120mg/l, pieces of tissues showed surprising capacity to survive and grow in the medium. It is rare in tissue culture experiments to use auxins concentration above 5mg/l . Increasing the sugar concentration also showed positive signs. The meristems were isolated in the early stages and were grown in medium containing different Cytokines. However, they tended to produce good roots and all attempts to manipulate these structures to produce shoots by varying the Auxins and Cytokinins ratio failed.

Conditions required for plant cells to grow *in vitro*:

- **Freedom** from competitions particularly from microorganisms for nutrition and other factors
- Availability of nutrients and removal of waste products for better growth of the tissue
- A controlled environment to maintain the cultures

Tissue culture offers significant benefits over traditional propagation methods.

- Much faster rates of growth can be induced *in vitro* than by traditional means.
- Multiplication of plants which are very difficult to propagate by cuttings or other traditional methods.
- Production of large numbers of genetically identical clones in a short time
- Seeds can be germinated with no risk of damping off/ predation.
- Under certain conditions, plant material can be stored *in vitro* for considerable period of time with little or no maintenance
- Tissue culture techniques are used for virus eradication, genetic manipulation, somatic hybridization and other procedures that benefit propagation, crop improvement, and basic research.

A paper entitled **"What makes clonal propagation of coconut difficult?"** was published in Proceedings Asia Pacific Conference on Plant Tissue and Agribiotechnology (APaCPA) 17-21 June 2007. In this research it was found that introduction of new varieties and hybrids with superior qualities are essential for crop improvement. However, to achieve this goal by conventional means is very slow. Tissue culture is the only tool available for speeding up the process.

Despite the success story, there are many constraints that hinder the progress in optimizing the clonal propagation protocol. What makes

clonal propagation of coconut difficult? The factors limiting the development of a clonal propagation protocol are:

1. Callogenesis and heterogeneous response of explants. The potential of various coconut tissues to undergo callogenesis has been tested. The results revealed that the embryogenic potential of coconut leaf explants is very low (< 10%) and limited to young leaves of a particular size (10-20 cm) of coconut seedlings (12-24 month old). Furthermore, the embryogenic capacity of leaf explants is also of short duration. This situation limits the use of coconut leaf for clonal propagation research.

2. Immature inflorescence is a promising explant as it contains numerous meristematic points. The success depends on the selection of inflorescence of correct maturity stage.

3. Lack of a proper marker to assess maturity. The best callusing reported so far is 30 %. Recent research unveiled the potential of unfertilized ovary as an initial explant for coconut cloning. The mean percentage of callusing with unfertilized ovary at correct developmental stage is 41 %.Further improvements to the culture conditions nearly doubled (76%) the callusing frequency.

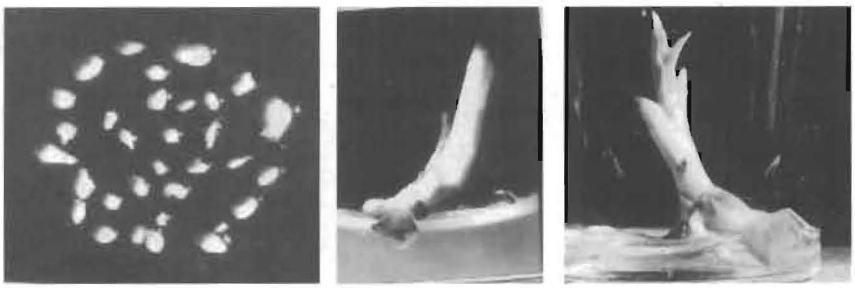


Fig -7 to 9 show a collection of embryoids that detech and fall into the meida and one of them germinating. Fig 9 shows a rare case of multliple shoots

4. Zygotic tissues like immature zygotic embryo and plumule (excised from mature zygotic embryo) hold potential for micro propagation. Unlike inflorescence and leaf tissues, the response of zygotic tissues is more consistent. However, the callusing frequency depends on embryo developmental stage. Under the best conditions callusing is usually above 75% in immature zygotic embryo and 55% in plumule explants.

5. Somatic tissues are the ideal explant for clonal propagation as the performance of mother palm is known. However, the response of these tissues are generally poor. Moreover, there is a marked difference among explants collected from different mother palms in terms of callus initiation. This might be due to difference in genotype or explant maturity.

6. Selection of a more responsive explant and culturing of tissues in media containing a range of hormone levels to suit the variable sensitivity of palms and the developmental stages of explants are some of the measures that can be applied to overcome this constraint.

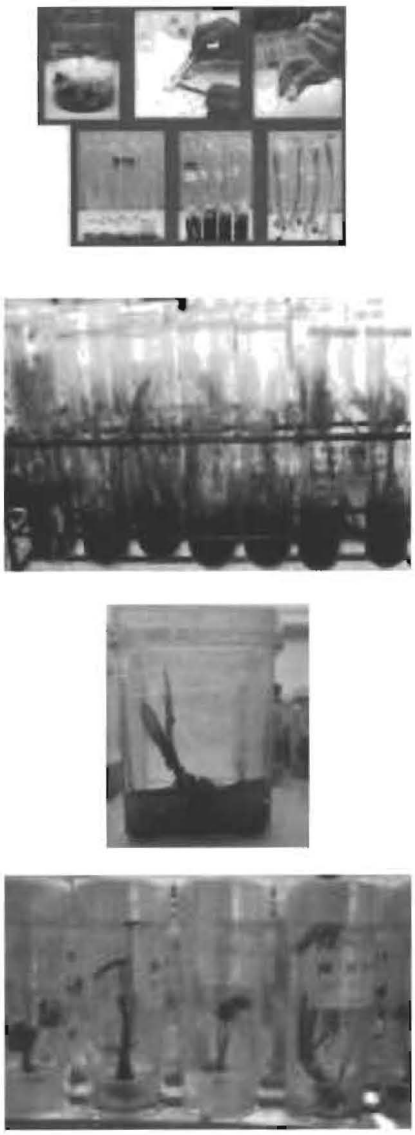
7. Effect of activated charcoal: Activated charcoal is an essential component of coconut tissue culture medium. It has strong adsorptive properties and its beneficial effects are attributed to the adsorption of phenols and other growth inhibitory substances. However, a major disadvantage of using activated charcoal is that it also can absorb plant regulators (hormones, vitamins) and some minerals (Cu and Zn). This creates undefined culture condi-tions which could lead to

Ovary Tissue culture experiment done in Srilanka.

variable tissue response and non-reproducible results. Different types/ brands/ batches of charcoal have different adsorptive capacities based on their origin, age, storage conditions and method of activation. This has a serious impact on callusing even from zygotic tissues. As a solution, a charcoal-free medium was developed. Addition of PVP (20 gl-1) and ascorbic acid (1100 ìM) to culture medium in place of charcoal gave rise to 60 % callus. However, the embryogenic potential of those calli was low. Use of a single type of charcoal under controlled conditions could be a way to overcome the problem. The use of custom-made coconut shell charcoal was found to be an effective solution as it is produced from a known and consistent source at a low cost. In order to minimize the indirect influence of activated charcoal, with regard to ABA level, currently the application of stress on somatic embryogenesis is being studied. Preliminary results show that heat stress (38 ÚC) for 2-3 days is capable of inducing somatic embryos in plumule-derived callus.

8. Callus multiplication. Coconut tissues generally produce highly heterogeneous compact callus. Establishment of cell suspensions is a useful tool for rapid multiplication of callus but it requires friable callus. However, despite the various treatments applied, no friable callus was obtained. The report by Perez-Nunez et al., (2006) on embryogenic callus multiplication by subdividing callus and repeated subculturing is an efficient tool for callus multiplication. Preliminary studies on the application of the method have produced encouraging results.

9. Somatic embryogenesis and plant regeneration. So far, the most frequent method used to induce somatic embryogenesis in coconut



Images of coconut tissue culture by coconut leaves

in Sri Lanka was the application of ABA (Fernando, 2001). The plant regeneration efficiency was further improved by incorporation of high agar induced water stress, PEG and AgNO3 in combination with ABA. However, the plant regeneration efficiency remained as low as 10% (Weerakoon, 2004). The results of histological studies showed that the presence of incomplete somatic embryos could be the reason for low plant regeneration efficiency. Further treatments applied to improve culture conditions for induction of complete somatic embryos and control treatment (ABA treatment) failed regeneration of plants. Detailed analysis of the procedure used revealed that the use of different types of charcoal at treatment development stage and treatment optimization stage might have caused inconsistent results.

10. Slow growth of regenerated plants. Growth of clonal plants in vitro and early ex vitro seemed to be very slow. When the growth of tissue-cultured plants was compared with embryo-cultured plants, despite being in in vitro for a prolonged period, clonal plants are smaller than embryo-cultured plants at the time of transplanting and they take longer time to reach field planting stage. Occasionally, it has been observed that callus originated from some individual plumules/ immature zygotic embryos could regenerate plants, at a high frequency including > 100 plants from a small callus independent of the treatment. This is a point to think if it is the genotype of the explant rather than the medium composition is the determining factor for obtaining positive results. This phenomenon complicates the improvement of the culture medium that is essential for induction and expression of somatic embryogenesis at a higher frequency. In order to minimize the indirect influence of activated charcoal, with regard to ABA level, currently the application of stress on somatic embryogenesis is being studied. Preliminary results show that heat stress (38 ÚC) for 2-3 days is capable of inducing somatic embryos in plumule derived callus.

The success of the breeding programmes is slow due to limitations attributed to its biology, size and perennial nature. Thus the potential of a clonal propagation

method of coconut to assist breeders is exciting. Coconut tissue culture research was initiated worldwide in early 1980s'. However, an economically viable or a practically sound protocol is yet to be developed. Current status of clonal propagation of coconut is in Sri Lanka. Since the inception of tissue culture research in 1983 at the Coconut Research Institute of Sri Lanka (CRISL), the response of various somatic and zygotic tissues to in vitro culture has been assessed. The work done over the years has indicated that clonal propagation of coconut is possible. It occurs through several steps; callogenesis, somatic embryogenesis, embryo maturation and germination. The resulting plants are genetically stable and their field performance is comparable to seed derived palms.

Although coconut is one of the most recalcitrant species to in vitro culture, the importance of developing a clonal propagation method is well accepted. Some of the difficulties encountered in realizing it have been identified. Efforts to standardize callusing medium and multiplication of embryogenic callus have given encouraging results. Use of the most suitable explant is one way of minimizing the genotypic effect on in vitro response of coconut. Further studies on the effect of stress on somatic embryogenesis might lead to the development of a reliable protocol for somatic embryogenesis as the dependence on ABA will be less. Culture medium should be fine-tuned to accelerate the growth of clonal plants in vitro and early ex vitro. Studies on gene transformation are a new avenue to be touched as in certain crops genes that affect in vitro regeneration have been identified. Genetic transformation using such genes could lead to a more efficient regeneration system of somatic embryogenesis. The only hope for coconut palm's asexual propagation lie on in vitro vegetative multiplication of high performance individuals using in vitro culture techniques and more particularly in somatic embryogenesis remains the only hope for a substantial improvement in the productivity of plantations. Unfortunately, however coconut is a highly recalcitrant species as far as tissue culture is concerned. In 1995, several groups involved in coconut regeneration research gathered together for the first time to start a joint effort to overcome the major difficulties encountered in coconut regeneration. Solid progress for the mastery of coconut regeneration has been made under this project funded by the European Community. Results obtained have allowed the regeneration of vitroplants in all the laboratories involved in the project. For the first time in coconut, reliable protocols for plantlet regeneration have been obtained and can be duplicated in different laboratories. This represents an important breakthrough for coconut regeneration obtained within the duration of the project. An international co-operation is needed on coconut tissue culture. Coconut is a highly recalcitrant crop poor in vitro tissue. In the past negative competition between the different teams led to an exchange of "advertising information" but a very poor exchange of scientific information. Coconut micro propagation require longterm research programmes with assured funding support which some countries cannot reliably provide without international assistance. This will increase research efficiency avoiding duplication of work and promote complementation and synergy of activities. Impact assessment information is to be carefully documented about the results obtained by the project. For the first time in coconut, reliable protocols for plantlet regeneration are available. Plantlets have been obtained through somatic embryogenesis by all the partners involved in the Project. Key factors for somatic embryogenesis in coconut have been identified. Putative protein markers for embryogenic tissues have been identified. Basic knowledge on in vitro tissue physiology have been increased. Links and exchanges between south and north partners have been strengthend. Capabilities of researchers from south countries were upgraded. Coconut growing countries involved in tissue culture are the beneficiaries and mainly the Philippines (the first coconut oil producer) and Mexico. Dissemination of the results through Technical Reports, publication of papers in international scientific journals. Due to lack of research, technology, lab and other infrastructure facilities in India Coconut Tissue culture is not get commercialized till date. Unless we are able to produce millions of plants over several years, or if that particular variety was rare, it would be unviable to propagate by tissue culture. Especially in case of trees, the R&D investment into finding the protocol for tissue culture, combined with long production time would make coconut more expensive than it is worth! At last it is hoped that India will be a member of this joint venture of research works and publications in next 25 years to boost the future of coconut tissue culture in India.