Part I. Papers presented to evaluate the status of research on coconut embryo culture and identify research gaps

COGENT and 1997 activities; workshop objectives and expected outputs - Pons A. Batugal

Current state of the art and problems with in vitro culture of coconut embryos - Florent Engelmann

State of research on coconut embryo culture and acclimatization techniques in UPLB - Aurora G. Del Rosario

State of research on coconut embryo culture and acclimatization techniques in the IDEFOR (Côte d'Ivoire) and ORSTOM/CIRAD laboratories (France) - J.L. Verdeil, V. Hocher, K. Triques, R. Lyakurwa, A. Rival, T. Durand-Gasselin, F. Engelmann, A. Sangare and S. Hamon

Status of research on coconut embryo culture and acclimatization techniques in India - Anitha Karun, Anuradha Upadhyay and V.A. Parthasarathy

Status of research on coconut embryo culture and acclimatization techniques in Indonesia - Nurhaini Mashud

Status of research on coconut zygotic embryo culture and acclimatization techniques in Mexico - C. Talavera, C. Oropeza, A. Cahue, J. Coello and J. Santamaría

Embryo culture activities at the Philippine Coconut Authority-Zamboanga Research Center (PCA-ZRC) - Ambrosio Raul R. Alfiler

Status of coconut (Makapuno) embryo culture and acclimatization techniques in VISCA, Baybay, Leyte, Philippines - Víctor M. de Paz

PCA's embryo culture technique in the mass production of Makapuno coconuts - Erlinda P. Rillo

Status of research on coconut embryo culture and acclimatization techniques in Papua New Guinea - Tore Ovasuru and Mathias Faure

Status of research on coconut embryo culture and acclimatization techniques in Sri Lanka - V R M Vidhanaarachchi, L K Weerakoon, S C Fernando, C K A Gamage and E S Santha

Status of research on coconut embryo culture and acclimatization techniques in Tanzania - Kennedy Mkumbo, Salustia Tembo and Reminister Marealle

COGENT and 1997 activities; workshop objectives and expected outputs - Pons A. Batugal

Senior Scientist and COGENT Coordinator, IPGRI, P.O. Box 236, UPM Post Office, 43400 Serdang, Selangor

Fellow coconut researchers, ladies and gentlemen:

Before I present the rationale and expected outputs of this workshop, please allow me to give you a brief description of the International Coconut Genetic Resources Network (COGENT), its goal and objectives.

COGENT is a global research network organized for and on behalf of coconut producing countries. It now has 35 coconut-producing member countries as shown in
Annex 1. The network was organized by the International Plant Genetic Resources Institute (IPGRI) in 1992 when the Consultative Group on International Agriculture Research (CGIAR) included coconut in its research agenda.

The goal of COGENT is to improve coconut production on a sustainable basis and to increase incomes in developing countries through improved cultivation of the coconut and efficient utilization of its products. COGENT aims to develop and implement an international mechanism to coordinate research activities of national, regional and global significance, particularly in germplasm exploration, collecting, conservation and enhancement. It also aims to establish a basis for collaboration on the broader aspects of coconut research and development.

IPGRI provides technical and administrative support to COGENT. Currently, it provides funding and support to member countries which undertakes various research projects and training activities. The activities for 1997 are shown in Annex 2 which includes this workshop.

As you may know, one of the priority projects of COGENT is the collecting, conservation and use of coconut genetic resources. Due to the recalcitrant and bulky nature of the coconut fruit, it has been difficult to collect and conserve representative germplasm, especially those from distant and isolated locations. The use of embryo culture techniques could alleviate the problem of collecting and exchange of germplasm because of the small size of the embryo material and for not being a carrier of some diseases. However, the technique has some limitations. Due to some deficiencies in the protocols used, the rate of survival from the embryo to the seedling stage has been quite low and the results in various laboratories have been variable. Thus, it is possible that for every embryo lost, an important diversity may have been lost.

To improve the situation, this workshop is being organized to assess the status of the coconut embryo culture and acclimatization technology, and to upgrade and standardize the protocols so that more coconut researchers could use it with better efficiencies. The objectives of the workshop are: 1) to present the latest results of coconut embryo culture and acclimatization research in various laboratories and assess the status of the technology; 2) to identify bottlenecks and areas for improvement in the technology; and 3) to develop a coordinated research agenda to remedy the identified technology constraints.

The expected outputs of the workshop are: 1) a status report on the deficiencies/constraints of the embryo culture and acclimatization technology based on reports presented; 2) draft project research proposals for the next two years aimed at solving current difficulties limiting the development and use of an optimized in vitro culture protocol which can easily be applied by non-specialists; 3) recommendations of suitable mechanisms to achieve greater collaboration among embryo culture researchers.

The tasks for this workshop are very challenging. However, with the capability and commitment of participants, I am optimistic that the workshop will be a success.

Annex 1. COGENT members and invitees

<table>
<thead>
<tr>
<th>Southeast and East Asia</th>
<th>South Asia</th>
<th>South Pacific</th>
<th>Africa/Indian Ocean</th>
<th>Latin America/Caribbean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Indonesia</td>
<td>2. India</td>
<td>2. Côte d'Ivoire</td>
<td>2. Costa Rica</td>
</tr>
</tbody>
</table>
| **Part I. Papers presented to evaluate the status of research on coconut embry...**

http://www2.bioversityinternational.org/publications/Web%5Fversion...
Annex 2. COGENT activities in 1997

Coconut Cadang-cadang viroid-like sequences meeting, Malaysia 21-23 April

The report on the detection of Coconut Cadang-cadang Viroid-like sequences in 1991 and the assumption that it could cause a serious threat to the coconut industry has virtually stopped coconut germplasm movement and the use of imported germplasm to develop improved varieties. The meeting was held to clarify the nature, pathogenicity and risks associated with Coconut Cadang-cadang viroid-like sequences (CCCVd-Ils.) after more research had been undertaken over the last five years. International participants consisting of pathologists, biotechnologists, safe germplasm movement specialists and coconut researchers attended the meeting to examine the issues based on available data. The proceedings of the meeting will be published soon.

Regional STANTECH trainers' courses: Africa Regional Course - Côte d'Ivoire, 16-15 June; LAC Regional Course - Jamaica, 16-26 July

Lack of well trained coconut researchers has hampered coconut germplasm conservation and use. To alleviate this constraint, two regional trainers' courses on standardized research techniques in coconut breeding (STANTECH) were held in Côte d'Ivoire for Africa and in Jamaica for LAC on 16-25 June and 16-26 July, respectively. The Côte d'Ivoire course, was attended by 9 country participants and the Jamaica course, 6 country participants. The participants were requested to train national researchers upon their return to duty station to provide a multiplier effect to the training courses. The Côte d'Ivoire course was hosted by the Marc Delorme Station Cocotier of IDEFOR while the Jamaica course was hosted by the Jamaica Coconut Industry Board. These courses were funded by the Common Fund for Commodities and IPGRI.

LAC regional project proposal formulation meeting, Jamaica, 7-12 July

The coconut industry in the LAC region suffers from low productivity and Lethal Yellowing Disease infection in some countries, resulting in low income for coconut farmers. To alleviate these constraints, the members of the LAC coconut research network decided during its meeting in Mexico last November 1996 to develop a collaborative regional research project. On 7-12 July, a project formulation meeting was held in Kingston, Jamaica to develop a regional coconut research project proposal for the LAC network. The meeting was hosted by the International Institute for Cooperation in Agriculture (IICA) at its Kingston office and co-funded by BUROTROP, IICA and IPGRI/COGENT. The draft proposal is currently being reviewed by member countries, IPGRI, COGENT, BUROTROP and IICA before revision and submission to a donor.

Coconut collecting and conservation training course, Philippines 1-12 September
Due to the bulky nature of the seednut and the perennial nature of the crop, germplasm collecting and conservation need to be efficient and effective. Hence, there is a need to train coconut researchers on efficient collecting and conservation strategies. On 1-12 September, the course was conducted at the Zamboanga Research Center of the Philippine Coconut Authority in Zamboanga City, Philippines. Eleven participants from 8 countries attended the course. This course is a preparation for the implementation of the proposed ADB-Phase 2 project in the Asia-Pacific region. The training course was funded by the Common Fund for Commodities.

**IFAD project meeting, Indonesia, 15-17 September**

The IFAD-supported project entitled "Sustainable use of coconut genetic resources to enhance incomes and nutrition of smallholders in the Asia-Pacific region" held its first project meeting on 15-17 September in Bogor, Indonesia. The meeting discussed the project proposals of 14 coconut-producing countries indicating ways to enhance farmers’ incomes and to promote germplasm conservation and use. It also discussed the IPGRI project on "Farmer participatory research to add value to coconut genetic resources and promote conservation and use of coconut diversity". The meeting was hosted by the Indonesian Agency for Agricultural Development (AARD) and funded by IFAD and ADB.

**CGRNAP annual review and planning meeting, Indonesia, 18-20 September**

The Asian Development Bank-supported IPGRI project entitled "Technical Assistance for a Coconut Genetic Resources Network for Asia and the Pacific (CGRNAP)" held its final annual project meeting on 18-20 September in Bogor, Indonesia, also hosted by AARD. In this meeting, the 13 country projects under the CGRNAP Phase 1 project and 23 project proposals under ADB Phase 2 project were successfully reviewed. The meeting also discussed the status of the International Coconut Genetic Resources Database and plans for the establishment of a multi-site International Coconut Genebank in 4 regions of the world. The meeting was hosted by the AARD and funded by the Asian Development Bank and IPGRI.

**International coconut embryo culture and acclimatization workshop, Philippines, 27-31 October**

The development and refinement of embryo culture and acclimatization techniques is imperative as we urgently need a complement to the use of seednuts for safe movement of germplasm. Hence, this workshop hosted by the PCA-Albay Research Center in Guinobatan, Albay, Philippines. The workshop is attended by 12 leading embryo culture practitioners from 10 countries of Asia-Pacific, Africa and LAC. The workshop is funded by the Common Fund for Commodities and the DFID.

This workshop will review the status of the technology, upgrade and standardize embryo culture and acclimatization techniques and develop coordinated experiments to test the upgraded technology upon return of the participants to their duty station. Results of experiments for testing the upgraded technology will be reported in a second workshop in 1999 to further upgrade the technology.

**Multilocation variety/hybrid trials workshop, Côte d'Ivoire, 10-12 November**

This is a joint workshop of the CFC-funded project on multilocation hybrid/variety trials involving Benin, Côte d’Ivoire, Tanzania, Brazil, Jamaica and Mexico, and the ADB-funded germplasm evaluation project involving Fiji, Tonga, Western Samoa, Vanuatu and Malaysia. The workshop will bring together the project leaders and regional coordinators to discuss work plans and to standardize and upgrade evaluation methodologies. The development of a common performance data set and a minimum data set of climatic and edaphic factors will also be discussed. This workshop will enable the countries to compare results and derive a wider and more meaningful application of research results in developing improved varieties and hybrids for...
low-income farmers. The workshop will be hosted by the Marc Delorme Station Cocotier in Côte d'Ivoire on 10-12 November and will be funded by the Common Fund for Commodities BUROTROP/CTA and IPGRI.

**COGENT Steering Committee meeting, Côte d'Ivoire, 13-15 November**

The 6th annual meeting of the committee will be hosted by Marc Delorme Station Cocotier in Côte d'Ivoire on 13-15 November. The meeting will be attended by the 11 members of the Steering Committee plus representatives from IPGRI, BUROTROP, CIRAD, CFC and other partner organizations. The meeting will discuss the progress reports of the five regional sub-networks, ongoing and planned activities and projects which include those funded by CFC, IFAD, ADB, the proposed multi-site International Coconut Genebank and the training needs of COGENT member countries. The meeting will be funded by the DFID, IPGRI and BUROTROP/CTA.

**Current state of the art and problems with in vitro culture of coconut embryos - Florent Engelmann**

IPGRI, Via delle Sette Chiese 142, 00145 Rome, Italy

**Introduction**

*In vitro* culture techniques have important applications for the collecting, exchange and conservation of coconut germplasm. Among the plant kingdom, coconut is one of the species with seeds of the largest dimensions. Moreover, there is no dormancy period and germination immediately follows maturation of the seed. These two characteristics drastically limit the amount of material which can be gathered during collecting missions. Simple and efficient *in vitro* field collecting techniques have been established by various research groups. They involve extracting the embryos from the nuts and inoculating them directly *in vitro* (Assy-Bah et al. 1987; Sossou et al. 1987; Karunaratne 1988; Rillo and Paloma 1991; Karun et al. 1993; Ashburner et al. 1995).

For germplasm exchange, the FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm recommend that coconut germplasm be distributed as zygotic embryos *in vitro* to reduce chances of introducing diseased material into disease-free areas (Frison et al. 1993). *In vitro* techniques have been used in some instances for the international exchange of coconut germplasm in the form of excised embryos inoculated *in vitro*.

As regards the conservation of coconut germplasm, preliminary experiments have indicated that it is possible to store zygotic embryos *in vitro* for one year in the growth room and to successfully germinate them afterwards (Assy Bah and Engelmann 1993). The feasibility of cryopreserving zygotic embryos in liquid nitrogen (-196°C) for long-term conservation of coconut germplasm has been demonstrated by Assy-Bah and Engelmann (1992).

Using *in vitro* techniques for collecting, exchanging and conserving coconut germplasm requires efficient protocols for *in vitro* germination and development of embryos into whole plantlets and for their acclimatization to *in vivo* conditions and further development into plants which can be transferred to the field. *In vitro* culture protocols for coconut zygotic embryos have been established by various coconut research institutes in Côte d'Ivoire, France, India, the Philippines and Sri Lanka (Assy-Bah 1986; Assy-Bah et al. 1989; Karun and Sajini 1993; Del Rosario and De Guzman 1976; Rillo and Paloma 1992; Karunaratne et al. 1991). These protocols have been applied by several additional coconut research institutes in Africa, Asia and Latin America with generally low success. The main problems and losses of material occur apparently during the acclimatization of plantlets to *in vivo* conditions.

The planned establishment of the multi-site international coconut genebanks (Rao and
Batugal 1998) and the various international breeding and testing programmes coordinated by COGENT would require extensive exchange of coconut germplasm. This should take place using in vitro cultured embryos to avoid risks of transmitting diseases in disease-free regions and to reduce shipment costs. The technology would also be useful in collecting missions especially in distant and isolated locations where transporting bulky seednuts would be a problem.

In this context, it was considered necessary to have a more precise idea of the performances, bottlenecks and problems with the implementation of the existing coconut embryo in vitro culture protocols, both in institutes where they have been established and in institutes where these protocols have been applied. This would allow the identification of priority research areas to be addressed by participants in this International Coconut Embryo Culture and Acclimatization Workshop at PCA Albay Research Center, Philippines from 27 to 31 October, 1997.

**Analysis of questionnaire on current application of coconut embryo in vitro culture techniques**

A questionnaire on the current application of coconut embryo in vitro culture techniques (see Attachment I) was sent to relevant institutes worldwide and the responses received from 11 institutes were carefully analyzed. The results reported were obtained on a total of more than 30 different varieties and hybrids.

**A. Collecting and introduction of embryos in vitro**

Seven out of the 11 institutes have inoculated embryos both in the field and in the laboratory, the 4 remaining institutes in the laboratory only. Contamination rates are very low to low (3 to 13% maximum) and no problems are reported at this stage.

**B. In vitro culture of embryos**

The protocols employed differ in the culture conditions employed: some protocols use solid or liquid medium only throughout the in vitro culture phase, others a succession of liquid/solid medium. The germination of embryos is generally performed in the dark, and the further development of plantlets under light conditions, but alternating light and dark periods from the culture initiation onwards is also mentioned. The culture media include the mineral solution of, or adapted from Eeuwens (1976) or that of Murashige and Skoog (1962). They vary mainly in the concentration of sucrose (from 30 to 60 g/l) and the presence of growth regulators (auxins), which are sometimes added to stimulate the production and development of roots.

There is a very large discrepancy between laboratories in the percentage of embryos which develop into whole plantlets and in the duration required to reach this stage: 40 to 86% of the embryos inoculated give whole plantlets within 3 to 14 months after inoculation. A very large heterogeneity in the response of embryos of a same batch is noted by several respondents. The only difference noted between varieties is that embryos of Tall varieties generally germinate and grow more rapidly than Dwarfs, as observed with seedlings.

The relatively poor results uncovered in terms of production of whole plantlets clearly indicate that there is scope for improvement of the in vitro culture protocol. The heterogeneous response of embryos may be partly due to the fact that embryos inoculated are at different maturity stages since they are generally sampled on open-pollinated nuts. The huge differences between laboratories in the average duration requested for producing plantlets ready for transfer in vivo can be partly explained by the heterogeneous response of embryos, but they are also due to the fact that laboratories transfer plantlets in vivo at very different developmental stages (from small plantlets with 5 cm-long roots and two small leaves to plantlets with 4-5 open leaves and a strong root system), and thus after very different in vitro culture periods.
C. Weaning of in vitro plantlets and further development in vivo

In all cases, plantlets are first transferred under high humidity, usually achieved by covering them with a polypropylene bag, followed by progressive adaptation to the surrounding conditions. Different potting mixtures and fertilizing solutions are employed. The success rate of plantlet establishment varies dramatically between laboratories, from 10% only to almost 100% of the plantlets transferred in vivo, with the majority of reports mentioning 60-70% of success. Not surprisingly, the good results are obtained by laboratories which have been working on coconut in vitro culture for a long time and have thus refined the weaning protocols, and the poor results come from laboratories performing their very first weaning experiments. This step of the process can thus be improved but, more importantly, precise guidelines must be produced and staff trained in the handling of in vitro plantlets, which are much more fragile than seedlings.

Very few systematic observations and measurements have been performed on plantlets beyond the acclimatization stage. A couple of reports mentioned that in vitro plantlets grow more slowly than seedlings.

D. General comments

The general comments received concerned the necessity of an overall improvement of the process, especially the establishment of plantlets, and the difficulties created by the great heterogeneity of the embryos inoculated. The importance of training staff to the technique was highlighted in one report.

Conclusion

The analysis of this questionnaire was very informative and allowed researchers to identify more precisely the current bottlenecks of the coconut embryo in vitro culture process. Interestingly, the establishment of plantlets in vivo, which was considered the major bottleneck, should indeed be improved but proved to be a major difficulty only in laboratories which were not familiar with handling of in vitro plantlets. As a complement to experiments aiming at increasing the efficiency of this step of the process, the publication of detailed technical guidelines and training of staff in the manipulation of plant material coming from in vitro culture should improve the situation.

The major new finding was that the in vitro culture process itself, i.e. from the inoculation of embryos in vitro to the production of whole plants ready for transfer in vivo is far from being fully efficient and there is scope for significant improvement. Finally, additional data should be collected on the development and growth in the field of plants coming from in vitro culture, in comparison with seedlings.

It is our hope that this analysis of the current state of the art and problems of coconut embryo in vitro culture will be useful to the Workshop participants in the planning of experiments aiming at solving the technical and scientific bottlenecks identified.

Acknowledgements

The contribution of all colleagues who have taken the time to fill and return the questionnaires is gratefully acknowledged.

References


**Attachment I. Questionnaire on the current application of coconut embryo *in vitro* culture techniques**

To: Marlene Diekmann/Florent Engelmann From:

IPGRI
Questionnaire on the Application of Coconut Embryo *in vitro* Culture Techniques

**A - collecting and introduction of embryos *in vitro***

A1 - Which method was used for collecting and introducing embryos *in vitro*?

- In case *in vitro* collecting was used, please describe briefly the technique and indicate average success rate of collecting and introduction *in vitro* and problems if any (e.g. contamination rate): __________
  
- In case embryos were inoculated in the laboratory, please describe briefly the technique and indicate average success rate of introduction *in vitro* and problems if any (e.g. contamination rate): __________

A2 - How many different varieties were used? ____________________________

A3 - Were there any differences in response between the varieties used? If yes, please specify. ____________________________

**B - *in vitro* culture of embryos**

Please describe briefly the technique used:

B1 - culture medium (or sequence of culture media): __________

B2 - light and temperature conditions: ____________________________

B3 - average duration required to produce fully developed plantlets: __________

B4 - average success rate of *in vitro* culture: __________

B5 - How many different varieties were used? ____________________________

B6 - Were there any differences in response between the varieties used? If yes, please specify. ____________________________

**C - weaning of *in vitro* plantlets and further development *in vivo***

C1 - Which conditions were applied for the establishment in soil following culture? __________

C2 - What was the rate of plantlet establishment in soil? __________

C3 - Did you observe differences in the development of *in vitro* plantlets compared to seedlings? If yes, please specify the nature of differences observed (e.g. delayed growth). __________

C4 - Did you observe any plant diseases in the plantlets? __________

C5 - On how many plants were the observations performed? __________

C6 - For how long were the observations on development of *in vitro* plantlets performed? __________

C7 - How many different varieties were used? __________

C8 - Were there any differences in response between the varieties used? If yes, please specify. __________
D - General Comments

Which problems do you see in the wide application of the method and which priority areas for can you suggest?

Status of research on coconut embryo culture and acclimatization techniques in UPLB - Aurora G. Del Rosario

Associate Professor, Department of Horticulture, College of Agriculture, University of the Philippines at Los Baños, College, Laguna, Philippines

Introduction

Embryo culture involves growing the embryo artificially by providing the necessary nutrients and conditions for its growth and development into a seedling that could be successfully established in the field.

The embryo culture technique in coconut (Cocos nucifera L.) has been successfully applied in the following areas:

1. The well recognised use of embryo culture to overcome the barrier of incompatible crosses and to rescue embryos that cannot continue to develop normally within the seednut has been achieved in coconut. This has been found to be the only means of germinating the Makapuno coconut (De Guzman and Del Rosario 1964; Del Rosario and De Guzman 1976).

2. In coconut germplasm collecting, conservation and exchange, the embryo culture technique could ease up and facilitate the limited exchange of germplasm because instead of the bulky seednuts, 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 (Assy Bah et al. 1987; Rillo and Paloma 1991; Ashburner and Thompson 1991).

3. With advances in technology and more knowledge on the toxins released by pathogens, embryos in culture could be screened for disease and pest reaction. Thus, germplasm could be evaluated at early stages without having to establish a plantation which would require a lot of space, time and manpower (Rillo and Paloma 1989).

This paper is a brief account of the status of the research on coconut embryo culture and acclimatization studies done at the University of the Philippines at Los Baños.

Available facilities

The University of the Philippines at Los Baños has facilities for *in vitro* culture work located in several buildings. Of these, one laboratory is devoted solely for mass propagation of Makapuno coconut by embryo culture. These include media preparation room with analytical balance, top loading balance, triple beam balance, stirrer/hot plate, pH meter, pressure cooker, autoclave, various types of glassware and chemicals. In the inoculating room, there are two laminar flow benches, a stereo microscope and a research microscope. There are two culture rooms with 200 square meter effective shelf area with controlled light and temperature facilities. For acclimatization, a 40 square meter greenhouse is equipped with misting facilities and air conditioner. A portion of the adjoining 200 square meter nursery area is provided with 50% and 30% shading facilities. Other units in the University have facilities for analytical work as well as for gas and liquid chromatography.

Development of the embryo culture technology
Early work on coconut embryo culture was initiated at the University of the Philippines with support from the National Research Council of the Philippines to overcome the problem of non-germination of the Makapuno coconut (De Guzman and Del Rosario 1964).

The normal coconut has a hard and crisp solid endosperm at maturity, while the Makapuno coconut is characterized by a soft, thick solid endosperm and jelly-like, viscous liquid endosperm that fills the nut cavity. It is a highly priced coconut used in the ice cream and pastries industries and for sweetened preserves. However, the yield of Makapuno-bearing palms, which are heterozygous for the character, is only 2 to 20% and the phenomenon is believed to be governed by a single recessive gene (Zuñiga 1953).

The Makapuno embryo is visually and anatomically similar to that of the normal coconut but does not germinate naturally because the abnormal endosperm, which supports germination of the embryo under normal conditions, rots. Thus, pure-bearing Makapuno coconut palms have not been obtained in nature. With the embryo culture technique, Makapuno seedlings have been successfully established in the field giving a yield of 80 - 100% Makapuno nuts.

The excised embryo was found to be capable of germinating when cultured on solid White's medium (De Guzman and Del Rosario 1964). Further improvement in germination and development of the embryos were obtained in Murashige and Skoog's (MS) medium with 15% coconut water and gibberellin (De Guzman 1970). Later, it was found that initial culture in White's liquid medium was very necessary for subsequent development upon transfer to MS agar medium (Balaga and De Guzman 1970). However, root development was very limited resulting in very low survival rate upon transfer to pots condition. Increasing the sugar level to 8% during a second solid phase of culture led to better root development and greater chances of survival (De Guzman et al. 1971). A subsequent study showed that increasing sugar level in White's medium during the initial liquid culture greatly improved germination and root development of embryos when transferred to MS agar medium with 4% sucrose (Del Rosario and De Guzman 1976). Part of the sugar acted as an osmotic agent.

Addition of 1% activated charcoal in the first solid culture greatly increased percent germination, rooting and shoot development. Also, decapitation of the root tips prior to transfer to a second solid medium resulted in higher percentage of lateral root formation, which are the functional and absorbing roots in vivo, thus enhancing their chances of survival ex vitro (De Guzman and Manuel 1977).

Germination was further enhanced with the addition of 195 mg KCl to the initial White's liquid medium (Miniano and de Guzman 1978). In 1983, Eeuwens's Y3 medium (Eeuwens 1976) was used in the first solid culture resulting in better growth of the seedlings in vitro. This is a special formulation for coconut tissue culture which contains high levels of potassium and chlorine needed in all stages of development of the coconut.

Substitution of agar with food grade agar "gulaman" bars as gelling agent in the solid phase of culture gave equally good germination and reduced production cost. Root development of the seedlings was further enhanced upon transfer to Y3 liquid medium with activated charcoal. When the seedlings have developed sufficient lateral roots, they are then transferred to greenhouse conditions and acclimatized to natural light and room temperature for 2 weeks.

**Acclimatization studies**

The success of any in vitro culture technology is measured not only in the number of plants regenerated or obtained in vitro but more importantly, the number of plants that have been transplanted and established in the field and grown to maturity. The major bottleneck in coconut embryo culture is the low survival rate after transplanting from the
culture bottles to potted soil in the greenhouse.

If the coconut embryo culture technology is to be of service in germplasm collecting and genetic conservation, breeding, pest and disease screening and commercial production or experimental work, it is necessary that the in vitro derived seedlings can be transplanted to the greenhouse and field without loss and with minimal cost and labor.

Coconut seedlings grown in vitro with 3-4 leaves and sufficient lateral roots are then acclimatized to natural light and the greenhouse environment for two weeks and then transplanted from the culture bottles to non-aseptic conditions in potted sand in the greenhouse. At this stage, the seedlings would experience severe physiological stresses when they must quickly adapt from heterotrophic nutrition in vitro to autotrophic existence in vivo. Changes in growing conditions such as temperature, relative humidity, and water loss contribute to the low survival ex vitro.

Studies on the changes in photosynthetic capacity and anatomical leaf characteristics during acclimatization to greenhouse conditions showed that leaves of embryo cultured coconut seedlings at transplanting stage have very low photosynthetic ability, low chlorophyll content, no carotenoid (Table 1), low stomatal index and were amphistomatous (Table 2) as compared to in situ grown seedlings (Malijan and Del Rosario 1986). This could be attributed to nutritional and environmental factors. Photosynthetic competence of the embryo cultured seedlings was found to begin 4 weeks after transplanting from culture vessels to pots. Full photosynthetic competence was attained 8 weeks after transplanting. This coincided with the full development of a new leaf under greenhouse conditions which was hypostomatous, with higher chlorophyll and carotenoid content.

**Coconut embryo culture protocol**

The current protocol used in our laboratory starts with the harvesting of mature green (9 to 11 month-old) Makapuno coconuts. After dehusking, the nuts are split crosswise and with the use of a cork borer, the embryo together with the surrounding endosperm, is extracted and collected. The collected embryos are washed thoroughly with soap and water. They are then sterilised in 5% sodium hypochlorite solution for 20 min.

Inside a transfer chamber, or laminar flow bench, decanting of the sodium hypochlorite solution is done. Using forceps and scalpel previously sterilised by flaming with an alcohol lamp, the embryos are excised from the endosperm cylinders in sterile Petri dishes or on sterile paper. The embryos are collected in a sterile flask (30 embryos per flask) and sterilised again with 1% sodium hypochlorite solution for 10 min. After decanting the sterilising solution, the embryos are rinsed three times with sterile distilled water. Embryos are inoculated individually in 18 x 150 mm test tubes containing 10 ml of Y3 liquid medium with 0.25% activated charcoal and 6% sucrose. They are incubated in the dark at 27-30°C and checked periodically for contamination.

After 4 weeks, the embryos enlarge and some show protrusion of the shoot and/or root. They are then transferred to 70 ml of Y3 agar medium with 0.25% activated charcoal and 6% sucrose in catsup bottles. They are incubated in lighted shelves at 27-30°C under a 9 h light/15 h dark photoperiod. After 4 to 6 weeks, 86% of the embryos germinate with varying rates of shoot and root development. The germinated embryos are then transferred every 4 to 6 weeks to freshly prepared medium of the same composition. The root tips are decapitated prior to inoculation. Well developed seedlings with at least 3 to 4 leaves are brought to the screenhouse and subjected to natural light and room temperature.

After 2 weeks, the seedlings are taken out of the culture bottles and washed thoroughly with water to remove the medium. They are dipped in 2 g/l fungicide solution (Dithane M-45) and transplanted to sterilised sand. They are covered with a plastic bag and exposed to 50% shade. The plastic bags are gradually loosened or lifted and after 8 weeks, the seedlings are transplanted to compost: sand (1:1) mixture and kept under...
30% shade. After 3 to 5 months, the seedlings can be transferred to field conditions.

References


Assy Bah, B. T. Durand-Gasselin, and C Pannetier. 1987. Use of zygotic embryo culture to collect germplasm of coconut (Cocos nucifera L.) Plant Genetic Resources Newsletter. 71:4-10


Thanh-Tuyen, N. T. and D. I. Apurillo. 1989. Philippine gulaman as a substitute for...
State of research on coconut embryo culture and acclimatization techniques in the IDEFOR (Côte d’Ivoire) and ORSTOM/CIRAD laboratories (France) - J.L. Verdeil¹, V. Hocher¹, K. Triques¹, R. Lyakurwa¹, A. Rival¹, T. Durand-Gasselin², F. Engelmann³, A. Sangare² and S. Hamon¹

¹ ORSTOM/CIRAD, Lab. GeneTrop, BP 5045, 34032 Montpellier cedex 1, France
² IDEFOR/DPO, Station M. Delorme, Port Bo*uet, 07 BP 13, Abidjan 13, Côte d’Ivoire
³ IPGRI, Via delle Sette Chiese 142, 00145 Rome, Italy

Introduction

Collecting and exchanging coconut germplasm is difficult and costly because of the weight and the size of the nuts (one of the biggest seeds in the plant kingdom). Because
coconut seeds are not dormant, the embryos germinate rapidly making germplasm storage difficult. In vitro culture of excised embryos represents an attractive way to simplify coconut germplasm exchanges and conservation. For this purpose, the ORSTOM-CIRAD tissue culture group, in collaboration with IDEFOR Côte d'Ivoire, initiated a research programme of this type 13 years ago.

Routine techniques for the collecting (Assy-Bah et al. 1987) and in vitro culture of zygotic embryos (Assy-Bah 1986; Assy-Bah et al. 1989) have been developed. This work was completed by the determination of conditions for the medium-term conservation of in vitro cultured zygotic embryos (Assy-Bah and Engelmann 1993) and cryopreservation (long-term conservation) of immature and mature coconut embryos (Assy-Bah and Engelmann 1992a, 1992b).

This paper describes the state of the art of coconut embryo culture in Montpellier, reflecting work carried out in the framework of a joint programme involving IDEFOR, CIRAD and ORSTOM. The limits of the current protocol will be presented and discussed. Studies conducted to increase the knowledge on vitroplant physiology in order to circumvent these bottlenecks will be presented.

Available facilities for embryo culture

The association between IDEFOR (Côte d'Ivoire) and ORSTOM-CIRAD (France) has allowed a pooling of complementary facilities.

Côte d'Ivoire has one of the world's most important coconut field collections containing more than 37 different ecotypes, each represented by at least 70 individuals and usually by several hundreds. IDEFOR, in collaboration with CIRAD, has developed coconut breeding programmes exploiting genetic variability within the species to obtain hybrids which perform better in traditional environments. These important field collection and breeding programmes make possible the rapid validation of the embryo culture technique on a wide range of different genotypes.

IDEFOR has also a tissue culture laboratory (mainly devoted to oil palm clonal propagation) where coconut embryo culture has been developed by Dr B. Assy-Bah. Near the tissue culture lab, a prenursery for acclimatization and a nursery are available for the study and development of vitroplant transfer to natural growing conditions.

This paper is dedicated to the memory of Dr Beatrice Assy-Bah, Senior Scientist in IDEFOR-DPO Côte d'Ivoire, who initiated and successfully conducted the IDEFOR-ORSTOM-CIRAD programme on coconut embryo culture and cryopreservation in France and Côte d'Ivoire between 1983 and 1996.

The ORSTOM-CIRAD lab has two culture rooms for coconut tissue culture. The lab has a complete range of equipment allowing analytical studies on vitroplant physiology: for histology, for endogenous plant growth regulator analysis (using HPLC and ELISA methods) and for biochemical analysis (nutrition, photosynthetic ability).

These complementary facilities can be used to increase the basic knowledge on coconut vitroplant physiology in order to optimize conditions for plantlet development and transfer to the ex vitro environment.

Description of protocol for in vitro embryo culture

Using the facilities previously described, much work has been carried out in the first instance by B. Assy-Bah in Côte d'Ivoire and in France and then subsequently, by the rest of the IDEFOR/OSTROM-CIRAD group. The main steps of the protocol are described below and summarized in Figure 1.

In vitro based embryo collecting, storage and transport methods
Collecting during a prospection mission

This method has been developed to gather embryos under field conditions during a collecting mission at a long distance from the tissue culture laboratory (Assy-Bah et al. 1987).

Sampling and disinfection of solid endosperm cylinders containing the embryo.
The first sampling step in the field consists of isolating and disinfecting the solid endosperm cylinder. This operation is carried out in the open air on a table which has been carefully washed and disinfected with commercial hypochlorite. Completely dehusked mature nuts (11-12 months) are split into two using a clean hammer. The solid endosperm cylinder containing the embryo is removed using a cork borer (Ø 20 mm). The cork borer and forceps used for this operation are previously disinfected by immersion in a bowl containing a commercial bleach solution (chlorine index 8%). A portable gas burner is used to sterilize the instruments. Batches of 30 cylinders are immersed for 20 minutes in 500 ml of commercial bleach (chlorine index 8%).

Direct inoculation into culture in the field. All manipulations are protected by a packing case resting on its side, near a gas burner flame. The forceps are heated in the flame and then cooled in the commercial bleach (chlorine index 8%) in which the endosperm cylinders are immersed. The embryos are isolated in a sterile Petri dish, rinsed once in sterile distilled water (15 ml in a 30 ml jar), then inoculated into culture on solid medium composition prepared and sterilized beforehand (agar 7 g/l). The caps of the culture tubes are held in place by plastic film. Once inoculation has been completed, the tubes are placed in the packing case and can be transported and stored easily.

Under the above conditions, the contamination rate is generally around 10 % against 4% for embryos inoculated in the germination liquid medium at the laboratory.

Inoculation under laboratory conditions

- Embryo storage and transport from the collecting site to the in vitro laboratory

Previous trials showed that immersion of the disinfected endosperm cylinders in a sterile KCl solution (16.2 g/l) provided the best conditions for their storage for a maximum period of 14 days. This gave enough time to reach the tissue culture laboratory to initiate culturing.

- Embryo excision before inoculation

After the storage period, the cylinders are re-sterilized by transferring them individually in a commercial bleach solution (chlorine index 8%) for 20 minutes. The embryos are then isolated in an air flow cabinet and rinsed in sterile distilled water before inoculation in a liquid medium.

Embryo conditioning for germplasm exchange

Embryo packaging has been developed for exchanging embryos by air freight. Endosperm cylinders are disinfected and embryos are excised under an air flow cabinet according to the protocol described previously. The embryos are then inoculated in polypropylene sterile tubes (15 x 100 mm) containing a solid waiting medium without sugar [Murashige and Skoog mineral elements (1962), Morel and Wetmore vitamins (1951), sodium ascorbate (100 mg/l), pH adjusted to 5.5 before adding the agar (7 g/l) and before autoclaving]. The inoculated tubes can easily be sent by air freight. It was demonstrated that embryos can be kept on the temporary solid medium for five days without altering their germination capacity.

When they arrive in the receiving lab, the embryos are transferred into a germination medium. It is interesting to note that the contamination rate between 0 and 5% using this type of packaging for expedition is generally very low.
**Culture conditions**

The culture medium used for embryo germination (MI 502) contains Murashige and Skoog mineral elements (1962), Morel and Wetmore vitamins (1951), sodium ascorbate (100 mg/l), sucrose (60 g/l) and neutralized activated charcoal (2 g/l; Sigma). The pH is adjusted to 5.5 before adding the charcoal and autoclaving (20 minutes at 110°C). The embryos are cultured on 20 ml of medium in 24 x 160 mm test tubes sealed with parafilm. They are incubated in a dark room at 27 ± 1°C. They are subcultured every 4-6 weeks into 20 ml fresh medium. Germinating embryos are kept in the dark until the first true leaf emerges (4 months for the more advanced embryos). As soon as the first true leaf is visible on the embryos and the root system is developed (at least one root with ramifications), plantlets are transferred in 100 ml Mi 502 liquid medium in one litre glass bottles under light conditions (ligth/12h dark photoperiod, 45 ± µmol/m2; using Sylvania gro-lux day light tubes).

Plantlets growing under light conditions are transferred every 4-6 weeks into large tubes (36x200 mm) containing fresh medium. They can be acclimatized when they display 3 to 4 unfolded green leaves (the more advanced plantlets reach the acclimatization stage 6-7 months after the initial inoculation).

**Performances and limits to the process**

**Contamination rates**

Contamination rates observed after one month of culturing are shown in Table 1 (for endosperm cylinders collected under lab conditions and under field conditions with storage during 14 days in a KCl solution). The low rates obtained after storage of solid endosperm cylinders may be due to the fact that further disinfection is carried out just before the embryos are isolated.

**Germination rate**

Elongation of the embryo is observed from the first weeks of culturing. After a phase during which elongation and weight increase occur, the gemmule appears at the end of the 1<sup>st</sup>-2<sup>nd</sup> months in culture (appearance of the first foliar sheaths). The percentages of embryos germinating after two (appearance of the first foliar sheaths) and six months (2 to 3 green leaves) in culture are shown in Table 2. Embryo storage in a KCl solution significantly reduces the germination rate.

However, it does not disturb further gemmule development and shoot growth. The results obtained indicated that the method developed to collect embryos in field conditions during a collecting mission far from the tissue culture labs can be used.

The IDEFOR/ORSTOM-CIRAD protocol has been successfully applied to several genotypes (PB121 hybrid, Malayan Yellow Dwarf, Cameroon Red Dwarf, Rennell Tall, Indian Tall). No significant influence of the genotype on the percentage of plantlets embryos reaching the acclimatization stage has been noticed.

**Effect of sucrose concentration on embryo germination and leaf development.**

Experimentations carried out using a range of sucrose concentrations (20, 30, 60, 90 and 120 g/l) showed that sucrose levels directly influence germination.

The percentage germination is higher with 60 g/l of sucrose. With this concentration, 70% of the embryos germinated (appearance of the first leaf sheath) within two months of culture against 44 % on the medium containing 20 g/l of sucrose. After four months of culture, the most satisfactory shoot growth was observed from plantlets with 60 g/l of sucrose treatment (Table 3).

**Haustorium development.** Using liquid medium, generally no haustorium growth and development was observed as it is often the case with solid medium (Assy-Bah et al.
When haustorial development is observed, cut it off after three months of embryo culture, when the gemmule is 2 - 4 cm in height. Haustorial removal will further improve the transplant survival in the greenhouse. This treatment probably forces the cultured embryos to rely more on their future organs (leaves and roots), thus increasing plantlet vigour.

**Rooting.** Rooting has often been a problem for coconut zygotic embryo culture. In the past, most of the germinated embryos either did not develop roots, or developed only a poor root system. Trials were conducted to induce neoformation of roots on 6-month old shoots cultured under light conditions. The best treatment was the basic MI 502 medium previously described supplemented with 20 mg/l of naphtaleneacetic acid (NAA) in presence of 2 g/l of activated charcoal.

Increasing the sucrose concentration (from 20 g/l that was initially used to 60 g/l) led to faster root system development during the first four months of culture under dark conditions.

Germinated embryos cultivated with 60 g/l sucrose are maintained under dark conditions until first leaf emergence and the appearance of secondary roots on the principal root. This new treatment now currently applied in IDEFOR-ORSTROM/CIRAD protocol allows the development of the root system without any NAA application.

**Findings**

One of the major problems encountered in coconut embryo culture is the great heterogeneity of embryo behaviour. Some of the embryos remain ungerminated while others reach the acclimatization stage. It is also interesting to emphasize that the development of *in vitro* plants is slower compared to the development of the seedlings (Table 2).

The heterogeneity among embryos could represent a bottleneck for the development of the technology on a large scale. The adapted strategy (to circumvent this difficulty) for the production of vitroplants will be further discussed below.

Bank collections for each conserved embryo is important and improvement of the protocol will be required. Hence, studies will be conducted to address these concerns.

**Acclimatization procedure and technique developed**

Once removed from the culture medium, the plantlets are rinsed with distilled water and then plunged for 5 min in a fungicide based on carbendazin (Benlate) to prevent fungal development. They are then transferred to sterile river sand.

By using a plastic bag (acrylic polypropylene) for covering each plantlet during the first two weeks, it is possible to maintain maximum relative humidity conditions. Plantlets are watered for the first month afterwhich, watering is supplemented with a nutritive solution applied every two days (see composition below).

**Composition of the nutritive solution used for acclimatizing plantlets (mg/l)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>274</td>
</tr>
<tr>
<td>Ca(NO₃)₂ 2H₂O</td>
<td>1095</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>137</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>274</td>
</tr>
<tr>
<td>(NH₄)₂ SO₄</td>
<td>137</td>
</tr>
<tr>
<td>KCl</td>
<td>2.74</td>
</tr>
<tr>
<td>H₂PO₃</td>
<td>3</td>
</tr>
<tr>
<td>MnSO₄ H₂O</td>
<td>15</td>
</tr>
</tbody>
</table>
ZnSO$_4$ $\cdot$ 7H$_2$O  2.74
(NH$_4$)$_6$Mo7O$_{4}$ 4H$_2$O  2.74
H$_2$SO$_4$  0.137
CuSO$_4$ 5H$_2$O  1.37
FESO$_4$ 7H$_2$O  24.9
EDTA  26.1

After two months of sand, the plantlets are transferred to forest leaf mould mixed with sand. At this stage, they are fed every two weeks with 50 ml of a N:P:K solution (8; 11; 14 ml/l) and every two months, 50 ml of chelated iron 6% (1 g f$^{-1}$) is added.

With this acclimatization procedure, the survival rate of plantlets is between 80 and 90% after two months. However, after a few months under natural conditions plantlet growth remains slow and heterogeneous.

**Studies of *in vitro* plantlet physiology**

*In vitro* culture protocols for coconut zygotic embryos allow the production of plantlets. However, the intrinsic quality of coconut vitroplants needs to be improved. For this purpose, the ORSTOM-CIRAD group has recently initiated studies to increase the knowledge on *in vitro* plantlet physiology to further improve the coconut embryo culture protocol.

**Photosynthetic ability of *in vitro* grown coconut plantlets derived from zygotic embryos**

During and after the transfer to *ex vitro* conditions, the photosynthetic ability of plantlets is an important factor in determining the success rates and further plantlet *ex vitro* growth. Studies were conducted in Montpellier to investigate the photosynthetic status of *in vitro* grown plantlets obtained by zygotic embryo culture combining various complementary approaches applied both *in vitro* and *in planta*.

The CO$_2$ exchange transpiration rates, chlorophyll concentrations and chlorophyll fluorescence emission were measured, thus providing accurate information on the activity of the photosynthetic apparatus based on the efficiency of photosystem II.

Also measured were the activities of the two carboxylase enzymes RubisCO (Ribulose 1,5-biphosphate carboxylase; involved in the photosynthetic reduction of CO$_2$) and the PEPC (Phospho-enolpyruvate carboxylase), which is representative of heterotrophic metabolism (for details of the techniques used see Rival *et al.* 1997).

Results showed that transpiration rates are similar in the *in vitro* cultured plantlets and in the autotrophic adult palms cultivated in the greenhouse. This suggests that stomatal opening is correctly regulated in *in vitro* coconut plantlets at the end of the *in vitro* culture process.

The establishment of photosynthetic metabolism was also demonstrated during the *in vitro* development of coconut plantlets. Several notable similarities have been observed between *in vitro* grown coconut plantlets and the adult autotrophic coconut palm (high level of photosystem II activity in the vitroplant one month after the transfer under light conditions, presence of mature well structured active chloroplasts). Nevertheless, a lower rate of net photosynthetic activity was recorded in *in vitro* grown plantlets as compared with the acclimatized palm. This could be explained by a lower RubisCO content and activity together with a lower chlorophyll content compared to the acclimatized palm.

This work will now be complemented by the monitoring of the parameters studied during the subsequent stage of plantlet acclimatization.
Study of the mobilization of the main organic nutrients by the haustorium during the in situ germination of coconut zygotic embryos

Results obtained in the previous study showed that in vitro cultivated coconut plantlets displayed an early initiation of a photosynthetic metabolism. Even if it can be improved (improvement of chlorophyll and RubisCO contents), the photosynthesis rate per unit of leaf area is not a limiting factor of vitroplant growth after acclimatization. Other limiting factors must therefore caused the slow development observed after acclimatization. A comparative study of plants grown from nuts and plants obtained in vitro suggested that insufficient leaf area and root system development could be the major factor limiting vitroplant growth and development after acclimatization. Therefore, increasing the leaf area of in vitro germinated embryos appears to be a major challenge for the improvement of coconut embryo culture. For this purpose, it is important to identify nutritional requirement for embryo germination.

One characteristics of coconut zygotic embryos is substantial development of the haustorium inside the nut cavity during germination. This organ invades the nut cavity and comes into contact with the reserves contained in the endosperm. It enables their hydrolysis and the mobilization of nutrients required for embryo germination. Some authors compared this organ to a «stomach» and enzyme secretion (lipases, proteases, saccharases) has even been detected (Bertrand 1994). Histological studies in the laboratory have allowed the characterization of the structure of this organ. In particular, digitations in the epidermal layer in contact with the nutrient reserves, and the existence of vascular bundles converging towards the embryonic axis were observed. This villosity displays numerous structural similarities to stomach villi in the digestive system of animals.

In order to complement the data currently available on this organ, chromatographic techniques available in the laboratory (Dussert et al. 1995; Magnaval et al. 1995) will be used to quantify the main organic nutrients (simple sugars, amino acids, fatty acids) which are present during nut germination.

These data will serve as a basis for the improvement of medium composition. The proposed study should lead to the development of a synthetic culture medium propitious to the harmonious development of in vitro plantlets, a stage which is currently a limiting factor in the application of in vitro culture to coconut.

Quantification of phytohormones in coconut seeds

Coconut seeds at different stages of development (3, 7, 9, 11 growth months and mature stage) were obtained from IDEFOR-DPO, Port Bouët, Côte d'Ivoire. At reception, they were dehusked and opened. Coconut milk, solid endosperm and embryos were sampled in order to conduct the hormone analysis.

As a first approach, analysis was concentrated on cytokinins (CK), as these factors are well known to play a role in morphogenetic events. The technique used for CK quantification was that developed by Prof. MIGINIAC laboratory (Paris VI) and recently adapted in the ORSTOM lab. It consists of three main steps: methanol extraction of the growth regulators, separation of the different forms by HPLC and a final quantification by immunoassay (ELBA) (Maldiney et al. 1986).

Different forms of cytokinins in the seed: iP, [9R] iP, Z and [9R] Z, and at different stages of the development were detected. The main results are that at three months, only the zeatin forms were detectable in coconut milk. Subsequently, considerable accumulation (the 4 different forms) occurred during the development of the nut both in coconut milk and in solid endosperm. The highest amounts were detected in solid endosperm at nine months, when the rate of deposition of endosperm in the nut is at its highest. Hence, it was believed that the hormones in question play a role in reserve accumulation. Most importantly, in the mature nut, high levels of cytokinins in the endosperm milk and embryo were found. These results might explain why the coconut...
Seed is recalcitrant. As germination can occur as soon as the nut is mature, cytokinins might be an important factor for germination. It might thus be very interesting to investigate the cytokinin behaviour in the seed at the beginning of germination.

**Embryo culture and acclimatization research activities planned for the next two years in the ORSTOM-CIRAD laboratory**

*Extension of the study of photosynthetic ability of in vitro grown coconuts to include vitroplants in the nursery* *(funding organization CIRAD, ORSTOM).* This study is partially support by the EC *(contract ERBTS3*CT940298).

*Study of the mobilization of key organic nutrients by the haustorium during in situ germination:* determination of the main organic compounds involved and application to zygotic embryo *in vitro* germination. Work performed in collaboration with CICY (Mexico) *(funding organization: ECOS-ANUIES, project S6E/669/96 ORSTOM-CIRAD).*

**Quantification of phytohormones in coconut seeds**

The study on the putative role of cytokinins during zygotic embryo germination, initiated in 1997, will be continued next year. The role of other plant growth regulators *(i.e. ABA, AIA)* will be investigated too.

**Influence of temporary immersion on embryo germination and plantlet development (RITA system)**

*In vitro* culture using temporary immersion *(immersion frequency can vary from 4 periods of 15 min per day to 1 period of 1 min per week)* offers all the advantages of a liquid medium without any of its drawbacks, asphyxia, vitrification and contact with toxic compounds.

Experiments carried out by CIRAD on banana, rubber tree, coffee, citrus and other species have shown that temporary immersion can dramatically improve *vitro* plant quality and development.

The CIRAD tissue culture group directed by Dr. C. Teisson has recently developed a culture device called «RITA» *(Récipient d'Immersion Temporaire Amélioré).* This culture device will be assessed for its usefulness in coconut embryo culture.

**Development of reliable in vitro and ex vitro hardening procedures to increase *vitro* plant vigour and growth after acclimatization**

It is necessary to develop a reliable hardening method for making plantlets more vigorous to improve their quality and *ex vitro* development.

**In vitro hardening**

Reliable *in vitro* hardening will be achieved by:

- Studying the influence of transferring plantlets to medium with low sugar concentration before acclimatization.

During *in vitro* growth and development, coconut plantlets show a transition from a heterotrophic to an autotrophic *(RubisCO-mediated)* mode of carbon fixation. Indeed, a marked decrease in PEPC, concomitant with substantial increase in RubisCO capacity, has been observed. In earlier studies, RubisCO capacity and content were found to be lower in plantlets than in the adult autotrophic coconut palm. This could explain the low rates of CO2 assimilation seen in *in vitro* grown plantlets. The high level of sucrose present in the culture medium *(60 g.L\(^{-1}\))* could affect RubisCO capacity. Indeed, exogenous carbohydrates have been reported to induce a depletion in RubisCO
efficiency (Van Huylenbroeck and Debergh 1996; Neuman and Bender 1989) and a lowering of photosynthetic rates (Serret et al. 1996). A reduction of the sucrose level in the culture medium at the end of the in vitro process could therefore allow an increase in photosynthesis, allowing a better regrowth of the plantlets after acclimatization.

- Studying the influence of the type of container closure used on embryo germination and plantlet development.

Depending on the type of closure used, an atmosphere of variable confinement can be created, thus giving rise to a high relative humidity, accumulation of CO₂ and ethylene, depletion of oxygenate...

The choice of container closure therefore influences the gaseous environment in the container and thereby, affects the quality of the plantlets produced. Different types of closure, namely: parafilm, clingfilm and micropore 3M (the latter is known to favour gas exchange) will be tested to improve vitroplant vigour and quality.

**Ex vitro hardening**

Studies will be conducted on ex vitro hardening (in collaboration with IDEFOR) in order to determine factors which influence the regrowth of the transplanted vitroplants. The influence of fertilizer application on plantlet development in the greenhouse, prenursery and nursery will be studied.

**Conclusion**

Studies conducted by the IDEFOR-CIRAD-ORSTOM group have led to the development of a simple protocol for coconut embryo culture. This protocol is based on the use of a single medium throughout the embryo germination period with the alternance of a dark phase followed by the transfer of the germinated embryo into light conditions.

On the basis of 100 zygotic embryos initially introduced in vitro, this protocol allowed the production of around 50 plantlets that were transferred under natural conditions (6-7 months after the inoculation) with a survival rate of between 80 and 90 % after two months. However, after a few months under natural conditions plantlet growth remained slow. Further studies will be required to improve the vigour of the produced plantlets in vitro and to determine the nutrient requirement for their development in the nursery.

One of the main difficulties encountered in coconut embryo culture is the great heterogeneity of embryo behaviour during in vitro germination. This depends on a wide range of uncontrollable factors such as embryo age, embryo developmental stage, physiological status, influence of the mother palm etc. In order to circumvent this important problem that can represent a bottleneck for the development of the technology for germplasm exchanges, a simple production strategy based on the selection of the more vigorous germinated embryo after two subcultures under dark conditions is proposed. According to the performance of the adopted embryo culture process, it is possible to select 50 % of the initially cultivated embryos (embryos with a developed gemmule and a primary root of 2 to 3 cm length bearing secondary roots). It is estimated that the 50 selected germinating embryos will bear 50 plantlets that will be transferred to natural conditions 4-5 months later. This choice might appear a severe one but it will allow a better subsequent management of vitroplant production (for germplasm exchange) with a lower production cost.

**References**


Assy Bah, B. 1992. - Utilisation de la culture in vitro d'embryons zygotiques pour la


Fig. 1. Overall scheme of the IDEFOR/CIRAD-OSTROM protocol for coconut embryo culture

Table 1. Comparison of the contamination rate\(^{(1)}\) of endosperm cylinders isolated under lab conditions or under field conditions, followed by storage for 14 days in a KCI solution.

<table>
<thead>
<tr>
<th>Replicate no.</th>
<th>Endosperm cylinders isolated under lab conditions</th>
<th>Endosperm cylinders isolated under field conditions and stored in a KCI solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of embryos cultured</td>
<td>% contamination</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>97</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>89</td>
<td>4.5</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Observations recorded after one month in culture (PB121 hybrid) (Assy-Bah 1992).

Table 2. Percentage\(^{(1)}\) of embryo germination \textit{in vitro} after 2 months (emission of the first foliar sheaths) and 6 months (appearance of at least one true leaf).

<table>
<thead>
<tr>
<th>Replicate no.</th>
<th>Time (month) spent in culture or germination conditions</th>
<th>Nut germination (control)</th>
<th>Embryo cylinders (1) isolated under lab conditions</th>
<th>Endosperm cylinders isolated under field conditions and stored in a KCI solution (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>36</td>
<td>6.0</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>30</td>
<td>8.8</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>40</td>
<td>20.9</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>32</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{(1)}\) % calculated on at least 75 embryos per treatment (PB121 hybrid).

\(^{(2)}\) Storage during 14 days.

Table 3. Influence of sucrose concentration on leaf emergence.

<table>
<thead>
<tr>
<th>Sucrose concentration (g/l)</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of embryos with at least 1 leaf(^{(1)})</td>
<td>46</td>
<td>49</td>
<td>53</td>
<td>50</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Calculated after 4 months of culture on at least 50 embryos per treatment (PB121 hybride) (Assy-Bah 1992).

Status of research on coconut embryo culture and acclimatization techniques in India - Anitha Karun, Anuradha Upadhyay and V.A. Parthasarathy

Biotechnology section, Central Plantation Crops Research Institute, Kasaragod Kerala,
India

Introduction

Research on coconut tissue culture was actively started in India by about six laboratories in early eighties but by nineties the research was pursued by the Central Plantation Crops Research Institute (CPCRI) only. Presently, the institute has well developed facilities for research on coconut biotechnology. The research team consists of a competent group of scientists headed by a principal scientist. Research on biochemical and molecular characterization of germplasm, coconut embryo culture and tissue culture work is being carried out by the group.

The institute has a well developed laboratory, greenhouse and field facilities. The detailed list of available facilities/equipment is given in Attachment 1.

Experiments conducted

The experiments conducted so far have led to the establishment of techniques for embryo culture and a protocol has been developed (Attachment 2). This technique has been successfully used to collect coconut germplasm from Indian Ocean Islands viz., Madagascar, Mauritius and Seychelles. The following is a brief summary of the various experiments conducted on coconut embryo culture.

Optimization of culture medium

In order to optimize the culture medium, two experiments were conducted with embryos excised from 8-month old nuts (cv. West Coast Tall or WCT). Ten embryos were used for each treatment/replication. The treatments were replicated eight times. The first experiment included three basal media (both as liquid and solid) viz., Y₃ (Eeuwens 1978), (30g/l sucrose), Y₃ (60g/l sucrose), Assy Bah's medium (Assy Bah et al. 1989) and MS (Murashige and Skoong 1962) medium. Y₃ medium supplemented with 60g/l glucose was used for the first month of inoculation while Y₃ medium with 30g/l of sucrose was used for subsequent culture. Solid medium was better than liquid medium for initial culture. The medium was supplemented with activated charcoal (1g/l). Table 1 presents the results of this experiment.

To ascertain the best hormone supplementation, a comparative experiment was conducted using NAA and IBA. NAA was supplemented at the rate of 0.5, 1.0 and 1.5mg/l while IBA was used at the rate of 1, 2 and 5mg/l. Y₃ medium supplemented with 1mg/l of BAP was used for the study. Supplementation of IBA (5 mg/l) and NAA (1mg/l) resulted in developing better rooting system. The media composition for coconut embryo culture is given in Attachment 3.

Age of embryos for retrieval

In order to ascertain the best time for excision of the embryos, experiments were conducted with cv. WCT using Y₃ medium supplemented with IBA (5 mg/l), NAA (1 mg/l), BAP (1 mg/l), activated charcoal (1 g/l) and sucrose (60g/l). Embryos were excised from 8, 9 and 11-month old nuts and cultured on the medium following the given protocol. It was observed that embryos excised from 9 and 11 month-old nuts germinated faster and had better germination rate. The growth of the plantlets was better as the embryos got older (Table 2).

Ex vitro studies

Studies indicated that plantlets were ready for transfer in vivo after 12 months of culture in vitro. A treatment with carbendazim (1 g/l) followed by a dip in IBA (1000 ppm) for 1 h improved establishment. The potting mixture consisted of soil, sand and coir dust at the ratio of 1:1:1. Initially, the plants were covered with polythene bags for 2-3 weeks. Hoagland solution was given once every 15 d.
Collecting germplasm using embryo culture

Collecting germplasm from Andaman Islands

In order to test the feasibility of using this procedure, embryo collecting from the germplasm block of World Coconut Germplasm Centre (WCGC) from Andaman Islands was performed. Eighty six embryos from six Pacific Ocean accessions were field collected and inoculated in 5 ml vials containing 1.5 ml sterile water. Subsequently, they were brought to CPCRI, Kasaragod and 15 d later, they were cultured using the standardized protocol. Initially the contamination rate was 3% only with very good germination and survival as shown in Table 3. Care must be taken to excise embryos from 9-month old and above germplasm for better germination and survival. Embryos must be singly inoculated in sterile water in individual vials.

Collecting germplasm from Indian Ocean Islands

Collecting of embryos from 3 Indian Ocean Islands viz., Madagascar, Mauritius and Seychelles was also carried out. A total of 1342 embryos representing 15 accessions were collected. The details of accessions, the number of embryos collected and developing embryos are given in Table 4.

The very high average germination percentage (72%) indicated that the collecting of embryos using the protocol developed is very efficient. The germinated embryos will be planted in a soil medium. So far there was no acclimatization problem.

List of planned experiments

Morphogenetic and physiological effect of antioxidants on coconut embryos

Objectives

a) To determine the effect of different antioxidants on nutrient uptake and plantlet physiology;

b) To find out the interaction between antioxidants and auxins/ cytokinins; and

c) To find out the best antioxidant and its concentration for effective plant growth and nutrient uptake.

Screening of coconut germplasm for drought tolerance

Objectives

a) To use embryo culture for effective screening of coconut germplasm for drought tolerance using PEG 6000 and sodium chloride. This experiment will be carried out in collaboration with the Division of Plant Physiology.

Funding agencies

1. Indian Council of Agricultural Research (ICAR), New Delhi

2. National Agricultural Technology Project (NATP)

3. CGRNP (ADB)

Conclusions

Researches on coconut biotechnology encompassing tissue culture, embryo culture and molecular markers are being carried out at CPCRI. The main achievements are the following:

1. Standardization of embryo collecting from field;
2. Standardization of culture media for embryo germination and rooting;
3. Standardization of acclimatization protocol; and

Further studies are in progress for standardization of protocol for uniformity of germination and growth of the embryo.

Acknowledgement

We are thankful to Dr M. K. Nair and Dr K. U. K. Nampoothiri, Past and Present Directors; Dr S.P. Ghosh, Deputy Director General (Hort.) ICAR and Dr R.N. Pal, Assistant Director General (Plantation) ICAR for their help.

References


Table 1. Performance of 8-month old embryos on different media

<table>
<thead>
<tr>
<th>Medium</th>
<th>State</th>
<th>Weight(g)</th>
<th>% Germination</th>
<th>% Survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y3 + 30g/L</td>
<td>Solid</td>
<td>1.15</td>
<td>42.40</td>
<td>40.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Liquid</td>
<td>1.44</td>
<td>52.40</td>
<td>50.00</td>
</tr>
<tr>
<td>Y3 + 60g/L</td>
<td>Solid</td>
<td>3.74</td>
<td>27.40</td>
<td>20.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Liquid</td>
<td>3.45</td>
<td>15.00</td>
<td>12.40</td>
</tr>
<tr>
<td>MS medium</td>
<td>Solid</td>
<td>1.31</td>
<td>20.00</td>
<td>12.50</td>
</tr>
<tr>
<td></td>
<td>Liquid</td>
<td>1.32</td>
<td>30.00</td>
<td>17.40</td>
</tr>
<tr>
<td>Assy Bah's</td>
<td>Solid</td>
<td>3.03</td>
<td>20.00</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>Liquid</td>
<td>3.84</td>
<td>12.40</td>
<td>7.40</td>
</tr>
</tbody>
</table>

* % survival from initial number inoculated

Table 2. Effect of age of the embryos on germination and morphogenesis

<table>
<thead>
<tr>
<th>Sl.no.</th>
<th>Character</th>
<th>8 months</th>
<th>9 months</th>
<th>10 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Percentage of germination, contamination and survival of field collected embryos from Andaman islands after 4 months under *in vitro* culture

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Accession name</th>
<th>No. of embryos</th>
<th>Germination (%)</th>
<th>Contamination (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Niu Uti</td>
<td>10</td>
<td>15</td>
<td>100</td>
<td>nil</td>
</tr>
<tr>
<td>2.</td>
<td>Niu Hako</td>
<td>12</td>
<td>13</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>Bora Bora (Local Tall)</td>
<td>16</td>
<td>17</td>
<td>58</td>
<td>nil</td>
</tr>
<tr>
<td>4.</td>
<td>Rangiroa Tall (Avataru)</td>
<td>17</td>
<td>19</td>
<td>100</td>
<td>nil</td>
</tr>
<tr>
<td>5.</td>
<td>Rangiroa Tall (Tiputa)</td>
<td>19</td>
<td>9</td>
<td>78</td>
<td>nil</td>
</tr>
<tr>
<td>6.</td>
<td>Nikkore</td>
<td>22</td>
<td>10</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4. Details of embryos collected from Indian Ocean Islands

<table>
<thead>
<tr>
<th>Acc no.</th>
<th>Varietal name</th>
<th>No. of embryos</th>
<th>% Germination after 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Collected</td>
<td>Developing</td>
</tr>
<tr>
<td>Mauritius</td>
<td>Pemba orange Dwarf</td>
<td>134</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Pemba Green Dwarf</td>
<td>113</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Pemba Yellow Dwarf</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Pemba Red Tall</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Dupays Tall</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Guelle Rose Tall</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Madagascar</td>
<td>Sambava Tall</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>West African Tall</td>
<td>150</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Sambava</td>
<td>Green Tall</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>----------</td>
<td>------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>105</td>
<td>3</td>
</tr>
<tr>
<td>10.</td>
<td>Comoros Tall</td>
<td>108</td>
<td>104</td>
</tr>
</tbody>
</table>

**Seychelles**

<table>
<thead>
<tr>
<th></th>
<th>Coco le rein Tall</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>116</td>
<td>113</td>
<td>1</td>
</tr>
<tr>
<td>11.</td>
<td>Coco le haul Tall</td>
<td>149</td>
<td>136</td>
</tr>
<tr>
<td>12.</td>
<td>Coco Blue Tall</td>
<td>51</td>
<td>48</td>
</tr>
<tr>
<td>13.</td>
<td>Coco Raisin Tall</td>
<td>163</td>
<td>153</td>
</tr>
<tr>
<td>14.</td>
<td>Coco Gra Tall</td>
<td>23</td>
<td>15</td>
</tr>
</tbody>
</table>

| Total no. | 1342 | 1262 | 45 | 51 |

| Average (%) | 3.5 | 3.8 | 72.00 |

**Attachment 1. List of facilities/equipment**

**A. Available equipment**

1. Vertical autoclave
2. pH meter
3. Inverted microscope
4. Growth chamber
5. CO₂ incubator
6. Micropipettes
7. Refrigerated centrifuge
8. Magnetic stirrer
9. Gas chromatograph
10. SLR camera with slide duplicator
11. Personal computer
12. Vacuum Filteration Unit
13. Horizontal laminar flow hood
14. Refrigerator
15. Culture trolleys
16. Hot air oven
17. Millipore Water purification system
18. Automatic dispenser
19. Thermal cycler
20. UV transilluminator
21. UV spectrophotometer
22. Photodocumentation system
23. Water bath
24. Dewar flask
25. Vertical gel electrophoresis unit
26. Horizontal gel electrophoresis unit with power pack

**B. Planned equipment/facility**

1. Deep freezer -20°C
2. Deep freezer -80°C
3. BOD incubator
4. Horizontal autoclave
5. Vertical laminar flow hood
Attachment 2. Protocol for embryo culture

A. Field collecting

Materials required

1. A folding portable inoculation hood.
2. Sterile conical flasks - 250 ml (5 Nos.)
3. Beakers - 500 ml (2 Nos.)
4. Long forceps (1 No.)
5. Alcohol 100 ml
6. Cotton/cheese cloth - 30 x 45 cm (100 Nos.)
7. Stain steel cork borer (diameter - 1.5 cm; length 20 cm)
8. Knives - big and small
11. Parafilm - 1 roll
12. Sterilized disposable gloves
14. Rubber bands - 500 g
15. Dehusker
16. Vials containing sterile medium
17. Measuring cylinder - 100 ml
18. Potassium permanganate - 500 g
19. Chlorine kit or Sodium hypochlorite: conical flask (1 L); Separating funnel (500 ml); reagent bottle - 500 ml.
20. Stickers for labelling
21. HCl

Methods

1. Dehusk and extract the embryo with the help of cork borer
2. Sterilize the surface of the inoculation hood with alcohol (95%)
3. Sterilize the embryos with disinfectant (Chlorine water-50% or 2% Na Hypochlorite) for 20 min
4. Wash the embryos 3-4 times with sterile water
5. Transfer the embryos into sterile water/medium.

B. In vitro culture and field establishment

1. Culture the embryos using retrieval medium (Annex 3);
2. Transfer the germinated embryos to rooting medium (Annex 3) for root induction and growth;
3. Transfer the plantlets with well developed roots to pots containing 1:1:1 of sterile sand, soil and coir dust;
4. Maintain high humidity by covering the plantlets in the pots with polythene bags or misting for 2-3 weeks;
5. Harden the plantlets by gradually introducing perforations in the polythene bags and subsequently removing the polythene bags during the night for 2 weeks;
6. After 3-4 months, transfer the plantlets to big polythene bags with soil and organic manure and keep it in a nethouse with 50% shade; and
7. Transfer the hardened plantlets to the field (total duration-from pot to polybag is 5-6 months).

Attachment 3. Media composition for embryo culture

A. Basal medium (Y3)

<table>
<thead>
<tr>
<th>SI.NO.</th>
<th>Chemical</th>
<th>Quantity (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NH₄Cl</td>
<td>535</td>
</tr>
<tr>
<td>2.</td>
<td>KNO₃</td>
<td>2020</td>
</tr>
<tr>
<td>3.</td>
<td>MgSO₄</td>
<td>247</td>
</tr>
<tr>
<td>4.</td>
<td>CaCl₂</td>
<td>294</td>
</tr>
<tr>
<td>5.</td>
<td>KCl</td>
<td>1492</td>
</tr>
<tr>
<td>6.</td>
<td>NaH₂PO₄</td>
<td>312</td>
</tr>
<tr>
<td>7.</td>
<td>KI</td>
<td>8.3</td>
</tr>
<tr>
<td>8.</td>
<td>H₃BO₃</td>
<td>3.1</td>
</tr>
<tr>
<td>9.</td>
<td>MnSO₄</td>
<td>11.2</td>
</tr>
<tr>
<td>10.</td>
<td>ZnSO₄</td>
<td>7.2</td>
</tr>
<tr>
<td>11.</td>
<td>NaMoO₄</td>
<td>0.24</td>
</tr>
<tr>
<td>12.</td>
<td>CuSO₄</td>
<td>0.16</td>
</tr>
<tr>
<td>13.</td>
<td>CoCl₂</td>
<td>0.24</td>
</tr>
<tr>
<td>14.</td>
<td>NiCl</td>
<td>0.024</td>
</tr>
<tr>
<td>15.</td>
<td>Inositol</td>
<td>100</td>
</tr>
<tr>
<td>16.</td>
<td>Pyridoxine HCl</td>
<td>0.05</td>
</tr>
<tr>
<td>17.</td>
<td>Thiamine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>18.</td>
<td>Biotin</td>
<td>0.05</td>
</tr>
<tr>
<td>19.</td>
<td>Na₂EDTA</td>
<td>37.3</td>
</tr>
<tr>
<td>20.</td>
<td>FeSO₄.7H₂O</td>
<td>13.9</td>
</tr>
</tbody>
</table>
B. Retrieval medium

\[ Y3 + NAA \,(0.5\,mg/l) + \,BAP \,(0.5\,mg/l) \]

C. Rooting medium

\[ Y3 + NAA \,(1.0\,mg/l) + \,IBA \,(5\,mg/l) \]

Status of research on coconut embryo culture and acclimatization techniques in Indonesia - Nurhaini Mashud

Researcher, Research Institute for Coconut and Palmae, Manado, Indonesia

Introduction

In Indonesia, coconut embryo culture has been performed since 1980 by several institutions. Using this technique, the Indonesian Biotechnology Research Institute for Estate Crops (IBRIEC) has produced kopyor coconut palms which were planted in its experimental garden. These palms produce about 95 to 100% 'true form' kopyor coconuts. The IBRIEC also tried to cultivate a piece of embryo on culture medium, but only callusing was achieved. Experiments conducted by other institutions have shown the most suitable media for coconut embryo culture.

The Research Institute for Coconut and Palmae (RICP) plans to conduct research on embryo culture. These studies will be funded by the Government of Indonesia and ADB Phase II. The research will produce coconut palms of several tall and dwarf cultivars, specifically, kopyor dwarf coconut palms for planting in Riau International Germplasm Collection.

In preparation to these researches, RICP is completing the embryo culture laboratory and nursery for acclimatization.

Current development of the coconut embryo culture laboratory

The RICP has a laboratory for coconut embryo culture consisting of three sterile rooms with different functions.

- Room 1 for storage of chemicals, media preparation and transfer of cultures;
- Room 2 for growth room; and
- Room 3 for maintenance.

The RICP is completing the embryo culture laboratory for handling the ongoing and planned researches on coconut embryo culture.

The other institute which perform the coconut embryo culture is IBRIEC. The institute has a complete laboratory for coconut embryo culture and other estate crops.

In vitro collecting and culture of embryos

Studies on coconut embryo culture are carried out by several research institutes namely, RICP, IBRIEC, Bogor Institute for Agriculture, Jogyakarta Training Center for Estate Crops, and the University of Jenderal Sudirman.

RICP

Coconut palms used in RICP experiments were from tall varieties. The nuts used for embryo culture were 11-12 months old. The nuts were dehusked, then split in half to reach the inner parts, from which the endosperm cylinder containing the embryo was
removed with a cork borer. The cylinder endosperm was put into a container (thermos) with coconut water.

In the laboratory, the endosperm cylinder was placed in a glass beaker, washed with water and sterilized using 95% alcohol. Furthermore, embryos were washed with 100% chlorox, and then rinsed with sterile distilled water to remove the chlorox. Using sterile forceps and scalpel, the embryos were extracted from the endosperm cylinder. The embryos were again sterilized with 10% chlorox for a minute and rinsed with sterile distilled water. The embryos were then either used for in vitro culture or stored in a sterile container.

The embryos were cultured in liquid modified Eeuwens medium for 4-5 weeks, then transferred to a fresh medium for 3-4 weeks. After which, the germinated embryos were transferred on solid medium. The results of the research indicated that embryos cultured in liquid modified Eeuwens medium had good growth.

**IBRIEC**

Coconut palms used in the experiments were tall coconut known as *kopyor* coconut and hybrid coconut. The embryos were taken from 9-12 month old nuts. The kopyor nuts were received from Beji Estate, PTP XVIII, Jepara, Central Java. The *in vitro* collecting procedure was the same as that of RICP.

The embryos were removed from the endosperm cylinder and sterilized in 5% hypochlorite for 10 min, then rinsed several times with sterile distilled water. The embryos were then cultured in two stages; first in liquid medium, next on solid medium.

The liquid media used in the experiment were White modified de Guzman (Wg), White modified Norstog (Wn), Eeuwens (Eu), and Heller (He). All the media contained 2% dextrose. The pH levels of the media were adjusted to 6.0 before autoclaving. The solid medium used was Murashige and Skoog (MS) enriched with 6% dextrose, 10 mg/l IAA, 0.5 mg/l IBA, and 5% active charcoal.

The embryos were inoculated in liquid media, and then shaken at 75 rpm for 12 h per day for six to eight weeks. Embryos germinated in the liquid media were transferred on solid medium (MS), and then incubated under an illumination of 1000 lux for 15 h/24, at a temperature of 24 - 26°C, and with a relative humidity of 60 to 70%. Germinated embryos were transferred to fresh solid medium every four week. This was perform until the germinated embryos had been cultivated for 8-12 weeks on solid medium.

Results showed that the embryos cultured in Wg, Wn, and Eu media grew 1.5 times longer *vis-a-vis* original size after the 12th day. The rapid growth of the embryos took place until the 26th day of culturing. Later, the growth rate of the embryos declined. High vigour of the embryos was observed in Wg, Eu, and Wn media. Inversely, the He medium was not effective in stimulating the growth of the embryos because of its low organic salts contents. Cytokinins, which are naturally found in coconut water, are necessary to grow coconut embryos *in vitro*.

The growth of shoot and root can be stimulated by adding 10 mg/l IAA in the solid medium. For growing lateral root, the medium was enriched with 0.5 mg/l IBA. The IBA is one of the plant regulators which cannot be translocated to the other parts of the plant. Balanced growth of shoots and roots was obtained in medium containing auxin.

After ten weeks on solid medium, the seedlings produced the second leaf and new lateral roots. The morphology of germinated embryos *in vitro* was the same as those *in situ* (Fig. 1).

After 14 weeks on the solid medium, seedlings were transferred to pots and placed in a glasshouse for two months of acclimatization.
The other experiment conducted by IBRIEC used a 12-13 month embryos of an old local variety taken from a smallholder coconut plantation in Tasikmalaya, West Java. Treatments were: He liquid medium, MS solid medium enriched with IAA (0 ppm, 5 ppm, 10 ppm and 15 ppm) for the first step, and MS solid medium enriched with IAA (0 ppm, 5 ppm, 10 ppm, 15 ppm) and dextrose (4%, 6%, 8%) for the second step. Embryos were cultured in liquid medium and shaken at 60 rpm for 12 hours per day for eight weeks. Embryos displayed good growth in liquid medium. The development of the embryonic part was faster than the haustorial part. In liquid medium, the haustorium colour changed to dark brown and it broke down in some parts.

After five to six weeks, selected germinated embryos were transferred to solid medium. On solid medium without IAA, growth of embryos was slower than in liquid medium. Even after four weeks on solid medium, some of the embryos failed to grow. On solid medium enriched with 10 ppm IAA, growth of embryos was faster than those cultured on the other solid medium. On medium enriched with 5 ppm IAA, growth of the germinated embryos was retarded. There was a 10% survival rate of embryos germinated on each medium.

After eight to ten weeks on the first solid medium, selected embryos were transferred to fresh solid medium enriched with dextrose (4, 6 and 8%). Dextrose with a 8% concentration had a good effect on root and shoot growth, but some of the embryos exhibited abnormal growth.

Some of germinated embryos cultured on MS medium enriched with 10 ppm IAA and subsequently, on MS medium enriched with 8% dextrose formed callus at the bottom part of embryo (Fig. 2). The formation of primary roots and other roots was retarded because of intensive callus growth.

After eight weeks on the second solid medium, the remaining normal embryos developed second leaves, new lateral roots, and pneumatophora with good growth. The morphology of the germinated embryos was normal (Fig. 3). However, the plantlets, when transplanted in the pots, survived for six weeks only.

**Bogor Institute for Agriculture**

Research on coconut embryo culture has been conducted using several media. Results indicated that shoot growth was faster with Eeuwens medium than with MS, NN, He, and SH media. Liquid medium gave better result than solid medium during the first 1.5 months of embryo growth.

A study conducted by Ermayanti in 1985 showed that the use of solid medium supplemented with 0.5 to 1.0% activated charcoal and incubation of embryos for nine weeks could stimulate the growth of embryo. Browning and inhibition of shoot growth were not observed.

Murtajiyanto (1986), using Eeuwens medium supplemented with 100 mg/l tyrosin, and 1 mg/l IBA, found out that the growth of complete embryos was better than the growth of embryos without haustorium.

**Jogyakarta Training Centre for Estate Crops**

An experiment conducted at Jogyakarta demonstrated that cystein, citric acid, and ascorbic acid could be used to reduce the oxidation degree of phenolic substances to further reduce the browning process.

**University of Jenderal Sudirman**

The University found out that Eeuwens medium was better than MS medium for coconut embryo culture. Treatment with 100 mg/l ascorbic acid and 150 mg/l citric acid allowed reduction of phenol oxidation but retarded embryo growth in length and
diameter. Treatment with 3 mg/l kinetin accelerated growth (length) of the embryo.

**Acclimatization technique**

Acclimatization of *in vitro* coconut plants was done in several stages. First, *in vitro* seedlings were planted in pots with 1:1:1 soil, sand and green manure medium. The pots were placed in a chamber with high intensity light (more than 1000 lux) and low relative humidity. They were then transferred to the greenhouse and later on in the nursery in plastic roofs. Finally, the surviving plants were transplanted to the field. It took 15 months from start of embryo culture to field planting of the seedlings, i.e. two months in liquid medium, six months on solid medium, and seven months in soil medium.

IBRIEC successfully produced kopyor coconut palms which were planted at the centre's experimental garden. There are currently 24 kopyor coconut palms producing 95% to 100% "true form" kopyor coconuts.

**Planned research activity on coconut embryo culture**

RICP plans to conduct coconut embryo culture research and acclimatization with funding support from the Government of Indonesia and ADB Phase II. Embryos of 14 coconut cultivars will be used in the research, namely: Bali Tall (BAT), Tenga Tall (TAT), Palu Tall (PUT), Sawarna Tall (SAT), Riau Tall (RUT), Mapanget Tall (MTT), Takome Tall (TET), Nias Yellow Dwarf (NYD), Bali Yellow Dwarf (BYD), Bali Green Dwarf (BGD), Jombang Green Dwarf (JGD), Sagerat Orange Dwarf (SOD), Salak Green Dwarf (SGD), and Raja Brown Dwarf (RBD).

The long term goal of the coconut embryo culture research is to produce dwarf kopyor coconuts. RICP will cross tall kopyor coconut and Salak Green Dwarf coconut to produce dwarf F₁ kopyor. Predictably, on the fourth year of the research, nuts from F₁ kopyor could be harvested as a source of embryos for *in vitro* culture.

**Conclusion**

In Indonesia, research on coconut embryo culture was conducted by several institutions, either research institutes or universities. By using embryo culture technique, IBRIEC has produced 14 kopyor coconut palms.

The research result of RICP indicated that embryos cultured in liquid modified Eeuwens medium had good growth. RICP plans to continue research on embryo culture and acclimatization and in the process, produce dwarf kopyor coconut. These researches will be funded by the Government of Indonesia and ADB Phase II.

RICP is completing the embryo culture laboratory and nursery for seedling acclimatization to handle the proposed coconut embryo culture research activities.

**References**


**Fig. 1. The development stages of kopyor coconut seedlings**

A. Embryo after excision from the endosperm  
B. Embryo after 4 weeks in liquid medium  
C. Seedling after 4 weeks on solid medium  
D. Seedling after 8 weeks on solid medium  
E. Seedling after 14 weeks on solid medium

**Fig. 2. Normal (left) and abnormal (right) growth of seedlings. Callus which inhibited the root growth was formed at the proximal end of the abnormal seedling**

**Fig. 3. Coconut seedlings with primary root and a pair of lateral roots from embryos cultured for eight weeks in the first transplant medium**

**Status of research on coconut zygotic embryo culture and acclimatization techniques in Mexico - C. Talavera, C. Oropeza, A. Cahue, J. Coello and J. Santamaría**

Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico

**Introduction**

Interest has increased on embryo culture technology for germplasm collection and exchange. This is particularly important for Mexico at the moment, since the devastating lethal yellowing disease (LYD) is already affecting most of the plantations in the Yucatán Peninsula (Carrillo and Piña 1990; Robert and Zizumbo 1991; Oropeza and Zizumbo 1997) and in the Pacific Coast (Oropeza et al. 1998), where most of the coconut growing areas are located (Fig. 1). The only way to deal with LYD efficiently is by replanting with resistant genotypes. It will be beneficial to introduce new genotypes to reinforce genetic improvement. However, there are current phytosanitary restrictions that severely limit germplasm introduction. Embryo culture technology presents a good alternative for safe movement of germplasm from country to country. Currently, the main limitation of embryo culture technology is the low embryo to plantlet efficiency.

Poor survival of in vitro-cultured plant materials in the field have been reported in coconut (Ashburner et al. 1995). It is therefore, imperative to evaluate the acclimatization efficiency of coconut vitroplants obtained from zygotic embryos when transferred to the field, and to assess their physiological competence in terms of controlling excessive water loss and their photosynthetic capabilities.

One way to improve the capacity of vitroplants to control water loss is to enhance the gas exchange within the culture vessels using membrane vents (Santamaría 1994). The rapid loss of water in coconut vitroplant leaves could be caused by the high levels of gases accumulated in the culture vessel. It is common to find high levels of gases inside culture vessels that could be detrimental to plant performance (Buddendorf-Joosten and Woltering 1994). It is, therefore, expected that ventilation might prevent the accumulation of gases and perhaps improve the acclimatization of vitroplants to the field. In fact, in Delphinium cultures, ventilation resulted in improved stomatal characteristics (Santamaría et al. 1993) and in Tagetes culture, ventilation has resulted not only in improved stomatal morphology and functionality but also in improved control of water loss and improved plant survival in the field without acclimatization (Santamaría et al. 1996). However, ventilation may lead to medium desiccation that would result in more frequent subculturing (Santamaría et al. 1996). If dissection is to be
avoided, one should look for a membrane that would allow gas exchange but be less permeable to water vapour.

The objectives of the present study on coconut are: a) to evaluate CICY’s protocol in terms of its efficiency to obtain acclimatized plants from zygotic embryos germinated in vitro; b) to evaluate the capacity of coconut vitroplants (obtained from zygotic embryos germinated in vitro) to control water loss, relative to that of coconut seedlings grown in the field; c) to determine if vitroplants would show a lower capacity to control water loss, define if the problem is associated with a poor cuticle development or associated with altered stomatal morphology or functionality; and d) to test the effect of ventilation in the culture vessel (using different types of membranes) on the capacity of vitroplants to control water loss.

Two institutions in Mexico have been involved in coconut in vitro culture, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) and Centro de Investigacion Cientifica de Yucatan (CICY). INIFAP started in 1977 and CICY in 1990.

Presently, only CICY is actively working in this field and therefore, most of the information in this paper relates to the activities in this institution.

Facilities and methodology

Facilities

CICY has in vitro culture facilities located in different buildings. The Biotechnology Unit is exclusively dedicated for purposes of culturing embryo in vitro. The infrastructure equipment and materials include:

**Facilities for media preparation.** Autoclaves, stirrer/hotplate units, pH meters, osmometer, analytical balances, toploading balances, dispensers, micropipettes, glassware, diverse materials and analytical chemicals.

**Facilities for in vitro culture.** Two rooms with six laminar flow cabinets each, seven culture rooms with controlled light and temperature conditions. Two of these culture rooms are solely for coconut cultures, each has an area of 10 m².

**Analysis.** Gas and liquid chromatographs with various types of detectors and spectrophotometers.

**Physiological studies.** Equipment to measure water relations, stomatal movements, stomatal conductance, respiration and photosynthesis rates. Controlled chambers to perform transpiration bioassays.

**Microscopy.** There are microscopes of different types and capacities, including an inverted fluorescence microscope, attached cameras and digital imaging equipment, and stereoscopes.

**Acclimatization facilities.** A 52 m² greenhouse with controlled mist generation that provides high relative humidity (80-90%); a larger greenhouse (400 m²) with ambient humidity, and nursery (800 m²), part of it shaded, and with watering facilities.

Protocols

**Collection.** Fruits were collected at Dzinzantun, Yucatán, about 80 km away from CICY. The variety used was the Green Malayan Dwarf. The fruits were cut in halves exposing the zygotic embryos surrounded by solid endosperm. Embryos were excised from the open nuts using a cork borer (1.6 cm diameter) and placed in distilled water.
**Embryo isolation.** Under aseptic conditions, the endosperm enclosing the embryo was washed in 70% ethanol for 1 minute rinsed three times with distilled sterile water, then rewashed in a 6% NaClO solution for 20 minutes and again rinsed three times with distilled sterile water. The embryos are then excised from the endosperm.

**Embryo culture.** Excised embryos were cultured in a Y3 medium (Eeuwens 1976) as modified by Rillo and Paloma (1992), i.e. without growth regulators or gelling agent, and containing activated charcoal (2.5 g 1-1, Sigma, USA). The medium formulation is shown in Table 1. The medium volume was 10 ml and the flask volume was 35 ml. The flasks were covered with aluminum foil. One zygotic embryo was cultured per flask. Cultures were kept in the dark at 27 ± 2 °C. After 6-8 weeks, when the embryos have germinated (plumule emergence), they were transferred to fresh medium (25 ml) in larger flasks (magenta box, 140 ml) and kept under photoperiod (16 h/8 h) at a light intensity of 45-60 rompt m-2 s-1 PAR. After another 6-8 weeks, the germinating embryos were transferred to double magenta boxes with 50 ml of medium, subcultured every 6-8 weeks until plantlets developed three leaves and secondary roots (Fig. 2).

**Capacity of leaves to control water loss.** Leaves of plants of all treatments were detached from the plant, placed on aluminum stands and weighed continuously for five hours. These transpiration decline bioassays were performed in a cabinet with 60% relative humidity, at 25°C and at a light intensity of 200 rompt m-2s-1.

**Cuticular integrity.** A similar transpiration decline bioassay to the one explained above was used except that the lower side of the leaves (only side containing stomata) were covered with Vaseline to block water loss from stomata. This was to allow water losses only through the cuticle from the upper side of the leaf.

**Stomatal morphology.** Peels were obtained from the lower side of leaves and observed under a light microscope to quantify stomatal length, density and pore aperture.

**Stomatal functionality.** Leaves were detached from plants and placed in a vial containing 10 ml of a MES (10 mM) solution or MES solution containing 10-8, 10-6 and 10-4M ABA. Vials were covered with aluminum foil to reduce evaporation of the leaf passing through the foil. Leaves were illuminated and weighed continuously for five hours during which the leaf area was determined. The transpiration rate was estimated as the amount of H2O transpired per leaf surface per second, after subtracting the water lost via evaporation from vials covered with aluminum foil but containing no leaves. These transpiration bioassays were performed in a cabinet with a relative humidity of 60% at 25°C and at a light intensity of 200 rompt m-2s-1.

**Ventilation tests.** Culture vessels (polycarbonate, Magenta GA7) were covered with conventional lids, (hard polypropylene, Magenta) or with a film of various materials such as filter paper Whatman no. 1, polypropylene film (Tresahan SCB, Hoescht), or polyvinyl chloride film (PVC, Kleen pack). Their diffusion characteristics were assessed by filling each vessel with a known concentration of ethylene and quickly monitoring the concentration of ethylene left in the vessel with time. Similarly, vessels with the various covers were filled with water and weighed. Vessels were reweighed daily for a week.

**Current research**

**Embryo conversion**

The embryos germinated eight weeks (plumule emergence) after being cultured *in vitro* are shown in Fig. 2a. During the following 32 weeks, they formed shoots and roots (Fig. 2b), that developed further to form leaves and secondary roots (Fig. 2c). Potted plants were obtained in the greenhouse eight weeks later (Fig. 2d). Most of the embryos germinated (82%), but not all developed further. Only 36% became plantlets. After acclimatization in the greenhouse, the proportion decreased to 29% (Fig. 3).
progress of plantlets beyond the greenhouse has not been followed formally, hence, no data is available of their performance in the nursery or in the field. It seems that in terms of efficiency, the major limiting step was the vitroplant development immediately after germination. Another problem observed was that chronologically germination was heterogeneous. While some embryos started germinating before the sixth week of culture, several others germinated after the 10th or more week of culture. As a result, the degree of development of embryos was far from homogeneous. Therefore, it is important to find ways to achieve early, uniform germination of the embryos and to achieve full development of the germinated embryos (see Future Research).

**Physiological competence**

**Capacity of leaves to control water loss.** At the end of 40 weeks in vitro, leaves from coconut vitroplants showed a lower capacity to control water loss compared to leaves from their field-grown counterparts. Leaves from vitroplants lost 40% of their original weight after only 5 hr of dehydration, whereas those from field-grown plants lost only 8% in the same time. The problem was not as serious as those reported for vitroplants from other species (Santamaria 1994, Santamaria and Kerstiens 1994) but sufficient to potentially cause a limited plant survival and quality when transferred to the field.

**Cuticular transpiration.** This poor control of water loss cannot be explained in coconut vitroplants by a poor cuticle development as has been suggested for other species (Sutter 1988). The application of petroleum jelly to the abaxial surface (the only surface with stomata) of the vitroplant leaves improved their water loss control to levels similar to those of leaves from field-grown plants.

**Stomatal morphology.** Evaluation of stomatal morphology (length and density) did not reveal any abnormality in stomata from vitroplants compared to those from field-grown seedlings. The stomatal aperture, however, was wider in vitroplants than in stomata from field-grown palms. Stomata from vitroplants in other species have not only shown wider stomatal pore apertures, but also larger stomata and high stomatal density compared to those from field plants (Santamaria et al. 1993).

**Stomatal functionality.** It was then possible that the poor control of water loss found in coconut vitroplants could be related to physiological impairment of their stomata. Experiments were conducted to assess the responses of leaves from coconut seedlings to ABA and compare them to that of vitroplants. The transpiration rates from leaves of field-grown seedlings were unaffected by ABA concentrations of 10-8 M but decreased dramatically in response to ABA 10-6 and 10-4 M. In vitroplants leaves, on the other hand, the transpiration rate was unaffected by ABA concentrations of 10-8 M and 10-6M decreasing only in response to 10-4 M ABA. Nevertheless, even at 10-4 M ABA, transpiration rates were not completely reduced (in fact they were as high as 40 µg cm-2 s-1). At the same ABA concentration, transpiration rates were nearly zero in leaves from field seedlings. A very similar pattern was found in vitroplants of delphinium using epidermal peels exposed to increasing concentration of ABA in the incubation medium. The vitroplants showed a very limited response to ABA whereas stomatal aperture in their field counterparts decreased as the concentration of ABA increased (Santamaria et al. 1993).

**Effect of ventilation**

**Cover permeability.** The permeability characteristics for the four covers tested, filter paper (Whatman no. 1), polypropylene film, PVC film (Kleen pack) film, and the conventional culture vessel lid are shown in Fig. 4. Results showed that: (i) filter paper (Whatman no. 1) covers were the most efficient in diffusing ethylene out of the vessel followed by the polypropylene film and the PVC film (Kleen pack) film that was almost as poor as the conventional culture vessel lid; and (ii) filter paper (Whatman no. 1) covers were the most efficient in diffusing water vapor out of the vessel followed by the PVC film (Kleen pack) and the polypropylene film that was almost as poor as the

Part I. Papers presented to evaluate the status of research on coconut em...
conventional culture vessel lid.

Therefore, the filter paper is very permeable for both ethylene and water vapor while polypropylene is quite permeable to ethylene but almost as efficient as the conventional lid in preventing water vapor to permeate out of the vessel. PVC, on the other hand, is quite impermeable to ethylene but permeable to water. From the biotechnological view point, the use of polypropylene is most appealing as a membrane for the ventilation of culture vessels as it allows the diffusion of gases out of the vessel (accumulation of excessive ethylene might be detrimental) without allowing medium desiccation.

**Capacity to control water loss.** The capacity of vitroplants to control water loss improved significantly when filter paper (Whatman no. 1) covers were used (Fig. 5a). The control of water loss shown by the leaves of vitroplants grown in vessels with Whatman covers was almost as efficient as that shown by the leaves from field palms. Vitroplants covered with the conventional lid showed the typical degree of water loss shown by vitroplants grown in sealed vessels. Vitroplants grown in vessels with Polypropylene and PVC covers showed an intermediate control of water loss compared with those with Whatman and those with conventional lids. The capacity of plants to control water loss in the various treatments was more related to their respective cover permeability to water than that to ethylene.

The improved capacity to control water loss shown by vitroplants grown with Whatman covers was not related to changes in stomatal density or length. However, the stomatal pore aperture shown by vitroplants grown in vessels sealed with Whatman No. 1 was narrower than that in the other vitroplants. The stomatal aperture in vitroplants from Whatman No. 1 was closer to that found in field-grown coconut palms (Fig. 5b). This was perhaps due to the ABA concentrations accumulated in the vitroplant leaves.

Leaves from vitroplants grown in vessels sealed with conventional covers showed less sensitivity to ABA than did field plants. Plants grown in all the 3 films tested ceased transpiring when exposed to high concentrations of ABA. However, vitroplants grown with Whatman covers did not transpire even in the absence of ABA. Therefore, the improved capacity to control water losses shown by vitroplants grown with Whatman covers was partly related to the closure of the stomata. It was possible that the endogenous concentrations of ABA were high in those plants. Vessels covered with Whatman lost more water from the medium than the rest of the treatments. It is possible that this medium desiccation may have promoted the accumulation of endogenous ABA in the plants. The increased levels of endogenous ABA would be sufficient to cause stomatal closure resulting to a better capacity to control water losses. It should be assessed if the improved capacity of the vitroplants to control water loss, as demonstrated in vessels with Whatman covers, would result in better survival in the field.

**Conclusions**

From the above results, it can be concluded that CICY's protocol is at the moment relatively inefficient in producing plants from the *in vitro* culture of zygotic embryos. The major losses occurred when the germinating embryos were-subcultured and placed under light for further growth and development. However, some other losses occurred when plants were transferred from the growth rooms to the greenhouse. It is likely that those losses will increase (both in terms of survival and plant quality) when those vitroplants are transferred from the greenhouse to the field. Coconut vitroplants derived from zygotic embryos cultured *in vitro* showed a reduced capacity to control water loss relative to that shown by field plants. The reason for this reduced capacity to control water loss was not related to abnormal development of stomatal morphology. It was more related to altered stomatal functionality, particularly to a lowered response to ABA. Ventilation resulted in increased capacity of vitroplants to control water loss particularly when filter paper was used. The reason for this may be that the medium desiccation promoted hardening probably due to increased endogenous concentrations.
of ABA. These results are promising as a means of hardening or acclimatizing in vitro plants.

**Future research**

As mentioned earlier, it seems that in terms of efficiency, the main limiting step of in vitro embryo development to plantlet formation is the growth process that occurs immediately after germination. It is where substantial effort should be made. This may include evaluating the action of growth regulators such as ABA that promotes embryo maturation (Rock and Quatrano 1995). This would allow embryos to fully mature and consequently, increase the conversion rate of a population. Also, gibberellins are known to promote germination of seeds of different species (Albert 1970; Chandra and Chauman 1976; Chin et al. 1988). Gibberellins could also be useful to shorten the timespan that all the embryos of a given batch take to germinate, resulting in uniform germination and development. This has been proven to work in other species (Chin et al. 1988). However, no previous studies have been reported on the effect of ABA on coconut embryo maturation or of gibberellins on coconut embryo germination.

In addition, further efforts should be done on evaluating plant survival during the acclimatization stages. At present, the evaluation of plant survival ex vitro had only been done with potted plants in the greenhouse. It is possible that further losses will occur when those plants are transferred to the field. There are plans to follow the development of the vitroplants at advanced stages of acclimatization and during their field establishment. The studies will include the evaluation of their capacity to control water losses and the development of photoautotrophic capacity.

The present culture system includes design features that may preclude proper embryo conversion, namely an immobile liquid medium where both embryos and plantlets remain flooded for months. Alternative systems might include designs that could provide a more dynamic environment limiting exposure of embryos and plantlets to liquid medium such as a bioreactor. This type of system might be an efficient tool in searching for ideal conditions for coconut embryo culture.

Experiments covering the ideas mentioned above, are currently being undertaken at CICY.

**Acknowledgment**

Results reported here are part of a study partially supported by the Commission of European Communities (Contract ERBTS3*CT940298).

**References**


Chandra, J. P. and P. Chauman. 1976. Notes on germination of spruce seeds with
gibberellic acid. Indian For 102:721-725.


Fig. 1. Main areas of coconut production (A) and distribution of lethal yellowing disease in Mexico in early 1998 (B)

Fig. 2. Views of the process of coconut embryo culture. Germinating embryos (a), developing shoots (b), developing plantlets (c), and acclimatized plantlets (d)

Fig. 3. Protocols for: (a) in vitro germination of coconut zygotic embryos and plantlet formation, and (b) ex-vitro acclimatization of the plantlets in a high humidity greenhouse. Figures on the right column are averages (± s.d.) of data obtained from 3 batches of embryos, of variable size (average size = 100)

Fig. 4. Time courses of the decrease in ethylene concentration (A), and of relative medium water loss (B), in vessels with different covers

Fig. 5. Time courses of the decrease in relative fresh weight (A) and stomatal pore
aperture (B) in leaves of field grown palms and vitroplants grown in vessels with
different covers. Results are means of 15 leaf samples in (A) and 60 stomata in
(B), per treatment
Part I. Papers presented to evaluate the status of research on coconut em...
Embryo culture activities at the Philippine Coconut Authority-Zamboanga Research Center (PCA-ZRC) - Ambrosio Raul R. Alfiler

Senior Science Research Specialist, PCA-Zamboanga Research Center, Zamboanga City, Philippines

Mass propagation of "Makapuno" coconut

The Makapuno is an expensive delicacy and its planting material is highly priced in the Philippines. Instead of the usual formation of a solid endosperm and coconut water, the solid endosperm of the makapuno coconut remains as a jelly, sometimes filling the entire cavity (Fig. 1). The Makapuno coconuts are found as a small proportion of normal fruit on a few palms, generally from the "Laguna Tall" ecotype. However, the occurrence of such coconuts appear to be a chance event. Although the Makapuno coconut contains an apparently normal embryo, it fails to germinate properly as the endosperm contains substances which are obviously lethal. Many tissue culture workers have reported success in germinating Makapuno embryos in vitro. However, although commercial exploitation is now taking place, plantlets from Makapuno embryos still command a high price.

Available facilities

As a part of a DOST/PCARRD-funded project, a Makapuno satellite laboratory (Fig. 2) has been established at PCA-ZRC to mass produce Makapuno seedlings for interested coconut farmers. A portion of the Training Centre at ZRC was renovated to accommodate an embryo culture laboratory with separate screened cleaning area, media preparation room and culture room (Fig. 2). Culture shelves, laboratory work tables and media, and labware cabinets were fabricated. The necessary electrical and plumbing fixtures were installed. Equipment for the laboratory include a laminar flow cabinet, pressure cooker, refrigerator, electronic top loading balance and air conditioners.

An ex vitro hardening station (Fig. 3) was constructed for the acclimatization of the in vitro-cultured seedlings prior to field planting. The elevated 10m x 6m x 5m screenhouse consists of three areas with decreasing light intensity for gradual hardening and exposure to ex vitro conditions. This was achieved by progressively decreasing the number of layers of the screenhouse netting.

Embryo culture of "Makapuno"

Harvesting of Makapuno coconuts is best done when the fruits are 10-11 months old (colour break stage). At this stage, the quality of the Makapuno meat is ideal for processing. The nut is dehusked and then split open. A cylinder of endosperm embedding the embryo is extracted (Fig. 4) using a large size cork borer (2 cm diameter). Since the embryos are to be brought to the laboratory facility immediately for culturing in vitro, the endosperm cylinders are temporarily placed in a clean container (glass or plastic).

In the laboratory, the cylinders are washed with detergent and water, rinsed very well and washed quickly in 95% ethanol. Then they are disinfected by soaking in 100% commercial bleach (5-6% sodium hypochlorite) for 20 minutes. The cylinders are then transferred to the laminar flow cabinet for excision and culture of the embryos. From hereon, aseptic procedures are strictly followed to avoid contamination.

The cylinders are rinsed in several changes of sterile water. Using a scalpel and forceps, the embryo is excised from the solid endosperm. Scalpels and forceps are regularly sterilized by dipping in 95% ethanol and flaming after each excision. After all the embryos have been excised, they are again disinfected with 10% commercial...
bleach for 1-2 minutes and rinsed several times in sterile water and finally in sterile distilled water. In the absence of a distilling apparatus at the ZRC laboratory, commercially available distilled drinking water is used for media and for all preparations needing distilled water.

Embryos are transferred singly into tubes containing Eeuwens's (Y3) liquid medium (Table 1) and cultured at 27-30°C with approximately 4000-5000 lux at 9-hour photoperiod (Fig. 5). After germination and growth, the plantlets are transferred to bigger growth vessels. Subculturing is done every month. Once the plantlets have developed enough secondary and tertiary roots, the culture vessels are transferred to the screenhouse for initial hardening and acclimatization. When the seedlings' roots developed poorly, a rooting hormone (NAA or IBA) is added to the media.

After 1-2 weeks, the seedlings are transferred to polybags (Fig. 6) containing sterile sand mixed with coir dust or coarse coir fiber. The seedlings are washed under tap and dipped in a fungicide (e.g. Captan, Vitigran Blue) solution before they are planted in polybags. High humidity level is maintained during the first three weeks by covering the polybagged seedling with a plastic bag supported by bamboo pegs. Every few days, the plastic covering is lifted a few inches to gradually expose and acclimatize the seedlings to screenhouse conditions. Exposure to full sunlight starts after about 6-7 weeks. Fertilizer is applied at the recommended rates for coconut seedlings after six weeks and every six months thereafter.

**Proposed programmes for embryo culture at PCA-ZRC**

**Mass propagation of "Makapuno" seedlings**

Production of in vitro grown Makapuno seedlings (Table 2) will be continued to provide sufficient planting materials to establish several one-hectare Makapuno demonstration farms. The purpose is to evaluate the field performance of in vitro-grown Makapuno coconuts under various agroclimatic conditions in the Philippines. Furthermore, the demonstration farms will serve as embryo sources for the continued propagation of in vitro Makapuno.

**Coconut germplasm collecting, conservation and exchange**

Collecting and conservation of coconut genetic resources are important and essential components in crop improvement programmes. The coconut seednut is characterized by considerable weight and volume. The lack of dormancy renders its transport conditions very difficult and expensive, and poses phytosanitary problems.

The use of in vitro techniques can facilitate the transport and offer some phytosanitary guarantees, when combined with appropriate disease indexing procedures. Germplasm exchange can be greatly facilitated with in vitro techniques. Embryos can be transported successfully around the world using mail or rapid delivery systems. For fairly short distances, embryos can either be transported as excised embryos in sterile liquid or in a core of endosperm leaving the embryos in situ. With adequate sterilization before and after excision from the core, the embryo could germinate successfully. Embryos which are not fully mature can be used and may germinate as well or better than mature embryos. Care should be taken, though, in extracting the endosperm cylinders with the embryo from the nut. The embryo should remain within the solid endosperm and should not be exposed through any crack in the endosperm.

In addition, cryopreservation of zygotic embryos can also play a major role in the conservation of coconut germplasm and exchange of genetic resources. Successful short-to medium-term cryopreservation of zygotic embryos have been reported. If successful methods for the long-term storage of coconut germplasm become available, costs and land required for conservation of coconut genetic resources would be reduced.
Table 1. Eeuwens's (Y3) medium

Macronutrients

- Potassium nitrate (KNO₃)
- Potassium chloride (KCl)
- Ammonium chloride (NH₄Cl)
- Sodium di-hydrogen phosphate dihydrate (NaH₂PO₄·2H₂O)
- Calcium chloride dihydrate (CaCl₂·2H₂O)
- Magnesium sulfate heptahydrate (MgSO₄·7H₂O)

Micronutrients

- Manganese sulfate tetrahydrate (MnSO₄·4H₂O)
- Zinc sulfate heptahydrate (ZnSO₄·7H₂O)
- Boric acid (H₃BO₃)
- Potassium iodide (KI)
- Copper sulfate pentahydrate (CuSO₄·5H₂O)
- Sodium molybdate dihydrate (Na₂MoO₄·2H₂O)
- Cobalt chloride hexahydrate (CoCl₂·6H₂O)
- Nickel chloride hexahydrate (NiCl₂·6H₂O)

Iron supplement

- Sodium EDTA dihydrate (Na₂EDTA·2H₂O)
- Iron sulfate heptahydrate (FeSO₄·7H₂O)

Vitamins

- Myo-inositol
- Thiamine-HCl
- Nicotinic acid
- Calcium D-pantothenate
- Biotin

Table sugar

Activated charcoal

Table 2. Status of "Makapuno" collection and seedling production at PCA-ZRC (As of 30 September 1997)

<table>
<thead>
<tr>
<th>Batch</th>
<th>Date Collected</th>
<th>Total No. of Embryos</th>
<th>% Germination</th>
<th>% Abnormal Embryos</th>
<th>Seedlings Transferred To Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aug. 26, 1996</td>
<td>69</td>
<td>26</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Aug. 27, 1996</td>
<td>54</td>
<td>56</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Sept. 9, 1996</td>
<td>17</td>
<td>64</td>
<td>65</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Oct. 1, 1996</td>
<td>3</td>
<td>100</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Oct. 7, 1996</td>
<td>44</td>
<td>68</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Oct. 9, 1996</td>
<td>48</td>
<td>21</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Oct. 17, 1996</td>
<td>44</td>
<td>39</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Oct. 22, 1996</td>
<td>14</td>
<td>14</td>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>
Fig. 1. Different types of Makapuno based on how much endosperm fills the nut cavity (from top-Type III, II and I)

Fig. 2. Makapuno satellite laboratory at PCA-Zamboanga Research Center:

a) exterior view,

b) interior view

Fig. 3. Makapuno screenhouse

Fig. 4. Extraction of the Makapuno embryo using a cork borer

Fig. 5. Different growth stages of Makapuno

Fig. 6. Polybagging and acclimatization of in vitro cultured Makapuno seedlings

Status of coconut (Makapuno) embryo culture and acclimatization techniques in ViSCA, Baybay, Leyte, Philippines - Victor M. de Paz

Researcher, Philippine Coconut Research and Development Foundation (PCRDF), Pearl Drive, Ortigas Complex, Pasig, Metro Manila, Philippines

Introduction

Embryo culture is found to be a practical technique in germplasm exchange. Excised and preserved embryo is more manageable to carry and transport than bulky seednut.

In the Philippines, embryo culture technique started in 1960's when the researchers of the University of the Philippines at Los Baños (UPLB) explored the possibility of growing pure Makapuno palms through embryo rescue. The Makapuno is considered an aberrant or mutant form of coconut with soft and glutinous endosperm instead of hard and fibrous nut as in normal coconut. It is a valued crop since it is used as ingredient in ice cream, pastries and other delicacies. Makapuno does not germinate in situ due to...
the incompatibility of the embryo with its highly perishable endosperm.

The major breakthrough was the successful in vitro growing of Makapuno embryos by Dr. E. V. de Guzman of UPLB (Balaga and de Guzman 1971). This paved the way to the field planting of pure Makapuno palm. However, in vitro grown Makapuno seedlings are very limited and the price is very prohibitive to an ordinary coconut farmer. Thus, it is necessary to increase the efficiency in the mass production of its planting materials to meet the present demand.

The Visayas State College of Agriculture (ViSCA) has developed new genotypes of Makapuno from Dwarf coconut x Tall Makapuno (Nuñez and De Paz 1996). These genotypes are early-bearers, highly-self and high yielding "Makapuno" palms. However, they do not respond well to in vitro growing condition used for Tall Makapuno. Hence, the Philippine Coconut Research and Development Foundation (PCRDF) ventured into mass production, coupled with improving the in vitro acclimatization techniques for higher survival rate, of these new Makapuno genotypes.

Facilities of ViSCA-PCRDF laboratory

The ViSCA-PCRDF laboratory based at ViSCA in Baybay, Leyte started two years ago. The laboratory facilities include a building with preparation, inoculation, and incubation rooms. The greenhouse is situated near the laboratory which can accommodate about 500 acclimatized seedlings. The 12 m x 40 m nursery, with seeding capacity of 2000 is well-secured with a concrete and cyclone wire fence. The equipment used for embryo culture are the following:

- Incubation shelves
- Hot plate magnetic stirrer
- Incubation cabinets
- Forceps
- Laminar air flow hood
- Cork borer
- Hot beads sterilizer
- Oven
- Analytical balance
- Air conditioner
- Pressure cooker
- Timer
- Standby generator
- Refrigerators
- pH meter

Growth and development of in vitro Makapuno genotypes

Characteristics of Makapuno genotypes used. UPMAC, which was the first pure Makapuno palm developed at UPLB through embryo culture technique, was used in this study in addition to the two new genotypes developed by ViSCA. UPMAC is tall in gross morphology with very low intraspadix overlapping of male and female phases (Table 1).

The new Makapuno genotypes are of two types: one is dwarf in gross morphology (VMAC 1) and the other is a D x T hybrid type (VMAC 2). Both are precocious which flower at 37 and 36 months after field planting, respectively. The dwarf type had 90% intraspadix overlapping of male and female phases, while the hybrid type had 76% (Table 1). Although planted near normal coconut trees, mean "Makapuno" yield of VMAC 1 was 99% while that of VMAC 2 was 90% (Table 1).

Culture media used in embryo culture of Makapuno genotypes. Two culture media formulations were used in culturing the different genotypes of Makapuno. These included Y3 basic (Eeuwens 1976) and Y3 modified, the latter representing the modified version of the micronutrient components of the former. The embryos from the three genotypes were grown in vitro using the two media formulations.

The different Makapuno genotypes had almost the same growth and development response in the two media formulations. However, more primary roots are developed
when embryos are grown in Y3 modified medium than in Y3 basic. Nevertheless, plantlets cultured in Y3 basic were heavier and had well-defined shoots than those grown in Y3 modified medium (Fig. 1). The survival rate did not differ much between the two media (Table 2). However, percent survival of plantlets (52.98%) in Y3 modified was higher compared with Y3 basic (30.78%). This 30.78% level of seedlings survival was consistent with the other laboratories of PCRDF using Y3 basic medium. The other laboratories of PCRDF in Manila and in Manuel S. Enverga University in Lucena observed a survival rate of 33 and 30%, respectively.

Other growth parameters like the number of leaves and plant height were affected by the genotype. Thus, it was evident that growth characteristics of coconut vary between genotypes (Assy Bah 1986 and Lineberger 1997).

Mass production of precocious and self-pollinating Makapuno

Mass production of precocious and self-pollinating Makapuno was done at the VISCA-PCRDF Laboratory, VISCA, Baybay, Leyte. It aimed to make new Makapuno genotypes available to interested individuals like investors and farmers who plan to venture into Makapuno business. One of the main objectives is to establish a five-hectare plantation as a showcase to farmers and as source of planting materials.

**In vitro and acclimatization techniques.** Embryos were excised from mature (9-10 months old) “Makapuno” nuts of VISCA-developed pure Makapuno hybrids.

Excised embryos were pre-sterilized and cultured singly *in vitro* using solid Y3 modified medium. Subsequent cultures using same medium were done 4-6 weeks interval until the plantlets reached 3 to 4-leaf stage and developed good root system. Successfully cultured seedlings in bottles were hardened in the greenhouse for two weeks. During potting, seedlings were dipped in fungicide (Benlate) solution for five minutes then planted in clay pots with sterilized soil medium composed of 2:1:1 ratio by volume of garden soil, compost and rice hull charcoal.

Potted seedlings were covered with plastic bags for 2-3 weeks before they were fully exposed to greenhouse condition (Fig. 2). Daily watering using distilled water was done for six weeks then followed up by tap water. Foliar fertilizer was applied periodically. After which, seedlings were transferred in bigger pots in the nursery with gradual exposure to sunlight until they were ready for field planting at 8-leaf stage.

**Survival rate of in vitro embryos or plantlets in acclimatized conditions.** An observation was made to determine the efficiency of the protocol adopted in the VISCA-PCRDF laboratory. There were 300 Makapuno embryos from both VMAC 1 and VMAC 2 genotypes used in the study. It was observed that mortality of cultures was prevalent in all growth stages, from initial culture up to field planting. This mortality was due to browning as well as contamination of cultures with bacteria and fungi. In the case of potted seedlings, mortality was due to some pathogens like *Erwenia sp.* Out of 300 embryos cultured *in vitro*, 139 seedlings survived which was about 46% of the total number of embryos cultured (Table 3).

**Planned research activities on Makapuno embryo culture**

PCRDF will undertake researches on the improvement of embryo culture and acclimatization techniques for Makapuno, specifically for VMAC3, VMAC 4 and VMAC5 genotypes. These include the modification of Y3 medium as well as evaluation of different sucrose levels on the physiological response of cultures.

Makapuno plantations in the Visayas regions will be established as sources of embryos for germplasm exchange as showcase of the commercial significance of the technology to the farmers.

**Conclusions and recommendations**
The research result of the PCRDF revealed that growth and development of *in vitro* Makapuno embryo vary between genotypes. Thus, improvement of the protocol should be made to attain balanced growth with high survival rate for plantlets of different genotypes.

Browning and contamination are the prevalent causes of the mortality of *in vitro* cultures. Investigation on the mode of entrance to the culture and characterization of the pathogens may be undertaken to come up with appropriate control measures.

**Acknowledgement**

I wish to thank the Technical Manager of PCRDF, Mr. Emil Carangdang and PCRDF CEO, Mr. Jose Eleazar for giving me the opportunity to attend this workshop and for the funding. A special acknowledgment is extended to Ms. Tessie C. Nuñez and Ms. J. T. Peña of the Regional Coconut Research Center, ViSCA for their valuable contributions.

**References**


**Table 1. Flowering and yield characteristics of different Makapuno genotypes**

<table>
<thead>
<tr>
<th>Code name</th>
<th>Age at initial flowering (mos.)</th>
<th>Intraspadix overlapping (%)</th>
<th>% &quot;Makapuno&quot; yield</th>
<th>Gross morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPMAC</td>
<td>71</td>
<td>very low</td>
<td>55</td>
<td>Tall type</td>
</tr>
<tr>
<td>VMAC1</td>
<td>37</td>
<td>90</td>
<td>99</td>
<td>Dwarf type</td>
</tr>
<tr>
<td>VMAC 2</td>
<td>36</td>
<td>76</td>
<td>90</td>
<td>D x T hybrid type</td>
</tr>
</tbody>
</table>

**Table 2. Growth characteristics of Makapuno seedlings from different genotypes grown *in vitro***

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of primary roots</th>
<th>No. of leaves</th>
<th>Plant height (cm)</th>
<th>Seedling weight (g)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y3 Basic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPMAC</td>
<td>2.00</td>
<td>2.83 b</td>
<td>22.23 abc</td>
<td>23.07 a</td>
<td>25.67</td>
</tr>
<tr>
<td>VMAC 1</td>
<td>1.87</td>
<td>3.00 b</td>
<td>24.63 a</td>
<td>24.97 a</td>
<td>26.67</td>
</tr>
<tr>
<td>VMAC 2</td>
<td>2.83</td>
<td>4.17 a</td>
<td>18.07 c</td>
<td>27.70 a</td>
<td>40.00</td>
</tr>
<tr>
<td>Mean</td>
<td>2.23 b</td>
<td>3.33</td>
<td>21.64</td>
<td>25.24 a</td>
<td>30.78</td>
</tr>
<tr>
<td>Y3 Modified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPMAC</td>
<td>3.67</td>
<td>2.33 b</td>
<td>23.97 ab</td>
<td>15.70 b</td>
<td>62.00</td>
</tr>
</tbody>
</table>
Means within a column and treatments having a common letter or those without letters are not significantly different at 5% level on LSD.

Table 3. Survival rate of Makapuno embryos/plantlets at different growth stages

<table>
<thead>
<tr>
<th>Stages of growth</th>
<th>Initial no. of embryo</th>
<th>No. of surviving embryos</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Stage (germination)</td>
<td>300</td>
<td>251</td>
<td>83.9</td>
</tr>
<tr>
<td>2nd passage (rooting stage)</td>
<td></td>
<td>203</td>
<td>81.0</td>
</tr>
<tr>
<td>3rd passage (complete plantlet stage)</td>
<td></td>
<td>162</td>
<td>79.5</td>
</tr>
<tr>
<td>Newly potted seedlings (clay pots)</td>
<td></td>
<td>141</td>
<td>87.5</td>
</tr>
<tr>
<td>Ready for planting (polyethylene bags)</td>
<td></td>
<td>139</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Fig. 1. Makapuno genotypes at potting, grown at different media formulations

Fig. 2. Acclimatized Makapuno seedlings in the greenhouse

PCA’s embryo culture technique in the mass production of Makapuno coconuts - Erlinda P. Rillo

Scientist II, Division Chief III, Philippine Coconut Authority, Albay Research Center, Banao, Guinobatan, Albay, Philippines

Introduction

Coconut is a very important crop in the Philippines as well as in many Asia-Pacific countries. It is called the Tree of Life' because of the many uses one can derive from it such as roofing materials, timber, fibres, nut water as drinks and oil. One third of the Philippine population depend on the coconut for their livelihood. Sixty percent (60%) of the coconut products traded in the world market is supplied by the Philippines.

Coconut is one of the recalcitrant crops that is difficult to conserve using the storage protocols used for most crops that produce seeds. Coconut germplasm have to be kept in field genebanks which are costly to maintain and are subject to the ravages of harsh climatic conditions, pests and diseases. Field genebanks require large areas too.

Embryo rescue in coconut

The embryo culture technology in coconut was first demonstrated by Dr. E. V. de Guzman at UPLB in the early 1960s when she successfully isolated and grew the embryo of the Makapuno coconut. Makapuno is a specialty coconut still quite rare in the, Philippines. It is characterized by a soft endosperm that almost fills the nut. There is no water to speak of. There are three types (I, II, and III) of Makapuno nuts based on how much endosperm fills the nut. Whether a particular type is borne by a tree all its productive life is still to be ascertained. The Makapuno phenomenon is reportedly governed by a single recessive gene, and, therefore, is a heritable trait.

Makapuno coconuts are generally borne by the Laguna Tall variety, although there are also reports that other varieties bear Makapuno coconuts too. Normal Makapuno
bearing trees only produce 2 to 17% Makapuno nuts depending on where they are planted. When planted as a solid plantation so that cross-pollination among them is virtually assured, higher yield is expected than when these are interplanted with other varieties. Proximity plays a great role in the Makapuno yield.

Makapuno coconuts could germinate in situ. Although it has a normal embryo, the abnormal status of the surrounding endosperm could not support its germination, and subsequent growth and development. This normal embryo could be extracted (rescued) and cultured in vitro (embryo culture) to become a high yielding, true-to-type Makapuno coconut. In vitro cultured Makapuno could yield from 75 to 100% Makapuno nuts depending on field planting proximity.

Although it has recently been demonstrated that coconuts can be cloned using immature inflorescence, collected non-destructively from the donor palm allowing for repeated collection of explants, leaves and plumules (the excised growing shoot of the embryo), the protocol is not standardized yet for general application. Cloning of elite varieties provides uniform, high yielding, early bearing, disease resistant planting materials. At the moment, embryo rescue technology is the only way to mass propagate the Makapuno coconuts.

Coconut is highly heterozygous and generally cross-pollinated. Since propagation, is until now, solely by seed, the progenies are highly variable. The heavy and bulky nature of coconuts are the big constraints in transporting propagation materials. In addition, the lack of dormancy results to early germination while still in transport. Hence, embryo culture is being developed as a means of transporting germplasm around the world for coconut improvement programmes. Embryos could be collected during prospection trips and cultured successfully in a laboratory after a few days under appropriate conditions.

A few researchers have developed coconut embryo culture protocols that could be applied under various conditions (Assy Bah et al. 1989; Rillo and Paloma 1991; Ashburner et al. 1995). Aside from facilitating germplasm exchange, inadvertent introduction of pests and diseases can also be minimized.

Embryo culture was also used to determine if the cadang-cadang disease could be transmitted through the nut or the embryo. Embryos from naturally infected nuts were collected and cultured in vitro for 6-8 months before these were bioassayed for the presence of the cadang-cadang viroid (CCVd). Table 1 shows the incidence of the viroid in vitro cultured diseased nuts (Pacumbaba et al. 1994). Purified CCVd was also inoculated to in vitro cultured coconut seedlings in the search for any resistant and/or tolerant coconut varieties (Rillo et al. 1989).

The coconut embryo culture protocol

Collection of the embryos

Ideally, 10-11 month old coconuts are harvested using a harvesting pole with a scythe attached to one end. Climbing is also a popular method of harvesting coconuts. With the use of a blunt instrument attached to a sturdy base, the nuts are husked by prying the husk off the nut. Splitting the nuts into halves is done by striking the more prominent line of the nut with the blunt side of a bolo. The embryo is embedded in the solid endosperm under the most prominent "eye" of the nut.

With the biggest cork borer (No. 10), the embryos still intact in the solid endosperm of the spliced nuts are extracted. The endosperm cores are pushed out of the cork borer using a piece of stick and collected in a clean container.

Pre-disinfection

After all the endosperm cylinders are extracted, these are washed in tap water and
quickly rinsed in 95% ethanol (EtOH) to remove the fats and then disinfected with 100% commercial bleach for 20 minutes. These are then washed in sterile water for 3 to 5 times to remove the bleach.

In case the embryos need to be transported after collection from the field, special packing procedures need to be done. Sterilized cylinders are transferred in sterile plastic bags with sterile moist cotton to keep them moist during transport. To keep the endosperm cylinders cold during transport, a styrofoam box containing some ice bags is used. However, airlines do not allow this. When this is the case, the cylinders are kept overnight in a refrigerator and transported in the same box without adding ice.

**Media preparation**

**Basal medium**

Results of an experiment comparing three medium formulations showed that the length of time to germinate embryos in liquid media was shortened from six weeks, using White's medium, to three weeks in Eeuwens' (Y3) medium (with or without AC) and in Murashige and Skoog's (MS) medium with AC (Rillo and Paloma 1990; Rillo and Paloma 1992). The use of these media resulted in higher weight gain of approximately 0.7 g and increased percent germination (30%, 23% and 43%, respectively in liquid media; 77%, 73% and 83%, respectively in solid media) (Rillo and Paloma 1990).

The Y3 medium formulation was specially formulated for coconut tissues by Dr C. J. Eeuwens of Wye College, University of London, in 1976. It was the third formulation that worked satisfactorily with coconut tissues, hence the number 3 affixed to the letter Y to designate the medium. The Y3 formulation supplemented with refined white sugar (45 g/l) and activated charcoal (2.5 g/l), has been used for coconut embryo culture work at the Albay Research Centre (ARC) laboratory. Further studies showed that tap water could substitute distilled water and the gelling agent could be eliminated in the culture medium. Adjustment of pH to 5.8 prior to sterilization is necessary for optimum growth of *in vitro* embryos (Areza et al. 1994). Unrefined light brown sugar could also substitute for refined white sugar to reduce production cost of Makapuno seedlings (Bonaobra III et al. 1996).

**Preparation of stock solution**

The Table below shows chemical composition and corresponding rates in formulating the Y3 culture medium for Makapuno embryos.

### Y3 culture medium for Makapuno

**Chemicals**

<table>
<thead>
<tr>
<th>To make 1 litre of Eeuwens (Y3) macronutrient stock solution (10x)</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weigh and dissolve separately in 50 ml double distilled water:</td>
<td></td>
</tr>
<tr>
<td>Potassium nitrate (KNO₃)</td>
<td>20.20</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>14.92</td>
</tr>
<tr>
<td>Ammonium chloride (NH₄Cl)</td>
<td>5.35</td>
</tr>
<tr>
<td>Sodium di-hydrogen phosphate dihydrate (NaH₂PO₄.2H₂O)</td>
<td>3.12</td>
</tr>
<tr>
<td>Calcium chloride dihydrate (CaCl₂.2H₂O)</td>
<td>2.94</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate (MgSO₄.7H₂O)</td>
<td>2.47</td>
</tr>
<tr>
<td>Mix solutions one after another with a magnetic stirring bar. Make up volume to 1 litre. Store in dark bottle.</td>
<td></td>
</tr>
</tbody>
</table>

**To make 1 litre Y3 micronutrients stock solution (100x)**

| Weigh and dissolve separately in 50 ml double distilled water:    |     |
| Manganese sulphate tetrahydrate (MnSO₄.4H₂O)                      | 1.120 |
### Zinc sulphate heptahydrate
(\(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\)) 0.720

### Boric acid
(\(\text{H}_3\text{BO}_3\)) 0.310

### Potassium iodide
(KI) 0.830

### Copper sulphate pentahydrate
(\(\text{CuSO}_4\cdot5\text{H}_2\text{O}\)) 0.025

### Sodium molybdate dihydrate
(\(\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}\)) 0.024

### Cobalt chloride hexahydrate
(\(\text{CoCl}_2\cdot6\text{H}_2\text{O}\)) 0.024

### Nickel chloride hexahydrate
(\(\text{NiCl}_2\cdot6\text{H}_2\text{O}\)) 0.0024

Mix solutions one after another with magnetic stirring bar. Make up volume to 1 litre.

**To make 1 litre Y3 iron source stock solution (100x)**

Weigh and dissolve together in 500 ml double distilled water:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium EDTA dihydrate</td>
<td>3.73</td>
</tr>
<tr>
<td>Iron sulphate heptahydrate</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Make up volume to 1 litre. Store in dark bottle.

**To make 1 litre Y3 vitamin stock solution (100x)**

Weigh and dissolve separately in 50 ml double distilled water:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-inositol</td>
<td>10.000</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.050</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.005</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>0.005</td>
</tr>
<tr>
<td>Ca-D-pantothenate</td>
<td>0.005</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Mix solutions one after another using a magnetic stirring bar. Make up volume to 1 litre. Store in dark bottle.

*Note: Stock solution (100x) of Myo-inositol can be prepared separately by dissolving 10 g in enough distilled water and making up the volume to 1 litre. The concentrations of stocks solutions are usually 10x for macro elements and 100x for micro elements and vitamins.*

The following are needed to prepare stock solutions:

- a. Chemicals, preferably of the highest grade available;
- b. Top-loading balance;
- c. Deionized or double distilled water;
- d. Beakers or suitable containers, graduated cylinders, magnetic stirrer;
- e. Dark bottles or plastic containers for storage of solutions; and
- f. Refrigerator to store chemicals and solutions.

Macro elements are usually completely soluble in water. Each chemical has to be dissolved one at a time in minimum amount of distilled water before mixing them. Each major solution is stored in plastic or glass container/s separately for convenient use. Stocks of vitamins, trace elements and organic supplements (FeEDTA, amino acids, etc.) should be stored in the refrigerator.

**Preparation of the Y3 culture medium**

**Materials:**

**Stock solutions of:**

- Macronutrients 10x
- Micronutrients 100x
- Myo-inositol 100x
Procedure:

To prepare one liter of Y3 liquid medium

- a. Measure out the following from the stock solutions:

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients</td>
<td>100 ml</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>10 ml</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>10 ml</td>
</tr>
<tr>
<td>Vitamins</td>
<td>10 ml</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

b. Weigh 45 g/l of sugar in the balance and dissolve in the above mixed solutions,

c. Using a volumetric flask or a graduated cylinder, make up the volume to one liter using distilled water.

d. Adjust the pH to 5.8 using 0.1-0.5M NaOH or 0.1-0.5M HCl.

e. Add 2.5g activated charcoal and stir.

f. Dispense 10 ml of the liquid medium into 25 x 150 mm test tubes, while stirring the medium constantly to evenly disperse the activated charcoal. For ketchup bottles, dispense 80 ml of the medium.

g. Cover with no. 4 rubber stopper with a 2 mm hole in the middle adequately stuffed with cotton.

h. Autoclave the medium at 121°C and at 15 psi for 15 minutes.

i. Cool before use.

Aseptic embryo culture technique

The exclusion of contaminating microorganism is an absolute necessity in tissue culture. Nutrient media, culture vessels and instruments used in manipulating the tissue and/or the plant material must be sterile. Cleanliness, efficient organization and routine sterilization of all materials reduce the risk of contamination.

Materials:

- Ethanol, 95%
- Zonrox (commercial household bleach), or 5-6% sodium hypochlorite
- Sterile distilled water
- Solid endosperm cylinders with coconut embryos
- Y3 liquid media
- Forceps, scalpel, Petri dishes, beakers, flasks
- Alcohol lamp or steri-beads sterilizer
- Laminar flow hood or wooden transfer box

Procedures:

To prepare sterile embryos for culture -
a. Wash the solid endosperm cylinders with tap water several times.

b. Rinse in 95% ethanol for 1-2 minutes. Decant.

c. Immerse in 100% commercial bleach (Zonrox) for 20 minutes in a clean beaker. Embryos which were pre-sterilized already prior to storage and transport are redecontaminated in 100% bleach for 5 minutes.

d. Inside the laminar flow hood decant bleach and rinse with sterile tap water at least 5 times.

To inoculate sterilized embryos into Y3 liquid medium -

a. Sterilize forceps, blades, and flasks either in the autoclave or oven (121°C at 15 psi for 15 minutes in the autoclave or 160-170°C for 1 hour in a sterilizing oven). Petri dishes lined with filter paper should be autoclaved.

b. Inside the laminar flow cabinet regularly dip the forceps, scalpels, scissors, etc. in 80% ethanol and sterilize them in the steri-beads or flame in an alcohol lamp for about 20 seconds. Let them cool on an aluminum instrument rack. Using these sterile instruments, excise embryos from the solid endosperm in the sterile Petri dishes lined with filter papers. Transfer embryos to sterile flasks.

c. After all embryos have been excised, disinfect them again in 10% bleach for 1 minute. Rinse with sterile distilled water for 3-5 times. Decant.

d. Transfer embryos on sterile Petri dishes lined with filter paper.

e. Inoculate singly onto test tubes containing Y3 liquid medium.

Culture conditions

a. Incubate cultures at 28-30°C with approximately 4000-5000 lux at 9-hr photoperiod.

b. Subculture to fresh medium at monthly interval.

c. Check periodically for contamination.

d. Embryos grow at different rates. Incorporation of 7-10 ppm NAA or IBA during the last subculture initiate more and longer roots. Generally, seedlings are ready for transplanting 6-8 weeks after initiation when enough roots have formed. The earliest recorded time to transfer ex vitro is 4-6 months (Rillo and Paloma 1992).

e. Altogether the culture period could be a year or more. Following these procedures using Makapuno embryos, 45% of the germinated embryos were successfully transplanted ex vitro.

Screenhouse practices

Plant establishment

Transplanting has to be done carefully, otherwise, a significant number of plants may be lost when transferred from aseptic tissue culture conditions to grow in an external environment. The internal anatomy and ultrastructure of seedlings propagated in vitro are different from that of greenhouse- or field-grown plants. Seedlings growing on sugar-supplemented medium in vitro produce only a small amount of their carbohydrate requirement through CO₂ fixation. When taken out of these culture conditions they have to adapt to the new environment and grow autotrophically. For embryo-cultured coconut seedlings, transfer shock is minimized by adapting the following procedure:
Materials:

- Sterile river sand
- Clear plastic bags or bell jars
- Bamboo sticks (when clear plastic bags are used)
- Polyethylene bags
- Fungicide (2.5 g/litre) solution

Procedure:

a. Take out the cultures from the laboratory to harden them in the screenhouse for one week.

b. After one week, pot them in sterilized river sand contained in small polyethylene bags. To sterilize the sand, place wet sand in autoclavable plastic bags and sterile in the autoclave at 121°C at 15 psi for 30 min. Alternatively, wet sand could be sterilized using a large vat on fire until sand is dried. Use when completely cool.

c. Take out hardened seedlings by breaking the bottles if sauce bottles are used. The roots have ramified, thus, it is not possible to take out the seedlings without breaking the bottle.

d. Wash out the media carefully. The liquid medium contains sugar that will attract ants if not washed completely. Dip quickly the seedlings in 2.5 g/l fungicide solution, e.g. Daconil.

e. Plant to sterilized sand.

f. To maintain high relative humidity, cover the seedlings with plastic bags. Support the plastic bags with bamboo pegs so that they will not sag on the leaves of the seedlings. Keep them covered for 5-7 days.

g. After this period, gradually expose the seedlings to screenhouse conditions by partially lifting the cover for a week.

h. On the third week, the plants can be fully exposed to screenhouse conditions.

i. Water the plants regularly and apply weekly dilute foliar fertilizer solution.

k. After 3 months, transfer the plants to bigger polyethylene bags using non-sterilized soil. After another 3-5 months, the plants can be transferred to the field. The plants should have 4-6 leaves by then. Field transplanting should be done during the cooler months to avoid the harsh dry weather during summer. Temporary shade of the plants will have to be provided i.e. using coconut fronds) to avoid/prevent transfer shock after field planting.

l. Provide the seedlings with the necessary cultural practices, particularly fertilization, for optimum growth response especially during the first three years. Makapuno palms are of the Tall type and should be planted with a distance of 8.5 or 9 m in a triangular pattern to maximize the land and allow intercropping particularly during the juvenile years of the palms. Embryo cultured Makapuno coconuts start flowering four years after field planting under optimum cultural management. Nut production should stabilize after six years.

Observations and modifications in the protocol

Last year, the sauce bottles which are being used as culture vessels were replaced with a squarish bottle but short for the growing coconut seedlings. However, it was possible
to extend the height with the use of autoclavable plastic bags to provide ample space during the last subculture. This innovation resulted to a significant increase in the number of seedlings successfully transplanted ex vitro as shown in Table 2.

The type of culture vessel plays a very important role during the culture period. If the right vessels are used such that the plants grow normally without any constraint, whether mechanical or physiological, ex vitro transplanting will not be difficult.

As shown in Table 3 Makapuno embryos have consistently shown a lower germination rate, suspectedly inherent in the Makapuno variety, compared to the normal coconuts (Table 4). Moreover, germinated embryos in vitro is no assurance that they will successfully establish ex vitro because some of them could still be lost along the subculture stages perhaps due to contamination and/or lack of enough and the right root formation.

A standardized embryo culture technology can very well be used for collecting, conserving and exchange of coconut genetic resources among the coconut growing countries. The risk of nut transmitted diseases will be minimized and bigger number of embryos could be transported at any one time without the problem of in situ germination.

Management requirements

An embryo culture laboratory will require the services of a tissue culture specialist to serve as technical consultant. It will require a full time laboratory technician who will do the embryo culture work from collection to ex vitro establishment and a labourer who will be responsible for the screenhouse maintenance activities.

It will also require tissue culture facilities with areas for washing, media preparation and sterilization, inoculation and growth rooms with culture shelves and adequate lighting. A modest-sized nethouse for the acclimatization and maintenance of the in vitro grown seedlings before field planting is also necessary. It should be equipped, at least, with the basic tissue culture equipment such as a laminar flow cabinet, autoclave (or domestic pressure cooker), gas stove, pH meter, macro and micro balances, distilling apparatus and air conditioning units. It must be provided with adequate glasswares, culture vessels and necessary chemicals. A reliable supply of water and electricity is mandatory. A stand-by generator will assure a continuous power supply during power failure.

The improved embryo culture technology of the PCA-ARC has been successfully transferred and adopted to mass produce in vitro true-to-type Makapuno coconuts. The Makapuno coconuts which are still rather rare in the Philippines (due to the lack of high yielding true-to-type planting materials) are important commercially in the ice cream and confectionery industries.

The country has now initiated a programme to develop the Makapuno industry. Hopefully, this will diversify the coconut industry which has long been dependent on copra and its by-products. At present, there are six satellite laboratories located in strategic locations in the country mass producing Makapuno coconuts using the above described embryo culture technology.

Summary

Embryo culture of coconut, which was first demonstrated in the rescue of the normal embryo of Makapuno coconuts, can be used to store and transport coconut germplasm which are heavy, bulky and are not dormant.

Embryos still embedded in endosperm cores are extracted from green mature coconuts, washed quickly in 95% ethanol, disinfected in 100% commercial bleach for 20 minutes and washed several times in sterile water before the embryos are excised
inside a clean bench. The embryos are again re-sterilized in 10% commercial bleach for a minute, washed and inoculated singly into culture tubes with Y3 liquid medium.

Depending on the rate of growth, 3-4 sub-cultures are done at 4 - 6 week intervals before the seedlings are ready for in vitro hardening and acclimatization for ex vitro transplanting. Rooting hormone may be added at the last sub-culture for root induction and improvement. Generally, the culture period is between 12 to 16 months.

The technique is now routinely used to mass produce the Makapuno coconut and to collect large numbers of embryos from remote prospecting places for transport to culture laboratories. It was also used in cadang-cadang transmission and resistance screening studies.

References


Table 1. Presence of the coconut cadang-cadang viroid in embryos and in in vitro grown plantlets originating from naturally infected palms

<table>
<thead>
<tr>
<th>Origin of seednuts</th>
<th>Embryos cultured in vitro (no.)</th>
<th>Embryos and plantlets with CCVda (no.)</th>
</tr>
</thead>
</table>
### Table 2. Percent germination and *ex vitro* survival of Makapuno coconuts cultured *in vitro* at ARC

<table>
<thead>
<tr>
<th></th>
<th>1995</th>
<th>1996</th>
<th>1996 (DOST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total collection</td>
<td>1701</td>
<td>465</td>
<td>304</td>
</tr>
<tr>
<td>% Germination</td>
<td>57</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Total no. of plantlets in the screenhouse</td>
<td>410</td>
<td>158</td>
<td>67</td>
</tr>
<tr>
<td>No. of plantlets that survived</td>
<td>285</td>
<td>147</td>
<td>59</td>
</tr>
<tr>
<td>% Survival</td>
<td>69.51</td>
<td>93.03</td>
<td>88.05</td>
</tr>
</tbody>
</table>

### Table 3. Percent germination of Makapuno coconuts grown *in vitro* in the various satellite laboratories

<table>
<thead>
<tr>
<th></th>
<th>No. of embryos</th>
<th>Percent germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRC</td>
<td>Aug. 1996 to Sept. 1997</td>
<td>2115</td>
</tr>
<tr>
<td>Pangasinan</td>
<td>Oct. 1995 to Sept. 1997</td>
<td>380</td>
</tr>
<tr>
<td>ARC</td>
<td>Aug. 1996 to Aug. 1997</td>
<td>2470</td>
</tr>
</tbody>
</table>

### Table 4. Percent germination of normal coconuts in the nursery.
*Source: ARC Quarterly Report July-September 1997*

<table>
<thead>
<tr>
<th>Population</th>
<th>Nuts shown</th>
<th>Germinated nuts</th>
<th>Percent germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Talls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Agta (ATA)</td>
<td>44</td>
<td>35</td>
<td>80</td>
</tr>
<tr>
<td>2. Basey (BAS)</td>
<td>60</td>
<td>53</td>
<td>88</td>
</tr>
</tbody>
</table>

---

*Table 3. Percent germination of Makapuno coconuts grown *in vitro* in the various satellite laboratories.*

*Table 4. Percent germination of normal coconuts in the nursery.*

*Source: ARC Quarterly Report July-September 1997*
Introduction

The safe movement of plant genetic materials has been a major concern for many countries in the world. Although man freely transported *Cocos nucifer* L. around the world because of its commercial importance, increased knowledge on pests and disease pathogens related to coconut made countries restrict this practice because of the quarantine risks. With the upsurge on the knowledge on biotechnology, minute sequences of plant pathogens have been detected on plant materials. This has prompted the development of techniques where not only the surface pathogen can be eliminated but most importantly, the virus, viroids, mycoplasm and other microorganisms within the plant materials can be avoided while transporting coconut materials from country to country. Embryo culture technique has provided this option as it became a useful tool which the breeders, and the conservationists can use to move coconut genetic material. In PNG, the need for embryo culture became necessary when the country decided to study and conserve its coconut biodiversity. The programme also extended to the introduction of exotic germplasm within and outside the region mainly to broaden the genetic base of the breeding material. Through the ACIAR project, viroid like sequence was discovered which made the position of embryo culture much more important and relevant. Consequently, protocols were developed and reported in several papers. Acclimatization protocol was not developed due partly to the short duration of the project and shortage of manpower.

The establishment of the International Coconut Genebank for the Pacific made it more urgent for this technique to be readily available to rescue embryos of important coconut germplasm from other countries. The advantage of embryo culture is that considerable savings can be made in transportation costs since one fruit is equivalent to 10 000 embryos (Harries 1982). There is a reduced quarantine risk due to the possible elimination of all the surface pathogens. Further, screening for draught tolerance and rescue of embryos from cultivars with desirable traits but have problem with germination could be made possible. Such ecotypes can be Makapuno which has difficulty germinating and therefore embryo culture provides an effective tool in ensuring that the embryos are rescued.

Facilities
The embryo culture facilities that were once used in ACIAR project are no longer in existence. Therefore, new facilities are planned for the Stewart Research Station in Madang. Project proposal was submitted through COGENT in September 1997 to secure funding for this project. Other laboratories at the University of Technology Lae are also doing some work on embryo culture.

**Work conducted on embryo culture**

In order to train personnel and develop protocols for embryo culture, several experiments were carried out under ACIAR funding from 1987 to 1993 on project, "Coconut Improvement". The combined efforts from both the Australian and PNG scientists and their resources resulted in publications of papers on relevant topics. In addition, a Ph.D thesis was conducted entitled, "Characterization, collection and conservation of Cocos nucifera L. in the South Pacific" which included embryo culture work.

**In vitro collection of embryos**

During the course of the research, a technique was developed on collecting and transporting embryos from remote locations to the laboratory. The transfer of cultured naked embryos in sterile distilled water was found to be better than plugs or whole nuts (Ashburner et al. 1994). There was less contamination and more embryos could be sent in a small package with transit time even as long as 14 days. Using plug or whole nut was too expensive because of its weight and bulkiness. If the source of materials was not far from their destination lab, embryos can be transported in endosperm plugs. A cork-borer is usually used to remove the plugs but sharp knife can also be used. Culturing of the embryo should take place only in a fully equipped laboratory, although some success has been achieved with field explanting using specialized equipment (Assy Bah et al. 1987). There were other protocols developed for coconut embryo collecting (Assy Bah et al. 1987; Sossou et al. 1987; Rillo and Paloma 1992a) and reference should be made to those techniques in conjunction with the one established by Ashburner et al. (1994).

**In vitro culture of embryo**

Work on *in vitro* culture of embryos resulted in the development of a liquid medium culture in the initial stage followed by a solid medium culture. The liquid medium was modified from a germination medium (Assy Bah 1986) by excluding the gelling agent. As soon as the embryos germinated in a liquid medium, they were transferred to a solid medium or growth medium (Eeuwens 1978). While in a solid medium, sub-culture to fresh medium was necessary depending on the plant vigour and age. At least one or two sub-cultures were sufficient. Overall, the performance of embryo germination and subsequent growth of the plantlets in liquid media was over 90% and in growth media, more than 80% survival was obtained in a number of trials. Further details on zygotic embryo culture of coconut is available in ACIAR Technical Reports 36 by Ashburner and co-workers (1993).

During various trials, several varieties were used. A large collection of Pacific varieties were sampled and transported to Melbourne where most of the *in vitro* culture trials were conducted. A number of publications resulted from work carried out in Melbourne. In PNG, the embryos of local varieties like Karkar, Markham and Raulawat Tall were used. Varietal differences in germination and growth of plantlets were evident but no further work was carried out to confirm the observations as the project was coming to an end.

**Acclimatization**

It is necessary to begin acclimatization in the laboratory by leaving the plantlets on the bench with lids open 24-48 hours before transferring them to a greenhouse. While in the acclimatization chamber, it is important that the roots are soaked in an anti-fungal
solution prior to planting. Excess medium substance on the roots should be thoroughly washed off as it provides good source of fungus growth. Humidity control and rate of evaporation within the poly tunnel must be well maintained.

Acclimatization facilities developed during the project was a simple unit built out of timber and covered with white transparent plastic to maintain humidity. Individual plantlets planted in polybags were placed inside this unit that has a lid opening from the top for regular inspection and watering. Each plantlet received a clear plastic bag that was placed over it in an inverted position. Full descriptions and illustrations can be obtained from ACIAR Technical Reports 36.

During the course of the embryo culture experiment, acclimatization was found to be the most tricky part. Under the conditions of the Papua New Guinea Cocoa and Coconut Research Institute (CCRI) facilities, the overall survival rate was 50% from the 100 plantlets transferred from culture room. The 50% loss covers failures from liquid or germination media culture through acclimatization stage. The 50% surviving plantlets were planted in polybags where another 5-10% were lost due to fungus infection of roots. The project was coming to an end so no further work was carried out to perfect the acclimatization techniques. Therefore, this area requires further research effort so that a suitable method can be developed to increase the survival rate of plantlets during acclimatization.

Infection by pathogenic or saprophytic fungi posed the greatest risk to the success of acclimatization therefore, a strict hygiene should be observed (Ashburner et al. 1994). This is perhaps one of the stages in acclimatization procedures that requires further investigation. Each country requirements may differ because of different types of pathogens or strains of pathogens present in individual country.

Additional studies

Studies on effects of plant hormones such as NM and Cytokinine and energy source (sucrose) were conducted at Horticultural Research Institute (HRI), Knoxfield by Australian scientists. Ashburner et al. (1993a) reported that addition of plant hormones had no effect on the development of roots and shoots. Hence, culturing embryos without plant hormones was recommended. Review on the subject is also presented in ACIAR Proceedings No. 53 (Ashburner et al. 1994).

The effect of solid and liquid phases in the basal medium was also investigated mainly to quantify the difference in growth and the development of coconut plantlets exposed at different times in an initial liquid medium before transferring to a solid medium. The embryo growth and germination, shoot and root growth were better after initial inoculation in the liquid medium, (Ashburner et al. 1991). Since then the liquid medium has always been included in subsequent trials.

The rates of photosynthesis of the embryo-derived plantlets were also investigated. It was found that the embryo cultured-derived plantlets had low photosynthetic capacities which may be the result of abnormal leaf anatomy and relatively low stomatal density. Ashburner (1994) reported that well developed shoot of about 160 mm in length and well developed root system were important factors to be considered in acclimatization procedures.

Planned research activities

The Coconut Breeding Section of CCRI plans to reactiviate the activity on embryo culture to facilitate transfer of plant genetic materials to the International Genebank. The major objective of the project is to build up the Institute's capability to carry out all aspects of embryo culture. The programme will involve the following:

1. Establishment of embryo culture laboratory;
2. Establishment of acclimatization facilities;
3. Training of local technical staff;
4. Experimentation with locally collected embryos; and
5. Importation of international germplasm.

The technology on embryo rescue and culture is very well established in many laboratories including the previous work in CCRI laboratory. However, acclimatization technology is somewhat lacking. Therefore, the approach and the methodology which CCRI hopes to adopt will be based mostly upon what will be developed from this workshop. Experiments will start with the locally available cultivars to establish the techniques. It will be followed by collections of local ecotypes. Embryos from other countries will be introduced only when the Coconut Breeding Section is confident that all the techniques on embryo culture can be successfully mastered. CCRI plans to achieve the following:

<table>
<thead>
<tr>
<th>Year</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 1</td>
<td>Set up embryo culture laboratory - purchase of equipment, chemicals and laboratory consumable.</td>
</tr>
<tr>
<td></td>
<td>ii) Staff recruitment and training in tissue culture at laboratory available in PNG (Unitech, LAES).</td>
</tr>
<tr>
<td></td>
<td>iii) Experimentation with embryo culture.</td>
</tr>
<tr>
<td>Year 2</td>
<td>Establishment and culturing embryos of local ecotypes.</td>
</tr>
<tr>
<td>Year 3</td>
<td>Collection and culturing embryos of local ecotypes.</td>
</tr>
<tr>
<td></td>
<td>ii) Introduction of selected international ecotypes.</td>
</tr>
</tbody>
</table>

It is expected that at the end of the project, CCRI will have a well established and functional embryo culture laboratory and acclimatization facilities with trained staff in order to carry its responsibility as the regional center for the International Coconut Genebank. The planned research activity on embryo culture and acclimatization will be funded by both COGENT and PNG CCRI. A project proposal titled, "Strengthening the Embryo Culture of the International Coconut Genebank for South Pacific (ICG-SP)" was submitted to COGENT.

Conclusion

Coconut embryo culture has many applications but as the current emphasis is on germplasm collecting, facilitating this activity through embryo culture is perhaps the foremost application (Frison et al. 1993). Embryo culture can exclude many harmful pests and diseases with germplasm, and embryo-derived plantlets may be screened for virus (Randles et al. 1992), viroid (Hanold and Randles 1991; Hodgson and Randles 1994) and MLO (Harrison et al. 1994; Rhode et al. 1993) when molecular techniques are applied.

The basic plant nutrients (macro and micro) for embryo culture plants have not changed much. Nearly all the researches used what was developed by Murashinge and Skoog (1962), together with the organics of Morel and Wetmore (1951). Germination medium first used by Assy Bah (1986) consisted of the same nutrients and growth medium by Eeuwens (1978). The addition of 6% sucrose (v/w) which mostly acts as an osmoticum (del Rosario and de Guzman 1976), also stimulates germination (Ashburner 1994). It was also reported by Rillo and Paloma in 1990 that addition of activated charcoal in the germination media is essential for optimum germination.

In the area of acclimatization, various results have been reported and perhaps attention should be made during this workshop to focus on those laboratories that had more success. Participants could learn from each other and hopefully better protocols can be found to maximize the survival rate of the seedlings in the acclimatization chamber.

References


Status of research on coconut embryo culture and acclimatization techniques in Sri Lanka - V R M Vidhanaarachchi, L K Weerakoon, S C Fernando, C K A Gamage and E S Santha

Coconut Research Institute, Sri Lanka

Introduction

The preliminary investigations on coconut embryo culture commenced in 1982 to accomplish successful in vitro germination and growth of mature zygotic embryos and to establish in vitro seedlings in soil. Several locally available cultivars were used for these studies.

Exchange of coconut germplasm is hampered by several constraints including the large size of the nut, lack of dormancy and phytosanitary regulations. These problems could be overcome if in vitro techniques are developed to facilitate the exchange of germplasm in the form of excised zygotic embryos.

Therefore, the main objective of embryo culture research is to develop in vitro methods for germplasm collecting and exchange. Embryo culture technology can also be applied to screen drought tolerant coconut germplasm and to rescue embryos of non-germinating types like Dikiri coconuts (Makapuno type).

This paper reports the results obtained from previous work and current research activities on coconut embryo culture.

Facilities

The Tissue Culture Laboratory of Coconut Research Institute (CRI), Sri Lanka was established in 1983. The laboratory is quite spacious and well planned. It is well equipped and has facilities for preparation and sterilization of culture media, aseptic culturing and incubation of cultures, and acclimatization of in vitro raised plantlets. Equipment needed for histology and biochemical analyses are also available.

**Equipment for media preparation:** Analytical and top-loading balances, glassware, stirrer/hot plate units, pH meters, micropipettes, ovens and autoclaves.

**Facilities for in vitro culture:** One culture room with 2 laminar flow cabinets and one large incubation room with light and temperature control devices.

**Acclimatization facilities:** A glasshouse with light and humidity control devices and a screenhouse.
**Equipment for histology:** Ovens, rotary microtome, light microscopes, inverted transmitted-light microscope, florescence microscope and stereomicroscope.

**Equipment for biochemical analysis:** HPLC, apparatus for SDS-PAGE and spectrophotometers.

**Embryo culture technique**

Embryo culture technique has been applied successfully for locally available varieties including tall (ordinary tall, *Dikiri* and *San Ramon* forms), dwarf (*pumila, eburnea* and *regia* - 3 colour forms), dwarf x tall and tall x tall.

Mature embryos (11-12 months postanthesis) are excised from the kernel and sterilized in 3% calcium hypochlorite for 5 minutes followed by rinsing in several changes of sterile distilled water. The embryos are cultured in glass tubes (30 X 200 mm) containing 10 ml of the liquid growth medium of which pH is adjusted to 6.0 before autoclaving at 121°C for 20 minutes. Tubes were then sealed with cotton wool plugs and incubated in the dark for two months. This is followed by incubation under 16 hr photoperiod of 6000 lux for 5-6 months. The incubation temperature was 30+1°C. The culture medium in each tube is replenished every month.

Under the present culture conditions, 65%-70% of the cultured embryos develop into complete seedlings when cultured in modified Euwens Y₃ (Karunaratne and Gamage 1985) liquid medium. The growth of the plantlets was significantly improved when the mineral, growth factor and sucrose content of the Y₃ formulation was doubled.

Poor rooting, spontaneous senescence of *in vitro* grown plants (specially with *Dikiri* coconuts) and a higher contamination rate of cultures during the latter part of *in vitro* development are some of the constraints encountered in embryo culture.

Experiments were carried out to assess the possibility of reducing cost of embryo culture. It was possible to substitute analar grade sucrose and potassium chloride (KCl) in the growth medium with commercial grade sucrose and fertiliser grade KCl without any negative effects on the growth of the plants as shown in Tables 1 and 2 (Fernando 1994). The feasibility of using tap water instead of deionized water and elimination of hormones (2,4-dichlorophenoxy acetic acid and 6-benzylaminopurine) in the medium are being tested at present to reduce the cost further.

Fully developed seedlings (7-8 months after culturing) having good shoot and root systems, are transplanted in small poly propylene bags (20 cm dia x 15 cm height) containing a pre-sterilized potting 1:1 mixture of river sand and coir dust. They are kept completely covered with poly propylene under low light conditions in the greenhouse for two weeks. Then the plants are gradually exposed to greenhouse conditions for about four weeks. Established seedlings are kept in the glasshouse for about three months until they produced new leaves. They are then transferred to bigger polybags (30 cm dia x 30 cm height) containing a potting 2:2:1 mixture of unsterilised top soil, dried cow dung and coir dust. The plants are exposed to direct sunlight for several weeks before they are planted in the field. During acclimatization, liquid nutrients are applied to the plants at two-week intervals. Plants are watered every other day. Under the present conditions, the success rate is about 60-65%. Experiments are in progress to improve this further by *in vitro* hardening (gradual removal of sucrose at the latter stages), using different potting mixtures (river sand only, river sand and compost, river sand, coir dust and dried cow dung), and different humidity levels.

Field evaluation of the embryo cultured plants (varieties tall and dwarf) has been carried out since 1987. No significant difference in growth parameters, floral structure and bearing pattern has been observed (Jayasekara and Premasiri 1987).

**Application of embryo culture technique**
1. Germplasm collecting and exchange

Two methods have been developed which are described below.

**Short-term preservation method.** The mature embryos are sterilized and cultured in screw capped vials (2 per vial with cotyledon buried) containing 10 ml of survival medium. The survival medium is agar based (0.8%) and consisting of one-half strength minerals, vitamins and growth factors of modified Eeuwens Y3 medium (Karunaratne et al. 1985), 60 g l⁻¹ sucrose and 0.25% activated charcoal. The cultured embryos are incubated in the dark at 30°C. When transferred to the germination medium (Karunaratne et al. 1985), 53%, 40% and 32% germination was observed after 2, 3 and 5 months of storage, respectively (Karunaratne 1988).

**Field explanting method.** Aseptic explanting and culturing are done in the field inside an inflatable glove box which can be easily taken to the field. Embryos are cultured directly in the germination medium placed in a vessel called the "Sossou flask" which is specially devised for long collection expeditions. The flask holds about five mature coconut embryos and the flask design prevents spilling of liquid medium and thus facilitates transportation. A case measuring 70X45X15 cm which a traveler can carry by hand can hold 100 flasks (Sossou and Kovoor 1987).

2. Embryo rescue of Dikiri coconuts

*Dikiri* coconuts containing soft, jelly-like endosperm, (similar to makapuno coconuts) do not germinate *in situ*. Embryo culture technique is applied successfully to rescue embryos of *dikiri* coconuts. Some of the *in vitro* raised *Dikiri* plants have been established in the field and their performance is monitored regularly.

3. In vitro screening for drought tolerant coconut germplasm

Studies have been conducted to test the feasibility of developing an *in vitro* method to screen large number of plants for water stress tolerance. Water stress conditions were simulated by incorporation of sodium chloride (NaCl) into the culture medium. The concentration of NaCl was progressively increased from 170 mM to 330 mM.

The results of the preliminary investigations indicated a higher rate of survival with putative drought-tolerant cultivars compared to drought-susceptible cultivars (Karunaratne et al. 1991). Seedlings which expressed different levels of tolerance to water stress conditions have been planted in drought-prone areas and performance are being monitored regularly.

The investigations on *in vitro* screening for drought tolerance is continued using polyethylene glycol (PEG) as the water stress simulant.

Future directions

1. **Physiological studies of the field-grown plants which were subjected to in vitro drought conditions.**

Physiological parameters such as leaf water potential, stomatal resistance, transpiration rate and cuticular wax content of the plants will be measured in order to compare their drought tolerance capacities under *in vitro* and field conditions. The results of this study will be useful in confirming the validity of the *in vitro* screening procedure.

2. **Studies on the development of specific markers for screening drought-tolerant coconut germplasm.**

Attempts will be made to identify and characterize any specific proteins synthesized in response to water stress using plants subjected to water stress under *in vitro* conditions. This could lead to the development of suitable protein markers for screening...
drought-tolerant coconut germplasm.

3. Studies on further improvement of soil establishment of in vitro-raised seedlings. Physiological parameters such as stomatal distribution and cuticular wax content of in vitro grown seedlings will be measured. The resulting information would aid in improving the survival rate of plants during acclimatization stage.


5. Cryopreservation Studies

At present, the conservation of coconut genetic resources is done through field collections. However, a long-term storage would facilitate the safe conservation of coconut germplasm. Therefore, cryopreservation of mature and immature zygotic embryos of coconut will be tried out as a long-term conservation option.

Acknowledgement

We wish to thank Mrs. S M Karunaratne for her valuable contribution in achieving the above progress. We would also like to thank the International Coconut Genetic Resources Network and the Coconut Research Board for giving us the opportunity to participate in this workshop and for providing the necessary funding support.

References


Table 1. Effect of commercial grade sucrose (S) and fertilizer grade KCl (K) on embryo germination and plant development

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination %</th>
<th>Plant development %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.9</td>
<td>54.1</td>
</tr>
<tr>
<td>S</td>
<td>83.9</td>
<td>53.1</td>
</tr>
<tr>
<td>K</td>
<td>79.8</td>
<td>45.0</td>
</tr>
<tr>
<td>K + S</td>
<td>79.8</td>
<td>62.8</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CV (%)</td>
<td>16.2</td>
<td>20.9</td>
</tr>
</tbody>
</table>

Table 2. Effect of commercial grade sucrose (S) and fertilizer grade KCl (K) on in vitro plant growth
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot height (cm)</th>
<th>Root length (cm)</th>
<th>Leaves/plant</th>
<th>Roots/plant</th>
<th>Plants with secondary roots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.6</td>
<td>3.6</td>
<td>1.4</td>
<td>2.4</td>
<td>69.8</td>
</tr>
<tr>
<td>S</td>
<td>8.9</td>
<td>3.5</td>
<td>1.4</td>
<td>2.4</td>
<td>79.6</td>
</tr>
<tr>
<td>K</td>
<td>9.7</td>
<td>3.1</td>
<td>1.4</td>
<td>2.2</td>
<td>77.0</td>
</tr>
<tr>
<td>K + S</td>
<td>9.4</td>
<td>3.2</td>
<td>1.5</td>
<td>2.1</td>
<td>75.2</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CV (%)</td>
<td>21.7</td>
<td>32.3</td>
<td>19.2</td>
<td>15.3</td>
<td>21.1</td>
</tr>
</tbody>
</table>

**Status of research on coconut embryo culture and acclimatization techniques in Tanzania - Kennedy Mkumbo, Salustia Tembo and Reminister Marealle**

Mikocheni Agricultural Research Institute (MARI)
Dar Es Salaam, Tanzania

**Introduction**

*In vitro* culture of zygotic coconut embryos had been developed as a tool to rescue the embryo of the Makapuno coconut (De Guzman 1970; Balaga and De Guzman 1971; Del Rosario and De Guzman 1976). Excision of embryos provides a useful option to conventional methods by lowering transportation costs, overcoming storage problems and meeting most of the quarantine requirements (Assy Bah 1986).

In this respect, embryo culture facilitates germplasm collecting, exchange, storage and conservation (Rillo and Paloma 1990, 1992; Assy Bah and Engelmann 1993). Moreover, the *in vitro* plantlets from excised embryos might be a tool for phytopathological studies on living material (Fisher and Tsai 1978). Virtually, all these engagements will depend on the success of *in vitro* culture of zygotic coconut embryos and subsequently, recovery of plantlets without significant loss of genetic materials.

The Plant Tissue Culture Laboratory of the Mikocheni Agricultural Research Institute (MARI) was established in 1992/93 with the following main objectives; to facilitate safe introduction and exchange of coconut germplasm; to conserve coconut genetic materials using *in vitro* techniques; to facilitate field collecting of coconut germplasm; and in the long run, to mass propagate individual palms with desirable traits.

The facilities available at MARI for embryo culture are listed in Attachment 1. This paper briefly reports the status of research on coconut embryo culture and acclimatization techniques in Tanzania.

**Experiments conducted on *in vitro* collecting and culture of embryos**

Since its establishment, the MARI laboratory has been mainly occupied with *in vitro* culture of mature zygotic coconut embryos. Different cultural procedures and protocols have been tried on different coconut varieties. This led to mastery of key components of embryo culture technique particularly with sterilization, excision, appropriate culture age, culture medium and growth conditions to weaning stage. No field collecting using *in vitro* techniques has been carried out in Tanzania. It is planned in the near future to test the protocol developed at the La Me tissue culture laboratory in the Ivory Coast.

**Explants and culture age of the embryo**

Different culture ages have been tested for coconut embryo culture. Seednuts of age 7-12 months old of different coconut varieties were initially used to establish the best culture age. Use of freshly harvested fruits’ endosperm cores with intact testa proved to
be of beneficial effect. This became apparent especially with respect to reduction of contamination rates (from 40 - 50% to 7 - 10%) and bleach phytotoxicity. At 9-12 months old, nuts (lower ages for dwarfs) gave optimal germination and their testa separated better from the shell. The 9-12 month maturity age was then adopted for routine zygotic coconut embryo culture.

**Surface sterilization and embryo excision**

Procedures for surface sterilization of endosperm cores and embryos are basically the same as those employed elsewhere in routine zygotic coconut embryo culture. Otherwise, solid endosperm cores are surface sterilised with 100% (undiluted solution) bleach (NaOCl) for 20 minutes. The embryos are freed from the cores using scalpels and forceps. Excised embryos are disinfected with a low strength (5 - 10%) bleach for one minute. These are washed with sterile distilled water for at least three changes before culturing into growth media.

**Inoculation and incubation**

Excised embryos are singly inoculated into a growth medium and transferred to the growth room. All these operations (excision and inoculation) are carried out in the laminar air flow cabinet.

Cultures are incubated at 29 - 30°C and at relative humidity of 30 - 50%. They are maintained in total darkness during pre-germination phase. After germination, embryos are transferred into a 12-hour photoperiod (only germinated embryos are transferred into light) room and subcultures are carried out after every four weeks.

**Culture medium**

During 1993/94, a series of experiments were conducted to test different types of media as used by various researchers working in the same field. These were essentially MS (Murashige and Skoog 1962) and Y3 (Eeuwens 1976) media with slight modifications. These were used in combination with different phytohormones. Addition of growth regulators into culture medium did not give significant influence on embryo germination and in subsequent plantlet recovery as compared to phytohormone-free medium. Some growth regulators (2,4-D and BAP or a combination of these) even inhibited germination. Exclusion of growth regulators in the culture medium was then adopted.

MS and Y3 liquid media as macro- and micro- nutrients respectively, Y3 vitamins with activated charcoal (2.5 g l⁻¹) and sucrose (45 g l⁻¹) proved to be optimal for embryo growth. Similar results were reported by Rillo and Paloma (1990). These types of media was adopted for routine culture of zygotic coconut embryos. Liquid medium was only used to initiate culture and the subsequent subcultures were in solid medium. Despite the fact that liquid medium gave accelerated growth rate and homogeneous gemmulation, germination speed during the post-gemmulation phase was not uniform. Lack of uniformity in embryo germination made it difficult to design proper experiments for weaning purposes because no single experiment could generate enough materials at the same stage of growth for weaning. Efforts were made to devise medium formulation in which two types of MS/Y3 medium formulated as liquid and solid, with some modifications as indicated below, were used;

<table>
<thead>
<tr>
<th>Medium formulation</th>
<th>Embryo placement</th>
<th>Additional material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid + Liquid (DP)</td>
<td>top</td>
<td>none</td>
</tr>
<tr>
<td>Solid (S)</td>
<td>top</td>
<td>none</td>
</tr>
<tr>
<td>Solid (SB)</td>
<td>bottom</td>
<td>none</td>
</tr>
<tr>
<td>Liquid (L) Control</td>
<td>top</td>
<td>one</td>
</tr>
<tr>
<td>Liquid (LC)</td>
<td>suspended</td>
<td>cotton wool</td>
</tr>
</tbody>
</table>
In this experiment, the favourable influences of the liquid medium to initial embryo growth did not favour post-germination development that allowed smooth proliferation of embryos to weaning stage. It was, therefore, not possible to attain maximum final germination percentage (Fig. 1). Longer stay of embryos in liquid medium seemed to lower germination percentage. This was more evident with submerged but disoriented embryos in liquid medium. They were slow in growth and lacked chlorophyll after germination. Some of them did not germinate at all. Ashbumer et al. (1991) reported similar results. Another key factor seemed to be the orientation of the embryo during growth in the liquid medium. This came from the fact that, if the embryos with their radicles and shoots were oriented to their natural posture after germination, growth proceeded normally. Germination laggards showed improved growth when sterile cotton wool was introduced into culture vessel to hold the embryo in position. Embryos (somatic or zygotic) of different plant species, especially monocots have been reported to exhibit strong response to germination in vitro if oriented to their natural posture (Mantell 1996 pers. comm.). However, the use of cotton wool complicated subsequent manipulations like subcultures and weaning.

There was no marked difference between the solid and liquid medium (used as single phase) in initial embryo weight increase but the effect was apparent when these were combined as a double phase (DP) medium (Fig. 1 and 2). Growth was more uniform in the solid(S) and DP media. An overlay of thin film of liquid medium over the solid phase demonstrated the beneficial effect of the DP medium in embryo growth. A thin film of liquid medium held the embryo automatically to its natural posture in its initial culture phase as it would be in the seednut. Advantageously, nutrients can be replenished by adding a layer of liquid medium above a semi-solid medium once it has become exhausted. This operation reduced labour costs and the stress imposed on explants during subculture. However, it required separate solid and liquid media preparation and dispensing of liquid medium into solid medium under sterile conditions.

Fig. 1 shows the development trend of Malayan Red Dwarf (MRD) embryos during the pre-germination phases in vitro. Double phase medium has been found to be more effective in inducing somatic embryogenesis in anther cultures (Johansson et al. 1982). In Hungary, the combined use of both solid and liquid medium has been patented as an improved method of in vitro mass propagation (Molnar 1987).

Embryos in SB medium showed least growth rate during pre-germination phase possibly due to lower oxygen diffusion rate (ODR) between the container atmosphere and the medium (Fig. 1). LC medium showed better support to weight increase than SB medium but was not superior to other types. Embryos in the SB medium were left in the same medium for 11 months and none of them germinated (Fig. 1). When these were transferred into solid media using the routine procedures, all of them germinated but died before they could develop the second scale leaf. This method, if refined could be one of potential avenues for in vitro storage of coconut embryos.

Based on these results, further experimentations were carried out using liquid, double phase and solid media using two coconut varieties, Malayan Red Dwarf (MRD) and East African Tall (EAT). The results are shown in Figures 2 and 3. It was apparent that all types of media used equally supported embryo growth in both varieties. From all types of media, it was possible to get over 80% germination 142 days after initial culture. However, embryo development after germination revealed two main features. First, embryos from dwarf variety germinated faster than embryos from tall variety (Fig. 3). Second, growth of dwarf variety embryos (expressed as rate of leaf production) after germination was faster compared to embryos from the tall coconut variety (Fig. 2). Although embryo development (in EAT) after germination was better in liquid medium, this was only possible when germination was successful and the embryo was in its natural orientation. These two conditions were important and necessary. Greater proportion of embryos in this type of medium remained ungerminated (Fig. 2). If the proportion of germinated embryos in the liquid medium is improved, this medium is...
even better than other types with respect to embryo development after germination.

Further experimentation confirmed these results which formed the basis for further decision on selection of culture medium. From these results, it is apparent that the three types of medium can be used successfully in dwarf varieties. However, these results are from two coconut varieties, the response of other varieties requires experimental confirmation. This will be confirmed in due course and results reported as appropriate.

**Acclimatization of embryo-derived plantlets**

Successful weaning of *in vitro* plantlets is an important aspect, otherwise all the applications of embryo culture may be obsolete. Survival is greatly enhanced by gradual acclimatization and hardening-off.

Ideal ramets for weaning are those with at least two foliage leaves and good root ramification. Experiments at MARI have shown that it is not necessary to have more than one root in the ramet for a successful *extra vitrum* establishment. High relative humidity in the first three weeks of establishment is important and necessary. River-sand proved to be effective in promoting root formation *in vivo*. Plantlets are kept in river-sand for two months and subsequently polybagged in top soil supplemented with farm yard manure. At this stage, one is sure of recovering more than 95% of the polybagged plantlets to field planting. Field planting may be done as soon as the plantlets have produced two to three leaves during *in vivo* period. However, plantlets may be left longer in the nursery so that they are not liable to damage during transplanting.

**Acclimatization techniques developed in Tanzania**

Little success has been achieved with the weaning of *in vitro* seedlings raised from zygotic coconut embryos. Since 1993 to the end of 1996, a total of 800 embryos have been cultured from which 55% germinated and 28% grew to weaning stage. However, only 1.3% survived to field planting stage (Table 1).

In the first acclimatization trials, sterile top soil mixed with sand was used. After dry heat sterilization the medium was put in polybags, plantlets were washed with lukewarm water to remove agar and then dipped in a fungicide solution containing 10 g l⁻¹ Ultra Dithane M₄₅, and planted in the weaning medium. The plantlets were planted singly and each was covered with a transparent plastic bag. Initially, 40 plantlets were set in the experiment. None of these reached field planting stage. Some modifications were made afterwards in which a humid room was used instead. Features of the room were as follows: the floor made of concrete was finely finished to make it impermeable to water. It was partitioned into beds by small concrete banks. These were built to hold sand and water (for humidification). All sides were lined with transparent polythene sheets. The intention was to keep relative humidity in the enclosure at around 90-100%. Hundred (100) ramets were planted in polybags containing sterile sand only and transferred to the humid room. Polybags were not in direct contact with the humidifying sand. All plantlets died after one month and there was a lot of fungal and algae growth in the humidifying sand. The temperature in the room in some cases reached 35°C. The experiment was repeated with no success. More experiments were conducted from which ten plantlets were successfully weaned and polybagged. Out of these, four plantlets were lost to termites and bud rot disease.

More experiments were carried out to improve moisture and temperature regime. Different materials and equipment were used and these included electric-run humidifiers, smaller humid chambers (locally assembled) and claypots. Results were obtained with varying success. However, in all these experiments, the critical period to *extra vitrum* establishment seemed to be between the second and fourth to fifth week after transfer to *in vivo* conditions (Table 1).
The number of seedlings that could be transferred to the field was determined by the population that survived the critical period. From this observation, it became evident that any weaning procedure intended to improve plantlet recovery should aim at improving the survival rate at the first few weeks extra vitrum. Speculative explanations for low survival rate extra vitrum may be due to: heavy dependence of plantlets on heterotrophic mode of life induced by culture conditions; rapid rot of the haustorial part that creates entry points for infectious microbes due to its parenchymatous cellular composition; and lack of wax deposition on leaves leading to uncontrolled transpiration rates. This is worsened by the presence of stomata on both sides of the leaves (Juma and Hornung 1997).

It then follows that, as much as one would struggle to produce good plantlets in vitro, procedures that could protect plantlets during the critical period should be in place.

Preliminary observations suggest that the longer the plantlet stay in culture, the more it becomes prone to unsuccessful establishment during the critical period.

In the mid 1996, experiments were initiated to refine the procedures based on previous results. Claypots and small humid chamber were selected for refinements. Claypots were chosen because they gave consistently better results contrary to humid chambers.

Claypots can withstand dry heat sterilization with the medium and are able to draw water from the reservoir by capillary. The latter eliminate the laborious operation of watering individual plants. This may as well act as a screening mechanism for possible contaminants that may be present in water.

Claypots give further advantage in that each plantlet is separated from the other and therefore, exclude cross-contamination from adjacent plantlets with infectious and transmissible pathogens. However, claypots are expensive (but reusable), laborious and time consuming to use in large-scale operation. They are heavy, especially when filled with sand, and require big dry heat oven for sterilization. They may require extended periods to cool before they can be used for plantlet transfer. Improperly baked pots crack when subjected to high sterilization temperatures.

The claypots

Claypots used are roughly 12 cm tall with a diameter of 12 cm and are locally available.

Procedure. Pots are two third filled with river-sand, sterilized at 150°C for 24 hours. They are left to cool for at least 12 hours. Water reservoir lined with plastic sheet is prepared and then filled with clean tap water. Pots are placed in the reservoir and left to soak the medium through capillary (Plate 1). Soaking takes about 20 minutes afterwhich, the medium is ready for plantlet transfer.

Plantlets are removed from the growth room and taken to the screenhouse. Gradual acclimatization may start when the plantlets are still in the culture vessels but this procedure seem to be unnecessary for embryo-derived coconut plantlets. Plantlets are removed from the culture medium, agar washed off and sprayed with a fungicide (Ultra Dithane M45) solution before transfer into claypots.

Individual plantlets are planted in their respective pots and labelled as necessary. They are then covered with a transparent plastic bag (top part sealed), fastened around the neck of the pot by a rubber-band.

The bag is held upwards to accommodate the plantlet by a stick tied to the bag and pot (Plate 2). Care must be taken not to leave the in vitro plantlets in open air for long spans as they will very quickly and hardly recover afterwards!

After three weeks, two holes are made in the plastic bag at opposite sides to reduce relative humidity. More holes are made after six weeks and the bag is completely
removed after eight weeks. At this stage, plantlets are ready for pricking-out.

**The humid chamber**

The chamber is made of metallic frame. Fifteen centimeters from the base (bottom), the frame is tied with metal beams that hold the four posts. The sand is placed in the lower part of the frame held in position by four wooden or metallic beams. Sand is filled to a depth that can accommodate the roots of the plantlet (about 10 cm deep). The frame is covered with transparent plastic sheets in all sides to the floor level to maintain high relative humidity during the first days of establishment. The sand may be sterile or not and it is watered together with the inner sides of the sheetings. At this juncture, the chamber is ready to be transferred with the *in vitro* plantlets. Preparation procedures of plantlets are as described above. Humid chambers are advantageous to claypots in that, setting the plantlets in the bed is less laborious but infectious microbes are easily spread to otherwise clean plantlets (Plate 3).

Gradual reduction of relative humidity in the chamber is done by folding up the sheetings. First folding to about 12 cm above the floor is done three weeks after transfer.

The sheetings are raised further in the sixth week and completely removed after two months. The plantlets are then ready for pricking-out into polybags with top soil and farm yard manure (Plate 4).

**Watering regime**

Watering in the claypots and humid chamber is done once in two weeks in the first four weeks. Water in the humid chamber is sprinkled, not flooded. During hardening-off, plantlets are watered as necessary to keep a minimum moisture regime in the weaning medium.

After pricking-out, they are watered in a skip-a-day regime. Two months later, fertilizers may be applied to sustain growth but this is not an automatic operation. Initially, 21 plantlets were set out in the claypots in which 62% were recovered for transfer to the field. On the other hand, only 10% plantlets out of 40 were successfully transferred to the field from the humid chamber.

These results compelled the investment of more efforts in the use of claypots. Another lot of 45 plantlets were set in claypots and same number in the humid chamber. Survival rate after 8 weeks was 30%.

At the time being, the Institute holds 58 embryo-derived plantlets that await field planting. Five are already in the field and are doing very well. It should be noted that plantlets in the polybags should be looked after properly because they are prone to diseases and pests, and physical damage.

More effort is being put to make establishment *extra vitrum* a success. Growth conditions *in vitro* and *extra vitrum* will be optimized to give a better final plantlet recovery.

**Additional experiments**

**Medium-term in vitro storage of zygotic coconut embryos.** Mature (11 to 12 months old) zygotic coconut embryos of the variety East African Tall were stored in *vitro* for six months. Various sucrose concentrations 0,2,4, 6 and 8 g l⁻¹ were tested on two types of media (MS/Y3 +/- activated charcoal). Growth during storage was assessed as length, width and weight increase. Embryos were retrieved after every two months for viability assessment. Plants were regenerated from zygotic embryos and plumular tissue excised from mature zygotic embryos. The recovery medium was MS/Y3 containing activated charcoal, Gelrite and 45 g l⁻¹ sucrose. Embryo growth and
germination were suppressed during storage. Browning, which is thought to be elicited by the presence of sucrose, was eliminated by the addition of activated charcoal to the medium. Viability of the embryos was retained throughout the storage period. Plumules gave higher germination rates than zygotic embryos, but seedling production was hampered by high levels of vitrification with the in vitro plant.

**Cryopreservation.** In Tanzania, coconut germplasm is conserved in field collections which occupy large areas. These are expensive to maintain and are prone to man-made and natural disasters. Long term storage is not possible due to recalcitrance of coconut seeds. In addition, coconut tend to germinate within a short period after maturity due to lack of dormancy.

It was envisaged that field collecting could be complemented with in vitro storage. Two attempts were made to test the protocol for cryopreservation as reported by Engelmann and Assy Bah (1992). In these experiments, mature and immature zygotic coconut embryos were used.

No single plantlet could be regenerated from cryopreserved embryos in all attempts. These experiments will be continued despite the problems related to cryo-injury and availability of liquid nitrogen. Modification of the available protocol with respect to pre-treatment (dehydration and cryoprotection), thawing and the recovery medium after storage will be given due attention. Given that most of the facilities are available, possibilities are high that a cryopreservation method will be worked out and developed in the near future.

**Conclusions and recommendations**

Embryo germination seems to pose little problem to in vitro techniques employing zygotic coconut embryo rescue/culture. The problem rest on lack of sustained growth after germination, especially after producing the second or third foliage leaf in vitro, and the period between nursery establishment and field planting.

Physiological and anatomical investigation of roots, leaves and apical bud meristematic activity during the transition period before field planting need to be looked at. Comparison may be made between the conventionally raised seedlings and the in vitro plantlets at the same stage of growth.

Studies should be carried out to establish when the seedling becomes independent of the nutrient reserve in the seednut. Preliminary results from experiments carried out in Tanzania indicate that an in vitro plantlet can only survive on its own after producing at least one foliage leaf. This could be of value especially in cutting down the time spent in culture.

Dwarf coconut varieties, exemplified by MRD, are fast growing in vitro as compared to tall varieties, e.g. EAT. Experiments may be set to establish why dwarfs perform better in vitro than tails. This has been observed in the weaning process as well. It is possible that nutrient requirements in vitro, in tails and dwarfs are different.

Zygotic coconut embryo culture techniques have been in place and practised for long now. It is imperative that these techniques and procedures are perfected to allow their application in other fields of coconut research. A research network is appropriate and called for.

Information exchange on advances made in this field need to be strengthened. It may be appropriate to make regular contributions (from different countries) in the BUROTROP bulletin. If possible, a section in the bulletin should be set aside for this purpose.

**References**
Table 1. Weaning survival of embryo-derived plantlets in Tanzania.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Initial No.</th>
<th>No. After 2 Weeks</th>
<th>No/After 5 Weeks</th>
<th>No. After 8 Weeks</th>
<th>% Success in Polybags</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>244</td>
<td>NA</td>
<td>NA</td>
<td>10</td>
<td>1%</td>
<td>4 lost to termites and bud rot 1*</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>54</td>
<td>24</td>
<td>24</td>
<td>43%</td>
<td>Termite attack and fungal disease killed 5 2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>21</td>
<td>14</td>
<td>13</td>
<td>43%</td>
<td>Leaf eating pests 3*</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>46%</td>
<td>All died of unknown disease 4*</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>35</td>
<td>5</td>
<td>4</td>
<td>10%</td>
<td>All in humid chamber</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>18</td>
<td>13</td>
<td>13</td>
<td>62%</td>
<td>Leaf eating pests 5*</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>76</td>
<td>32</td>
<td>27</td>
<td>30%</td>
<td>Death mainly due to fungal disease 6*</td>
</tr>
</tbody>
</table>

1* various methods  
2* all in humid chamber  
3* all in humid chamber  
4* all in claypots  
5* all in claypots  
6* 45 in claypots (success 46%) and 45 in humid chamber (13 % success)

Fig. 1. Average weight of embryos at initial culture and after 2 weeks in culture using different types of medium formulation

![Fig. 1. Average weight of embryos at initial culture and after 2 weeks in culture using different types of medium formulation](image)

Fig. 2. Rate of leaf production of coconut embryos of Malayan Red Dwarf (MD, MS, ML) and East African Tall (ED, ES, EL) 142 days after initial culture. Note: Whenever quoted, S denotes solid, D - double phase and L - liquid media

![Fig. 2. Rate of leaf production of coconut embryos of Malayan Red Dwarf (MD, MS, ML) and East African Tall (ED, ES, EL) 142 days after initial culture. Note: Whenever quoted, S denotes solid, D - double phase and L - liquid media](image)
Fig. 3. Germination (%) of Malayan Red Dwarf (Mn, MS, ML) and East African Tall (ED, ES, EL) embryos 142 days after initial culture

Plate 1. Plantlets before potting or putting in the humid chamber

Plate 2. Plantlets in claypots

Plate 3. Plantlets in a locally assembled humid chamber

Plate 4. Plantlets in polybags containing top soil and farm yard manure

Attachment 1. Facilities available in the Plant Tissue Culture Laboratory of the MARI

<table>
<thead>
<tr>
<th>Facility</th>
<th>Type</th>
<th>Quantity</th>
</tr>
</thead>
</table>

http://www2.bioversityinternational.org/publications/Web%5Fversion...
<table>
<thead>
<tr>
<th>Equipment</th>
<th>Brand/Model</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical balance</td>
<td>KERN 870</td>
<td>1</td>
</tr>
<tr>
<td>Autoclave (Steam sterilizer)</td>
<td>WEBECO C</td>
<td>1</td>
</tr>
<tr>
<td>Bead sterilizer</td>
<td>STERI 250</td>
<td>1</td>
</tr>
<tr>
<td>Computers</td>
<td>SIEMENS NIXDORF, Toshiba</td>
<td>2</td>
</tr>
<tr>
<td>Cool box</td>
<td>Coleman Thermoelectric</td>
<td>1</td>
</tr>
<tr>
<td>Dehumidifiers</td>
<td>ED305 K6</td>
<td>2</td>
</tr>
<tr>
<td>Dissector</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Dissection Microscope (with light source)</td>
<td>Nikon SMZ- IB</td>
<td>1</td>
</tr>
<tr>
<td>Distillation apparatus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single still</td>
<td>IKA-DEST M3000, GFL 2002</td>
<td>2</td>
</tr>
<tr>
<td>Double still</td>
<td>TQS 31500</td>
<td>1</td>
</tr>
<tr>
<td>Dry heat oven</td>
<td>Memmert</td>
<td>1</td>
</tr>
<tr>
<td>Freezers</td>
<td>LIEBHERR OKO super, BOSCH economic-froster</td>
<td>2</td>
</tr>
<tr>
<td>Hot plate (without magnetic stirrer)</td>
<td>EGD</td>
<td>1</td>
</tr>
<tr>
<td>Hot plates (with magnetic stirrer)</td>
<td>IKAMAG RCT</td>
<td>2</td>
</tr>
<tr>
<td>Humidifiers</td>
<td>Defensor AG model 505</td>
<td>3</td>
</tr>
<tr>
<td>Ice machine</td>
<td>SCOTSMAN AF-10</td>
<td>1</td>
</tr>
<tr>
<td>Incubators</td>
<td>Rubath Apparate GmbH</td>
<td>2</td>
</tr>
<tr>
<td>Laminar flow hood</td>
<td>Clean Air Deutchland</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DLF/REC4 KL2A</td>
<td></td>
</tr>
<tr>
<td>Liquid nitrogen refrigerators</td>
<td>Taylor-Wharton 34HC, Taylor-Wharton 3XTL</td>
<td>1</td>
</tr>
<tr>
<td>Microscope (with light source)</td>
<td>WILL V 200</td>
<td>2</td>
</tr>
<tr>
<td>Microtome</td>
<td>Reichert-Jung 1165/Rotocut</td>
<td>1</td>
</tr>
<tr>
<td>pH meter</td>
<td>WTW pH 522</td>
<td>1</td>
</tr>
<tr>
<td>Pipette washer</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Portable UV light source</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Power generators</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Precision balance</td>
<td>KERN P115</td>
<td>1</td>
</tr>
<tr>
<td>Pressure cookers</td>
<td>CENTROclav, Prestige</td>
<td>2</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>AEG SANTO</td>
<td>1</td>
</tr>
<tr>
<td>Sealing machine</td>
<td>Polystar 100GE</td>
<td>2</td>
</tr>
<tr>
<td>Shaker</td>
<td>Gerhardt RO 30</td>
<td>1</td>
</tr>
<tr>
<td>Thermohygrometer</td>
<td>3.015/1, Durotherm</td>
<td>2</td>
</tr>
<tr>
<td>Water heater</td>
<td>AEG</td>
<td>1</td>
</tr>
<tr>
<td>Wax melting plate</td>
<td>MEDAX SP-12</td>
<td>1</td>
</tr>
</tbody>
</table>

**Required Facilities:**

- Improvement of the screenhouse
- Incorporation of temperature regulators in the growth room
- Purchase of an automatic media dispenser
- Facilities for ELISA
- Additional laminar air flow cabinet
- Glassware
- Additional facilities and materials for histological studies