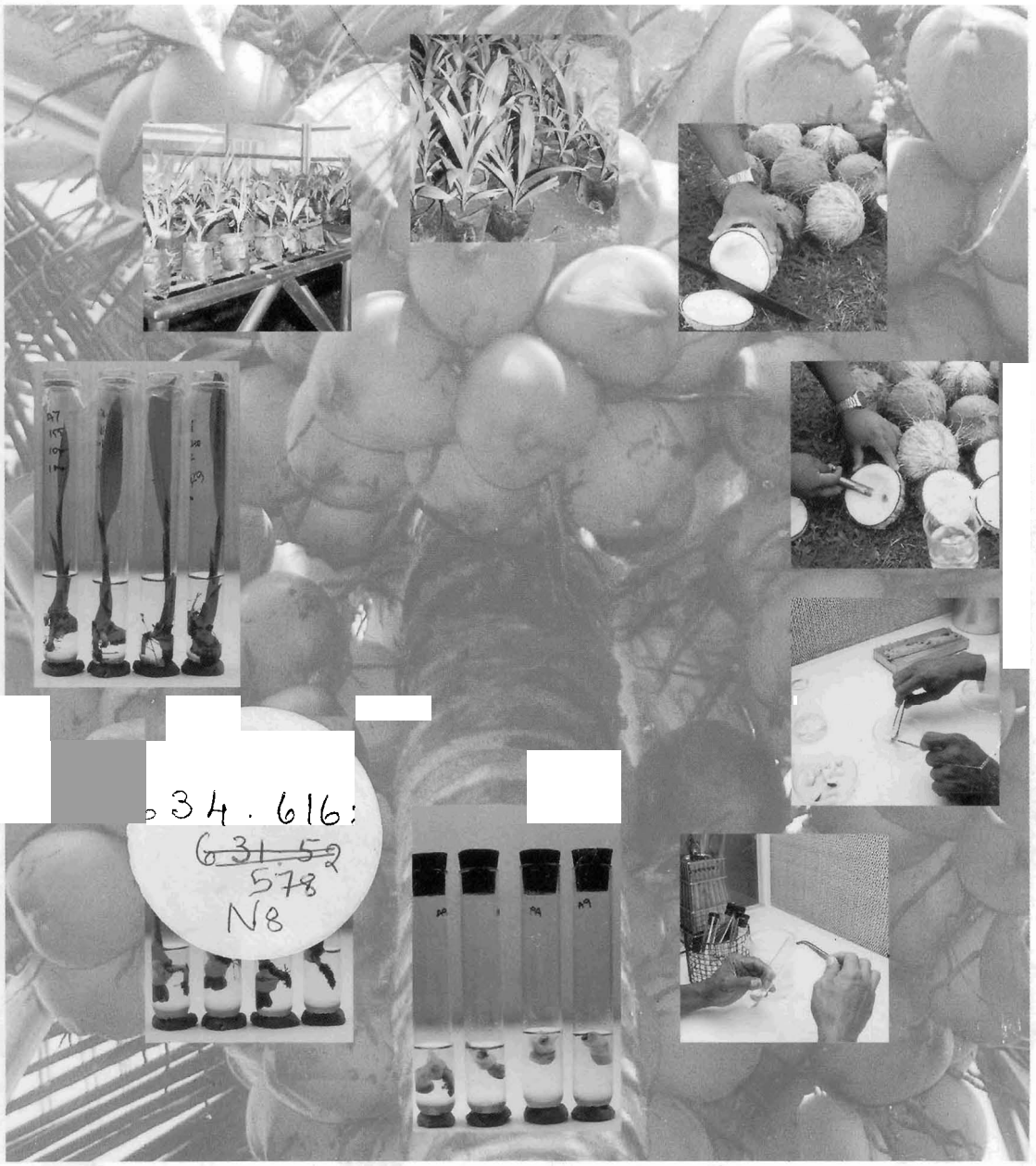
Coconut Embryo *In Vitro* Culture

Proceedings of the first workshop on Embryo Culture
27 – 31 October 1997
Banao, Guinobatan, Albay, Philippines

Pons A. Batugal *and* **F. Engelmann,** *editors*



COGENT

International Plant Genetic Resources Institute
IPGRI

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COGENT and 1997 activities; workshop objectives and expected outputs

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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 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

Southeast and East Asia	South Asia	South Pacific	Africa/Indian Ocean	Latin America/ Caribbean
Current members				
1. China	1. Bangladesh	1. Cooke Is.	1. Bénin	1. Brazil
2. Indonesia	2. India	2. Fiji	2. Côte d'Ivoire	2. Costa Rica
3. Malaysia	3. Pakistan	3. Kiribati	3. Ghana	3. Cuba
4. Myanmar	4. Sri Lanka	4. Papua New Guinea	4. Kenya	4. Guyana
5. Philippines		5. Solomon Is.	5. Mozambique	5. Haiti
6. Thailand		6. Tonga	6. Nigeria	6. Jamaica
7. Vietnam		7. Vanuatu	7. Seychelles	7. Mexico
		8. Samoa	8. Tanzania	8. Trinidad -Tobago
Invitees				
		9. Tuvalu	9. Comoro	9. Colombia
		10. Marshall Is.	10. Madagascar	10. Dominican Republic
				11. Ecuador
				12. El Salvador
				13. Guatemala
				14. Panama
				15. Venezuela

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-ls.) 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 gemplasm. 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 México, 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

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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.

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

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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

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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 whic h 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.

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Table 1. Rate of photosynthesis, and pigment content of *in vitro* cultured coconut plantlets during successive stages of culture *in vitro* and after transplanting*

Growth stage	Photosynthetic rate (Mg/Sq Dm/Hr)	Chlorophyll content (Ug/G)	Carotenoid content (Ug/G)	Chl : Car ratio
2-leaf**	9.22b	125.5e	0.0 d	122.5 : 0
4-leaf	2.26c	215.9bc	0.0 d	215.9 : 0
2 WAT***	13.78a	271.0b	23.9 cd	11.3 : 1
4 WAT	3.37c	280.4b	56.2 bcd	5.0 : 1
8 WAT	6.28bc	304.7a	82.9 bc	3.7 : 1
25 WAT	4.86c	224.1b	116.9 b	1.9 : 1
45 WAT	3.87c	174.2cd	194.2 a	0.9 : 1

* Means followed by the same letter are not significantly different at 5% level
** In vitro
*** Weeks after transplanting
(Malijan and Del Rosario 1986)

Table 2. Stomatal indices in the abaxial and adaxial epidermis of *in vitro* cultured coconut plantlets during *in vitro* culture and after transplanting.*

Growth stage	Adaxial epidermis	Abaxial epidermis
2-leaf **	0.002 b	0.010 b
4-leaf	0.853 a	4.700 a
2 WAT***	0.764 a	4.264 a
4 WAT	0.813 a	5.440 a
8 WAT	0.000 b	4.820 a

* Means followed by the same letter are not significantly different at 5% level
** In vitro
*** Weeks after transplanting
(Malijan and Del Rosario 1986)

State of research on coconut embryo culture and acclimatization techniques in the IDEFOR (Côte d'Ivoire) and ORSTOM/CIRAD laboratories (France)

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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 Béatrice 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).

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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 1st–2nd 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.* 1989). 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 suvival 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)

KNO_3	274
$Ca(NO_3)_2\ 2H_2O$	1095
$KH_2\ PO_4$	137
$MgSO_4\ 7H_2O$	274
$(NH4)_2\ SO_4$	137
KCl	2.74
H_3PO_3	3
$MnSO_4\ H_2O$	15
$ZnSO_4\ 7H_2O$	2.74
$(NH_4)_6\ MO7OZ_4\ 4H_2O$	2.74
H_2SO_4	0.137
$Cu\ SO_4\ 5H_2O$	1.37
$FESO_4\ 7H_2O$	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 gl^{-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 CO2) 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 (ELISA) (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) occured 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 rôle 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 vitroplant 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 vitroplant 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 CO_2 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.

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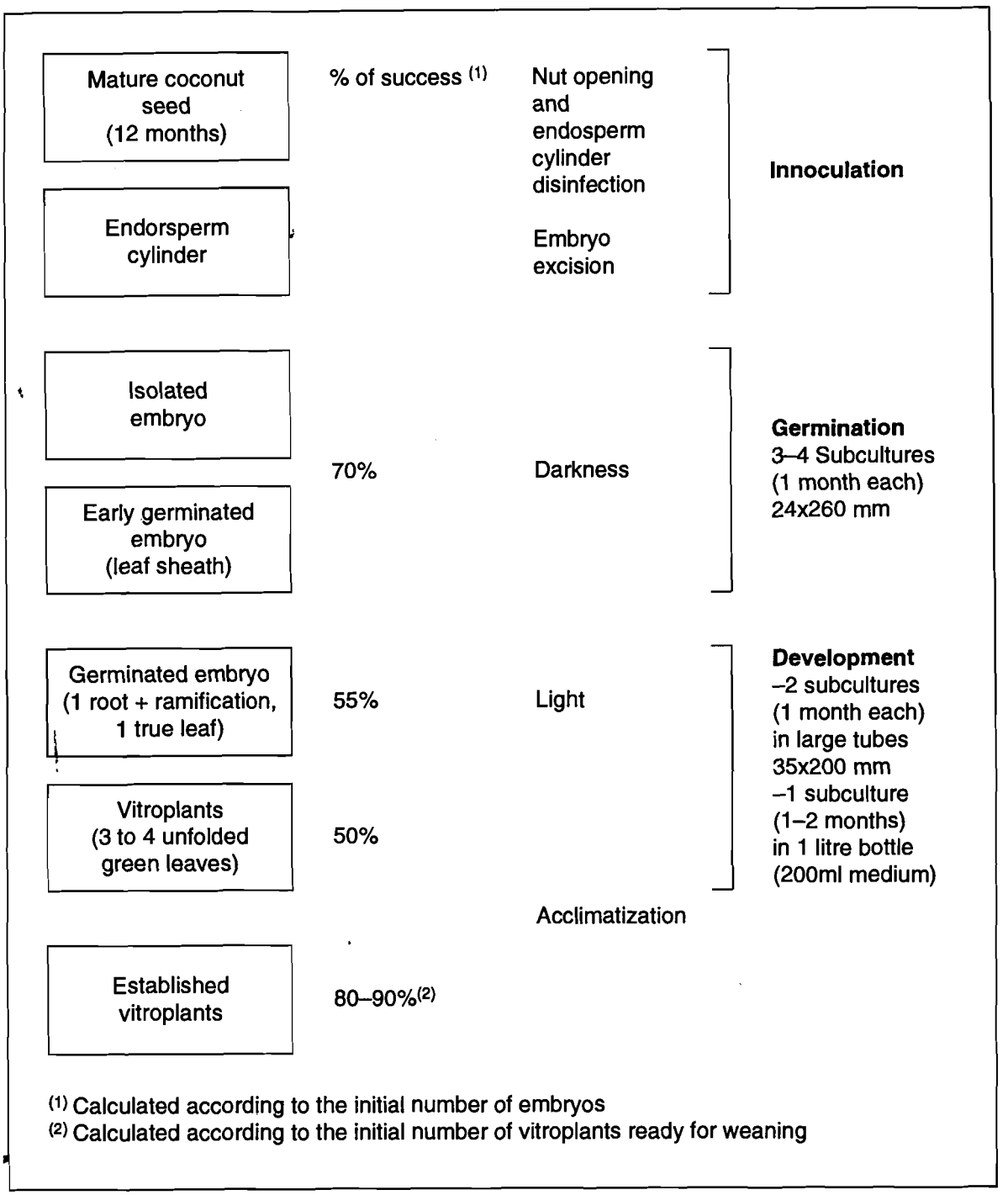

Fig. 1. Overall scheme of the IDEFOR/CIRAD-OSTROM protocol for coconut embryo culture

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 KCl solution.

Replicate no.	Endosperm cylinders isolated under lab conditions		Endosperm cylinders isolated under field conditions stored in KCl	
	No. of embryos cultured	% contamination	No. of embryos cultured	% contamination
1	96	3.1	97	3.1
2	99	8.1	89	4.5
3	97	6.2	91	4.4
4	89	4.5	90	8.9

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

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

Replicate no.	Time (month) spent in culture or germination conditions	Nut germination (control)	Embryo cylinders (1) isolated under lab conditions	Endosperm cylinders isolated under field conditions and stored in a KCl solution (2)
1	2	36	6.0	5.3
	6	97	63.3	28.9
2	2	30	8.8	1.2
	6	96	54.1	16.2
3	2	40	20.9	0.0
	6	88	34.4	23.0
4	2	32	3.5	2.0
	6	86	43.9	24.6

(1) % calculated on at least 75 embryos per treatment (PB 121 hybrid).
(2) storage during 14 days.

Table 3. Influence of sucrose concentration on leaf emergence.

	Sucrose concentration (g/l)				
	20	30	60	90	120
% of embryos with at least 1 leaf (1)	46	49	53	50	8

(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

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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.*, Y_3 (Eeuwens 1978), (30g/l sucrose), Y_3 (60g/l sucrose), Assy Bah's medium (Assy Bah *et al.* 1989) and MS (Murashige and Skoong 1962) medium. Y_3 medium supplemented with 60g/l glucose was used for the first month of inoculation while Y_3 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. Y_3 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 Y_3 medium supplemented with IBA (5 mg/l), NAA (1 mg/l), BAP (1mg/l), activated charcoal (1g/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 5ml 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. CGRNAP (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
4. Standardization of protocol for short-term storage of embryos.

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.

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

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

* % survival from initial number inoculated

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

Sl.no.	Character	8 months	9 months	10 months
1.	% germination	60	74	83
	At the time of transfer			
2.	Root volume (ml)	4.78	3.65	5.15
3.	Plant height (cm)	21.10	22.26	24.74
4.	No. of leaves	3.41	3.88	3.70
	After 8 months in pots			
5.	Plant height(cml.)	28.71	31.61	32.76
6.	Root volume (ml)	10.34	10.41	12.77
7.	Leaf lenght (cm)	24.79	32.64	24.60
8.	No. of leaves	4.50	4.43	4.48

Table 3. Percentage of germination, contamination and survival of field collected embryos from Andamans islands after 4 months under *in vitro* culture

Sl. no.	Accession	Acc no.	No. of embryos	Germination (%)	Contamination (%)	Survival (%)
1.	Niu Ui	10	15	100	nil	60.00
2.	Niu Hako	12	13	100	2	92.32
3.	Bora Bora (Local Tall)	16	17	58	nil	52.94
4.	Rangiroa Tall (Avatoru)	17	19	100	nil	84.21
5.	Rangiroa Tall (Tiputa)	19	9	78	nil	66.66
6.	Nikkore	22	10	100	1	80.00

Table 4. Details of embryos collected from Indian Ocean Islands

Acc no.	Varietal name	No. of embryos				% Germination after 3 months
		Collected	Developing	Contaminated	Damaged	
	Mauritius					
1.	Pemba orange Dwarf	134	127	4	3	65.38
2.	Pemba Green Dwarf	113	97	10	6	69.04
3.	Pemba Yellow Dwarf	25	14	–	–	71.42
4.	Pemba Red Tall	29	24	1	4	70.83
5.	Dupays Tall	66	63	–	3	53.33
6.	Guelle Rose Tall	60	50	3	7	83.33
Madagascar						
7.	Sambava Tall	68	68	–	–	89.70
8.	West African Tall	150	145	–	5	70.37
9.	Sambava Green Tall	115	105	3	7	75.23
10.	Comoros Tall	108	104	–	4	75.75
Seychelles						
11.	Coco le rein Tall	116	113	1	2	80.55
12.	Coco le haut Tall	149	136	11	2	80.55
13.	Coco Blue Tall	51	48	3	–	88.60
14.	Coco Raisin Tall	163	153	7	3	62.23
15.	Coco Gra Tall	23	15	2	6	68.70
	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. CO_2 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
6. Uninterrupted power supply
7. Generator
8. Ice machine
9. Vacuum oven
10. Submarine gel electrophoresis unit
11. Environmental shaker
12. Greenhouse

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
9. Scalpel – 1 No.
10. Plastic tray – 1 No.
11. Parafilm – 1 roll
12. Sterilized disposable gloves
13. Scissors – 2 Nos.
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)

Sl.No.	Chemical	Quantity (mg/l)
1.	NH_4Cl	535
2.	KNO_3	2020
3.	$MgSO_4$	247
4.	$CaCl_2$	294
5.	KCl	1492
6.	NaH_2PO_4	312
7.	KI	8.3
8.	H_3BO_3	3.1
9.	$MnSO_4$	11.2
10.	$ZnSO_4$	7.2
11.	$NaMoO_4$	0.24
12.	$CuSO_4$	0.16
13.	$CoCl_2$	0.24
14.	NiCl	0.024
15.	Inositol	100
16.	Pyridoxine HCl	0.05
17.	Thiamine HCl	0.5
18.	Biotin	0.05
19.	Na_2EDTA	37.3
20.	$FeSO_4.7H_2O$	13.9

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

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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. Afterwhich, 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 glasshouse 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 F_1 kopyor. Predictably, on the fourth year of the research, nuts from F_1 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.

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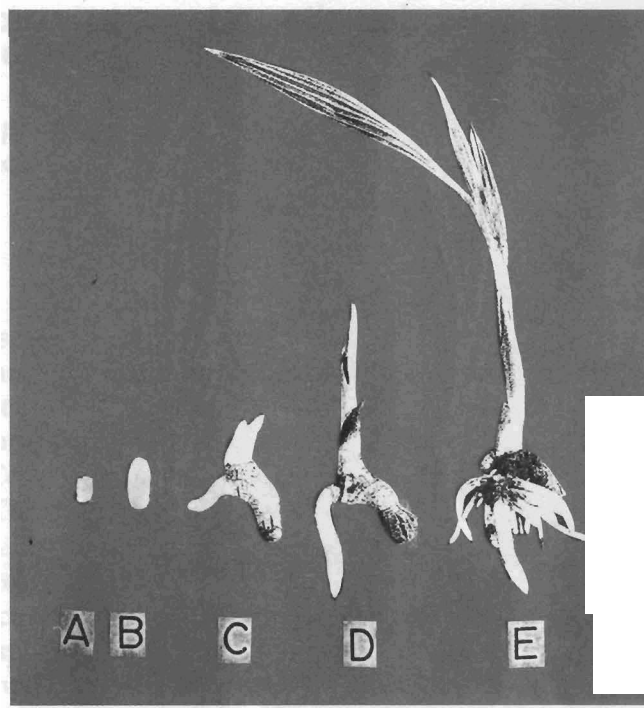

Fig. 1. The development stages of kopyor coconut seedlings
A. Embryo after excision from the endosperm
B. Embryo after 4 weeks in liquid medium
C. Seedling after 4 weeks on solid medium
D. Seedling after 8 weeks on solid medium
E. Seedling after 14 weeks on solid medium

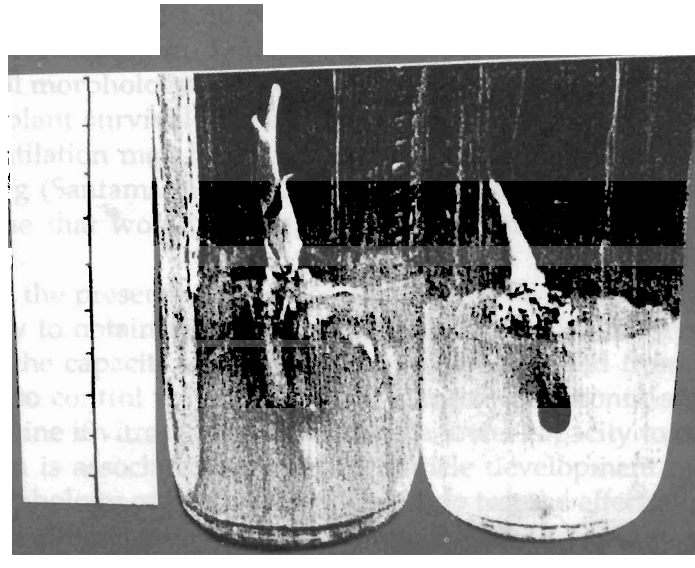

Fig. 2. Normal (left) and abnormal (right) growth of seedlings. Callus which inhibited the root growth was formed at the proximal end of the abnormal seedling

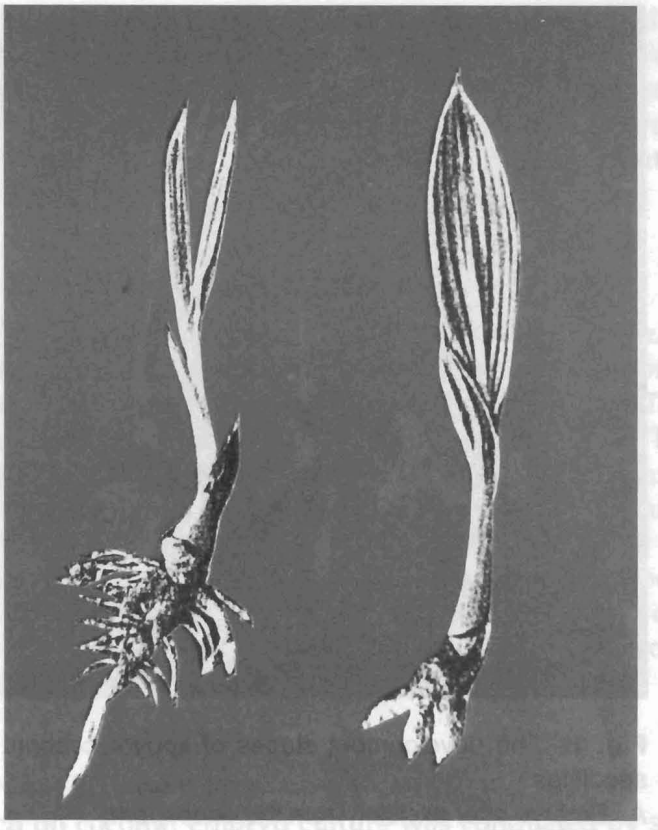

Fig. 3. Coconut seedlings with primary root and a pair of lateral roots from embryos cultured for eight weeks in the first transplant medium

Status of research on coconut zygotic embryo culture and acclimatization techniques in México

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Introduction

Interest has increased on embryo culture technology for germplasm collection and exchange. This is particularly important for México 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 México have been involved in coconut *in vitro* culture, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) and Centro de Investigacíon Científica de Yucatán (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 m^2.

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 m^2 greenhouse with controlled mist generation that provides high relative humidity (80–90%); a larger greenhouse (400 m^2) with ambient humidity, and nursery (800 m^2), 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 l–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 µmol 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 µmol 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 H_2O 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 µmol 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 (Tresaphan 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). The 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 10^{th} 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 (Santamaría 1994, Santamaría 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 (Santamaría *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-2s-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 (Santamaría *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 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 to 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 biorreactor. 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

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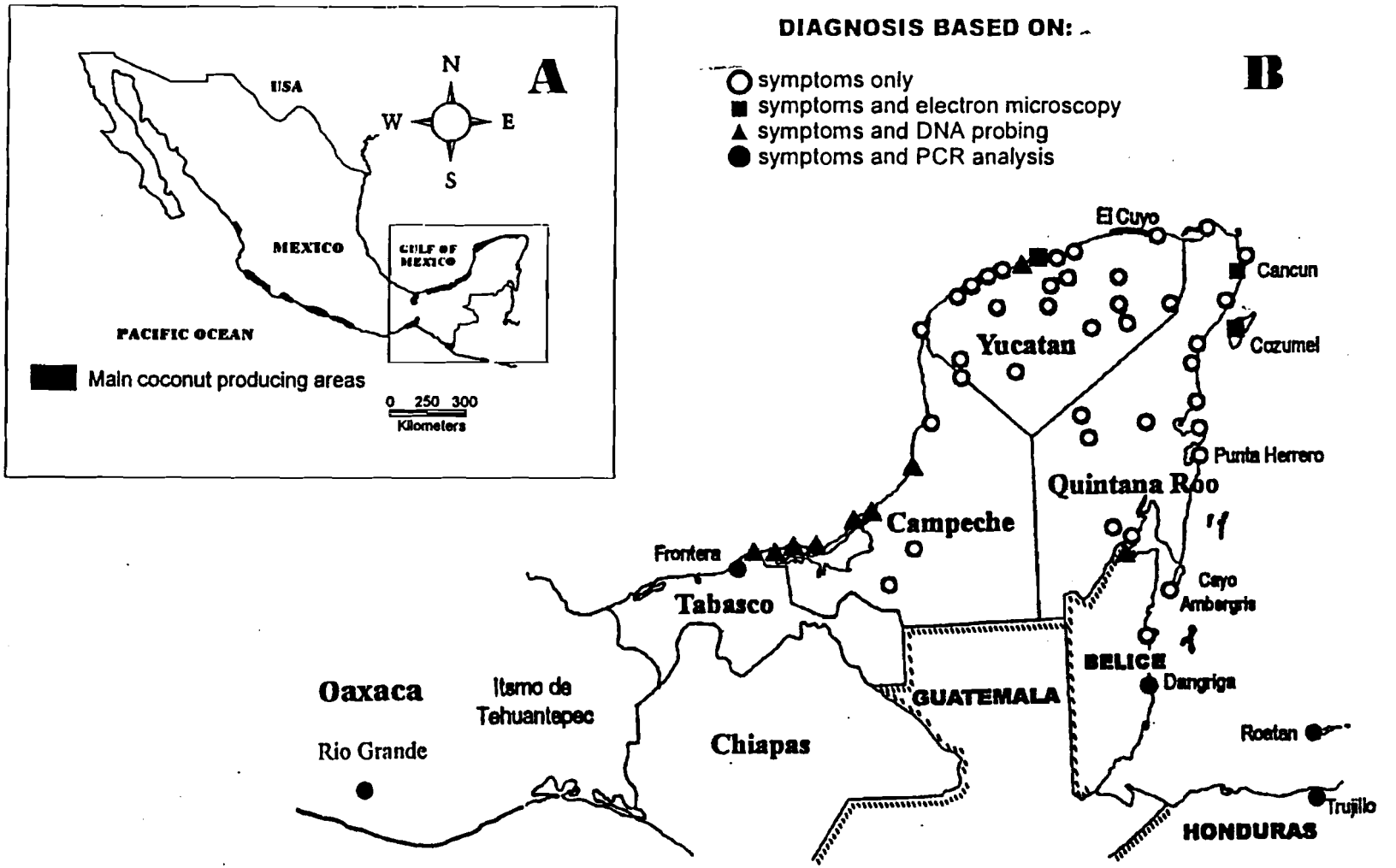

Fig. 1. Main areas of coconut production (A) and distribution of lethal yellowing disease in México in early 1998 (B)

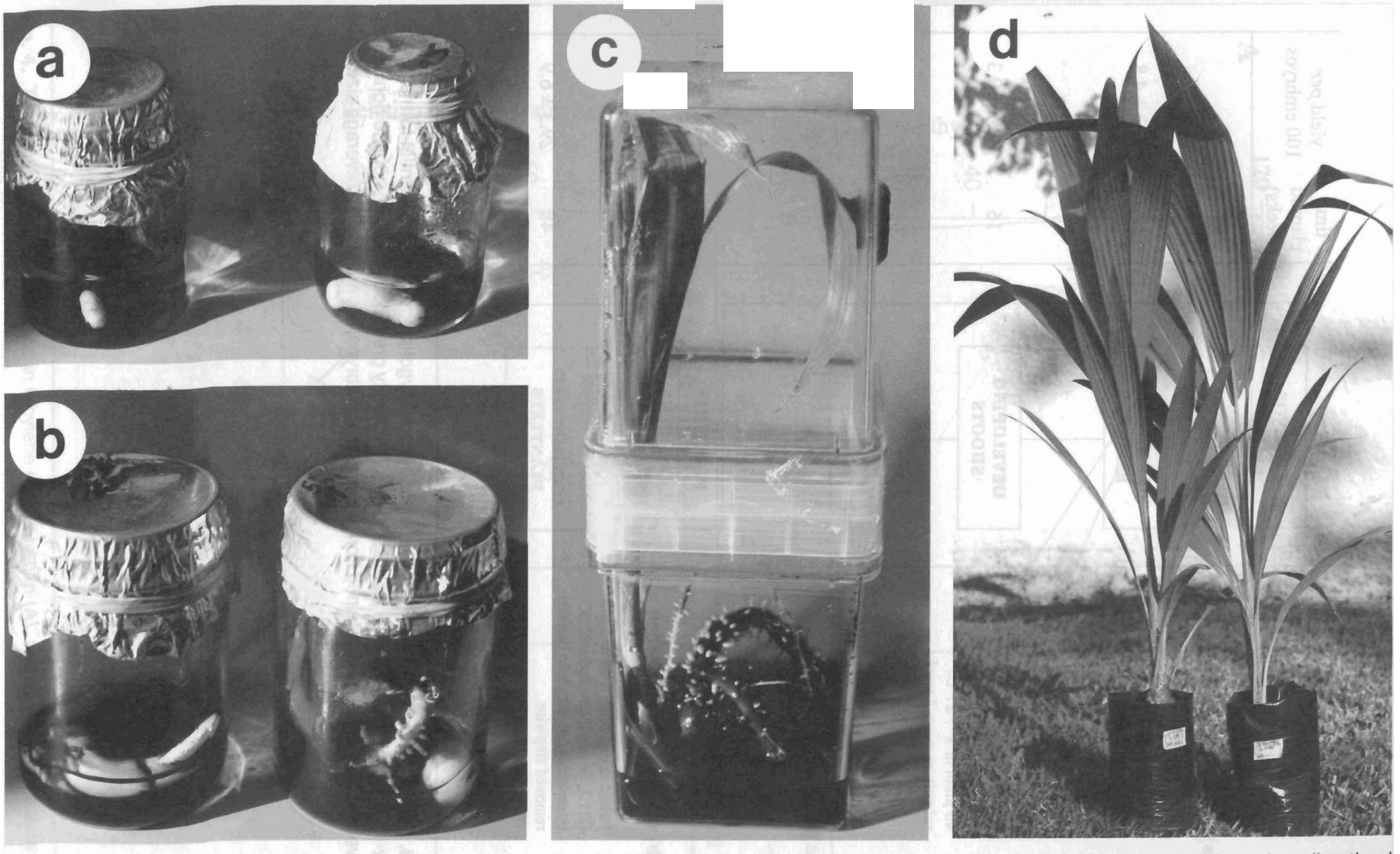

Fig. 2. Views of the process of coconut embryo culture. Germinating embryos (a), developing shoots (b), developing plantlets (c), and acclimatized plantlets (d)

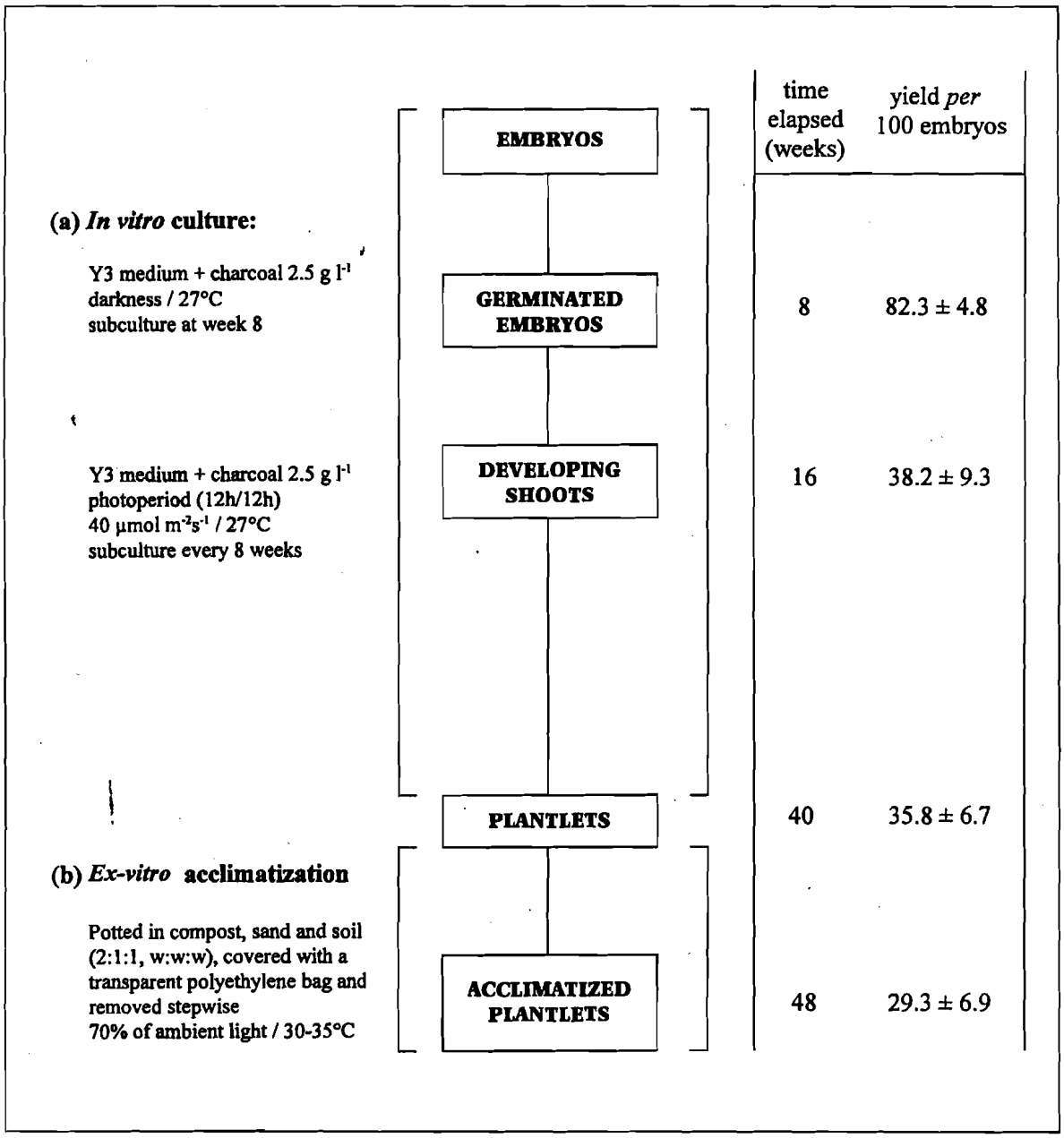

Fig. 3. Protocols for: (a) *in vitro* germination of coconut zygotic embryos and plantlet formation, and (b) *ex-vitro* acclimatization of the plantlets in a high humidity greenhouse. Figures on the right column are averages (± s.d.) of data obtained from 3 batches of embryos, of variable size (average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

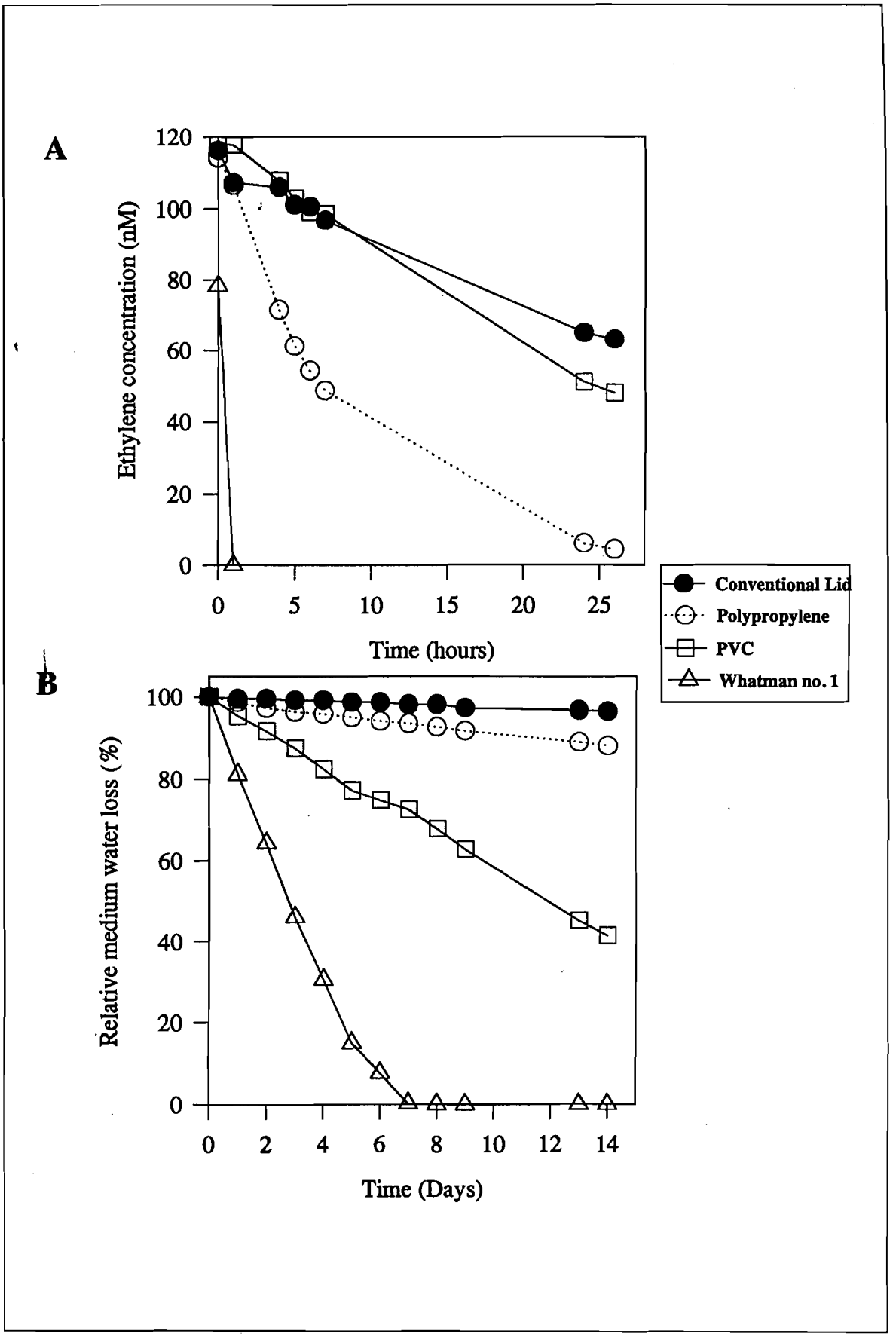

Fig. 4. Time courses of the decrease in ethylene concentration (A), and of relative medium water loss (B), in vessels with different covers

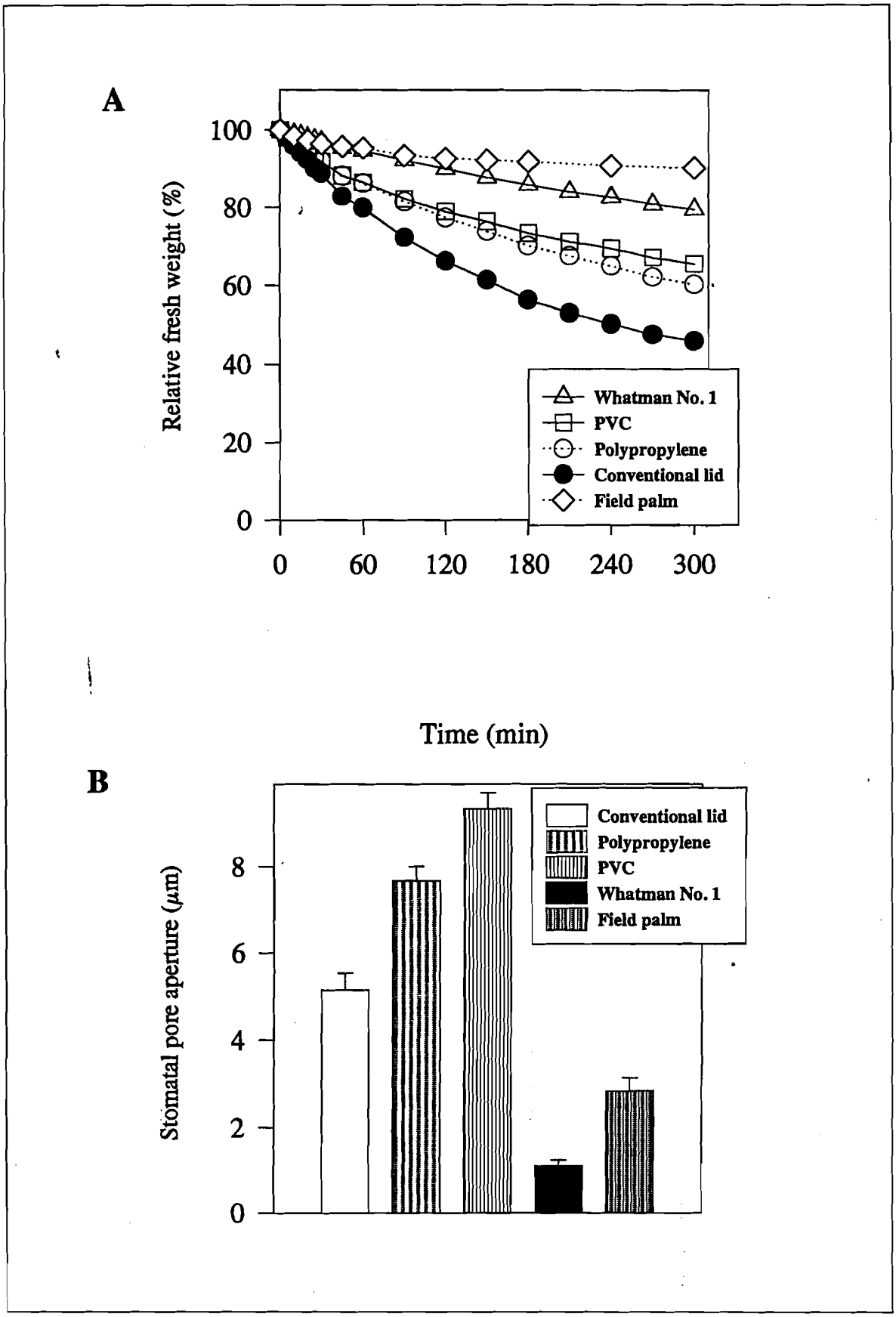

Fig. 5. Time courses of the decrease in relative fresh weight (A) and stomatal pore aperture (B) in leaves of field grown palms and vitroplants grown in vessels with different covers. Results are means of 15 leaf samples in (A) and 60 stomata in (B), per treatment

Embryo culture activities at the Philippine Coconut Authority-Zamboanga Research Center (PCA-ZRC)

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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 ve ry 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 f or 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 (KNO_3)
Potassium chloride (KCl)
Ammonium chloride (NH_4Cl)
Sodium di-hydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)
Calcium chloride dihydrate ($CaCl_2.2H_2O$)
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)
Micronutrients
Mangnese sulfate tetrahydrate ($MnSO_4.4H_2O$)
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)
Boric acid (HBO_3)
Potassium iodade (KI)
Copper sulfate pentahydrate ($CuSO_4.5H_2O$)
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)
Nickel chloride hexahydrate ($Ni\ Cl_2.6H_2O$)
Iron supplement
Sodium EDTA dihydrate ($Na_2EDTA.2H_2O$)
Iron sulfate hetahydrate ($FeSO_4.7H_2O$)
Vitamins
Myo-inositol
Thiamine-HCl
Nicotinic acid
Calcium D-pantothenate
Biotin
Table sugar
Activated charcoal

Fig. 2. Makapuno satellite laboratory at PCA-Zamboanga Research Center: a) exterior view, b) interior view

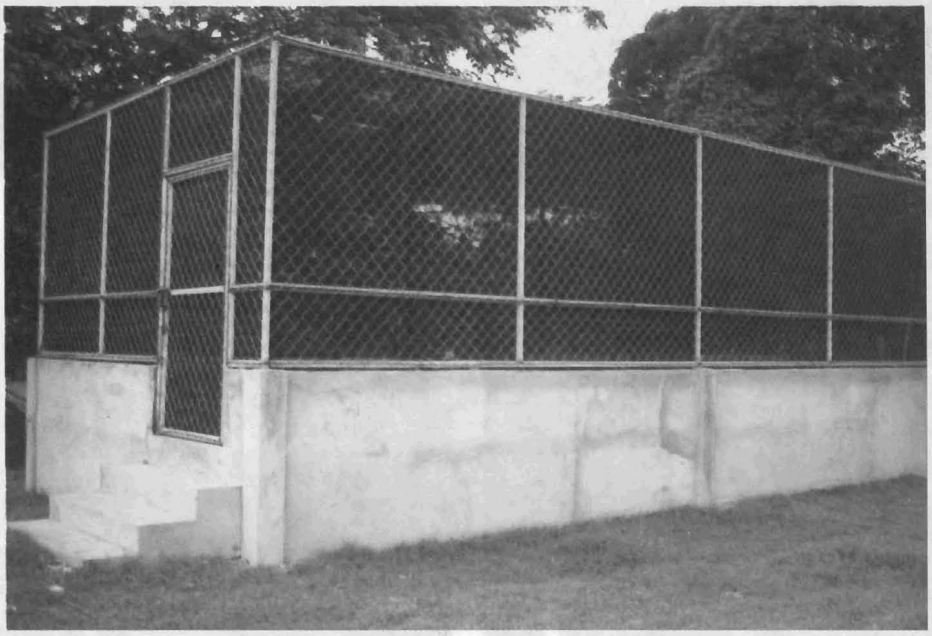

Fig. 3. Makapuno screenhouse

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

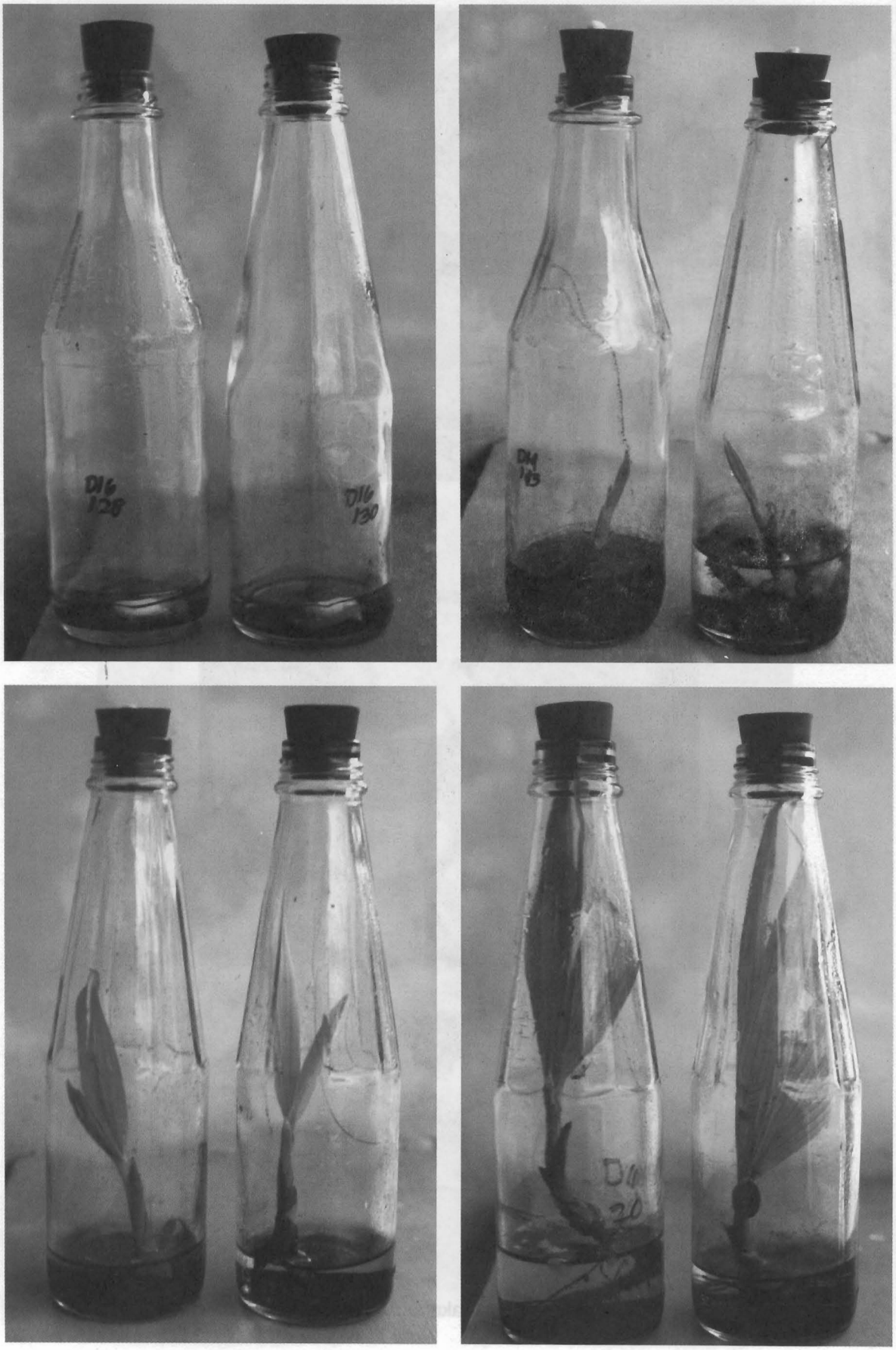

Fig. 5. Different growth stages of Makapuno

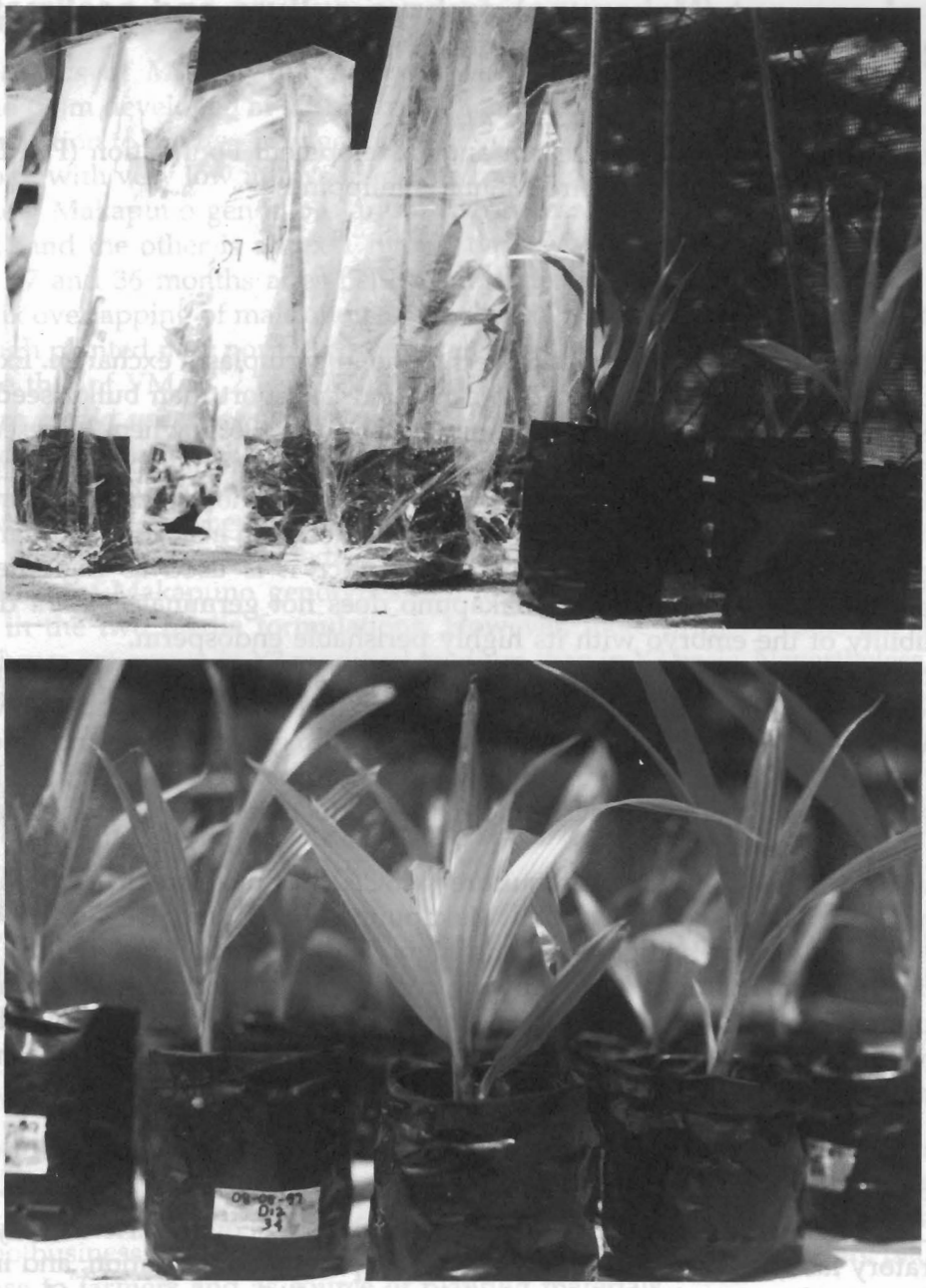

Fig. 6. Polybagging and acclimatization of *in vitro* cultured Makapuno seedlings

Status of coconut (Makapuno) embryo culture and acclimatization techniques in ViSCA, Baybay, Leyte, Philippines

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

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 screenhouse 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. Afterwhich, 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 effficiency 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.

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Table 1. Flowering and yield characteristics of different Makapuno genotypes

Code name	Age at initial flowering (mos.)	Intraspadix overlapping (%)	% "Makapuno" yield	Gross morphology
UPMAC	71	very low	55	Tall type
VMAC 1	37	90	99	Dwarf type
VMAC 2	36	76	90	D x T hybrid Type

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

Treatments	No. of primary roots	No. of leaves	Plant height (cm)	Seedling weight (g)	Survival rate (%)
Y3 Basic					
UPMAC	2.00	2.83 b	22.23 abc	23.07 a	25.67
VMAC 1	1.87	3.00 b	24.63 a	24.97 a	26.67
VMAC 2	2.83	4.17 a	18.07 c	27.70 a	40.00
Mean	2.23 b	3.33	21.64	25.24 a	30.78
Y3 Modified					
UPMAC	3.67	2.33 b	23.97 ab	15.70 b	62.00
VMAC 1	3.30	2.30 b	23.70 ab	24.50 a	36.67
VMAC 2	3.10	4.23 a	19.40 bc	23.20 a	60.00
Mean	3.36 a	2.96	22.36	21.13 b	52.89
C.V. (%)	27.99	18.69	12.12	16.40	76.72

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

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

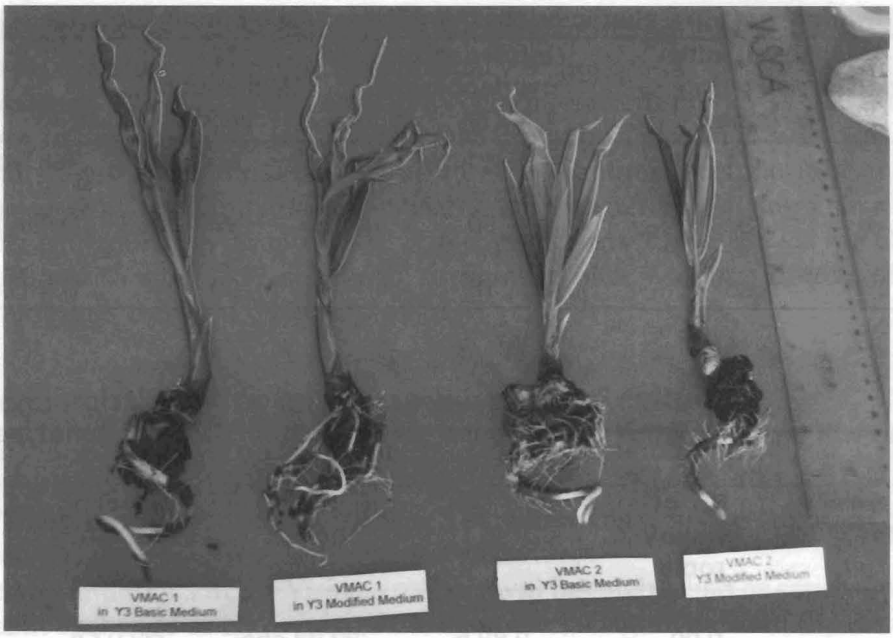

Fig. 1. Makapuno genotypes at potting, grown at different media formulations

Fig. 2. Acclimatized Makapuno seedlings in the greenhouse

PCA's embryo culture technique in the mass production of Makapuno coconuts

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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	g/l
To make 1 litre of Eeuwens (Y3) macronutrient stock solution (10x)	
Weigh and dissolve separately in 50 ml double distilled water:	
Potassium nitrate (KNO_3)	20.20
Potassium chloride (KCl)	14.92
Ammonium chloride (NH_4Cl)	5.35
Sodium di-hydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)	3.12
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	2.94
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	2.47
Mix solutions one after another with a magnetic stirring bar. Make up volume to 1 litre. Store in dark bottle.	
To make 1 litre Y3 micronutrients stock solution (100x)	
Weigh and dissolve separately in 50 ml double distilled water:	
Manganese sulphate tetrahydrate ($MnSO_4.4H_2O$)	1.120
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	0.720
Boric acid (H_3BO_3)	0.310
Potassium iodide (KI)	0.830
Copper sulphate pentahydrate ($CuSO_4.5H_2O$)	0.025
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.024
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	0.024
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	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:	
Sodium EDTA dihydrate ($Na_2EDTA.2H_2O$)	3.73
Iron sulphate heptahydrate ($FeSO_4.7H_2O$)	1.39
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.:	
Myo-inositol	10.000
Thiamine-HCl	0.050
Nicotinic Acid	0.005
Pyridoxine-HCl	0.005
Ca- D-pantothenate	0.005
Biotin	0.005
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
– Vitamins	100x
– FeEDTA	100x
– Table Grade Sugar	45.0 g/l
– AC (Activated Charcoal)	2.5 g/l

Procedure:

To prepare one liter of Y3 liquid medium –

a. Measure out the following from the stock solutions:

Stock	Volume
Macronutrients	100 ml
Micronutrients	10 ml
Myo-inositol	10 ml
Vitamins	10 ml
FeEDTA	10 ml

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 0.1–5M 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^{0}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 CO_2 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:
a. Sterile river sand
b. Clear plastic bags or bell jars
c. Bamboo sticks (when clear plastic bags are used)
d. Polyethylene bags
e. 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 prospection places for transport to culture laboratories. It was also used in cadang-cadang transmission and resistance screening studies.

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

Origin of seednuts	Embryos cultured *in vitro* (no.)	Embryos and plantlets with CCVda (no.)
Albay		
San Miguel Island	17	4
Paulog, Ligao	29	4
Camarines Norte		
Calasgasan Daet	3	0
Vinzons	18	0
Camarines Sur		
Atulayan Island	25	0
Calabanga	10	0
Pasacao	19	0
Sipocot	22	3
Tinambac	25	0
Catanduanes	2	0
Samar		
Calbayog	29	3
Sta. Margarita	29	1
Quezon		
General Nakar	39	0
Infanta	39	1
Balute, Real	59	0
Total	**365**	**14**

[a] Detected by MHA-dotblot.

Table 2. Percent germination and e*x vitro* survival of Makapuno coconuts cultured *in vitro* at ARC

	1995	1996	1996 (DOST)
Total ollection	1 701	465	304
% Germination	57	52	48
Total no. of plantlets in the screenhouse	410	158	67
No. of plantlets that survived	285	147	59
% Survival	69.51	93.03	88.05

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

		No. of embryos	Percent germination
ZRC	Aug. 1996 to Sept. 1997	2 115	50.31
Pangasinan	Oct. 1995 to Sept. 1997	380	60.90
ARC	Aug. 1996 to Aug. 1997	2 470	52.33
Leyte	Dec. 1996 to Aug. 1997	186	78.14

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

Population	Nuts shown	Germinated nuts	Percent germination
Talls			
1. Agta (ATA)	44	35	80
2. Basey (BAS)	60	53	88
3. Tinambacan (TIN)	67	63	94
4. Laoang (LAO)	64	60	94
5. Guiuan (GUT)	61	57	93
6. Lope de Vega (LOP)	61	49	80
7. Borongan (BOR)	61	50	82
8. San Policarpio (POL)	60	55	92
9. Catbalogan (CAB)	39	34	87
Dwarfs			
1. Guiuan (GUD)	27	13	48
2. Sta. Margarita (MAR)	19	18	95
Total	**562**	**487**	

Status of research on coconut embryo culture and acclimatization techniques in Papua New Guinea

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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 micro-organisms 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 Cytokinnine 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 160mm 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 reactivate 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:

Year		Activity
Year 1	i)	Set up embryo culture laboratory – purchase of equipment, chemicals and laboratory consumable.
	ii)	Staff recruitment and training in tissue culture at laboratory available in PNG (Unitech, LAES).
	iii)	Experimentation with embryo culture.
Year 2	i)	Establishment and culturing embryos of local ecotypes.
	ii)	Introduction of selected international ecotypes.
Year 3	i)	Collection and culturing embryos of local ecotypes.
	ii)	Introduction of selected international ecotypes.

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 Rosarlo and de Guzman 1976), also stimulates germination (Ashburer 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.

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Status of research on coconut embryo culture and acclimatization techniques in Sri Lanka

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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 steriomicroscope.

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

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 Eeuwens Y_3 (Karunaratne and Gamage 1985) liquid medium. The growth of the plantlets was significantly improved when the mineral, growth factor and sucrose content of the Y_3 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 fertilizer 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 screenhouse for two weeks. Then the plants are gradually exposed to screenhouse 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 unsterilized 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 Y_3 medium (Karunaratne *et al.* 1985), 60 gl^{-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 70X45 X15 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.

4. ***Development of a medium-term preservation technique for coconut germplasm using immature zygotic embryos of coconut.***
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.

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

Treatment	Germination %	Plant development %
Control	78.9	54.1
S	83.9	53.1
K	79.8	45.0
K + S	79.8	62.8
Significance	NS	NS
CV (%)	16.2	20.9

Table 2. Effect of commercial grade sucrose (S) and fertilizer grade KCl (K) on *in vitro* plant growth

Treatment	Shoot height (cm)	Root length (cm)	Leaves/ plant	Roots/ plant	Plants with secondary roots (%)
Control	9.6	3.6	1.4	2.4	69.8
S	8.9	3.5	1.4	2.4	79.6
K	9.7	3.1	1.4	2.2	77.0
K + S	9.4	3.2	1.5	2.1	75.2
Significance	NS	NS	NS	NS	NS
CV (%)	21.7	32.3	19.2	15.3	21.1

Status of research on coconut embryo culture and acclimatization techniques in Tanzania

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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 (NaOCI) 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 gl^{-1}) and sucrose (45 gl^{-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;

Medium formulation	Embryo placement	Additional material
Solid + Liquid (DP)	top	none
Solid (S)	top	none
Solid (SB)	bottom	none
Liquid (L) Control	top	one
Liquid (LC)	suspended	cotton wool

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. l). 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. Ashburner *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 geminated 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 $10gl^{-1}$ Ultra Dithane M_{45}, 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 unsuccessfull 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 M_{45}) 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 wilt 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 plantlest 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 effforts 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 gl^{-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 gl-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 talls. This has been observed in the weaning process as well. It is possible that nutrient requirements *in vitro*, in talls 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.

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

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

[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)

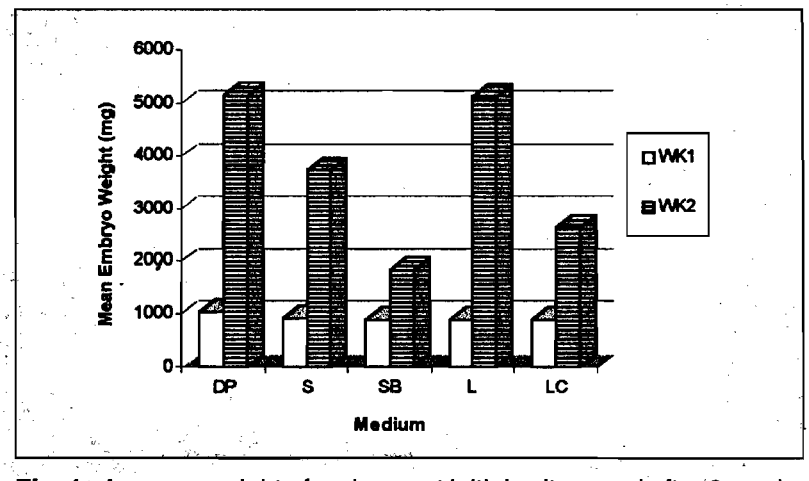

Fig. 1. Average weight of embryos at initial culture and after 2 weeks in culture using different types of medium formulation

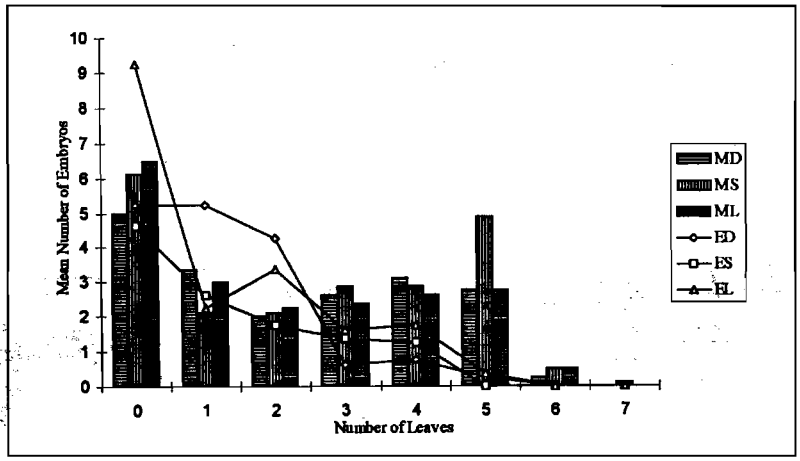

Fig. 2. Rate of leaf production of coconut embryos of Malayan Red Dwarf (MD, MS, ML) and East African Tall (ED, ES, EL) 142 days after initial culture. *Note: Whenever quoted, S denotes solid, D – double phase and L – liquid media*

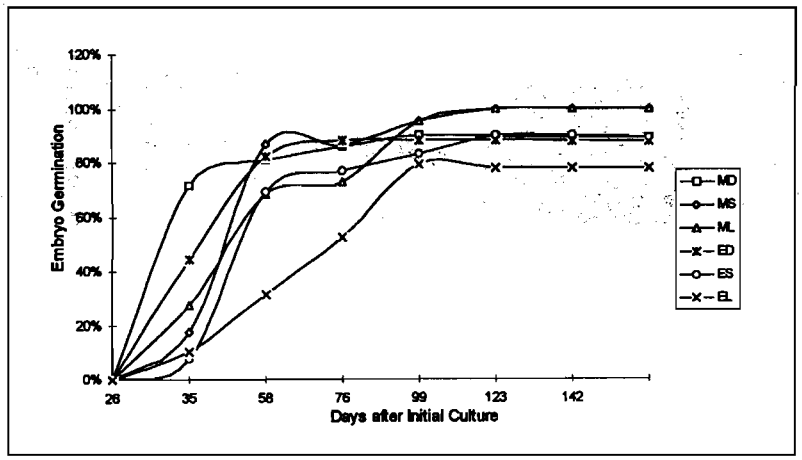

Fig. 3. Germination (%) of Malayan Red Dwarf (Mn, MS, ML) and East African Tall (ED, ES, EL) embryos 142 days after initial culture

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

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

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

Part II
Proposed coordinated research to address identified research gaps

IMPROVING *IN VITRO* COCONUT ZYGOTIC EMBRYO CULTURE

Project for Brazil

Project Leaders
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Country : Brazil
Project Title : Improving *in vitro* coconut zygotic embryo culture

I. Introduction

The first works with the culture *in vitro* of the coconut were accomplished with zygotic embryos (Cutter and Wilson 1954; Abrahams and Thomas 1962 and Luck *et al.* 1966). Some success has been obtained with this methodology, that can be used for collecting, exchange and germplasm conservation and for propagation of rare hybrids (Rillo and Paloma 1990; Jagadeesan and Padmanabhan 1982; Gupta *et al.* 1992; Ashburner *et al.* 1996). The embryo donor plants more frequently mentioned have been of hybrid PB 121. In most of the experiments, the embryos are collected from mature fruits, i.e. 11–12 months after fertilization, when they already begin to lose the color of the peel. Different formulations have been used to guarantee the germination *in vitro* of the embryos but MS medium (1962), with addition Morel and Wetmore vitamins (1951) and activated charcoal seems to work in a satisfactory way (Assy Bah 1986; Assy Bah *et al.* 1989; Rillo and Paloma 1990; Ashburner *et al.* 1996). Ashburner *et al.* (1991) considered it advantageous to pre-inoculate the embryos in liquid media. This procedure accelerated the germination but it was not shown to be essential.

Example of another practical application of the culture of embryos, besides germplasm exchange, was related by Karunaratne *et al.* (1991). These authors accomplished *in vitro* screening for germplasm adapted to drought. They used modifications of the medium formulated by Eeuwens (1976) where the potassium chloride was substituted with chloride of sodium. The embryos were cultured for three weeks into media with increasing levels of chloride of sodium, until most of the plants presented severe symptoms of water stress, such as retardation of growth of the aerial part and necrosis of some tissues. Depending on the genotype, some plants could resist up to 230 mM of chloride of sodium. They were considered tolerant at high levels of salinity and prone to survival in dry atmospheres. In this same work, PEG and manitol were tested as inductants of osmotic stress but they were shown toxicant in very low concentrations, preventing the continuity of the experiments. The authors however, illuminate, if these agents were added to solid or liquid media. Zaid and Hughes (1995), working with date palm embryos added PEG 8.000, for a final concentration of 20% p/v, to the liquid media for culture and they observed beneficial effects related to the adaptability and acclimatization of the plantlets *in vivo.*

In CPATC, germinating the embryos *in vitro* has been done, although in a discontinued way and not systematic, especially in improving the adaptability of the plantlets to the natural conditions of cultivation (Siqueira *et al.* 1994). Losses in the stage of acclimatization are due mainly to difficulties in inducing the establishment of a root system efficient enough to guarantee the plant survival (Assy Bah *et al.* 1989; Ashburner *et al.* 1993). The decrease in the amount of epicuticular wax in the leaves, as demonstrated for date palm plants (Phoenix dactylifera) (Zaid and Hughes 1995) could also be an important factor. For the coconut tree, Karunaratne *et al.* (1991) showed high sensibility to presence of PEG, but they did not explain whether it was added to solid or liquid media. Other experiments, on coconut, indicated the possibility of using high sucrose concentrations (Assy Bah *et al.* 1989), that it would act in way similar to the polyetileneglycol and the use of the auxin, naphtalene acetic acid (Assy Bah *et al.* 1989; Ashburner *et al.* 1993). As additional symptom of the tolerance to the stress caused by the addition of chloride of sodium to the culture media, Karunaratne *et al.* (1991) cited the growth of a larger number of roots in plantlets that survived.

In general, growth conditions, favored temperatures between 27 and 28 °C and also of 30–31°C. The luminous intensity frequently used for the development of the aerial part of the plantlets was 90 mmol/m2/s, or 3,000 Lux, supplied by white fluorescent lamps, with photoperiods varying from 12 to 16 hours.

II. Justification

Initial research in coconut tissue culture was made with zygotic embryos. Relative success has been obtained with embryo culture, in several countries, but no definitive protocol has been published. This technique can be useful for germplasm collecting, change and conservation and is, obviously, scientifically significant.

At CPATC, some progress has been made with germination and *in vitro* establishment of coconut platelets. However, acclimatization has always been difficult because satisfactory root development hasn't been common.

Some of the researchers at CPATC were involved with tissue and zygotiuc embryo culture, in the past few years and some three or four plants grown from *in vitro* cultured embryos can be seen here in. Nowadays we are re-beginning researches on tissue culture and the laboratory in its structuring phase.

III. Objectives

To validate three protocols for *in vitro* culture of mature coconut zygotic embryos in terms of high germination and *in vivo* plant establishment rates.

IV. Methodology

Initially, the MYD and RIT embryos (11–12 month old after pollination) are going to be collected at the CPATC Coconut Genebank.

Protocols to be tested would be:
- Protocol developed at CPATC – Brazil
- Protocol developed at UPLB-Philippines (Annex 2)
- Protocol developed at CPCRI-India (Annex 3)

Protocol developed at CPATC:
1. Collect mature embryos and extract endosperm plugs containing embryos
2. Drive aseptic treatment, 30 seconds in 70% ethanol and 20 minutes in commercial bleach (2–3 % active chlorine)
3. Extract embryos from endosperm plugs in the flow cabinet and pass them quickly in 10% commercial bleach and three times in sterile water
4. Inoculate embryos in MS solid media, plus vitamins and 60% sucrose
5. Drive transfers each 45 days
6. If necessary, at the 20th week add 200 uM NAA to the media and at the 24th week add 400 uM, till plants had developed a good root system
7. When plantlets had reached 25 cm with a good root system and at least one well developed leaf, they can be transferred to sterile sand, in the nursery, and after that two month or so, to the soil.

Treatments will include 3 protocols, 2 genotypes, and 3 replications with 25 embryos per replication, for a total of 450 embryos/genotype. Data will be analyzed statistically.

Parameters observed will be: germination rate, contamination rate, shoot and root appearance, number of leaves and roots at transfer to pot, survival rate in the nursery, number of leaves per month and height before planting.

V. Activities

Activity	Period
Embryo collecting and inoculation in germination test media (100 embryo/month)	September/1998 – February/1999
Subculturing and maintaining	September/1998 – August/1999
Transfer to pot and acclimatization	August/1999 – July/2000

VI. Budget (US$)

Item	External			National counterpart		
	Y1	Y2	Total	Y1	Y2	Total
Personal				3240	10 000	13 240
Supplies/Material		1500	1500	1000	1700	2700
Travel		2450	2450	2450	–	2450
Others		350	350	1550	3700	5250
Total		**4300**	**4300**	**8240**	**15 400**	**23 640**

This project will involve hiring, at least one trainee for the Tissue Culture Lab at CPATC, for the duration of the project period. An arrangement shall be made to harvest mature fruits each week from the Genebank, 135 km from Aracaju. The project schedule was planned to begin in July/1998 and to end in July/2000.

EMBRAPA/CPATC could be responsible for the entire budget in the first year and for personel salary and wages, as well as supplies and material for the second year. However, we are going to need the COGENT sponsorship for international travel.

INCREASING THE EFFICIENCY OF EMBRYO CULTURE TECHNOLOGY TO PROMOTE GERMPLASM COLLECTING

Project for Cuba

Project Leader
Jorge Rolando Cueto

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Country : Cuba
Project Title : Increasing the efficiency of embryo culture technology to promote germplasm collecting

I. Justification and rationale

Collecting and exchanging of coconut germplasm are difficult and costly because of the considerable weight and size of the seednut, and the rapid loss of viability. Zygotic embryo culture is an alternative method because it avoids the high cost of transportation, tedious quarantine requirements, and the risk of accidental introduction of pests in particular, pathogens. Often symptomless, viruses pose a special risk. In order to minimize this risk, effective testing (indexing) procedures are required to ensure that distributed material is free of pests that are of quarantine concern.

The Instituto de Investigaciones de Cítricos y otros Frutales is conducting research on *in vitro* culture embryos to develop an appropriate methodology for collecting exotic varieties of coconut. The project leader has been working in this research area for two years.

II. Objectives

1 To compare the efficacy of four embryo culture protocols (Annexes 1 to 4);
2 To develop embryo culture *in vitro* protocol appropriate to the desired genotype;
3 To study the effect of growth regulators on embryo plantlet survival *in vitro* and *ex vitro*; and
4 To investigate the effect of different concentration of sucrose in growth medium on plantlet survival *in vitro* and *ex vitro*.

III. Methodology

Four protocols which were agreed during the International Coconut Embryo Culture Workshop in Albay, Philippines in October 1997 will be tested on a Dwarf (Malayan Yellow Dwarf) and a Tall variety of coconut ("Criollo" variety, similar to "Jamaica Tall").

Uniform mature embryos from the two varieties will be used in the experiment. The experiment will include steps in field collecting of endosperm cylinder with zygotic embryos until the acclimatization procedure. The experiment will test 4 protocols as described in Annexes 1–4. Sufficient number of embryos will be obtained so that after disinfection and elimination of infected embryos, there will be at least 10 healthy embryos left for each of the 3 replications. Parameters to be studied are:

- Germination percentage
- Contamination percentage
- Oxidation percentage
- Percentage of the latent embryos and/or abnormal embryos
- Rate of development of the explants
- Length of the main root
- Average number or secondary roots developed *in vitro*
- Plant height
- Survival percentage in nursery and field conditions.

Sampling, disinfection and transfer of embryo to test tube will follow the procedures in the STANTECH Manual. The following steps will be followed:

Step 1 – Sampling: Mature coconut fruit (11 – 12 month of age) will be collected in the field. The coconut albumen with the embryo will be extracted using a cork borer or similar equipment.

Step 2 – Disinfection: The coconut albumen cylinders with the embryos will be disinfected. Different desinfection methods will be evaluated.

Treatment 1 – After all the endosperm cylinders are extracted, they will be washed in tap water and in 95% ethanol (quickly) to remove the fats and then will be disinfected with 100% commercial bleach for 20 minutes.

Treatment 2 – The coconut albumen cylinders with the embryos will be disinfected in 5% NaOCl for 20 minutes. The embryos will then be isolated, sterilized in 1% NaOCl for 10 minutes and then washed three times with sterile distilled water.

Treatment 3 – After all the endosperm cylinders are extracted and disinfected with 50% comercial bleach for 20 minutes and then washed in tap water two times, the cylinders will be maintained in the antioxidant agent solution (citric acid 250 mg/l) until inoculation into medium.

Treatment 4 – The cylinders will be sterilized by transferring to a filtered solution of sodium hypochlorite (70% active chlorite) for 20 minutes. The embryos will then isolated in an air flow cabinet and rinsed in sterile destilled water before inoculation into liquid medium.

Step 3 – Embryo transfer: The embryo transfer will be done in a laminar flow cabinet. The albumen core or cylinder surrounding the embryo will be cut carefully to isolate the embryo which will then be washed with sterile water before putting it in the desired culture medium. Four culture media types will be tested and the details are shown in Annexes 1–4.

Step 4 – Acclimatization/Adaptation: The different plants obtained *in vitro*, will be grown using several substrates: irrigated sand with nutrient solution, sand + compost (1:1), and soil + sand + compost (1:1:1).

During acclimatization in the test tube, the following ambient conditions will be maintained: 65% relative humidity and 25°C ± 1 temperature at natural light.

The plantlets will be transferred into a greenhouse in different substrates with high humidity using the plastic bag. The temperature would then be between 26–37°C. Gradually, the plastic bag would be loosen; then transferred to the screenhouse in polythene bag at ± 37°C and 85% relative humidity. Finally, the plantlets will be exposed pass to nursery condition (±37°C and 85% relative humidity) at full sunlight. The results will be statistically analyzed.

To investigate the effect of growth regulators and the relations between different concentrations of sucrose in growth medium on plantlet survival *in vitro* and *ex vitro*, the following treatment combinations will be evaluated in the 4 Protocols.

Growth regulator	Embryo germination	Plantlets rooting
BAP	0.5 mg/l	–
IBA	7 mg/l	5 mg/l
NAA	0.5 mg/l	1 mg/l
Sucrose	60 g/l	30 g/l

IV. Expected outputs

1. An efficient protocol for embryo culture and acclimatization with high percentage of plant recovery and survival post in vitro.
2. Comparative data on the performance of the Dwarf vs Tall variety to the different in vitro protocols and acclimatization treatments.
3. Data on the response of the two varieties on two different growth regulator treatments and different concentrations of sucrose in growth medium on plantlets survival *in vitro* and *ex vitro* according to Protocol 1, 2, 3 and 4.

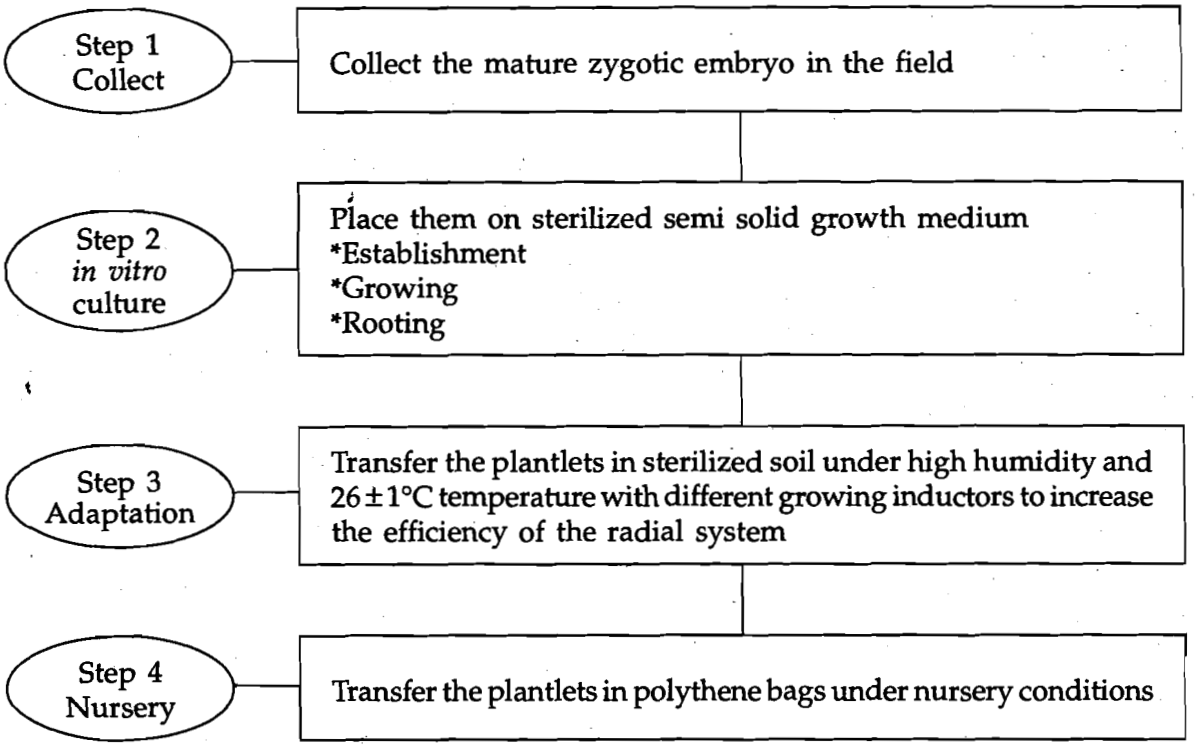
V. Project activities

No.	Activity	Year 1		Year 2	
		1st.Semester	2 nd.Semester	1st.Semester	2nd.Semester
1	Collecting embryos from two varieties and inoculation in the germination media.				
2	Subculture monthly at different phases: germination, growing and rooting, to use some growing inductors into culture medium, and record data.				
3	Transfer of plantlets to sterilized soil for adaptation *ex vitro* condition into the greenhouse where humidity and temperature are controlled. Record data.				
4	Transfer of plantlets to pots and hardenning in the nethouse/ greenhouse. Data recording.				
5	Final data analysis and report writing.				

VI. Budget (US $)

Item	External			National counterpart		
	Y1	Y2	Total	Y1	Y2	Total
Personnel	500	1500	2000	2000	2500	4500
Supplies/Material	1750	1000	2750	–	–	–
Travel	250	–	250	100	150	250
Other	–	–	–	800	800	1600
Total	**2500**	**2500**	**5000**	**2900**	**3450**	**6350**

Appendix 1. Scheme of work



IMPROVING THE EFFICIENCY OF COCONUT ZYGOTIC EMBRYO CULTURE: A PHYSIOLOGICAL AND BIOCHEMICAL APPROACH

Project proposal of ORSTOM/CIRAD-CP, France

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Country : France
Project Title : Improving the efficiency of coconut zygotic embryo culture: a physiological and biochemical approach

I. Justification and rationale

In vitro culture of excised embryos represents an attractive way to simplify coconut germplasm exchange and conservation. Even if routine techniques for collecting and *in vitro* culture of zygotic embryos (protocol of Assy Bah, see Attachment 1) have been developed in ORSTOM (in collaboration with IDEFOR), some problems remain. One of the major difficulties encountered in coconut embryo culture is the heterogeneity of the embryo behaviour, which highly decreases the success rate of the method (only 45 to 50% of the initial number of embryos will reach the stage of acclimatized plantlets). It is also important to emphasize that the development of *in vitro* plants (during the *in vitro* phase and after acclimatization) is slower compared to the development of seedlings.

In order to circumvent these bottlenecks, studies to increase our knowledge on vitroplant physiology were conducted in Montpellier. We investigated the photosynthetic status of *in vitro* grown plantlets obtained by zygotic embryo culture combining various complementary approaches. Results demonstrated the establishment of a functional photosynthetic metabolism during the *in vitro* development of coconut plantlets (for details see Triques *et al.* 1997 – Plant Science 127:39–51). However, a lower rate of net photosynthesis was recorded in *in vitro* grown plantlets. This could be due to the lower level of leaf surface observed on the *in vitro* plantlets (compared to seedlings of the same age). Preliminary analyses of endogenous cytokinin content revealed a high level of this type of hormones in the mature seed, which could affect nutrient mobilisation during the germination of the coconut seed. We think now that there could be some deficiencies in the culture media used for embryo culture (e.g. lack of sugar, lipids, hormones) and our aim is to perform some physiological analyses in order to provide information on the nutrition status of the seedlings.

II. Objectives

To generate data to help explain the physiological basis of *in vitro* growth and identify what is lacking in current protocols to improve the coconut zygotic embryo *in vitro* culture process.

- To explain the role of the haustorium in the nutrient supply for the germinating nut.
- To explain the role of endogenous growth regulators during the germination of the nut.
- To determine the photosynthetic capacity of naturally germinating seeds.
- To improve the culture system by experimenting the temporal immersion system (RITA) and by improving the hardening and weaning phases.

III. Methodology/approach

The approach will include the use of a range of techniques (see Attachment 2) we can access in order to measure different physiological/biochemical parameters. To compare the results with those of other laboratoriess, we will mainly use the MYD genotype to conduct our experiments. We may also use the PB 121 hybrid (IDEFOR-IRHO). The materials will be provided by IDEFOR, with which we collaborate on a joint breeding project. Results would then be shared with other collaborating countries in the COGENT Coconut Embryo Culture Project Network in order to discuss with them how to improve the *in vitro* culture technology and trials based on our observations.

In addition, ORSTOM/CIRAD-CP could provide (according to availability of funds) some training for the network participants who would like to learn some of the techniques we are using.

IV. Project activities

A. Ongoing projects

1) Study of the mobilisation of the main organic nutrients by the haustorium during *in situ* germination

Analyses will be performed in order to determine the main organic compounds (sugars, lipids and amino acids) involved during the germination of whole seeds. This work will be performed in collaboration with CICY (Mexico). First results are expected within one year.

2) 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. In addition, as the role of ABA on (somatic and zygotic) embryo maturation was highlighted during the 1997 Workshop and as some of the participants will start to introduce ABA in the culture medium (to improve embryo development synchronization), we will start to investigate ABA quantification in the nut (endosperm and embryo) in order to provide further information on this growth regulator changes during nut development. First results are expected within one year.

3) Extension of the study on photosynthetic ability of *in vitro* grown coconut to vitroplants in the nursery in comparison with seedlings in the field (funding organisation CIRAD, ORSTOM) This study is partially supported by the EC (contract ERBTS3*CT940298)

This work will be performed in collaboration (through complementary approaches) with CICY, UPLB and MARI under the present project network. Participants would design complementary approaches in order to have a full description of what is happening in vitroplantlets and in seedlings in the field. First results could be available within one year.

4) Characterization of the Makapuno nut at the biochemical, histological and genetic level

As Makapuno coconut embryo culture is very important in the Philippines, characterization of the Makapuno nut (biochemical, structural, genetical) would provide important information to understand the peculiarity of Makapuno (compared to the normal nut). A study was initiated by PCA and ORSTOM in 1997 under an ORSTOM grant (provided to Cristeta CUETO from PCA for a 2-month training period). Analyses of endosperm lipids, protein, oil and sugar contents have been started. In addition, examination using transmission and scanning electron microscopy of the ultrastructural features of the endosperm was initiated. Results of microscopy examinations will be useful to observe and characterise the Makapuno endosperm cell structure.

PCA and ORSTOM/CIRAD-CP are actually looking for a grant extension in order to have C. Cueto to carry on the work.

B. Future projects (depending on funding and staff availability)

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

Experiments carried out in CIRAD notably on banana, rubber tree, coffee and citrus showed that temporary immersion can highly improve vitroplant quality and development. The CIRAD tissue culture group directed by Dr. C. Teisson has recently

proposed a culture device called RITA (Recipient d'Immersion Temporaire Amélioré). This culture device will be experimented for coconut embryo culture and this project will be funded by ORSTOM/CIRAD-CP.

2) *In vitro* plantlets root anatomy and measurement of nutrient uptake throughout the *in vitro* process

The aim is to determine if the root system develops normally under *in vitro* conditions and nutrient uptake analyses would provide data to help explain how they are functioning. This project will be (according to availability of grant/funds) conducted in collaboration with UPLB.

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

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

In vitro hardening

Ways of promoting reliable *in vitro* hardening will be tested by:

- studying the influence of transferring plantlets to medium with low sugar concentration before acclimatization ; and by
- studying influence of the type of container closure on embryo germination and plantlet development.

Ex vitro hardening

Studies will be conducted on *ex vitro* hardening (in collaboration with IDEFOR) in order to determine factors which influence the growth of the transplants. The influence of fertilizer application on plantlet development in the greenhouse, pre-nursery and nursery will be studied.

V. Expected outputs

Study of the nutrients supplied by the haustorium to the seedlings and hormone analyses would provide information which might be used to improve the culture medium and to design new culture sequences.

Photosynthetic analyses on seedlings under natural conditions would provide data on the needed rate of photosynthesis for the *in vitro* plantlets to be able to grow normally. Photosynthetic analysis on vitroplanlets can also provide information on the exact growth chambers/greenhouse conditions we have to use for the *in vitro* plantlets. The first results might be available within one year.

Characterization of the Makapuno nut would provide important information for a better management of this genotype and might be started in 1998 if we can get a grant. The other experiments (test of RITA system, root anatomy, nutrient uptake) would be run only if funds and sufficient staff are available. These studies will be mainly funded by ORSTOM, CIRAD-CP, the European Community and the ECOS project.

Attachment 1. Protocol used at ORSTOM (Assy Bah, slightly modified)

Using the facilities previously described, much work has been carried out first by B. Assy-Bah in Côte d'Ivoire and in France and then completed by the rest of the group.

1. In vitro based embryo sampling, storage and transport methods

Sampling during a collecting mission

This method was developed for collecting embryos in the field located far from the tissue culture laboratory (Assy-Bah and Pannetier. 1987.)

- *Sampling and disinfecting 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 hypochlorite.

Completely de-husked mature nuts (11–12 months) are split cross-wise into two using a clean hammer or bolo. A 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 3% chlorogenic sodium hypochlorite solution. A portable gas burner is used to sterilize the instruments.

Batches of 30 cylinders are immersed for 20 min in a 500 ml of a calcium hypochlorite solution (70% active chlorine; 45g/l).

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

Previous trials showed that immersion of the disinfected endosperm cylinders in a sterile KCI solution (16.2 g/l) provided the best conditions for their storage for a maximum period of 14 d. This gave enough time to return to the laboratory to start the culturing operation.

- *Embryo excision before inoculation*

After the storage period, the cylinders are re-sterilised by transferring them individually to a filtered solution of calcium hypochlorite (70% active chloride; 45 g/l) during 20 min.

The embryos are then isolated under an air flow cabinet and rinsed in sterile distilled water before inoculation in liquid medium.

Embryo conditioning for export

This type of 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 previously described. 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 (7.5 g/l) and before autoclaving]. The inoculated tubes can easily be sent by air freight. It was demonstrated that the embryos can be kept on the temporary solid medium during 5 d without alteration of their germination capacity. When they arrive in the receiving lab, the embryos are transferred directly on the germination medium.

2. Embryo 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), neutralized activated charcoal (2 g/l) (Sigma). The pH is adjusted to 5.5 before adding the charcoal and autoclaving (20 min at 110°C). The embryos are cultured in 24 x 160 mm test tubes containing 20 ml of medium sealed with plastic parafilm. They are incubated in a dark room at 27± 1°C.

They are subcultured every 4–6 weeks on 20 ml of fresh medium. The germinating embryos are kept in the dark until the first true leaf emerges (3 – 4 months for the more advanced embryos). As soon as the first true leaf and the root system are developed (at least one root with ramifications), plantlets are transferred on 100 ml of Mi 502 liquid medium in one Lglass bottles under light (photoperiod of 12 h light/12 h dark, 45 + µmol/m2/s; Sylvania gro-lux day light tubes). Bottles are covered with foam caps surrounded by aluminium foil and sealed with parafilm.

Plantlets growing under light conditions are transferred every 4–6 weeks in large tubes (36x200 mm) on 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).

3. Acclimatization procedure

The protocol is performed in a tropical greenhouse where humidity and temperature are controlled.

Once removed from the culture medium the plantlets are carefully rinsed with distilled water and then plunged for 5 min in a fungicide solution based on carbendazin (Benlate, 2 g/l) to prevent fungal development. They are then placed on 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. Progressively the plastic bag is opened. Plantlets are watered with water only for the first month and then a nutritive solution is applied every 2 d (see composition below).

Composition of the nutritive solution used for acclimatization plantlets (mg/L)

KNO3	274
Ca(NO3)2 2H20	1095
KH2PO4	137
MgSO4 7H20	274
(NH4)2 S04	137
KCI	2.74
H3PO3	3
MnSO4 H20	15
ZnSO4, 7H20	2.74
(NH4)6 M07OZ4 4H20	2.74
H2SO4	0.137
Cu SO4 5H20	1.37
FeSO4 7H20	24.9
EDTA	26.1

After two months on sand, the plantlets are transferred to forest leaf mould.

At this stage, they are fertilized every two weeks with 50 ml of a N:P:K solution (8;11;14; 2 ml/1). Every two months 50 ml of chelated iron 6% (1 g/l) is also added.

Attachment 2.

- Facilities for tissue culture
  - 2 culture rooms (one dark, one with photoperiod)
- Tropical greenhouse
- Study of vitroplant physiology
  - Nutrients analyses : sugars (HPLC), lipids (CPG), minerals (ionic chromatography) and amino acids (HPLC)
  - Exogenous growth regulators (in the medium) analyses (HPLC)
  - Endogenous plant growth regulators (abscisic acid, auxin, cytokinins) analysis (by HPLC and ELISA methods)
  - Photosynthetic parameters analyses
    Enzyme (PEPC, RubisCO) characterization (enzyme activity, quantification by immunorocket and immunoblots techniques)
    Chlorophyll quantification and their capacity measurement transpiration and photosynthetic rate measurement
  - Histology (optical and electron microscopy)

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INCREASING THE EFFICIENCY OF EMBRYO CULTURE TO PROMOTE GERMPLASM COLLECTING

Project for India

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Country : India
Project Title : Increasing the efficiency of embryo culture to promote germplasm collecting

I. Justification and rationale

Exchange of coconut germplasm in the form of embryos circumvent the problems arising due to large nut size, short duration of dormancy and phytosanitary regulations. Different protocol for embryo culture *in vitro* and subsequent field establishment has been published with varying degree of success (10%–70%). A protocol with the success comparable with that obtained *in situ* is most desirable for efficient germplasm collecting and conservation. The role of growth regulators like ABA and GA3 in coconut embryo maturation and germination is yet to be studied and it might allow the use of immature embryos for germplasm collecting. Much remain to be understood about the genotype effect on *in vitro* embryo germination.

At Central Plantation Crops Research Institute (CPCRI), an efficient protocol for field collecting and *in vitro* culture of embryo, and subsequent field establishment has been standardized. This protocol was recently used to collect 1342 embryos representing 15 accessions from 3 Indian Ocean Islands. These embryos are growing in culture with average germination of 72%.

II. Objectives

The objective of the project is to validate the protocol for *in vitro* culture of mature and immature embryos with high germination and suitability for large number of varieties. Specifically, the project aims:

1. T o compare the efficacy of four (4) different embryo culture protocols (Annexes 1–4);
2. To study the effect of genotype on *in vitro* embryo culture; and
3. To study the effect of ABA, GA3 and/or osmoticum on embryo germination.

III. Methodology

Mature embryos of West Coast Tall (WCT) will be used with 4 different available protocols (Annexes I–4). The protocol standardized at CPCRI will be used for culture of mature embryos from Rennel Island Tall, Malayan Yellow Dwarf (MYD), Chowghat Green Dwarf (CGD) and WCT (as control) to study the varietal effect. A link with the other laboratories (PCA-Albay Research Center, Philippines; UPLB, Philippines and CICY, Mexico) has been established for these two experiments. RIT and MYD are common for all these laboratories. Experiment will include 3 replications with 4 embryos per replication. Parameters to be studied are germination rate, contamination rate, number of leaf, number and volume of roots, plant height suitable for weaning and percent survival in nursery and field. Varying concentrations of ABA, GA_3 and osmoticum like PEG, Mannitol will be used with 10 and 12 month-old WCT embryos and their effect on embryo germination will be monitored. The protocol developed at CPCRI will be used for this experiment. Link with CICY, Mexico; CRISL, Sri Lanka; RICP, Indonesia has been established for this experiment.

IV. Expected output

1. Validation of the protocol suitable for wide range of genotypes and with high success rate.
2. Understanding of effect of ABA, GA3 and osmoticum on maturation/germination of embryo.
3. Identification of appropriate growth regulators/osmoticum for enhancing immature embryo germination.

V. Project activities

	Activities	Year 1	Year 2
1.	Collecting of embryo from different accessions and inoculation in the germination media	—	
2.	Subculture, maintenance and root induction of different cultures *in vitro*, recording data on germination contamination, etc.	—	
3.	Transfer of plantlets to the pots and hardening in the laboratory, recording data before and after hardening	—	
4.	Transfer of plantlets to polythene bags and hardening in the nethouse / greenhouse, recording data	—	
5.	Final data analysis and report writing	—	

VI. Budget (US$)

Item	External			National counterpart		
	Y1	Y2	Total	Y1	Y2	Total
Personnel	500	1500	2000	3240	3780	7020
Supplies/Material	1750	1000	2750	–	–	–
Travel	250	–	250	–	–	–
Others	–	–	–	1000	1000	2000
Total	**2500**	**2500**	**5000**	**4240**	**4780**	**9020**

Country : Indonesia
Project Title : Increasing the efficiency of embryo culture technology to promote coconut germplasm collecting, conservation and exchange

I. Justification and rationale

Embryo culture is an important technique for coconut germplasm collecting, conservation and exchange. To safeguard the coconut industry, exchange of germplasm has been regulated by quarantine procedures to prevent introduction of pests and lethal diseases. However, for some diseases, germplasm exchange through aseptic is permitted. The different protocols of embryo culture *in vitro* and *ex vitro* have been published, but the success rate is in varying degrees in different laboratories. For efficient germplasm collecting, conservation and exchange, protocols with high success rate are needed. Also, growth regulators have an important role in the success of embryo culture technique in terms of maturation and germination of embryo. In particular, the role of ABA and GA3 need to be studied.

Under COGENT, Indonesia serves as the host country for the International Coconut Genebank for Southeast Asia. As a host country, it will receive from and send germplasm in the form of embryos to COGENT member countries. Thus, there is a need to upgrade the embryo culture technology to make it efficient as a tool for safe movement of germplasm.

There are some coconut accessions that performed well in coconut development programme. The accessions are namely, Mapanget Tall (MTT), Malayan Yellow Dwarf (MYD), and common accession that is Renelle tall (RLT) planted in field collection of RICP.

II. Objectives

1. To upgrade the equipment and supplies capability protocol of embryo culture laboratory at RICP.
2. To compare four promising embryo culture protocols (Annexes 1–4) which were agreed at the International Embryo Culture workshop in the Philippines on 28–30 October 1997.
3. To evaluate varietal responses to *in vitro* embryo culture protocols.
4. To evaluate the effect of ABA and GA3 on embryo germination.

III. Methodology

Mature embryos of MTT, RLT, and MYD will be used as test materials for the four different available protocols (PCA-Philippines, UPLB-Philippines, CPCRI-India, ORSTOM-France), Annexes 1–4. The best protocol will be used for culture of mature embryos of the three accessions to evaluate the varietal effect. The experiment will consist of 3 replications with 20 embryos per each replication. The parameters to be observed are germination rate, contamination rate, appearance of shoot and roots, number of leaves, number and volume of roots, and survival rate in nursery. The effect of plant regulator will be tested in MTT with 9, 10 and 11 month-old embryos, using the best protocol identified above.

IV. Expected output

1. Suitable embryo culture protocol for wide range of genotypes with high success rate.
2. Appropriate growth regulators for increasing the germination of immature embryos.

V. Project activities

Activities	Year 1	Year 2
1. Collecting of embryos from different accessions and inoculation in germination media.	x	–
2. Subculture in different cultures *in vitro;* data recording of germination andcontamination rate.	x	x
3. Transfer plantlets to the pots for weaning; data recording of survival rate.	–	x
4. Transfer plantlets to the polybags and hardening in screenhouse.	–	x
5. Data analysis and reporting.	–	x

VI. Budget (US$)

Item	External			National counterpart		
	Year 1	Year 2	Total	Year 1	Year 2	Total
1. Personnel	–	500	500	500	500	1000
2. Supplies/Material	2500	1900	4400	1500	–	1500
3. Travel	–	–	–	–	1000	1000
4. Others	–	100	100	300	200	500
Total	**2500**	**2500**	**5000**	**2300**	**1700**	**4000**

IMPROVEMENT OF EMBRYO CULTURE ÉFFICIENCY FOR THE SAFE MOVEMENT OF COCONUT GERMPLASM

Project for Mexico

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Country : Mexico
Project Title : Improvement of embryo culture efficiency for the safe movement of coconut germplasm

I. Justification and rationale

Mexico is presently affected by the devastating lethal yellowing disease which could be overcome to some extent by replanting resistant material. The identification of resistant varieties in Mexico is being carried out, but it is necessary to test germplasm from other parts of the world to widen the genetic basis for coconut improvement. Unfortunately, the introduction to Mexico of these materials is not possible at the moment due to phytosanitary constraints, except if carried out using the embryo culture technique.

However, most of the current protocols for embryo culture have low efficiencies for acclimatized plantlet production. Some of the protocols presented in the International Embryo Culture and Acclimatization Workshop (PCA Albay Research Center, Philippines, October 27–31, 1997) are promising – by CPCRI (India), OSTROM (France), PCA (Albay,Philipines) and UPLB (Los Baños, Philippines). However, they need to be validated for applicability to different genotypes and their efficiency.

The present project proposes to validate the above mentioned protocols using embryos of three different varieties, and to define medium formulation and culture conditions to improve the embryo culture efficiency.

II. Objectives

1. To compare the best four currently available embryo culture protocols (Annexes 1–4).
2. To study the effect of genotype on *in vitro* embryo culture.
3. To study the effect of ABA, PEG and GA3 on the germination and development of mature *in vitro*-cultured embryos.
4. To evaluate the addition of cocofiber or vermiculite to the embryo culture system after germination to determine if root and subsequent whole plantlet development is improved.

III. Methodology/approach

A. Methodology

1. Preparation of plant materials.
2. Application of embryo culture protocols (see Annexes 1–4).
3. Addition of growth regulators and osmoticum.
4. Monitoring of germination and plantlet development.
5. Introduction of cocofiber to the system.
6. Evaluation of root and shoot development and functioning.
7. Statistical analysis of gathered data.

B. Approach

1. To compare the four protocols, embryos (ds 10) of one single variety (MYD) will be used. Within 35 weeks during the course of the evaluation, identification of the best protocol should have been made.
2. This protocol will then be tested on three varieties, MYD, Renell tall and a local tall to determine varietal effect.

3. Tests will also be conducted to evaluate the effect of ABA and PEG on the promotion of maturation and germination of embryos ds 10. Embryos will be pre-treated with these chemicals at different concentrations and transferred to germination medium. For germination, the best performing protocol in (1) will be used.
4. The effect of different concentrations of GA3 on germination will be tested on MYD embryos using the best protocol in (1).
5. Using the best protocol in (1), an experiment will be carried out to test the addition of cocofiber or vermiculite to absorb the medium and support root development, but most of all to provide aeration. In this condition, root development will be evaluated in comparison with the standard protocol, in terms of morphological changes: growth in size, leaf production, primary, secondary and tertiary root formation, and root histology.

Activities (1) and (2) will be carried out jointly with similar experiments performed by each member of the network, using a common variety in most cases and additional different varieties for each member. Activities (3) and (4) will be carried out jointly with CRI (Sri Lanka) and CPCRI (India), and Activity (5) will be carried out jointly with CRI (Sri Lanka) also using different varieties in each case.

IV. Expected output

1. Information regarding the performance of each protocol and varietal effect.
2. Appropriate treatments to improve maturation and germination for increasing the efficiency of embryo culture are identified.
3. Effect of cocofiber to improve root and shoot development.

V. Project activities

Activities	Semester				Collaboration with other members of the network
	1	2	3	4	
1	X	X	X		All members
2		X	X	X	All members
3		X	X	X	CRI (Sri Lanka), CPCRI (India)
4		X	X	X	CRI (Sri Lanka), CPCRI (India)
5	X	X	X	X	CRI (Sri Lanka)

VI. Budget (US$)

Item	External			National counterpart		
	Y1	Y2	Total	Y1	Y2	Total
1. Personnel				yes	yes	
2. Supplies/Material	2500	2500	5000	1500	1500	3000
3. Travel						
4. Others				1000	1000	2000
Total	**2500**	**2500**	**5000**	**2500**	**2500**	**5000**

Ongoing research

There are other projects being carried out at CICY or about to start that are complementary to the present proposal.

(a) **Studies on the composition of the main nutrients in the reserve tissues and the haustorium during germination of the coconut.** The nutrients to be analyzed are: sugars, lipids and amino acids). This is in collaboration with ORSTOM/CIRAD (France). Funds come from the Commission of the European Communities (ERBTS3*CT940298) and the programme France-Mexico ECOS-ANUIES. In this study, the concentrations of the main nutritive components of the solid endosperm, liquid endosperm, haustrorium and plantlet will be determined during germination. Morphological and anatomical changes will also be studied. The project will start in 1998.

(b) **Studies on the development of physiological competence in plantlets derived from coconut zygotic embryos cultured *in vitro*.** This will be a collaborative effort with ORSTOM/CIRAD (France), UPLB (Phil) and MARI (Tanzania) under the present network. Funds for ORSTOM/CIRAD and CICY are from the Commission of the European Communities (ERBTS3*CT940298). In this study the physiological competence in terms of photosynthetic capacity (photosynthetic rate, photosynthetic pigments and photosynthetic enzyme activities) and the water control capacity (cuticle integrity, stomata morphology and functionality and transpiration) are evaluated in developing plantlets derived from zygotic embryos cultured *in vitro*. Also, studies are carried out on the effect of various factors (ventilation, sugar concentration, light intensity) on the acquisition of physiological competence. This project is already in progress in ORSTOM with regards to photosynthesis studies. Studies on ventilation and stomata performance have already started at CICY. The rest of the activities in CICY will start in 1998.

(c) **Germination of coconut zygotic embryos in a bioreactor.** Funds come from CONACYT (Mexico). In this study small bioreactors are used to determine the optimal conditions for embryo germination and development. This system will avoid the permanent flooding contitions of the reported embryo culture techniques. The system also allows for controlled gas exchange.

Future research

(a) **Maturation of immature coconut embryos *in vitro*.** Immature embryos at different stages of development will be treated with ABA and PEG to promote reserve accumulation and overall maturation. This study is part of the project that will start in 1998 funded by CONACYT, Mexico and in collaboration with Indonesia.

(b) **Quantification of phytohormones (cytokinins, auxin and ABA) in coconut seednuts.** This will carried out in collaboration with ORSTOM/CIRAD. Funds will be partially provided by the programme France-Mexico ECOS-ANUIES.

In the above mentioned studies, exchange of personnel has taken place both ways and it will continue during 1998. The concerned researchers, technicians and postgraduate students will be jointly supervised by both institutions.

UTILIZATION OF EMBRYO CULTURE TECHNOLOGY FOR GERMPLASM CONSERVATION: DEVELOPMENT OF MEDIUM-TERM CONSERVATION FOR COCONUT ZYGOTIC EMBRYOS

Project for Philippines

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Country : Philippines
Project Title : Utilization of embryo culture technology for germplasm conservation: development of medium-term conservation for coconut zygotic embryos

I. Application

Embryo culture technology is of great importance for germplasm collecting, conservation and exchange of recalcitrant species such as coconut. Collection and transport of coconut germplasm in the form of embryo cultures instead of seednuts has many advantages. Medium-term storage (several months to 1–2 years), however, is necessary in some instances such as collecting in distant places or longer expedition time, site for *in situ* conservation is not yet ready, or when quarantine restrictions have been imposed.

Medium-term storage can be achieved by maintaining cultures under slow growth conditions through limiting physical environment and culture medium composition (Withers 1990). Earlier studies conducted by Assy-Bah and Englemann (1993) have indicated that coconut embryos can be stored for 6 and 12 months with 100 and 50% germination of stored embryos, respectively. In addition, storage protocols need to be tested on a range of genotypes. Studies to prolong storage duration beyond 12 months are needed.

II. Objectives

1. To validate the efficiency of published protocols for coconut embryo culture.
2. To study the effect of media modifications as a means to prolong the storage duration of embryo cultures.

III. Methodology

1. *Validation of embryo culture protocols*
The 4 published protocols (Annexes 1–4) for coconut embryo culture (PCA, UPLB, CPCR-India, CIRAD/ORSTOM) will be tested using chemicals, supplies and environmental conditions used at the Institute of Plant Breeding (IPB). Mature coconut (10–11 months) of Laguna Tall will be collected, embryos will be excised and cultured *in vitro*. A total of 100 embryos (25 embryos/replication, 4 replications) will be used for each protocol.

2. *Media modification to prolong storage duration*

a. The effect of different concentrations of sucrose in the presence or absence of activated charcoal on the survival and growth of stored embryos will be determined. Embryos of 4 cultivars (2 tall, 2 dwarf) will be kept in culture for 6, 12, 18 and 24 months following the procedure of Assy Bah *et al.* (1989). Observations on survival and growth while in storage medium will be made . After storage, embryos/plantlets will be transferred to recovery medium. Survival and growth of embryos will be taken after 3 months on recovery medium.

b. The effect of different concentrations of osmotically active substances (sorbitol and mannitol) on survival and growth of stored embryos will be studies. Embryos of 2 cultivars (1 tall, 1 dwarf) will be kept in culture for 6 and 12 months. Observations on survival and growth while in storage and recovery medium (as described in methodology 2) will be taken. (This part of the project will be in collaboration with the CPCR-India and CRI-Sri Lanka).

c. The effect of different concentrations of growth inhibitor, ABA, on the survival and growth of stored embryos will be studied. Embryos of 2 cultivars (1 tall, 1 dwarf) will be cultured for 3 and 6 months. Observations on the survival and growth of embryos while in storage and recovery medium as described in methodology 2 will be taken. (This part of the project will be in collaboration with CPCR-India and CRI-Sri Lanka).

IV. Expected outputs

1. Embryo culture protocol that can be adapted by IPB.
2. Culture medium that can be used for medium-term conservation.

V. Project activities

Activities/Time schedule	Y1				Y2			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
1. Validation of embryo culture protocols (germination to acclimatization)	———	———	———	———	———	———	———	
2. Experiments on culture media modification								
a – effect of sucrose	———	———	———	———	———	———	———	———
recovery of stored embryos				———	———	———	———	———
b – effect of osmotica			———	———	———	———	———	———
recovery of stored embryos					———	———	———	———
c – effect of ABA						———		
recovery of stored embryos							———	

VI. Budget (US$)

Item	External		Grand total
	Y1	Y2	
Personne			
Part-time Technician (Honorarium)	500	500	1000
Supplies and materials	1000	1000	2000
Travel	500	500	1000
Others (Contract labor)	500	500	1000
Total	**2500**	**2500**	**5000**

IMPROVEMENT OF COCONUT EMBRYO CULTURE EFFICIENCY FOR GERMPLASM COLLECTING AND CONSERVATION

Project for Philippines

Project Leader
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Implementing Agency

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Country : Philippines
Project Title : Improvement of coconut embryo culture efficiency for germplasm collecting and conservation

I. Rationale

The coconut embryo culture technique developed to rescue the non-germinating Makapuno has also been very useful in germplasm collecting and conservation as well as in breeding for disease and drought resistance. However, the technology is beset with problems on low germination and survival rates during acclimatization of the heterotrophic plantlets from the laboratory conditions in the greenhouse and in the field. Success rate in the greenhouse is largely dependent on the physiological status and photosynthetic ability of the young seedlings.

The development of coconut embryo culture technology has led to the development of protocols in various laboratories. Validation of these protocols have to be undertaken under laboratory condition and genotype. In addition, effect of culture medium, effect of photoperiod and [illegible] would lead to a better understanding of [illegible] a basis for increasing its photosynthetic

[illegible] coconut varieties to various coconut embryo

[illegible] status of the culture media during germination and [illegible] *in vitro*

[illegible] different photoperiod regimes on the growth and development [illegible] *in vitro*

[illegible] coconut embryos and plantlets *in vitro* and during greenhouse conditions.

[illegible]thetic capacity [illegible] CO2 enrichment *in vitro* and during acclimatization [illegible]onditi

[illegible]arieties [illegible] Makapuno
in vitro [illegible] PCA, CPCRI and ORSTOM
[illegible] in three re[illegible] to be
[illegible] number of le[illegible], number
[illegible] survival [illegible]

[illegible] *in*
[illegible]M-

Effect of photoperiod

Coconut embryos at different stage of *in vitro* culture will be subjected to 0, 9, 12, 16 hr photoperiod. Data on percent germination, shoot length, number of leaves, root length, number of roots, number of transplantable seedlings and percent survival in the greenhouse will be gathered and evaluated.

Gas exchange

Changes in levels of CO_2, O_2 and ethylene will be determined at different stages of *in vitro* development of embryo-cultured coconut seedlings and during acclimatization to greenhouse conditions. Photosynthetic capacity of the seedlings will also be measured. This will also be in collaboration with ORSTOM-CIRAD and CICY

t of C02 enrichment

In vitro hardening to enhance photosynthetic competence will be done by C02 enrichment of the seedlings *in vitro* and during acclimatization to greenhouse conditions. Different concentrations of CO_2 will be introduced inside the culture vessels and in plastic bags in the greenhouse at different durations . Photosynthetic capacity of the seedlings as affected by CO_2 treatment will be measured.

IV. Expected output

1. Identification of a coconut embryo culture protocol with the highest transplantable seedlings and survival in the greenhouse.
2. Information on the performance and number of weanable seedlings and survival of MYD, Rennel Tall, Laguna Tall and Makapuno in the four embryo culture protocols evaluated.
3. Data to understand the changes in nutrient status of the culture medium during germination and seedling development *in vitro.*
4. Data to understand the response of coconut embryos and seedlings *in vitro* to different photoperiod regimes.
5. Data to understand the gas exchange status of coconut embryos *in vitro* and during acclimatization to greenhouse conditions.
6. Increased percentage of transplantable seedlings and survival *ex vitro* by C02 enrichment *in vitro* during acclimatization.

V. Project activities

Activities/Time schedule	Year 1				Year 2		
	Q1	Q2	Q3	Q4	Q1	Q2	Q3
Embryo collection							
In vitro culture							
Acclimatization							
Photoperiod studies							
Gas exchange studies							
CO_2 enrichment							
Nutrient status							
Data analysis							
Preparation and submission of report							

VI. Budget (US$)

Item	External			National		
	Year 1	Year 2	Total	Year 1	Year 2	Total
Personnel	500*	500*	1000	8400	8400	16800
Supplies/ materials	1000	1000	2000	1000	1000	2000
Travel	1000		1000			
Others		1000	1000			
Total	**2500**	**2500**	**5000**	**9400**	**9400**	**18800**

* Part time technician

DEVELOPMENT OF AN IMPROVED EMBRYO CULTURE PROTOCOL FOR COCONUT

Project for Philippines

Project Leaders
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Implementing Agencies

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Country : Philippines

Project Title : Development of an improved embryo culture protocol for coconut

Budget (US$)

Item	Year 1	Year 2	Total	Year 1	Year 2	Total
Personnel	500*	500*	1000	8400	8400	16800
Supplies/materials	1000	1000	2000	1000	1000	2000
Travel	1000		1000			
Others	[illegible]		[illegible]			

* Part time technician

I. Rationale

The embryo culture technology [illegible] conservation, [illegible] exchange of [illegible] to mass produce the non-germinating [illegible] Makapuno coconut in the Philippines, Dikiri in Sri Lanka and Kopyor in Indonesia. Many countries involved in embryo culture have developed their own embryo culture protocol with varying rates of success in terms of germination rates, seedling growth and *ex-vitro* survival rates. It is still possible to optimize the technology to attain highest success in recovery from embryo to field establishment.

II. Objectives

1. To compare and validate the various embryo culture working protocols from different laboratories (Annexes 1–4);
2. To develop a "hybrid" embryo culture protocol with the highest possible efficiency; and
3. To establish collaborative mechanism among 10 participating laboratories in the DFID project through an Embryo Culture (EC) project network, as agreed during the Philippine EC workshop.

III. Methodology

The different working protocols that would be compared will include the protocols developed in India, Ivory Coast, Philippines (Philippine Coconut Authority and University of the Philippines at Los Baños).

The different working protocols will be tried using the following coconut varieties:

Malayan Yellow Dwarf
Rennel Island Tall
Makapuno (Laguna Tall)
Catigan Green Dwarf
Tagnanan Tall

The following parameters will be observed:

a) % germination after [illegible]
b) number and size of leaves [illegible]
c) number and volume of [illegible]
d) seedling height at weaning
e) seedling weight at weaning
e) duration of developmental stage before weaning
f) number of successfully grown and weaned seedlings

There will be three replicates [illegible] per replicate.

The project leader (E. Rillo) [illegible] network coordinator to inform the members [illegible] participants through an EC project newsletter, technical advice and information dissemination.

IV. Expected output

1. Comparative recovery success rates of the various protocols using [illegible] parameters: % germination, duration of developmental period, % [illegible]
2. Developed a "hybrid" embryo culture protocol incorporating the most efficient [illegible] from the various working protocols.
3. The developed "hybrid" protocol will be used in the mass production of the [illegible] coconut. It will also enable the availability of a duplicate protocol for [illegible] collecting and exchange of coconut germplasm.
4. A well-coordinated EC project network.

V. Project activities

	Y1	Y2
1. Sampling	x	
2. Inoculation	x	
3. Subculture	x	
4. *In-vitro* hardening		x
5. *Ex vitro* hardening		x
6. Transfer to *ex-vitro* medium		x
7. Fertilization		x
8. Field planting		x
9. Submit reports		x

VI. Budget (US$)

Item	External		National counterpart		Grand
	Y1	Y2	Y1	Y2	total
Personnel			6000	7000	13000
Supplies and materials	1100	1100	400	400	3000
Travel	500	500	300	300	1600
Others	1000	1000	100	100	2200
Total	2600	2600	6800	7800	19800

[illegible] EMBRYO CULTURE TECHNOLOGY TO
[illegible] PROMOTE COCONUT GERMPLASM
[illegible] COLLECTING AND EXCHANGE

Project for Sri Lanka

Project Leader
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INCREASING THE EFFICIENCY OF EMBRYO CULTURE TECHNOLOGY TO PROMOTE COCONUT GERMPLASM COLLECTING AND EXCHANGE

Project for Sri Lanka

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Country : Sri Lanka
Project Title : Increasing the efficiency of embryo culture technology to promote coconut germplasm collecting and exchange

I. Justification

Coconut growers in Sri Lanka have been assisted by the Coconut Research Institute, in respect to recommendations on improved varieties, viable agronomic practices, crop protection strategies, fertilizer application, etc. Improved varieties (CRIC 60 and CRIC 65) were produced by hybridization of locally available genotypes. Further improvements will be hampered due to the narrow genetic diversity of the available coconut germplasm. Quarantine regulations has prevented not only the introduction of pest and lethal diseases but also germplasm exchange. Germplasm exchange is now permitted in the form of aseptic embryos. To facilitate germplasm exchange as embryos, the present embryo culture protocol has to be validated and upgraded in order to achieve the maximum possible survival rate.

There is an urgent need for breeding for tolerance to a disease of unknown aetiology (also known as leaf scorch decline) and for drought tolerance.

II. Objectives

1. To validate selected protocols (Annexes 2–3) and improve the present protocol used at the CRISL.
2. To evaluate the effect of ABA and/or osmoticum and GA3 on the germination and growth of embryos.
3. To evaluate the effect of added substrate on the efficiency of weaning.

III. Methodology

1. Protocols used in CPCRI, India and UPLB, Philippines will be compared with the protocol currently used in CRISL (Attachment 1) with the variety Sri Lankan Tall. The best protocol will be tested with varieties MYD, RIT (received from PNG) and Dikiri. Percentage germination and development of embryos, plant vigour at weaning and percentage survival at hardening will be evaluated.

2.a) Pre-treatment with ABA and/or osmoticum in different combinations will be tested as pre-treatment, with the best protocol selected using 10 month-old embryos of Sri Lankan Tall and Dikiri. The selected treatments will be also tested with retrieval media for preserved embryos.

b) Different concentrations of GA_3 will be tested in germination medium compared to the selected protocol with mature Sri Lankan Tall and Dikiri embryos. The study will be conducted in collaboration with CICY, Mexico and CPCRI, India. The germination percentage and uniformity of seedlings will be evaluated.

3. Rooting substrates such as coir dust, coir fibre and vermiculite will be compared against the control protocol using *in vitro* raised seedlings of Sri Lankan Tall and Dikiri at weaning stage in collaboration with CICY, Mexico. Parameters such as root growth, morphology, effect on shoot system will be evaluated.

Statistical design – The experiment will be conducted following the randomized complete block design (RCBD) and results will be statistically analyzed.

IV. Expected output

1. An efficient protocol will improve survival rate of *in vitro* raised seedlings.
   Present survival rate – 40 – 50%
   Expected survival rate – above 90%
   The experiments will be conducted with embryos of exotic varieties. This will also be an opportunity to receive exotic germplasm.
2. The expected survival rate with the best selected protocol will be further improved by pre-treating of embryos with pre-ripening agents for uniform maturity. The outcome will be also useful in retrieval of embryos stored for short term. Germination induced by GA3 and uniform maturity at germination will lead to higher survival rates and uniform seedlings at weaning.
3. Good root system and other morphological characters functioning better, eventually leads to better shoot development and vigorous seedling at weaning. This will facilitate hardening and improve survival rate.

V. Project activities

Activities	Year 1	Year 2
1. Evaluation of protocols using SL Tall	———	
2. Evaluation of best protocol using MYD, RIT, Dikiri	———	———
3. Evaluation of maturation and germination treatments	———	———
4. Evaluation of rooting. Substrates		———

VI. Budget (US$)

Item	External			National counterpart		
	Y1	Y2	Total	Y1	Y2	Total
Personnel	500	1200	1700	150	150	300
Supplies	1800	1100	2900	2000	1200	3200
Travel	200	200	400	–	–	–
Other	–	–	–	150	150	300
TOTAL	**2500**	**2500**	**5000**	**2300**	**1500**	**3800**

Attachment 1. Composition of modified Eeuwens Y3 medium (X2)

Compound	Concentration	
KNO_3	40	mM
KCl	40	mM
NH_4Cl	20	mM
$NaH_2PO_4.2H_2O$	4	mM
$CaCl_2.2H_2O$	4	mM
$MgSO_4.7H_2O$	2	mM
$FeSO_4.7H_2O$	100	mM
NaEDTA	200	mM
$MnSO_4.4H_2$	100	mM
KI	100	mM
$ZnSO_4.7H_2O$	50	mM
H_3BO_3	100	mM
$COCl_2.H_2O$	2	mM
$Na_2 MoO_4.2H_2O$	2	mM
$CuSO_4.5H_2O$	2	mM
$NiCl_2.6H_2O$	0.2	mM
L glutamine	1370	mM
L Arginine	1148	mM
L Asparagine. H_2O	1334	mM
m inositol	1110.2	mM
Pyridoxine.HCl	10	mM
Thiamine.HCl	6	mM
Nicotinic acid	16.2	mM
6 Benzylamino purine	10	mM
2,4 D	0.2	mM
Sucrose	60	g/l
Activated charcoal	2.5	g/l

INCREASING THE EFFICIENCY OF *IN VITRO* CULTURE OF ZYGOTIC COCONUT EMBRYOS TO PROMOTE GERMPLASM COLLECTING

Project for Tanzania

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Implementing Agency

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Country : Tanzania
Project title : Increasing the efficiency of *in vitro* culture of zygotic coconut embryos to promote germplasm collecting

I. Justification and rationale

Historically, coconuts in Tanzania were introduced from India, Maldives and Malaysia. It is believed that from these introductions, natural selection and introgression thereafter have produced distinct sub-populations that are adapted to different biotic and abiotic conditions.

Some collecting has been done on very limited su-populations and location, and collected germplasm have been conserved. Lethal disease (LD), drought and pests present the major obstacles to coconut development in Tanzania. Compared to other varieties, the local East African Tall (EAT) has shown a comparatively better performance with regard to drought. No variety has so far shown resistance to LD. A number of hybrids have been produced and tested, but their ongoing evaluation does not show a promising future.

Widening the coconut genetic pool may be a pre-requisite for further breeding activities. This can be achieved through more germplasm collecting, exchange, introduction and conservation. These processes are frequently hampered by quarantine regulations to prevent introduction of pests and diseases. Embryo culture offers safe alternative for local and international germplasm exchange, collecting and introduction. Despite its long existence, application of this technique has been limited by low survival rate of plantlets *in vitro* and *ex vitro*. Detailed and/or conclusive physiological and histological study to explain this phenomenon is scanty. The role of sucrose to final plantlet recovery *in vitro* and *ex vitro is* not well understood as well. Parallel studies of *in vitro* and conventionally grown plantlets is imperative to establish the cause of growth deviations exhibited by *in vitro* plantlets. This project is tailored towards that direction.

II. Objectives

1. To validate the best available protocols in comparison to the currently used protocol at the MARI.
2. To study the physiological and histological integrity of roots and leaves of parallel grown embryos and seednuts *in vitro* and *in situ*, respectively. This study will be carried out in collaboration with ORSTOM (Institut Francais de Recherche Scientifique pour le Development) and CICY (Centro de Investigacion Centifica de Yucatan, A.C.).
3. To establish the main *in vitro* conditions and practices that lead to physiological and histological malfunctioning of plantlets *in vitro* and *ex vitro*.
4. To correlate the research findings of the study to the survival rate *in vitro* and *ex vitro*.
5. To use the research findings as a basis to improve plantlet recovery *in vitro* and *ex vitro*.
6. To investigate the effect of different concentrations of sucrose in growth medium on plantlet survival *in vitro* and *ex vitro*.

III. Methodology

Validation of protocols

Cultural conditions and practices will be adopted from the best protocols of the PCA-ARC (Philippine Coconut Authority – Albay Research Centre), CPCRI (Central Plantation Crops Research Institute – India), UPLB (University of Philippines at Los Banos) and ORSTOM (Annexes 1–4). These will be compared to the protocol used at MARI (Mikocheni Agricultural Research Institute – Dar Es Salaam).

Rennel Tall, Malayan Yellow Dwarf, Pemba Red Dwarf (local dwarf) and East African Tall (local tall) will be the varieties of choice for validation experiment.

Physiological and histological studies

Culture and sowing age for parallel experiments will be 10 – 11 month old nuts. Parallel sampling and data collection will be carried out *in situ* and *in vitro.* Root samples will be taken from primary and secondary roots at different growth stages. Sampling at transition period *(ex vitro* to field planting) will also be carried out at regular intervals. First, second and third foliage leaf produced *in vitro* and *in situ* will be collected and prepared for physiological and histological studies. First, second and third foliage leaves produced *ex vitro* will be sampled and their physiological and histological integrity investigated. The same will be done for the first, second and third scale leaves produced *in vitro* and *in situ.*

Physiological parameters will be measured using Leaf Chamber Analyser (Type LCA-4) and these will include photosynthetic rate, sub-stomatal (CO_2) carbon dioxide concentration, transpiration rate, stomatal conductance and resistance, energy conversion factor, PAR on leaf surface, boundary layer resistance to water vapour, leaf surface temperature and CO2 concentration in the growth room and atmosphere.

The anatomy of roots, leaves and stems will be investigated with the aid of microscope. This will entail fixation, dehydration, infiltration with paraffin wax, embedding, sectioning, staining and photography. Additionally, wax deposition on leaves and meristematic activity of the root tips and apical bud at different growth stages will be investigated.

Experimental design and data analysis

The experiment will be laid out in a Complete Randomised Block Design and data will be analysed statistically. Thirty (30) embryos from each of the three varieties will be cultured in three replications using five protocols.

IV. Expected output

1. Better understanding of cultural practices and conditions that lead to propagation of abnormal plantlets *in vitro* established.
2. Basic knowledge on detrimental effects of cultural conditions and practices unveiled.
3. Results utilized in the optimization of culture medium and *in vitro* conditions to improve plantlet recovery.
4. Loss of valuable genetic materials collected, introduced, rescued or exchanged put to a minimum.
5. Role of sucrose to *in vitro* plantlets survival established.
6. Academic pool in coconut research enriched with valuable findings.

Milestones

1. 500 embryos of each of the four varieties cultured *in vitro.*
2. 500 seednuts of each of the four varieties set in nursery bed.
3. 5 plantlets from each replication for each variety sampled and investigated with physiological parameters regularly and undestructively both *in vitro* and *in vivo.*
4. 5 plantlets from each replication for each variety sampled and investigated with histological parameters regularly *in vitro* and *in vivo.*
5. Computer data input, analysis and interpretation at each sampling.
6. 1800 plantlets transferred from the lab to *in vivo* conditions after 6 – 8 months.

7. 1800 plantlets acclimatised for two to three months and polybagged.
8. 1800 seedlings polybagged after three months from the nursery bed.
9. Report/paper prepared and presented to the COGENT (International Coconut Genetic Resources Network)/IPGRI (International Plant Genetic Resources Institute) and possibly published in an international journal.

V. Time-activity schedule

Activity	Year 1				Year 2			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
1. Procurement of materials and supplies	xxx				xxx			
2. Collection of embryos from different accessions, culture and sowing	xxx	Xxx						
3. Subculture maintenance and sampling *in vitro* and *in situ*	xx	Xxx	xxx	xxx				
4. Data collection	xxx	xxx	xxx	xxx	xxx	Xxx	xxx	
5. Transfer of plantlets to *in vitro* conditions				xxx				
6. Sampling *in vitro* and *in situ*				xxx	xxx	Xxx	xxx	
7. Data analysis and report write-up							xxx	xxx

VI. Budget (US$)

Item	External		Internal	
	Y1	Y2	Y1	Y2
1. Personnel*	1000	1000	1000	1000
2. Materials/supplies	1000	1000	1000	1000
3. Travel	300	300	300	300
4. Others	200	200	200	200
Total	**2500**	**2500**	**2500**	**2500**

*May necessitate additional contract labour.

INCREASING THE EFFICIENCY OF COCONUT EMBRYO RESCUE TO FACILITATE COLLECTING AND THE SAFE MOVEMENT OF GERMPLASM

Project for Papua New Guinea

Project Leader
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Implementing Agency

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Country : Papua New Guinea
Project Title : Increasing the efficiency of coconut embryo rescue to facilitate collecting and safe transfer of germplasm

I. Background and justification

The main purpose of embryo culture is to facilitate germplasm collecting and safe exchange. Coconut germplasm tend to be found in isolated geographical locations. In order to capture the maximum genetic variability within/among populations, one must collect sufficient number of nuts for conservation. However, the current technique can only rescue 9–12 month old nuts which means, at any one time, up to 3 bunches (average of about 15 nuts) can be harvested from the palm. More nuts can be harvested if one can decrease the physiological age at which the embryos can be successfully rescued. In addition, the percent survival of embryo-derived plants is quite low and variable from one laboratory to another, resulting in loss of precious diversity after collecting missions. Thus, there is a need to increase the efficiency of embryo culture and acclimatization techniques to remedy the above problems.

II. Objectives

1. To compare the efficacy of four embryo culture protocols – PCA, UPLB, CPCRI, ORSTOM/CIRAD (Annexes 1–4) on a selected variety.
2. To establish minimum age when the embryos can be successfully rescued during germplasm collection.
3. To collect maximum genetic diversity within/among populations using embryo rescue.

III. Methodology and approach

Establishing the laboratory facilities/equipment and training of staff for embryo culture will be of first priority. The four protocols already developed by PCA (Philippines), UPLB (Philippines), CPCRI (India) and ORSTOM/CIRAD (France) will be tested on a selected variety, eg. Local Tall, using embryos of different ages, 7–12 months. Observations on survival and abnormalities will be made.

IV. Expected output

Year 1 CCRI will have a well established and functional embryo culture laboratory and acclimatization facilities with trained staff to carry its responsibility as the regional centre for the International Coconut Genebank for the South Pacific (ICG-SP).

Year 2 Four embryo-rescue protocols verified on Local Tall. Successful acclimatization procedure developed or verified.
Protocols developed to rescue embryos from bunches of 9–12 months old Local Tall. Local ecotypes collected and embryos rescued using the best protocol. Exotic ecotypes introduced through embryo culture.

V. Activities and schedule

Year 1

a) Set up embryo culture laboratory - purchase of equipment, chemicals and laboratory consumables (Funding mainly from ICG-SP project).
b) Staff recruitment and training in tissue culture laboratory available in PNG (Unitech, LAES).
c) Verifying the efficacy of 4 protocols on one Local Tall and one Local Dwarft variety.

Year 2

a) Continue verification of 4 protocols.
   Establishment and culturing of embryos of Local Tall at various physiological ages, 9-12 months.
b) Acclimatization of embryo-rescued seedlings.
c) Collecting and culturing embryos of local ecotypes.
d) Introduction of selected international ecotypes.

VI. Budget (US$)

Item	External			National counterpart[1]		
	Y1	Y2	Total	Y1	Y2	Total
Personnel						
Supplies/materials	2,500	2,500	5,000			
Travel						
Others						
TOTAL	**2,500**	**2,500**	**5,000**			

(1) The Budget is not indicated

Annex 1. Coconut embryo culture protocol at PCA

Field extraction of embryos

Using a cork borer, part of the solid endosperm embedding the embryo is extracted from the splitted nuts. The endosperm cylinders are collected in a clean container.

Pre-sterilization

After all the endosperm cylinders are extracted, these are washed in tap water and in 95% ethanol quickly to remove the fats and then disinfected with 100 % commercial bleach (Zonrox) for 20 minutes. These are then washed three times with sterile water to remove the bleach. The step is best done in a clean room with still air to minimize contamination.

Media preparation

A. Preparation of stock solution
The concentrations of stock solutions are usually 10 x for macro elements and 100 x for micro elements and vitamins.

B. Preparation of the Y3 culture medium (see Annex 5)

Materials

Stock solutions of:

- Macro-nutrients 10 x
- Micro-nutrients 100 x
- Myo-inositol 100 x
- Vitamins 100 x
- Fe-EDTA 100 x
- Table grade sugar 45 g/l
- Activated charcoal (AC) 2.5 g/l

1. Weigh out 45 g/l sugar in the balance and dissolve in the solution above.
2. Using a volumetric flask or a graduated cylinder, make up the volume to one liter using distilled water.
3. Adjust the pH to 5.8 using 0.1–5 M NaOH or 0.1–0.5 M HCl.
4. Add 2.5 g activated charcoal and stir.
5. Dispense 10 ml of the liquid medium, into 150 x 25 mm test tubes, while stirring the medium constantly to evenly disperse the activated charcoal. For ketchup bottles, dispense 80 ml of the medium.
6. Cover with no. 4 rubber stopper with a 2 mm hole in the middle adequately stuffed with cotton.
7. Autoclave the medium at 121°C at 15 psi for 15 minutes.
8. Cool before use.

Asceptic techniques

A. Preparation of sterile embryos for culture

1. Wash the solid endosperm cylinders with tap water several times.
2. Rinse in 95% ethanol for 1-2 minutes. Decant.
3. Immerse in 100% commercial bleach (Zonrox) for 20 minutes in a clean beaker. For embryos which were pre-sterilized already prior to storage and transport, immerse these in 100% bleach for 5 minutes.
4. Inside the laminar flow hood after sterilization, decant bleach and rinse with sterile distilled water at least 5 times.

B. Culture of sterilized embryos

1. 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 the oven). Petri dishes lined with filter paper should be autoclaved.
2. Inside the laminar flow, frequently dip the forceps and scalpels, scissors, etc. in 80% ethanol and sterilize them in the sterile-beads or flame in an alcohol lamp for 20 seconds. Let them cool on an aluminium instrument rack. Using these sterile instruments, excise embryos out from the solid endosperm in the sterile Petri dishes lined with filter papers. Transfer embryos to sterile flasks.
3. 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.
4. Transfer embryos on sterile Petri dishes lined with filter paper.
5. Inoculate singly into test tube containing Y3 liquid medium.

C. Culture conditions

1. Incubate cultures at 28–30°C with approximately 4,000–5,000 lux at 9 hr photo-period (15 hr. dark and 9 hr. light).
2. Subculture to fresh medium at monthly interval.
3. Check periodically for contamination.
4. Embryos grow at different rates. Generally, 6–8 weeks after roots and shoots are formed. The earliest recorded time to transfer *ex vitro* is 4 months.
5. All together, the culture period could be a year or more.

Screenhouse practices

Materials

1. Sterile river sand
2. Clear plastic bags/bell jars
3. Bamboo sticks (when clear plastic bags are used)
4. Polyethylene bags
5. Fungicide (2.5 g/l) solution

Procedure

1. Take out the seedlings from the laboratory to harden them in the screenhouse for one week.
2. After one week, pot them in sterilized river sand and contained in small polyethylene bags.
3. Take out hardened seedlings.
4. Wash out the media carefully. The liquid medium contains sugar that will attract ants if not washed completely. Dip the seedlings in 2.5 g/l fungicide solution, e.g. Daconil.
5. Plant to sterilized sand.

6. To maintain high relative humidity, cover the seedlings with plastic bags. Support the plastics with bamboo pegs so that they will not sag on the leaves of the seedlings. Keep them covered for 5–7 days.
7. After this period, gradually exposed the seedlings to screenhouse conditions by partially lifting the cover for a week.
8. On the third week, the plants can be fully exposed to screenhouse conditions.
9. Water the plants regularly and apply dilute foliar fertilizer solution weekly after it has developed new leaves.
10. After 3 months, transfer the plants to bigger polyethylene bags using non-sterilized soil.
11. After another 3–5 months, the plants can be transferred to the field. The plant should have 4 – 6 leaves by then.
12. Provide the seedlings with the necessary cultural practices for optimum growth response especially during the first three years.

Annex 2. Coconut embryo culture protocol at UP Los Baños

1. Collect endosperm cylinders with embryos.
2. Sterilize in 5% NaOCl for 20 minutes.
3. Excise embryos from endosperm cylinders.
4. Sterilize embryos in 1% NaOCl for 10 minutes.
5. Rinse three times with sterile distilled water.
6. Inoculate in liquid Y3 medium (see Annex 5) + 60 g/l sucrose.
7. Incubate in the dark.
8. After 4 weeks, transfer to solid Y3 medium + 60 g/l sucrose. Incubate at 9 hours photoperiod at 25–29°C.
9. After 4–6 weeks, transfer germinated embryos to liquid Y3 medium + 6% sucrose. Decapitate root tips of embryos prior to inoculation.
10. For seedlings with slow shoot and root development, it is necessary to transfer to fresh medium after 4–6 weeks.
11. Well-developed seedlings with 3 to 4 leaves and profuse lateral roots should be brought to the screenhouse and subjected to natural light.
12. After 2 weeks, take seedlings out of culture medium.
13. Wash with water to remove culture medium.
14. Dip in fungicide (Dithane M-45 2 g/l).
15. Transplant to sterilized sand.
16. Cover with plastic bag and expose to 50% shade.
17. Gradually loosen/lift plastic bag.
18. After 3–4 weeks, transplant to compose sand (1:1) mixture.
19. Keep under 30 % shade.
20. After 3 months, expose to full sun conditions.

Annex 3. Coconut embryo culture protocol at CPCRI, India

I. Field collections

Materials

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 95 % (100 ml)
6. Cotton/cheese cloth: 30 x 45 cms (100 Nos.)
7. Stainless steel cork borer (diameter: 0.5 cm; length 20 cm)
8. Knives: big and small
9. Scalpel: 1 No.
10. Plastic tray: 1 No.
11. Parafilm: 1 roll
12. Sterilized disposable gloves
13. Scissors: 2 Nos.
14. Rubber bands: 500 gm
15. Dehusker
16. Vials containing sterile medium
17. Measuring cylinder: 100 ml
18. Potassium permanganate; 500 gm
19. Chlorine kit klonal flask (1 liter); separating funnel
20. Gummed labels
21. HCl

Methods

1. Dehusk and extract the embryo with cork borer.
2. Surface sterilize the inoculation hood with 95% alcohol.
3. Sterilize the embryos with desinfectant (Clorine water–5%) for 20 minutes.
4. Wash the embryos 3–4 times with sterile water.
5. Transfer the embryos into sterile water/medium.

II. In vitro *culture*

1. Inoculate the embryos in solid retrieval medium (Y3 + 60 gm/l sucrose + NAA (0.5 mg/l) + BAP (0.5 mg/l)) and incubate in dark until germination (average germination time of mature embryo is 20–25 days for dwarf and 35–40 days for tall) and transfer to light with 16 hr photoperiod (Temp. 27–29°C, RH 65–70%).
2. Subculture every 4–5 weeks. Reduce the sucrose concentration to 30 g/l.
3. Germinated embryos (with two leaves and primary root, after almost 4 months of inoculation) are transferred to liquid rooting medium (Y3 + 30 g/l sucrose + NAA (1ppm) + IBA (5 ppm).
4. Subculture to same medium after every 4 – 5 weeks. Transfer to wide mouth and longer tubes, whenever necessary.
5. Plantlets with well-developed secondary and tertiary root and shoot system (3–4) leaves, 20–25 cm height, 5–6 ml root volume) are ready for transfer to small pots. Potting mixture consists of (1:1:1) sterilized (autoclaved) soil : sand : decomposed coir.
6. Pre treat the plantlets with Carbendazim (1 g/l) and IBA (1,000 ppm) for 1 hour each and transfer to the pots.

III. Acclimatization

1. Cover the plantlet with polyethylene bag for 2–3 weeks and keep it indoor at room temperature but with artificial light.
2. Supply Hoaglands solution once in 15 days.
3. Irrigate to keep potting mixture moist.
4. After 3 weeks, harden the plantlets by gradual introduction of perforations in polyethylene bags.
5. After 2 weeks, remove the polyethylene bags during night for 2 weeks.
6. After 2 weeks, remove the polyethylene bags completely and let it stay indoor for 1 week.
7. Transfer to bigger pots and keep in a nethouse with 50% shade.
8. After 3–4 months, transfer the plantlets to big polyethylene bags with soil and organic manure and keep in a nethouse with 50% shade.(Total duration from pot to polybag is 5–6 months). Irrigate regularly and apply recommended dose of fertilizer, whenever necessary.
9. After 4–5 months, plantlets can be transferred to the field.

Annex 4. Coconut embryo culture protocol at ORSTOM

I. In vitro *based embryo sampling, storage and transport methods*

1. 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 had been carefully washed and disinfected with 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 (diameter 20mm). The cork borer and forcep used for this operation are previously disinfected by immersion in a bowl containing a 3% chlorogenic sodium hypo-chlorite solution. A portable gas burner is used to sterilize the instruments. Batches of 30 cylinders are immersed for 20 minutes in a 500 ml of a calcium hypochlorite solution (70% active chlorine: 45 g/l).

2. Embryo storage and transport from collecting site to the in vitro *laboratory*

Immersion of the disinfected endosperm cylinders in a sterile KCl solution (16.2 g/l) provides the best conditions for their storage for a maximum period of 14 days. This gives enough time to return to the laboratory to start the culturing operation.

3. Embryo excision before inoculation

After the storage period, the cylinders are re-sterilized by transferring them individually to a filtered solution of calcium hypoclorite (70% active chlorite: 45 g/l) for 20 minutes. The embryos are then isolated in an air flow cabinet and rinsed in sterile distilled water before inoculation into liquid medium.

II. Embryo culture conditions

The culture medium used for embryo germination (MI 502) contains Murashige and Skoog mineral element (1962), Morel and Wetmore (1951), sodium ascorbate (100 mg/l), sucrose (60 g/l), neutralized activated charcoal 92 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 in 24 x 160 mm test tubes containing 20 ml of medium sealed with plastic parafilm. They are incubated in a dark room at 27°C.

They are subculture every 4–6 weeks into 20 ml of fresh medium. The germinating embryo is kept in dark until the first true leaf emerges (3 – 4 months for the more advanced embryos). As soon as the first true leaf and the root system is developed (at least one root with ramifications), plantlets are transferred into 100 ml MI 502 liquid medium in one liter glass bottles under light (12 hours per day 45 µmol/m2; Sylvania gro-lux day light tubes). Bottles are covered with foam caps surrounded by aluminium foil and sealed with parafilm.

Plantlets growing under light conditions are transferred every 4 – 6 weeks in large tubes (36 x 200 mm) into fresh medium. They can be acclimatized when they displayed 3 to 4 unfolded green leaves (the more advanced plantlets reach the acclimatization stage 6–7 months after the initial inoculation).

III. Acclimatization procedure

The protocol is performed in a tropical greenhouse where humidity and temperature are controlled. Once removed from the culture medium, the plantlets are carefully rinsed with distilled water and then plunged for 5 minutes in a fungicide based on carbendazin (Benlate, 2 g/l) to prevent fungi development. They are then placed on sterile river sand. By using plastic bags (Acrylic polypropylene) for covering each plantlet during the first

two weeks, it is possible to maintain maximum relative humidity conditions. Progr... the plastic is opened. Plantlets are watered with water alone for the first month and ... a nutritive solution is applied every two days.

Composition of the nutritive solution used for acclimatization plantlet (mg/l)

KNO3	274.00
Ca(NO3)2.2H2O	1095.00
KH2PO4	137.00
MgSO4.7H2O	274.00
(NH4)2.SO4	137.00
KCl	2.74
H3PO3	3.00
MnSO4.H2O	15.00
ZnSO4.7H2O	2.74
(NH4)6Mo7Oz4.4H2O	2.74
H2SO4	0.137
CuSO4.5H2O	1.37
FeSO4.7H2O	24.90
EDTA	26.10

After 2 months of sand, the plantlets are transferred to forest leaf mould. At this stage, they are fertilized every 2 weeks with 50 ml of a N:P:K solution (8;11;14;2 ml/l). Every 2 months, 50 ml of chelated iron 6% (1 g/l) is also added.

Annex 5. Media composition for coconut embryo culture (mg/l)

Chemical	PCA Y3	UPLB Y3	CPCRI Y3	ORSTOM Y3
NH_4NO_3	–	–	–	1650
NH_4Cl	535	535	535	–
KNO_3	2020	2020	2020	1900
$MgSO_4.7H_2O$	247	247	247	370
$Cacl_2.2H_2O$	294	294	294	440
KCl	1492	1492	1492	–
KH_2PO_4	–	–	–	170
$NaH_2PO_4.2H_2O$	312	312	312	–
KI	8.3	8.3	8.3	0.83
H_3BO_3	3.1	3.1	3.1	6.2
$MnSO_4.4H_2O$	11.2	11.2	11.2	22.3
$ZnSO_4.7H_2O$	7.2	7.2	7.2	8.6
$CuSO_4.5H_2O$	0.25	0.25	0.16	0.025
$CoCl_2.6H_2O$	0.24	0.24	0.24	0.025
$NaMoO_4.H_2O$	0.24	0.24	0.24	0.025
$NiCl.6H_2O$	0.024	0.024	0.024	–
$Fe_2SO4.7H_2O$	13.9	41.7	13.9	24.9
Na_2EDTA	37.3	55.8	37.3	26.1
Myo-inositol	100	–	100	100
Pryridoxine HCl	0.05	0.05	0.05	1.0
Thiamine Hcl	0.05	0.05	0.5	1.0
Nicotinic Acid	0.05	0.05	–	1.0
Ca-D-pantothenate	0.05	–	–	1.0
Biotin	0.05	0.05	0.05	0.01
Folic acid	–	0.05	–	–
Glycine	–	1.0	–	–
Na Ascorbate	–	–	–	100
BAP*	–	–	0.5	–
NAA*	–	–	0.5	–
NAA**	–	–	1.0	–
IBA**	7.0**	–	5.0	–
Agar	–	7.0g/l		
liq/sol/liq	7.0g/l			
sol/sol/liq	–			
Activated charcoal	2.5g/l	2.5g/l	1g/l	2g/l
Sucrose	45g/l	60g/l	60g/l	60g/l
PH	5.8	5.6	5.6	5.5

* except for agar, sucrose and AC
** germination medium
*** rooting

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