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Coconut Embryo *In Vitro* Culture: Part II

COGENT

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CICY

Florent Engelmann
Pons Batugal
Jeffrey Oliver
editors

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Citation:
F Engelmann, P Batugal and JT Oliver, editors. 2002. Coconut Embryo *In Vitro* Culture Part II: IPGRI-APO, Serdang, Malaysia.

Cover Pictures: Dr. Carlos Oropeza

ISBN 92-9043-536-4

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Foreword

The International Plant Genetic Resources Institute (IPGRI) established the International Coconut Genetic Resources Network (COGENT) in 1992 to promote the sustainable conservation and use of coconut genetic resources. To implement this mandate, COGENT encouraged coconut producing countries to become members of the network and serve as partners in this diversity conservation effort. To date, COGENT has 38 member-countries, consisting of four countries in South Asia (Bangladesh, India, Pakistan and Sri Lanka); seven in Southeast and East Asia (China, Indonesia, Myanmar, Malaysia, the Philippines, Thailand and Vietnam); eight in the South Pacific (Cooke Islands, Fiji, Kiribati, Papua New Guinea, Samoa, Solomon Islands, Tonga and Vanuatu); nine in Africa and the India Ocean (Benin, Côte d'Ivoire, Ghana, Kenya, Madagascar, Mozambique, Nigeria, Seychelles and Tanzania); and ten in Latin America and the Caribbean (Brazil, Colombia, Costa Rica, Cuba, Guyana, Haiti, Honduras, Jamaica, Mexico and Trinidad-Tobago).

To conserve precious coconut germplasm for current and future use, COGENT member countries have conserved 1,370 accessions in 28 genebanks and collection sites in 23 countries and have provided passport and characterization data to the COGENT International Coconut Genetic Resources Database (CGRD). Data on molecular marker characterization of coconut accessions are also now being generated by COGENT and partner institutions and incorporated into the CGRD. This database is updated yearly and disseminated to coconut breeders worldwide. Through the CGRD, coconut breeders can effectively identify the appropriate coconut germplasm that they could use in their breeding programmes.

To further ensure effective conservation and use of coconut germplasm, COGENT is establishing a multi-site International Coconut Genebank (ICG) which is hosted by India for South Asia, Indonesia for Southeast and East Asia, Papua New Guinea for the South Pacific and Côte d'Ivoire for Africa and the Indian Ocean. Negotiations are underway for Brazil to host the ICG for Latin America and the Caribbean. The ICG will conserve, evaluate and provide information and access to 200 of the most important coconut germplasm of each region. The ICG is part of the International E*x Situ* Collections under the International Undertaking on Plant Genetic Resources (now the International Treaty) of the Food and Agriculture Organization. COGENT is also promoting *in situ* conservation as part of its poverty reduction project in coconut growing communities sponsored by the Asian Development Bank (ADB). It is also developing cryopreservation technologies for long-term conservation of coconut germplasm.

As membership in COGENT commits each country to access to its germplasm and information, coconut breeders can thus request for germplasm for breeding and research purposes. To promote the exchange and use of germplasm by coconut producing countries, COGENT has developed a pest-risk assessment protocol to provide plant quarantine officers more information as basis for developing better quarantine decisions for the safe movement of coconut germplasm. In addition, IPGRI/COGENT is improving the coconut embryo *in vitro* culture technology to increase its efficiency for higher seedling recovery. In 1997, IPGRI/COGENT organized the First International Workshop on Coconut *In Vitro* Culture in Albay, Philippines. Co-organized by the Philippine Coconut Authority and funded by the Common Fund for Commodities (CFC), the workshop gathered together coconut embryo *in vitro* technology practitioners from 12 countries to identify technology gaps, upgrade existing *in vitro* technology and propose research to further develop the technology. The result of this workshop was published in the *Coconut Embryo* In Vitro *Culture*: *Proceedings of the First Workshop on Embryo Culture, 27–31 October 1997, Banao, Guinobatan, Philippines*, edited by P.A. Batugal and F. Engelmann. Based on the research proposals submitted by the participants, IPGRI/

COGENT awarded projects to 12 researchers who conducted their researches from 1998 to 2001. These projects were funded by the Department for International Development (DFID) of the UK. The initial results of these research projects were reported during the 2nd International Coconut I*n Vitro* Culture Workshop, hosted by the Centro de Investigacion Cientifica de Yucatan (CICY) and funded by DFID. These results are reported in this publication.

The result of this coconut embryo *in vitro* culture work is important for five main reasons: First, it has made available to coconut producing countries *in vitro* technologies that were previously not available to many coconut research institutes worldwide; Second, the improved technology has been used to train coconut researchers in Bangladesh, China, Indonesia, Malaysia, Philippines, Pakistan, Fiji, Papua New Guinea, the Philippines, Samoa, Thailand, Tonga, Vanuatu and Vietnam; Third, due to the improved technology, 20 countries can now use the technology for collecting germplasm from isolated and distant locations, thus facilitating collecting and conservation; Fourth, it has aided in rapidly multiplying and enhancing the access to previously limited quantities of high-priced soft-endosperm coconut varieties of Indonesia, the Philippines and Sri Lanka which could increase by about five times the incomes of coconut farmers and their households; and lastly, the improved technology will enhance the safe exchange of coconut germplasm and thus promote the use of coconut diversity to benefit resource-poor coconut farmers.

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Background on the development and implementation of the coconut embryo *in vitro* culture project

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Introduction

The efficient use of coconut genetic resources has been hampered by difficulties in collecting and exchanging germplasm. Within the plant kingdom, coconut is the species with the largest seeds. Moreover, it has no dormancy period and germination immediately follows maturation of the seeds. These characteristics drastically limit the amount of material that can be gathered during collecting missions. Germplasm exchange is even made more difficult due to the high risk of introducing pests and diseases as well as the high cost of transporting whole nuts.

With coconut, *in vitro* techniques have been used by various groups to address these problems (Engelmann 1998). Simple and efficient *in vitro* protocols have been developed for collecting coconut germplasm in the field, which involve extracting the embryos from the nuts and inoculating them *in vitro*. *In vitro* techniques have also been used in some instances for the international exchange of coconut germplasm in the form of excised embryos inoculated *in vitro*. Preliminary experiments have also shown that medium-term conservation of coconut embryos is feasible *in vitro*, as well as their long-term conservation through cryopreservation (using liquid nitrogen at −196°C).

Using *in vitro* techniques for collecting, exchanging and conserving coconut germplasm requires efficient protocols for *in vitro* germination of embryos, development of embryos into whole plantlets, acclimatization to *in vivo* conditions and further development into seedlings which can be outplanted in the field. This becomes especially significant with the establishment of the multi-site International Coconut Genebank or ICG (Rao and Batugal 1998) and the implementation of the various international breeding and testing programmes coordinated by the International Coconut Genetic Resources Network (COGENT), which rely heavily on *in vitro* techniques for collecting and exchanging germplasm.

The coconut embryo *in vitro* culture project

In vitro culture protocols have been developed by various coconut research institutes worldwide (Batugal and Engelmann 1998). However, a survey conducted by the International Plant Genetic Resources Institute (IPGRI) in 1996 revealed the relatively low overall efficiency of existing protocols (Engelmann 1998). It was therefore decided to organize a workshop to assess more precisely the status of coconut *in vitro* culture and to upgrade and standardize the protocols, so that coconut researchers could use them with better efficiencies (Batugal 1998). The first International Workshop on Coconut Embryo Culture held at PCA in Albay, Philippines on October 1997 documented and précised the information generated from the initial survey. Results of the first workshop revealed large discrepancies between the performances of the existing *in vitro* culture protocols, with only 14 to 55% of inoculated embryos developing

into plantlets *in vivo*. These differences were also strongly accentuated by the experience of the research teams utilizing these protocols. An important discovery from the analysis of the existing protocols was that the main bottleneck was not the acclimatization of plantlets, as initially thought, but the low efficiency of embryo germination and plantlet development *in vitro*. Findings also showed that these protocols differed greatly in the culture conditions, the composition and sequence of media employed, the developmental stage selected for weaning of plantlets and the number and type of coconut varieties tested, which were limited.

Based on these findings, two main objectives were identified for the UK Department for International Development (DFID)-funded research project established during the first workshop:

- Improve the maturation and germination rates of embryos and their development into whole plantlets; and
- Determine and select the most efficient *in vitro* culture protocol and test it with a large number of varieties.

The research activities implemented by the project participants focused on the following:

1. Improvement of maturation and germination rate of embryos by determining the effects of growth regulators (ABA, GA_3), osmoticums (PEG, mannitol) and amino acids (proline).

2. Improvement of plantlet development.
   - 2.1 Anatomical and physiological studies of plantlets
     - Histology of root and leaf development
     - Functionality assessed by measuring root absorption capacity and leaf transpiration, as well as photosynthetic capacity (photosynthetic rate)
   - 2.2 Study of atmospheric composition in culture vessels
     - Changes in CO_2, O_2 and ethylene in culture vessels in relation with plantlet development, monitored and modified if necessary
   - 2.3 Effect of CO_2 enrichment on development and acclimatization of plantlets
     - Effect of CO_2 enrichment at the end of *in vitro* culture period or immediately after transfer of plantlets *in vivo*
   - 2.4 Effect of photoperiod on germination of embryos
     - Effect of different lighting regimes on the germination of embryos
   - 2.5 Effect of rooting substrates
     - Efficiency of rooting substrates (e.g. vermiculite, coconut fibre) compared with the standard protocols

3. Comparison of the efficiency of four selected protocols (CPCRI, India; PCA-ARC and UPLB, Philippines; and IRD-CIRAD, France) and experimentation with different varieties (MYD and RT as common control; additional varieties in the screening experiments selected by each participating institute).

4. Medium-term conservation of embryos using *in vitro* storage.

A rapid survey of the reports submitted by project participants at the time of completion of the first phase of the project, which are all published in these Proceedings, allow some preliminary observations of the results achieved. *In vitro* culture of coconut embryos and production of plantlets are now operational in all participating laboratories. Success rates have also significantly improved, with 31 to 80% of inoculated embryos developing into plantlets *in vivo*. A large diversity of coconut

germplasm has been employed since the tissue culture protocols have been tested with over 20 varieties. These tests have revealed a very strong genotypic effect in response to *in vitro* culture. Although the overall performances of the *in vitro* culture technique have greatly improved, an optimal protocol has not been idenitified due to the high variability of the responses obtained in different laboratories.

Additional experiments have provided other important information. It has been demonstrated that GA_3 has a positive effect on the germination of embryos and on the further development of plantlets. The position of the embryo on the medium also seems to be extremely important as embryos placed with the plumule directed upwards displayed the highest germination percentages. Finally, the "hybrid protocol " proposed by one participating laboratory, which combines the most efficient steps of the four protocols tested, seems to hold good promises for further increasing the performances of coconut embryo *in vitro* culture.

Conclusion

During 2nd International Coconut *In Vitro* Culture Workshop held in Merida, Mexico, results obtained were extensively analyzed and discussed with the aim of identifying the most important topics to be further studied and refined in the planned second phase of the project. It is our hope that through the successful implementation of this global project, the upgraded and standardized embryo *in vitro* culture technology will rapidly make a significant impact on the establishment of the multi-site ICGs and on the development of breeding and testing programmes by facilitating the conservation and exchange of coconut germplasm.

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Table 1. Summary of results obtained in eight laboratories with *in vitro* culture of coconut embryos, before and after the implementation of the DFID/CFC-funded project.

Country/ Laboratory	Before project									After project								
	Initial number of embryos	Germinated *in vitro* (%)		Recovered *in vitro*		Recovered in nursery		Recovered after field transplanting		Initial number of embryos	Germinated *in vitro*		Recovered *in vitro*		Recovered in nursery		Recovered after field transplanting	
		No.	%	No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	No.	%
Sri Lanka	2000	1140	57	920	46	740	37	629	31	561	389	69	254	45	158/ 300	53	na	na
Philippines (PCA)	2085	1161	56	770	37	770	37	770	37	4287	3258	76	2079	48	na	na	na	na
Vietnam na		na	na	na	na	na	na	na	na	na	450	373	83	309	69	na	na	na
Mexico	300	247	83	107	36	87	29	na	na	741	504	68	na	na	na	na	na	na
Cuba	200	147	74	59	29	0	0	0	0	240	134	56	48	20	na	na	na	na
Indonesia	54	na	na	8	15	na	na	na	na	847	625	74	324	38	159	49	na	na
India	1677	1239	74	na	na	na	na	215/ 409	52	960	835	87	605	63	521	54	na	na
Philippines (IPB)	na	na	na	na	na	na	na	na	na	256	150	59	49	19	22	9	na	na

Note: Figures indicated in the Table are based on all the data provided by participants in the Summary Tables included in their respective contributions.
na: Data not available at the time of publication.

RESEARCH REPORTS OF PARTICIPATING COUNTRIES

Increasing the efficiency of embryo culture to promote germplasm collecting in India

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Introduction

Collecting and exchanging coconut germplasm is difficult and expensive because of the bulkiness of the seed. Due to a very short dormancy period, the seeds germinate during transit and cannot be stored even for short periods. Embryo culture techniques provide a more efficient and safe alternative to seed for the movement of coconut germplasm. *In vitro* culture protocols for coconut zygotic embryos have been established by various coconut research institutions for different cultivars. To improve/upgrade these existing protocols for mature (11 months after fertilization) and immature (9 months after fertilization) embryos and to obtain high survival rates, the International Coconut Embryo Culture and Acclimatization Workshop was conducted at the Philippine Coconut Authority (PCA), Philippines from 27 to 31 October 1997.

Objectives

Based on the recommendations of the workshop, experiments were initiated at the Central Plantation Crops Research Institute (CPCRI), Kasaragod with the following objectives:

1. To compare the efficacy of four embryo culture protocols (Annexes 1.1–1.4): PCA, Philippines; UPLB, Philippines; CPCRI, India; and IRD, France; and to study the genotypic effect on *in vitro* embryo culture;
2. To study the effect of growth hormones (GA_3 and ABA) on embryo maturation and subsequent germination; and
3. To study the effect of osmotica (mannitol and polyethylene glycol) and of proline on embryo maturation and subsequent germination.

Six experiments were conducted at CPCRI to study:

1. Response of cultivars to *in vitro* culture protocols;
2. Effect of GA_3 on embryo maturation and germination;
3. Effect of ABA on embryo maturation and germination;
4. Effect of mannitol on embryo maturation and germination;
5. Effect of proline on embryo maturation and germination; and
6. Effect of polyethylene glycol on embryo maturation and germination.

Materials and methods

Comparative experiments of the four embryo culture protocols were initiated in October 1998. These involved two varieties each of the tall and dwarf genotypes. The test embryos of West Coast Tall (WCT) and Laccadine Ordinary (LCT) were harvested from CPCRI, while the embryos from Chowghat Orange Dwarf (COD) and Malayan Yellow Dwarf (MYD) were taken from the International Coconut Genebank-South Asia (ICG-SA), Kidu.

On the other hand, the experiments on the effects of growth regulators (GA_3 and ABA) and osmotica were started in March 1999. They were carried out using both mature (11-month old) and immature (9-month old) WCT embryos, also from CPCRI.

Culture conditions

Inoculated embryos of different genotypes were incubated in the dark at 27± 2°C, under 85% RH. Upon germination, cultures were transferred to a 16h light/8h dark photoperiod.

Four media were used for germination- three were liquid and one (CPCRI) was solid. Agar at 5.5 g/L was added to the CPCRI medium for gelling. Whatman No. 1 filter paper bridges were immersed in liquid medium to support the embryos. Sub-culturing was done at 30–45 days intervals. Observations including weight of the embryos, numbers of leaves, length of shoot and roots as well as abnormalities were recorded at every subculture.

Physiological parameters

Gas exchange parameters were measured immediately after taking out plantlets from the culture tubes and just before their transfer in pots for acclimatization. This was done under culture room conditions at high humidity level, using a portable photosynthesis measurement system (ADC LCA 4, UK). The PAR incidence on leaflet surface was 55±2 $mol.m^{-2}.s^{-1}$, boundary layer resistance to H_2O was 0.2 $mol.m^2.s^{-1}$ and the input air relative humidity was 80% at 22°C. At least ten observations were recorded in each treatment. Standard procedures of statistical analysis were followed using the SPSS software.

Experimental details

A. Protocol and variety interaction

There were 16 treatment combinations tested: four protocols (PCA and UPLB, Philippines; CPCRI, India; and IRD, France) and four cultivars (MYD, LCT, COD and WCT) with three replications using 20 embryos per treatment.

B. Effect of growth hormones on germination and survival

1) Effect of GA_3

Eight treatment combinations of GA_3 (1.0, 0.5, 0.1 and 0.05 µM) were evaluated using mature (11-month old) and immature (9-month old) embryos, with 20 embryos per treatment.

2) Effect of ABA

Three levels of ABA (10, 20 and 30 µM) were compared using mature and immature embryos, with 20 embryos per treatment.

C. Effect of osmotica on maturation and germination of zygotic embryos

1) Effect of mannitol

Three levels of mannitol at 0.2, 0.3 and. 0.4 M were tested using mature and immature embryos, with 20 embryos per treatment.

2) Effect of proline

Two levels of proline, 10 and 20 µM, were tested using mature and immature embryos, with 20 embryos per treatment.

3) Effect of polyethylene glycol (PEG)

Four levels of polyethylene glycol (PEG) at 0.5%, 1%, 2% and 3% were tested with 10 mature and 10 immature embryos. A control treatment (without PEG) was also used for comparison.

All the above experiments were replicated three times.

Experimental Results

A. Interactions between protocol and variety

Forty-five (45) days after inoculation

Swelling of the embryos and protrusion of the plumule were observed after 15–20 days in the dwarf cultivars and after 35–45 days in the tall varieties. Browning of the embryos was noted in cultures on IRD medium (Plate 1). This might be due to the high concentration of salts in the medium (MS) compared to the Y3 medium. The germination percentages, 45 days after inoculation, are presented on Table 1. Germination and initial growth were faster in liquid medium. Dwarf varieties germinated faster and the highest germination percentage was noted in the CPCRI medium. Results of this comparative study showed that Eeuwens Y3 medium was more efficient for *in vitro* culture of coconut embryo.

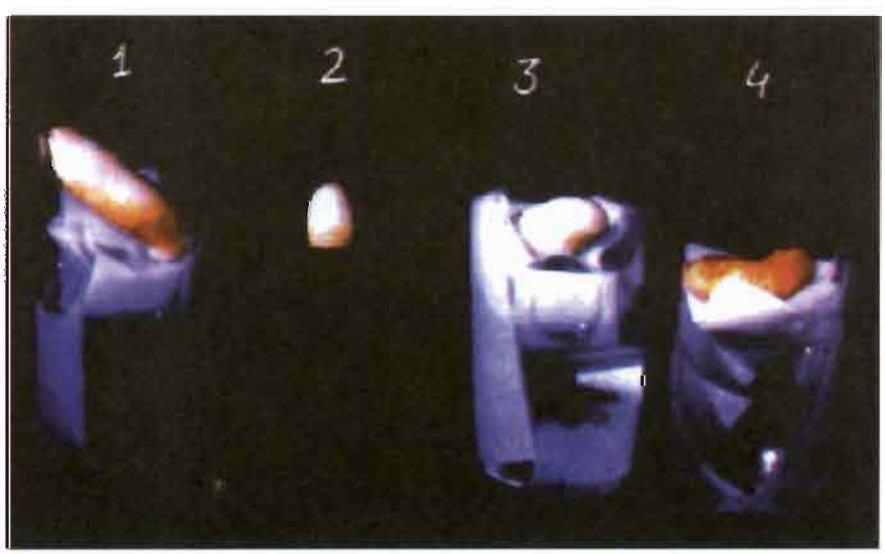

Plate 1. Germination of LCT embryos 30 days after culture with the four protocols tested (1-PCA, Philippines; 2-CPCRI, India; 3-UPLB, Philippines; 4-IRD, France). Note the browning of embryos with the IRD protocol.

Genotypic variation was noted in these experiments. Additional results from CPCRI, Kasaragod also showed genotypic variation between cultivars collected from the Indian Ocean Islands. Pemba Orange and Pemba Yellow cultivars were very sensitive to *in vitro* culture. Around 70% of the Pemba Orange and 90% of the Pemba Yellow plantlets exhibited necrosis *in vitro* or during *ex vitro* establishment. Other accessions collected from the Indian Ocean Islands also exhibited necrosis but with a lower percentage.

A potting mixture of sand and soil in equal proportions was found to be necessary for *ex vitro* establishment of Pemba Orange plantlets. Better recovery of plantlets would have been achieved if embryos were initially cultured on solid medium with the shoot apex upwards before culturing them in a liquid medium.

Sixty (60) days after inoculation

The germination percentage of embryos of tall varieties increased 45–60 days after inoculation (Table 1). No significant difference in germination was observed among treatments after 60 days. All protocols gave an average germination rate of between 85% and 89%. The differences among cultivars and the cultivar x protocol interaction were significant. Maximal germination was observed in MYD (96.7%) and the lowest in WCT (76.7%). Germination for COD was 90.8% and LCT 85.8%. The response of MYD was comparable with all protocols.

Table 1. Effect of culture protocol employed on the germination of embryos of the four cultivars tested

Cultivar	Germination (%) 45 days after inoculation					Germination (%) 60 days after inoculation				
	CPCRI	UPLB	PCA	IRD	Mean	CPCRI	UPLB	PCA	IRD	Mean
LCT	16.66	36.20	57.62	42.27	38.19	71.66	90.00	91.87	90.00	85.88
WCT	15.00	42.59	43.33	37.28	34.55	78.33	70.00	81.67	76.66	76.66
COD	86.66	36.66	63.33	63.33	62.5	93.33	91.67	86.67	91.67	90.83
MYD	96.33	88.33	98.33	80.00	90.75	98.33	96.66	95.00	96.67	96.66
Mean	53.66	50.94	65.65	55.72		85.41	87.1	88.80	88.75	

CD (5%) for comparison of germination at 60 days after inoculation = 9.81; CV = 6.30%

Characterization of *in vitro* growth

The data on weight of embryos at 60, 120 and 200 days after inoculation, on the length of shoots 120 and 200 days after inoculation, and on the length of roots 200 days after inoculation were subjected to the Multivariate tests for repeated observations as well as to univariate split block. The univariate ANOVA revealed differences between protocols in weight of embryos (Table 2), but the former multivariate test revealed only significant cultivar x time interaction. The protocol x time interaction was highly significant for the linear trend. No three-way interaction was observed. The lowest weight gain of embryos was found with the CPCRI protocol. It might be due to the solid state of the medium. Maximum weight gain was noted with the UPLB, IRD and PCA protocols, respectively. All the varieties showed low weight increase with the CPCRI protocol. Weight of the embryos was higher with liquid than solid medium. There was no cultivar x time interaction for weight gain. Plate 2 shows the comparison of growth of LCT embryos on the four media tested two months after inoculation.

Table 2. Mean weight of embryos of the four varieties tested after culture with the four protocols at various durations

Protocol / Duration	Variety / Weight(g)				Mean
	LCT	COD	WCT	MYD	
CPCRI					
60 days	0.49	0.25	0.53	0.33	0.40
120 days	0.85	0.65	0.95	0.93	0.85
200 days	1.21	0.72	1.72	1.24	1.22
UPLB					
60 days	1.02	0.79	0.98	0.86	0.91
120 days	1.96	1.70	1.84	1.79	1.82
200 days	3.39	3.25	2.87	3.33	3.21
PCA					
60 days	1.19	0.78	0.94	0.62	0.88
120 days	2.48	1.48	1.92	1.41	1.82
200 days	4.25	3.79	2.78	2.40	3.31
IRD					
60 days	1.27	0.77	1.07	0.66	0.94
120 days	2.56	1.63	1.85	1.17	1.80
200 days	4.22	3.10	3.08	2.33	3.18

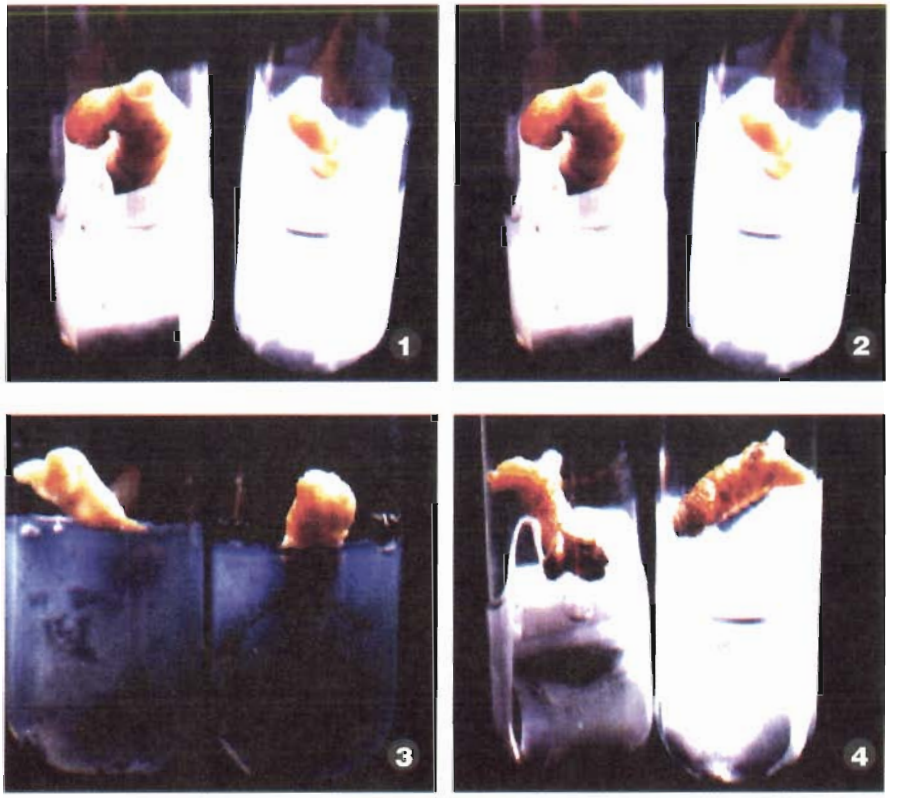

Plate 2. Germination of LCT embryos 60 days after culture with the four protocols tested (1-PCA, Philippines; 2-UPLB, Philippines; 3-CPCRI, India; 4-IRD, France). Note browning of embryos with the IRD protocol.

The percentage of plants having both a shoot and roots four months after inoculation is shown in Table 3. Root formation was lowest with the CPCRI protocol and highest with the IRD protocol. CPCRI recommends supplementation with IBA at 5 mg/L and NAA at 1 mg/L in the root induction medium. The CPCRI plants were sub-cultured on this medium. Plate 3 shows the growth of plants before transfer to root induction medium while Plate 4 shows the plants seven months after inoculation.

Table 3. Effect of culture protocol on the percentage of plantlets of the four varieties tested with shoot and roots formed 120 days after culture initiation

Cultivar	Protocol							
	CPCRI		UPLB		PCA		IRD	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
LGT	76.66	20.00	68.33	68.33	88.30	80.00	81.16	73.33
WCT	58.33	31.66	63.33	51.66	66.66	40.00	71.66	60.00
COD	70.00	23.33	73.33	53.38	81.66	51.66	81.66	68.33
MYD	95.00	28.33	91.66	65.00	85.00	46.66	90.00	81.66
Mean	74.99	25.83	74.16	59.59	80.41	54.58	81.12	70.80

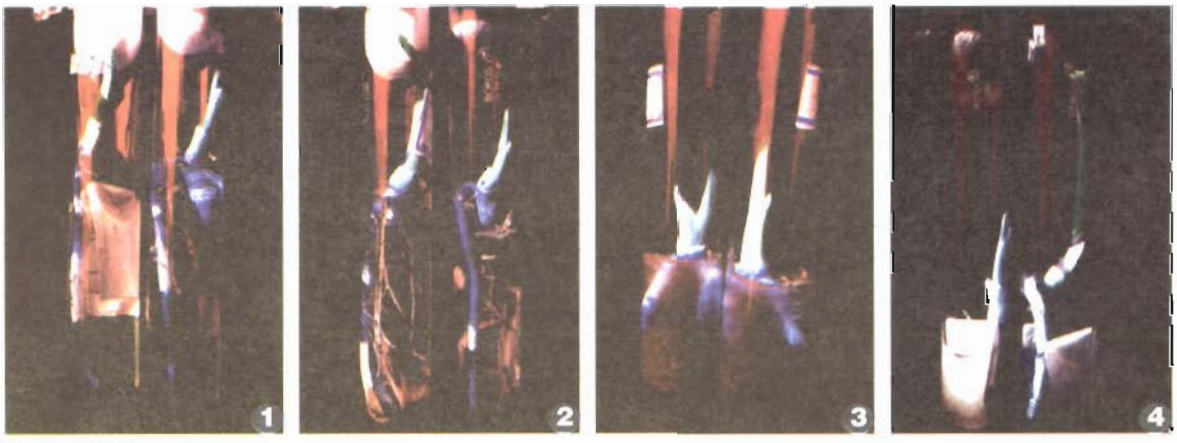

Plate 3. Plantlets produced from LCT embryos four months after culture with the four protocols tested (1-PCA, Philippines; 2-UPLB, Philippines; 3-CPCRI, India; and 4-IRD, France). Note the longer roots produced with the PCA and UPLB media.

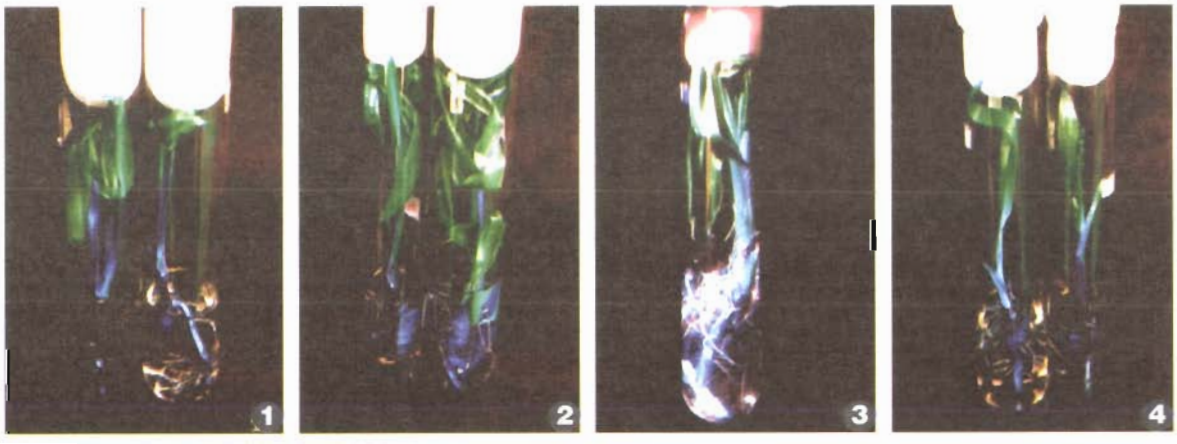

Plate 4. Plantlets produced from LCT embryos seven months after culture with the four protocols tested (1-PCA, Philippines; 2-UPLB, Philippines; 3-CPCRI, India [transferred from solid to liquid medium]; and 4-IRD, France).

Hyperhydricity

Hyperhydricity was noted only in liquid medium (Plate 5). The percentage of hyperhydric plantlets is presented in Table 4. No abnormalities were noted on solid or CPCRI medium.

Plate 5. Abnormalities observed during *in vitro* culture of embryos (1-MYD embryo in IRD medium; 2-MYD embryo in PCA medium; 3-COD embryo in UPLB medium; and 4-COD embryo in IRD medium). Hyperhydricity was noted only in the liquid medium.

Table 4. Percentage of hyperhydric plantlets among the four varieties on the four culture media tested, 60 days after inoculation

Variety	Protocols				
	CPCRI	UPLB	PCA	IRD	Mean
LCT	0.00	1.66	8.33	10.00	4.99
WCT	0.00	3.33	13.33	11.66	7.08
COD	0.00	30.00	46.66	21.66	24.58
MYD	0.00	18.33	20.00	10.00	12.08
Mean	0.00	13.33	22.08	13.33	

Ex vitro studies

A total of 90 plantlets from the various treatments were planted in pots. As in the CPCRI protocol, the plantlets were treated with 1 g/L Bavistin/Carbendazim and the roots were immersed in a solution of IBA (1000 ppm) for one hour. At the time of transfer, data on shoot length, number and dimensions of leaves, root length, number of roots, collar girth and root volume were collected. Significant differences among protocols were noted with respect to plant height, volume of roots, and length and width of leaves (Table 5). No significant differences were noted among cultivars and there was no protocol x cultivar interaction.

Table 5. Average measurements of plantlets produced with the four protocols tested at the time of transfer to pots

Protocol	Plant height (cm)	Root volume (cm^3)	Leaf length (cm)	Leaf width (cm)
CPCRI	20.54	4.18	14.27	1.68
IRD	18.50	2.78	12.54	1.47
PCA	23.73	5.11	16.50	1.93
UPLB	21.15	4.37	13.91	1.72

Several physiological parameters of acclimatization including gas exchange, chlorophyll content and several other biochemical parameters were also recorded. This study is in progress.

Transplanting shock

The potted plants exhibited transplanting shock within two weeks following transfer. Plants produced according to the UPLB and IRD protocols experienced 66% and 50% loss, respectively (Plate 6). This might be due to the higher concentration of sucrose and mineral salts of the basal medium.

Plate 6. Plantlets exhibiting transplanting shock two weeks after transfer to pots.

In vitro and *ex vitro* plantlet physiology

The net photosynthetic rate (Pn) of plantlets, measured immediately after they were taken out of the culture tubes, was negative in all four cultivars with the four culture media tested (Table 6). Transpiration rates (E) were similar in plantlets of all the four cultivars. However, stomatal conductance (gs) was higher in MYD compared to other cultivars. Stomatal conductance was less than 0.01 $mol.m^{-2}.s^{-1}$ for the other cultivars. The type of medium used for culturing plantlets influenced seedling photosynthetic rate (Table 7). Feedback inhibition of photosynthesis due to the presence of sugars in the culture medium was lowest in plantlets grown on UPLB medium and highest in those raised on the PCA medium. Transpiration rate and stomatal conductance were higher in plantlets raised on UPLB medium, while a lower transpiration rate was observed in plantlets grown on PCA medium.

Table 6. Variation in gas exchange parameters among plantlets of the four varieties tested

Cultivars	Pn ($\mu mol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	Gs ($mol.m^{-2}.s^{-1}$)
WCT	-0.258 ± 0.145	0.106 ± 0.024	<0.01
LCT	-0.252 ± 0.081	0.115 ± 0.023	<0.01
MYD	-0.306 ± 0.168	0.127 ± 0.023	0.010 ± 0.001
COD	-0.228 ± 0.088	0.100 ± 0.027	<0.01

Pn = Net photosynthetic rates
E = Transpiration rates
Gs = Stomatal conductance

Table 7. Influence of culture medium on the gas exchange parameters of plantlets of the four cultivars tested

Medium	Pn ($\mu mol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	Gs ($mol.m^{-2}.s^{-1}$)
UPLB	–0.024 ± 0.006	0.152 ± 0.009	0.0156 ± 0.0026
PCA	–0.536 ± 0.104	0.062 ± 0.016	<0.01
IRD	–0.228 ± 0.034	0.119 ± 0.013	<0.01
CPCRI	–0.256 ± 0.041	0.115 ± 0.017	<0.01

Pn = Net photosynthetic rates
E = Transpiration rates
Gs = Stomatal conductance

It was found that the highest percentage of surviving plantlets was obtained with the PCA protocol, irrespective of the cultivar, followed by those with the CPCRI and UPLB protocols. The highest plantlet mortality both *in vitro* and *ex vitro* was obtained with the IRD protocol. Overall results showed a marked improvement over those obtained before the initiation of the project (Table 8). This is evidenced by the high survival rates of plantlets with the different protocols (Table 9).

Table 8. Summary of results achieved with *in vitro* culture of coconut embryos before the implementation of the project as of March 2000

Own							Indigenous						Exotic					
Sl. No.	Variety	Age of embryos	No. of embryos collected	Germination (%)	Contamination (%)	Survival in field (%)	Acc. No.	Variety	No. of embryos	Germination (%)	Contamination (%)	Survival in field (%)	Acc. No.	Variety	Embryos collected	Germination (%)	Contamination (%)	Survival in field
1	West Coast Tall (WCT)	8	26	84.6	3.36	58.2	10	Bora Bora Tall	17	58.2	0.0	23.52	1	Pemba Orange	134	65.38	3.0	–
		9	30	70	3.39	60.0	12	Niu Ui	15	100	0.0	26.66	2	Pemba Green	113	69.04	8.8	1.8
		11	30	70	3.39	60.0	16	Rangiroa Tiputa Tall	9	77.7	0.0	33.33	3	Pemba Yellow	25	71.42	0.0	–
2	Chowghat Orange Dwarf (COD)	11	240	70	3.3	61.25	17	Nikkara	10	100	1	10	4	Pemba Red Tall	29	70.83	3.4	6.9
							19	Rangiroa Avatoru Tall	19	100	0.0	0	5	Dupays Tall	66	53.33	0.0	–
							22	Niu Hako	13	100	2	36.9	6	Gulle Rose	60	83.33	5.0	10.0
											23.1		7	Sambava Tall	68	87.7	0.0	8.8
													8	West African Tall	150	70.37	0.0	1.3
													9	Sambava Green	111	75.23	2.7	6.3
													10	Comorose Tall	110	75.75	0.0	4.5
													11	Coco Lerin	116	80.55	0.8	–
													12	Coco Lehaut	149	80.55	7.4	–
													13	Coco Bule	51	88.6	5.9	25.5
													14	Coco Rasin	63	62.23	11.1	3.2
													15	Coco Gra	23	68.7	8.7	4.3

Recently, 46 plantlets from the first set of experiments were planted at ICGB-SA Kidu.

Table 9. Summary of results achieved with *in vitro* culture of coconut embryos using the four protocols tested during the project

Sl. No.	Protocol	Cultivar	Embryos inoculated	Embryos germinated (60 days) (%)	Embryos contaminated (%)	*In vitro* plantlets		At time of transfer to pots			No. of plants potted (%)	No. of plantlets established	Mean survival in pot
						Shoot (%)	Root (%)	No. of leaves	Collar girth (cm)	Root vol. (ml)			
1	PCA Philippines	LCT	60	91.67	5.00	88.3	80.00	2.14	3.7	6.07	47	39	
		WCT	60	81.67	6.66	66.66	40.00	2.2	3.1	5.2	40	55	
		COD	60	86.67	1.66	81.66	51.66	3.25	3.5	6.0	31	27	
		MYD	60	95.00	15.00	85.00	46.66	4.0	3.3	6.15	36	29	
		Mean		88.75	7.10	80.41	54.58	2.89	3.38	5.86	38	32.5	58.33
2.	UPLB Philippines	LCT	60	90.00	6.66	68.33	68.33	2.1	3.03	4.38	37	23	
		WCT	60	70.00	6.66	63.33	51.66	2.2	2.93	4.74	41	32	
		COD	60	91.67	8.33	73.33	53.38	3.0	3.46	6.0	35	31	
		MYD	60	96.66	6.66	91.66	65.00	3.3	3.03	4.45	34	29	
		Mean		87.08	7.10	74.16	59.59	2.65	3.11	4.39	37	28.75	47.90
3.	CPCRI, India	LCT		71.66	3.33	76.66	20.00	2.3	2.93	4.16	40	33	
		WCT	60	78.33	3.33	58.33	31.66	1.3	2.70	4.50	48	38	
		COD	60	93.33	6.66	70.00	23.33	3.0	3.00	4.00	42	36	
		MYD	60	98.33	3.33	95.00	28.33	2.66	2.70	4.83	39	23	
		Mean		85.41	4.16	74.99	25.83	2.32	2.83	4.37	41	32.5	54.16
4.	IRD, France	LCT	60	90.00	10.00	81.16	73.33	2.62	3.70	2.62	28	21	
		WCT	60	76.66	8.23	71.66	60.00	2.16	3.35	4.66	32	18	
		COD	60	91.67	8.33	81.66	68.33	2.25	3.25	5.87	39	23	
		MYD	60	96.67	5.00	90.00	81.66	3.32	3.16	2.83	36	28	
		Mean		88.75	7.91	81.12	70.80	2.59	3.37	3.99	32.5	22.5	37.50

B. Effect of growth hormones

Effect of GA_3

A total of 480 embryos (240 each of mature and immature) were cultured on media with 0.05, 0.1, 0.5 and 1.0µM GA_3. During the first phase of the experiment (90 days), the percentage of germination and growth parameters including shoot and root length after 45 days and 70 days in culture were recorded. It was observed that embryos germinated with all levels of GA_3 tested, irrespective of their maturity stage (Plate 7 and Table 10). Therefore, addition of GA_3 in the culture medium had no effect on the maturation of immature embryos. The analysis of variance revealed that growth of immature embryos was significantly higher than that of mature ones. However, the relative growth of mature and immature embryos with different levels of GA_3 was not uniform at the 70th day in culture and a significant maturity x GA_3 interaction was observed (Table 11). Immature embryos showed maximum growth on the 70th day with the medium supplemented with the highest level of GA_3. Growth of mature embryos was relatively lower but better with 0.5 and 1.0µM GA_3 (Table 10).

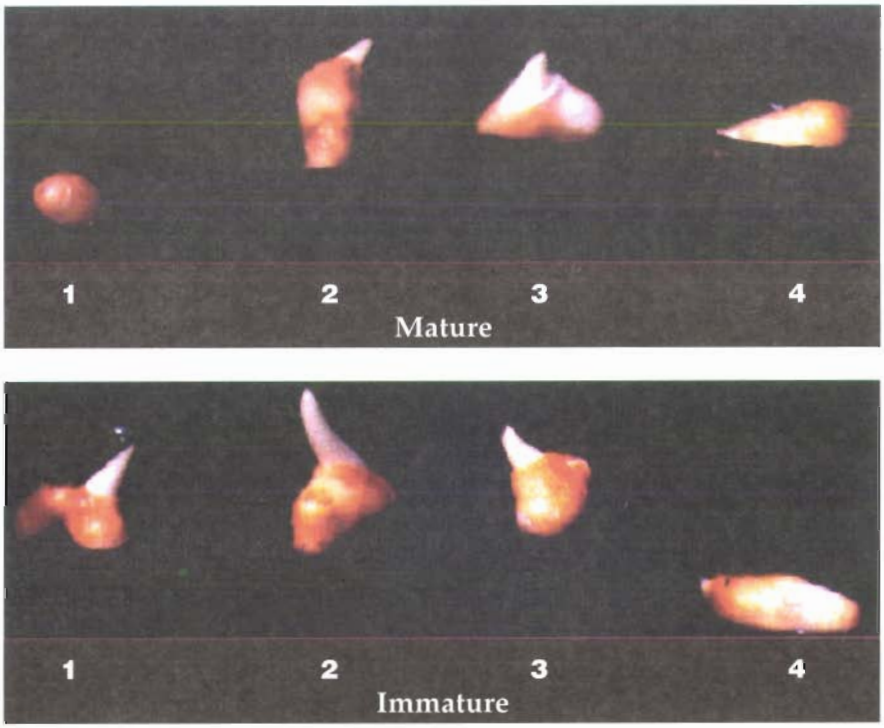

Plate 7. Effect of various GA_3 concentrations ([1] 1 µM, [2] 0.5 µM, [3] 0.1 µM and [4] 0.05 µM) on the germination of mature and immature WCT embryos 45 days after inoculation.

Table 10. Effect of GA3 on the germination of mature (M) and immature (IM) embryos, and on the length of roots and shoot of plantlets after 45 and 70 days in culture

GA^3 (µM)	Germination (%)		Shoot length (cm)				Root length (cm)			
			45th day		70th day		45th day		70th day	
	M	IM	M	IM	M	IM	M	IM	M	IM
1.00	70.00	73.30	0.19	0.15	0.40	0.62	0.11	0.14	0.33	0.61
0.50	51.70	61.00	0.20	0.16	0.35	0.54	0.15	0.23	0.30	0.38
0.10	83.30	75.80	0.23	0.35	0.27	0.53	0.14	0.27	0.15	0.24
0.05	62.50	58.10	0.15	0.19	0.26	0.35	0.08	0.11	0.07	0.07

Table 11. Summary of ANOVA showing the levels of significance for the various growth parameters studied

Source of Variation	Shoot length		Root length	
	45th day	70th day	45th day	70th day
Maturity	0.046	0.048	0.001	0.000
GA3 level	0.071	0.018	0.383	0.066
Maturity x GA3	0.232	0.028	0.466	0.040

After 90 days on medium containing GA_3, cultures were transferred to retrieval medium without GA_3. The weight gained by the cultures was recorded at the time of transfer. The length of shoot and roots at the 120th and 150th days in culture were also recorded. The level of GA_3 in the medium, the maturity of embryos and their interaction had a significant effect on the weight gain of embryos. The weight gain of immature embryos was equal at all levels of GA_3 except at the lowest level (data not shown). In the case of mature embryos, maximum weight gain was achieved with 0.1µM GA_3. There was no significant difference in the growth characters of embryos, which had been cultured with different levels of GA_3. Comparisons were also made after removing the effect of initial growth (analysis of covariance with length of shoots and roots at 70th day as covariates). Again, no significant difference was observed among the different treatments.

Effect of ABA

The overall growth of cultures during the first 90 days with medium supplemented with different levels of ABA was lower, compared to that of embryos grown with GA_3 in the previous experiment. Since root growth was negligible in the initial phase of the experiment, only the length of shoots was recorded after 60 days. Growth in retrieval medium was evaluated by measuring the length of shoots and roots 40 days after transfer to retrieval medium. The log-linear analysis of the number of embryos germinated revealed a significant effect of ABA concentration (Fig. 1). Concentration of ABA at 10–20 µM increased germination of immature embryos, contrary to its

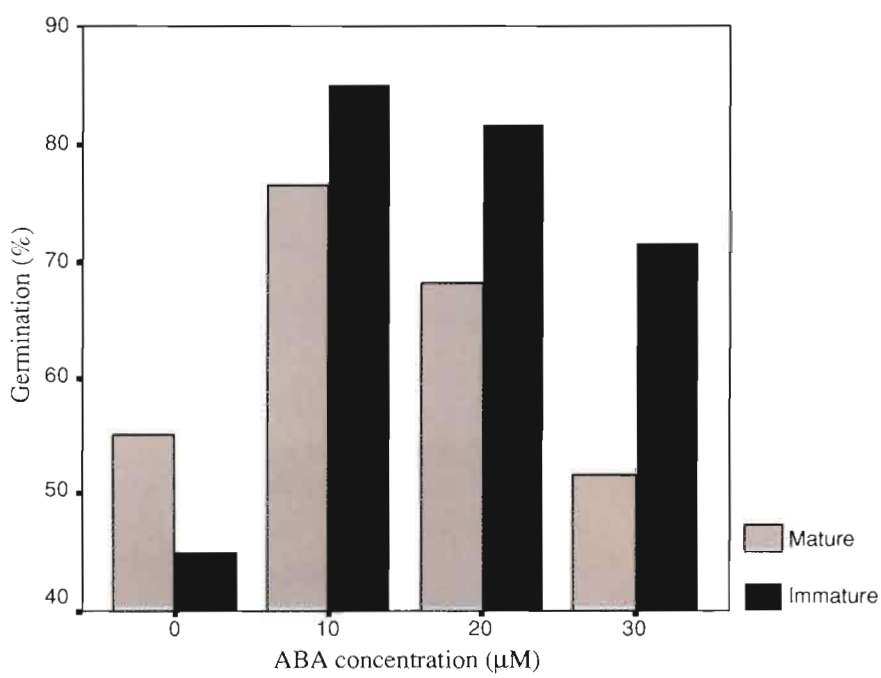

Fig.1. Germination of mature and immature embryos on media with different concentrations of ABA.

expected inhibitory effect. Significant differences in shoot length 60 days after inoculation were noted (Table 12). All three concentrations of ABA tested increased germination for immature embryos. A significant interaction between maturity of embryos and levels of ABA was also observed. The addition of ABA in the medium contributed to an increase in weight of both mature and immature embryos.

Table 12. Summary of ANOVA showing levels of significance for the various morphological characters studied

Source of variation	Shoot length (60^{th} day)	Weight gain (90^{th} day)	Root length (130^{th} day)	Shoot length (130^{th} day)
Maturity	0.599	0.597	0.708	0.785
ABA level	0.087	0.054	0.030	0.264
Maturity x ABA	0.045	0.244	0.012	0.086
CV %	33.83	19.44	42.81	39.12

For all parameters measured, the ABA concentration of 30 µM produced the lowest values (Table 13) among the ABA treatment levels. Growth of immature embryos treated with 10µM ABA was better than those with other concentrations. The poor growth and germination of embryos cultured on media with the highest ABA concentration suggest that further studies on using higher levels of ABA to induce quiescence of embryos still need to be done.

Table 13. Effect of ABA on the growth of mature (M) and immature (IM) embryos

ABA concentration (µM)	Length (cm) of shoot (60^{th} day)		Weight gained (g) (90^{th} day)	Length of shoot (cm) (130^{th} day)		Length of roots (cm) (130^{th} day)	
	M	IM	*	M	IM	M	IM
0	0.37	0.13	0.39	1.27	0.34	1.50	**
10	0.46	0.88	0.42	1.03	1.60	0.17	0.93
20	0.42	0.62	0.42	1.10	1.35	1.02	0.49
30	0.34	0.45	0.31	1.03	0.95	0.57	0.43

* Mean weight gain for immature and mature embryos.

** No root growth observed.

Comparison of treatments after removing the effect of initial growth (analysis of covariance with the length of shoot and root after 60 days as covariates) showed no significant maturity x ABA interaction for length of shoot after 130 days. This suggested that incorporation of ABA in the initial culture medium had no adverse effect on the growth and development of plantlets in the retrieval medium (Plate 8)

Plate 8. Effect of ABA concentrations (10, 20 and 30 µM) on the germination and maturation of mature and immature WCT embryos after 45 days of culture.

ABA and GA_3 levels were tested independently for their effect on germination and growth of coconut embryos. Further studies are needed to observe any effect of these growth hormones employed in combination and at various concentrations. The effect of a combination of growth hormones and osmotica (i.e. mannitol, polyethylene glycol and praline) should also be investigated.

C. Effect of osmotica on zygotic embryos

Effect of mannitol

Mature and immature embryos were exposed to three mannitol concentrations, 0.2, 0.3 and 0.4M. After 90 days in culture, no embryos germinated except the controls (Plate 9).

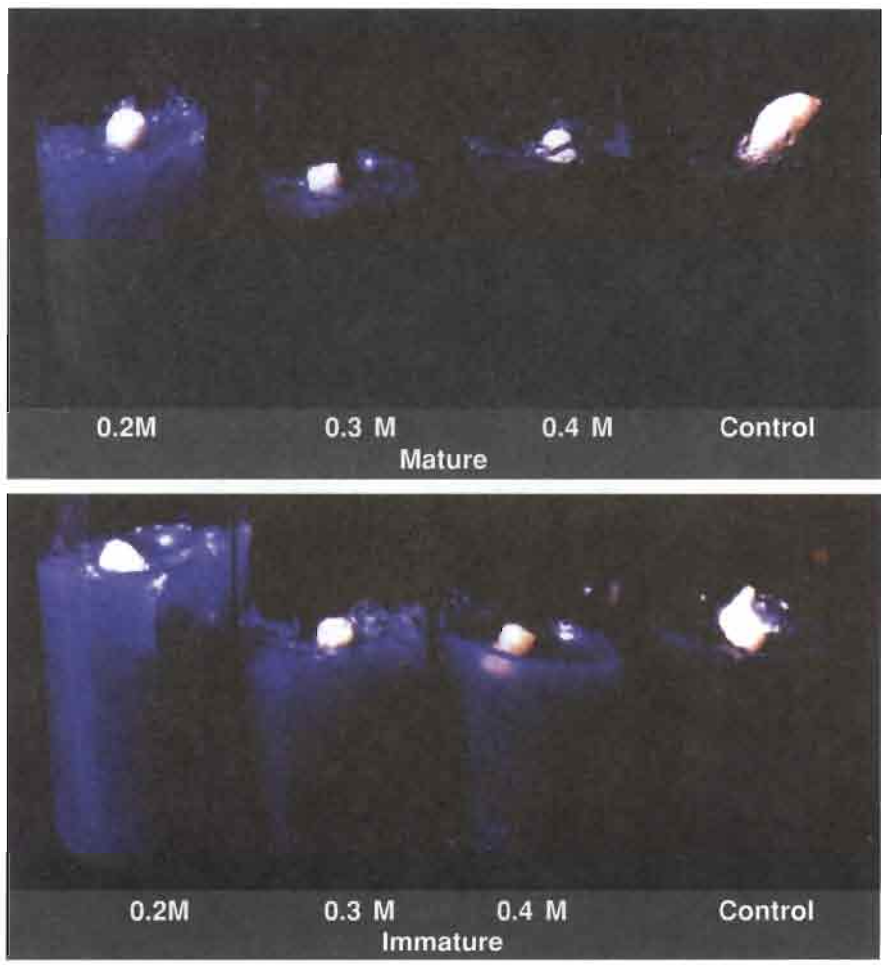

Plate 9. Effect of mannitol concentration on the germination of mature (top) and immature (bottom) embryos of variety WCT, 45 days after inoculation.

However, upon transfer to retrieval medium, some of the embryos treated with mannitol germinated (Table 14). Germination of both immature and mature embryos decreased in line with increasing mannitol concentrations. Increasing the mannitol concentration resulted in a decrease in the number of hyperhydrous immature embryos, whereas no marked effect was noted on hyperhydricity of mature embryos. Severe browning was also noted in immature embryos treated with mannitol (Fig. 2).

Table 14. Effect of mannitol on the germination and hyperhydricity of mature and immature embryos after 90 days of culture

Mannitol concentration	Germination (%)		Hyperhydricity (%)	
	Mature	Immature	Mature	Immature
Control	100.0	100.0	0.0	10.0
0.2 M	60.0	48.3	15.0	20.0
0.3 M	48.3	28.3	25.0	10.0
0.4 M	20.0	1.7	20.0	6.7

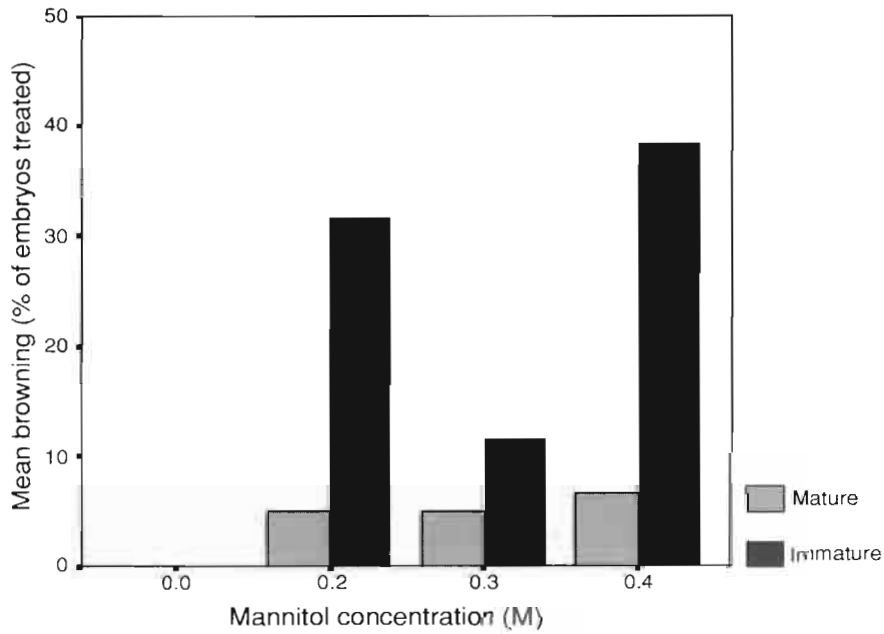

Fig. 2. Effect of mannitol concentration (M) on the browning of mature and immature embryos in the initial culture medium.

Effect of proline

Two proline concentrations (10 and 20 µM) were tested to determine their effect on maturation and subsequent germination of embryos. Germination and browning of embryos were recorded 30 days after inoculation. Germination percentage at the time of transfer to retrieval medium (after 90 days in proline medium) is shown in Table 15. No significant differences in germination percentage between the two proline concentrations tested were noted (Plate 10). However, germination of mature and immature embryos was significantly different. Plant growth varied between mature and immature embryos with significant differences noted in several parameters (Table 16). With mature embryos, significant differences among the praline concentration levels were noted for shoot length and fresh weight of plantlets (Plate 11 and Table 17). The increase in shoot length and fresh weight of plantlets was observed to be proportionate to the increase in proline concentration.

Table 15. Effect of proline on the germination of mature and immature embryos on retrieval medium after 90 days of culture

Maturity stage of embryos	Proline concentration (µM) 0	10	20	Mean
Mature	96	93	93	94
Immature	100	100	98	99

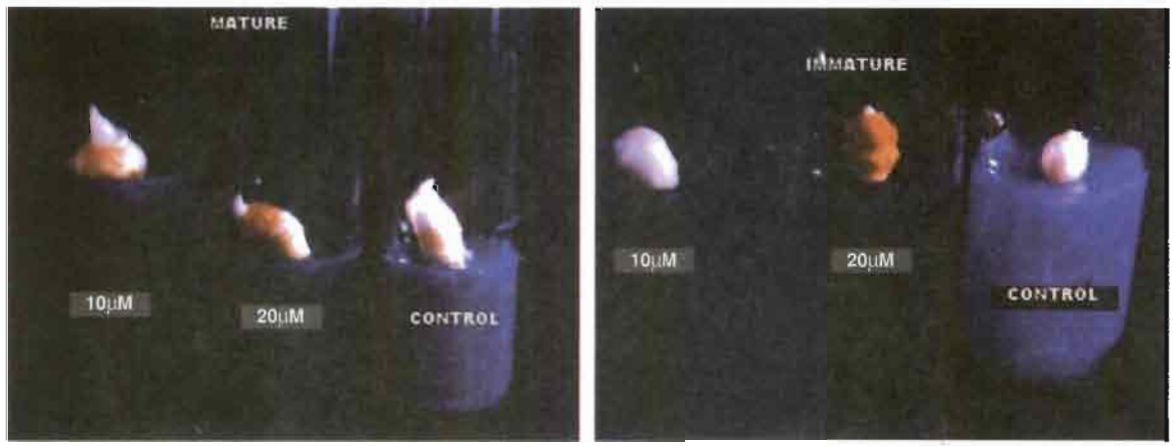

Plate 10. Effect of proline concentration (0, 10, 20µM) on germination of mature and immature embryos of cultivar WCT after 45 days of culture.

Table 16. Data showing significant differences between embryos at different maturity stages after 90 days of culture on medium with proline

Maturity stage of embryos	Abnormality (%)	Root length (cm)	Shoot length (cm)	Weight (g)
Immature	9.0	0.86	1.84	0.47
Mature	2.0	1.40	2.34	0.93

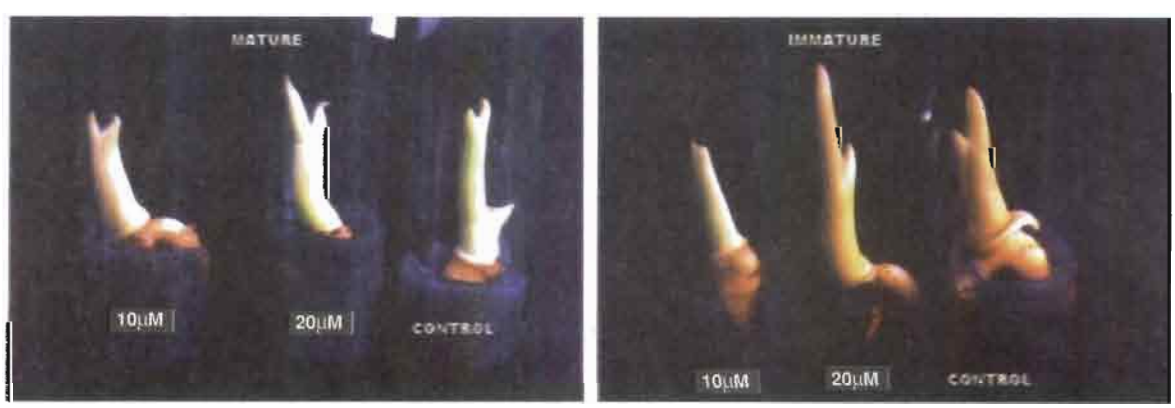

Plate 11. Effect of proline concentration (0, 10, 20 µM) on germination of mature and immature embryos of cultivar WCT after 45 days of culture on retrieval medium.

Table 17. Growth parameter differences among mature embryos cultured for 90 days on medium treated with different proline concentrations

Parameter measured	Proline concentration (µM)		
	0	10	20
Shoot length (cm)	1.51	2.17	2.58
Weight (g)	0.61	0.66	0.84

Effect of polyethylene glycol (PEG)

Mature and immature embryos were subjected to five PEG (0, 0.5, 1, 2 and 3%). Germination was observed in all four treatments (Plate 12). The germination percentages at the time of transfer (after 90 days in PEG medium) on retrieval medium are shown on Table 18. Hyperhydricity was higher in mature embryos (Plate 13) and increased in line with increasing PEG concentrations (Table 18).

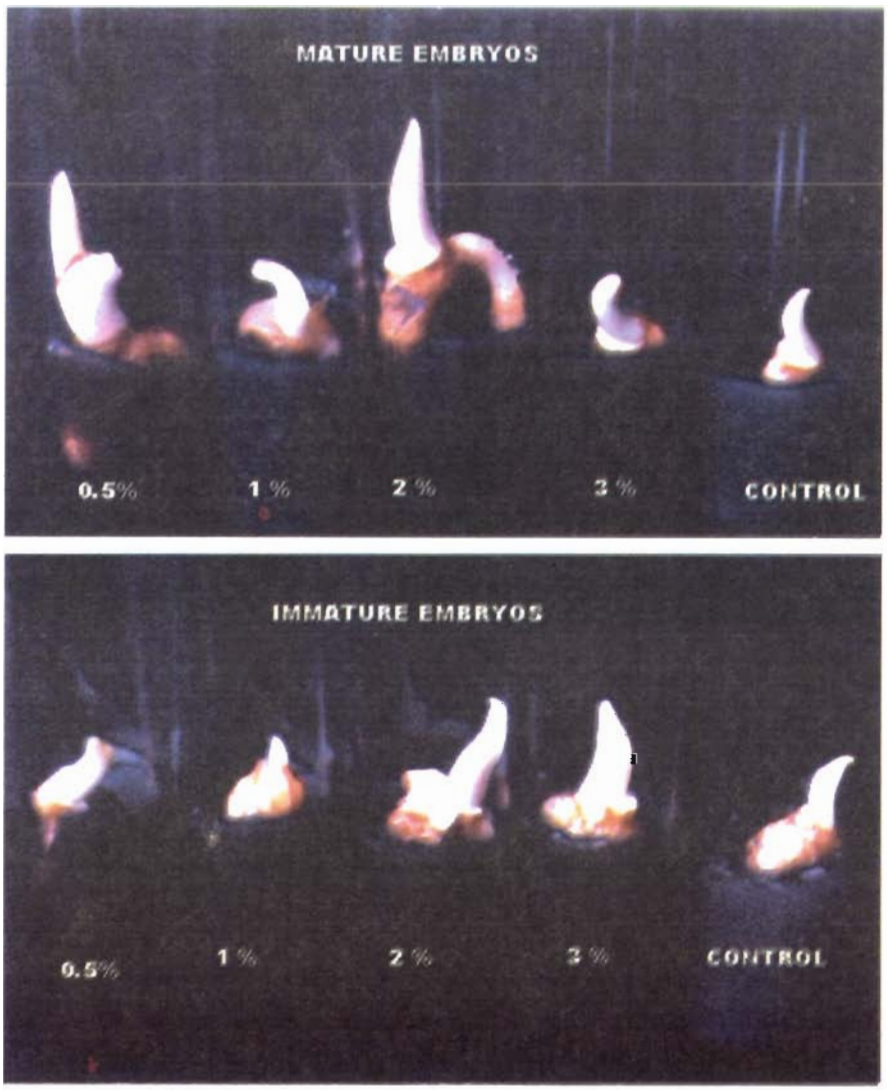

Plate 12. Effect of different PEG concentrations (0, 0.5, 1.0, 2.0, 3.0%) on germination of mature and immature embryos of WCT cultivar after 45 days of culture.

Table 18. Effect of different PEG concentrations on the germination and on the occurrence of hyperhydricity in mature and immature embryos

PEG concentration (%)	Germination (%) (after 90 days in PEG medium)		Hyperhydricity (%)	
	Mature	Immature	Mature	Immature
0	80.0	40.0	13.3	0.0
0.5	76.7	50.0	36.7	0.0
1.0	83.3	56.7	30.0	16.7
2	83.3	56.7	73.3	20.0
3	80.0	40.0	70.0	10.0

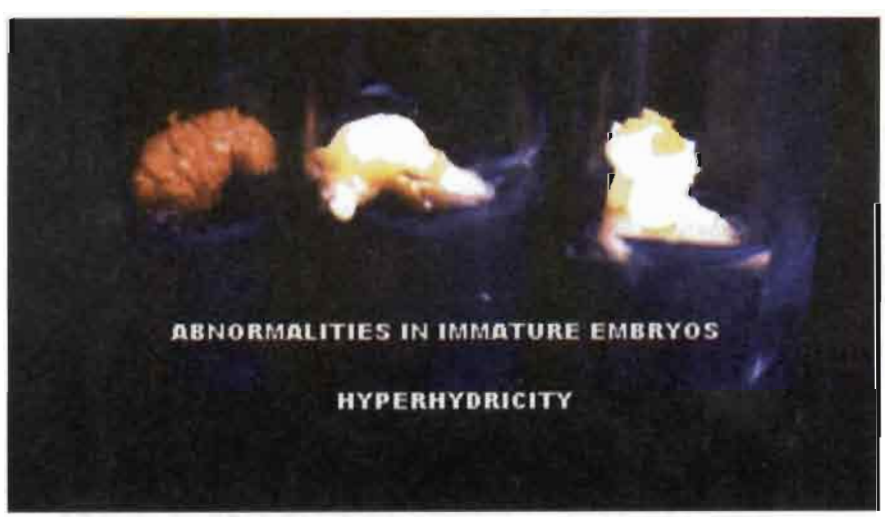

Plate 13. Hyperhydric mature and immature embryos observed during the experiment on the effect of PEG concentration.

Hierarchical log-linear models revealed that only the age of embryos was found to be significant with regard to germination. The interaction between the PEG concentration and the maturity of embryos was not significant. On the other hand, the multivariate test on shoot length and plantlet weight revealed significant differences among the tested PEG concentrations as well as between mature and immature embryos (Table 19).

Table 19. Effect of different PEG concentrations on the shoot length (cm) of mature and immature embryos and on corresponding fresh weight (g) of plantlets produced

PEG concentration	Shoot length (cm)		Plantlet weight (g)	
(%)	Immature	Mature	Immature	Mature
0	1.03	0.63	0.41	0.50
0.5	1.01	0.67	0.42	0.47
0.1	1.24	1.04	0.37	0.80
2.0	1.03	0.84	0.38	0.55
3.0	0.59	0.61	0.26	0.73

Conclusion

At CPCRI, India, six experiments were conducted to compare four embryo culture protocols (UPLB, Philippines; PCA, Philippines; CPCRI, India; and IRD, France) and to study the effect of two growth regulators and osmotica on embryo germination and culture of four cultivars: West Coast Tall (WCT); Laccadive Ordinary (LCT); Chowghat Orange Dwarf (COD); and Malayan Yellow Dwarf (MYD).

Findings showed that interactions between the protocols employed and the development rate of embryos were highly significant. Among the four protocols tested, the CPCRI protocol resulted in slow germination and plantlet growth as compared to the other three protocols. Hyperhydricity of embryos were noted only in liquid medium. Transplanting shock was higher with plantlets produced with the UPLB and IRD protocols. It was also found out that plantlet survival rate was highest with the PCA protocol, irrespective of the cultivar, followed by those with the CPCRI and UPLB protocols. On the other hand, plantlet mortality, *in vitro* and *ex vitro*, was highest with the IRD protocol.

Five other experiments were conducted to study the effect of growth hormones and osmotica on the maturation and germination of mature and immature embryos. Different concentrations of GA_3, ABA, mannitol, PEG and proline were used as treatments. The studies show that there was no interaction between the GA_3 concentration and culture duration on shoot length. Growth of shoots depended largely on the maturity of embryos. However, for root development, there was a significant interaction between maturity and GA_3 concentration. It was also observed that there is a significant interaction between maturity of embryos and the ABA concentration for initial shoot length (60 days) and root development (120 days). Initial observations with mannitol showed that all the three levels of mannitol tested inhibited germination. The interaction between mannitol level and embryo maturity significantly affected hyperhydricity in both mature and immature embryos. PEG and proline did not affect germination.

Growth of plantlets was also found to be influenced by the age of the embryos. Significant differences among levels of proline were observed for shoot length and weight gain. An increase in proline concentration resulted in a proportionate increase in shoot length and weight. The multivariate tests showed significant differences among PEG levels as well as between mature and immature embryos. Shoot length significantly varied with PEG levels. Significant differences in weight increase were noted between embryos at different maturity levels.

The physiological parameters of acclimatization (i.e. net photosynthetic rates, stomata conductance and transpiration rates) were measured at the time of transfer of plants to pots. Net photosynthetic rates were negative in all four cultivars raised with the four media. Stomata conductance was generally less than 0.01 $mol.m^{-2}.s^{-1}$. Transpiration rates varied significantly with medium. Transpiration rates and stomatal conductance were higher in seedlings raised on UPLB medium, whereas those raised on PCA medium had lower transpiration rates.

Constraints

Funds allocated for this project were limited (US$5,000) and biochemical studies could not be carried out under this scheme. However, plant tissues for future biochemical studies were preserved in buffer solutions.

Future plans

Experiments aimed at testing higher concentrations of ABA (40-60μM) and other gibberellic acids such as GA_{4-7} have been initiated to define the optimal concentrations of these growth regulators in inducing the development of coconut embryos.

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Increasing the efficiency of embryo culture technology to promote coconut germplasm collecting and exchange in Sri Lanka

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Introduction

The Coconut Research Institute of Sri Lanka (CRISL) provides technical assistance to coconut farmers regarding improved varieties, viable agronomic practices, crop protection strategies, the use of fertilizer and other related subjects. Despite the inherent constraints to the breeding of palm species, CRISL has achieved significant progress in crop improvement using conventional breeding techniques. Two improved varieties, the CRIC 60 and the CRIC 65, have been produced through the hybridization of locally available germplasm.

Since 1950, Sri Lanka has recognized the importance of introducing coconut germplasm from other countries in supplementing its coconut diversity. However, importing germplasm could inadvertently bring in new pests and diseases as germplasm exchange procedures between countries have yet to be laid down. In this regard, embryo culture technology is a very valuable tool for the efficient collecting, exchanging and conserving of germplasm. It could be successfully employed to eliminate diseased stocks and cut down transport costs as well. Furthermore, this technology would facilitate the rescue of embryos of the non-germinating but economically important coconut cultivar such as the Dikiri. However, techniques used for *in vitro* culture of embryos and *ex vitro* hardening of resulting plants would have to be optimized in order to achieve maximum recovery of embryos for field establishment. To this end, the embryo culture protocol presently used at CRISL will have to be validated and refined in order to achieve the highest possible success rate.

Objectives

The study aims to:

1. Validate selected protocols and improve the protocol presently used at the CRISL;
2. Evaluate the effect of added substrates on efficiency of weaning; and
3. Evaluate the effect of ABA and/or osmoticum and GA_3 on the maturation and germination of embryos.

Activities

A. Preliminary studies: Comparison of Y3 and 2Y3 media

Since 1985, the 2Y3 medium (modified Eeuwens Y3 liquid medium at double strength with 0.25 % activated charcoal and 6% sucrose) has been used at CRISL for mature zygotic embryo culture of coconut. As most of the participating countries under the DFID-funded embryo culture project used the Eeuwens Y3 medium at single strength, it was decided to test the performance of embryos cultured in 2Y3 (modified Eeuwens Y3 liquid medium at single strength with 0.25% activated charcoal and 6% sucrose)

and the Y3 media. This was done as a preliminary study prior to testing selected embryo culture protocols from different laboratories.

The experiment was replicated six times using mature embryos of the cultivar Sri Lanka Tall (SLT), with fifteen embryos in each treatment. Embryo germination, plant vigour at the time of soil establishment and *ex vitro* survival of plants raised in the two media were evaluated.

B. Validation of selected embryo culture protocols

1. Comparison of four different embryo culture protocols

An experiment was conducted to compare the embryo culture media used at CPCRI, India and UPLB, Philippines with the Y3 and 2Y3 media used at CRISL, Sri Lanka and to select the best protocol for application. The composition of each of the four media is given in Appendix 1.

a) SLT cultivar

The experiment used mature embryos of the cultivar SLT used and was replicated four times, with 25 embryos per treatment. The germination and subsequent growth of embryos and *ex vitro* survival of plants raised in the four different media were evaluated.

b) Dwarf cultivar

The use of MYD for testing the above four protocols (as mentioned in the original proposal) was not possible as MYD was not available in Sri Lanka. Therefore, permission was obtained to use the cultivar Sri Lanka Green Dwarf (SLGD) instead of MYD. The evaluation of three protocols (Y3CRI, UPLB and CPCRI) using SLGD was initiated in October 1999. The experiment was replicated twice with 25 embryos per treatment. The germination and subsequent growth rate of embryos in each medium were recorded.

2. Testing of the best protocol with Dikiri

Based on the results of the comparison of four different embryo protocols, Y3CRI was selected as the best protocol for application. A total of 111 Dikiri embryos were cultured in the Y3CRI medium and the germination, subsequent growth of embryos and *ex vitro* survival of plants were recorded.

C. Addition of substrates to promote root development

An experiment, using several rooting substrates in the growth medium, was initiated to test the feasibility of improving the survival rate of *in vitro*-cultured seedlings. Three different rooting substrates (coir fiber, vermiculite and absorbent cotton wool) were added to the growth medium (2Y3CRI) at the final stage of *in vitro* culture. The media were tested against the control (without any rooting substrate). Fifteen SLT embryos were allocated for each treatment without any replicate.

D. Effect of gibberellic acid (GA_3) on the germination of embryos

1. Study on the effect of GA_3 on embryo germination

Based on the results of the experiment comparing the four culture protocols, it was evident that the Y3CRI medium (liquid) should be used as the basal medium for these GA_3 tests. Three levels of GA_3 (0.046, 0.23, 0.46 µM) were tested against the control. Sterilization of GA_3 was done by co-autoclaving it with the other media components. The embryos were cultured in media containing different levels of

GA_3 and after four weeks, they were transferred to regular Y3CRI medium without GA_3. The germination of embryos in the different treatments was evaluated.

a) SLT cultivar
Mature embryos of the SLT cultivar were used and the experiment was replicated four times. In two replicates, 50 embryos were allocated for each treatment but in the other two replicates the number of embryos per treatment was 45 and 28.

b) Dikiri
Due to the scarcity of Dikiri nuts, the experiment was replicated only twice. The number of embryos allocated for each treatment in the two replicates was 15 and 11.

2. Mode of sterilization of GA_3
For the above experiments, GA_3 was autoclaved. In order to test whether the mode of sterilization would affect the activity of GA_3a preliminary experiment was conducted using filter-sterilized GA_3.

a) Experiment I (preliminary)
Due to the scarcity of Dikiri nuts, ungerminated Dikiri embryos from previous experiments were used with only two levels of GA_3 (0.046 and 0.23 µM). Twenty embryos were used for each of the two treatments without replicates. Germination of embryos was recorded after four weeks in culture. Since only a few embryos germinated in the media containing 0.046 µM GA_3, the remaining ungerminated embryos were transferred to the media containing 0.23 µM GA_3. Germination was recorded after another four weeks.

b) Experiment II
Since the results of the preliminary experiment indicated that filter-sterilized GA_3 at the concentration of 0.23 µM had a positive effect on embryo germination, the present experiment was set up to test whether the germination of Dikiri embryos could be further improved by increasing the concentration of GA_3. Thus, two levels of GA_3 (0.23 and 0.35 µM) were tested against the control (without any GA_3). Twenty-five embryos were allocated for each treatment without any replicates. The germination of embryos was recorded after four weeks. The embryos that germinated were transferred to regular Y3CRI medium without any GA_3 while the ungerminated ones were treated with the same level of GA_3 until germination.

E. Effect of abscisic acid (ABA) on maturation of embryos

An experiment was conducted to study the effect of ABA on the maturation of embryos cultured *in vitro*. Immature SLT nuts (9 and 10 months old) were used to test three levels of ABA (5, 10, 20 µM) against the control (without any ABA). Filter-sterilized ABA was incorporated into the Y3CRI medium (both solid and liquid) and immature embryos were cultured in the different treatments. Twenty-five embryos were allocated for each treatment. The experiment was repeated with 30 embryos per treatment. Most of the embryos cultured on solid medium enlarged rapidly and attained the size of mature embryos faster. With some embryos, this occurred within three days of culturing on the ABA medium. Subsequently, all these embryos were transferred to the Y3CRI liquid medium without ABA. The enlargement of embryos cultured in the liquid medium was not as rapid as that in the solid medium. When the embryos matured, they were transferred to the Y3CRI liquid medium with no ABA. The effect of each treatment on germination of embryos was evaluated.

Materials and methods

Cultivars used

Sri Lanka Tall (SLT), Sri Lanka Green Dwarf (SLGD) and Dikiri

Sterilization and culturing of embryos

The embryos, excised from the kernel, were sterilized in 3% calcium hypochlorite for five minutes and then rinsed in sterile distilled water several times. The embryos were then cultured in 10 ml growth medium in 30 X 200 mm size glass test tubes sealed with cotton wool plugs. The culture media were sterilized by autoclaving at 121°C for 15 minutes.

Culture conditions

The cultures were incubated in the dark during the first 10 weeks and then transferred to light (16 h light/8 h dark photoperiod; 10.5 µM. m^{-2}. s^{-1} light intensity [PAR]). The incubation temperature was 30 ± 1°C.

Acclimatization

The procedure used for acclimatization of *in vitro*- raised plants is given in Appendix 2.

Statistical Design

The Randomized Complete Block Design (RCBD) was used for all the experiments.

Data collected

- Percentage germination of embryos
- Length of shoots
- No. of leaves/*in vitro* plant
- No. of primary roots/*in vitro* plant
- Extent of secondary root growth
- No. of plants transferred to soil
- Rate of *ex vitro* survival

Results and discussion

A. Preliminary Studies

Comparison of Y3 and 2Y3 media

As shown in Table 1, germination of the SLT cultivar embryos in the Y3 medium was significantly higher than that in the 2Y3 medium. Furthermore, the vigour of plants grown in the Y3 medium, at the time of transplanting in soil, was significantly higher than the plants grown in the 2Y3 medium. The survival of the plants during acclimatization was also compared. More than 95% of the plants grown on Y3 medium survived, while only 60% of the seedlings grown in 2Y3 medium survived *ex vitro*. These results clearly indicated that the Y3 medium was superior to the 2Y3 medium for the culture of mature zygotic embryo of coconut. The other important aspect was that the cost for the production of embryo-cultured plants could be greatly reduced by the use of Y3 medium, as the minerals, vitamins and other growth factors were

at single strength when compared to those in the 2Y3 medium. As a result of profuse root growth, the plants raised in the Y3 medium could be transplanted within 5–6 months. Plants raised in the 2Y3 medium were ready for transplanting only after 7–9 months. Thus, *in vitro* growth period can be shortened with the use of Y3 medium.

Table 1. Effect of two different culture media on the germination and *in vitro* growth of coconut zygotic embryos

Culture medium	Germination length (%)	Shoot area (cm)	Total leaf primary roots (cm^2)	No. of per plant	Extent of secondary root growth
Y3	84.7	31.2	94.5	3.0	5.7
2Y3	65.8	24.8	64.8	2.1	4.5
Significance	*	**	*	**	**
CV%	15.2	11.7	42.0	13.5	10.1

* P = 0.05
** P = 0.01

B. Validation of selected embryo culture protocols

Comparison of four different embryo culture protocols

SLT cultivar

In this experiment, two culture media developed at CRISL (Y3CRI and 2Y3CRI) were tested. The results of the preliminary studies (Table 1), however, had already indicated that the Y3CRI medium was better for growth of *in vitro* cultured embryos and *ex vitro* survival of plants. This was further confirmed by the results of the present study. Therefore, when discussing the results of the present study, comparisons will be made only among Y3CRI, UPLB and CPCRI media.

Germination of embryos

As summarized in Table 2, no significant difference was observed in the germination rate of embryos cultured in the different media. However, it took longer for embryos to germinate on the CPCRI medium than on the other two media. This may be due to the solid state of the CPCRI medium. As nutrients are not so efficiently absorbed from solid medium as they are from liquid medium, the embryos cultured on liquid medium will take longer to germinate. Another factor which might have affected the rate of germination was the amount of activated charcoal present in the medium. The content of charcoal in the Y3CRI and UPLB media (0.25%) is higher than that present in the CPCRI medium (0.1%). Activated charcoal enhances embryo growth as it absorbs toxic compounds from the medium and the cultured tissues. This might have contributed to the more rapid growth of embryos in these two liquid media. However, the initial slow growth of embryos on CPCRI medium did not affect the overall growth of *in vitro* plants.

Ex vitro *survival*

No significant difference in the germination, *in vitro* growth and *ex vitro* survival of the embryos was found among the Y3CRI, CPCRI and UPLB media (Table 2). *Ex vitro* survival of plants grown in all three media was very high. Even though the plants raised in Y3CRI medium had better root growth than those in the other two media, this difference was not reflected in the *ex vitro* survival of the plantlets. Thus, in terms of *ex vitro* survival, the three media appeared to be equally good.

Table 2. Effect of four different culture media on the germination, *in vitro* growth and *ex vitro* survival of SLT embryos

Protocol	Germination (%)	No. of leaves/ *in vitro* plant	No. of roots/ *in vitro* plant	Plants potted (%)	*Ex vitro* survival of plants (%)
Y3CRI	86.00[a]	2.08[a]	3.00[a]	66.80[a]	98.00[a]
2Y3CRI	84.00[a]	2.18[a]	2.53[b]	68.64[a]	60.75[b]
UPLB	82.20[a]	1.98[a]	2.53[b]	66.84[a]	93.75[a]
CPCRI	73.00[a]	1.80[a]	2.20[b]	71.54[a]	98.25[a]
CV%	14.57	9.79	12.00	18.64	9.20

* Within a column, mean values followed by the same letter were not significantly different at the 5% significance level.

Root and shoot growth

One month after culture initiation, emergence of the radicle was observed in 76% of the embryos cultured in the Y3CRI medium, whereas in the UPLB and CPCRI media it was only 64% and 23%, respectively (Table 3). At the end of the second month, the percentage of embryos showing emergence of radicle was comparable in the UPLB and Y3CRI media but was lower in the CPCRI medium. Shoot emergence was faster in the Y3CRI medium than in the other two media, though two months after culture the percentage of embryos with emerging shoots was high, even in the other two media.

Table 3. Rate of shoot and root growth of embryos germinated in three different culture media

Protocol	Embryos with emergence of radicle (%)		Embryos with shoot emergence (%)		Embryos with secondary root formation (%)		Rooted plants with at least 1 leaf (%)
	after 1 month	after 2 months	after 1 months	after 2 months	after 2 months	after 3 months	after 4 months
Y3CRI	76.0	90.0	46.3	97.5	51.0	81.0	64.4
UPLB	64.0	91.5	6.3	83.5	40.0	75.0	50.0
CPCRI	23.2	53.6	2.9	75.4	19.4	53.7	45.5

In the Y3CRI and UPLB media, secondary root growth was observed in the embryos during the second and third month of culture initiation. Secondary root growth was slower in the CPCRI medium. However, towards the end of the fourth month, the shoot and root growth of the plants were comparable in the three media, with the percentage of rooted plants with at least one leaf being almost similar in all the three media.

No significant difference was observed among the three media in terms of the number of leaves/*in vitro* plant at soil establishment. However, the number of primary roots/*in vitro* plant was significantly higher in the Y3CRI medium than in the other two media (Table 2).

It was noted that the growth rate of embryos was faster in theY3CRI medium than in the other two media during the initial period of culture. This could be due to the differences in culture medium composition as the Y3CRI medium was rich in amino acids like asparagine, arginine and glutamine which were absent in the other two media (Appendix 1). Furthermore, the concentration of certain vitamins (pyridoxine, thiamine and nicotinic acid) was higher in the Y3CRI medium.

SLGD cultivar

Germination of embryos

Results showed germination of SLGD embryos in the CPCRI medium seemed slow (Table 4). Even after five weeks, none of the embryos had germinated in the CPCRI medium. After twelve weeks in culture, the germination percentage was observed to be much higher with the UPLB medium than with the other two media.

Table 4. Percentage germination of SLGD embryos in three different media after five and twelve weeks in culture

Protocol	% Germination after 5 weeks	% Germination after 12 weeks
Y3CRI	22.0	36.6
UPLB	48.8	81.8
CPCRI	0.0	36.4

As shown in Table 4, germination of embryos in both Y3CRI and CPCRI media was poor. The percentage of embryo germination of the two cultivars, SLT and SLGD in the UPLB medium was found to be similar. On the other hand, the germination of SLGD embryos cultured in Y3CRI and CPCRI media was much lower than SLT (Tables 2 and 4).

Shoot and root growth

It was difficult to make any accurate comparison of the performance of embryos cultured in the Y3CRI medium with that in the other two media since a substantial number of embryos cultured in the Y3CRI medium were contaminated. Observations, therefore, were made using a few clean embryos. However, the data available indicated that root growth was better in Y3CRI medium than in the other two media (Table 5). Shoot growth in the CPCRI medium was better than in the Y3CRI medium. Even though germination was poor, the overall growth rate of embryos was higher in Y3CRI medium.

Shoot growth of embryos was much higher in the CPCRI medium than in the UPLB medium. The rate and extent of root growth was low in both the CPCRI and UPLB media. However, the number of primary roots per plant was slightly higher in the UPLB medium than in the CPCRI medium.

Table 5. Shoot and root growth of SLGD embryos in three different culture media

Protocol	Shoot length (cm)	No. of leaves/ *in vitro* plant	No. of roots/ *in vitro* plant
Y3CRI	16.6*	1.6*	2.0*
UPLB	5.2	0.7	1.3
CPCRI	21.7	1.8	0.86

* 67% of the germinated embryos in Y3CRI medium were contaminated; therefore, observations were made using only a few "clean" embryos.

Selection of the best protocol

During the first few months of culture, there was a higher growth rate seen in embryos cultured in the Y3CRI medium than in the other two media. Furthermore, the number of primary roots/*in vitro* plant was also significantly higher in the Y3CRI medium. Because of these two factors, the Y3CRI medium was selected as the basal medium for subsequent experiments. It should be noted, however, that in terms of percentage of germination of embryos, percentage of plants potted and *ex vitro* survival, all three media were found to be equally effective.

Testing of the best protocol with Dikiri

As summarized in Table 6, the growth of Dikiri embryos in Y3CRI medium was found to be satisfactory. When the varieties SLT and Dikiri were compared, the germination percentage of Dikiri embryos in Y3CRI medium was lower than that of SLT (Tables 2 and 6). Attempts were made to improve the germination percentage of embryos. As shown in the results of subsequent experiments, germination of Dikiri embryos could be improved by treatment with GA_3.

The percentage of plants potted was higher with the Dikiri than with the SLT but *ex vitro* survival of plants was observed to be higher with SLT than with Dikiri (Tables 2 and 6).

Table 6. Effect of Y3CRI medium on germination of Dikiri embryos, their *in vitro* growth and *ex vitro* survival

Germination	65.8 %
Number of leaves/*in vitro* plant	1.8
Number of roots/*in vitro* plant	2.5
Plants potted	78.1%
Ex vitro survival rate of plants	86.0%

C. Addition of substrates to promote root development

In the preliminary tests, the addition of the three rooting substrates had no effect on embryo root formation. As indicated by earlier results, root growth, plant vigour at weaning and *ex vitro* survival of plants grown in Y3CRI, UPLB, and CPCRI media were very high. The experiment was discontinued since it was felt that root induction would not be a problem if these media were used in culturing embryos.

D. Effect of GA_3 on germination of embryos

As shown in Tables 7 and 8, no significant difference was observed in the germination of both SLT and Dikiri embryos in the four treatments tested. Even though the difference was not statistically significant, germination percentage of embryos (of both varieties) treated with 0.46 µM GA_3 was markedly higher than that in the other treatments. The positive effect of GA_3 at this concentration might have been masked by the high coefficient of variation (CV: 20.14 and 16.24). One of the reasons for the high CV could be the high palm to palm variation seen in coconut. Individual embryos could vary greatly in their vigour. Due to the scarcity of the Dikiri nuts, only a few embryos were allocated for each treatment, which might also have contributed to the high CV value.

Table 7. Effect of different concentrations of autoclaved GA_3 on the germination of mature embryos of SLT cultured *in vitro*

Protocol	Concentration of GA_3 (µM)	Germination (%)
Y3CRI	0.00	60.13
"	0.046	63.57
"	0.23	55.99
"	0.46	73.86
Significance		ns
CV (%)		20.14

Table 8. Effect of different concentrations of autoclaved GA_3 on the germination of Dikiri embryos

Protocol	Concentration of GA_3 (μM)	Germination (%)
Y3CRI	0.0	65.1
Y3CRI	0.046	58.3
Y3CRI	0.23	37.9
Y3CRI	0.46	70.0
Significance		ns
CV (%)		16.24

For the above experiments, GA_3 was co-autoclaved along with the other medium components. This might have lowered the GA_3 activity due to partial degradation during autoclaving. Therefore, higher levels of autoclave-sterilized GA_3 should be tested in subsequent experiments. This view is further supported by the results obtained from the experiment where filter-sterilized GA_3 was tested with Dikiri embryos. The results indicated that germination of Dikiri embryos was considerably increased when treated with filter-sterilized GA_3 at 0.23 μM (Table 9). Furthermore, germination of embryos was very rapid in this treatment as most of the embryos germinated within two to three weeks of culture initiation. Thus, it indicated that the mode of sterilization of GA_3 was also important in defining effective levels of GA_3 for enhancing embryo germination.

Table 9. Effect of two concentrations of filter-sterilized GA3 on the germination of Dikiri embryos

Concentration of GA3 (μM)	Initial number of embryos	Number of embryos germinated	Germination (%)
0.046	20	3	15.0
0.230	37	29	78.4

In the experiment where GA_3 levels were varied up to 0.35M, the germination rate of Dikiri embryos in all treatments was relatively low when compared to the rate observed in the other experiments (Table 10). The exact reason for the poor germination of Dikiri embryos in this experiment is not known. There was a seasonal variation in Dikiri embryo germination in the past and that might have been one of the reasons for the low germination rate observed. However, the germination of embryos treated with 0.23 and 0.35 μM GA_3 was higher than that of the control, indicating the positive effect of GA_3 on embryo germination (Table 10). Of the two concentrations of GA_3 tested, the 0.35 μM concentration was shown to be more effective in promoting germination of embryos. The experiment is being repeated to check whether this effect is consistent.

Table 10. Germination percentage of Dikiri embryos treated with different concentrations of filter-sterilized GA_3

Concentration of GA_3 (μM)	Germination (%)
0.00	27.3
0.23	47.6
0.35	50.0

Further experiments should be carried out to determine the optimal concentration of GA_3 for enhancing embryo germination. As the objective of the above experiments was to study the effect of GA_3 on embryo germination, determining the optimal concentration of GA_3 in the culture medium after germination of embryos was not incorporated. Therefore, further experiments could be conducted to study the effect of GA_3 on subsequent shoot and root growth in germinated embryos.

E. Effect of ABA on maturation of embryos

In the first experiment, a substantial number of SLT embryos in some treatments were contaminated. Hence, observations were based on a few embryos making it difficult to interpret the results and clarify the effect of the different concentrations of ABA on embryo maturation. Results of a second similar experiment revealed that ABA in liquid medium was not effective in promoting embryo maturation or in inducing embryo germination (Table 11). However, when incorporated in a solid medium, ABA enhanced germination of embryos and the positive effect was more pronounced with higher concentrations of ABA (10 and 20 µM). The highest percentage (76.7%) of germination for nine-month old embryos was achieved with 10 µM ABA, whereas 20 µM ABA gave the highest percentage of germination (80%) with ten-month old embryos. The results also indicated that the number of immature embryos cultured on solid medium was higher than that in the liquid medium, even in the control (without ABA).

Another factor, which could affect embryo maturation, is the duration of ABA treatment. In the present study, the embryos were kept in ABA-containing media only for a short period of time (3 days to 2 weeks). Further experiments are necessary to determine the optimal concentration as well as the duration of ABA treatment.

The effect of ABA on maturation of Dikiri embryos could not be studied due to the scarcity of the Dikiri nuts. Since all of the Dikiri nuts collected were used for the experiments on GA_3, ABA experiments on these nuts were not carried out.

Table 11. Germination percentage of SLT embryos (from 9 and 10- month old nuts) treated with different concentrations of ABA

Concentration of ABA (µM)	State of the medium	Germination (%)	
		Maturity of nut	
		9 months	10 months
0	Liquid	3.3	13.3
	Solid	40.0	43.3
5	Liquid	3.3	6.6
	Solid	53.3	56.7
10	Liquid	6.6	6.6
	Solid	76.7	70.0
20	Liquid	16.6	13.3
	Solid	56.7	80.0

Constraints

The scarcity of Dikiri nuts was a major constraint in conducting experiments with this particular cultivar as test material. In a single collecting mission, only a limited number of nuts could be collected as Dikiri palms are restricted to a small region of the country. Thus, very few Dikiri embryos were allocated per treatment. This could have contributed to the high variation observed in some of the experiments.

High palm to palm variation and variation within a bunch is inherent to coconut. This factor also contributed to the high coefficient of variation in some of the experiments.

The high contamination rate in certain experiments was also a problem in evaluating the effects of various treatments. As most of the embryos had to be discarded, the number of embryos available for evaluation was significantly reduced.

Conclusion and recommendations

The project aimed to validate and refine the current embryo culture protocol used at CRISL for future application in germplasm collecting and exchange. The experiments resulted in important findings that could be applied to increase the efficiency of the CRISL protocol.

The embryo culture medium used routinely at CRISL (before commencing the present project) was the 2Y3CRI. The fact that the other participating countries used Eeuwens Y3 medium at single strength led us to the development of Y3CRI medium, which was shown to be much better than the 2Y3CRI medium. With the use of Y3CRI medium, it was possible to improve the *in vitro* growth and *ex vitro* survival of plants.

For the SLT cultivar, no significant differences in the parameters studied (germination percentage, number of leaves per plant, number of plants potted and *ex vitro* survival of plants among the three media) were found. However, root growth and growth rate of embryos were better in Y3CRI medium than in the other two media. Based on these observations, the Y3CRI medium was selected as the best basal medium to be used for subsequent experiments.

The three media (Y3CRI, UPLB and CPCRI) were compared using the SLGD embryos. Results revealed that the germination of embryos was highest in the UPLB medium. However, shoot growth was poor in the UPLB medium, whereas a higher rate of shoot growth was observed in the other two media. Root growth was satisfactory in the Y3CRI medium, while poor rooting was observed in embryos cultured in UPLB and CPCRI media. Tables 12 and 13 present the summary results of the embryo culture studies conducted in Sri Lanka.

Experiments also revealed the positive effect of GA_3 on embryo germination, especially if the GA_3 was filter-sterilized.

Preliminary results on the effect of ABA on embryo maturation indicated that ABA enhances *in vitro* maturation of immature embryos. This finding could lead to the recovery of embryos from any immature nut during germplasm collecting, thereby maximizing variability within populations. In some of the experiments, the effect of certain treatments might have been masked by high contamination and variation. Had it not been for this drawback, more valuable information could have been generated.

Table 12. Summary results of embryo culture experiments of DFID project in Sri Lanka: Comparison of four embryo culture protocols

Protocol	Cultivar	Initial No. of embryos	No. of embryos germinated	No. of leaves/ *in vitro* plant	No. of roots / *in vitro* plant	No. of *in vitro* plants potted	No. of plants established in screen-house
Y3CRI	SLTall	100	86	2.08	3.00	58	57
2Y3CRI	,,	100	84	2.18	2.53	58	34
UPLB	,,	100	80	1.98	2.53	52	49
CPCRI	,,	100	73	1.80	2.20	53	52
Y3CRI	SLGD	50	15	1.6*	2.0*	5*	5*
UPLB	,,	50	36	0.7	1.3	–	–
CPCRI	,,	50	16	1.8	0.86	08	–
Y3CRI	Dikiri	111	73	1.80	2.50	57	49

* 67% of the germinated embryos were discarded due to contamination and observations were made using only a few embryos.

Table 13. Summary results of embryo culture studies at CRISL as of 30 August 2000

Parameters/Cultivars	Culture protocols used			
	Before start of project	UPLB	CPCRI	Own*
Number of embryos inoculated				
1. SLT	> 1000	100	100	100
2. Dikiri	> 1000			111
3. SLGD	–	50	50	50
Contamination (%)				
1. SLT	10–15	23	11	17
2. Dikiri	10–15			5.4
3. SLGD	–	14	12	18
Germination (%)				
1. SLT	60–70	80	73	86
2. Dikiri	40–60			65.8
3. SLGD	–	81.8	36.4	36.6
Whole plants *in vitro* (%)				
1. SLT	45–50	52	53	58
2. Dikiri	40–50			51.4
3. SLGD	–	32	26	10*
Average duration of *in vitro* culture (weeks) to obtain whole plantlets				
1. SLT	28–36	20–28	24–30	20–24
2. Dikiri	28–36			20–24
3. SLGD	–	28–36	28–32	24–28
Survival after transfer to nursery (%)				
1. SLT	35–40	49	52	57
2. Dikiri	35–40			51.4
Survival after transfer to field (%)				
1. SLT	80–90	Field planting	not done	
2. Dikiri	60–70			

* Most of the plants were contaminated; percentage was calculated based on the remaining embryos.

Literature cited

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Ranasinghe, C. S., L. K. Weerakoon, Y. M. H. Liyanage and D. T. Mathes. 1999. Physiological aspects of *in vitro*-grown coconut (*Cocos nucifera* L.) plants during acclimatization. CORD 15(2):46-67.

Appendix 1

Composition of different embryo culture media (mg/l)

Chemical	2Y3CRI	Y3CRI	UPLB	CPCRI
KNO3	4040	2020	2020	2020
KCl	2984	1492	1492	1492
NH4Cl	1070	535	535	535
NaH2PO4.2H2O	624	312	312	312
CaCl2.2H2O	588	294	294	294
MgSO4.7H2O	494	247	247	247
FeSO4.7H2O	27.8	13.9	41.7	13.9
NaEDTA	74.6	37.3	55.8	37.3
MnSO4.4H2O	22.4	11.2	11.2	11.2
KI	16.6	8.3	8.3	8.3
ZnSO4.7H2O	14.4	7.2	7.2	7.2
H3BO3	6.2	3.1	3.1	3.1
CoCl2.6H2O	0.48	0.24	0.24	0.24
Na2MoO4.2H2O	0.48	0.24	0.24	0.24
CuSO4.5H2O	0.32	0.16	0.25	0.16
NiCl2.6H2O	0.048	0.024	0.024	0.024
L-glutamine	200	100	–	–
L-Arginine	200	100	–	–
L-Asparagine.H2O	176	88	–	–
Myo-inositol	200	100	–	100
Pyridoxine.HCl	2.0	1.0	0.05	0.05
Thiamine.HCl	2.0	1.0	0.05	0.5
Nicotinic acid	2.0	1.0	0.5	–
Biotin	–	–	0.05	0.05
Folic acid	–	–	0.05	–
Glycine	–	–	1.0	–
BAP	1.1	1.1	–	0.5
NAA[1]	–	–	–	0.5
NAA[2]	–	–	–	1.0
IBA[2]	–	–	–	5.0
2,4-D	0.02	0.02	–	–
Agar	–	–	7.0g/l	7.0g/l
Sucrose	60g/l	60g/l	60g/l	60g/l
Activated charcoal	2.5g/l	2.5g/l	2.5g/l	1g/l

[1] Germination medium

[2] Rooting medium

Appendix 2

Acclimatization procedure developed at CRISL

1. Remove the plants from the test tubes and rinse with water to wash media off the roots.

1. Drench the plants in a mixture of fungicide (0.025 % Benlate) and diluted nutrient solution for a few hours. Then, transfer the plants to clear polypropylene bags (23 cm X 46 cm) filled with about 10 cm of unsterilized potting medium (river sand and compost, 1:1). Moisten potting medium with a dilute nutrient solution.

2. Cover the plantlets with clear plastic bags to maintain high relative humidity. Keep the plants in a house shaded by a layer of coir mat with a light intensity of PAR 640–750 $\mu M.\ m^{-2}.\ s^{-1}$.

3. After one week, partially cut the plastic bag cover to reduce relative humidity. A week later, cut off the plastic bag cover to fully expose the plants. Water the plants as required.

4. Three weeks after transplanting, cut down the upper part of the polypropylene bags to slightly above the level of the potting soil. Water the plants regularly and apply liquid fertilizer every ten days.

5. After about three to four months (when the plants are about 40–50 cm in height), repot the plants in larger polybags (25 cm dia X 30 cm height) containing a potting mixture of top soil, compost, river sand, dried 'cowdung' and coir dust at 2:1:1:1:1/2 ratio.

6. Keep the plants in the shade house for another month before transferring them to a shed of green mesh with a higher light intensity of PAR 1000–1200 $\mu M.\ m^{-2}.s^{-1}$. After about two months, expose the plants to direct sunlight. Apply liquid fertilizer weekly. When the plants reach a height of about 75 cm (with four to six leaves), outplant them in the field.

Development of an improved embryo culture protocol for coconut in the Philippines

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Background and rationale

In the Philippines, the late Dr. Emerita V. de Guzman of UPLB used White's medium when she started her work on coconut embryo culture to rescue the non-germinating but economically valuable Makapuno cultivar. This variety is characterized by a soft endosperm that almost fills the nut (hence the name "Makapuno" meaning "almost full" in Filipino). There is little or no liquid within the nut. In order to get the true-to-type Makapuno coconut, the embryo of the Makapuno nut has to be cultured *in vitro*. Mature palms from cultured embryos could yield 75 to 95% of Makapuno nuts compared to the "kabuwig" nuts (ordinary nuts growing together with the Makapuno nuts in a bunch). Because of the growing interest in the Makapuno, the original embryo culture technique needs to be improved and adopted for the culture of the Makapuno embryos. Results of studies conducted by Rillo and Paloma in 1990 revealed that coconut embryos grew best in the Y3 medium without hormones. The Y3 medium was developed by Eeuwens (1976) in England for coconut tissue culture. Assy Bah (1986) modified and adopted the MS medium for her coconut embryo culture work in France and Côte d'Ivoire. Subsequently, other laboratories (UPLB and CPCRI) adopted the Y3 medium for growing coconut embryos. At the moment, there are eight government-funded and eight private Makapuno embryo culture laboratories in the Philippines. It is envisioned that Makapuno will diversify and revive the coconut industry, which has long been dependent on copra and its by-products.

The International Coconut Genetic Resources Network (COGENT) is mandated to promote the use of a broader genetic variability via the exchange of seednuts to improve coconut varieties and hybrids. Such an exchange would incur high expense in the shipment of the heavy and bulky nuts. Besides, there is the probability of introducing pest and disease propagules to countries importing the seednuts. To overcome these constraints, it was suggested that the coconut embryo technology which has been recommended in the FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm (Frison *et al.* 1993) be used as a tool for collecting, conserving and exchanging germplasm.

To look into the possibility of developing a working protocol for coconut embryo culture, COGENT organized the "First International Coconut Embryo Culture and Acclimatization Workshop" in the Philippines in October 1997. At the workshop, it was decided that the various coconut embryo culture protocols used in the different laboratories be evaluated, and research gaps identified and addressed. Each of the participating countries submitted a proposal, which might be able to address the identified research gaps. The Philippines was assigned the task of developing an improved coconut embryo culture protocol, possibly a "hybrid" among the best protocols available, for possible adoption by the COGENT-member countries.

Study 1. Comparison of four embryo culture protocols for coconut

Objectives

The study aims:
1. To compare and validate four embryo culture protocols, namely the PCA-ARC, CPCRI, UPLB and IRD protocols; and
2. To develop a "hybrid" embryo culture protocol with the highest possible efficiency.

Materials and methods

Embryo collecting

A study comparing the *in vitro* performance of various coconut cultivars in four culture media (UPLB, CPCRI, IRD and PCA-ARC) was conducted at the Philippine Coconut Authority, Albay Research Center (PCA-ARC). Details of each protocol are provided in Annexes 1.1 to 1.4 with a summary of media composition in Annex 1.5.

Mature embryos of four tall varieties (Laguna, Makapuno, Tagnanan and Rennel Island Tall) and one dwarf variety (Malayan Yellow Dwarf) were collected from the PCA-Zamboanga Research Center (ZRC) and from the Albay Research Center (ARC). The Makapuno embryos were collected from *in vitro* grown Makapuno palms planted at the ARC.

The embryos from the PCA-ZRC used for replicate 1 were disinfected with 100% commercial bleach, rinsed with sterile water, packed in sterile moistened plastic bags and refrigerated overnight before they were flown to ARC. Upon arrival at ARC, the embryos were disinfected following the disinfection procedure for endosperm cores, as described in the PCA-ARC protocol (Annex 1.4).

Embryo culture

The embryos and seedlings that developed were maintained in the laboratory according to the procedures described in each protocol.

Ex vitro establishment

For *ex vitro* plantlet establishment, the protocol developed at the PCA-ARC was adopted for all seedlings produced during the study (Annex 1.4).

Statistical analysis

The experiment was set up using a 5 x 4 factorial experiment involving five varieties and four protocols with three replications. Replicate 1 was initiated in March 1998 while replicates 2 and 3 were initiated in July 1998. The following data were gathered:
- Percentage germination after 1, 2, and 3 months from initial culture;
- Percentage shoot and root formation;
- Percentage root formation;
- Percentage secondary and tertiary root formation;
- Percentage complete seedlings (rooted seedlings with at least one true leaf);
- Number of leaves at weaning time;
- Duration of developmental stage before weaning; and
- Number of successfully grown and weaned seedlings.

Transformed (arcsine) data analyzed for variance and means were compared using the Duncan Multiple Range Test (DMRT).

Results and discussion

Medium

Three protocols (PCA-ARC, UPLB and CPCRI) used the Y3 formulation as a base, with their respective modifications, except for the IRD protocol which used a modified MS medium. The basic differences among the four protocols are presented in Table 1.

Contamination

Results of the four protocols validated showed no significant difference in terms of contamination percentage among PCA-ARC, UPLB, CPCRI and IRD cultures (Table 2). This indicated that the decontamination procedures for these three protocols could possibly be adopted as routine procedures for the culture of coconut embryos. Among the test cultivars, MAKT (Makapuno) exhibited the least contaminated embryos. No significant difference was seen in the interaction between the different protocols and the various cultivars.

Germination

The embryos of cultivars Laguna (LAGT), Rennel Island Tall (RIT), Tagnanan (TAGT), Makapuno (MAKT) and Malayan Yellow Dwarf (MYD) cultured in the four media exhibited varying germination rates one month after initial culture (Table 3). Regardless of the cultivar, embryos cultured in the PCA-ARC medium showed the highest germination percentage one month after inoculation. This was significantly higher than those achieved with the IRD medium. Further incubation of the embryos (second and third months) in their respective media and culture conditions resulted in an increase in germination percentage. The UPLB medium (84%) showed the highest percentage, followed by CPCRI (83.33%) then PCA-ARC and IRD. The higher sugar level, the incorporation of gelling agent in CPCRI and UPLB media and the higher concentration of iron in UPLB medium might have contributed to the increase in germination percentage during the second and third months of culture. Apparently, continuous incubation of cultures in either liquid medium/continuous light or solid medium/continuous darkness during the first four months of culture period might not be beneficial for the embryos and the developing seedlings. Incubation of embryos on solid medium in the dark during the germination stage and in liquid medium under light during the plantlet development stage might have simulated the situation of the embryos during germination *in situ*. Earlier work on coconut embryo culture had shown that germination could be improved by culturing embryos on solid medium. De Guzman *et al.* (1971) observed that after eight weeks in White's liquid medium, Makapuno embryos increased in size but remained ungerminated until after their transfer to solid medium. The low germination percentage noted with the IRD medium could be attributed to an insufficient chlorine concentration in its macronutrient source, compared to the three other protocols using the Y3 formulation. Chlorine proved to be essential for the growth and development of coconut palms.

Regardless of the protocol, there were significant differences among the cultivars. The highest germination percentages on the third month were noted with the LAGT (81.66%) and MAKT (81.25%) embryos.

Shoot and root emergence

Simultaneous shoot and root emergence, noted one month after culture initiation, ranged from 11 to 42% (Table 4). At that time, the highest percentage of cultures with both shoots and roots formation was noted with the PCA-ARC protocol, followed by the UPLB, CPCRI and IRD protocols. During the next three months, the number of cultures with both shoot and roots increased with all protocols. At the end of the fourth month, the highest number of embryos with both shoot and root formed were in the

UPLB medium. Shoot and root emergence of embryos cultured with the PCA-ARC, CPCRI and IRD media were significantly slower compared to those grown in the UPLB medium. The cultivar Makapuno consistently exhibited the most number of cultures with both emerging shoot and root, followed closely by the MYD and LAGT. There was no significant interaction between protocols and cultivars.

Root and leaf formation

Regardless of the cultivar, primary root formation with or without shoot emergence was noted in 34–74% of the embryos, one month after culture initiation, with the four protocols (Table 4). Among the media tested, the highest significant root formation observed in the fourth month (85.89%) was noted in embryos cultured in the UPLB medium. Despite the presence of 0.5 ppm NAA, cultures in CPCRI medium had the lowest percentage of primary root formation. Secondary and tertiary root formation was noted in the third month after culture initiation (Table 5). The highest secondary and tertiary root growth percentage was seen in the UPLB cultures and the lowest in the IRD. Secondary root formation was significantly highest in the MAKT embryos.

In terms of the average number of primary roots formed *in vitro* (Table 6), the highest count was noted in seedlings cultured in the UPLB medium (2.46), followed by the IRD and PCA-ARC. The CPCRI medium, with its supplement of BAP and NAA throughout the culture period, produced the lowest average number (1.80) of primary roots formed *in vitro*.

Average leaf production was highest in the seedlings produced with the CPCRI protocol, followed by PCA-ARC, UPLB and IRD (Table 6). Among the cultivars, TAGT, MYD and RIT had the most leaves in the fourth month, while LAGT and MAKT had the least leaves per *in vitro* plant. There was no significant interaction between protocols and cultivars.

Complete seedling development

Emergence of the first true leaf was noted in the third month. Regardless of the culture medium, the highest percentage of complete seedlings developed was noted in the MAKT at 75.42% (Table 7), while the highest percentage of complete seedling development was obtained with the UPLB protocol. The UPLB protocol produced significantly more seedlings (84.32%) compared to the other protocols. Incubating the germinated seedlings in the Y3 medium with its higher amount of macro-elements, particularly chlorine, might have influenced the growth and development of the seedlings. Moreover, incubation of the seedlings in the liquid medium might have allowed a more efficient uptake of nutrients and better gas exchange among the plant cells than those incubated on a solid medium. The influence of light on photomorphogenesis and photosynthesis of the seedlings *in vitro* could have contributed to the better performance of cultures in UPLB, CPCRI and PCA-ARC media since these cultures were exposed to light at the beginning of or earlier in the culture process than the IRD protocol.

Ex vitro establishment

Seedlings with balanced shoot and root development were transplanted using the PCA-ARC protocol for *ex vitro* establishment five months after culture initiation. Coconut seedlings from the five test cultivars transplanted to and kept in the screenhouse were then 5–18 months old.

Table 7 indicated that 62.80%, 59.02% and 38.53% of seedlings cultured following the UPLB, CPCRI and PCA-ARC protocols, respectively, were transplanted 5–7 months after culture initiation. Only a few seedlings (25.12%) cultured using the IRD protocol were transplanted within the same period.

Except for TAGT, the data showed no significant differences among the other four cultivars in terms of percentage of seedlings transplanted *ex vitro*. The highest percentage (54.49%) was noted in the MAKT.

The highest recovery rate was noted with the UPLB protocol (42.35%) followed by the CPCRI and PCA-ARC, while the lowest recovery was noted with seedlings cultured in IRD medium (6.97%) (Table 7). Significant difference in the recovery percentage among the cultivars was highest (48.96%) with the MAKT. MAKT embryos cultured using CPCRI protocol had the highest recovery of 69.17% (Table 8). Recovery percentage is computed based on the number of established seedlings over the initial number of embryos

On the other hand, *ex vitro* survival was highest in seedlings cultured using the PCA-ARC protocol (77.07%) (Table 7). MAKT embryos cultured using PCA-ARC protocol had the highest survival percentage *ex vitro* of 98.04% (Table 8). One of the factors that might have contributed to this could have been the sugar content. The other three media had a 1.5% higher sugar concentration than the PCA-ARC. Triques *et al.* (1997) reported that a high level of sucrose in the culture medium (60g/L) could affect the RuBisCO capacity. It has also been reported that exogenous carbohydrates induced depletion in the RuBisCO efficiency (Hidder and Desjardins 1994; 1995) and the photosynthetic rate (Serret *et al.* 1996). A reduction of the sucrose level in the culture medium during the last subculture could therefore induce an increase in photosynthesis, probably *via* an increase in the RuBisCO efficiency (Triques *et al.* 1997). The lower sugar concentration of the PCA-ARC medium might have contributed to the earlier adaptation of the plantlets to autotrophic conditions in the screenhouse. Nevertheless, with the PCA-ARC, it had been observed that a further lowering of sugar concentration to the 45 g/L level or lower during the last subculture stages was detrimental to the *ex vitro* survival of the seedlings (Bonaobra and Rillo, unpubl.).

Summary and conclusion

Regardless of the cultivar, the highest germination of embryos three months after culture initiation was noted with UPLB and CPCRI media. Subsequent seedling development, based on the shoot and root development, and percentage of transplanted and recovered seedlings, were consistently better with the UPLB protocol than with the other three protocols. Results showed that Y3 medium was better for embryo germination and subsequent development of seedlings *in vitro* than the modified MS medium. It was also observed that myo-inositol, as adopted in the UPLB protocol, could be omitted in the culture medium. At least one subculture of embryos on solid medium, as observed with the UPLB and CPCRI protocols, was essential for germination and subsequent seedling development. The results of *ex vitro* survival showed that the PCA-ARC protocol for *ex vitro* establishment could be adopted regardless of the *in vitro* culture protocol employed. Thus, for further trials, the PCA-ARC protocol was adopted for *ex vitro* establishment. The significant differences observed among cultivars in the parameters considered could be attributed to the highly variable nature of coconut, it being a highly cross-pollinated crop. A summary of the results obtained is presented in Table 9.

Study 2. Development of a "hybrid" coconut embryo culture protocol

The results of the comparative study on the four coconut embryo culture protocols (Annexes 1.1 – 1.4), showed germination was highest with the UPLB and CPCRI protocols. The UPLB medium was also shown to be most favorable for subsequent seedling development *in vitro*. For *ex vitro* seedling establishment, the PCA-ARC protocol was the most efficient. Based on these results, a 'hybrid' protocol needs to be formulated to obtain the highest efficiency possible for use in coconut germplasm collecting, conservation and exchange.

Objectives

1. To compare the effects of myo-inositol and BAP/NAA on the *in vitro* performance of Laguna Tall (LAGT) and Malayan Yellow Dwarf (MYD);
2. To shorten the culture period of seedlings *in vitro;*
3. To increase the percentage of seedlings transplanted *ex vitro* and the percentage of survival in the screenhouse/nursery; and
4. To develop a highly efficient hybrid protocol.

Materials and methods

Embryo collecting

Mature coconut embryos of Laguna Tall and Malayan Yellow Dwarf were collected from the PCA-Albay Research Center.

Media

Based on the results of Study 1, a 'hybrid' protocol was initiated, using Eeuwens' (Y3) formulation as base with a higher iron concentration (UPLB) in combination with the UPLB and PCA-ARC vitamin sources, 7 g/L agar, and 1 g/L acid-washed activated charcoal. Germinated embryos were maintained on semi-solid medium for two passages (second and third month). Sugar concentration was maintained at 60g/L for the first four months and reduced to 45 g/L for subsequent transfers.

Four treatments in the presence or absence of myo-inositol (PCA-ARC and UPLB) and a combination of 0.5 ppm BAP/NAA (CPCRI) were tested. The treatments were:

T1	– myo-inositol	– BAP/NAA
T2	+ myo-inositol	– BAP/NAA
T3	– myo-inositol	+ BAP/NAA
T4	+ myo-inositol	+ BAP/NAA

Embryo culture

The embryos were disinfected and cultured following the aseptic techniques of the PCA-ARC protocol (Annex 1.4).

Ex vitro establishment

For *ex vitro* establishment, the protocol developed at PCA-ARC was used for all seedlings produced in the study (Annex 1.4).

Statistical analysis

The experiment was laid out using a 2 x 4 factorial experiment involving two varieties and four medium treatments with three replications. For germination, 50 embryos per replicate were used. For subsequent growth and development analysis, 30 seedlings

per replicate in Treatments 1, 2 and 4, and 27 seedlings per replicate for Treatment 3 were used. The following data were gathered:

Germination (first three months in culture)
- Percentage germination
- Percentage shoot and root formation
- Percentage root formation
- Percentage secondary and tertiary root formation

Growth and development (up to ten months in culture)
- Percentage complete seedlings (rooted seedlings with at least one true leaf; based on germinated embryos)
- Number of leaves at weaning time
- Number of primary roots at weaning time
- Duration of developmental stage before weaning

Ex vitro establishment

- Number of transplanted seedlings [%TP] = $\frac{\text{No. of transplanted seedlings}}{\text{No. of inoculated embryos}} \times 100$
- Number of recovered seedlings [%RS] = $\frac{\text{No. of transplanted seedlings}}{\text{No. of inoculated embryos}} \times 100$
- Number of successfully grown and weaned seedlings % survival *ex vitro* = $\frac{\text{No. of established seedlings}}{\text{No. of transplanted seedlings [TP]}} \times 100$

Transformed (arcsine) data were analyzed using the analysis of variance, and means were compared using the Duncan Multiple Range Test (DMRT).

Results and discussion

Contamination

Among the different media evaluated, the PCA-ARC protocol adopted for disinfecting the zygotic embryos showed no significant difference in terms of contamination percentage (Table 10) either in the presence or absence of myo-inositol (PCA-ARC and UPLB) or with a combination of 0.5 ppm BAP/NAA (CPCRI). Response of the test cultivars also showed no significant difference.

Germination

Statistical analysis showed significant differences between the two test cultivars during the germination stage. MYD exhibited a higher germination (Table 11), simultaneous shoot and root formation (Table 12), leaf production (Table 14) and secondary and tertiary root formation (Table 16). Treatment 4 favored early leaf production as manifested by the emergence of a spear-shaped leaf. Further tertiary root formation was observed in a few cultures of MYD one month after culture initiation (Table 16). Both leaf production and tertiary root formation were noted in LAGT only three months after culture initiation. These differences could be attributed to the genotypic differences between the dwarf and tall cultivars.

Regardless of the cultivar, germination and shoot emergence were observed four weeks after culture initiation. Although no significant difference was noted among the treatments one month after the start of the experiment, the highest germination percentages were noted in T4 and T2, followed by T1 and T3 (Table 11). Germination increased to 74–85% on the second month with the highest percentages in T1 and T2, followed by T4. Lowest germination was noted in T3. Only a slight increase in germination percentage was observed three months after culture initiation.

Shoot and root emergence

Nearly half of the cultures displayed simultaneous shoot and root formation one month after culture initiation (Table 12). MYD had a higher percentage of cultures with simultaneous shoot and root formation than LAGT. Except for T3 which gave the lowest percentage of cultures with simultaneous shoot and root formation, no significant difference was noted among T1, T2 and T4 (Tables 12 and 13). T3 was not significantly different from T4. T1 exhibited the highest percentage of cultures (85.33% and 87.33%) with simultaneous shoot and root formation during the second and third month, respectively.

Leaf formation

In a few cultures of the MYD in T2-T4, leaves emerged earlier than the LAGT whose leaf emerged only in the third month (Tables 14 and 15). Although early leaf formation was noted in T2-T4, the highest percentage of seedlings with at least one true leaf was noted in T2, three months after culture initiation. It was, however, not significantly different from T1. Although late leaf formation was noted in T1, this treatment was able to surpass T3 and T4 during subsequent months. In the third month, 54–70% of the cultures displayed at least one true leaf (Table 14).

Root formation

Based on the results collected, the treatments favored early primary root formation in both cultivars, with averages of 73–87% of the embryos having formed roots within the first month (Table 16). After one month in culture, T2 had the highest percentage of cultures with roots followed by T1, T4 and T3. The percentage of rooted seedlings only slightly increased in subsequent months. No significant difference was noted among cultures in T1, T2 and T4. T3, however, produced significantly lower rooted seedlings than the other three treatments on the third month. It was noted that there was a higher percentage of rooted cultures than those with leaves during the first three months. T2 also gave the highest percentage of cultures with at least one primary root. The emergence of secondary and tertiary roots was earlier in cultures using hybrid protocol (Study 2 noted in the first and second months) than in those on standard protocols (secondary and tertiary roots noted in the third month of *in vitro* culture). Among the treatments, the highest percentages of cultures with secondary and tertiary roots were noted in T4 and T1.

Growth and development *in vitro* until five months in culture

Plant height, leaf production and number of primary roots were used as indicators of seedling growth and development (Table 17). After five months in culture, no significant differences among the treatments were noted among these indicators.

Although there was no significant difference among treatments, T1 gave the highest percentage of seedlings with primary roots and leaves five months after culture initiation. As mentioned earlier, leaf production in cultivar LAGT started only in the third month of culture.

Complete seedling development was significantly better with MYD than LAGT (Table 18). Regardless of the treatment, 86.17% of MYD embryos developed into complete seedlings. No significant difference was noted among T1, T2, and T4. The lowest percentage of complete seedling development was observed in T3.

Ex vitro establishment

Transplanting of the LAGT and MYD seedlings *ex vitro* started five months after culture initiation. Within 5–7 months, 62–77% of the seedlings produced with T1 – T4 were transplanted *ex vitro* (Table 18). This was higher compared to those obtained with the PCA-ARC, CPCRI and UPLB protocols (38–63%) for the same period. Based on data obtained ten months after culture initiation, a higher number of the MYD seedlings were potted *ex vitro*, compared to the LAGT. Except for T1, which was significantly different from T3, no significant differences were noted among T1, T2 and T4. Recovery percentages were not significantly different among the four treatments.

Adopting the PCA-ARC protocol for *ex vitro* establishment showed no significant differences among the four treatments in terms of *ex vitro* survival (Table 18). Mean ranges of 57–68% and 88–93% recovery and *ex vitro* survival, respectively, were noted with the four treatments.

Effects of myo-inositol and BAP/NAA

During the initial development stage, significant differences in terms of germination percentages were noted between embryos cultured in the medium with (T3, T4) and without (T1, T2) BAP/NAA. Higher germination was noted in the medium without BAP/NAA (Table 11). Higher root formation was noted in the medium with myo-inositol and without BAP/NAA (Table 16). Leaf production was significantly higher in the medium with myo-inositol (T4) during the first month *in vitro* (Tables 14 and 15). However, the difference was insignificant afterwards. During the third month, leaf production was significantly lower in the media (T3, T4) with BAP/NAA. The percentage of cultures showing simultaneous shoot and root formation was significantly higher in the media (T1, T2) without BAP/NAA (Table 12). Data also showed low germination and complete seedling formation with T3 (Tables 11 and 18, respectively).

Data obtained during the subsequent incubation period were not significantly different whether with or without myo-inositol and BAP/NAA. Hence, embryos could be cultured satisfactorily in a medium without myo-inositol and without BAP/NAA. The results of Study 2 are summarized in Table 19.

Comparison of *in vitro* and *ex vitro* performance of cultures with various treatments

Table 20 presents some of the combined results of Studies 1 and 2.

Contamination

Results of the coconut embryo culture survey conducted by IPGRI in 1997 showed that percentage contamination was 1–2% with PCA-ARC protocol, 2–3% with CPCRI and <1% with IRD protocol (Table 21). For the validation of the four protocols, mature embryos were collected from the Zamboanga Research Center as well as from the Albay Research Center. A slight increase in contamination percentage was noted with the PCA-ARC (3.70%), UPLB (3.28%), CPCRI (3.65%) and IRD (2.08%) protocols due to long distance handling of some of the embryos (Table 2). In the hybrid protocol, 5-9% of the embryos were contaminated probably because of improper handling (Table 10).

Germination

It was noted that germination of LAGT and MYD embryos was almost similar in the CPCRI and IRD media (Fig. 1). LAGT germination was consistently higher than MYD with the PCA-ARC, UPLB, CPCRI, and IRD media. Compared to the 'hybrid' protocol (T1-T4), the MYD germination was consistently higher than that of the LAGT. Regardless of the cultivar, 75–90% of the embryos germinated with T1–T4 during the third month. This was higher compared to those obtained with the four standard protocols (52–84%) with cultures of the same age. The higher germination percentages obtained with CPCRI, UPLB and T1-T4 compared to the PCA-ARC may be attributed to the higher sugar content used in the first three months and to the culture stage on solid medium in all initial test protocols.

Shoot and root emergence

Simultaneous formation of shoot and root was higher in the four hybrid treatments (74–85%) during the second month (Table 12) than in the four original protocols (29–77%) (Table 4).

Root formation

The formation of secondary and tertiary roots was earlier in cultures using 'hybrid' protocol (Study 2 noted in the first and second months) than those using the standard protocols (secondary and tertiary roots noted in the third month of *in vitro* culture).

Ex vitro performance of the seedlings

The results of the coconut embryo culture survey (IPGRI 1997) showed that the average duration required to produce fully developed plantlets was 8–14 months with the PCA-ARC protocol, 6–12 months with CPCRI and 7–10 months with the IRD protocol (Tables 20 and 21). Comparing the four original protocols and the hybrid protocols, fully developed plantlets ready for *ex vitro* establishment could be obtained earlier (*i.e.* after at least 5 months of *in vitro* culture) as observed in both studies (Table 20). This reduction in the duration of the *in vitro* culture period is clearly a significant improvement.

There were differences among the treatments when comparing the percentage of complete (Fig. 2), transplanted (Figs. 3, 4), recovered (Fig. 5) and *ex vitro* survival (Fig. 6) of the LAGT and MYD seedlings that were obtained from the different protocols (PCA-ARC, UPLB, CPCRI, IRD, T1, T2, T3, T4).

Among the four standard protocols, complete seedling formation was highest in the UPLB medium at 84.32% (Table 7). This data was comparable to the percentages noted with 'hybrid' protocols T1 (80.33%), T4 (79.67%) and T2 (79.33%) media (Table 18).

The four 'hybrid' protocols (T1, T2, T3, and T4) exhibited significant differences in percentage of transplanted seedlings (77, 67.33, 62, and 72%, respectively) (Table 18). These data were comparable to the percentages noted with CPCRI (59.02%) and UPLB (62.80%) media (Table 7).

A higher percentage of recovered seedlings (57–67%) (Fig. 5) is noted from the four hybrid treatments (Table 18) than from among the PCA-ARC, UPLB, CPCRI or IRD protocols (6–42%) (Table 7).

The results of the coconut embryo culture survey (IPGRI 1997) showed that the percentage of *ex vitro* survival of seedlings transplanted was 65–75% with the PCA-ARC protocol, almost 100% with CPCRI and 90% with the IRD protocol (Table 21).

With the validation of the four protocols using five varieties from the ZRC and the ARC, survival with the PCA-ARC protocol increased to 77.07%; and to 69.16% with the UPLB protocol. On the other hand, survival with the CPCRI and IRD protocols was reduced to 50.98% and 19.50%, respectively (Table 7). The source and age of embryos, location of the laboratory, operator's efficiency and other factors could probably have influenced the results. With the 'hybrid' protocol, 88–93% (Fig. 6) survival of seedlings was achieved 3–6 months after transplanting (Table 18) compared to 19–77% with the PCA-ARC, UPLB, CPCRI or IRD protocols (Table 7).

Summary and conclusion

It can be concluded that coconut zygotic embryos grow and develop satisfactorily in Eeuwens (Y3) (1976) nutrient formulation supplemented with the iron source of the UPLB protocol ($FeSO_4.7H_2O$, 41.70 mg/L and Na_2EDTA, 55.80 mg/L), combined with the vitamin source of the UPLB and PCA-ARC protocols (Pyridoxine HCl, 0.05 mg/L; Thiamine HCl, 0.05 mg/L; Nicotinic acid, 0.05 mg/L; Ca-D pantothenate, 0.05 mg/L; Biotin, 0.05 mg/L; Folic acid, 0.05 mg/L; and glycine, 1 mg/L); table grade sugar at 60 g/L during the first three months, then reduced to 45 g/L for the subsequent transfers; and activated charcoal (acid washed) (1 g/L). Embryos initially prefer liquid medium and, with poor germination, embryos need to be subcultured on semi-solid medium with agar (7 g/L) during the second to third month *in vitro*. The PCA-ARC protocol is satisfactory for *ex vitro* transplanting.

The results further suggest that myo-inositol, BAP and NAA can be omitted from the culture medium without any detrimental effects on the growth and development of the embryos. These results confirm the non-usage of myo-inositol in the UPLB protocol throughout the various stages of the protocol. Further, BAP and NAA are also not necessary in the Y3 formulation if the medium is to be used to culture coconut embryos as also shown by Rillo and Paloma (1990).

The recommended protocol is currently being tested using the Makapuno embryos. Since significant improvement was noted when culturing the LAGT and the MYD embryos with the modified protocol, it is recommended that the proposed 'hybrid' protocol be tested with other tall, dwarf and hybrid coconut varieties. This will validate the conceptualized 'hybrid', protocol, which seems to be superior to the four original protocols tested. A proposal for a Phase II of this project has been prepared to validate the recommended 'hybrid' protocol using other coconut cultivars and hybrids.

The 'hybrid' protocol has shortened the duration of the *in vitro* culture as well as improved embryo development, resulting in higher recovery of *ex vitro* plantlets. This protocol will be very beneficial for improving coconut germplasm collecting and exchange. In the Philippines, the hybrid protocol will benefit the mass production of *in vitro* cultured Makapuno embryos, which is now being done in eight government and eight private laboratories all over the country. Other high value soft endosperm coconut mutants such as the Lono in the Philippines, Kopyor in Indonesia and Dikiri Pol in Sri Lanka could also be cultured within shorter periods using this protocol.

A proposal to exploit the embryo culture technology to mass produce these high value soft endosperm coconut mutants is now being prepared and it will definitely make use of this optimized 'hybrid' embryo culture protocol. In the long run, new uses and markets could be developed for these types of coconuts which have, until now, not been exploited due to their scarcity. It will be an altogether new scenario with this new technological development.

Constraints, lessons learned and recommendations

1. The efficiency of the technique is greatly affected by the age and quality of the nuts and embryos. The embryos collected are not exactly of the same age; 10–11 month-old embryos respond better *in vitro*. The best embryos are those collected from tagged nuts.
2. Embryos from hand-fertilized palms respond better *in vitro*. Embryos from hand-fertilized palms are more plump and vigorous. Due to the shortage of embryos from the ARC, some of the embryos used in these experiments were obtained from farmers' fields.
3. Experienced technicians will be needed properly handle the embryos and transfer the cultures at the appropriate time. This is very important for any transfer of technology. Trained recipients for such work should also be identified.
4. Another 'hybrid' protocol is currently being tested. This test involves transferring the embryos to a particular medium protocol at a specific culture stage where they are observed to have given the best response *in vitro*. This has been discontinued due to a lack of embryos.
5. Considering the variability in coconuts, it would have been best if all the treatments had been set up at the same time to reduce unnecessary variance. However, this was not possible due to shortage of embryos of the varieties required.

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Table 1. The basic differences among the four *in vitro* culture protocols for coconut embryos employed in the study

	PCA-ARC	UPLB	CPCRI	IRD
Medium	**Y3**	**Y3**	**Y3**	**MS**
Micronutrients				
Iron Source		Higher conc. of FeSO4.7H2O –3x Na2EDTA-1.5x	Lower concentration of CuSO4 .5H2O	
Myo-inositol		No myo-inositol		
Vitamins		W/o Ca D-pantothenate, with folic acid & glycine	No nicotinic acid & Ca D-pantothenate	
Sucrose (g/L)	45	60	60 initiation 30 germination	60
Hormones	IBA for rooting		BAP and NAA for initiation & developmentNAA and IBA for rooting	
Activated charcoal (g/L)	2.5 (acid washed)	2.5 (acid washed)	1.0 (acid washed)	2.0 (neutralized)
pH	5.8	5.6	5.6	5.5
State of the medium	Liquid all throughout	Liquid-solid-liquid	Solid-liquid	Liquid all throughout
Photoperiod	9h light/ 15h dark	Dark for the 1st month, thereafter	Dark for 20–25 days thereafter 9h light/15h dark	Dark until first true leaf emerges, 16h light/8h dark then 12h light/12h dark

Table 2. Effect of culture protocol on the embryo contamination of the five coconut cultivars

Protocol/Cultivar	Contamination (%)
Protocol	
PCA-ARC	3.70 a
UPLB	3.28 ab
CPCRI	3.65 ab
IRD	2.08 b
Cultivar	
MYD	3.00 ab
LAGT	4.99 a
TAGT	2.52 ab
RIT	2.87 ab
MAKT	2.50 b
Protocol x cultivar	ns
Mean	3.18
CV (%)	31.87
Before start of the project at ARC using MAKT embryos	1 – 2

ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 3. Effect of culture protocol on the embryo germination of the five coconut cultivars

Protocol/Cultivar	Average germination (%)			
	Before start of the project at ARC	1st month	2nd month	3rd month
Protocol		**	***	***
PCA-ARC		45.56 a	65.70 b	69.52 b
UPLB		37.34 a	80.80 a	84.00 a
CPCRI		31.48 a	74.29 ab	83.83 a
IRD		13.67 b	35.33 c	52.04 c
Cultivar		***	*	*
MYD		25.04 bc	54.52 c	66.84 b
LAGT		54.55 a	73.95 a	81.66 a
TAGT		27.02 bc	61.41 abc	67.09 b
RIT		14.50 c	57.34 bc	64.90 b
MAKT	1161/2085 (55.68%)	38.96 ab	72.92 ab	81.25 a
Protocol x cultivar		ns	ns	ns
Mean		32.01	64.03	72.35
CV (%)		41.23	23.60	20.52

***= significant at 0.01% level; ** = significant at 0.1% level; * = significant at 5% level; ns = not significant.

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 4. Effect of culture protocol on emergence of shoot and root, and primary root formation of the five coconut cultivars

Protocol/Cultivar	Shoot and root emergence (%)		
	1st month	2nd month	4th month
Protocol	**	**	**
PCA-ARC	42.20 a	59.82 b	66.65 b
UPLB	35.96 a	77.01 a	84.54 a
CPCRI	21.72 b	46.80 b	60.86 b
IRD	11.17 b	29.24 c	52.01 c
Cultivar	**		
MYD	22.93 bc	52.30 ab	69.56 ab
LAGT	44.09 a	57.57 ab	67.97 ab
TAGT	24.96 b	49.90 ab	57.16 b
RIT	12.23 c	41.89 b	58.97 b
MAKT	34.58 ab	64.44 a	76.42 a
Protocol x cultivar	ns	ns	ns
Mean	27.76	53.22	66.02
CV (%)	39.92	26.63	20.11
	Primary root formation (%)		
Protocol	**	**	**
PCA-ARC	58.22 b	66.53 b	70.41 b
UPLB	73.85 a	83.53 a	85.89 a
CPCRI	36.32 c	50.27 c	60.71 b
IRD	34.54 c	50.39 c	62.60 b
Cultivar	ns		
MYD	54.60	67.57 ab	74.05 a
LAGT	47.30	59.15 ab	69.60 ab
TAGT	46.48	53.81 b	58.65 b
RIT	49.01	58.91 b	66.39 ab
MAKT	56.25	73.96 a	80.83 a
Protocol x cultivar	ns	ns	ns
Mean	50.73	62.68	69.90
CV (%)	19.72	19.26	18.35

** = significant at 1% level
ns = not significant
In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 5. Effect of culture protocol on the mean secondary and tertiary root formation of the five coconut cultivars

Protocol/Cultivar	Secondary root formation (%)		Tertiary root formation (%)	
	3rd month	4th month	3rd month	4th month
Protocol	**	**	**	**
PCA-ARC	32.57 b	36.90 bc	10.10 bc	15.68 c
UPLB	64.85 a	70.96 a	30.74 a	41.98 a
CPCRI	30.71 b	40.64 b	14.23 b	28.40 b
IRD	17.40 c	26.46 c	4.22 c	10.29 c
Cultivar	*	**		
MYD	37.09 ab	45.02 b	16.20 ab	25.85 ab
LAGT	35.72 b	43.62 b	20.32 a	27.03 ab
TAGT	30.64 b	35.67 b	10.62 b	17.67 b
RIT	29.29 b	36.91 b	9.47 b	18.64 ab
MAKT	49.17 a	57.50 a	17.50 ab	31.25 a
Protocol x cultivar	ns	ns	ns	ns
Mean	36.38	43.74	14.82	24.09
CV (%)	29.26	23.83	61.04	42.46

** = significant at 1% level; * = significant at 5% level; ns = not significant
In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 6. Effect of culture protocol on the average number of roots and leaves produced per seedling of the five coconut cultivars

Protocol/Cultivar	Average number of leaves/ *in vitro* plant at 4th month	Average number of roots/ *in vitro* plant at 4th month
Protocol	**	**
PCA-ARC	2.91 a	2.25 a
UPLB	2.63 b	2.46 a
CPCRI	3.02 a	1.80 b
IRD	2.43 b	2.35 a
Cultivar	**	**
MYD	2.90 a	2.95 a
LAGT	2.61 bc	1.91 b
TAGT	2.91 a	1.98 b
RIT	2.85 ab	2.11 b
MAKT	2.47 c	2.14 b
Protocol x cultivar	ns	ns
Mean	2.75	2.22
CV (%)	10.95	21.10

** = significant at 1% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5 % level.

Table 7. Effect of culture protocol on *in vitro* and *ex vitro* performances of seedlings of the five coconut cultivars (*Ex vitro* results have been collected 3–18 months after transplanting of plantlets)

Protocol/Cultivar	Complete seedlings at 10th month (%)	Transplanted seedlings (%)	Recovered seedlings (% survival after transfer to screenhouse)	Survival *ex vitro* (%)
Protocol	**	**	**	**
PCA-ARC	66.65 b	38.53 b	30.07 b	77.07 a
UPLB	84.32 a	62.80 a	42.35 a	69.16 a
CPCRI	69.24 b	59.02 a	33.28 b	50.98 b
IRD	52.48 c	25.12 c	6.97 c	19.50 c
Cultivar	*		**	**
MYD	70.94 ab	49.04 ab	26.15 b	47.18 b
LAGT	75.22 a	48.46 ab	24.53 b	46.23 b
TAGT	57.16 b	38.24 b	20.76 b	44.52 b
RIT	62.11 ab	41.58 ab	20.42 b	45.89 b
MAKT	75.42 a	54.49 a	48.96 a	87.06 a
Protocol x cultivar	ns	ns	**	**
Mean	68.17	46.36	28.17	54.17
CV (%)	20.82	22.79	23.66	24.18
Before start of the project at ARC using MAKT embryos		770/2085 (36.93%)	625/2085 (29.98%)	625/770 (81.17%)

** = significant at 1% level; * = significant at 5% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 8. Interaction effect of the culture protocol and coconut cultivars on the *ex vitro* performances of seedlings noted 3-18 months after transplanting of plantlets

Protocol x cultivar **	Recovered seedlings (%)			
	PCA-ARC	UPLB	CPCRI	IRD
MYD	22.18 a	31.34 b	48.58 b	2.49 b
LAGT	27.12 a	46.58 ab	21.57 c	2.86 b
TAGT	33.45 a	36.28 b	10.94 c	2.38 b
RIT	26.75 a	35.03 b	16.12 c	3.80 b
MAKT	40.83a	62.50a	69.17a	23.33a
Protocol x cultivar **	**Survival *ex vitro* (%)**			
MYD	67.57 b	55.47 b	58.09 b	7.58 b
LAGT	76.48 b	63.66 ab	36.89 b	7.78 b
TAGT	72.27 b	62.77 ab	34.57 b	8.46 b
RIT	70.98 b	72.96 ab	32.11 b	7.50 b
MAKT	98.04 a	90.93 a	93.09 a	66.18 a

** = significant at 1% level

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 9. Summary results of Study 1: Comparison of four coconut embryo culture protocols

Protocol	Cultivar	Initial no. of embryos	No. of germ'd embryos	No. of contaminated embryos	No. of leaves/*in vitro* plant	No. of roots/*in vitro* plant	No of potted plants	No. of plants estab'd in the screenhouse
PCA-ARC	LAGT	134	105	9	2.90	1.98	47	38
	TAGT	196	142	3	3.15	1.99	94	67
	RIT	180	108	2	2.85	1.91	70	48
	MAKT	120	95	4	2.62	2.44	50	49
	MYD	149	105	4	3.02	2.95	60	40
	Total	**779**	**555**	**22**	**14.54**	**11.27**	**321**	**242**
UPLB	LAGT	114	108	8	2.30	2.17	84	53
	TAGT	195	170	4	2.94	2.18	111	74
	RIT	182	136	8	2.64	2.51	98	65
	MAKT	120	116	4	2.36	2.38	82	75
	MYD	154	126	4	2.92	3.04	115	52
	Total	**765**	**656**	**28**	**13.16**	**12.28**	**486**	**319**
CPCRI	LAGT	132	120	5	2.94	1.41	74	27
	TAGT	191	137	8	3.28	1.74	54	19
	RIT	173	135	8	3.31	1.61	87	28
	MAKT	120	107	4	2.46	1.83	89	83
	MYD	158	146	5	3.13	2.42	136	75
	Total	**774**	**645**	**30**	**15.10**	**9.01**	**440**	**232**
IRD	LAGT	129	67	3	2.32	2.07	27	3
	TAGT	200	91	4	2.28	2.00	46	5
	RIT	189	104	6	2.61	2.40	57	9
	MAKT	120	72	0	2.43	1.90	42	28
	MYD	131	66	5	2.50	3.40	27	5
	Total	**769**	**400**	**18**	**12.14**	**11.77**	**199**	**50**

Date of report: 25 February 2000

Table 10. Effect of different media on the embryo contamination of LAGT and MYD cultivars

Medium/Cultivar	Contamination %
Medium	ns
– myo inositol – BAP/NAA (T1)	5.67
+ myo inositol – BAP/NAA (T2)	7.33
– myo inositol + BAP/NAA (T3)	8.67
+ myo inositol + BAP/NAA (T4)	9.33
Cultivar	ns
LAGT	8.17
MYD	7.33
Medium x cultivar	ns
Mean	7.75
CV	30.58%

ns = not significant

Table 11. Effect of different media on the embryo germination of LAGT and MYD cultivars during the first three months

Medium/Cultivar	Germination (%)		
	1st mo	2nd mo	3rd mo
Medium	ns	*	*
– myo inositol – BAP/NAA (T1)	45.67	85.67 a	89.67 a
+ myo inositol – BAP/NAA (T2)	49.33	85.67 a	86.67 a
– myo inositol + BAP/NAA (T3)	44.67	74.00 b	75.00 b
+ myo inositol + BAP/NAA (T4)	49.67	81.33 ab	83.00 ab
Cultivar	**	**	**
LAGT	41.33 b	77.17 b	78.17 b
MYD	53.33 a	86.17 a	89.00 a
Medium x cultivar	ns	ns	ns
Mean	47.33	81.67	83.58
CV (%)	10.14	8.38	8.04

** = significant at 1% level; * = significant at 5% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 12. Effect of different media on shoot and root formation of LAGT and MYD cultivars

Medium/Cultivar	Shoot and root formation (%)		
	1st month	2nd month	3rd month
Medium	ns		*
– myo inositol – BAP/NAA (T1)	45.00	85.33 a	87.33 a
+ myo inositol – BAP/NAA (T2)	51.00	84.67 a	85.33 a
– myo inositol + BAP/NAA (T3)	46.33	74.33 b	75.00 b
+ myo inositol + BAP/NAA (T4)	49.67	81.67 ab	83.00 ab
Cultivar	**	**	**
LAGT	41.17 b	76.83 b	77.83 b
MYD	54.83 a	86.17 a	87.50 a
Medium x cultivar	**	ns	ns
Mean	48.00	81.50	82.67
CV (%)	13.74	6.63	5.99

** = significant at 1% level; * = significant at 5% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 13. Interaction effect of medium treatment and genotype on the shoot and root formation during the first month

Medium x cultivar **	Shoot and root formation (%) (1st month)	
Medium	LAGT	MYD
– myo inositol – BAP/NAA (T1)	40.00 ab	50.00 b
+ myo inositol – BAP/NAA (T2)	40.00 ab	62.00 a
– myo inositol + BAP/NAA (T3)	33.33 b	59.33 ab
+ myo inositol + BAP/NAA (T4)	51.33 a	48.00 b

** = significant at 1% level

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 14. Effect of different media on the mean leaf production percentage of the LAGT and MYD cultivars during the first three months

	Leaf production (%)		
	1st month	2nd month	3rd month
Medium	*	ns	**
– myo inositol – BAP/NAA (T1)	0.00 b	31.00	69.00 a
+ myo inositol – BAP/NAA (T2)	1.00 ab	25.00	70.00 a
– myo inositol + BAP/NAA (T3)	0.33 b	24.67	54.00 b
+ myo inositol + BAP/NAA (T4)	2.00 a	19.00	56.00 b
Cultivar	**	**	**
LAGT	0.00 b	0.00 b	44.00 b
MYD	1.67 a	49.83 a	80.50 a
Medium x cultivar	*	ns	ns
Mean	0.83	24.92	62.25
CV (%)	129.61	73.06	8.27

** = significant at 1% level; * = significant at 5% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 15. Interaction effect of medium treatment and coconut cultivar on leaf production during the first month

Medium x cultivar *	Leaf production (%)	
Medium	LAGT	MYD
– myo inositol – BAP/NAA (T1)	0.00 a	0.00 c
+ myo inositol – BAP/NAA (T2)	0.00 a	2.00 b
– myo inositol + BAP/NAA (T3)	0.00 a	0.67 bc
+ myo inositol + BAP/NAA (T4)	0.00 a	4.00 a

* = significant at 5% level

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 16. Effect of different media on the root formation of the LAGT and MYD cultivars during the first three months

Medium/Cultivar	Primary root formation (%)		
	1st month	2nd month	3rd month
Medium	**	*	*
– myo inositol – BAP/NAA (T1)	82.67 a	86.67 a	88.00 a
+ myo inositol – BAP/NAA (T2)	86.67 a	89.00 a	89.33 a
– myo inositol + BAP/NAA (T3)	73.00 b	76.67 b	76.67 b
+ myo inositol + BAP/NAA (T4)	84.00 a	86.00 a	86.67 a
Cultivar	*	**	**
LAGT	78.83 b	80.50 b	81.33 b
MYD	84.33 a	88.67 a	89.00 a
Medium x cultivar	ns	ns	ns
Mean	81.58	84.58	85.17
CV (%)	5.46	5.82	5.93
Secondary root formation (%)			
Medium	ns	ns	ns
– myo inositol – BAP/NAA (T1)	19.00	48.67	63.67
+ myo inositol – BAP/NAA (T2)	12.33	50.00	62.67
– myo inositol + BAP/NAA (T3)	9.33	45.67	56.67
+ myo inositol + BAP/NAA (T4)	10.33	47.67	64.33
Cultivar	**	**	**
LAGT	3.67 b	34.67 b	48.50 b
MYD	21.83 a	61.33 a	75.17 a
Medium x cultivar	ns	ns	ns
Mean	12.75	48.00	61.83
CV (%)	69.09	11.11	10.28
Tertiary root formation (%)			
Medium	ns	ns	ns
– myo inositol – BAP/NAA (T1)	0.00	12.67	35.00
+ myo inositol – BAP/NAA (T2)	0.00	17.00	31.67
– myo inositol + BAP/NAA (T3)	0.00	14.33	27.00
+ myo inositol + BAP/NAA (T4)	1.67	15.67	30.00
Cultivar	ns	**	**
LAGT	0.00 a	0.00 b	6.83 b
MYD	0.83 a	29.83 a	55.00 a
Medium x cultivar	ns	ns	ns
Mean	0.42	14.92	30.92
CV (%)	353.27	71.77	19.80

** = significant at 1% level; * = significant at 5% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 17. Effect of different media on shoot length, number of leaves and roots of the LAGT and MYD cultivars

	Shoot length (cm)			
	2nd month	3rd month	4th month	5th month
Medium	ns	ns	ns	ns
– myo inositol – BAP/NAA (T1)	2.50	7.32	16.30	23.77
+ myo inositol – BAP/NAA (T2)	2.48	7.52	16.64	24.30
– myo inositol + BAP/NAA (T3)	2.50	7.74	16.73	24.18
+ myo inositol + BAP/NAA (T4)	2.59	8.36	17.07	24.75
Cultivar	**	**	**	ns
LAGT	2.14 b	6.43 b	14.74 b	23.88
MYD	2.89 a	9.05 a	18.64 a	24.61
Medium x cultivar	ns	ns	ns	ns
Mean	2.52	7.74	16.69	24.25
CV (%)	18.58	10.26	7.86	7.32

	No. of leaves produced				
	1st month	2nd month	3rd month	4th month	5th month
Medium	ns	ns	ns		ns
– myo inositol – BAP/NAA (T1)	0.01	0.29	0.93	1.41 b	2.24
+ myo inositol – BAP/NAA (T2)	0.01	0.28	0.97	1.42 b	1.95
– myo inositol + BAP/NAA (T3)	0.01	0.26	0.89	1.48 ab	2.13
+ myo inositol + BAP/NAA (T4)	0.01	0.22	0.87	1.62 a	2.07
Cultivar	**	**	**	**	**
LAGT	0.00 b	0.01 b	0.73 b	1.29 b	1.84 b
MYD	0.01 a	0.52 a	1.10 a	1.68 a	2.36 a
Medium x cultivar	ns	ns	ns	ns	ns
Mean	0.01	0.26	0.92	1.48	2.10
CV (%)	161.42	86.57	14.36	9.98	14.56

	No. of primary roots produced				
	1st month	2nd month	3rd month	4th month	5th month
Medium	ns	ns		ns	ns
– myo inositol – BAP/NAA (T1)	0.99	1.67	2.02 b	2.40	2.49
+ myo inositol – BAP/NAA (T2)	0.85	1.72	2.08 ab	2.41	2.47
– myo inositol + BAP/NAA (T3)	1.05	1.78	2.08 ab	2.40	2.46
+ myo inositol + BAP/NAA (T4)	1.04	1.83	2.22 a	2.46	2.35
Cultivar	ns	**	**	**	**
LAGT	1.01	1.60 b	1.94 b	2.16 b	2.22 b
MYD	0.96	1.91 a	2.25 a	2.68 a	2.67 a
Medium x cultivar	ns	ns	ns	ns	ns
Mean	0.98	1.75	2.10	2.42	2.44
CV (%)	20.89	9.62	6.42	7.70	8.70

** = significant at 1% level; ns = not significant

In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 18. Effect of different medium treatments on *in vitro* and *ex vitro* performance of LAGTand MYD seedlings

	Complete seedlings (%)	Number of leaves per *in vitro* seedling at transplanting	Number of root per *in vitro* seedling at transplanting	Transplanted seedlings (%) (No. of potted plantlets/initial no. of embryos x 100)	Recovered seedlings (%) (No. of established seedlings/initial no. of embryos x 100)	Survival *ex vitro* (%) (No. of established seedlings/no of potted plantlets x 100)
Medium		*	ns		ns	ns
T1	80.33 a	2.61 a	2.01	77.00 a	67.67	88.03
T2	79.33 a	2.44 ab	1.86	67.33 ab	60.67	90.47
T3	67.67 b	2.14 b	1.74	62.00 b	57.33	93.53
T4	79.67 a	2.47 ab	1.95	72.00 ab	67.33	93.42
Cultivar	**	**	**	**	**	ns
LAGT	67.33 b	1.66 b	1.48 b	60.00 b	55.17 b	92.46
MYD	86.17 a	3.17 a	2.29 a	79.17 a	71.33 a	90.26
Medium x cultivar	ns	ns	ns	ns	ns	ns
Mean	76.75	2.42	1.89	69.58	63.25	91.36
CV (%)	8.96	10.71	15.80	9.73	11.01	7.02

* = significant at 5% level; ** = significant at 1% level; ns = not significant
In a column, means followed by a common letter are not significantly different at DMRT 5% level.

Table 19. Summary results of Study 2: Development of a 'hybrid' coconut embryo culture protocol

	Cultivar	Initial No. of embryos	No. of germ'd. embryos	No. of contaminated embryos	No. of leaves/*in vitro* plant	No. of roots/ *in vitro* plant	No. of potted plants	No. of plants estab'd. in the screen-house
T1	LAGT	150	128	3	2.04	3.04	102	95
	MYD	150	140	14	3.19	3.71	129	108
Total		**300**	**268**	**17**			**231**	**203**
T2	LAGT	150	120	14	1.79	3.04	90	53
	MYD	150	141	8	3.09	4.06	112	52
Total		**300**	**261**	**22**			**202**	**105**
T3	LAGT	150	100	16	1.17	2.45	72	64
	MYD	150	127	10	3.11	4.09	114	108
Total		**300**	**228**	**26**			**124**	**172**
T4	LAGT	150	121	16	1.63	2.52	96	89
	MYD	150	129	12	3.31	4.13	120	113
Total		**300**	**250**	**28**			**216**	**202**

Date of report: 25 February 2000

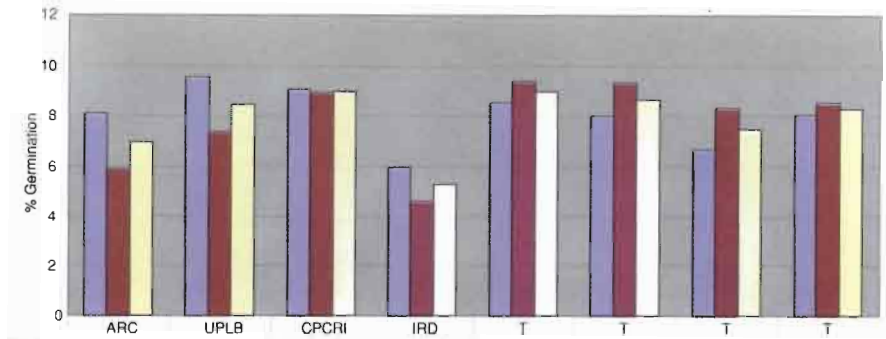
Table 20. Summary of results obtained by the PCA-ARC studies on the improvement of *in vitro* culture protocol for coconut zygotic embryos (as of 25 February 2000)

Parameter	Cultivar	Before start of the project at PCA-ARC	Protocol				Hybrid protocol			
			UPLB	PCA-ARC	CPCRI	IRD	T1	T2	T3	T4
Initial number of embryos cultured	MYD		154	149	158	131	150	150	150	150
	LAGT		114	134	132	129	150	150	150	150
	TAGT		195	196	191	200				
	RIT		182	180	173	189				
	MAKT	2085	120	120	120	120				
No. of embryos germinated (%)	MYD		126 (73.52)	105 (58.71)	146 (89.21)	66 (45.90)	140 (94.00)	141 (93.33)	127 (83.33)	129 (85.33)
	LAGT		108 (95.45)	105 (81.01)	120 (90.65)	67 (59.55)	128 (85.33)	120 (80.00)	100 (66.67)	121 (80.67)
	TAGT		170 (81.08)	137 (72.69)	137 (72.69)	91 (43.89)				
	RIT		136 (73.27)	108 (58.83)	135 (77.43)	104 (50.06)				
	MAKT	1161/2085 (55.68)	116 (96.67)	107 (89.17)	107 (89.17)	72 (60.83)	129 (86.00)	112 (74.67)	114 (76.00)	120 (80.00)
No. of fully developed *in vitro* plantlets (%)	MYD		115 (64.07)	60 (31.97)	136 (83.95)	27 (16.16)	102 (68.00)	90 (60.00)	72 (48.00)	96 (64.00)
	LAGT		84 (74.35)	47 (35.27)	74 (59.28)	27 (24.94)				
	TAGT		111 (56.23)	94 (46.79)	54 (27.88)					
					46 (22.30)					
	RIT		98 (50.99)	70 (38.14)	87 (50.04)	57 (27.18)				
	MAKT	770/2085 (36.93)	82 (66.33)	50 (40.46)	89 (74.17)	42 (35.00)				
Average duration of *in vitro* culture to obtain whole plantlets	MYD LAGT TAGT RIT MAKT	8-14 mo or 32-56 wk	At least 5 mo or 20 wk		At least 6 mo or 24 wk		At least 5 mo or 20 wk			
% Survival after transfer to nursery	MAKT	100								
% Survival after transfer to field	MAKT	100								

- * Data based on material collected between June 1997 and May 1998.
- All results, except for ex vitro survival, expressed as a function of number of embryos inoculated.
- Survival after transfer to screenhouse (recovered seedlings) refers to the number of established seedlings/number of inoculated embryos.
- Ex vitro survival refers to the number of established seedlings/number of transplanted seedlings.
- The culture medium used in the hybrid protocol includes Eeuwen's formulation with higher iron concentration (UPLB) and a combination of UPLB and ARC vitamin sources, 7 g/L agar, and 1 g/L acid-washed activated charcoal. Germinated embryos were maintained on semi-solid medium for two passages (second and third month). Sugar concentration was maintained at 60g/L for the first four months and reduced to 45 g/L for subsequent transfers. Four treatments were tested, which considered the presence or absence of myo-inositol (PCA-ARC and UPLB) and of a combination of 0.5 ppm BAP/NAA (CPCRI). The treatments were: T1 – myo-inositol – BAP/NAA, T2 + myo-inositol, – BAP/NAA, T3 – myo-inositol, + BAP/NAA, and T4 + myo-inositol + BAP/NAA.

Table 21. Coconut embryo culture survey results (IPGRI 1997)

Parameters	Culture Protocol	Data
% Contamination	PCA-ARC	
	UPLB	
	CPCRI	2 – 3%
	IRD	<1%
Average duration required to produce fully developed plantlets	PCA-ARC	8 – 14 mos
	UPLB	
	CPCRI	6 – 12 mos
	IRD	At least 7 mos; Ave. duration of *in vitro* culture is 10 mos.
Were there any differences in response between varieties used?	PCA-ARC	
	UPLB	
	CPCRI	Yes
	IRD	
Rate of plantlet establishment in the soil (*ex vitro* survival)	PCA-ARC	65 – 75%
	UPLB	
	CPCRI	Almost 100%
	IRD	90%



	ARC	UPLB	CPCRI	IRD	T	T	T	T
LAGT	81.0	95.4	90.6	59.5	85.3	80.0	66.6	80.6
MYD	58.7	73.5	89.2	45.9	94.0	93.3	83.3	85.3
Mean	69.8	84.4	89.9	52.7	89.6	86.6	75.0	83.0

Figure 1. Germination percentage of LAGT and MYD embryos cultured using the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) protocols three months after culture initiation.

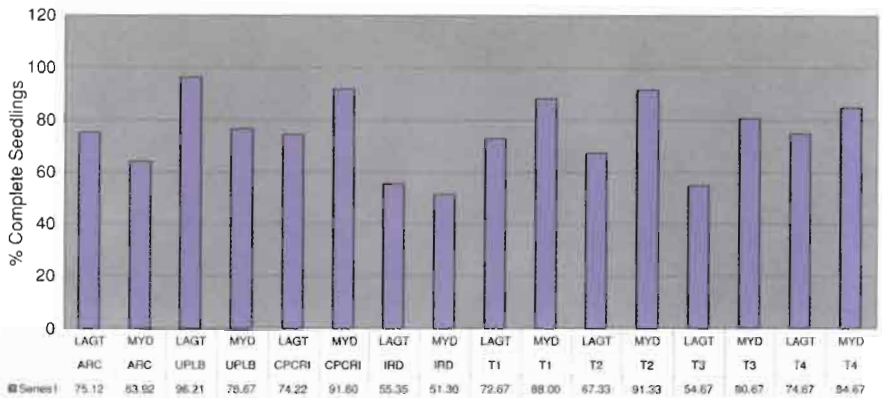
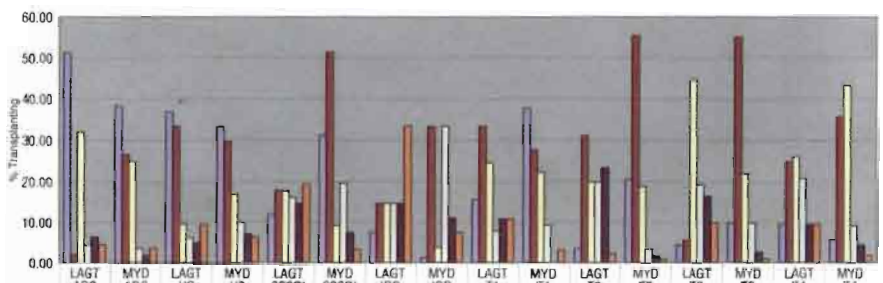


Figure 2. Percentage of complete seedlings of LAGT and MYD seedlings cultured with the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) protocols.



	LAGT ARC	MYD ARC	LAGT UP	MYD UP	LAGT CPCRI	MYD CPCRI	LAGT IRD	MYD IRD	LAGT T1	MYD T1	LAGT T2	MYD T2	LAGT T3	MYD T3	LAGT T4	MYD T4
5 mo	51.06	38.33	36.90	33.33	11.94	31.06	7.40	1.11	15.69	37.69	3.33	20.54	4.17	9.65	9.38	5.83
6 mo	2.12	26.66	33.33	29.72	17.91	51.51	14.81	33.33	33.33	27.69	31.11	55.36	5.56	55.26	25.00	35.83
7 mo	31.91	25.00	9.52	17.11	17.91	9.09	14.81	3.70	24.51	22.31	20.00	18.75	44.44	21.93	26.04	43.33
8 mo	4.25	3.33	5.95	9.90	16.41	19.69	14.81	33.33	7.84	9.23	20.00	3.57	19.44	9.65	20.83	9.17
9 mo	6.38	1.66	4.76	7.20	14.92	7.57	14.81	11.11	10.78	0.00	23.33	1.79	16.67	2.63	9.38	4.17
10 mo	4.25	3.33	9.52	6.30	19.40	3.03	33.33	7.40	10.78	3.08	2.22	0.89	9.72	0.88	9.38	1.67

Figure 3. Monthly percentage of *ex vitro* transplanting of LAGT and MYD seedlings 5 to 10 months after culture initiation using the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) media.

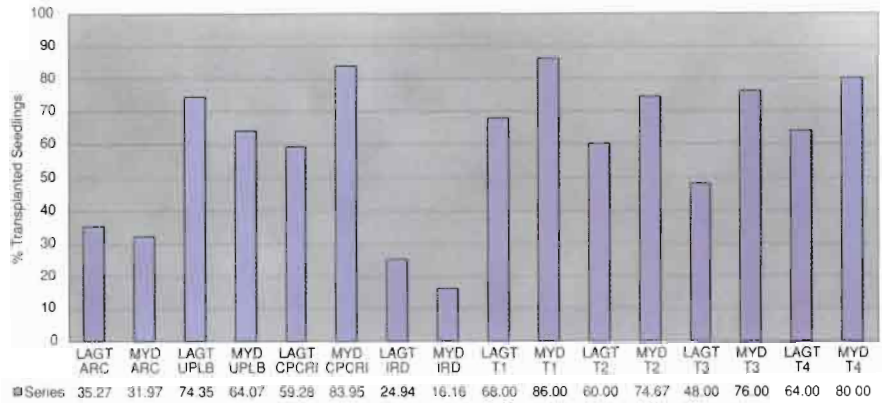


Figure 4. Percentage of transplanted seedlings of LAGT and MYD cultured with the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) protocols.

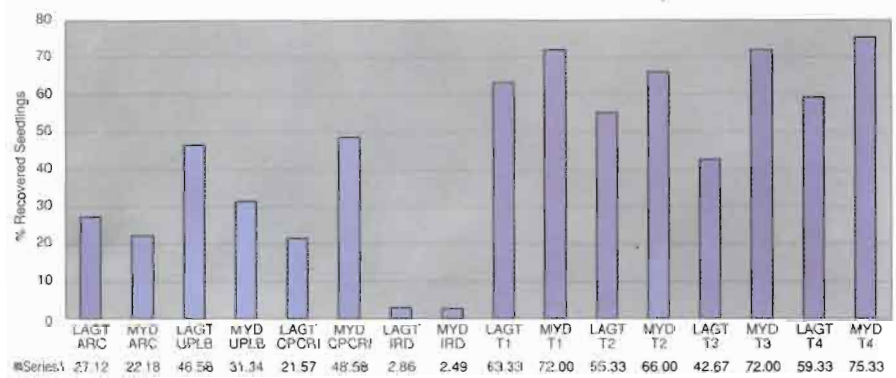


Figure 5. Percentage of recovered seedlings of LAGT and MYD cultured with the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) protocols.

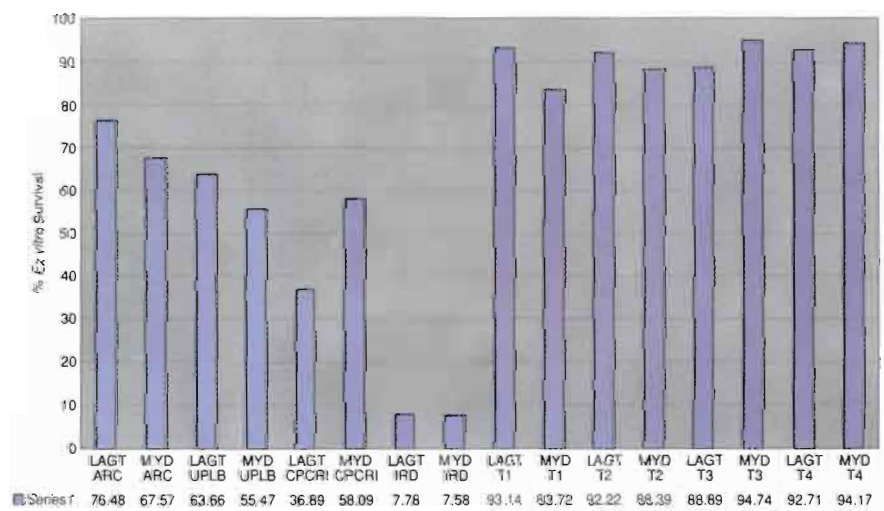


Figure 6. Percentage of *ex vitro* survival of LAGT and MYD seedlings cultured with the PCA-ARC, UPLB, CPCRI, IRD and the hybrid (T1, T2, T3, T4) protocols.

Utilization of embryo culture technology for germplasm conservation: Development of medium term conservation for coconut zygotic embryos in the Philippines

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Background and rationale

Embryo culture technology has many important applications for coconut improvement. It is the only means of rescuing potentially abortive embryos of high value mutant coconuts such as the Makapuno (De Guzman and Del Rosario 1964). It is the basis for *in vitro* screening for drought tolerance (Karunaratne *et al.* 1991) and coconut Cadang Cadang viroid resistance (Rillo *et al.* 1988). Embryo culture is a very useful technique for the transport and safe exchange of germplasm for coconut improvement programmes. The technique would lower the costs of transporting materials and eliminate problems associated with the storage of mature nuts. Embryo culture would also satisfy most phytosanitary requirements that presently preclude any coconut germplasm transfer (Ashburner and Thompson 1993). Furthermore, embryo culture can be the basis for *in vitro* conservation of coconut.

Traditionally, coconut germplasm resources are maintained in field collections. Maintenance under field conditions is very costly and exposes the germplasm to risks such as natural calamities (typhoon, drought, floods, pests and diseases) which may wipe out some accessions or even entire collections. *In vitro* conservation can serve as a back up system for field conservation and as a ready source of materials for germplasm exchange. For long-term *in vitro* conservation for coconut, the ultimate aim is cryopreservation, *i.e.* storage in liquid nitrogen (Assy-Bah and Engelmann 1993). Medium-term conservation for coconut would be most useful for some specific applications such as: 1) *in vitro* collecting missions in distant or isolated places, 2) maintaining materials collected from disease infected areas while waiting for phytosanitary clearance, and 3) the safe exchange of germplasm materials to be brought to designated international or national coconut genebanks, or to be used for coconut breeding programmes.

For medium-term conservation (several months to two years), the aim is to reduce the growth rate in culture so as to extend the interval between subcultures. This can be achieved by modifying the culture environment conditions, particularly by lowering the temperature, e.g. to 10–15°C in the case of banana (Banarjee and De Langhe 1985) and/or by modifying the culture medium composition. In most developing countries where continuous power supply is a problem, modification of the culture medium to reduce growth is a better approach than using low temperature.

Objectives

The main objective of the project was to develop medium-term conservation protocols for coconut zygotic embryos. Specifically, the project aims to:

- Test the efficiency of published coconut embryo culture media and to identify a workable embryo culture protocol that can be used as the basis for culture and recovery of *in vitro* stored materials; and
- Develop a minimal growth protocol based on culture media modification for medium-term storage of coconut zygotic embryos.

Study 1. Validation of the efficiency of published coconut embryo culture protocols

The improvements done to the coconut embryo culture technique by the different tissue culture laboratories resulted in a number of established protocols. At present, four established coconut embryo culture protocols (Annexes 1.1 – 1.4) are being used for different genotypes and under various culture environment conditions.

The objective of this study was to test the efficiency of these published media and to identify a medium, which could be used as a standard protocol for culture and recovery of *in vitro* stored embryos.

The plant materials used were mature embryos (10–12 months after pollination) obtained from seednuts of the cultivar Laguna Tall and Makapuno coconuts. Embryos excised from freshly harvested or stored mature seednuts were surface-sterilized following the procedure developed at the Philippine Coconut Authority, Philippines (Rillo 1995). Solid endosperm cylinders containing the embryos were soaked in detergent, washed in tap water and surface-sterilized in 100% commercial bleach for 20 minutes. Embryos excised from the endosperm cylinders were further sterilized in 10% commercial bleach for one minute and rinsed 3–4 times in sterile water.

Coconut embryos (about 5 mm long) were cultured with five different published culture media (UPLB, PCA, CPCRI 1 and 2, and IRD), while the Makapuno embryos were cultured with the UPLB, PCA and IRD medium. The composition of the five media is as follows:

- UPLB medium, with a pH of 5.6 and containing Eeuwens (Y3) macro and micro nutrients, vitamins and amino acids, sucrose (60 g/L) and activated charcoal (2.5 g/L), was used in a liquid/solid/liquid sequence;
- PCA medium, with a pH of 5.8 and containing Y3 macro and micro nutrients, vitamins and amino acids, sucrose (45 g/L) and activated charcoal (2.5 g/L), was used continuously in the liquid state;
- CPCRI-1 medium with a pH of 5.6 and containing Y3 macro and micro nutrients, vitamins and amino acids, sucrose (60 g/L) + BAP (0.5 mg/L), NAA (0.5 mg/L) and activated charcoal (1.0 g/L), was employed in a solid/solid/liquid sequence.
- CPCRI-2 medium was the same CPCRI-1 medium with an addition of IBA (5.0 mg/L); and
- IRD medium with a pH of 5.5 and containing Murashige and Skoog's macro and micro nutrients, vitamins and amino acids, sucrose (60 g/L) and activated charcoal (2g/L), was used continuously in the liquid state. In all the media, the pH was adjusted using NaOH or HCl.

Embryos were cultured in culture vessels containing 20 ml of medium. Initially, the embryos were kept in the dark for four to six weeks and, after germination, they were transferred to the growth room at 25±2°C under a photoperiod of 16h light/8h dark with a light intensity of 17–35 $mmol.m^{-2}.s^{-1}$. The experiment was set up in two trials. A total of 35 – 40 Laguna Tall embryos and 20 – 24 Makapuno embryos were used. Observations on germination and subsequent growth of embryos were made regularly.

For acclimatization and potting out, the standard procedures used by the Institute of Plant Breeding (IPB), UPLB were followed. Plantlets ready for potting were transferred from the growth room to the main laboratory and then to the greenhouse where they were exposed to natural light for two weeks.

Plantlets were removed from the culture vessels, washed in tap water to remove all medium adhering to the plantlets and roots, and then dipped in fungicide (Benlate, 1 g/L) before they were transplanted in sterilized soil-coir dust mixture (1:1, v/v). The plantlets were covered with plastic bags and exposed to 50% shade. After two weeks, the plastic bags were gradually lifted and then removed completely after four

weeks. Watering was done when needed. Hoagland solution was applied six weeks after potting and then once every two weeks, until the plantlets were fully established. The plantlets were transferred to bigger bags three months after potting.

Study 2. Development of medium-term storage protocol for coconut embryos

Culture media manipulations that were studied included:

- Modification of the medium components (e.g. activated charcoal and sucrose concentration) critical for growth;
- Use of the growth retardant abscisic acid (ABA);
- Adjustments made in the osmotic concentration of the medium by the addition of mannitol or sorbitol; and
- Reduced amount of nutrients available for the embryos.

The plant materials used were mature embryos obtained from seednuts of the cultivars Laguna Tall, Tagnanan Tall, Malayan Yellow Dwarf and Coco Niño. The embryos were excised and cultured following the procedures described in Study 1.

The effects of different minimal growth treatments were studied, such as:

- Sucrose concentration (20, 40, 60, 80, 100 g/L) in the presence or absence of 2.5 g/L activated charcoal;
- ABA concentration (0, 0.1, 0.5, 1.0 and 2.0 mg/L);
- Mannitol or sorbitol concentration (0, 0.05, 0.1, 0.2 and 0.3 M); and
- Reduced strength of nutrient concentration (1x, 1/2x, 1/4x, 1/8x and 1/10x).

All treatments contained Y3 macro and micronutrients, vitamins and amino acids. Except for the experiment on sucrose and activated charcoal, all media were supplemented with 60g/L sucrose and 2.5 g/L activated charcoal.

Embryos were cultured in 15 to 30 ml (depending on the size of vessel) culture medium. Two replications with 10 to 16 embryos per replication were used for each media treatment. All cultures were maintained under a photoperiod of 16h light/8h dark with light intensity of 17–35 $mmol.m^{-2}.s^{-1}$ at 25±2°C. Embryos were kept in storage for six months or longer. All measurements on growth parameters were made during storage of embryos and only the embryos that developed into plantlets were transferred to recovery medium at the end of each storage period.

Results and discussion

Study 1. Validation of the efficiency of published coconut embryo culture protocols

The efficiency of established embryo culture protocols was tested using the Laguna Tall cultivar. The medium developed at CPCRI supported the highest percentage germination (85.7%) of cultured coconut embryos (Table 1). This medium also produced the highest percentage of embryos developing into whole plantlets ready for potting. The UPLB and PCA media showed the same percentage of germination (65.0%). However, the percentage of embryos that developed into whole plantlets was higher with the UPLB medium. The IRD medium gave the lowest germination percentage (57.4%) and whole plantlet development (5.7%).

The average duration *in vitro* to obtain whole plantlet ranged from 32.2 to 34.8 weeks. The shortest duration *in vitro* (26 weeks) was obtained using the UPLB medium. The percentage of survival after potting ranged from 0 to 14.3% of inoculated embryos. Plantlets produced using the IRD medium did not survive after potting.

Table 1. Response of Laguna Tall embryos on five published embryo culture protocols

Parameters	Protocols tested in the current project				
	Own (before start of project)	UPLB	PCA	CPCRI-1	CPCRI-2	IRD
Contamination (%)		25.0	22.5	34.3	40.0	28.6
Germination (%)		65.0	65.0	85.7	74.3	57.4
Whole plantlets *in vitro** (%)		27.4b	15.0c	40.0a	28.6b	5.7c
Ave. duration *in vitro* (weeks) to obtain whole plantlets*		32.8	34.8	33.2	34.8	32.8
Survival after nursery (%)		12.5	10.0	14.3	14.3	0
Survival after transfer to field (%)		–	–	–	–	–

Note: All percentages were expressed as a function of the number of embryos inoculated.
*Refers to whole plantlet with 1 to 2 fully expanded leaves and with extensive root system.
Means with different letters are significantly different at P=0.01.

For Makapuno embryo culture (Table 2), the IRD medium gave the highest percentage of germination (37.0%), although the highest percentage of surviving whole plantlets was obtained using the UPLB medium (16.7%). Only 12.5 % of these whole plantlets managed to survive potting and establishment in the soil.

Table 2. Response of Makapuno embryos on three published embryo culture protocols

Parameters	Protocols tested in the current project			
	Own (before the start of project)	UPLB	PCA	IRD
Number of embryos inoculated	None			
Trial 1		19.0	16.0	23.0
Trial 2		5.0	4.0	4.0
Total		24.0	20.0	27.0
Contamination (%)		54.2	50.0	55.6
Germination (%)		29.2	25.0	37.0
Whole plantlets *in vitro** (%)		16.7	0	7.4
Ave. duration *in vitro* (weeks) to obtain whole plantlets*		31.7	–	35.5
Survival after nursery (%)		12.5	–	0
Survival after transfer to field (%)		–	–	–

Note: All percentages were expressed as a function of the number of embryos inoculated.
*Refers to whole plantlet with 1 to 2 fully expanded leaves and with extensive root system.

Although germination percentage was generally high (about 57.4 to 85.7% for Laguna Tall and 25 to 37% for Makapuno embryos), the percentage of embryos developing into whole plantlets with 1–2 fully expanded leaves and extensive root system ready for potting was low (5.7 to 40% and 0 to 16.7% for Laguna Tall and Makapuno, respectively). Some of the germinated embryos exhibited abnormal growth with the formation of shoot without roots, very limited elongation of the shoot with extensive roots or excessive enlargement of the haustorium (Fig. 1).

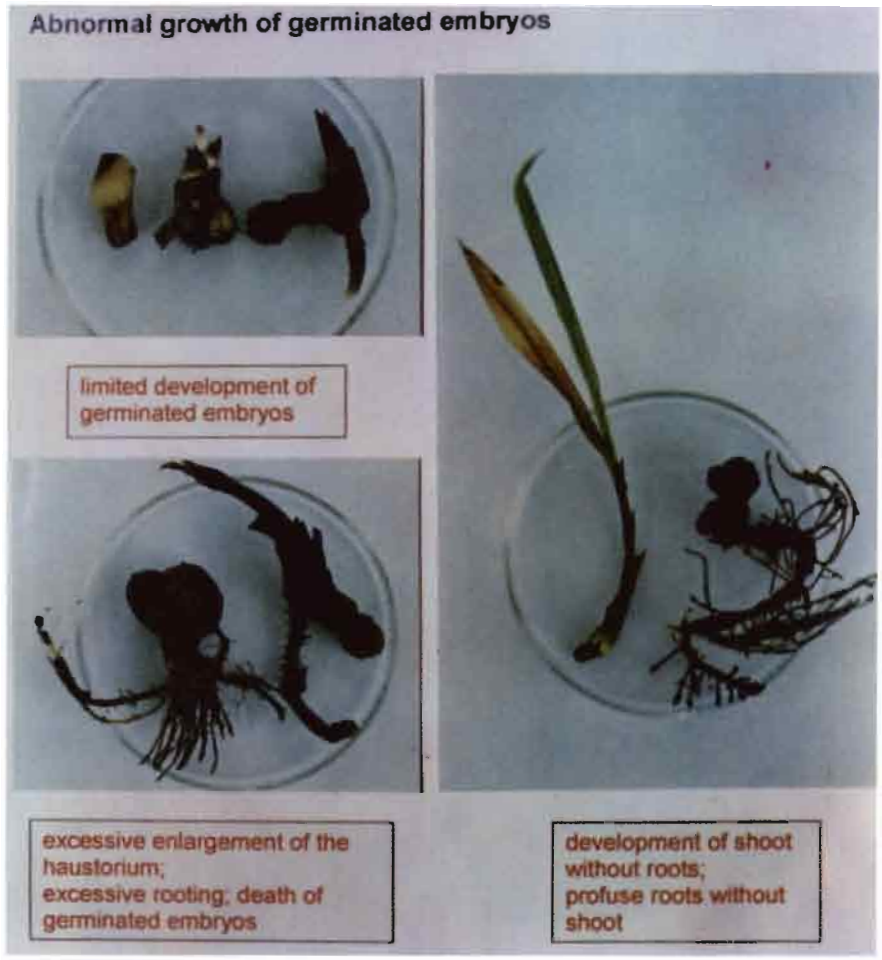

Fig. 1. Germinated embryos showing abnormal growth.

Some germinated embryos did not develop further even after regular transfers to fresh medium. These results indicated that whole plantlet development was a critical stage in the culture of coconut embryos. Furthermore, high contamination, particularly during subcultures, contributed to the low efficiency of the established techniques. Coconut embryo culture requires extended culture duration and regular transfers to fresh medium. For such a culture system, contamination was a major problem and the contamination was higher in protocols requiring changes in the state of the medium (*i.e.* solid to liquid or liquid to solid) or when transferring plantlets with well-developed leaves and roots.

Study 2. Development of medium-term storage protocol for mature coconut zygotic embryos

Effect of sucrose in the presence or absence of activated charcoal

The addition of activated charcoal in the medium was critical for the survival, germination and subsequent growth of cultured embryos of Laguna Tall, Malayan Yellow Dwarf and Coco Niño (Table 3). Absence of activated charcoal resulted in a significant reduction in germination of embryos, even in the medium with higher sucrose concentration. In some instances, the absence of activated charcoal in the medium resulted in the death of cultured embryos, particularly after a prolonged culture storage (after 9 months). For the three cultivars, Laguna Tall, Malayan Yellow Dwarf and Coco Niño, increasing the concentration of sucrose in the presence of activated charcoal resulted in a corresponding increase in germination during storage. The highest germination response was obtained with 60 – 100 g/L sucrose in the presence of activated charcoal.

As a minimal growth treatment, the use of low concentration of sucrose (20g/L) in combination with activated charcoal showed promising results. This treatment combination showed lower germination percentage and limited shoot and root growth of embryos germinated during storage (Fig. 2, Table 3). Surviving plantlets after nine months of storage with this treatment combination resumed normal growth upon transfer to recovery medium (Fig. 3). Further observations on embryos cultured on this minimal growth treatment are needed to fully assess the recovery of stored embryos.

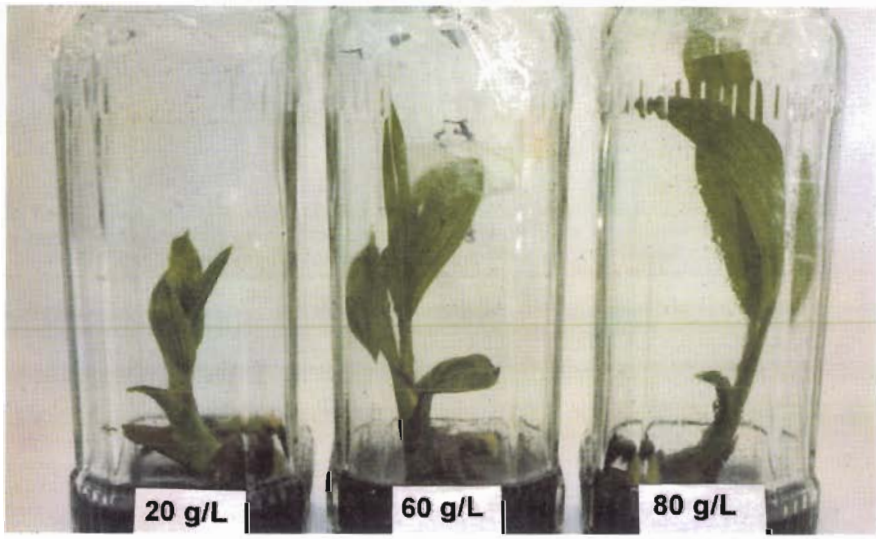

Fig. 2. Growth and development of coconut embryos cultured on Y3 medium supplemented with different concentrations of sucrose in the presence of activated charcoal.

Table 3. Effect of sucrose concentration in the presence or absence of activated charcoal in the medium on survival, total germination and shoot and root elongation of cultured embryos of tall and dwarf genotypes after nine months of storage

Cultivar	Growth parameter	Without activated charcoal					With 2.5 /L activated charcoal					Mean
		Sucrose concentration (g/L)										
		20	40	60	80	100	20	40	60	80	100	
Laguna Tall	% Total germinated embryos	10.0	32.5	28.6	14.0	20.0	46.5	32.5	58.3	70.0	80.0	**39.2a**
	% Germinated embryos with shoot and root elongation	0	0	28.6	14.0	20.0	46.5	32.5*	55.0	60.0	66.6	
	% Dead embryos	80.0	67.5	71. 4	86	60.0	0	0	0	10	20	
Malayan Yellow Dwarf	% Total germinated embryos	0	42.8	26.6	33.3	12.0	80.0	71.4	71.4	65.0	80.0	**48.3a**
	% Germinated embryos with shoot and root elongation	0	21.4	0	0	0	60.0	28.6	71.4*	30.8*	40.0	
	% Dead embryos	100.0	75.0	64.0	50.0	88.0	0	0	0	10.0	20.0	
Coco Niño	% Total germinated embryos	0	25.0	33.3	18.3	20.0	50.0	75.0	60.0	66.7	71.4	**41.9a**
	% Germinated embryos with shoot and root elongation	0	8.3	0	9.0	20.0	33.3	50.0	40.0	66.7	57.1	
	% Dead embryos	80.0	50.0	66.0	72.0	80.0	17.0	25.0	20.0	16.0	14.0	
Mean	**%Total germinated embryos**	**3.3d**	**33.4c**	**29.9c**	**21.9c**	**17.5cd**	**58.3b**	**59.6b**	**63.2ab**	**67.2a**	**77.1a**	

*Whole plantlets obtained from this treatment were potted in the soil.

Means with the same letter are not significantly different at P=0.05.

Observations were taken from two replications with 10 to 15 embryos per replication. Measurements were taken from remaining clean cultures in storage.

Fig. 3. Recovery and normal growth of embryos after nine months of storage on medium supplemented with 20g/L sucrose + activated charcoal upon transfer to standard Y3 medium.

Effect of ABA

Addition of ABA at 0.1 to 2.0 mg/L showed no significant growth inhibitory effect (Fig. 4 and Table 4) for cultivars Laguna Tall and Malayan Yellow Dwarf. The addition of ABA, instead, further enhanced germination and subsequent growth of germinated embryos. Thus, in some ABA treatments (Table 4), whole plantlets were obtained in about 26 weeks of culture (without transfer) on the same medium. Plantlets cultured in some ABA treatments were potted out several weeks earlier than the control treatment.

For coconut embryos, ABA treatment may not be effective in reducing growth *in vitro*. Possibly, the concentrations tested were not high enough to inhibit growth. Higher ABA concentrations might be needed to inhibit growth.

Fig. 4. Growth and development of coconut embryos cultured on Y3 medium supplemented with different concentrations of ABA.

Table 4. Effect of ABA concentration on germination, and shoot and root elongation of cultured embryos of tall and dwarf coconut genotypes after nine months of storage

Cultivar	Growth parameter	ABA concentration (mg/L)					Mean
		0	0.1	0.5	1.0	2.0	
Laguna Tall	% Total germinated embryos	70.5	68.9	76.4	71.25	63.5	**70.1a**
	% Germinated embryos with shoot and root elongation	58.3	58.3*	76.4*	52.9*	55.0*	
Malayan Yellow Dwarf	% Total germinated embryos	77.1	84.6	62.5	90.0	60.0	**74.8a**
	% Germinated embryos with shoot and root elongation	57.1*	60.0*	62.5*	50.0*	57.1*	
Mean	**% Total germinated embryos**	**73.8a**	**76.7a**	**69.4a**	**80.6a**	**61.7a**	

*Whole plantlets obtained from this treatment were potted in the soil.
Means with the same letter are not significantly different at P=0.05.
Observations were taken from two replications with 10 to 16 embryos per replication. Measurements were taken from remaining clean cultures in storage.

Effect of osmotic adjustment induced by mannitol or sorbitol

Addition of osmotically active substances like sorbitol or mannitol significantly affected the germination of cultured coconut embryos of Tagnanan Tall, Laguna Tall and Malayan Yellow Dwarf (Tables 5 and 6).

As a minimal growth treatment, mannitol showed greater inhibitory effect than sorbitol, particularly at the 0.3 M concentration (Fig. 5). Further observations on the recovery of embryos stored on this minimal growth treatment are needed.

Sorbitol, at all concentrations tested, enhanced the germination of cultured embryos of Tagnanan Tall, Laguna Tall and Malayan Yellow Dwarf (Table 5). As a growth-limiting agent, sorbitol at 0.3 M significantly reduced shoot elongation but not root growth (Fig. 5). Higher concentrations of mannitol (0.2 or 0.3M) significantly reduced germination during storage as well as the subsequent growth of germinated coconut embryos (Table 6 and Fig. 6). Lower concentrations of sorbitol or mannitol (0.05 to 0.1M) however, induced earlier germination and faster growth of germinated embryos during storage. Some embryos cultured on low concentrations reached the whole plantlet stage earlier than the control treatment.

Table 5. Effect of sorbitol concentration on germination and shoot and root elongation of cultured embryos of tall and dwarf coconut genotypes after six months of storage

Cultivar	Growth parameter	Sorbitol concentration (M)					Mean
		0	0.05	0.1	0.2	0.3	
Tagnanan Tall	% Total germinated embryos	81.8	82.6	76.4	82.6	77.1	**80.1ab**
	% Germinated embryos with shoot and root elongation	63.3*	70.0*	64.0*	82.6	58.0	
	% Germinated embryos with shoot and root elongation	38.0*	55.0	50.0	44.4	20.0	
Malayan Yellow Dwarf	% Total germinated embryos	76.4	83.3	92.8	87.5	72.0	**82.4a**
	% Germinated embryos with shoot and root elongation	60.7*	83.3*	80.0	80.0	17.0	
Mean	**% Total germinated embryos**	**76.7a**	**82.8a**	**79.7a**	**74.8a**	**62.5a**	

*Whole plantlets obtained from this treatment were potted in the soil.
Means with the same letter are not significantly different at P=0.05.
Observations were taken from two replications with 10 to 16 embryos per replication. Measurements were taken from remaining clean cultures in storage.

Table 6. Effect of mannitol concentration on germination and shoot and root elongation of cultured embryos of tall and dwarf coconut genotypes after six months of storage

Cultivar	Growth parameter	Mannitol concentration (M)					Mean
		0	0.05	0.1	0.2	0.3	
Tagnanan Tall	% Total germinated embryos	81.8	80.0	80.0	43.7	28.6	**62.8a**
	% Germinated embryos with shoot and root elongation	63.3*	70.0*	66.0*	31.4	14.0	
Laguna Tall	% Total germinated embryos	71.9	71.4	73.2	43.5	42.9	**60.6a**
	% Germinated embryos with shoot and root elongation	38.0*	42.8*	46.4	43.5	42.9	
Malayan Yellow Dwarf	% Total germinated embryos	76.4	1.2	94.7	79.4	58.3	**78.0a**
	% Germinated embryos with shoot and root elongation	60.7*	68.7*	57.8	21.0	5.8	
Mean	**%Total germinated embryos**	**76.7a**	**77.5a**	**82.6a**	**55.5b**	**43.2b**	

*Whole plantlets obtained from this treatment were potted in the soil.
Means with the same letter are not significantly different at P=0.05.
Observations were taken from two replications with 10 to 15 embryos per replication. Measurements were taken from remaining clean cultures in storage.

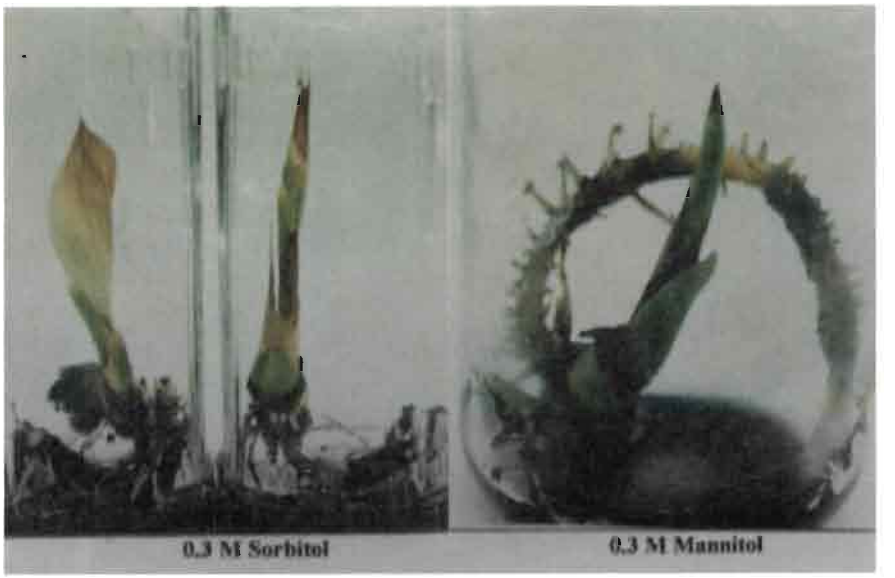

Fig. 5. Growth and development of coconut embryos cultured on Y3 medium supplemented with 0.3 M sorbitol or mannitol.

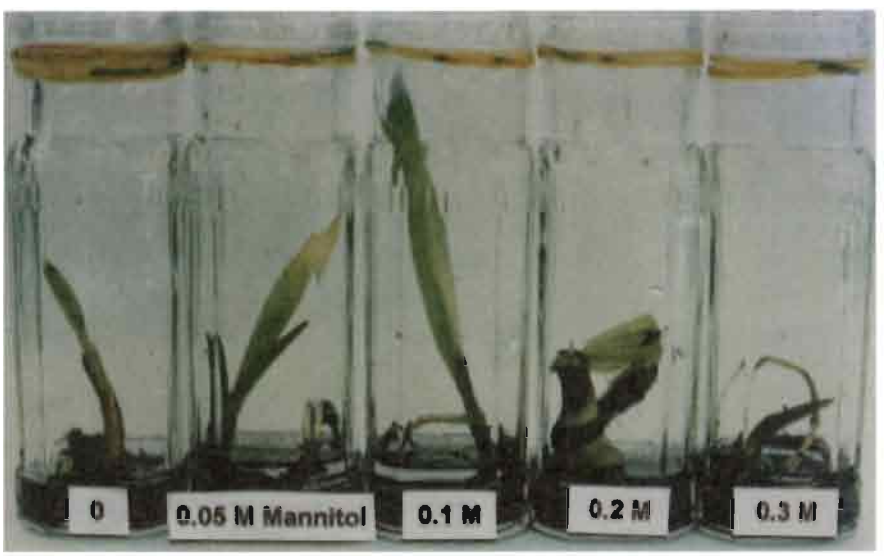

Fig. 6. Growth and development of coconut embryos cultured on Y3 medium supplemented with different concentrations of mannitol.

Effect of reduced nutrient concentration

Malayan Yellow Dwarf embryos cultured on medium with reduced nutrients showed significant decrease in germination percentage during storage compared to embryos cultured on standard medium (Table 7). Embryos cultured on full strength (1x) and half strength (1/2x) Y3 medium showed no significant differences in germination percentage while embryos cultured on reduced amounts of nutrients (1/4x to 1/10x) exhibited significantly lower percent germination (32.5% to 45.0%). Root and shoot growth of embryos was also inhibited with reduced concentrations of Y3 medium. Further observations on the recovery and subsequent growth of embryos are needed to assess the growth limiting effects of reduced amount of nutrients.

Table 7. Effect of different basal nutrient concentrations on germination and subsequent growth of cultured embryos of Malayan Yellow Dwarf after six months of storage

Growth	Basal nutrient concentration (strength)				
parameter	1x	1/2x	1/4x	1/8x	1/10x
Total germinated embryos (%)	70.0 a	65.0a	45.0ab	32.5b	34.0b
Germinated embryos showing shoot and root elongation (%)	70.0	65.00	36.0	25.4	26.9

Means with the same letter are not significantly different at P=0.05

Observations were taken from two replications with ten embryos per replication. Measurements were taken from the remaining clean cultures.

Constraints, lessons learned and recommendations

1. The efficiency of the published embryo culture protocols was generally low. Two major factors might have contributed to the low efficiency of the established techniques. The first was the abnormal development of germinated embryos. Moreover, these abnormalities (Fig. 1) were observed with all media tested, indicating some inherent differences in the cultured embryos. Further studies on embryo culture should focus on achieving normal plantlet development and to identify the possible sources of variations or differences in *in vitro* response. The second major constraint was the high contamination occurring during the initial culture (particularly for stored nuts like the Makapuno nuts bought from the market or Makapuno embryos obtained from the processing plant), the subculture process (particularly during transfer of embryos/plantlets from different states of media) or in transferring plantlets with well-developed roots and leaves. Contamination is a problem in most tissue culture laboratories, especially in the tropical developing countries.

2. For medium-term storage experiments, the non-availability of a higher number of embryos was a problem. The UPLB coconut genebank could provide only a limited number of embryos and most of the embryos used in the project were obtained from the PCA, Zamboanga City and other coconut research stations. This problem caused some delays in setting up some experiments. All future activities requiring embryos from different genotypes should be fully coordinated with national coconut genebanks so that they can provide more embryos of different genotypes required for the project.

3. The best medium for coconut embryo culture can be adapted and used as the basis for culture recovery of stored embryos and for other coconut embryo culture research at IPB. The techniques and experiences learned from this project would greatly help IPB in future coconut tissue culture researches.

4. To fully assess the response of embryos to the different media manipulations being studied for medium-term conservation, a longer observation period (*i.e.* six months or longer) would be required. Observations on embryos cultured on promising minimal growth treatments (low level of sucrose + activated charcoal and mannitol treatment) and other treatment combinations should be continued.

5. The minimal growth treatments identified in the project should be further evaluated using a wide range of genotypes and a higher number of embryos (stored at different durations). The effectiveness of minimal growth treatment should be evaluated in terms of recovery after storage.

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Increasing the efficiency of embryo culture technology to promote coconut germplasm collecting, conservation and exchange in Indonesia

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Background and rationale

Embryo culture is an important technique for coconut germplasm collecting, conservation and exchange. To safeguard the coconut industry, quarantine procedures regulate the exchange of germplasm to ensure that they are not contaminated by pests or lethal diseases. However, germplasm exchange in the form of aseptic cultures is permissible. Different protocols have been published for embryo culture *in vitro* and *ex vitro*, but the success rate varies among laboratories. It is therefore necessary to upgrade embryo culture technology to make it a safe and efficient tool for exchanging germplasm. Growth regulators play an important role in the success of embryo culture in terms of maturation and germination of embryos. In particular, the role of abscisic acid (ABA) and gibberellic acid (GA_3) needs to be studied. The protocols used for *in vitro* culture of embryos and *ex vitro* culture of plantlets have to be validated in order to achieve the highest possible success rate, from the germination of embryos to *ex vitro* establishment of plantlets.

Under COGENT, Indonesia hosts the International Coconut Genebank for Southeast Asia (ICG-SEA) and as such, the country will be a hub for the exchange of coconut embryos among COGENT member countries. Therefore, the Research Institute for Coconut and Palmae (RICP) of Indonesia participated in Phase I of the DFID-funded research project on "Increasing the Efficiency of Embryo Culture Technology to Promote Coconut Germplasm Collecting, Conservation and Exchange" sponsored by COGENT.

Objectives

The objectives of the RICP research are to:

1. Upgrade the equipment, supplies and operational potentials of the embryo culture laboratory at RICP;
2. Compare four embryo culture protocols (Annexes 1.1 to 1.4) that were identified at the International Embryo Culture Workshop in the Philippines held from 28 to 30 October 1997;
3. Evaluate the response of different coconut varieties to *in vitro* embryo culture protocols; and
4. Evaluate the effect of GA_3 and ABA on embryo germination.

Materials and methods

A. Evaluation of four embryo culture protocols using three coconut cultivars

Three varieties, namely the Rennel Tall (RLT, introduced), Mapanget Tall (DMT, local) and Nias Yellow Dwarf (GKN, local) were used to compare the four promising embryo

culture protocols. Mature embryos of RLT and GKN were collected from Tenga, Minahasa, North Sulawesi, whereas the DMT embryos were collected from Kima Atas Experimental Garden, RICP, Manado, also in North Sulawesi.

The embryos were cultured in the laboratory according to the procedures described in each protocol. *Ex vitro* establishment was also done following these protocols.

The research was conducted following a 4 x 3 factorial experiment with three replications, and with 20 embryos per treatment combination. The parameters observed included germination, contamination, number of leaves/*in vitro* plant and survival in nursery.

B. Evaluation of varietal response to two different embryo culture protocols

Results of the first experiment indicated that the CPCRI and UPLB protocols were the most efficient for stimulating growth of RLT, DMT and GKN embryos. Based on this, the two protocols were used to further evaluate the varietal response, using embryos of these three coconut varieties.

This research was conducted following a 2 x 3 factorial experiment with three replications, with 20 embryos per treatment combination. The parameters observed included germination, contamination, number of leaves/*in vitro* plant, number of roots/*in vitro* plant, number of transplanted seedlings and survival in nursery.

C. Effect of GA_3 on embryo maturation and germination

DMT embryos of three different ages (9, 10 and 11 months old) were cultured with five different GA_3 concentrations: 0, 20, 40, 60, and 80 ppm.

The research was conducted following a 3 x 5 factorial experiment resulting in 15 treatment combinations. Each treatment, consisting of 20 embryos, was replicated three times. The parameters observed were the same as in the previous experiment.

D. Effect of GA_3 and ABA on embryo maturation and germination

DMT embryos of three different ages were also used. The same concentrations of GA_3 as in the previous experiment were employed with supplementary concentration of ABA at a constant level of 0.075 ppm.

This research was conducted following a 5 x 3 factorial experiment with three replications using 20 embryos per treatment. The parameters observed were the same as in the previous experiment.

Results and discussion

A. Evaluation of four embryo culture protocols using three coconut varieties

Germination

Three months after inoculation, the RLT, DMT and GKN embryos showed different responses in terms of germination with the four media tested. The highest germination rate was obtained with the CPCRI protocol (86.11%), followed by UPLB protocol (83.89%), both of which were significantly different from those achieved with the IRD and PCA protocols (Table 1). This might be related to the higher level of sugar and iron as well as the presence of agar in the CPCRI and UPLB protocols. In addition, the UPLB medium also contained a high level of iron.

Sugar is a source of energy that enhances the growth of embryos. Embryos could grow better when cultured on media containing agar, as in the CPCRI media. Culture of embryos on solid medium in the dark during the germination phase and in liquid

medium under light conditions during the further growth phase resulted in a higher germination and plantlet production rate.

In the liquid medium, the embryos increased in size but germinated slowly. This lowered the germination rate in the liquid medium.

Contamination

Contamination was generally high but consistently and significantly lower when using the CPCRI protocol (10–25 %, Table 1). Contamination highly depends on the sanitary status of the laboratory, the equipment available, and the expertise of workers.

Table 1. *In vitro* and *ex vitro* growth of coconut embryos and seedlings of the RLT, DMT and GKN varieties using the PCA, UPLB, CPCRI and IRD protocols

Protocol	Coconut Variety	Germination (%)	Contamination (%)	No. of		Transplanted seedlings (%)	Surviving seedlings in screen-house (%)
				Leaves/*in vitro* plant	Roots/*in vitro* plant		
PCA	RLT	71.67 b	46.67 e	3.67 bc	3.67 d	38.33 cd	26.67 d
	DMT	66.67 a	48.33 e	3.67 bc	3.67 d	41.67 e	28.33 d
	GKN	73.33 b	21.67 b	4.00 c	3.67 d	36.67 c	25.00 cd
UPLB	RLT	85.00cd	41.67 d	3.67 bc	3.67 d	50.00 h	35.00 e
	DMT	80.00 c	45.00 de	3.00 a	3.00 bc	45.00 f	33.33 de
	GKN	86.67 d	30.00 c	4.00 c	4.00 d	63.33 h	35.00 e
CPCRI	RLT	86.67 d	25.00 b	3.33 a	2.67 a	40.00 de	16.67 b
	DMT	83.33 cd	20.00 b	3.00 a	2.00 a	41.67 e	15.00 b
	GKN	88.33 d	10.00 a	3.00 a	2.00 a	48.33 g	20.00 bc
IRD	RLT	66.67 a	48.33 e	3.33 a	3.33 e	30.00 b	18.33 b
	DMT	60.00 a	33.33 c	3.00 a	3.00 bc	26.67 a	8.33 a
	GKN	68.33 b	43.33 de	3.33 a	3.33 c	25.00 a	6.67 a
	CV (%)	8.05	11.85	19.77	22.40	9.22	31.58

Note: Numbers followed by different letters are significantly different at the 5% level based on Least Significant Difference (LSD).
RLT = Rennel Tall; DMT = Mapanget Tall/Local Tall; GKN = Nias Yellow Dwarf/Local Dwarf

Data were derived using the following formula:

$$\text{Contamination (\%)} = \frac{\text{No. of contaminated embryos}}{\text{No. of inoculated embryos}} \times 100$$

$$\text{Transplanted seedlings (\%)} = \frac{\text{No of transplanted seedlings}}{\text{No. of inoculated embryos}} \times 100$$

$$\text{Survival in screenhouse (\%)} = \frac{\text{No. of surviving seedlings}}{\text{No. of inoculated embryos}} \times 100$$

Number of leaves and roots

Embryos cultured with the UPLB and CPCRI protocols produced plantlets earlier than those with the PCA and IRD protocols. The average duration of *in vitro* culture to obtain a whole plantlet was different between the tall and the dwarf varieties. Embryos of RLT and DMT produced seedlings within 27–28 weeks and GKN within 24–26 weeks.

Leaf production of plantlets of the three varieties was higher with the PCA and UPLB protocols, compared to the CPCRI and IRD protocols (Table 1). With the PCA and UPLB protocols, the plantlets produced more roots, so the plantlets grew better and produced more leaves.

Primary root formation was observed four weeks after culture initiation on the four media. Root formation was highest with the PCA and UPLB media (Table 1). The number of roots/*in vitro* plant was lower (2.00–2.67) with the CPCRI medium compared to the three other media.

Ex vitro survival

The seedlings were transplanted in the screenhouse six months after the initial culture of GKN embryos and seven months after for the RLT and DMT.

The seedlings transplanted had balanced shoot and root development. The percentage of transplanted seedlings was based on the number of inoculated embryos. Embryos from the UPLB protocol produced the highest percentage of transplanted seedlings at 45–63.33% (Table 1).

Survival rate of seedlings in the screenhouse was based on the number of surviving seedlings of inoculated embryos. The highest survival rate (33.33–35.00%) was found with the UPLB protocol, followed by PCA, CPCRI and IRD (Table 1).

Embryos cultured with the UPLB protocol produced good roots and shoots, which explained why transplanted seedlings survived better with this protocol. Although the germination percentage of embryos with the PCA protocol was lower than that of the CPCRI, embryos cultured with the PCA protocol produced good roots and shoots. As a result, the number of transplanted and surviving seedlings with the PCA protocol was also high.

B. Evaluation of varietal response to two different embryo culture protocols

Germination

Embryos of the RLT, DMT and GKN gave a good response in terms of germination to both protocols tested (Table 2). In this experiment, the germination percentage of DMT embryos with the two protocols increased from 80.00% (UPLB) and 83.67% (CPCRI) in the first experiment (Table 1) to 83.67% (UPLB) and 86.33% (CPCRI) in the second experiment.

Table 2. *In vitro* and *ex vitro* growth of embryos and seedlings of the RLT, DMT and GKN varieties using the UPLB and CPCRI protocols

Protocol	Coconut Variety	Germination (%)	Contamination (%)	No. of		Transplanted seedlings (%)	Surviving seedlings in screen-house (%)
				Leaves/*in vitro* plant	Roots/*in vitro* plant		
UPLB	RLT	84.67 a	15.00 a	3.67 a	4.00 b	63.33 c	40.00 c
	DMT	83.67 a	15.00 a	3.67 a	4.00 b	50.00 b	38.33 c
	GKN	86.67 a	18.33 b	3.00 a	3.33 b	66.67 c	46.67 d
CPCRI	RLT	86.67 a	20.00 b	3.00 a	2.33 a	50.00 b	28.33 b
	DMT	86.67 a	15.00 ab	3.00 a	2.33 a	43.33 a	20.00 a
	GKN	88.33 a	15.00 b	3.00 a	2.00 a	51.67 b	25.00 b
	CV (%)	8.05	11.85	19.77	22.40	9.22	31.58

Note : Numbers followed by different letters are significantly different at the 5% level based on LSD.

Contamination

Compared with the first experiment, contamination of embryos dropped from 10-48% (Table 1) to 15–20% in the second experiment (Table 2). This was due to improvements in the equipment and skills of workers.

Number of leaves and roots/*in vitro* plant

The three varieties produced the same number of leaves with both protocols, *i.e.* about three leaves per *in vitro* plant (Table 2). Leaves started to appear eight months after culture initiation, though only leaves that were green at that time were counted.

Root growth in the RLT, DMT and GKN embryos was slow with the CPCRI protocol. The shoots were produced earlier than the roots, so that the embryos were not able to absorb nutrients from the media quite well. With the UPLB protocol, roots were generally formed earlier than shoots. Most likely, the capability of embryos to absorb nutrients was higher due to the presence of roots. *In vitro* plantlets with good growth of shoots and roots were produced on this medium.

Ex vitro survival

The percentage of seedlings of the three varieties was higher (50.00–66.67%) with the UPLB protocol than with the CPCRI protocol (Table 2). This was due to the better growth of *in vitro* plantlets with the UPLB protocol compared to the CPCRI protocol. By contrast, although embryo germination with the CPCRI protocol was high, the formation of roots was low; therefore, the number of transplanted seedlings that survived was lower with the CPCRI than with the UPLB and PCA protocols.

The low survival rate of seedlings produced with the IRD protocol might be a result of insufficient macronutrient concentration, especially chlorine. The UPLB, PCA and CPCRI protocols used a nutrient-rich modified Y3 medium, whereas the IRD protocol used a modified MS medium with lesser nutrients.

The survival rate of seedlings produced with the UPLB protocol after transfer to the screenhouse was also high. It could be concluded that when seedlings are healthy and vigorous when transplanted, they could survive well under *ex vitro* conditions. Another reason could be the high concentration of iron in the UPLB medium. Iron is required for synthesis of chlorophyll and is part of the structure of cytochromes and other molecules that are important in electron transfer (Anonymous 1984).

C. Effect of GA_3 on embryo maturation and germination

Germination

Embryo germination was observed three months after culture initiation. The results showed that when the medium was supplemented with GA_3, regardless of concentration, immature embryos germinated as fast as mature ones (10 and 11 months old) (Table 3). This indicated that GA_3 stimulated germination of immature embryos. Generally, for seeds of monocotyledonous species like coconut, GA_3 can be used effectively to stimulate germination of dormant seed (Moore 1979). Gibberellic acid enhances germination in certain coconut genotypes (Krishnamoorthy 1975, in Gardner *et al.* 1985).

Immature embryos are considered physiologically dormant (Gardner *et al.* 1985). A deficiency of growth-promoting substances or the lack of the proper balance between hormones could cause embryo dormancy (Krishnamoorthy 1981). Most dormancy mechanisms could be broken by growth promoting substances such as GA_3 (Wattimena 1987). Maturation and ripening as well as fruit-setting and growth of some fruits are subject to GA_3 control (Leopold & Kriedemann 1975).

In this experiment, contamination was low in all varieties, ranging between 10.67 and 38.33% (Table 3).

Table 3. Germination of immature and mature DMT embryos in UPLB medium supplemented with GA3, and subsequent development of seedlings *in vitro* and *ex vitro*

Concentration of GA_3 (ppm)	Age of embryos (months)	Germination (%)	Contamination (%)	Transplanted seedlings (%)	Surviving seedlings in screenhouse (%)
0	9	58.33 a	13.22 bc	18.33 a	6.67 a
	10	83.67 d	12.67 abc	23.33 a	13.33 bc
	11	83.33 d	12.33 abc	55.00 d	51.67 f
20	9	80.00 b	38.33 e	23.33 a	8.33 ab
	10	86.67 d	11.33 ab	30.00 b	16.67c
	11	83.33 d	10.67 a	55.00 d	51.67 f
40	9	83.33 e	21.67 d	31.67 b	13.33 bc
	10	96.67 f	11.67 ab	35.00 b	18.33 cd
	11	81.67 c	11.67 ab	53.33 d	51.67 f
60	9	88.33 e	20.00 d	46.67 c	16.67 c
	10	93.33 g	14.67 c	48.33 c	23.33 d
	11	81.67 c	11.33 ab	55.00 d	50.00 e
80	9	83.33 d	13.67 bc	46.67 c	15.00 bc
	10	81.67 c	13.33 bc	46.67 c	23.00 d
	11	80.00 b	10.33 a	51.67 d	51.67 f
CV (%)		7.89	3.87	18.44	26.65

Note: Numbers followed by different letters are significantly different at the 5% level of LSD.

Ex vitro survival

Although immature embryos germinated as fast as mature embryos in media supplemented with GA_3, they resulted in a lower percentage of transplanted seedlings (23.33-46.67%) than the mature embryos (51.67–55.00%) (Table 3). The lower percentage of transplanted seedlings from immature embryos was caused primarily by a higher percentage of germinated embryo browning (43.67%) than the immaturity of the embryos (less than 11.00% for 10 and 11 month-old embryos).

In medium supplemented with 60 ppm GA_3, germination reached 88.33%, while percentage of transplanted seedlings reached 46.67%. Increasing GA_3 concentration to over 60 ppm significantly reduced germination, though percentage of transplanted seedlings was not different from that noted of a medium with 60 ppm GA_3. Mature embryos, treated with GA_3 at all concentrations, had the highest survival rate.

D. Effect of GA_3 and ABA on maturation and germination of embryos

Increasing the GA_3 concentration from 0 to 60 ppm in the presence of 0.075 ppm ABA in the medium raised germination of immature embryos from 36.67% to 86.67%, which was significantly higher than that of the other treatments (Table 4).

Contamination was even lower (11.33–14.67 %) compared with previous experiments.

Table 4. Germination of immature and mature embryos of the DMT variety in UPLB medium supplemented with ABA and GA3, and subsequent development of seedlings *in vitro* and *in vivo*

Concentration of		Age of embryos (months)	Germination (%)	Contamination (%)	Transplanted seedlings (%)	Surviving seedlings in screenhouse (%)
ABA (ppm)	GA_3 (ppm)					
0.075	0	9	36.67 a	14.67 d	20.67 a	10.00 a
		10	73.33 bcd	13.33 bcd	26.67 cd	15.00 a
		11	78.33 bcd	13.67 bcd	36.33 hi	33.33 b
	20	9	61.67 b	12.33 bcd	20.33 ab	8.33 a
		10	66.67 b	14.67 d	28.33 cde	15.00 a
		11	76.67 cde	12.33 abc	40.00 ij	33.33 b
	40	9	61.67 b	12.67 abc	25.33 bc	11.67 a
		10	78.33 de	13.67 bcd	31.67 efg	13.33 a
		11	78.33 de	11.33 a	41.67 j	31.67 b
	60	9	86.67 f	13.67 bcd	30.67 defg	13.33 a
		10	73.33 bcd	13.67 bcd	31.67 efg	16.67 a
		11	70.00 bc	12.67 abc	35.00 gh	35.00 b
	80	9	81.67 ef	12.00 ab	30.33 de	11.67 a
		10	75.00 cde	14.00 cd	33.33 fg	16.67 a
		11	66.67 b	11.67 ab	35.00 gh	35.00 b
CV (%)			12.22	5.76	16.96	19.29

Note: Numbers followed by different letters are significantly different at the 5% level of LSD.

Ex vitro survival

The percentage of transplanted seedlings obtained from immature embryos was high in media supplemented with 0.075 ppm ABA and 60-80 ppm GA_3 (30.33–30.67%).

The highest survival rate of seedlings produced from immature embryos after transfer to the screenhouse was 13.33 % with a medium supplemented with 0.075 ppm ABA and 60 ppm GA_3.

Although the germination percentage of immature embryos was comparable to that of mature (10 and 11 month-old) embryos, they produced a lower number of transplanted seedlings because of browning affecting 35% of them. In addition to browning, the *ex vitro* survival rate of transplanted seedlings produced from immature embryos was also low.

Constraints and conclusions

Table 5 presents the summary of results of the RICP studies on embryo culture protocols. The highlights of observations are as follows:

- High contamination in the first experiment was a problem. Fortunately, in the second, third and fourth experiments, contamination decreased dramatically.
- A high percentage of immature embryos were lost due to browning.

- Regardless of the variety, the highest germination percentage was noted with the CPCRI protocol but the percentages of transplanted and of surviving seedlings were higher with the UPLB protocol.

- GKN embryos gave a high germination percentage with the CPCRI medium as well as a high number of roots per *in vitro* plant and of transplanted and surviving seedlings with the UPLB protocol. Generally, the three coconut varieties tested responded well with the UPLB protocol, especially on the growth of germinated embryos.

- The incorporation of 60 ppm GA_3 in the medium induced a higher percentage of germination and transplanted seedlings.

- The introduction of ABA and GA_3 into the medium resulted in increased germination of immature embryos, but germination was lower than in medium supplemented with GA_3 only.

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Table 5. Summary table for coconut embryo culture studies at RICP as of 22 August 2000

Parameter	Cultivar	Culture protocol used					
		Before start of project	UPLB	PCA	CPCRI	IRD	Own
Number of embryos inoculated	RLT	–	60	60	60	60	
	DMT	54	60	60	60	60	
	GKN	–	60	60	60	60	
	Kenari	–	–	–	–	–	90
	Kopyor*	–	–	–	–	–	37
Contamination (%)	RLT	–	41.67	46.67	25.00	48.33	
	DMT	–	45.00	48.33	20.00	33.33	
	GKN	–	30.00	21.67	10.00	43.33	
	Kenari	–	–	–	–	–	15.33
	Kopyor*	–	–	–	–	–	–
Germination (%)	RLT	–	85.00	71.67	86.67	66.67	–
	DMT	–	80.00	66.67	83.33	60.00	–
	GKN	–	86.67	83.33	83.33	68.33	–
	Kenari	–	–	–	–	–	80.33
	Kopyor*	–	–	–	–	–	–
Whole plantlets *in vitro* (%)	RLT	–	58.33	50.00	52.00	40.00	–
	DMT	14.81	55.00	50.00	51.67	40.00	–
	GKN	–	66.67	53.33	60.00	41.67	–
	Kenari	–	–	–	–	–	41.11
	Kopyor*	–	–	–	–	–	–
Average duration of *in vitro* culture (weeks) to obtain whole plantlets	RLT	–	28	27	28	40.00	–
	DMT	29	28	28	28	40.00	–
	GKN	–	24	25	26	41.67	–
	Kenari	–	–	–	–	–	41.11
	Kopyor*	–	–	–	–	–	–
Survival after transfer to the screen-house (%)	RLT	–	41.67	35.00	33.33	25.00	–
	DMT	–	46.67	38.33	33.33	25.00	–
	GKN	–	45.00	41.67	36.67	28.33	–
	Kenari	–	–	–	–	–	26.67
	Kopyor*	–	–	–	–	–	–
Survival *ex vitro* (%)	RLT	–	71.43	70.00	64.52	41.67	–
	DMT	–	80.65	76.67	64.52	41.67	–
	GKN	–	71.05	78.13	66.67	48.00	–
	Kenari	–	–	–	–	–	21.00
	Kopyor*	–	–	–	–	–	–
Survival after transfer to nursery (%)	RLT	–	48.00	47.62	48.00	40.00	–
	DMT	–	52.00	52.17	52.00	45.00	–
	GKN	–	55.56	48.00	55.56	45.45	–
	Kenari	–	–	–	–		21.00
	Kopyor*	–	–	–	–		–

Note: Kopyor in initial culture; there are no germinated embryos so far.

Coconut embryo culture in Vietnam

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Introduction

Coconut is one of the most important oleaginous crops in the world. Studies have been conducted to improve coconut production through breeding, germplasm collecting and maintenance and *in vitro* culture. Coconut embryo culture is considered to be a reliable method for coconut germplasm exchange and conservation. In connection with this, two international workshops on coconut embryo culture have been organized by COGENT to review the technical advancement and application aspects of coconut zygotic embryo culture.

Coconut embryo culture technology has also been used successfully to propagate some special varieties of coconut. In the Philippines, coconut embryo culture has become a standard technique in the propagation of the Makapuno cultivar.

Coconut has long been cultivated on large areas of the central coastal regions and the Mekong River Delta in Vietnam. Coconut cultivation plays a vital role in the economy and nutrition of many farming families in the country as almost all the parts of the coconut can be used for the production of high-value items such as handicrafts, confectionaries, construction materials, medicines and the like.

In Vietnam, the Oil Plant Institute (OPI) is the national institute responsible for coconut development. In the past, two major coconut development programmes have been implemented by OPI: the UNDP-funded Programme on Coconut Improvement in 1985 and the COGENT-funded Programme on Coconut Germplasm Evaluation and Conservation in 1995. Under these programmes, a field collection of coconut varieties was established at the OPI's Dong Go Experiment station (Ben Tre province). An *in vitro* tissue culture laboratory was also set up at the OPI headquarters in Ho Chi Minh City to assist in the maintenance of rare or low germinating coconut varieties such as the Dua (aromatic), Makapuno and Xiem.

Work on coconut embryo culture started in 1993. With COGENT's assistance from 1998 onwards, embryo culture in Vietnam has greatly improved. This report deals with the results of the 1998–1999 COGENT-supported studies on coconut embryo culture carried out at the OPI.

Methods

Two experiments were conducted:

1. Selection of the most suitable medium from among four embryo culture protocols (Annexes 1.1–1.4): PCA, UPLB, CPCRI and IRD (Engelmann 1998); and
2. The use of modified PCA medium for embryo culture of 11 varieties which eight were the common varieties like the Malayan Yellow Dwarf, Ta (Dong Go), Ta (Binh Thanh), EO, Dau, Lang-Co, Quang-Nam and the Khia and the other three being rare varieties like the Makapuno (known to local farmers as "waxy nuts"), Dua (aromatic) and Xiem.

The following procedures were used for these two experiments.

Explant preparation

The husks of freshly harvested, mature coconuts were removed and the nuts split open. The cylindrical portion of the coconut meat encasing the embryo was removed from each of the endosperms. These cylindrical portions were then thoroughly washed in a large beaker placed under running tap water.

Sterilization of the explants

The explants were sterilized by immersion in 95% alcohol for two minutes, then in a bleach solution of 10% NaOCl for 20 minutes. They were then carefully washed five times in sterile distilled water under the laminar flow hood.

Preparation of medium

Test tubes, containing 10 ml of medium mixed with 2.5 g/L of charcoal, were plugged with rubber stoppers whose small holes were filled with cotton before they were sterilized by autoclaving at 121°C for 15 minutes.

In vitro culture

The embryos, extracted from the endosperm cylinders under the laminar flow hood, were placed in sterilized Petri dishes before they were put into the prepared culture tubes. Temperature condition of the culture was maintained at 28 ± 2°C under an illumination of 4000 lux with 9h light/15h dark photoperiod.

Results and discussion

Selection of medium for coconut embryo culture

Embryos of three varieties, Ta (Dong Go), Dau and Eo, were cultured following four culture protocols developed by the Philippine Coconut Authority (PCA), University of the Philippines at Los Baños (UPLB), Central Plantation Crops Research Institute (CPCRI) and the Institut de Recherche pour le Developpement (IRD). Observations were made four and eight weeks after inoculation. Results are presented in Figs. 1–6.

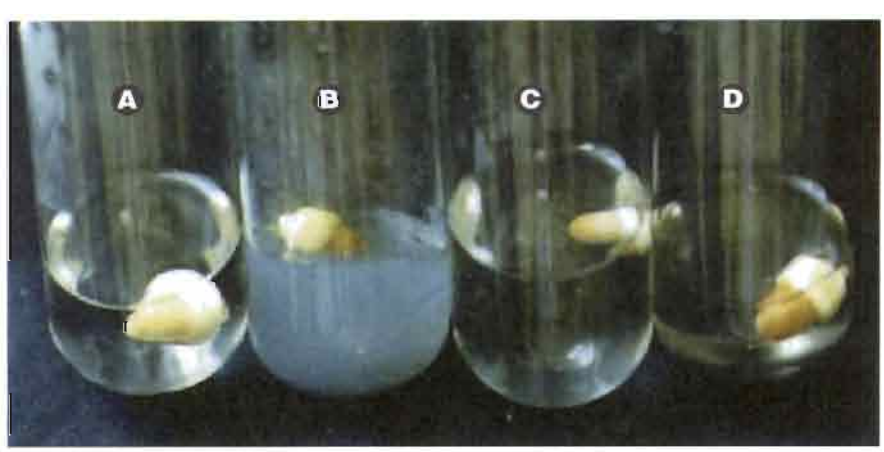

Fig. 1. Development of embryos of TA (Dong Go) variety on four media after four weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

Fig. 2. Development of embryos of TA (Dong Go) variety on four media after 8 weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

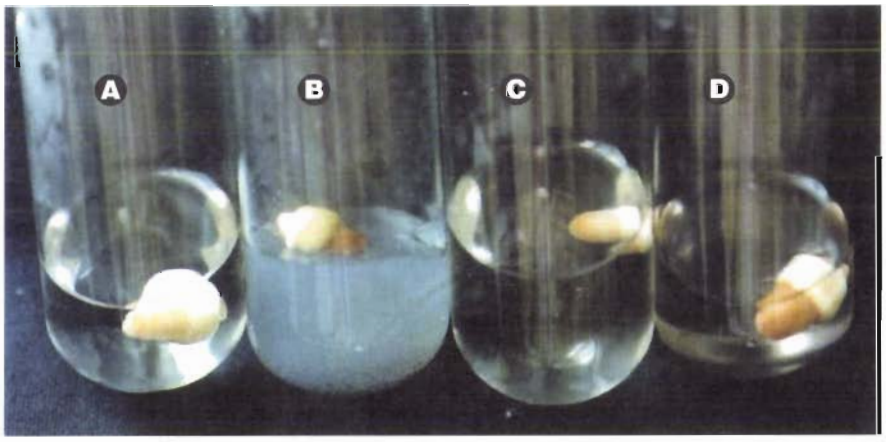

Fig. 3. Development of embryos of Dau variety on four media after 4 weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

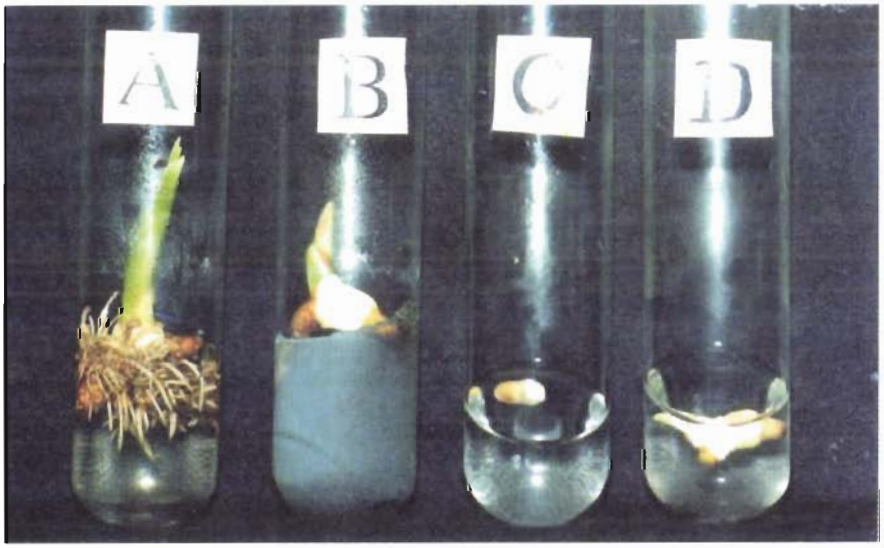

Fig. 4. Development of embryos of Dau variety on four media after 8 weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

The PCA medium with 7mg/L IBA was seen to be the best medium for embryo development with varieties Ta (Dong Go) and Dau after four weeks in culture. With the PCA medium, these two varieties developed strong shoots and roots after eight weeks of culture.

However, EO embryos did not develop any shoot or root even after 12 weeks in culture. The concentration of 7 mg/L IBA in the medium, which induced profuse root formation in other varieties, might have been too high for the EO variety (Figs. 5, 6 and 7).

Fig. 5. Development of embryos of EO variety on four media after 4 weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

Fig. 6. Development of embryos EO variety on four media after 12 weeks in culture (A) PCA; (B) UPLB; (C) CPCRI; (D) IRD.

Fig. 7. Excessive embryo root formation on PCA medium containing 7 mg/L IBA.

The use of modified PCA medium for embryo culture of eight common coconut varieties

As mentioned, the use of 7 mg/L IBA led to excessive root formation (Fig. 7) in some varieties of embryos cultured. In this experiment, the IBA concentration was lowered to 2 mg/L. The findings were as follows:

- The culture of embryos of the eight common coconut varieties on modified PCA medium supplemented with 2mg/L IBA proved to be highly successful;
- All embryos were swollen one week after culture initiation (Fig. 8);
- Embryos began to germinate 2–3 weeks after culture initiation (Fig. 9), depending on the variety and the maturity of the embryos. After 6–7 weeks, shoots and roots appeared (Fig. 10); and
- The germination rate and intensity were highest with the Ta (Dong Go) variety (Fig. 11), followed by the Malayan Yellow Dwarf (Fig. 12) and the Ta (Binh Thanh) (Fig. 13). Specific data are shown in Table 1.

Fig. 8. Swollen embryo one week after culture initiation.

Fig. 9. Germinated embryo 2 to 3 weeks after culture initiation.

Fig. 10. Embryo with shoot and root 6 to 7 weeks after culture initiation.

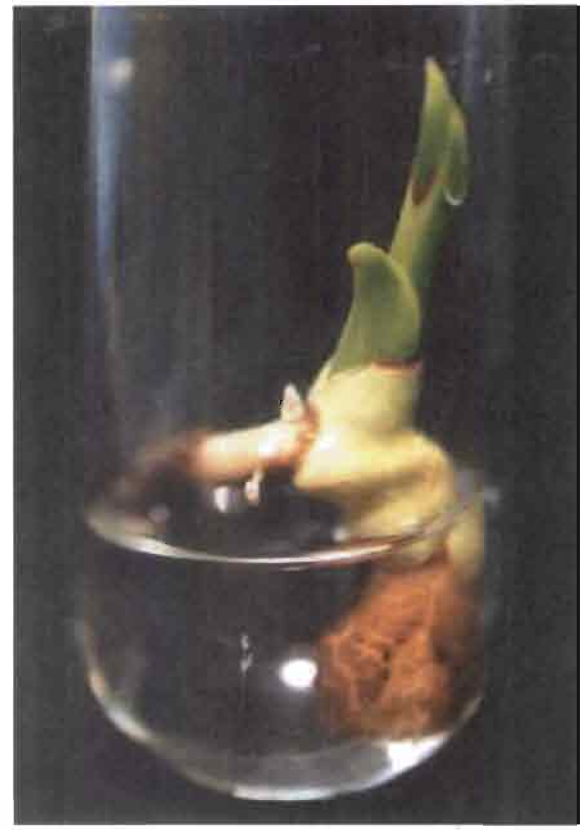

Fig. 11. A developed TA (Dong Go) embryo.

Fig. 12. MYD embryo with newly developed shoot.

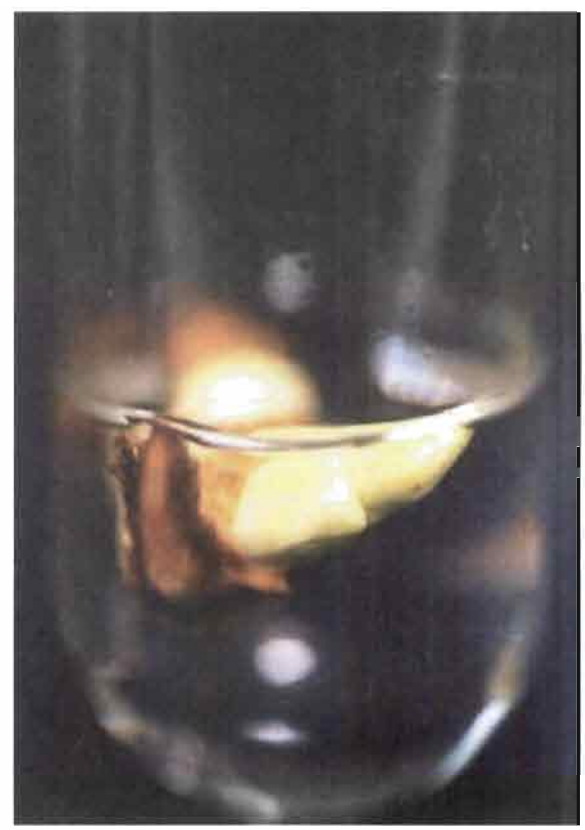

Fig. 13. Germination of a TA (Binh Thanh) embryo.

Table 1. Plant height, root length and stem diameter of *in vitro* plantlets of three coconut varieties on modified *PCA medium (six and seven months after culture initiation)

	Plant height (cm)		Root length (cm)		Stem diameter (cm)	
	6 mos	7 mos	6 mos	7 mos	6 mos	7 mos
Ta (Dong Go)	18.2	19.8	8.2	11.7	1.2	1.3
MYD	14.8	15.8	11.0	11.5	0.9	1.1
Ta(Binh Thanh)	13.0	15.0	5.0	5.5	0.8	0.9

* Instead of 7 mg/L IBA, 2 mg/L IBA concentration was used.

After six to seven months of culture on the modified PCA medium, plantlets of the Ta (Dong Go) variety had the highest shoot growth, root length and shoot diameter, followed by the Malayan Yellow Dwarf and Ta (Binh Thanh). These variations may be due to differences in the maturity of the embryos inoculated.

Embryos from the other coconut varieties, including the Dau, EO, Lang – Co, Quang-Nam and Khia also grew well on the modified PCA medium (Figs. 14–17).

Fig. 14. A Dau embryo showing strong shoot and root development.

Fig. 15. Different stages of *in vitro* growth of Dau coconut embryos.

Fig. 16. Strong shoot development of an *in vitro* EO plantlet.

Fig. 17. An EO embryo showing profuse root growth.

The best results were obtained with embryos from 11- to 12-month old nuts.

In liquid medium, all of the embryos of the Ta (Dong Go) variety floated while some embryos of the Malayan Yellow Dwarf and the Ta (Binh Thanh) sank. Batugal (pers. comm. 1999) attributes this to the maturity of the nuts used.

It is possible that the embryos which sank could have germinated better on a shaker or on shallower liquid medium which would allow more oxygen to reach the germinating embryos.

The lower IBA concentration in the PCA culture medium had the beneficial effect of balancing the development of both the shoots and roots. It also enhanced fast germination of the embryos with a fairly strong shoot growth (Fig. 11).

These observations might be valid only for the coconut varieties planted under natural conditions in Vietnam. More experiments will be conducted using the other varieties to validate the findings.

The results of the tests on the Xiem, Makapuno and Dua (Aromatic) varieties are shown in Figs. 18–24.

The modified PCA medium with 2 mg/L IBA was a good medium for embryo culture but an increase in vitamin concentration might be needed for good germination and growth of embryos, especially with the aromatic variety Dua.

Fig. 18. A Xiem plantlet showing balanced leaf and root growth.

Fig. 19. Different stages of Xiem embryo development *in vitro*.

Fig. 20. A Vietnam Makapuno with waxy endosperm.

Fig. 21. Makapuno embryos in different stages of development.

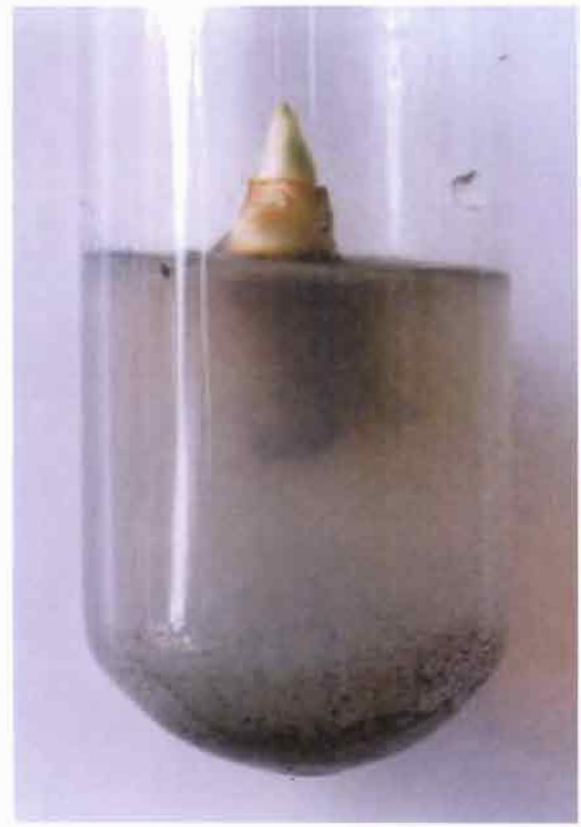

Fig. 22. Endosperm of a Dua variety.

Fig. 23. Swollen Dua embryo in culture.

Fig. 24. A Dua (aromatic) embryo showing strong shoot growth.

Makapuno in Vietnam

In Vietnam, local Makapuno types have been collected since 1995 (Vo Van Long 2000). There are two types of nuts on a "Makapuno" plant:

- Nuts having normal development pattern; and
- Nuts without separable liquid, known to local farmers as "waxy nuts".

It was observed that embryos of the Makapuno nuts are longer than those of normal nuts. However, the outer appearance of these nuts is similar.

Conclusion

- Among the four media tested, the PCA medium was proven to be the best for coconut embryo culture.

- The modified PCA medium with 2mg/L IBA gave the best embryo development performance regardless of the varieties tested.

- The embryo culture technique has been successfully applied to the cultivation of the Vietnamese Makapuno coconut, Dua (an aromatic variety fairly hard to germinate) and other rare coconut varieties. In total, embryos from 11 Vietnam coconut varieties have been cultured successfully.

- Successful coconut embryo culture of the above mentioned rare coconut varieties has greatly contributed to the development of desired varieties, thus promoting their export and local consumption.

- Knowledge of the embryo culture technique greatly enhances Vietnam's participation in the Regional Coconut Genebank.

- Results of the coconut embryo culture in Vietnam (Table 2) will be used in the production of new varieties of coconut and Vietnam's participation in regional coconut research activities through the support of COGENT, the University of Queensland (Australia) and other international institutions.

Table 2. Summary results of coconut embryo culture studies in Vietnam

	Protocols tested in the current project				
	UPLB	**PCA**	**CPCRI**	**IRD**	**Own***
Number of embryos inoculated					
Dau	30	30	30	30	
EO	30	30	30	30	
Ta (Dong Go)	30	30	30	30	30
Malayan Yellow Dwarf					30
Ta (Binh Thanh)					30
Contamination (%)					
Dau	3.3	0	0	0	
EO	0	0	3.3	6.6	
Ta (Dong Go)	0	3.3	0	0	3.3
Malayan Yellow Dwarf					3.3
Ta (Binh Thanh)					6.6
Germination (%)					
Dau	90.1	93.3	66.6	83.3	
EO	70.0	80.0	73.3	70.0	
Ta (Dong Go)	93.4	96.6	70.0	86.6	96.7
Malayan Yellow Dwarf					90.1
Ta (Binh Thanh)					86.8
Whole plantlets *in vitro* (%)					
Dau	83.3	86.6	50.1	80.0	
EO	63.3	70.0	66.6	60.0	
Ta (Dong Go)	86.6	90.0	53.4	66.7	93.4
Malayan Yellow Dwarf					83.3
Ta (Binh Thanh)					80.0
Average duration of *in vitro* culture (weeks) to obtain whole plantlets					
Dau	33	31	37	33	
EO	39	38	40	39	
Ta (Dong Go)	32	30	36	32	28
Malayan Yellow Dwarf					30
Ta (Binh Thanh)					33

* Own: PCA medium modified by supplementing 2mg/l of IBA instead of 7mg /l.

Acknowledgment

The author wishes to thank Dr. Pons Batugal, COGENT Coordinator, for his support in directing the research activities. The author would also like to express her sincere thanks to Prof. DSc Phan Lieu, Head of International Programmes of the Oil Plant Institute (OPI) for his constructive comments, his assistance in the preparation of this paper and his critical reading of the manuscript.

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Improvement of *in vitro* techniques for collecting and exchanging coconut germplasm in Papua New Guinea

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Background and rationale

Research work on tissue culture of coconut in Papua New Guinea started in 1986 under the Australian Centre for International Agriculture Research (ACIAR)-funded "Coconut Improvement Project". This was concluded in 1993 and the research results were published in various journals and the ACIAR Technical Bulletins. Some of these results will be useful to the proposed project on building and equipping the embryo culture laboratory to facilitate the regional genebank at CCRI – Stewart Research Station. The embryo culture protocol developed then may be tested together with other protocols.

The coconut embryo culture facility is needed to facilitate the introduction and exchange of selected populations of coconut for the genetic improvement programmes of the host country as well as for the other countries in the same region. Since PNG has been nominated as the host country for the International Coconut Genebank for the South Pacific (ICG-SP), the coconut embyro culture facility must be properly established and sufficiently equipped for the purpose of introducing and exchanging germplasm. An acclimatization facility of sufficient size to cater for the genebank requirements is yet to be established. In addition, training of staff on embryo culture is essential for the continuity and successful management of the project.

Many of the advantages offered by the embryo culture method include the exchange of disease-free materials and the lower freight charges for shipping isolated embryos rather than the bulky nuts (Assy Bah *et al.* 1987; Assy Bah and Engelmann 1993; Asburner *et al.* 1996). The method of Ashburner *et al.* 1996 has been successful in the past, but the acclimatization procedure needs to be refined. Results of this method have shown that the success rate from isolated embryo culture to seedling at nursery stage was 50% under the Kerevat conditions (Ashburner *et al.* 1996). Thus, more work is required in perfecting the technique before adopting it as a tool for germplasm collecting and exchange. Adopting a protocol with a higher success rate is desirable even though it may not reach the 100% survival mark. Maintaining a sufficient amount of diversity could be achieved by increasing the size of samples collected.

The method used for collecting coconut embryos is well described by Rillo and Paloma (1991) and various other researchers (Assy-Bah *et al.* 1987; Ashburner and Thompson 1991; Karun *et al.* 1993; Asburner *et al.* 1996). In collecting embryos from distant locations, the Stewart Research Station (SRS) laboratory plans to adopt the method of Rillo and Paloma (1991).

Objectives

The project aims to:

1. Set up and equip a coconut embryo culture laboratory;
2. Train local technical staff on embryo culture;

3. Compare two embryo culture protocols (PCA 'hybrid' protocol and CPCRI, India) and select the more suitable one for local conditions;
4. Establish acclimatization facility; and
5. Start introducing selected populations via zygotic embryos in liquid medium.

Activities

The modification work recommended for the existing laboratory and office space to facilitate embryo culture work started in October 1999 and was completed in January 2000. A new sink was installed and tiles were laid in all the rooms. Glass windows were fixed and sealed to minimize air movement; a new air conditioner was installed in the culture room and existing cupboards without doors were fitted with new ones. A work bench and two lighted culture shelves were installed in the preparation and culture rooms, respectively. The existing main door was replaced with double doors.

The basic laboratory chemicals, glassware and equipment are now in place to start the actual embryo culture work (see Appendices 1 and 2 for a full listing). The laboratory now has a storage room for glassware, a transfer room for asceptic culturing of embryos, a culture room for incubation, a laminar flow unit, and facilities for media preparation and sterilization.

An Assistant Research Officer who will assist Mr. Afred Kembu, Research Officer, will be recruited and given on-the-job training.

To compare and select the suitable embryo culture protocol for the SRS, samples of locally collected embryos were cultured in July 2000. The PCA 'hybrid' (Appendix 3) and CPCRI (Annex 1.2) protocols were used and are being tested for suitability to SRS conditions.

Materials for constructing the acclimatization facility are being purchased. Construction will start as soon as these materials become available.

Results and discussion

Papua New Guinea is yet to carry out the comparison of suggested culture techniques from various countries or laboratories. However, from the findings of the organizations which have conducted the studies, the PCA 'hybrid' (PCA+UPLB protocols) and CPCRI protocols have been identified as the most promising. With the completion of the laboratory, these two methods can be further compared to select the one suitable under PNG (SRS) conditions. There are no results yet because the first batch of embryos were cultured only in July 2000.

Planned research activities on coconut embryo culture

Stage 1. The SRS laboratory will have to adopt and perfect the coconut embryo *in vitro* culture and plantlet acclimatization techniques. Based on the results of the comparative studies of various protocols done by other laboratories, only the PCA 'hybrid' and the CPCRI protocols will be tested. Initially, SRS will compare these protocols and select one for adoption. Once sufficient expertise is acquired, the introduction of the selected genetic materials will start.

Stage 2. Build acclimatization and nursery facilities.

Stage 3. Start introducing the selected populations and facilitate the exchange of germplasm via zygotic embryos between PNG and other countries. After the acclimatization stage, seedlings will be transplanted to the field at the International Genebank.

Listed below are the accessions approved by the COGENT member countries in the South Pacific Region for the centre to import:

Pacific accessions	No. of embryos	Source country
1. Rotuma Tall	250	Fiji
2. Tonga Tall	250	Tonga
3. Kiribati Tall	250	Kiribati
4. Rangiroa Tall	250	Tahiti
5. Vanuatu Tall	250	Ivory Coast
6. Western Samoa Tall	250	Western Samoa
7. Samoan Yellow Dwarf	250	Western Samoa
8. Niu Leka Green Dwarf	250	Fiji
9. Fiji Tall	250	Fiji
10. Niu Vai	250	Western Samoa
11. Niu Afa	250	Western Samoa
12. Christmas Islands Tall	250	Kiribati
13. Kiribati Green Dwarf	250	Kiribati
14. New Caledonia Tall	250	New Caledonia
15. Vanikoro Tall	250	Solomon Island
16. Solomon Tall	250	Solomon Island
17. Niu-Bubu	250	PNG & Solomon
Other accessions		
18. Cameroon Red Dwarf		Ivory Coast
19. Pilipog Green Dwarf		Indonesia
20. Tacunan Green Dwarf		Philippines
21. Aromatic Green Dwarf		Philippines
22. Catigan Green Dwarf		Thailand
23. Brazilian Green Dwarf		Philippines
24. West African Tall		Ivory Coast
25. Sri Lankan Tall		Ivory Coast
26. PanamaTall		Sri Lanka
		Jamaica

Schedule of activities

Year	Activities
2000	i) Adopt and perfect the embryo culture technique suitable for local conditions
	ii) Establish the acclimatization facility
2001	i) Start introducing populations from the region
	ii) Initiate operation of acclimatization and nursery facilities
2002	i) Continue embryo culture research on selected populations
	ii) Field plant the plantlets from early introductions

Conclusion

The embryo culture laboratory and most of the equipment and consumables are now properly set up and ready for use.

A modest acclimatization facility is being constructed. Meanwhile, the laboratory has started testing the PCA 'hybrid' and CPCRI culture methods using embryos from coconuts at the station.

The introduction and exchange of germplasm will start soon after the embryo culture protocols currently being tested have been confirmed.

Acknowledgement

I wish to thank the Acting Director of PNGCCRI and the Section Head – for Coconut Breeding – for their confidence in me, and for giving me the opportunity to attend this workshop. Thank you Mr T. Ovasuru and Dr J. Ollivier for your help, too.

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Appendix 1

Facilities and Equipment at SRS embryo culture laboratory

A. Currently available equipment and facilities

Facilities for media preparation and sterilization	Quantity (pcs.)	Cost (US$)
1. Pressure cooker	1	1188.64
2. Gas burner/cylinder	1	69.96
3. Hot air oven	1	1082.04
4. Water still	1	799.25
5. Semi-Micro balance	1	1074.55
6. pH meter	1	177.11
7. Hot plate/stirrer	1	264.73
8. Refrigerator	1	611.77
9. Working bench	1	1241.23
10. Glass desiccator	1	97.45
11. Pipette controller	1	55.44
12. Spatulas	4	40.40
13. Various types of glassware	449	846.04
14. Chemicals	32	1027.80
Facilities for *in vitro* culture		
15. Laminar flow cabinet	1	3800.52
16. Culture trolley	1	26.20
17. Chair	1	126.74
18. Bunsen burner	1	7.83
19. Forceps	5	30.47
20. Scalpel blades/handles	4xPkt/100 blades & 4 handles	63.61
21. Two lighted culture shelves	2	1197.34
22. Timer	2	14.74
23. Air conditioner	1	786.33
24. Thermometer max/min	1	11.83

B. Equipment to be procured

1. Hot beads sterilizer
2. Standby generator
3. Vertical autoclave
4. Uninterrupted power supply unit
5. Automatic dispenser
6. Light microscope / stereo microscope
7. Laminar flow cabinet
8. pH meter
9. Additional glassware
10. Vortex mixer

Appendix 2

Chemicals for SRS embryo culture laboratory

Chemical name	Quantity	Year Purchased	Cost (Kina)	Cost (US$)
1. Potassium nitrate	500g	1999	109.53	35.87
2. Potassium chloride	250g	1999	41.58	13.62
3. Ammonium chloride	500g	1999	81.61	26.73
4. Calcium chloride dihydrate	500g	1999	105.23	34.46
5. Magnesium sulphate Heptahydrate	500g	1999	89.03	29.16
6. Ammonium nitrate	500g	1999	93.13	30.50
7. Potassium phosphate Monobasic	500g	1999	131.39	43.03
8. Manganese sulphate	500g	1999	97.81	32.03
9. Zinc sulphate heptahydrate	100g	1999	53.30	17.46
10. Boric acid	100g	1999	40.80	13.36
11. Potassium iodide	100g	1999	99.77	32.67
12. Cupric sulphate	250g	1999	69.50	22.76
13. Molybdic acid	100g	1999	97.23	31.84
14. Nickel chloride	100g	1999	50.37	16.50
15. EDTA	100g	1999	100.55	32.93
16. Ferrous sulphate heptahydrate	50g	1999	19.13	6.27
17. Myo-inositol	100g	1999	121.05	39.64
18. Thiamine HCl	25g	1999	53.89	17.65
19. Nicotinic acid	100g	1999	44.12	14.45
20. Pyridoxine HCl	25g	1999	81.22	26.60
21. Calcium D-pantothenate	100g	1999	108.75	35.62
22. Sodium phosphate	100g	1999	53.49	17.52
23. Cobalt chloride hexahydrate	100g	1999	148.57	48.66
24. Glycine	100g	1999	53.89	17.65
25. L-ascorbic acid	500g	1999	196.60	64.39
26. Folic acid	5g	1999	42.56	13.94
27. Biotin	1g	1999	196.02	64.20
28. BAP	1g	1999	50.37	16.50
29. NAA	25g	1999	40.41	13.23
30. IBA	1g	1999	27.33	8.95
31. Charcoal	500g	1999	74.97	24.55
32. Agar	1kg	1999	468.18	153.33

Appendix 3

PCA 'hybrid' protocol for coconut embryo culture

Medium Components		Composition	
			1x (mg/L)
Macro	Eeuwens (Y3)	NH4Cl	535.00
		KNO3	2020.00
		MgSO4.7H2O	247.00
		CaCl2.2H2O	294.00
		KCl	1492.00
		NaH2PO4.2H2O	312.00
			1x (mg/L)
Micro	Eeuwens (Y3)	KI	8.30
		H3BO3	3.10
		MnSO4.4H2O	11.20
		ZnSO4.7H2O	7.20
		CuSO4.5H2O	0.250
		CoCl2.H2O	0.240
		NaMoO4.H2O	0.240
		NiCl.6H2O	0.024
			1x (mg/L)
EDTA	UPLB	Fe2SO4.7H2O	41.70
		Na2EDTA	55.80
			1x (mg/L)
Vitamins	UPLB + ARC	Pyridoxine HCl	0.05
		Thiamine HCl	0.05
		Nicotinic acid	0.05
		Ca-D-pantothenate	0.05
		Biotin	0.05
		Folic acid	0.05
		Glycine	1.00
Table grade sugar	60 g/l	60 g/l from culture initiation until seedlings have developed shoots and roots (until the 3rd to 4th month)	
	45 g/l	For maintenance prior to transplanting to the soil	
Activated charcoal (acid washed)	1 g/l		
Gelling agent (Sigma agar)	7 g/l		
State	Liquid/solid/solid/ liquid	• Culture initiation (So) = liquid • 1st Subculture (S1) = solid • 2nd Subculture (S2) = solid • 3rd Subculture (S3) = liquid until the seedlings are ready to be transplanted to the soil.	

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

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Introduction

Embryo culture has been practised for a very long time, with reports dating back to the early works of Cutter and Wilson in 1954, Abrahams and Thomas in 1962 and Ventura in 1966. Researchers in the Philippines have subsequently shown interest in the *in vitro* culture of coconut embryos, with special emphasis on the Makapuno type of coconut. The *in vitro* culture of Makapuno has been studied extensively by de Guzman (1970), Balaga *et al.* (1971), Rosario *et al.* (1976) and later by Rillo *et al.* (1992). Researchers, notably Assy Bah (1986, 1987, 1989), Ashburner *et al.* (1991, 1993) and Sugimura *et al.* (1994) have extended their studies to include embryos from normal coconut seed nuts with compatible endosperm. Different tall and dwarf varieties have been used for research and the development of their embryos into seedlings has been reported on various occasions (Fisher & Tsai 1978; Iyer 1982; Gupta *et al.* 1984; Karunaratne 1985; Assy Bah 1986; Sugimura *et al.* 1994).

The use of *in vitro* techniques is considered as one of the most appropriate procedures for germplasm collecting and exchange. For this reason, COGENT organized the first "International Coconut Embryo Culture Workshop" in the Philippines to evaluate the different protocols in coconut embryo culture and acclimatization used in the different countries. At this workshop, the Mikocheni Agriculture Research Institute (MARI) in Tanzania was tasked to investigate some physiological parameters related to plantlet survival *in vitro* and *ex vitro*. Together with this, the best protocols presented at the workshop were to be validated and compared to the protocol often used at MARI.

Objectives

- To validate and compare other available protocols with the protocol currently used at MARI;
- To study the physiological integrity of leaves of *in vitro* plantlets and of *in vivo* germinated seedlings;
- To find out the main *in vitro* conditions and practices that lead to physiological disorders of *in vitro* and *ex vitro* plantlets;
- To correlate the research findings of the study and the survival rate of *in vitro* and *ex vitro* plantlets; and
- To use the findings of the research as basis to improve *in vitro* and *ex vitro* plantlet recovery.

Materials and methods

Validation of protocols

Three varieties, Malayan Yellow Dwarf (MYD), Pemba Red Dwarf (PRD) – a local dwarf and East African Tall (EAT) – a local tall, were used to validate the protocols used

by the different laboratories. Mature nuts were split open transversely using a machete. The part of the solid endosperm encasing the embryo was extracted from the split nuts with a cork borer. The endosperm cores were surface-sterilized by immersing them in 100% NaOCl for 20 minutes. The embryos were then freed from the cores and disinfected with low strength (10%) bleach for one minute. They were washed with sterile distilled water three times before inoculation *in vitro.* Excised embryos were individually inoculated in 500 cc bottles containing 30 cc of growth medium, and then transferred to the growth room.

These activities (excision and inoculation) were carried out in the laminar airflow cabinet, which was wiped thoroughly with 70% ethanol to prevent contamination. Sixty embryos were cultured *in vitro* for each variety and protocol. Culture conditions and methods were adopted from the best protocols viz.: CPCRI (Central Plantation Crops Research Institute – India, denoted as C), UPLB (University of Philippines at Los Baños, denoted as U), PCA (Philippine Coconut Authority, denoted as P), and IRD (Institut Français de Recherche Scientifique pour le Développement, denoted as O). These were compared with the protocol used at MARI (Mikocheni Agricultural Research Institute – Dar es Salaam, denoted as M) as presented in Appendix 1. Except for the O medium that incorporated modified MS medium, the four other protocols used basically the Y3 recipe with their respective modifications (Annexes 1.1 – 1.4). The same number of seed nuts (10 – 11 months old) was sown *in vivo* to parallel the *in vitro* experiment.

Physiological studies

Parallel sampling and data collecting were carried out *in vitro* and *ex vitro.* Physiological parameters and environmental factors associated with the performance of plantlets *in vitro, in vivo* and *ex vitro* were noted and recorded. Data were taken from 49 *in vitro,* 38 *ex vitro* plantlets and 91 *in vivo* seedlings. The parameters recorded include photosynthetic rate (PS), transpiration rate (TR), stomata conductance (SC), and environmental factors such as the sub-stomatal carbon dioxide concentration (CSS), photosynthetically active radiation (PAR) on leaf surface, carbon dioxide concentration in the growth room (CCR), screenhouse (CCS) and atmosphere (CCA), and leaf surface temperature (LST) inherent to these parameters. These parameters were measured using a Leaf Chamber Analyser (Type LCA-4). During the operation, the *in vitro* grown plantlets were temporarily removed from the culture vessel and only one fully unfolded leaf was used for each of the physiological measurement set up. Roots were maintained in the liquid according to medium requirements during this time. Values were recorded after a 2–3 minute stabilization period. At least two measurements were taken on two different leaves for each plantlet. The parameters were measured on clear cloudless days. Data on these parameters were collected on a monthly basis. Final results for the experiment to validate the embryo germination, environmental parameters related to physiological performance of plantlets and seedlings *in vitro, in vivo* and *ex vitro* are presented in this report.

Acclimatization procedure

The successful weaning of *in vitro* plantlets is an important aspect without which all the applications of embryo culture could be rendered obsolete. Survival of plantlets is greatly enhanced by their gradual acclimatization and hardening-off.

To study these two parameters, clay pots were filled two-thirds full with river sand and sterilized at 150°C for 24 hours and then cooled for at least 12 hours. These pots were then placed in reservoir containing the medium, which the soil will soak up via capillarity for about 20 minutes. While these pots were soaking up the medium, ramets

with at least two leaves and good ramification were selected. After the physiological measurements were taken from the *in vitro* plantlets, the plantlets were transferred to the screenhouse where they were taken out of the culture vessels. The plantlets were washed to remove the medium before they were sprayed with a fungicide (Ultra Dithane M_{45}) solution and transplanted in the prepared clay pots.

Individual plantlets in their respective pots were labeled and then covered with a transparent plastic bag placed upside down and fastened at the neck of the pot with a rubber band. The bag was propped upright by a stick to accommodate the plantlet. Watering in the clay pots was done once in two weeks during the first four weeks. On the third week, holes were made in the plastic bag to reduce the relative humidity within. More holes were made after six weeks and the bag was completely removed after the eighth week. Pricking was conducted at this period of time. After this stage, watering regime changed to every other day. Two months later, fertilizers were applied to sustain growth.

Experimental design and data analysis

Initially, the experiment was laid out following a Complete Randomized Block Design. It was expected that data would be analyzed statistically. However, the rate of contamination, especially after the measurement of physiological data, made it difficult to carry out statistical analysis. Related to this, the results were expressed as means of the values obtained in each category (parameter). PS and TR data were transformed (logarithmic transformation) for presentation.

Results

Germination rates for EAT, MYD and PRD

The East African Tall *in vitro*

Despite the low growth rate and final germination percentages *in vitro* (Table 1), all the various types of media tested seemed to support embryo growth equally (Fig. 1).

Table 1. Final germination of EAT, MYD and PRD embryos *in vitro* in different types of medium 65 days after culture initiation

Medium	Variety	Total No. of cultured embryos	Contamination (%)	Surviving embryo (non germinated) (%)	Germinated with shoots only (%)	Embryos with roots & shoots (%)	Total germinated embryos (%)
IRD	EAT	60	24.0	40.0	21.4	14.6	36
	PRD	60	9.8	18.2	20.0	52.0	72
	MYD	60	17.6	38.4	5.7	38.3	44
PCA	EAT	60	30.0	25.0	26.2	18.8	45
	PRD	60	12.3	29.7	20.0	38.0	58
	MYD	60	16.8	18.2	29.5	35.6	65
UPLB	EAT	60	28.0	52.0	6.2	13.8	20
	PRD	60	25.0	20.0	15.0	40.0	55
	MYD	60	15.3	16.7	23.0	45.0	68
MARI	EAT	60	26.5	37.5	31.0	5.0	36
	PRD	60	17.0	25.0	20.5	37.5	58
	MYD	60	15.0	20.0	20.0	45.0	65
CPCRI	EAT	60	29.9	26.1	8.6	36.4	45
	PRD	60	36.0	49.0	12.1	12.9	25
	MYD	60	32.0	48.0	6.3	14.7	20

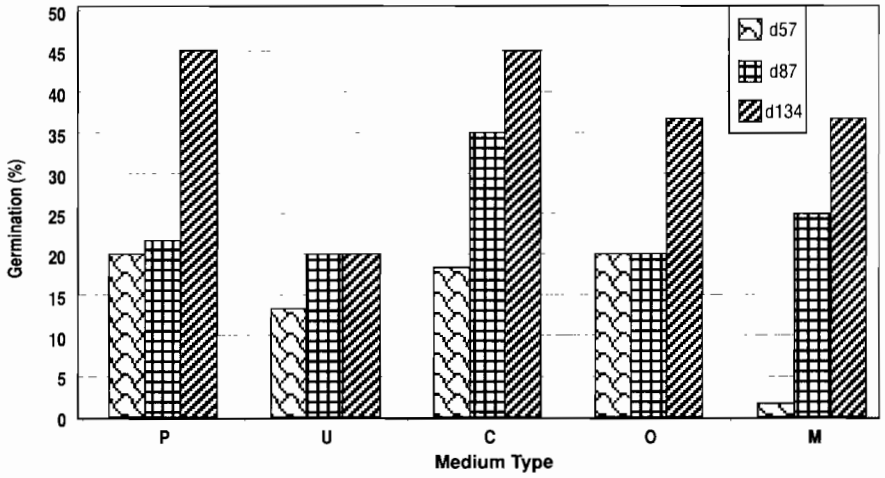

Fig. 1. Germination percentage of EAT embryos *in vitro* overtime in different types of media (d57, d87 and d134 are the number of days after initiation of culture *in vitro,* they stand for the same wherever cited in the text).

Among the five types of media, U showed the lowest final germination (20%), while the values of the other four media were higher but not exceeding 45%. Media P and C gave the highest final germination percentages for EAT embryos.

Malayan Yellow Dwarf *in vitro*

Compared to the EAT, the MYD embryos showed higher germination rates with all types of media except C (Fig. 2). After three months *in vitro*, the MYD embryos cultured in the P, U, O and M media showed higher germination percentages than the EAT cultured for four months (Figs. 1, 2 and 3). MYD initially germinated more rapidly before the first passage, but the rate slowed down in the subsequent passages. However, despite showing a similar trend in C and O, these media seemed less favourable to *in vitro* growth of MYD embryos. The media P, U and M were the best for the MYD *in vitro* embryo germination.

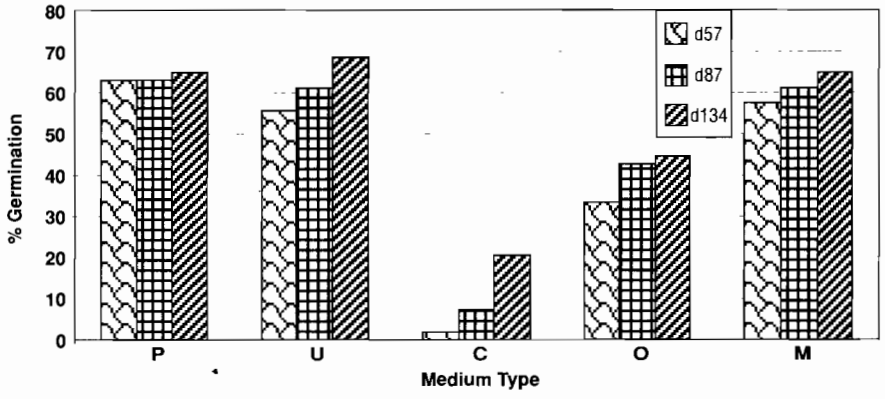

Fig. 2. Germination percentage of MYD embryos *in vitro* over time in different types of media.

Pemba Red Dwarf *in vitro*

As was apparent with the MYD, the C medium showed the least support to PRD embryo growth *in vitro*. The PRD variety grew best in the IRD medium with 72% final germination rate (Fig. 3, Table 1). The other media, except CPCRI, also showed better support though the rates were not as high as that in the IRD medium.

No further germination was observed after 165 days of incubation *in vitro*. Apparently, extending the incubation period of cultures in either liquid or solid medium under continuous light or darkness beyond 165 days did not improve the germination of embryos.

PRD, MYD and EAT *in vivo* germination

The three varieties showed similar trends in germination rate and final germination *in vitro* and *in vivo*. However, the rate exhibited by PRD *in vitro* (Fig. 3) was higher than *in vivo* (Fig. 4). Although the EAT germinated much more slowly than the MYD, it reached the highest final germination percentage (78%), compared to both the MYD (61%) and the PRD (38%), at 134 days after sowing.

A comparison between *in vivo* and *in vitro* germination revealed that EAT performance was better *in vivo* (78%) than *in vitro* (45%). MYD showed similar response to both types of culture practices (*in vivo*, 61% and *in vitro*, 64%; Figs. 3 and 4).

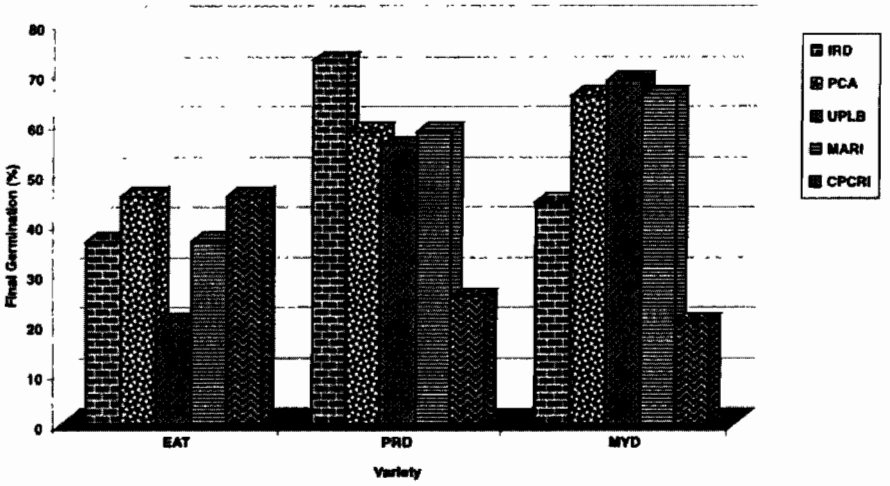

Fig. 3. Final germination of EAT, PRD and MYD embryos *in vitro* in different types of medium 165 days after culture initiation.

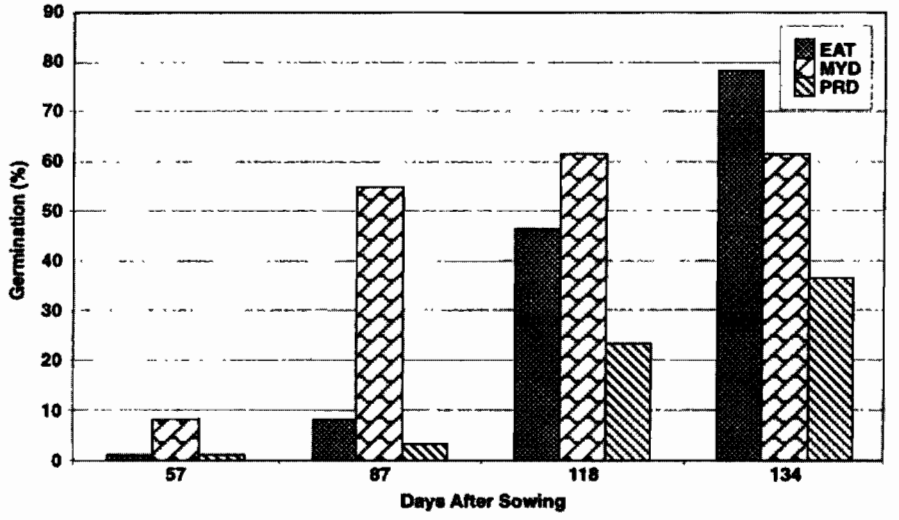

Fig. 4. Percentage germination of coconut varieties *in vivo.*

Ex vitro establishment of plantlets

The survival percentage (Table 2) is based on the number of plantlets established *ex vitro* from each variety and protocol. The number of surviving *ex vitro* seedlings was highest when the UPLB protocol was used. This could be due to the high iron content of the medium compared to that in the other protocols. While the PRD showed the highest germination percentage in the IRD medium (Table 1), it had the lowest survival rate *in vitro* (Table 2). This could be due to the higher chlorine content in the IRD than in the other media.

Table 2. Actual plantlet survival (%) in different types of media

Variety/Medium	UPLB	PCA	CPCRI	IRD	MARI
EAT	10.0	11.7	25.0	11.7	3.3
PRD	31.0	30.0	8.3	8.3	31.7
MYD	38.3	27.0	11.7	28.3	36.7

Physiological studies

The results of the physiological studies showed that, over a period of time, there was a general trend of carbon dioxide increase in the growth room. In the growth room, the average CO_2 concentration was at about 400 µmol.mol^{-1}. The CO_2 concentration increased to 450 µmol.mol^{-1} on day 225 and on day 231, it was more than double the value recorded on day 225 (Figs. 5a, b and c and Table 3).

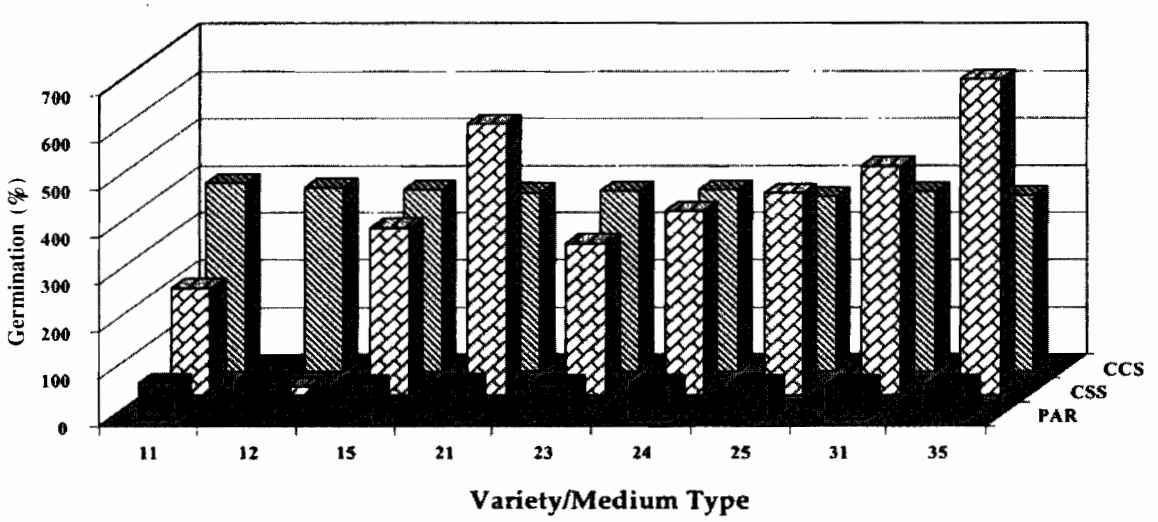

Fig. 5a. *In vitro* performance of coconut varieties with respect to physiological parameters in different types of media 205 days after initial culture. On the x-axis, the first digit represents the variety and the second is the medium type where, 1 = EAT, 2 = MYD and 3 = PRD; medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB, and 5 = PCA. Units for PAR are µmol.m^{-2}.s^{-1} and for CSS and CCS are µmol.mol^{-1}.

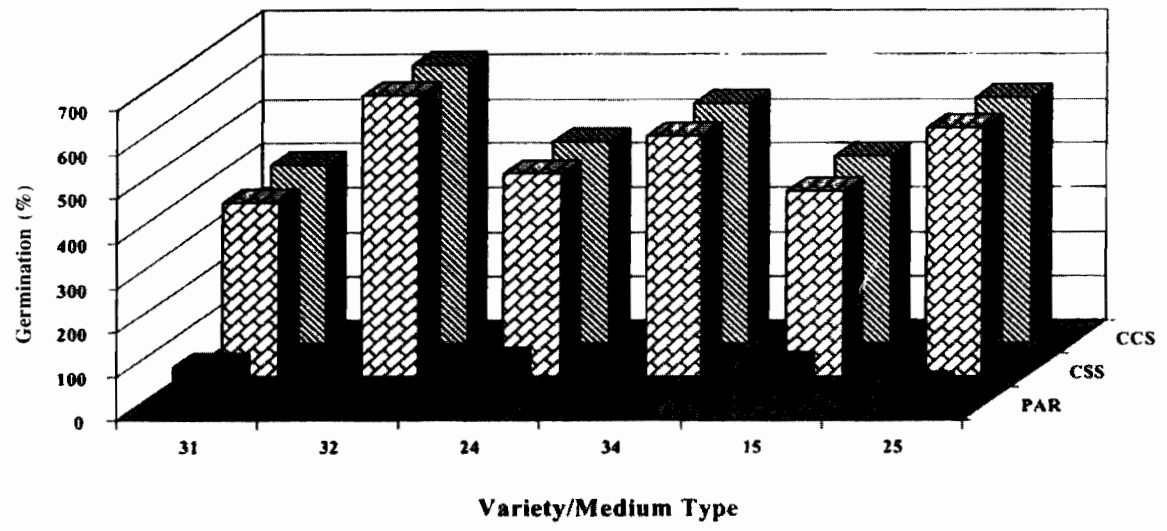

Fig. 5b. *In vitro* performance of coconut varieties with respect to physiological parameters in different types of media 225 days after initial culture. (On the x-axis, the first digit represents the variety and the second is the medium type. 1 = EAT, 2 = MYD and 3 = PRD; medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB, and 5 = PCA. Units for PAR are $\mu mol.m^{-2}.s^{-1}$ and for CCS are $\mu mol.mol^{-1}$).

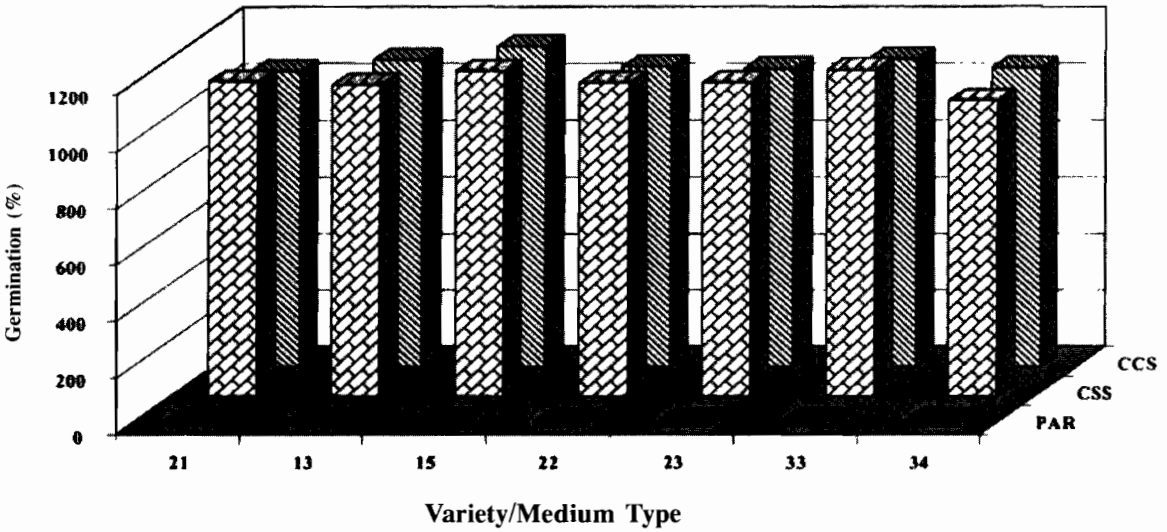

Fig. 5c. *In vitro* performance of coconut varieties with respect to physiological parameters in different types of media 231 days after culture initiation. (On the x-axis, the first digit represents the variety and the second is the medium type. 1 = EAT, 2 = MYD and 3 = PRD; medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB, and 5 = PCA. Units for PAR are $\mu mol.m^{-2}.s^{-1}$ and for CSS and CCS are $\mu mol.mol^{-1}$).

Table 3. *In vitro* performance of coconut varieties with respect to physiological parameters in different types of medium over time

Time/Variety/ Medium	TR*	PS*	PAR	CSS	CCR	LS	SC
111	0.12	35.88	75.00	226.00	398.40	32.90	
112	0.01	13.99	80.00	17.20	389.20	33.10	
115	0.01	4.18	73.00	353.75	384.45	32.95	
121	0.00	1.65	76.00	574.25	378.05	33.05	
123	0.02	3.06	72.67	319.80	380.90	32.57	
124	0.46	0.00	73.00	388.65	384.10	32.20	
125	0.28	1.00	73.00	426.60	369.40	32.50	
131	0.03	4.77	71.50	483.78	378.88	32.35	
135	0.02	0.67	71.67	668.03	371.23	32.77	
231	1.02	12.80	100.20	392.24	402.54		
232	0.47	0.53	15.50	636.90	627.65		0.24
224	0.86	0.17	112.60	459.72	456.54		0.20
234	0.51	2.72	44.67	544.50	543.00		8.89
215	0.94	15.02	102.25	418.43	422.90		3.06
225	0.53	6.52	56.00	561.94	555.16		0.30
321	0.47	51.70	20.00	1111.40	1043.60	27.30	0.17
313	0.50	1.58	20.00	1098.53	1084.63	27.83	0.20
315	0.86	17.96	20.00	1147.90	1132.20	28.20	0.45
321	0.43	3.46	18.13	1034.39	1001.18	26.55	0.31
322	0.21	8.64	20.00	1107.20	1059.10	27.70	0.06
323	0.27	6.62	20.00	1108.80	1053.90	27.60	0.06
333	0.55	0.30	20.00	1153.46	1088.83	27.84	0.21
334	0.33	0.14	20.00	1044.10	1057.30	27.70	0.10

*Transformed data

Legend: PS, Photosynthetic rate; TR, transpiration rate; SC, stomata conductance; CSS, sub-stomatal carbon dioxide concentration; PAR, photosynthetically active radiation on leaf surface; CCR, carbon dioxide concentration in the growth room; CCS, carbon dioxide concentration in the screenhouse; CCA, carbon dioxide concentration in the atmosphere and LST, leaf surface temperature. Time 1 = 205; 2 = 225 and 3 =231 days after culture initiation; Variety 1 = EAT, 2 = MYD, 3 = PRD; Medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB and 5 = PCA.

The sub-stomatal carbon dioxide concentration (CSS) in *in vitro* plantlets increased over time with atmospheric carbon dioxide concentration, irrespective of the medium and variety used. Seedlings *in vivo* showed a similar trend (in CSS), but the values were lower compared to the *in vitro* plantlets. The trend was similar in all varieties over the recording period (Figs. 5a, b and c, Fig. 6 and Table 4). The increase in CSS *in vivo* did not influence the photosynthetic rate, which remained more or less constant, regardless of changes in concentration of sub-stomatal carbon dioxide (data not shown).

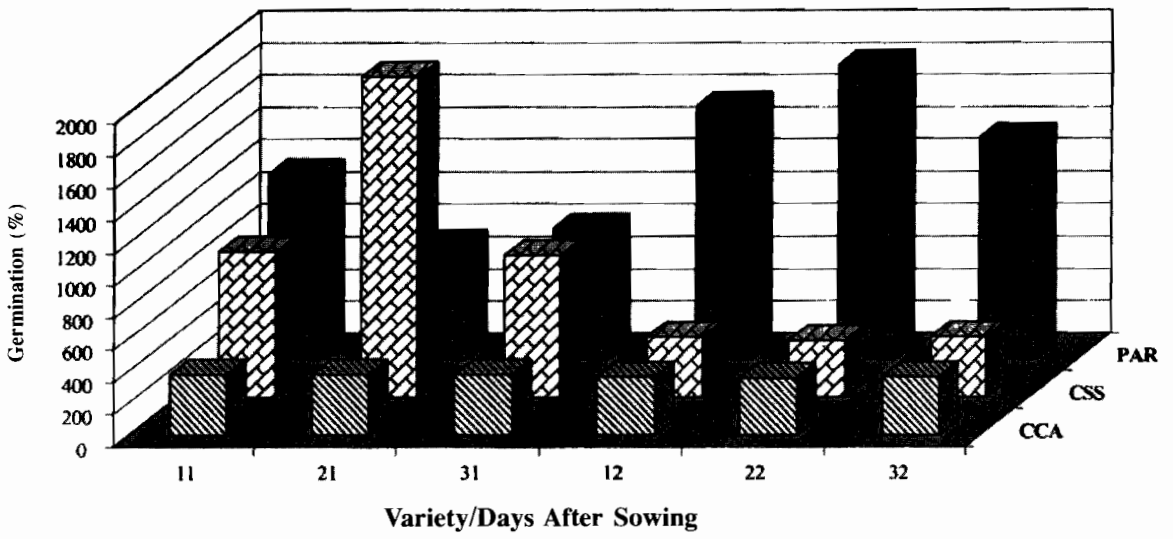

Fig. 6. Physiological performance of seednuts germinated *in vivo*. On the x-axis, the first digit represents the variety: 1 = EAT, 2 = MYD and 3 = PRD; and the second digit represents the number of days after sowing: 1 = 205, 2 = 225. Units for PAR were $\mu mol.m^{-2}.s^{-1}$ and for CSS and CCA were $\mu mol.mol^{-1}$.

Table 4. Physiological performance of coconut varieties *in vivo*

Variety/month	TR*	PS*	PAR	CSS	CCA
11	0.13	3.89	1145.29	904.38	374.66
21	0.10	2.17	707.33	1985.05	375.12
31	0.11	3.51	815.33	879.48	370.81
12	0.48	5.01	1562.94	372.52	354.31
22	0.44	5.99	1819.17	350.28	352.30
32	0.54	3.85	1365.55	371.46	358.74

*Transformed data

Legend: On the first column, the first digit represents the variety; 1 = EAT, 2 = MYD and 3 = PRD; and the second digit represents the number of days after sowing [1 = 205, 2 = 225]. Units for PAR were $\mu mol.m^{-2}.s^{-1}$ and for CSS and CCA were $\mu mol.mo1^{-1}$.

Photosynthesis *in vivo* was proportionately related to the stomata conductance levels (data not shown) but was not affected by the atmospheric carbon dioxide concentration. Of the five media tested *in vitro*, the photosynthetic rate was higher in plantlets grown in media O, U and P for MYD and PRD. EAT reacted poorly to all types of media over the entire recording period. Photosynthetically, media C and M showed poor support in all varieties. Higher levels of CSS observed in *in vitro* plantlets did not have any additional effect on the photosynthetic rate of the plantlets (Figs. 5a, b and c).

The photosynthetic rate *ex vitro* (Table 5) remained low and comparable to the *in vitro* rates before transfer from the growth room. In general, all varieties, irrespective of the medium type, showed poor photosynthetic rates in the first month of their growth *ex vitro* (Fig. 7).

Table 5. Physiological parameters of coconut embryo-derived plantlets after transfer *ex vitro*

Variety/Medium	TR*	PS*	PAR	CSS	CCS	LST
11	0.44	6.68	59.00	396.55	387.55	33.00
12	0.48	10.56	62.00	370.00	379.10	33.20
15	0.54	2.77	68.00	361.20	366.45	32.60
21	0.57	6.25	59.00	386.27	378.53	32.90
22	0.52	30.25	71.00	380.00	397.30	32.40
23	0.54	1.82	38.00	419.40	415.60	33.20
24	0.41	0.81	79.25	370.72	371.66	33.10
25	0.61	47.53	83.00	392.69	385.44	33.01
32	0.58	16.93	59.00	434.00	421.20	33.15
34	0.49	7.13	63.00	377.20	385.13	32.97
35	0.67	0.18	55.25	378.50	380.45	32.73

* Transformed data

Legend: PS, Photosynthetic rate; TR, transpiration rate; SC, stomata conductance; CSS, sub-stomatal carbon dioxide concentration; PAR, photosynthetically active radiation on leaf surface; CCR, carbon dioxide concentration in the growth room; CCS, carbon dioxide concentration in the screenhouse; CCA, carbon dioxide concentration in the atmosphere and LST, leaf surface temperature. Variety 1 = EAT, 2 = MYD and 3= PRD; Medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB and 5 = PCA.

PAR levels *in vitro* were less than 50% lower than the values recorded *in vivo*. The photosynthetic rate increased with PAR values, and likewise low PAR reduced the rate of photosynthesis in *in vitro* plantlets (Figs. 5a, b and c and Fig. 6).

The transpiration rate *in vitro* was consistently low in all varieties cultured in all types of media compared to those recorded with *in vivo* seedlings (Fig. 7). However, higher levels were observed when PAR increased. TR rates of *ex vitro* plantlets remained comparable to the rates observed *in vitro*. TR values, both *in vitro* and *ex vitro*, were far below the values observed in the *in vivo* seedlings.

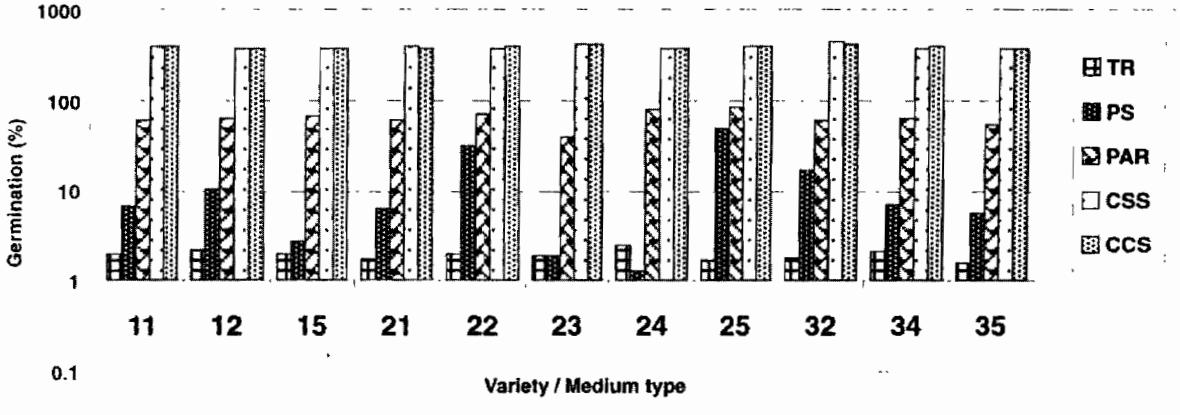

Fig. 7. Physiological parameters of coconut embryo-derived plantlets after transfer *ex vitro*. On the x-axis, the first digit represents the variety and the second the medium type where, 1 = EAT, 2 = MYD and 3 = PRD; medium types are 1 = IRD, 2 = CPCRI, 3 = MARI, 4 = UPLB, and 5 = PCA. Units for TR, PS and PAR were $\mu mol.m^{-2}.s^{-1}$ and for CSS and CCS were $\mu mol.mol^{-1}$.

Discussion

The embryos of the three coconut varieties cultured in five different media showed different germination rates and final germination percentages (Figs. 1, 2 and 3). It was apparent, from the results, that the five types of media were suitable for varieties MYD and PRD with respect to *in vitro* germination (Fig. 3). The poor germination of EAT embryos in all media tested and their low survival rate after transfer to the nursery could attributed to the special nutritional requirements for this variety (and possibly for other tall varieties in general). Some nutrients served for *in vitro* growth of the EAT embryos were probably at sub-optimal levels. A different medium type might therefore be required for EAT. As a result, only a few plantlets could be recovered from both *in vitro* and *ex vitro* cultures. The results implied that different coconut varieties could have different nutritional requirements for *in vitro* growth. The difference between the required amounts could be small but crucial. It is therefore necessary and important to develop a 'consensus' or 'universal medium' that contain the optimum nutritional levels for all coconut varieties *in vitro* so that the embryo culture technology can be applied more effectively for germplasm collecting.

Seednut germination *in vivo* gave better results compared to *in vitro* germination of embryos. Since seednuts sown *in vivo* came from the same lot as those cultured *in vitro*, the cultural practices was seen to have induced a difference in germination (*in vivo vs in vitro*). The lower *in vivo* germination observed with the PRD variety resulted from termite attack on some seednuts in the nursery before germination. Unless *in vitro* culture of embryos is required for germplasm exchange or collecting, raising coconut seedlings *in vivo* might be preferred.

The analysis of physiological data showed that plantlet performance *in vitro* and *ex vitro* was poor. The comparison among the five media types with respect to embryo germination, the photosynthetic and survival rates revealed that media O, U and P were better than media C and M. However, these media were better only for dwarf varieties, *i.e.* MYD and PRD. EAT performed poorly in all media types for all parameters observed *in vitro*. *In vivo* seedlings were better than *in vitro*-derived seedlings for all physiological parameters. Under *in vitro* and *ex vitro* conditions, the plantlets showed very low rate of photosynthesis. This could mainly be due to the corresponding low PAR values observed under those conditions and probably to the not so well developed and functioning stomata.

In vitro plantlets seemed to depend heavily on the carbon source supplied in the growth medium. This explains why growth could be sustained, even though photosynthesis was very low in *in vitro* plantlets. The effect was probably inherent to the formation of structurally inefficient photosynthetic machinery during growth *in vitro*. This probably had a significant impact on the survival of plantlets *ex vitro*. The majority of plantlets (as indicated by the survival rate in Table 1) were unable to adjust to autotrophic life when transferred *ex vitro*. As the photosynthetic machinery was not functional, no carbon source was available to the *ex vitro* plantlets so their chances of survival were low.

The low transpiration rate or its absence in some plantlets was considered a phenomenon that endangered their survival *in vitro*. However, despite this, plantlets showed sustained growth indicating that there was delivery of water, mineral nutrients and carbon source from the medium matrix. This could be attributed to the role of roots as observed by Peter *et al.* (1999). When transpiration is very slow or absent, the roots secreted ions into the xylem bringing about the water potential gradient. This enabled water movement by osmosis. It was probable that this phenomenon was common *in vitro*, especially when plantlets were in a heterotrophic mode of life. Since photosynthetic rate was generally extra-ordinarily low in *in vitro* plantlets, osmotic movement delivered the carbon source and nutrients from the medium (source) to

the growing parts of the plants. The cause of this phenomenon could be a combination of factors such as the abnormal stomata and high stomata resistance resulting from the increased boundary layer (due to high sub-stomatal CO_2 concentration). High relative humidity and still air in the culture vessel acted in combination to dramatically reduce transpiration in *in vitro* plantlets. Plantlets raised under these conditions could hardly survive the harsh *ex vitro* conditions.

The higher concentration of carbon dioxide in the growth room and in the sub-stomatal chambers could be equated to CO_2 enrichment. Since plantlets were ubiquitously supplied with essential nutrients and moisture, it was expected that the photosynthetic rate would rise considerably. However, this was not the case, mainly because, as mentioned earlier, the other factors (PAR, stomata functioning) played a more important role in the process.

The results obtained in the experiment indicated that the best medium types were not as efficient as expected. It might be relevant to continue searching for a "consensus medium" that would stand all the conditions to support embryo growth *in vitro* and *ex vitro*. This would reduce the risk of losing valuable genetic materials obtained during collecting missions, transfers and introductions. Alternatively, the *in vitro* conditions and procedures should be tailored to suit particular varieties if the development of the "consensus medium" proved to be difficult.

Conclusion

In as much as coconut germplasm collecting and exchange remain paramount to coconut improvement, the application of zygotic coconut embryo culture to this field of study requires thorough investigation of *in vitro* conditions that currently lead to low plantlet recovery *ex vitro*. The optimization of *in vitro* manipulation procedures and conditions need to be re-addressed. Medium composition may be a subset of many conditions that could affect plantlet survival *ex vitro*.

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Changes in culture conditions and medium formulation to improve efficiency of *in vitro* culture of coconut embryos in Mexico

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Introduction

Lethal yellowing (LY) is a devastating disease that has killed most coconut (*Cocos nucifera* L.) palms in the Yucatán Peninsula and has already started to affect the rest of México and other countries in Central America (Oropeza and Zizumbo 1997). One way of dealing with this disease is to make palms LYD-resistant through genetic improvement.

To attain this, appropriate coconut genotypes from other parts of the world must be introduced into México. To do this safely, embryo *in vitro* culture technique is recommended to ensure that the embryos germinate and convert into plantlets in a controlled environment. To date, most protocols exhibited low efficiency, with approximately 40% embryo conversion to plantlets compared to about 80% in seeds (Batugal and Engelmann 1998). This limits the application of the technique since the low plantlet production may represent unwanted genetic selection. These protocols used either liquid medium or solid medium during the initial weeks of culture when embryos germinate. The best results reported so far (conversion above 80%) have been from cultures on solid medium (Karun *et al.* 1998). The reason for this, however, was not clear. On the other hand, V.M. de Paz (pers. comm.) said that when embryos were cultured on solid medium, the percentage of germination was higher when they were placed with the plumule end upwards rather than downwards. No research has been done to test this systematically. In addition, the use of gibberellic acid (GA_3) has been found to promote *in vitro* germination of Kentia palm (*Howea forsteriana*) seeds (Chin 1988), while abscisic acid (ABA) has been reported to promote embryo maturation (Li *et al.* 1997).

This paper reports comparative studies on the use of solid medium and liquid medium, the position of embryos during culture, the effect of GA_3 on the germination of coconut zygotic embryos and their subsequent conversion to plantlets as well as on the different protocols tested with various genotypes.

Materials and methods

Plant material

For most studies, embryos were obtained from the Malayan Green Dwarf (MGD) coconut palms Yucatán, México. Other varieties used were Malayan Red Dwarf (MRD) obtained locally and Malayan Yellow Dwarf (MYD) obtained from two different sources, locally (different small sources) and from the Instituto Nacional de Investigaciones Forestales, Agricolas Y Pecuarias (INIFAP) in the State of Quintana Roo. At the collecting site, the fruits, 12–14 months after pollination, were cut transversely with a machete to expose the embryo surrounded by a solid endosperm. The endosperm enclosing the embryo was excised from the open nuts using a (1.6 cm diameter) cork borer (Fig. 1).

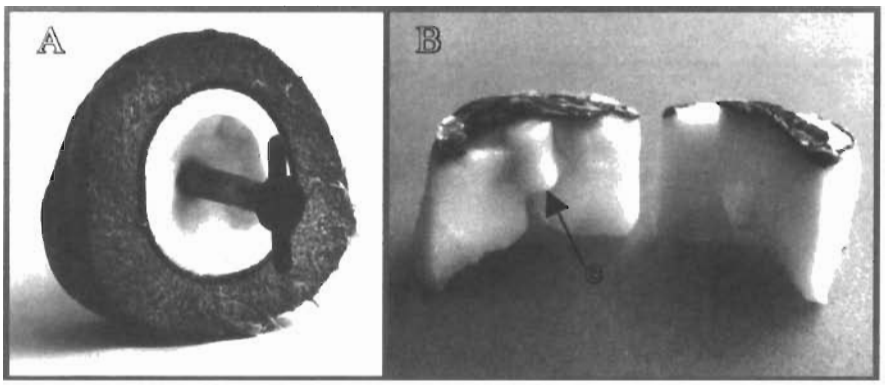


Fig. 1. A coconut fruit cut transversely (A) for extraction of an endosperm cylinder (B) containing the embryo (e).

Sterilization

As the endosperm cylinders (Fig. 1B) were extracted at the collecting site, they were placed in a 0.6% NaOCl solution. The cylinders were then surface-sterilized at the collecting site with 70% ethanol for three minutes, rinsed three times with distilled sterile water, washed in a 3% NaOCl solution for 20 minutes and rinsed three times with distilled sterile water. Once in the laboratory, under aseptic conditions, the endosperm cylinders were again washed in 70% ethanol for three minutes and rinsed three times with distilled sterile water, washed in a 3% NaOCl solution for 20 minutes and finally rinsed three times with distilled sterile water. The embryos were excised from the endosperm (Fig. 1B) and washed in a 0.6% NaOCl solution for 10 minutes before rinsing them with distilled sterile water three times.

Culture media and conditions for CICY protocol

All chemicals were obtained from Sigma (USA). Embryos were cultured in 50-ml culture vessels containing 10 ml of Y3 medium (Eeuwens 1976) as modified by Rillo and Paloma (1992) with the addition of activated charcoal (2.5 g/L). The pH of the medium was adjusted to 5.75 before the medium was autoclaved for 20 minutes at 120°C. During the first four weeks of culture, embryos were kept in the dark at 27 ± 2°C. They were then transferred to 25 ml of fresh liquid medium in 145-ml culture vessels and subsequently to 50 ml of fresh liquid medium in 500-ml vessels when the plantlets started to grow. The cultures were kept under a photoperiod (16 h light/ 8 h dark) with a light intensity of 45-60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR and subcultured every six weeks until plantlets developed three bifid leaves and three primary roots. At the end of the *in vitro* culture phase, isotactic isopropylene bags were placed above the 500-ml vessels to allow more room for the plantlets to grow (according to E. Rillo, unpublished). Flasks and covers used are shown in Fig. 2.

The experimental treatments included: (a) addition of gelling agent (3 g/L gelrite); (b) placing embryos in different positions when cultured on solid medium or supporting each embryo with a Sorbarod or floating ring when cultured in a liquid medium; (c) the addition of gibberellic acid (GA_3) at different concentrations (0.046, 0.46, 4.6 and 46 μM); (d) the addition of abscisic acid (ABA)/polyethyleneglycol (PEG) at different concentrations; and (e) the use of inert support materials for plantlet development (agrolite, vermiculite and cocopeat). For practical purposes, treatments with solid medium followed by liquid medium are referred to in the text as solid to liquid (S-L), and treatments with liquid medium followed by liquid medium are referred to as liquid to liquid (L-L).

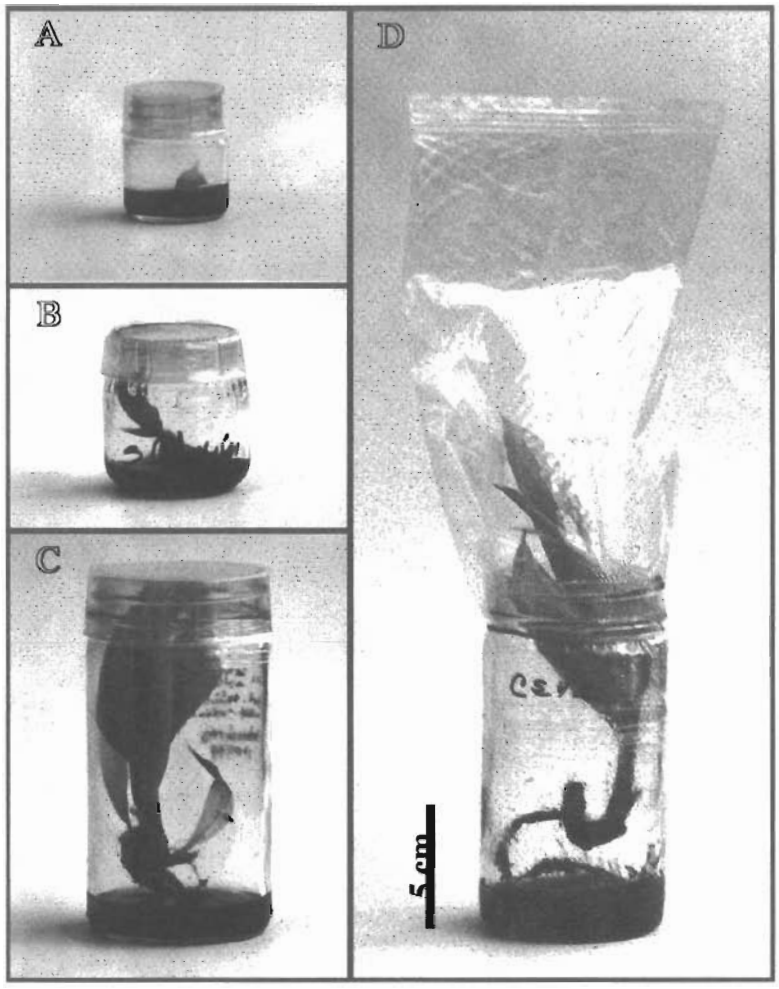


Figure 2. Containers for coconut embryo *in vitro* culture. Larger size containers were used as development progressed: embryo germination (A), shoot development (B), early plantlet development (C) and late plantlet development (D). The 5-cm bar stands for all the containers.

Culture media and conditions; other protocols

The other protocols tested were those from UPLB (Philippines), CPCRI (India), IRD-CIRAD (France) and PCA (Philippines); details of the protocols in Annexes 1.1 to 1.4 are according to reports by Batugal and Engelmann (1998). The protocols were followed as closely as possible, except for the sterilization procedure, which was done in all cases as described above.

Plantlet acclimatization

Plantlets were transferred to black polyethylene bags containing a mixture of peatmoss and soil (1:1) for acclimatization in a glasshouse.

Respiration rate and KCN treatment

Respiration of embryos was measured as O_2 consumption (µmol/h/g fw) using a Gilson 5/6H Oxygraph after 1, 3, 15 and 30 days of culture on solid medium. For this, embryos were transferred from the culture medium to a buffer solution (200 µM HEPES, pH 7) in the equipment cuvette. Measurement of respiration and percentage of germination were carried out with or without the respiration inhibitors KCN (0.0005 to 50 µM), sodium azide (0.0005 to 0.5 µM) and 2,4-dinitrophenol (2,4-DNP, 0.0005 to 0.5 µM).

Discussions

Effect of gelling agent and embryo position for germination

To study the effect of the gelling agent on the percentage of germination, coconut embryos cultured on medium with the gelling agent (S) were placed in three different positions: upward (SU), horizontal (SH) and downward (SD) (Fig. 3A). Embryos cultured in the medium without gelling agent (L) were placed only in the horizontal position.

Germination of embryos in L was lower than that in S where the embryos were cultured with the plumule end in either the upward (SU) or horizontal (SH) position (Fig. 3, Table 1). Germination was highest with the SU embryos. The same pattern was observed for the conversion of embryos to plantlets (Fig. 3B and Table 1) and the plantlet development (Fig. 4A and B). Whole plantlets were obtained from 70% of the embryos after only six months of culture. It was noted that germination and performance in general were better on solid medium than in liquid medium. In all the above mentioned cases, after four weeks of culture with or without the gelling agent, embryos were transferred to liquid medium. Earlier trials had shown that if embryos were retransferred to solid medium, their development was retarded and most embryos failed to develop into plantlets (data not shown).

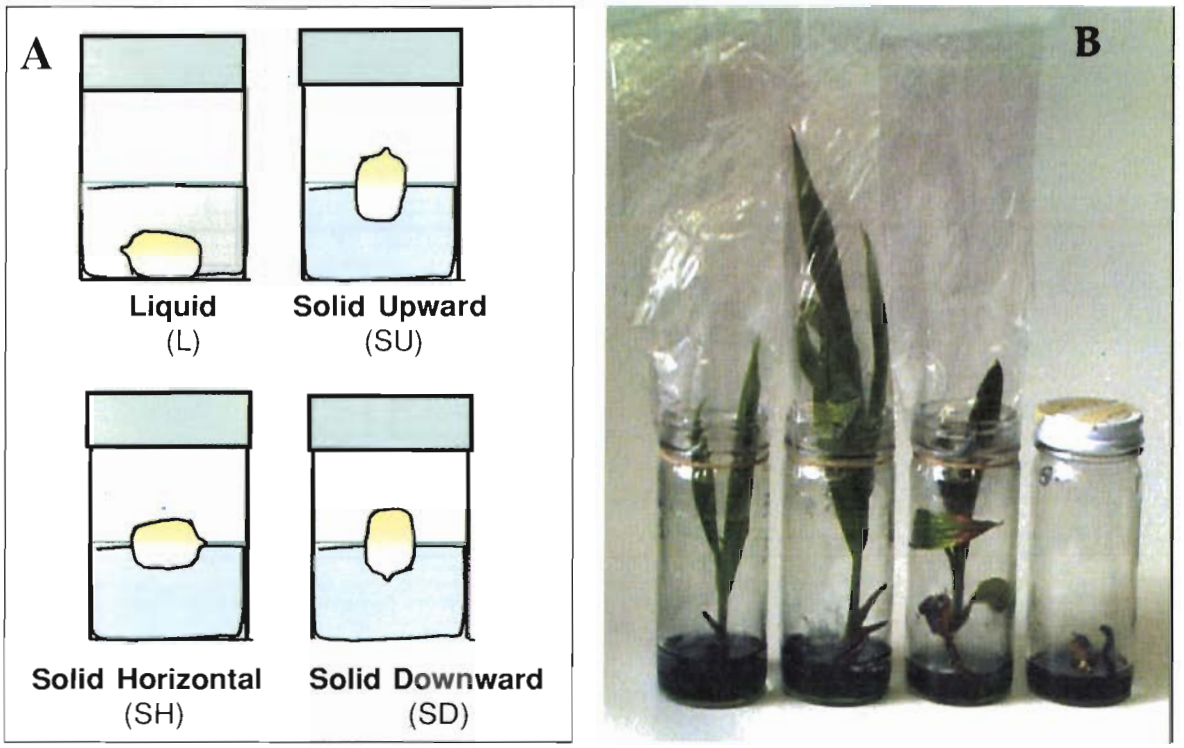


Fig. 3. Effect of the position of embryo in cylture and gelling agent (3 g/L gelrite) on the *in vitro* germination of MGD zygotic embryos (A) and their conversion into plantlets after six months (B).

Table 1. Effect of types of medium and the position of embryos during culture on percent germination and conversion to plantlets

Parameters	Liquid	Solid		
		Upward	Horizontal	Downward
Germination (%)	66 c	93 a	88 b	56 c
Conversion (%)	45 c	88 a	59 b	37 c

Data shown are means (3 batches; n = 20 embryos per batch). Different letters within rows denote significant differences.

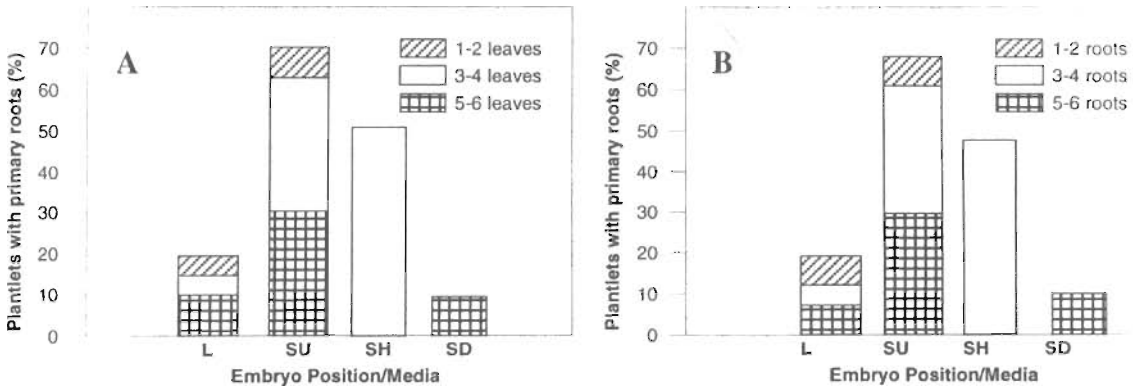
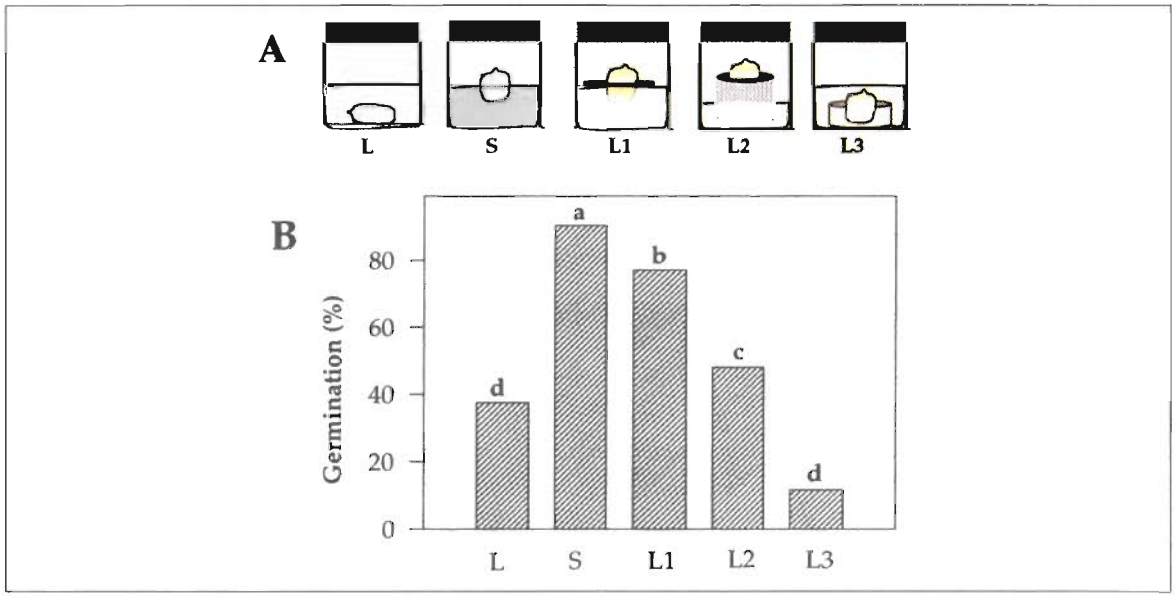


Fig. 4. Effect of gelling on the *in vitro* development of MGD zygotic embryos after six months of culture. (A) Plantlets with bifid leaves; (B) Plantlets with primary roots. Data shown are percentages from 20 plantlets analysed. Embryo position and media are denoted by: L, liquid; SU, solid upward; SH, solid horizontal; and SD, solid downward.

The beneficial effect of the initial culture on solid medium depended on the position of the embryos. Embryos in SD had a performance as low as those in L. In both cases, the plumule end of the embryo was submerged. Whether this was important or not was tested as shown in Fig. 5A.

Embryos cultured in liquid medium with the plumule in the upward position performed better than those in treatments in which the embryos were submerged either in a horizontal or upward position (Fig. 5B). Performance was significantly better when the plumule end of the embryos was kept on the surface of the medium.



Figures 5a and 5b. *In vitro* MGD zygotic embryo germination under different treatments (A): L, embryos submerged in liquid medium; S, embryos kept on the surface on solid medium; L1, embryos in liquid medium kept on the surface with a floating ring; L2, embryos in liquid medium kept on the surface with a Sorbarod; L3, embryos in liquid medium kept submerged with a Sorbarod. Embryos were kept standing in the upward position in treatments S, L1, L2 and L3, and horizontal in liquid medium. Solid medium contained gelling agent (3 g/L gelrite). (B) Germination percentages after month of culture. Data shown are means (3 batches; n = 20 embryos per batch). Different letters denote significant differences.

Aerobic respiration in germinating embryos

It is known that some seeds need O_2 and aerobic respiration for their germination (Bewley and Black 1983). When O_2 was removed from the container by replacing the container air atmosphere with N_2, germination of coconut zygotic embryos in S dropped from above 80% to about 12% (Fig. 6). This suggests a requirement for air for their germination. To test the need for aerobic respiration for germination, embryos were cultured in media supplemented with respiration inhibitors KCN. Embryos showed respiration (4.6 ± 1.2 µmol O_2/h/g fw) without KCN, but it dropped to below basal levels when 5 or 50 µM KCN was added (Table 2). Germination dropped from 100% without KCN to 0% when KCN was added. Lower KCN concentrations (0.0005 – 0.5 µM) produced similar results (data not shown). When the embryos were transferred from medium containing 0.0005 µM KCN at week two of culture to medium without the inhibitor, over 50% of them germinated in the following two weeks. Similar results (data not shown) were observed when other respiration inhibitors (sodium azide and 2,4-DNP) were used. Taken together, these results and those reported in the previous section support a requirement for aerobic respiration for coconut zygotic embryos to germinate successfully.

Treatment	Liquid	Solid	Solid + N2
Germination (%)	10 b	87 a	12 b

Figure 6. Effect of replacement of container air atmosphere by N2 on the percentage germination of MGD zygotic embryos. Shown are embryos cultured for four weeks on solid medium, as compared with embryos cultured with solid or liquid medium with normal air atmosphere. Embryos on solid medium were placed on the surface of the gel with the plumule end upwards. Different letters denote significant differences.

Table 2. Effect of KCN on the germination and respiration of MGD zygotic embryos cultured on solid medium with the plumule end upwards

KCN (µM)	Germination (%)		Respiration (µmoles O_2 / h / g fw)		
	15 days	30 days	0 day	15 days	30 days
0	100	100	1.39 ± 0.45	1.62 ± 0.45	4.6 ± 1.2
5	0	0	1.39 ± 0.45	0	0
50	0	0	1.39 ± 0.45	0	0

Addition of GA_3 to the medium

Initial observations after four weeks of culture (Fig. 7) showed that the percentage of germination of coconut embryos in media containing GA_3 was higher than that in media without this hormone, regardless of whether a gelling agent was present or not. The best GA_3 concentrations were at 0.46 and 4.6 µM for liquid medium and solid medium, respectively. Given the results of the observations, an experiment was carried out to test the different treatments in combination with a gelling agent, the upward embryo position and the effect of GA_3 on the germinating embryo.

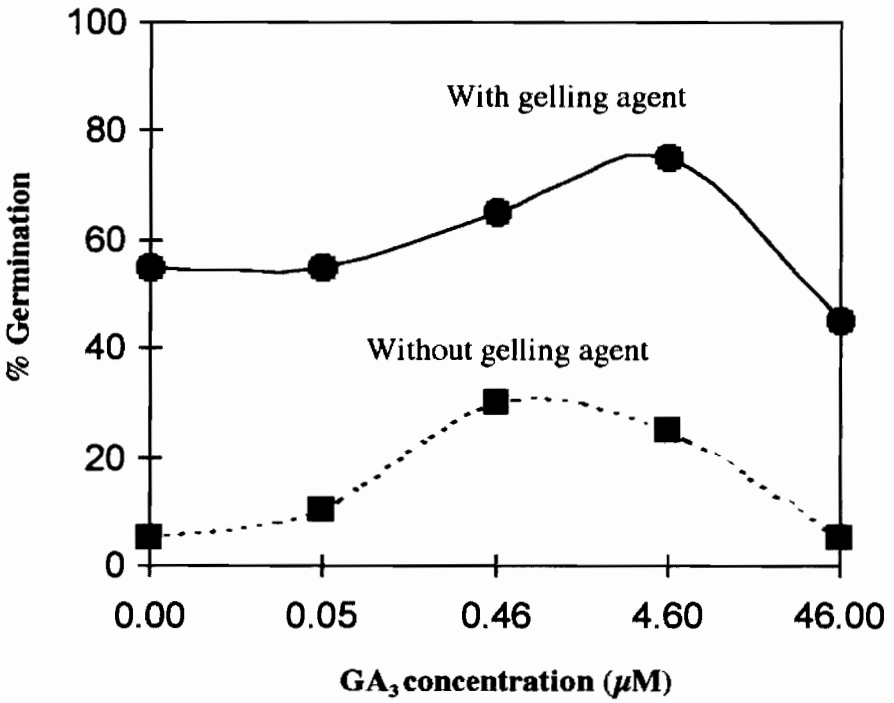


Figure 7. Effect of GA3 concentration in media with or without gelling agent (gelrite 3 g/L) on *in vitro* germination of MGD zygotic embryos. Data taken after four weeks of culture. When using medium with gelling agent, embryos were placed with plumule axis upwards. Points represent means (n = 20).

Again, the results showed that the percentage of germination on solid medium (S-L) was higher than that in the liquid medium (L-L), irrespective of the presence of GA_3 (Fig. 8A). When GA_3 was added, embryos germinated sooner in both the solid and liquid medium (Fig. 8A). The best treatment was seen in the solid to liquid cultures with GA_3 which promoted the germination of over 90% of the embryos. This treatment also showed just as good a result – nearly 90 % - in the conversion of the embryos to plantlets whereas in the liquid to liquid treatment (containing GA_3 as well), the conversion rate of the embryos to plantlets was only 45%. The corresponding treatments without GA_3 showed lower percentages of conversion. A second experiment with more replicates showed the same pattern but with lower means (Fig. 8B). Plantlets of each treatment were transferred to *ex vitro* conditions when they had three bifid leaves and three main roots. No losses have been reported yet in each treatment after three months (Table 3).

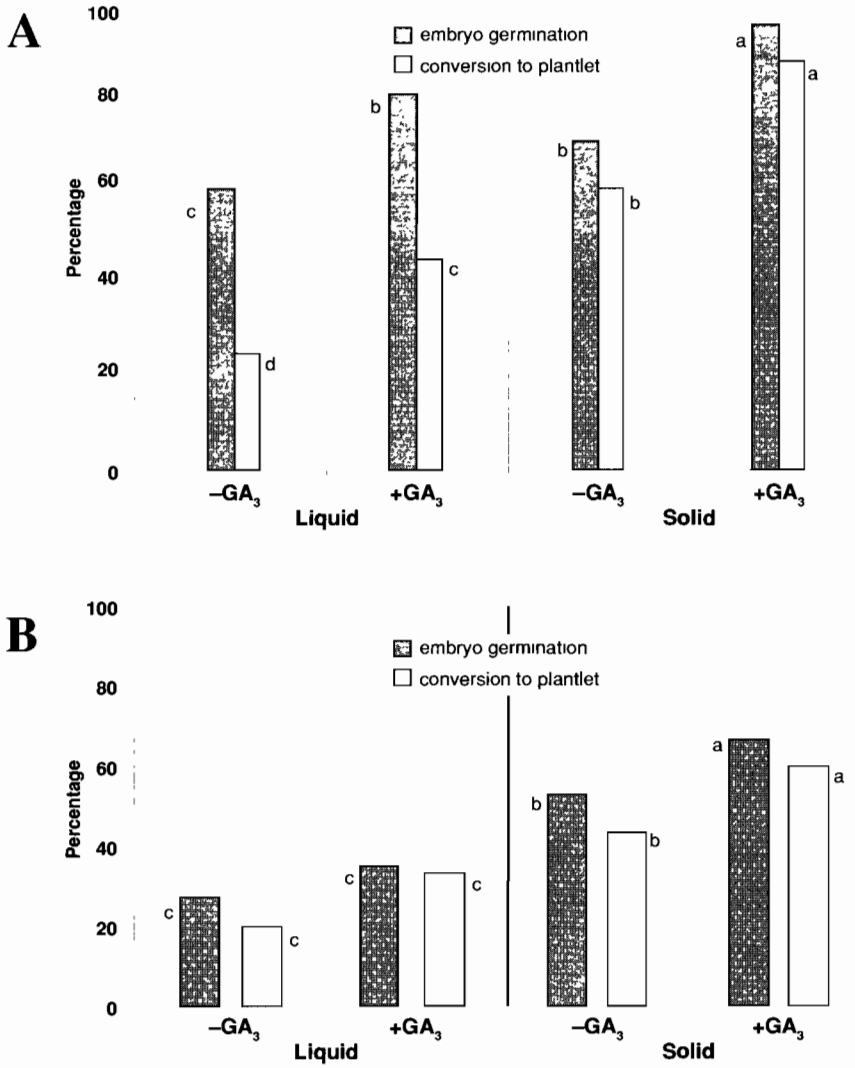


Figure 8. Effect of four different treatments on *in vitro* germination of MGD zygotic embryos and their conversion to plantlets after 33 weeks of culture (2 trials, A+B). Treatments are medium with or without GA_3, and in each case with (solid) or without (liquid) gelling agent (3 g/L gelrite). Embryos on solid medium were cultured for the initial four weeks on this medium, then transferred to liquid medium for the rest of the experiment. Those in liquid medium were also transferred to liquid medium for the rest of the experiment. When using a medium with gelling agent, embryos were placed with the plumule axis upwards. GA_3 concentration was 4.6 µM for liquid medium and 46 µM for solid medium. Data points are means: (A) n = 20; (B) 3 batches, n = 20 for each.

Table 3. Survival after three months in *ex vitro* conditions of plantlets obtained from different *in vitro* culture treatments of MGD zygotic embryos

Treatment		Survival	
Medium[a]	GA_3 (μM)	n[b]	%
L	0	10 / 10	100
L	0.45	15 / 15	100
S	0	33 /33	100
S	4.46	42 / 42	100

(a) L = liquid Y3 medium (without gelrite), S = solid Y3 medium (with gelrite).

(b) Plantlets were transferred from *in vitro* culture when they had three bifid leaves and three main roots. The number of plantlets vary because treatments produced different numbers of developed plantlets.

Effect of ABA/PEG on embryo germination

The phytohormone ABA promotes maturation of embryos. If coconut zygotic embryos from nuts of a particular age are not all at the same maturity level, ABA could be useful to synchronize their physiological status, germination and plantlet development. An experiment was initiated to test the effect of ABA/PEG on plantlet development but all plants were lost due to contamination. Another experiment was thus initiated. Results showed that the use of ABA and PEG, whether independently or in combination at all concentrations tested, decreased the percentage of embryo germination (Table 4). These results, when compared to those reported by UPLB, showed an increased germination rate with the ABA treatment, particularly at the 2.2 μM concentration level. As the present experiment continues, the effects of the various treatments on plantlet development will be observed.

Table 4. Effect of ABA/PEG application on the *in vitro* germination of MGD zygotic embryos

ABA (μM)	Percent Germination		
	0 PEG (g/L)	10 PEG (g/L)	30 PEG (g/L)
0	98.6	52.6	50.0
2.2	65.0	30.0	33.3
22.5	75.0	40.0	25.0
45.0	38.0	31.5	45.0

Figures derived based on n=20.

Use of support materials

Plantlets (MGD) were transferred from liquid medium without any support material to liquid medium with a support material. Three different types of support material such as the agrolite, vermiculite and cocopeat were used in the test to see if root and plantlet development in general could be improved (Fig. 9A). The results showed that plantlet development was poorer when any of these support materials was used (Fig. 9). These support materials affected both the root and shoot development (Fig 9B). Plantlet survival in the presence of all the support materials during the two-month test was also poor – 25% with agrolite, 12% with cocopeat and 0% with vermiculite – compared to 100% (n=20) survival of control plantlets growing in liquid medium without any support material. The results thus obtained were the opposite of what were expected. In view of this, no further tests were carried out on the use of support materials.

Comparison of different protocols

The first set of tests using different protocols was started in December, 1998. Unfortunately it was affected by the mite / contamination problem in the culture rooms. A second set of tests followed in 1999. The protocols (Annexes 1.1 – 1.4) tested were those from CPCRI (India), PCA (Philippines), UPLB (Philippines) and IRD-CIRAD (France) as reported at the International Embryo Culture and Acclimatization Workshop (Batugal and Engelmann 1998). Within the CPCRI protocol itself, three concentrations – 0.25, 0.5 and 1 mg/L for each NAA and 6-BAP – of growth regulators were tested. The CICY protocol used, herewith referred to as 'modified protocol', included modifications in relation to the protocol, referred to as 'own before start of project', used previously. These modifications were: (a) initial four week culture on solid medium instead of liquid medium; (b) during this period, embryos were placed on the medium surface with the plumule end facing upwards; and (c) during the last weeks of the in vitro culture, isotactic isopropylene bags were placed above the flasks to accommodate further growth. The genotype used was the MYD (from Dzindzantun, Yucatán). Results shown in Tables 5 and 6 indicated that the best germination rates (70.7 – 82.3%) were obtained with the CPCRI protocol with any of the growth regulator concentrations used (Table 5). This performance was followed by the PCA protocol exhibiting an early 70% germination rate compared to the other protocols with a germination rate higher than 50%.

The proportion of embryos developing shoots (data not shown) was highest for the CPCA protocol (60%); intermediate for the CPCRI protocol (48%), UPLB protocol (47%) and the IRD-CIRAD protocol (41%) and lowest for the CICY modified protocol (about 30%). However, development was very slow with only shoots obtained with most of the protocols after 68 weeks. Presently, whole plantlets have been obtained only with the CICY modified (28%) and the CPCRI (2.1%) protocols (Table 5). Plantlet development was faster with the CICY modified protocol (Fig. 10). In the case of the CICY protocol, these results differed from those reported in previous sections when evaluations were carried out with MGD, suggesting a genotypic effect. The duration for the growth of the whole plantlets was also very long compared to that observed for MYD in the test reported in Table 6. This could probably be related to the fact that the nuts for each test were obtained from different locations, resulting in G x E variance.

The nuts were obtained locally from different small sources for the protocol test (Table 5), whereas for the genotype tests (Table 6), they were obtained from a single source (INIFAP) in a remote location. The test results, obtained with MYD embryos, cannot be compared with those obtained with the CICY 'own before start of project' protocol as the latter used MGD embryos. Nevertheless, the test results showed that the protocol performance was affected by the genotype used.

Figure 9. Effect of the use of support materials on coconut (var. Malayan Green Dwarf) *in vitro* plantlet development: (a) no support, (b) agrolite, (c) vermiculite, and (d) cocopeat. Plantlets with two bifid leaves and at least one primary root grown in liquid medium without support material were cultured for two months in medium with a support material.

Fig. 10. Plantlet development after nine months of culture of MGD zygotic embryos using the CICY protocol (a) and the CPCRI protocol with two different growth regulator concentrations (b) and (c).

Table 5. Summarized results of protocol comparison studies in CICY using MYD embryos

Country: México	**Laboratory:** CICY	**Researcher:** Carlos Oropeza
Date submitted: August 28, 2000	**Genotype used:** Malayan Yellow Dwarf (Dzidzantun, Yucatán.)	

	Culture protocol used					
	CICY Own before start of project	**Protocols tested in the current project**				
		UPLB	**PCA**	**CPCRI1**	**ORSTOM**	**CICY (modified)**
Number of embryos inoculated	ND	100	99	a) 98 b) 96 c) 99	99	100
Contamination (%)	ND	5 (0.02)	2 (0.05)	a) 5.1 (0.05) b) 3.1 (0.03) c) 4.0 (0.025)	8 (0.13)	4 (0.05)
Germination (%)	ND	54 (0.21)	69.7 (0.10)	a) 77.5 (0.163) b) 82.3 (0.110) c) 70.7 (0.125)	60 (0.13)	52 (0.08)
Developing shoots [2]	ND	42.0 (0.17)	53.3 (0.08)	a) 26.5 (0.22) b) 27.0 (0.30) c) 25.2 (0.14)	32 (0.07)	0
Whole developing plantlets [3]	ND	0	0	a) 0 b) 0 c) 0	0	4 (0.77)
Whole plantlets ready for transfer to *ex vitro* (%) [4]	ND	0	0	a) 2 b) 2.1 c) 0	0	24 (0.43)
Total whole plantlets *in vitro* (%)	ND	0	0	a) 2 b) 2.1 c) 0	0	28
Average duration of *in vitro* culture (weeks) to obtain whole plantlets	ND	ND	ND	ND	ND	68
Survival after transfer to nursery (%)	ND	ND	ND	ND	ND	92
Survival after transfer to field (%)	ND	ND	*ND*	ND	*ND*	ND

(1) a, b and c denote 0.25, 0.5 and 1 mg/L of each NAA and BAP. (2) Shoots have developing leaves but not developing roots. (3) Plantlets with several leaves and one or two main roots. (4) Plantlets with at least three bifid leaves and at least three main roots. ND = not determined yet.

Table 6. Summarized results of protocol comparison studies in CICY using three genotypes

Country: México **Laboratory:** CICY **Researcher:** Carlos Oropeza

Date submitted: August 28, 2000 **Genotypes used:** MGD, MYD and MRD

	Culture protocol used						
	CICY Own before start of project	**Protocols tested in the current project**					
		CPCRI			**CICY (modified)**		
		MGD	**MYD**	**MRD**	**MGD**	**MYD**	**MRD**
Number of embryos Inoculated	300	125	150	94	141	131	100
Contamination (%)	ND	7.6	17.3	4.2	5	0	0
Germination (%)	82.3 (0.06)	98.4 (0.5)	46.7 (0.27)	39.4 (0.14)	98.6 (0.024)	62.9 (0.055)	62 (0.45)
Whole developing plantlets [2]	ND	13.8 (0.05)	7.3 (0.02)	2.1 (0.14)	39.2 (0.1)	17 (0.2)	0
Whole plantlets ready for transfer to *ex vitro* (%) [3]	ND	0	0	0	46.4 (0.08)	20 (0.1)	11 (0.6)
Total whole plantlets *in vitro* (%)	35.8 (0.19)	13.8 (0.11)	7.3 (0.56)	2.1 (0.16)	85.6 (0.02)	37 (0.02)	11 (0.6)
Average duration of in vitro culture (weeks) to obtain whole plantlets ready for transfer [4]	Over 40	ND	ND	ND	31	30	31
Survival after transfer to nursery (%)	81	ND	ND	ND	100	ND	ND
Survival after transfer to field (%)	ND	ND	ND	ND	ND	ND	ND

(1) MGD, Malayan Green Dwarf (Dzidzantun, Yucatán.); MYD, Malayan Yellow Dwarf (Chetumal, Q. Roo); MRD, Malayan Red Dwarf (San Crisanto, Yucatán.). (2) Plantlets with several leaves and one or two main roots. (3) Plantlets with at least three bifid leaves and at least three main roots. (4) Average duration given is not the definitive one since it was calculated based only on the data for current whole plantlets ready for transfer *ex vitro*, and there are other whole developing plantlets yet to reach that stage of development. ND = not determined.

As a follow up of the work described above (Table 6), a test with MYD, MRD and MGD genotypes using the CPCRI (growth regulators at 0.5 mg/L) and CICY protocols was initiated. The test was intended to include other genotypes (MYD x Tall hybrids) but they were not available when the test started. Results showed similar germination rates for MGD. However, it was observed that there was a higher germination percentage for the MYD and MRD embryos using the CICY protocol than with the CPCRI. The percentage of whole plantlets ready for transfer *ex vitro* and total whole plantlets was higher with the CICY protocol than with the CPCRI protocol for the three genotypes used (Table 6). The total number of whole *in vitro* plantlets was highest for the MGD variety, intermediate for the MYD and lowest for the MRD, with both of the protocols. Therefore, according to these results, the performance of both protocols was dependent on genotype. This corresponded with what was reported by other laboratories working on the same project (Second DFID Project Progress Report, 1999). Results obtained with the current CICY modified protocol showed a higher percentage of both the germination and total whole plantlets obtained compared to those obtained previously with the CICY 'own before start of project' protocol (using MGD embryos). This showed that an improvement has been achieved during the course of the project (Table 6). At the time this report was compiled, our study showed that the average duration to obtain the whole plantlets cultured *in vitro* (ready for transfer *ex vitro*) cannot be compared to the earlier report (Table 6) as the latter was calculated based only on the data for whole plantlets ready for transfer to *ex vitro*. There were other whole plantlets which have yet to reach this stage of development.

Results

The various tests showed that germination and performance in general were better on solid medium. In all cases, after the fourth week of culture, with or without the gelling agent, the embryos that were transferred to a liquid medium survived and developed into plantlets. In earlier studies done, it was noted that when the embryos were transferred to the liquid medium, their developent was retarded and most of the embryos failed to develop into plantlets. It was also noted that the performance was significantly better when the embryos were placed horizontally with the plumule facing upwards on a solid medium.

Both past and present results show that there is a requirement for aerobic respiration for coconut zygotic embryos to germinate. Results showed that with the addition of GA_3, embryos germinated sooner regardless of whether they were cultured on solid or in liquid medium. The solid to liquid treatment, with GA_3 added, promoted the germination of over 90% of the embryos and a good conversion rate of 90% of the embryos to plantlets as compared to about 45% conversion in the liquid to liquid treatment. In both treatments, plantlets having three bifid leaves and three main roots upon transfer survived after three months.

The presence of ABA or PEG, by itself or together and at all concentrations tested, decreased the germination rate of the embryos. As this result contradicted the previous report by UPLB which showed an increased germination rate with ABA, the present study will continue.

The shoot and root systems of plantlets transferred from liquid medium with no support material to liquid medium with support materials like agrolite, vermiculite and cocopeat showed poor plantlet development. Plantlet survival rate was poor with all support materials – 25% with agrolite, 12% with cocopeat and 0% with vermiculite – compared to a 100% survival rate of control plantlets in liquid medium without the support materials.

Problems encountered

The two main problems encountered were:

- **Contamination**
  At the end of 1998, an arthropod infestation and an associated contamination in the laboratory affected not only the experiment on GA_3, which had been running for over six months, but also an experiment to test the effects of ABA/PEG that had just started. Most cultures in these experiments were lost.

- **Limited nut availability**
  This was specifically a problem for the test genotypes. Lethal yellowing, coupled with the demand for tender coconut water, had dramatically decreased the number of palms and consequently the number of nuts available for the experiments.

Conclusions

- The results of the experiments show that the coconut embryo culture technique used with the MGD embryos can improve the percentage of germination and conversion to whole plantlets if the embryos are cultured: (a) on a solid medium during the first four weeks of culture and then transferred to a liquid medium for the rest of the culture duration; (b) by placing them on the surface of the gel with the plumule end upwards; and (c) on a medium with GA_3.
- Evidence indicated that aerobic respiration is needed for coconut zygotic embryos to germinate.
- Results of comparing protocols using three test cultivars showed that the type of protocol used had a genotypic effect on performance.

Acknowledgements

The authors wish to thank Miguel Fernández and Nelson Torres of CICY for the technical assistance rendered; COGENT and CONACYT-SISIERRA (México) for the partial financial support given; and INIFAP CIR-SUR for providing some of the plant materials used.

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Increasing the efficiency of the embryo culture technology to promote germplasm collecting in Cuba

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Background and rationale

Tissue culture techniques have important applications for coconut germplasm collecting, conservation and exchange. In the plant kingdom, coconut is the species with the largest seeds. However, coconut seeds are recalcitrant and do not have a dormancy period so that germination takes place almost immediately after ripening. Both characteristics impede the use of seeds for genetic resources conservation for this species.

The establishment of coconut germplasm collections, and the breeding and evaluation programmes initiated by COGENT require intensive germplasm exchange (Rao and Batugal 1998). The *in vitro* culture technology significantly facilitates this exchange because it prevents the introduction of pest and diseases to regions where they do not exist. This technology is also very useful for collecting germplasm in distant and isolated places where the transport of such large fruits pose a major problem.

Besides these advantages, *in vitro* culture is very useful in rescuing non-germinating embryos, as in the case of the Makapuno mutant. This technique also facilitates the study of disease transmission because it offers the possibility of working with living tissues.

Objectives

1. To compare the efficacy of four different embryo culture protocols;
2. To develop embryo culture *in vitro* protocols appropriate to the desired genotypes;
3. To study the effect of selected growth regulators on plantlet survival *in vitro* and *ex vitro;* and
4. To investigate the effect of different sucrose concentrations in the growth medium on plantlet survival *in vitro* and *ex vitro*.

Activities conducted

Embryo culture

The study compared the *in vitro* performance of two coconut cultivars with four different embryo culture protocols (Anexes 1.1 – 1.4). Mature coconut embryos of Criollo Tall and Malayan Yellow Dwarf were collected from palms planted at Baracoa, Guantanamo province located 1200 km away from the experimental station.

The embryos and plantlets subsequently developed were cultured according to the procedures described in each protocol.

Ex vitro plantlet establishment

For *ex vitro* plantlet establishment, the procedures developed in the Instituto de Investigaciones de Cítricos y otros Frutales (IICF) were adopted in all experiments with the four protocols.

Statistical analysis

Treatments included four protocols and two genotypes replicated three times with ten embryos per replicate, i.e. a total of 120 embryos/genotype. The following data were gathered:

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

Data collected were analyzed statistically. A bifactorial analysis was applied for protocols and coconut varieties. Differences among means were compared using the Duncan Multiple Range Test.

Results and discussion

Fig. 1 presents the contamination percentages obtained with each of the protocols tested using fruits collected in Baracoa. The best test result was obtained with the UPLB protocol (20% contamination).

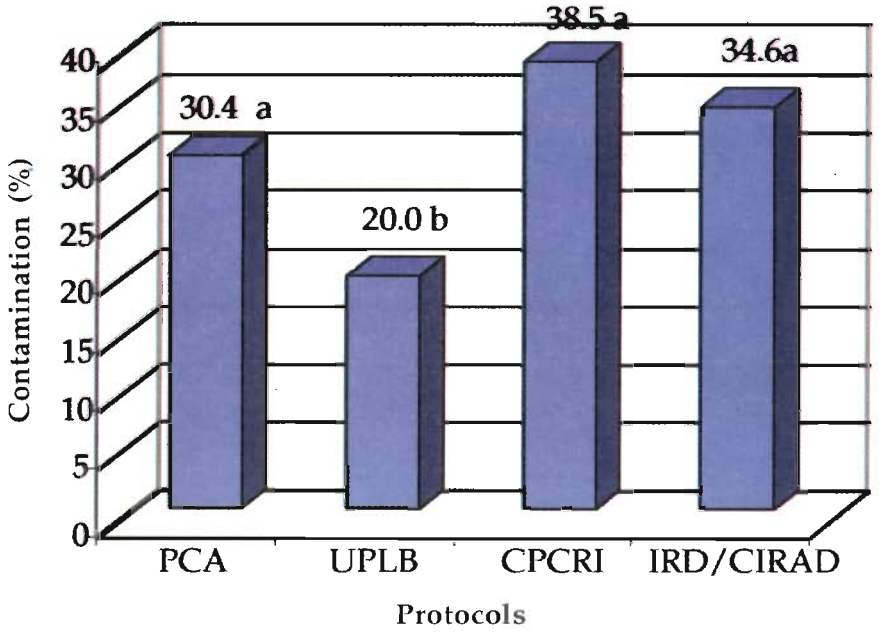

Fig. 1. Percentage of contamination of embryos extracted from fruits collected in Baracoa.

Although contamination percentages were within the range reported by Assy Bah (1986) and Del Rosario (1998), they were high for the test explants. Mkumbo *et al.* (1997) mentioned this problem pointing out that it generally appears when extractions of the endosperm cylinders are not performed immediately after collecting the fruit. For this test, the fruits used were harvested from orchards located in Baracoa, Guantánamo, 1200 km away from the laboratory. This might have been the cause of such high contamination values. However, it is important to mention that the seednuts were kept inside the fruits so that they were well protected. The testa was also kept intact.

Considering that these values might have been affected by the storage time of fruits before embryo extraction, the process was repeated with fruits picked at a nearby orchard.

The results showed no contamination of embryos either with the PCA or the UPLB protocols (Fig. 2). The CPCRI protocol produced a 21% contamination by bacteria and fungal colonies. However, in this experiment, the IRD (Institut de Recherche pour le Développement) protocol produced 93.3% contamination, which was much higher than the result obtained in the previous experiment.

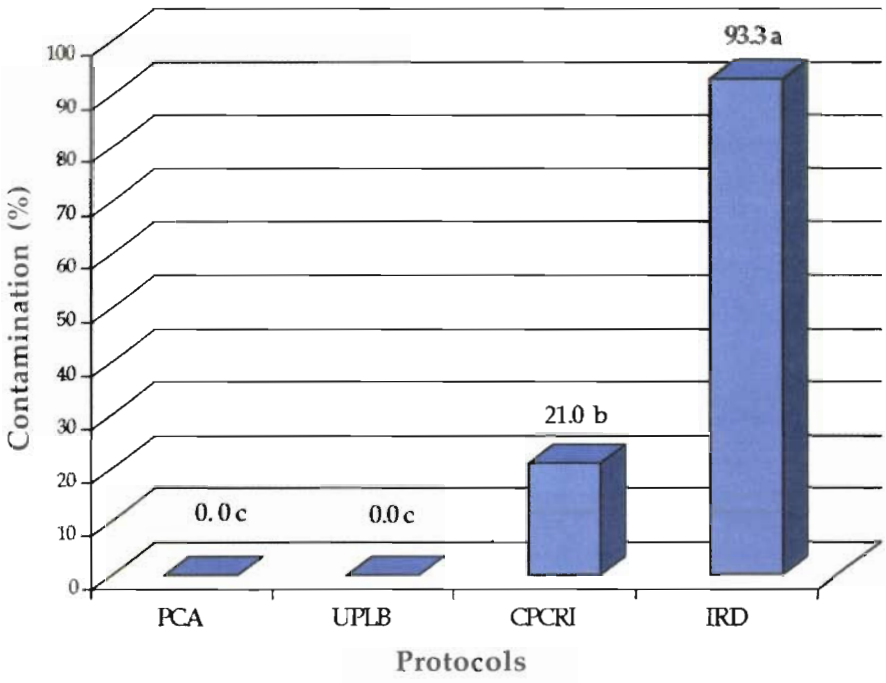

Fig. 2. Percentage of contamination of embryos extracted from fruits collected in an orchard close to the tissue culture laboratory.

These results confirmed what Mkumbo *et al.* (1997) had indicated regarding the need of having fresh fruits available for laboratory work. This also suggests that where there are difficulties with fruit transportation, the embryo *in vitro* inoculation process should preferably be carried out *in situ*.

Oxidation

Oxidation was observed only in some zones of the explants where, as a result of the extraction process, some mechanical damages had occurred but with no effect on the embryo growth.

In vitro culture

Table 1 shows the percentage of surviving, germinated, abnormal and latent embryos in the four protocols after three months in culture. Highly significant differences were found in the interaction between protocols and cultivars. The two cultivars showed differences in behaviour with the four protocols evaluated. The UPLB protocol produced the highest percentage of surviving and germinated embryos with both cultivars. This suggests the effectiveness of the culture medium used by UPLB. The CPCRI protocol had the highest percentage of abnormal embryos. This suggests that some of the components of this culture medium promote abnormalities in embryo development. The number of latent embryos was highest with the IRD protocol for both varieties, showing this protocol as the least efficient.

Table 1. Percentages of surviving, germinated, abnormal and latent embryos of the two coconut varieties cultured using four different protocols

Parameter	Cultivar	PROTOCOL				C.V.
		PCA	UPLB	CPCRI	IRD	
Surviving embryos (%)	Tall	95.50a	97.01a	91.69c	82.14d	4.30
	Dwarf	94.95ab	96.87a	93.59b	91.13c	
Germinated embryos	Tall	66.20c	77.10a	33.30e	46.60d	1.97
(%)	Dwarf	71.30b	78.10a	29.90f	47.40d	
Abnormal embryos (%)	Tall	8.45d	6.61e	33.39b	8.84d	4.06
	Dwarf	5.85e	6.27e	43.39a	18.13c	
Latent embryos (%)	Tall	16.90d	13.30e	25.00b	26.70a	4.20
	Dwarf	17.80d	12.50e	20.30c	25.60ab	

Means with different letters are significantly different.

Fig. 3 shows embryo growth after four weeks of *in vitro* culture using the developmental stages defined by Fremond *et al.* (1968). The UPLB protocol produced 64 % of embryos at Stage 5 with well-developed roots and shoots.

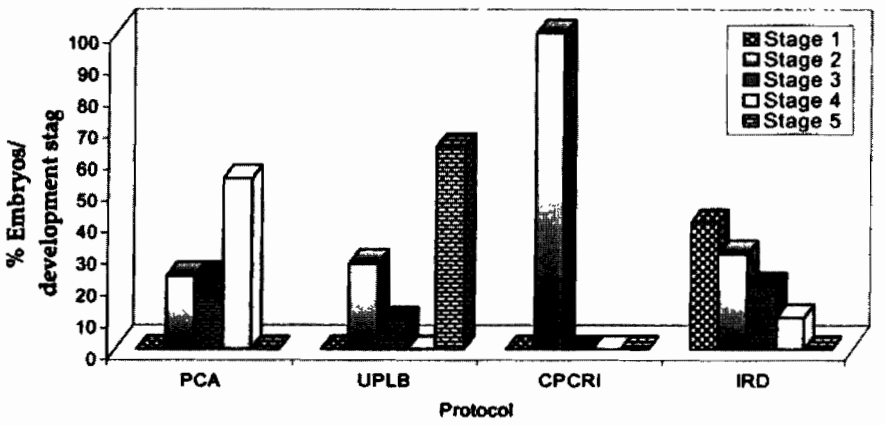

Fig. 3. Percentage of embryos at different development stages after four weeks in culture using the four protocols.

Differentiation of embryos

Table 2 presents the percentage of embryos differentiated and plantlets established from germinated embryos after eight months in culture. Highly significant differences were found in protocol-cultivar interactions. Percentages were low for both parameters observed. The UPLB protocol produced 64% of differentiated embryos for both genotypes. A similar response was obtained with the IRD protocol as regards the varietal effect, but the values were much lower for the two variables. The CPCRI protocol gave the highest percentage of non-germinated but surviving swollen embryos. However, no differentiation occurred and no plantlets could be produced. In most cases, it was observed that swelling of cultured embryos resulted in delayed germination or a latent non-germinated state.

Table 2. Percentages of differentiated embryos and established plantlets obtained with the different protocols

Parameter	Cultivar	Protocol				C.V.
		PCA	UPLB	CPCRI	IRD	
Differentiated embryos (%)	Tall	54.0c	64.0a	0 e	10.0d	3.24
	Dwarf	59.0b	64.2a	0 e	10.8d	
Established plantlets (%)	Tall	21.5b	30.5a	0 d	5.25c	10.98
	Dwarf	28.7a	31.4a	0 d	5.80c	

Means with different letters are significantly different.

Table 3 presents the average dimensions of the plantlets before their transfer *ex vitro*. The average length of the leaf and the main root obtained were similar in all conditions. The UPLB protocol produced the embryos with the longest secondary roots.

Table 3. Development of *in vitro* plantlets produced with the four protocols before their transfer *ex vitro*

Parameter	Protocol				C.V.
	PCA	UPLB	CPCRI	IRD	
Length of leaf (cm)	17.0a	16.80a	0 b	16.40a	8.20
Length of primary root (cm)	5.8a	5.60a	0 b	4.60a	25.83
Length of secondary root (cm)	1.0b	1.70a	0 d	0.35c	10.72

Means with different letters are significantly different.

A very low percentage of the plantlets developed *in vitro* survived to acclimatization. The root system developed by plantlets with all protocols was not sufficient to ensure their adaptation during the acclimatization stage (Table 4).

Table 4. Summary results of coconut embryo culture studies in IICF

Parameter/Cultivar	Culture protocol tested				
	Own (before start of project)	UPLB	PCA	CPCRI	IRD
Number of embryos inoculated					
1.Local Tall	200	30	30	30	30
2.Local Dwarf		30	30	30	30
Contamination (%)					
1. Local Tall	44	–	–	21.50	93.30
2. Local Dwarf		–	–	21.50	93.30
Germination (%)					
1. Local Tall	73.80	77.10	66.20	33.30	46.60
2. Local Dwarf		78.10	71.30	29.90	47.40
Whole plantlets *in vitro* (%)					
1. Local Tall	29.50	30.50	21.50	–	5.25
2. Local Dwarf		31.40	28.70	–	5.80
Average duration of *in vitro* culture (weeks) to obtain whole plantlets					
1. Local Tall	24	24-32	24-32	24-32	24-32
2. Local Dwarf		24-32	24-32	24-32	24-32
Survival after transfer to nursery (%)					
1. Local Tall	–	30.5	21.5	–	5.25
2. Local Dwarf		31.4	28.7	–	5.80
Survival after transfer to field (%)	None for all varieties/protocol				

The results indicated that the Y3 liquid medium induced a more rapid germination of embryos and development of plantlets (Fig. 3). This might have been due to the high levels of potassium and chloride, which are required for the growth of coconuts. The Murashige and Skoog (1962) medium, although rich in salts (see Annex 1.5), might not have contained sufficient levels of those compounds. These results are similar to those obtained by Miniano and De Guzman (1978). Liquid medium also contributed to such a response. The PCA and UPLB protocols produced plantlets at more advanced stages after the first four weeks in culture. The liquid condition may favour embryo orientation, which is a determining factor in monocotyledonous plants (Mantell 1996 as cited by Mkumbo *et al.* 1997). Mkumbo *et al.* (1997) indicated that liquid medium stimulates root and leaf growth and inhibit haustorium development. It can thus be inferred that under these conditions, the absorption of nutrients and the gaseous exchanges are more efficient with the liquid medium than on the solid medium.

Explants on solid Y3 medium (CPCRI protocol) grew in volume but did not differentiate, showing response similar to that reported with solid MS medium (Capote *et al.* 1995). This medium induced a significant number of abnormal explants and

plantlets with haustorium, the removal of which is recommended in the methodology developed by Assy Bah (1986). This behaviour might have been due not only to its solid medium condition, but also to the effect of growth regulators, which were present in the medium. After four weeks in culture on the CPCRI medium, 100% of the embryos were at growth Stage 2. This retarded growth was maintained throughout the experiment and no plantlets were obtained. This result differed from that obtained with the PCA and UPLB protocols which produced 54% of embryos at Stage 4, and 64% at Stage 5, respectively.

Based on these results, it was deduced that growth regulators (ANA, IBA and BAP) did not favour embryo differentiation. However, analyses carried out by Verdeil *et al.* (1997) had shown that the highest cytokinin concentrations were measured in 9-month old fruits, which produced high-germinated embryos (Child 1974). This indicates that cytokinins might have played a determining role in this process. It is possible that a more in-depth study is required to actually determine the effect of growth regulators, not only quantitatively, but also qualitatively. It might thus be very interesting to study the evolution of growth regulators in seeds at the onset of germination.

Despite the fact that liquid medium induced accelerated growth and homogeneous gemmulation, germination rate during the post-gemmulation phase was not uniform. Mkumbo *et al.* (1988) devised a medium formulation in which two types of MS/Y3 medium were employed in liquid and solid form, respectively. In this experiment, the favourable influences of liquid medium on initial embryo growth had no positive effect on post-germination development. This was also observed when the PCA and UPLB protocols were compared. However, when cultured explants (UPLB protocol) were transferred to solid medium, they started to develop a large quantity of secondary roots and their shoots grew rapidly.

Conclusions

- Liquid Y_3 medium induces germination more rapidly while solid medium is better for the growth phase of plantlets because it favours the development of secondary roots.
- An extended culture of embryos in liquid medium seems to reduce germination percentage.
- The UPLB protocol produces a higher percentage of established plantlets.

Recommendations

- More in-depth analysis of the physical and chemical elements that may favour development of the root system that will allow a better *ex vitro* adaptation of plantlets;
- Further studies on the effects of growth regulators to accurately determine their action, optimal concentrations and application period;
- More studies on the germination process of fruits in natural conditions; and
- Carryout experiments to determine the effects of the composition of the culture media at different stages of embryo *in vitro* culture.

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State of zygotic coconut embryo culture and cryopreservation research at IRD/CIRAD, France

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Introduction

The coconut palm is the plant species with the largest seed in the plant kingdom. In addition, coconut seeds are highly recalcitrant as regards their storage behaviour and they display no dormancy. As such, the seeds present real constraints for germplasm conservation and exchange (Hocher *et al.* 1999). *In vitro* culture of excised embryos is an effective way to overcome these constraints. For the last thirteen years, the IRD-CIRAD tissue culture group, in collaboration with CNRA (formerly IDEFOR), Côte d'Ivoire (Verdeil *et al.* 1998), has developed a research programme on *in vitro* culture of coconut zygotic embryos and somatic embryogenesis.

This paper describes the various important inputs made in coconut zygotic embryo culture at IRD/CIRAD since the first International Embryo Culture and Acclimatization Workshop held in the Philippines in 1997. It also presents the limits of the current *in vitro* culture protocol and the results of the studies conducted to increase IRD-CIRAD's knowledge of the physiology of *in vitro* plantlets.

New inputs in embryo culture and plantlet acclimatization research

In vitro plantlet physiology

Extension of the study on photosynthetic ability of in vitro *grown plantlets*

In vitro culture protocols for coconut zygotic embryos enhance the production of plantlets. However, the intrinsic quality of coconut *in vitro* plantlets needs to be improved. For this purpose, the IRD-CIRAD group has initiated studies to acquire more knowledge on the physiology of *in vitro* plantlets in order to optimize the coconut embryo culture protocol (Hocher *et al.* 1998).

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

The present constraint in coconut embryo culture is the slow growth of germinating embryos. Research will have to be carried out to overcome this constraint. Previous studies on the mechanisms involved in the *in vitro* photosynthesis process and on the establishment of carbon photoautotrophy of plantlets performed in Montpellier have shown that:

- Transpiration rates were similar in the *in vitro* cultured plantlets and in the autotrophic adult palms cultivated in the greenhouse. The number of stomatas on leaves of plants grown *in vitro* and those *in vivo* was similar. This suggested that the stomatal opening was correctly regulated in the *in vitro* plantlets at the end of the *in vitro* culture process.
- Several similarities had been observed between the *in vitro*-grown coconut plantlets and the adult autotrophic coconut palms. They both had a high level of photosystem II activity; *in vitro* plantlets, transferred one month after light conditions, also had mature, well-structured and active chloroplasts in the leaves.

- There was, however, a lower rate of net photosynthetic activity in the *in vitro* plantlets as compared to the palms grown *in vivo*. This could be explained by the lower activity and content of the RubisCO and chlorophyll in the *in vitro* grown plantlets compared to palms grown *in vivo* (Triques *et al.* 1997, 1998).

Study of the mobilization of the main amino acids by the haustorium during germination of coconut embryos

The photosynthetic ability of coconut plantlets transferred to *ex vitro* conditions is seen to be one of the important factors determining the success of their acclimatization and further growth. Studies were conducted in Montpellier to investigate the photosynthetic status of *in vitro* grown plantlets derived from zygotic embryo culture. These studies combined various complementary approaches applied both *in vitro* and *in planta*. The results obtained in this study showed that *in vitro* coconut plantlets displayed early initiation of photosynthetic metabolism. Even if it could be improved by an increase in chlorophyll and RubisCO content, the photosynthetic rate per unit of leaf area was not a limiting factor for growth of *in vitro* plantlets after acclimatization.

Therefore, there must have been other limiting factors causing 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 growth and development of *in vitro* plantlets after acclimatization. Thus, increasing the leaf area of *in vitro* plantlets appeared to be the major challenge for the improvement of coconut embryo culture. For this purpose, it was important to identify the nutritional requirements for embryo germination and leaf development.

One characteristic of coconut zygotic embryos is the substantial development of the haustorium (distal part of the cotyledon) inside the nut cavity during germination. This organ invades the nut cavity and comes in contact with the reserves contained in the endosperm. It enhances 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 IRD/CIRAD laboratory enabled the characterization of the structure of this organ; in particular, the digestive system in the epidermal layer, which is in contact with the endosperm cells containing the nutrient reserves. There are vascular bundles converging towards the embryonic axis. This villosity shows numerous structural similarities like those of the stomach *villi* in the digestive system of animals.

A study was conducted to analyze the type of nutrients (amino acids, lipids, sugars, etc.) present in the haustorium and how they were transferred to the germinating embryos. Presented here are the first results obtained with the analysis of the amino acids and sugar. Experiments for the analysis of the other types of nutrients are still ongoing.

Material and methods

In March 1997, 160 Malayan Yellow Dwarf nuts from IDEFOR, Côte d'Ivoire were sown in the IRD tropical greenhouse. In the first year, a sample of six nuts was taken every two weeks. Various parameters such as the weight of the nut, the albumen (solid and liquid), the haustorium, the number of leaves, roots, etc. were measured. The albumen (liquid and solid) and haustorium were frozen and stored at –80°C. Before analysis, the haustorium was lyophilized and crushed into powder. Chromatographic techniques available in the IRD/CIRAD Laboratory (Magnaval *et al.* 1995, modified by JL Verdeil) were used to quantify the main amino acids present in the haustorium during germination.

Results

Figure 1 shows the changes in weight of the coconut water, solid endosperm and haustorium during germination of the coconut seed. Before the 8th week, no significant change was recorded. It was interesting to note that around the 16th week, while the coconut water has been totally consumed, the haustorium had simultaneously reached its maximal weight (Nowak 1999).

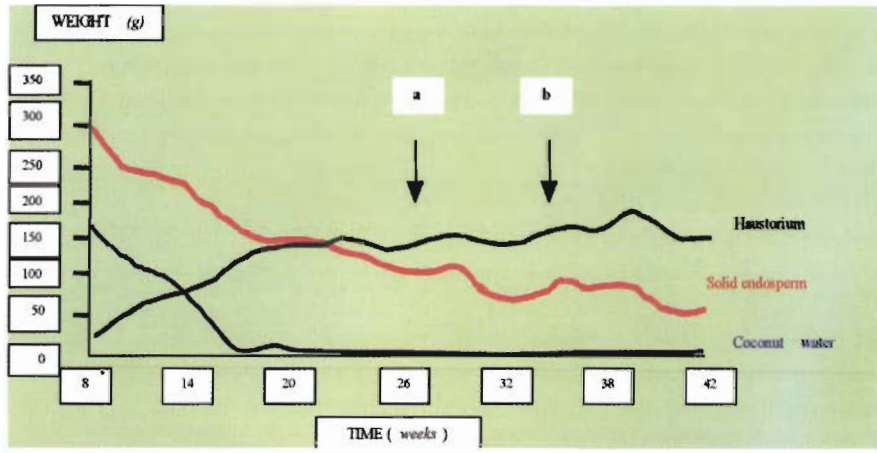

Fig. 1. Changes in weight of coconut water, solid endosperm and haustorium during coconut seed germination: (a) indicates the development of the first bifid leaf; (b) indicates the development of the first adult leaf with leaflet initialization.

Evolution of the amino acid concentration during coconut seed germination

Amino acids detected in coconut haustorium

The following free amino acids (AA) were detected in the coconut haustorium: alanine (Ala), valine (Val), histidine (His), proline (Pro), threonine (Threo), glutamate (Glu), tyrosine (Tyr), leucine (Leu), iso-leucine (Ileu), phenylalanins (Phe), aspartate (Asp), glycine (Gly), serine (Ser), lysine (Lys) and methionine (Met).

These acids were classified according to their concentration (Table 1), which differed drastically from each other. It was noted that only the serine level was higher than 50 µmol/g DW. The other AA could be separated into two groups: the first one with AA concentration between 50 and 25 µmol/g and the second one with AA concentration lower than 25 µmol/g.

Table 1. Concentration (µmol/g DW) of the different amino acids present in the haustorium of coconut seeds

≤25 µmol/g	>25 to ≤ 50 µmol/g	≥ 50 µmol/g
Val, Lys, Met, Ala, Glu, Leu, Ileu, Phe	His, Asp, Pro, Gly, Threo, Tyr	Ser

Changes in concentration of amino acids detected in coconut haustorium

The evolution of the concentration of the different amino acids during germination will not be presented in detail here. However, it is important to mention that a preliminary analysis of the results show three phases in the changes observed:

- An increase in the quantity of Ala, Ileu, Lys, Val, His, Asp, Pro, Threo, Tyr, Leu, Phe, Ser between the 8th and 16th weeks. This could be related to the development of the haustorium (Fig. 1);
- An increase in Gly, Leu, Lys, Met, Phe, Ser, Threo, Tyr and Val in the 26th week. The development of the first bifid leaves and the increase in the concentration of these AA could be correlated; and
- A second increase for some AA (Ala, Asp, Glu, His, ILeu, Leu, Phe, Ser, Threo, Tyr and Val) in the 34th week, which could be related to the development of the first adult leaf (Fig. 1).

Conclusion

The results obtained were indicative of the important role of the AA in the different phases of coconut seed germination and plantlet development. This study should be further supported by additional analyses in the quantification of AA in the albumen and in the coconut water.

There is a need to test the effect of an addition of the following combination of amino acids (on the basis of the ratio of AA concentrations measured in the haustorium and in the albumen calculated from collected data): Ser (150 mg/l), His (30 mg/l), Asp (30 mg/l), Pro (30 mg/l), Gly (30 mg/l), Threo (30 mg/l), Tyr (30 mg/l) and Val, Lys, Met, Ala, Glu, Leu, ILeu, Phe (15 mg/l each) in the culture medium.

This combination could reflect the AA content in the haustorium at the beginning of germination under natural conditions and should serve as a basis for the improvement of medium composition for *in vitro* germination of coconut embryos.

Quantification of sugars and minerals in haustorium during coconut embryo germination

The haustorium played a paramount role in the endosperm reserve hydrolysis and mobilisation during embryo germination. However, the nature and the composition of the compounds absorbed by the haustorium and transferred to the embryonic axis remained unknown. The quantification of the main sugars (inositol, sorbitol, glucose, fructose, sucrose) and the main minerals was done by HPLC during the course of the embryo germination and plantlet development (sampling of six nuts every two weeks for 46 weeks).

Sugars

The main sugars detected inside the haustorium during embryo germination were sucrose, fructose, glucose and inositol. Sorbitol, which was detected at a high concentration (around 6g/l) in coconut water, was not detected in the haustorium. The sucrose concentration showed a regular decrease in the haustorium from 300mg/g lyophilized haustorium to 75mg/g 42 weeks after embryo intiation. The glucose and fructose concentrations inside the haustorium showed a similar evolution after a constant phase, when their concentration remained stable (around 50mg/g lyophilized haustorium). However, the fructose concentration increased to 100mg/g of lyophilized haustorium while glucose increased to 150mg/g of lyophilized haustorium 42 weeks after sowing. Inositol showed an increase until the 16th week after sowing (up to 8.75mg/g lyophilized haustorium), followed by a consistent decrease to 2.5mg/g of lyophilized haustorium 42 weeks after sowing. The inositol

concentration found in the haustorium was significantly higher than that normally added to the *in vitro* culture media. The amounts of glucose and fructose were also high, corresponding to the concomitant hydrolysis of sucrose, the concentration of which decreased consistently.

Since inositol seemed to have played an important role in *in situ* seed germination, it was decided to test its influence on *in vitro* germination of embryos using a range of concentrations between 100mg and 2g/l.

Minerals

Chloride and nitrate, quantified by HPLC using the protocol described by Dussert *et al.* (1995), were the main anions detected in the haustorium during embryo germination (concentrations were three- and five-times that of the sulfate and phosphate levels, respectively). Surprisingly, 42 days after sowing, the concentrations of Na^+, K^+, Mg^{++} and Ca^{++} (Na^+ = 2.93 ± 0.03 mg/g; K^+ = 29.2 ± 0.29 mg/g; Mg^{++} = 4.66 ±0.11 mg/g; Ca^{++}=3.01 ± 0.04 mg/g) did not show any significant variation in the haustorium during the course of germination.

This study would be supplemented by the quantification of minerals inside the coconut endosperm (liquid and solid) to see if the data obtained could reflect a continuous flux of cations and anions through the haustorium during embryo germination and seedling development.

A statistical analysis is being done to see if the sugar and mineral concentrations measured in the haustorium would be in any way related to the morphological data in the number of roots, shoot and root length, etc. as recorded during embryo germination and plantlet development.

Since the concentration of chloride and nitrate anions was high during *in situ* embryo germination, it was decided to add more KCl and $CaNO_3$ to the culture medium to enhance the *in vitro* germination of zygotic embryos.

Influence of light quality on embryo germination

Light quality was known to have had an important influence on plant morphogenesis. A trial had been conducted to study embryo germination under various light regimes using adapted Sylvania Grolux and Coolwhite fluorescent tubes (blue, red, and white). The experiment was carried out in November 1999 using LT embryos (100 embryos per light treatment) provided by E. Rillo of the PCA, Philippines.

Preliminary results showed that under red light, embryos look etiolated (long shoots and narrow pale green leaves). Under blue light, shoots were shorter than those under red light but they had dark green leaves. This study is still evaluating the chlorophyll content and photosynthetic capacity of leaves in the different treatments.

Quantification of cytokinins during the seed development

Most of the work done on phytohormones in coconut had focused on cytokinins, because of their importance in morphogenesis, and on the availability of a technique for measuring their concentration as developed by Prof. Miginiac of the University Paris VI, France.

Coconut seeds at different stages of development (3, 7, 9, 11 months after anthesis, and mature nuts) were obtained from CNRA, Port Bouët, Côte d'Ivoire. Upon receipt, the nuts were husked and split open. The coconut milk, solid endosperm and embryos were analysed for their hormone. The three main steps for this analysis included methanol extraction of the growth regulators, separation of the different forms by HPLC and the final quantification of cytokinins by immunoassay (ELISA) (Maldiney *et al.* 1986).

Results

The analysis showed the following:

- Different forms of cytokinins : isopentenyladenine (iP), [9R] iP, zeatine (Z) and [9R] Z were detected in the seeds at different stages of development;
- Only the zeatin formed in the coconut milk was detectable in the third month;
- A considerable amount of the four different forms of cytokinins accumulated in both the coconut milk and in the solid endosperm during the development of the nuts; and
- The highest amount of cytokinins was detected in the solid endosperm of the 9-month old nuts when the rate of endosperm development was at its highest.

Comments

- The cytokinins detected at different stages of development might have helped build the reserve accumulation.
- High levels of cytokinins were found in the endosperm water and embryos of mature nuts. This could explain the recalcitrant storage behaviour of coconuts, as the cytokinins seem to have stimulated germination.

Conclusion

Cytokinins played an important role in the germination of coconut embryos. In this regard, Indian coconut researchers added cytokinin cocktails to the culture media. Such an addition of cytokinin cocktails should be used very carefully as there might be some possible adverse effects on the subsequent development of plantlets. To prevent this, the use of cytokinin precursors such as adenin sulfate and other adenine-derived components is recommended to safeguard the germination step.

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

The temporary immersion process (Fig. 2) developed by CIRAD using the so called 'RITA' container successfully tested on numerous plants was applied to the Malayan Yellow Dwarf zygotic embryos provided by the Marc Delome Station (CNRA, Port Bouêt, Côte d'Ivoire). Two trials were performed under different experimental conditions:

Trial 1 – Comparison between continuous immersion and temporary immersion (2 x 5 min/day), with culture medium containing MS macro- and micro-elements and GA_3 (10^{-4}M); and

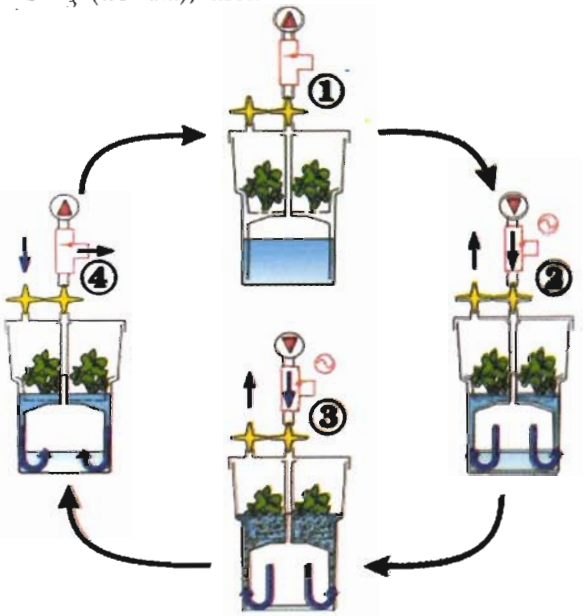

Figure 2. Schematic presentation of the operation of the RITA temporary immersion culture system.

Stand-by: Long stage **(1)** Plants are placed on polyurethane foam disc; **Flooding**: Short stage, ranging from 1min/day to 4 times 15min/day; **(2)** Sterile air is overpressured in the lower container which pushes the liquid medium into the upper container holding the plants; **(3)** A sterile airflow continuously agitates and oxygenates the medium and renews the air inside the vessel; **(4)** When the airflow is stopped, the pressure in the two parts of the container adjusts and the liquid medium returns to the bottom of the vessel by gravity. The plants remain covered by a film of medium by capillary attraction.

Trial 2 – Comparison between continuous immersion and temporary immersion, with culture medium containing Eeuwens macro- and micro-elements with increased chloride concentration (10g/l NaCl) and GA_3 ($0.5\ 10^{-4}$M).

Results

During these preliminary experiments, there was no significant improvement with temporary immersion in comparison with the classical process as only one frequency and duration of the immersion phase was tested. It was noted that temporary immersion was a good system to prevent the onset of any oxidation-related problems. A better development of embryos cultured with macro- and microelements of Eeuwens was noted compared with those cultured with the MS mineral solution. Additional experiments are underway.

Conclusion

It was too early to draw any conclusion on the potential of temporary immersion to improve the *in vitro* germination and development of coconut embryos.

Influence of gibberellic acid on zygotic embryo germination and plantlet development

Two trials on the influence of GA_3 on the germination of coconut zygotic embryo were conducted in Montpellier. Two GA_3 concentrations were used (10^{-4}M and $0.5\ 10^{-4}$M) with a control without any growth regulators. Experiments were carried out using the 502 medium described by Assy-Bah (1992) with 2g/l activated charcoal. The percentages of embryo germination were 85% ($GA_3\ 10^{-4}$M), 87% ($GA_3\ 0.5\ 10^{-4}$M) and 90% (control). Observations made four months after transfer of plantlets *in vivo* showed that GA_3 promoted shoot growth (shoot length of $GA_3\ 10^{-4}$M was 18 ± 4 cm; $GA_3\ 0.5\ 10^{-4}$M, 15 ± 5 cm and without Ga_3 only 9 ± 2 cm). However, shoots developed with the highest GA_3 level was etiolated (with long and very narrow leaves). A further experiment using lower concentrations of GA_3 will be conducted in Montpellier.

Cryopreservation of coconut germplasm

In the 1980s, a cryopreservation protocol was established for whole zygotic embryos (Assy-Bah 1992). It was demonstrated that cryopreservation of zygotic embryos was possible with high survival percentages using the pregrowth-desiccation technique, but the number of genotypes tested was limited (Assy-Bah & Engelmann 1992). Further research was therefore needed. The limited volume of the embryos did not allow experimenting with other techniques such as those based on encapsulation. Besides, embryos did not survive as a whole but only in the meristematic zones where the shoot and root poles could withstand exposure to the liquid nitrogen. Based on these observations, new cryopreservation protocols have been tested using plumular tissues (*i.e.* the caulinary meristem with one or two-leaf primordia). To implement this new programme, IRD funded a three-year PhD program of studies and a one year research grant. Ms. Oulo N'Nan (CNRA, Côte d'Ivoire) started her PhD thesis on cryopreservation of coconut zygotic embryos, initiating the work on freezing of plumular tissues. Plumules appear to be an interesting material for cryopreservation because of their small size (around 1 mm) and their structure (presence of many meristematic cells). During her first research in Montpellier in 1999, Ms. Oulo N'Nan carried out experiments with plumules using the encapsulation/ dehydration technique, which had been successfully used on various plants (cassava, coffee, yam, etc.) in the laboratory. Misterbino Borges (Cuba) performed additional trials in 2000 by under the same IRD grant.

Plumules, which had been excised and encapsulated in alginate beads, were treated with various sucrose concentrations then dehydrated in air-tight boxes containing silica

gel. The first results of cryopreservation experiments and of optic and electronic histological analyses are summarized below.

Materials and method

The plant material consisted of zygotic embryos taken from 10-12 month old nuts of the MYD variety from the Marc Delorme Station (CNRA), Port-Bouët, Côte d'Ivoire. After disinfection (Verdeil *et al.* 1998), caulinary apices (around 1 mm long) were excised from these embryos in the laminar air flow cabinet under a stereo binocular (Fig. 3).

Caulinary apices (plumules) extracted from zygotic embryos were precultured in test tubes for three days. This was done to allow the embryos to recover from excision stress and to guard against potential contaminations. Preculture is followed by encapsulation of plumules in alginate beads of 4 to 5 mm in diameter. Encapsulated plumules were pretreated in liquid medium with various sucrose concentrations (0.5M, 0.75M, 1M, 1.2M) for three days. Twenty encapsulated plumules in a 125 ml Erlenmeyers flask containing 30ml liquid medium were placed on a rotary shaker set at 91 rpm in complete darkness.

After the sucrose pretreatment, the beads were quickly surface-dried on sterile paper and placed over 40g of silica gel in 125ml airtight boxes and dehydrated for 8, 10 and 16 hours down to a bead moisture content of between 0.14 and 0.20 gH_2O/ g DW. The evolution of water content of alginate beads was measured as described on yam (Malaurie 1998; Malaurie *et al.* 1998).

From each batch of 20 beads, 10 were transferred on to a culture medium as desiccation control and the other 10 were placed in a 2ml propylene sterile cryotube and immersed directly in liquid nitrogen. After freezing in liquid nitrogen, encapsulated plumules were rewarmed for 40 minutes in the laminar air flow cabinet, before being transferred to test tubes (one plumule per tube) containing 20 ml of growth medium.

For photonic microscopy, histological sections were stained with fushin and naphthol blue black (Fisher 1968) to show the cell structure with glucides stained pink by fushin and proteins and the nucleus blue by naphthol blue black (Buffard-Morel *et al.* 1992). For electronic microscopy, plumules were fixed by immersion for two hours in a 0.1M calcodylate buffer (pH 7.2) containing 2% glutaraldehyde and 1% cafein (Verdeil *et al.* 2001).

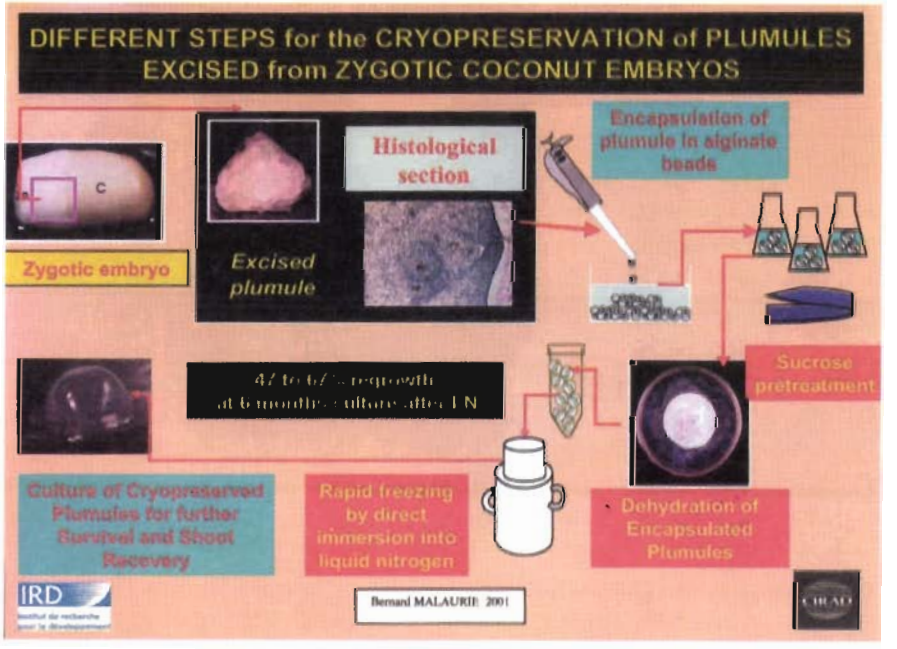

Fig. 3. Successive steps of the encapsulation/ dehydration protocol developed for cryopreserving coconut plumules.

Results

Preliminary results showed that cryopreservation of coconut plumules by encapsulation/dehydration was feasible (Fig. 3). After six months recovery growth period, survival of cryopreserved plumules reached 67% after pretreatment with 0.75M and 1M sucrose followed by 8 to 16 hours of dehydration (Malaurie 2001; Malaurie & Borges 2001). Most of the work had focused on histo-cytological study of plumules after each step of the cryopreservation process.

Effect of sucrose pretreatment and dehydration on plumule development

Observations in photonic microscopy showed that regardless of the sucrose pretreatment and the dehydration period used, the condensation of the chromatin was seen to have been enhanced and the nucleus changed from a spherical to an oval shape. Cells from the meristematic dome, compared to those of leaf primordia, showed fewer injuries after a dehydration period of between 8 to 16 hours when pretreated with up to 1M sucrose. However, a higher sucrose concentration (1.2M) with the longest dehydration period tested (16 hours) produced great damages to all types of cells. Electronic microscopy showed that after the sucrose pretreatment, the nucleoplasmic ratio of most of the meristematic cells tended to decrease and the nuclei became more oval. These modifications were, however, limited. Contrary to that, after dehydration, most of the meristematic cells were plasmolysed, with a denser and retracted cytoplasm and the nuclei were often pycnotic.

Effect of cryopreservation on plumule development

Observations under photonic microscopy showed that cryopreservation did not induce additional damages to those noted after sucrose pretreatment and dehydration.

Conclusion

This first report on cryopreservation of plumules by encapsulation/dehydration was based on a limited amount of test materials since only 300 plumules of one genotype from Côte d'Ivoire and 400 plumules from Mexico and Santo Domingo had been employed (Malaurie & Borges 2001). Since these preliminary experiments, further trials have been conducted to establish a process which could be used routinely with an extended genotypic diversity. Experiments are now being done monthly in Montpellier using embryos procured from the Marc Delorme Station (CNRA, Côte d'Ivoire).

General conclusion

The CIRAD-IRD group had conducted studies, which had led to the development of a simple protocol for coconut embryo culture. This protocol was based on the use of a single medium throughout the embryo germination and plantlet development periods with the succession of a culture phase in the dark for germination followed by a transfer of the germinated embryos to light conditions for plantlet growth and development.

From the 100 embryos introduced *in vitro*, about 50 plantlets were transferred to natural conditions 6-7 months after the inoculation, with a survival rate of between 80 and 90%. However, plantlet growth, under natural conditions, remains slow. Therefore, the efficiency of the *in vitro* culture protocol needs to be improved. Based on the results of the experiments described in this paper, the following are suggested:

- The nature and concentration of compounds present in the haustorium at the beginning of germination under natural conditions should serve as a basis for improving the composition of the culture medium for coconut embryos;

- In particular, inositol, chloride and nitrate anions present in high quantities in the haustorium showed large variations in their concentrations during germination. The effect of inositol at concentrations ranging between 100 mg/l and 2 g/l should thus be tested and concentrations of KCl and KNO_3 should be increased in the culture medium;
- Euwens mineral elements should be used instead of the MS elements as they proved to have induced a better development of the embryos; and
- The preliminary results obtained with plumule cryopreservation should be confirmed using different coconut varieties.

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Makapuno embryo culture: The Philippine Coconut Research and Development Foundation experience

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Introduction

The Philippine Coconut Research and Development Foundation, Inc. (PCRDF) started its involvement in Makapuno embryo culture in 1978 (PCRDF, The First Decade, 1975-1985). The *in vitro* culture of coconut embryos had been developed as a tool to rescue the embryos of Makapuno. The technology was initiated by the late Dr. Emerita de Guzman in 1970, then a professor at the University of the Philippines at Los Baños (UPLB). Knowing the full potential of the technology, PCRDF supported Dr. de Guzman in the mass propagation of Makapuno. After her demise, PCRDF continued with the project, this time with Dr. Aurora del Rosario who previously had been Dr. de Guzman's project assistant. During this second phase, success rates were not encouraging enough to interest private entrepreneurs to adopt the technology.

In 1995, after learning about the works and success in the research of Mrs. Erlinda Rillo, Scientist II and Division Chief III of the Philippine Coconut Authority (PCA), Albay Research Center (ARC), PCRDF made an ambitious move to test the viability of Makapuno as a commercial crop. Makapuno seedlings were bought from PCA and planted in a 10-hectare demonstration farm to showcase the technology. Two hectares were intercropped with Pili, a nut indigenous to the Philippines; another two hectares were interplanted with banana and the rest with other intercrops. At present, the Makapuno plants in the first three hectares have started to flower.

Encouraged by the work of Mrs. Rillo, PCRDF decided to set up a satellite laboratory at the Enverga University in Lucena. Subsequently, PCRDF forged a joint venture agreement with the Visayas State College of Agriculture (VISCA) in southern Philippines to mass propagate the dwarf, self-pollinating Makapuno hybrid that had been developed.

Several adverse issues, however, affected PCRDF's activities. First, the cadang-cadang disease prevented expansion and transfer of the PCA Makapuno seedlings out of the Bicol region. Second, VISCA had only four mother plants as a source of their embryos at the time when there was a problem of sourcing good and reliable suppliers of Makapuno nuts in Lucena, Quezon.

In 1996, after 20 years of being a mere funding agency for coconut R&D, PCRDF decided to build up its own potentials as a research institution, dedicating 100% of its work to coconut embryo culture. An R&D center was set up to conduct studies on several modules such as microbiology and biotechnology; product research and development; Makapuno and tissue culture; instruments and wet chemistry; and a small pilot facility.

Makapuno laboratory at the PCRDF central laboratory

PCRDF had its own technical personnel for the Makapuno embryo culture trained by Mrs. Rillo at the PCA-ARC. In the first year of operation, not only were the Makapuno embryos expensive, reliable sources of these embryos were difficult to find. Simultaneously, another satellite laboratory was set up in the province of Batangas

(50 km south of Manila) to culture Makapuno embryos as well as the 'lakatan' banana variety and cutflower tissues. In Batangas, reliable and good sources of Makapuno nuts were available. Because of the low success rate (25%) in 1997, PCRDF decided to further review and study the culture protocol experimenting with different culture medium, systems and procedures. By the end of 1998, PCRDF had achieved a success rate of between 40 and 45%; and in 1999, it was up to 76%.

Methodology and observations

Stages of Activities

For evaluating performance following the modified Y3 media formulations, the activities were divided into various stages of development. The stages and their duration are as follows:

Stage	Activity	Duration (months)
I	Inoculation	1 – 1.5
II	Germination/development of roots and shoots (1st to 5th scale leaf)	1 – 1.5
III	Plantlets	4 – 5.5
IV	Acclimatization/Screenhouse	3 – 4.5
V	Nursery	3
VI	Field Planting	
		12 – 16

Factors considered and observations

The effects of solid *versus* liquid media on the germination rates as well as the effects of variation in the media sugar concentrations, light and hormones on embryo development were studied and compared.

Effects of solid medium on gemination rate

- Minimal swelling/enlargement of embryos;
- Germination rate as high as 100%;
- Shorter germination period (1 to 1.5 months); and
- Less or very minimal contamination.

Effects of liquid medium on gemination rate

- General swelling/enlargement of embryos;
- Germination rate of only 60 to 75%;
- Longer germination period (two to three months); and
- High chances of contamination.

The amount of agar was also varied as follows:

Stage I = 7 g/L
Stages II and III = 4 g/L

Effects of variation in the sugar concentration

Sugar is one of the media components that greatly influence the development of embryos (Pierik 1987). Ordinary table sugar was used in the following concentration:

Stage I	=	60 g/L
Stages II and III	=	40 g/L

Effects of light

Other than the standard quality checks for the procedures and media formulations, the effects of light on the germination of the embryos were also considered.

One advantage of PCRDF was that it had several satellite laboratories, which compares notes on problems and their possible solutions. These laboratories used the same media, modified Y3 in Stages I to III. Initially, the behaviour of Makapuno embryos in the 1st and 2nd stages in relation to photoperiodism (Thomas and Vince-Prue 1997) were evaluated, such as long period of darkness or direct lighting versus indirect lighting conditions in Stage I, and the effects of cool light in combination with ordinary fluorescent daylight bulbs. Studies on the effects of exposing the embryos to IR followed by FR on embryo growth are also planned.

Effects of hormones

To enhance the development of roots and shoots, NAA and BAP were added as follows:

Stage II	=	10 ppm BAP
Stage III	=	17 ppm NAA

To prevent browning, 2.5 g/L activated charcoal was added in Stages I to III.

Screenhouse for acclimatization and hardening

The screenhouse enclosed in double layers of # 6 mesh screens reinforced with cyclone wires had an elevated floor covered with gravel on the top and sand at the bottom to prevent water logging. The space within was divided into two sections – one with plastic roofing and the other without.

All the seedlings in the big test tubes were left inside the screenhouse with roof for two weeks before they were transferred to the polybags or clay pots with sterile river sand. The roots were washed using soap scraping to thoroughly remove the remaining solid media. Then the roots were dipped in a Benlate solution (2.5 g/l) before planting. They were covered with autoclavable plastic bags for a week or two. After four to six weeks, the seedlings were transferred to the section with no roofing. In order to maintain good atmospheric environment, misting devices were provided to supply the moisture/water requirement for the young seedlings. Underground and untreated water was used to water the plants. One and a half months later, the seedlings were transferred to the nursery where they were kept until they were ready for field planting three months after. During the period, urea was added at the rate of one teaspoon per gallon of water once every two weeks.

Nursery management

Seedlings were placed initially in a partially shaded area for two months with continued fertilization, then they were totally exposed to the sun.

Flow chart of activities		Success rates in 1999 (%)
Extraction of Makapuno embryo		
↓		
Inoculation	(Stage I)	99 – 100
↓		
Germination	(Stage II)	97.4
↓		
Plantlets	(Stage III)	86.3
↓		
Acclimatization Screenhouse	(Stage IV)	95.4
↓		
Nursery	(Stage V)	96.8
↓		
Field Planting	(Stage VI)	100

Based on the above success rates, the final plantlets survival rate was 76.85 percent.

Other activities

During the time when liquid media was still being used, the four to seven-month old ungerminated embryos were segregated. Some of these embryos germinated, and the one that survived was field planted. Unfortunately, it was no longer possible to identify the plant since the farmer who planted it mixed it with the others already in the field.

Attempts were also made to split 20 normal coconut embryos after one month in solid modified Y3 medium, which resulted in a 77.5% germination (31 out of 40 halves) rate. Some of the Stage II observations showed:

- Generally stunted and slow growth;
- Curling of leaves and rapid elongation of primary roots; and
- Browning.

Future activities

With this encouraging development, there is still much work to be done to further improve the success rates of the Makapuno embryo culture. These include:

- Determining the effects of growth hormones in combination with coconut milk and other growth promoting substances (Weaver 1972; Narayanaswamy 1994; George 1993);
- Studying the soil, plant, water and atmosphere equilibria/relations;
- Determining the cause of embryo swelling in liquid medium and suberization;
- Determining the possible effects of adding mannose in culture media on the development of explants;
- Determining the cause of stunted growth in the split embryos;
- Performing agronomic studies on Makapuno seedlings from split embryos; and
- Comparing the use of technical- versus reagent-grade chemicals in embryo culture media.

Conclusion

PCDF's trial and error experiments proved beneficial to its culture activities. Varying the sugar concentrations, adding specific hormones such as NAA and BAP as well as solidifying the media by the addition of agar increased the success rate of the Makapuno embryo culture to 76%. Based on PCDF's current culture practices and success rate, the estimated cost for each embryo cultured Makapuno seedling was P(esos)175.00 or US $4.30 (Table 1).

Among the various stages of development, Stage III was the lowest in terms of survival due, probably, to its long duration. It was also determined that a long exposure of embryos to dark conditions (Stage I) neither shorten nor improve their germination significantly.

All of PCDF's satellite laboratories, including the Central Laboratory where the embryo culture module is located, are equipped with state-of-the art facilities. Sanitation, safety and personnel standards are constantly being improved.

The most common problems encountered were browning and contamination by yeast, bacteria (i.e. enterobacter) or mold. No chemical treatments were added to the media since contamination varied from time to time.

One of the most important aspects for the continued success of the project is well-trained and dedicated personnel who should be given proper support and encouragement.

Table 1. Estimated production cost of an embryo cultured Makapuno seedling (in Philippine pesos)

	Stages of development					
	I	II	III	IV	V	VI
Macronutrient	4.663	4.663	4.663	–	–	–
Micronutrient	0.082	0.082	4.663	–	–	–
Myo-inositol	1.860	1.860	1.860	–	–	–
Vitamins	0.196	0.196	0.196	–	–	–
FeEDTA	0.099	0.099	0.099	–	–	–
Agar	16.100	9.200	9.200	–	–	–
Sugar	1.440	0.960	0.960	–	–	–
NAA	–	–	0.498	–	–	–
BAP	–	7.650	–	–	–	–
Water	4.000	4.000	4.000	10.00	10.00	10.00
Total	**28.440**	**28.710**	**21.528**	**10.00**	**10.00**	**10.00**
No. of culture tubes	100	35	20	–	–	–
Cost per culture	0.568	1.640	5.390	–	–	–
Cost of potting materials	–	–	–	5.00	5.00	5.00
Estimated energy consumption per embryo	1.50	1.50	1.50	–	–	–
Total	**2.068**	**3.140**	**6.890**	**15.00**	**15.00**	**5.00**

A.	Estimated cost per seedling (at 100 % success rate)	P57.098	
B.	Actual cost per seedling (at 76% success rate)	75.129	
	+ direct and indirect labor		
	+ building/equipment depreciation	100.00	
		P 175.00	(at US$1 = P40.65)

Acknowledgement

I would like to thank Dr. Pons Batugal of COGENT, IPGRI and CGIAR for their kind assistance and for giving me the opportunity to participate in this international workshop on Coconut Embryo Culture. Thanks also to Mrs. Erlinda Rillo of the Philippine Coconut Authority – Albay Research Center, for sharing her technology with us; to Dr. Carlos Oropeza for his help in obtaining my visa to enter Mexico and to my Vice Chairman/CEO Mr. Jose R. Eleazar Jr. for allowing me to represent PCRDF.

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Embryo culture activities at the University of Queensland, Australia

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Rationale and objectives

Presently, coconut (*Cocos nucifera* L.) germplasm collecting relies on the harvest and transport of the bulky fruits. For most collectors, this is not cost-effective and, in some cases, leads to the introduction of pests and diseases to new areas. The isolation, surface sterilization and transportation of small pieces of endosperm containing the embryos (referred to as endosperm plugs) could overcome these problems but the technique requires sterile culture facilities to be available at or near the field collecting site. Often, sterile culture facilities are not available as most collecting sites are in remote locations. Some attempts have been made to use equipment that can be easily transported to the field. However, these techniques also require a second, often damaging, laboratory surface sterilization treatment. More recently, Ashburner *et al.* (1993) developed a method for coconut germplasm collecting that only involves the isolation and transport of naked embryos. This method is suitable for mutant coconut forms but suffers from embryo loss through contamination. Thus, there is a need for a technique that not only isolates individual embryos but also protects them from contamination before they are to be cultured. The use of a low-temperature treatment is a well-known method to suppress microorganism multiplication and to retain tissue health. It could be assumed that the use of an appropriate low-temperature treatment during embryo transport from the field to the laboratory may be a way of reducing subsequent embryo contamination. However, it is also possible that such a treatment could damage coconut embryos.

The objective of the present study is to test a new, simple method for remote site coconut germplasm collecting. Specifically, the low-temperature treatment will be investigated as a way of suppressing microorganism activity during transport prior to application of the standard tissue culture treatment.

Methods

Development of a germplasm collecting protocol

Coconut (*Cocos nucifera* L.) fruits imported from Western Samoa were purchased from a local supermarket. The fruits were split open and the endosperm plugs (2.5 cm diameter surrounding the embryo) were removed by a clean but non-sterile method. This technique, suitable for field collecting, used a cork borer that had been cleaned with 70% ethanol and washed with tap water. The embryos were then removed from the plugs with clean dissecting instruments, weighed and individually placed, without surface sterilization, into sterile polycarbonate screw cap culture tubes (8.0 x 2.5 cm diameter) containing 10 ml of a filter-sterilized ascorbic acid solution (1 mg l^{-1}). Any visibly damaged or abnormal embryos were discarded prior to incubation in ascorbic acid. The embryos were incubated in the dark at one of two temperatures (5 or 26 $\pm$ 1°C) for 0, 1, 2 and 4 days. After the appropriate temperature treatment and incubation time, 10 replicate embryos were weighed and their health assessed. Health assessment

was achieved by placing them individually into sterile stoppered (Subaseal stoppers) glass tubes (4.8 x 2.5 cm diameter), and incubating in the dark at 26 ± 1°C for 12 hours. Embryo-free tubes were set up as controls. After the first four hours of incubation, a 0.5 ml headspace gas sample was removed from each of the treatments and controls and analysed for carbon dioxide. The removed gas samples were replaced with sterile air and the vessels incubated for eight hours more before a second headspace gas sample was taken for ethylene analysis. A gas chromatograph fitted with a flame ionization detector (95°C) was used for ethylene analysis, while a thermal conductivity detector (65°C) was used for carbon dioxide analysis. The ascorbic acid solutions remaining in the polycarbonate tubes after embryo health assessment were shaken and viewed under a microscope for microorganism density using a counting chamber. The remaining 9 ml of ascorbic acid solution in each of the tubes was subjected to electrical conductivity and pH analyses at 26 ± 1°C to assess further aspects of the embryos' health. After health assessment, embryos were surface sterilized using sodium hypochlorite and washed in sterile deionized water. They were then individually placed in sterile polycarbonate screw cap tubes (8.0 x 2.5 cm diameter) containing 10 ml of an autoclaved (120°C, 15 min) liquid culture medium consisting of Y3 basal nutrient medium (Eeuwens 1976) supplemented with Morel and Wetmore vitamins (Morel and Wetmore 1951), sucrose (175 mM) and activated charcoal powder (2.5 g l^{-1} agar, Sigma Chemical Co, St. Louis, USA) of similar constitution, in similar culture vessels, placed under the same environmental conditions for a further four weeks. During this period, embryos were scored for germination, and after six weeks of growth, they were evaluated by measuring fresh weight. Subculture onto media of a similar type was undertaken every four weeks and, after four such subcultures, the seedlings were planted into soil.

Remote site germplasm collecting

The protocol developed and tested in the laboratory was field evaluated in West Lombok, Indonesia. The coconuts used in this study were the cultivar Batu Layar from smallholder plantations located along the island's west coast road. Mature fruits were harvested from randomly sampled trees, dehusked, split and the endosperm plugs removed with a non-sterile but clean cork borer. The embryos were then isolated from the plugs and placed (in lots of 150) in six sterile polycarbonate screw cap tubes (11.0 x 4.5 cm diameter) each containing 50 ml of a sterile ascorbic acid solution (1 mg l^{-1}). Three of these tubes were placed immediately on a cotton wool layer sitting over bags of ice housed in an insulated plastic cooler tank. In a preliminary study, this apparatus had been shown to provide a constant temperature of 5 ± 2°C inside the culture tube for at least four days. The other three tubes were placed in a similar tank but without ice, providing an average temperature, when closed, of 26 ± 5°C. Both tanks were then transported (*c.* 20 km; the collecting and transportation took about 6 hours) to the University of Mataram. Once in the laboratory, the embryos from the tank with ice were removed and placed individually, without surface sterilization, into sterile polycarbonate, screw cap culture tubes (8.0 x 2.5 cm diameter) containing 10 ml of a filter-sterilized ascorbic acid solution (1 mg l^{-1}). The embryos were incubated in the dark in a refrigerator (5 ± 2°C). The embryos from the second tank were similarly treated but placed on a laboratory shelf (*c.* 28/25 ± 2°C, day/night) in the dark. After 0, 1, 2 and 4 days of incubation, tubes from both storage treatments were removed, the embryos taken out, surface sterilized, weighed and individually cultured, first in liquid, then onto solid medium (as described above). The percentage of embryos that had become contaminated was assessed and the germination rate of the remaining embryos determined six weeks after the start of the culture.

Experimental design and statistical analysis

Both studies were undertaken following a completely randomized design. In the first study on protocol development, each incubation period consisted of 10 replicate tubes each containing one embryo. In the second study on remote site germplasm collecting, each incubation period had three replications, each consisting of 50 tubes with an embryo in each tube. The data from both experiments were statistically analysed for variance and mean and then compared with the Student-Newman-Keuls test using SigmaStat. The data of contamination, germination rate and microbial density underwent a square-root transformation prior to statistical analysis.

Results and discussion

Development of a germplasm collecting protocol

The initial fresh weight of the embryos at the time of isolation was 170 ± 10 mg and this did not significantly change during the 4-day incubation period at 5°C or 26°C (data not shown). The embryos incubated at 5°C showed no visible morphological changes: they remained white with a firm texture. In contrast, the embryos incubated at 26°C turned yellow and the texture soft, the intensity of both these features increasing with time. The embryo incubation solution kept at 5°C remained clear while that from around the embryos kept at 26°C became turbid. The pH and electrical conductivity of both incubation solutions significantly increased during the course of incubation (Fig. 1*A*, *B*); however, over the 4-day period, the increase in both parameters was seen to be significantly greater at 5°C than at 26°C. Microorganism density in the 5°C incubation solution did not significantly increase during the 4-day period. However, at 26°C, microorganism density increased 10-fold (Fig. 1*C*). In the control, there was no microorganism density increase detected. The remaining viable embryos from both temperature treatments germinated and grew in a manner similar to those cultured fresh. In addition, there was no significant difference in individual embryo growth (as measured by fresh weight gain) six weeks after the start of cold incubation as compared to the 26°C treated embryos (Fig. 1*F*). All the plants produced had normal morphology, indicating that the cold treatment had not damaged the embryos nor upset their subsequent growth potential.

Remote site germplasm collecting

All embryos incubated at 5°C remained white and firm while those under the conditions without temperature control turned yellow and soft, with the colour intensity and texture increasing with time. Although detailed analysis of the microorganism density in incubation solutions was not undertaken, visual observations indicated that the incubation solution kept at 5°C remained clear over the incubation period, while the solution from the treatment without temperature control became turbid. The highest rate of embryo contamination (35%) was observed when the embryos were incubated without temperature control while the cold-treated embryos exhibited a very low rate of contamination (<5%; Fig. 2). The germination and subsequent growth of the uncontaminated embryos from both temperature treatments were similar (Fig. 2). Since no change occurred in the incubation solutions taken from the control tubes without embryos, the increase in pH and electrical conductivity (Fig. 1*A*, *B*) may indicate that under both temperature conditions, solutes were released from the embryos being incubated. Since this leakage was greatest at 5°C, this could indicate that chilling injury had occurred. This is not surprising since similar low-temperature treatments had been shown to affect coconut and other tropical crops in a similar way. The occurrence of chilling injury was further indicated by the higher ethylene production (Fig. 1*E*) and the lower respiration rate (Fig. 1*D*) under this condition as compared to the

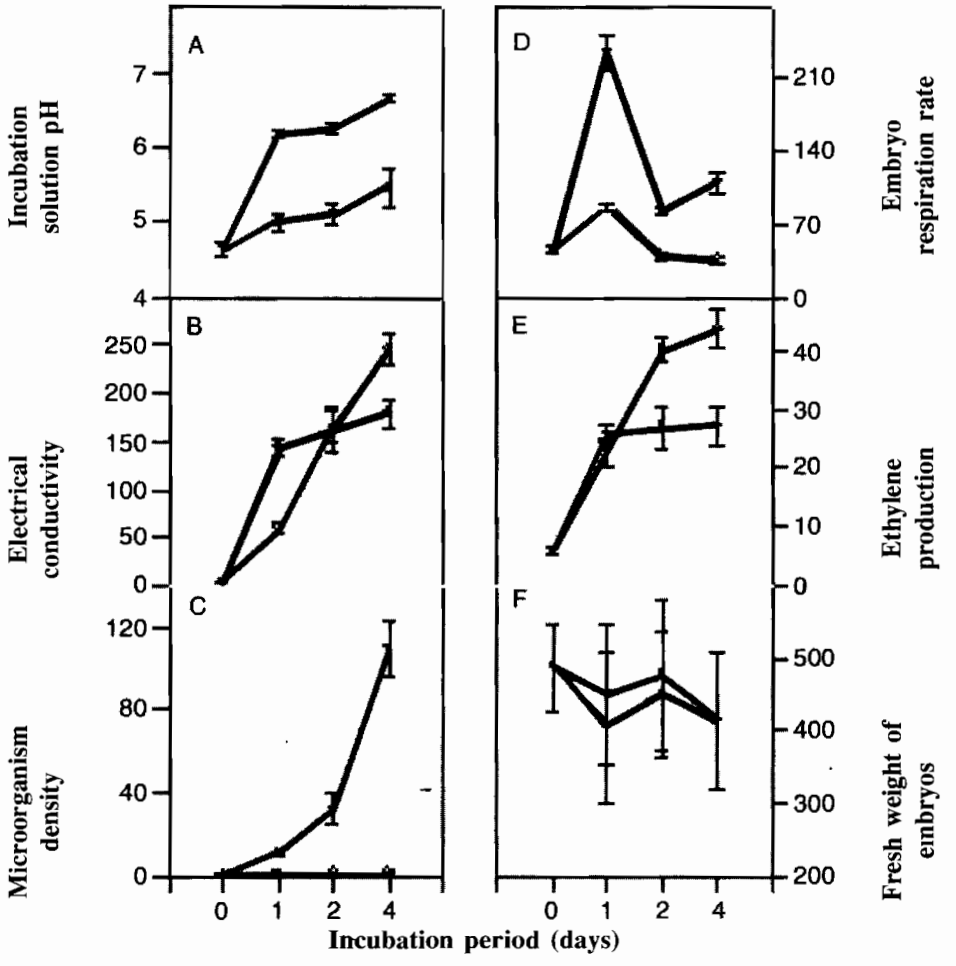

Fig. 1. Condition of the incubation solution (A, B and C) and coconut embryos (D, E and F) following incubation at 5 ± 1°C (ô) or 26 ± 1°C. After the indicated time, the solutions were analyzed for pH (A), electrical conductivity (B) and microorganism density (C). Embryo respiration rate (D) and ethylene production (E) were measured after transferring the embryos from the solution to sealed tubes and incubating at 26 ± 1°C in the dark. The fresh weight (F) of embryos was recorded after six weeks of culture on a tissue culture medium. All data points are means ± s.e. (vertical bars).

warmer incubation condition (Fig. 1*E*). Even though some chilling injury might have occurred, this was not sufficient to prevent the low-temperature-treated embryos from germinating and growing in a fashion identical to embryos that were not low-temperature treated (Figs. 1 and 2). The preservation of the health of the embryos under the low-temperature treatment could be because this condition prevented microorganism multiplication (Fig. 1C) similar to that observed on many other occasions. As the indicators of chilling injury used here were still increasing after four days, it might be unwise to store coconut embryos for much longer at 5°C. Under most germplasm collecting conditions, four days would be ample time to return the embryos to a tissue culture laboratory for surface sterilization and culture.

The advantage of the present remote-site germplasm collecting technique is that no sterilization or aseptic culture is required in the field. This is different from most other previously described techniques for coconut germplasm collecting. In addition, the present technique can be undertaken by unskilled workers in the field and requires little equipment and chemicals (*c.* 3 kg in total weight for the isolation and transportation of at least 500 embryos).

Conclusions

A new technique for coconut (*Cocos nucifera* L.) germplasm collecting was evaluated in the laboratory and tested in the field in West Lombok, Indonesia. The technique involved the non-sterile isolation of embryos and incubation in sterile absorbic acid solution (1 mg l^{-1}) at 5 ± 1°C in the dark. During this incubation period, the embryos could be transported and/or stored for a period of up to four days without embryo viability loss. Following this period the embryos were surface sterilized with sodium hypochlorite (1.5% w/v) for 20 minutes, washed with sterile water and cultured in a liquid Y3 basal nutrient medium supplemented with Morel and Wetmore vitamins, sucrose (175 µM) and activated charcoal (2.5 g l^{-1}). After two weeks, the embryos were sub cultured onto a solid medium of similar constitution to encourage germination. Germinated embryos grew and produced healthy plants with normal morphology. Despite mild chilling injury as indicated by elevated ethylene production and solute leakage, the transported embryos retained viability with normal morphology. Using the low-temperature incubation treatment, the microorganism density in the ascorbic acid solution was kept low whereas that around other embryos kept at higher temperatures (25°C) increased. Even though the embryos were exposed to a low- temperature treatment for up to four days, they were able to germinate (93% viable) and grow similar to freshly cultured embryos.

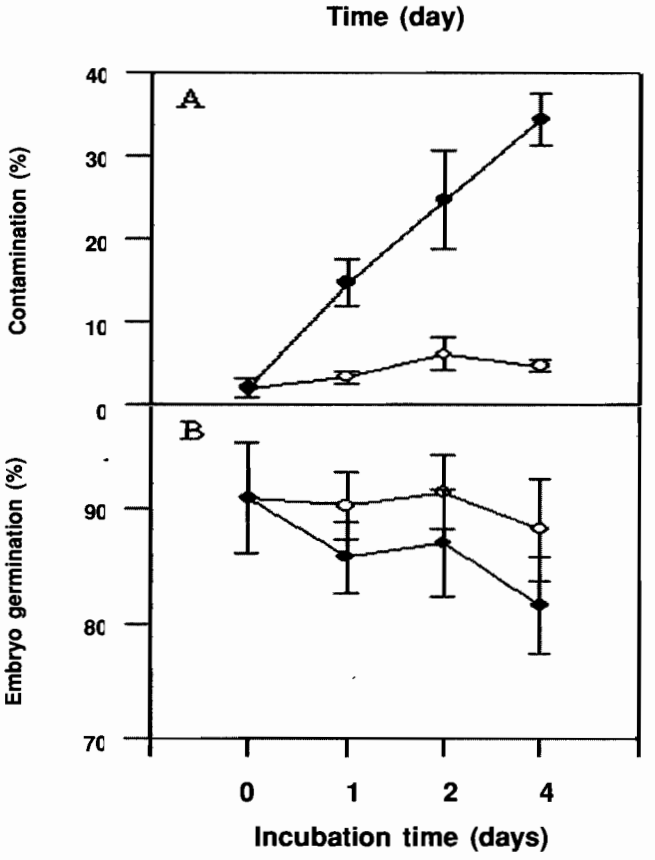

Fig. 2. Condition of field-collected coconut embryos following incubation at 5 ± 1°C (ô) or without temperature control 18/25 ± 2°C. After the indicated time and culture on a tissue culture medium for six weeks, embryo contamination (A) and germination (B) were determined. All data points are means ± s.e. (vertical bars).

Acknowledgements

The authors thank the Australian Agency for International Development (AusAID) for a scholarship awarded to Y.M.S. Samosir. Special thanks to Dr Parman and Dr Mansur Ma'shum, University of Mataram, West Lombok, Indonesia, for their cooperation during the field trials.

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ANNEXES

Annex 1

Embryo Culture Protocols

Annex 1.1. Coconut embryo culture protocol at the University of the Philippines at Los Baños (UPLB), Philippines

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 (3) times with sterile distilled water.
6. Inoculate in liquid Y3 medium + 60g/L sucrose.
7. Incubate in the dark.
8. After 4 weeks, transfer to solid Y3- medium + 60g/L sucrose. Incubate under 9 hours light/14 hours dark photoperiod at 25-29°C.
9. After 4-6 weeks, transfer germinated embryos to liquid Y3 medium + 60g/L 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 are brought to the screenhouse and subjected to natural light.
12. After 2 weeks, take seedlings out of culture bottles.
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 compost: sand (1:1) mixture.
19. Keep under 30% shade.
20. After three months, expose to full sun conditions.

Annex 1.2. Coconut embryo culture and field establishment protocol at the Central Plantations Crop Institute (CPCRI), India

1. Inoculate the embryos in solid retrieval medium (Y3 medium + 60g/L sucrose + NAA (0.5 mg/L) + BAP (0.5 mg/L) and incubate in the dark till germination (average germination time of mature embryos is 20–25 days for Dwarf and 35–40 days for Tall) and transfer to light with 16 hours photoperiod (Temperature 27–29°C, Relative Humidity 65–70%).
2. Subculture every 4–5 weeks. Reduce the sucrose concentration to 30g/L.
3. Germinated embryos (with two leaves and primary root, almost four months after inoculation) are transferred to liquid rooting medium (Y3 + 30g/L sucrose + NAA [1ppm] + IBA [5ppm]).
4. Subculture on same medium 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.
6. Potting mixture consists of autoclave-sterilized soil: sand: decomposed coir dust (1:1:1).
7. Pre-treat the plantlets with Carbendazim (1g/L) and IBA (1000 ppm) for 1 hour each and transfer to the pots.

Acclimatization

1. Cover the plantlets with polyethylene bags for 2–3 weeks and keep them indoor at room temperature with artificial light.
2. Supply Hoagland's solution once every 15 days.
3. Irrigate to keep potting mixture moist.
4. After three weeks, harden the plantlets by gradually perforating the polyethylene bags.
5. After two weeks, remove the polyethylene bags at night for two weeks.
6. After two weeks, remove the polythene bags completely and keep plantlets indoor for one week.
7. Transfer plantlets to bigger pots and keep them in net house with 50% shade.
8. After 3–4 months, transfer the plantlets to big polythene bags containing soil and organic manure and keep them in a net house 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 1.3. Coconut embryo culture protocol at IRD/CIRAD-CP/IDEFOR, France

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

Sampling during a collection mission

This method has been developed to collect embryos in field conditions during a collecting mission a long distance away 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 on a table, which has been carefully washed and disinfected with hypochlorite in the open air. —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 (Δ 20mm), which must be -disinfected by immersion in a bowl containing a 3% chlorogenic sodium hypochlorite solution before use. A portable gas burner is used to sterilize the instruments.

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

- **Embryo storage and transport from the collecting site to the in vitro laboratory**
  Previous trials have shown 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 return to the laboratory to start the -culturing operation.

- **Embryo excision before inoculation**
  After the storage period, the cylinders are re-sterilized by immersing them in a filtered solution of calcium hypochlorite (70% active chlorine; 45 g/L) for 20 minutes. The embryos are then isolated under an air-flow cabinet and rinsed in sterile distilled water before inoculation onto liquid medium.

B. 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 described above. The embryos are then inoculated in sterile polypropylene 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 for -five days without any alteration that might affect -their germination capacity. When they arrive at- the destination- laboratory, the embryos are transferred on the germination medium.

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

These embryos- are sub cultured every 4-6 weeks onto 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 to 100 ml Mi 502 liquid medium in one L glass bottles under light (12 hours light/ 12 hours dark; light intensity 45 ± µmol/m^2; Sylvania gro-lux day light tubes). Bottles are covered with foam caps surrounded by aluminum foil and sealed with paraffin.

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

D. 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 five minutes in a Carbendazin-based fungicide solution (Benlate, 2 g/L) to prevent fungi development. They are then planted on sterile river sand. A plastic bag (acrylic polypropylene) is placed over each plantlet during the first two weeks to maintain maximum relative humidity. The plastic cover is progressively opened. Plantlets are watered regularly for the first month and then a nutritive solution is applied every two days (see composition below).

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

KNO_3	274.00
$Ca(NO_3)_2\ 2H_2O$	1095.00
KH_2PO_4	137.00
$MgSO_4\ 7\ H_2O$	274.00
$(NH4)_4\ SO_4$	137.00
KCl	2.74
$H_3\ PO_3$	3.00
$MnSO_4\ H_2O$	15.00
$ZnSO_4\ 7\ H_2O$	2.74
$(NH_4)_6\ MO7Oz_4\ 4\ H_2O$	2.74
$H_2\ SO_4$	0.137
$Cu\ SO_4\ 5H_2O$	1.37
$FESO_4\ 7\ H_2O$	24.90
EDTA	26.10

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

Annex 1.4. Coconut embryo culture protocol at the Philippine Coconut Authority-Albay Research Station (PCA-ARC)

Embryo collecting and pre-sterilization

Ideally, 10 to 11 months old coconuts are harvested. Coconuts that are younger than 10 months do not respond favorably to *in vitro* conditions to produce robust Makapuno coconut palms.

After all the endosperm cylinders are extracted, they 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. The cylinders are then washed three times with sterile water to remove the bleach. This 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 10x for macro-elements and 100x for micro-elements and vitamins.

B. Preparation of the Y3 culture medium

• Macronutrients	10x
• Micronutrients	100x
• Myo-inositol	100x
• Vitamins	100x
• FeEDTA	100x
• Table Grade Sugar	45g/L
• AC (Activated Charcoal)	2.5g/L

1. Weigh 45 g/L of sugar and dissolve it in the above solution.
2. Using a volumetric flask or 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 volumes of 10 ml of liquid medium into 150 x 25 mm test tubes_while stirring constantly to disperse the activated charcoal evenly. If using 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.

Aseptic 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 for -storage and transport, immerse these in 100% bleach for 5 minutes.
4. Inside the laminar flow hood after sterilization, decant bleach and rinse samples 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 steri-beads or flame with an alcohol lamp for 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 the 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 in test tubes containing Y3 liquid medium.

C. Culture conditions

1. Incubate cultures at 28-30°C under approximately 4,000-5,000 lux with a 15 hours dark/9 hours light photoperiod.
2. Subculture to fresh medium at monthly intervals.
3. Check periodically for contamination.
4. Embryos grow at different rates. Generally, after 6-8 weeks in culture roots and shoot are formed. The earliest recorded time to transfer a plantlet *ex vitro* is 4 months.
5. Altogether, the culture period could be a year or more.

Screenhouse practices

A. Materials

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

B. 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- in small polyethylene bags.
3. Take out hardened seedlings.
4. Wash out the medium carefully. The liquid medium contains sugar that will attract ants if not washed completely. -Dip the seedlings in 2.5 g/L fungicide solution.
5. Plant in sterilized sand.
6. To maintain high relative humidity, cover the seedlings with plastic bags. Support the plastics bags with bamboo pegs so that they will not collapse -on the leaves of the seedlings. Keep them covered for 5-7 days.
7. After this period, gradually expose the seedlings to screenhouse conditions by partially lifting the plastic 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 diluted foliar fertilizer solution weekly new leaves develop.
10. After 3 months, transfer the plants to bigger polyethylene bags containing -non-sterilized soil.
11. After another 3-5 months, when 4 to 6 leaves appear, the plants can be transferred to the field.
12. Provide the seedlings with the necessary cultural practices for optimum growth response especially during the first three years.

Annex 1.5. Coconut embryo culture media composition (mg/l)

SL.NO	Chemical	PROTOCOLS			
		PCA	U PLB	C PCRI	IRD/ORSTOM
		Y3	Y3	Y3	MS
MACRONUTRIENTS					
	NH4NO3	-	-	-	1650
	NH4Cl	535	535	535	-
	KNO3	2020	2020	2020	1900
	MgSO4.7H2O	247	247	247	370
	CaCl2.2H2O	294	294	294	440
	KCl	1492	1492	1492	-
	KH2PO4	-	-	-	170
	NaH2PO4.2H2O	312	312	312	-
MICRONUTRIENTS					
	KI	8.3	8.3	8.3	0.83
	H3BO3	3.1	3.1	3.1	6.2
	MnSO4.4H2O	11.2	11.2	11.2	22.3
	ZnSO4.7H2O	7.2	7.2	7.2	8.6
	CuSO4.5H2O	0.25	0.25	0.160	0.025
	CoCl2.6H2O	0.24	0.24	0.24	0.025
	NaMoO4.H2O	0.24	0.24	0.24	0.025
	NiCl.6H2O	0.024	0.024	0.024	-
	Fe2SO4,7H2O	13.9	41.7	13.9	24.9
	Na2EDTA	37.3	55.8	37.3	26.1
ORGANICS					
	Myo-inositol	100	-	100	100
	Pyridoxine HCl	0.05	0.05	0.05	1.0
	Thiamine HCl	0.05	0.05	0.5	1.0
	Nicotinic Acid	0.05	0.5	0.5	1.0
	Ca-D-panthothenate	0.05	-	-	1.0
	Biotin	0.05	0.05	0.05	0.01
	Folic acid	-	0.05	-	-
	Glycine	-	1.0	2	-
	Na Ascorbate	-	-	-	100
	BAPk	-	-	0.5	-
	NAAk	-	-	0.5	-
	NAAkk	-	-	1.0	-
	IBAkk	7.0	-	5	-
	Agar	-	7.0g/L liq/sol/liq	5.5g/L sol/sol/liq	-
	Activated charcoal	2.5g/L	2.5g/L	1g/L	2g/L
	Sucrose	45 g/L	60g/L	60 g/L * 30 g/l **	60 g/L
	Table sugar	45 g/L		30 g/L	
	pH	5.8	5.6	5.7	5.5

* Germinating medium

** Rooting medium

Annex 2

Workshop Objectives and Programme

Second International Coconut Embryo Culture Workshop

Centro de Investigacion Cientifica de Yucatan (CICY)
14-17 March 2000

Workshop objectives

1. To review recent research on coconut embryo culture and acclimatization and upgrade protocols for increased efficiency;
2. To develop research and development project proposals for submission to appropriate donors; and
3. To strengthen research collaboration among the members of the Coconut Embryo Culture Project Network.

Programme

Tuesday, 14 March

Opening Program

0900 - 0915	Opening remarks	Dr. Carlos Oropeza
0915 - 0945	Remarks on behalf of IPGRI and COGENT and presentation of workshop objectives	Dr. Pons Batugal *COGENT Coordinator*
0945 – 1000	Welcome remarks, introduction of CICY and official inauguration of the workshop	Dr. Alfonso Larque *CICY Director General*
1000 - 1030	Coffee break	
1030 - 1100	Special presentation: CICY's research on physiology of vitroplants	Dr. Jorge Santamaría

Presentation of Research Reports

1100 - 1130	Philippine Coconut Authority	Mrs. Erlinda Rillo
1130 -1200	Philippine Coconut Research and Development Foundation	Mr. Emile Carandang
1200 - 1230	Discussion	
1230 – 1330	Lunch	

1330 – 1400	Research Institute for Coconut and Palmae, Indonesia	Mrs. Nurhaini Mashud
1400 – 1430	Central Plantation Crops Research Institute, India	Dr. V.A. Parthasarathy
1430 – 1500	Cocoa and Coconut Research institute, PNG	Mr. Alfred Kembu
1500 – 1530	Discussion	
1530 – 1600	Coffee break	
1600 – 1630	Coconut Research Institute, Sri Lanka	Dr. L. K. Weerakon *(By Dr. Pons Batugal)*
1630 – 1700	Oil Plant Institute, Vietnam	Mrs. Vu Thi My Lien *(By Dr. Pons Batugal)*
1700 - 1730	Discussion	

Wednesday, 15 March

0830 – 0900	Centro de Investigación Científica de Yucátan, Mexico	Dr. Carlos Oropeza
0900 – 0930	Instituto de Investigaciones de Cítricos y otros Frutales, Cuba	Ms. Maricela Capote
0930 – 1000	Centro de Pesquisa Agropeuaria dos Tabuleiros Costeiros (CPATC), Brazil	Ms. Paula Angelo *(By Dr. Pons Batugal)*
1000 – 1030	Discussion	
1030 – 1100	Coffee break	
1100 – 1130	Mikocheni Agricultural Research Institute, Tanzania	Mr. Raphael Sallu
1130 – 1200	Centre National De Recherche Agronomique, Côte d'Ivoire	Dr. Kouassi Koffi II Nazaire
1200 – 1230	Discussion	
1230 – 1330	Lunch	
1330 – 1400	Secretariat of the Pacific Community, Fiji	Dr. Mary Taylor
1400 - 1430	International Research for Development, France	Dr. Bernard Malaurie
1430 – 1500	Institute of Plant Breeding, Philippines	Dr. Olivia Damasco

1500 – 1530	University of Queensland, Australia	Dr. Steve Adkins
1530 – 1600	Discussion	
1600 – 1630	Coffee break	
1630 – 1730	Summary presentation of research outputs Discussion on upgrading the embryo culture protocols Creation of a task force to upgrade protocols	

Thursday, 16 March

8300 – 1000	Visit to CICY
1000 – 1730	Field trip
2000	Workshop Dinner at Hotel Los Aluxes

Friday, 17 March

0830 – 0910	Report of Task Force on upgrading protocols and discussion

Presentation of Proposed Future Work

0910 – 0930	Philippine Coconut Authority	Mrs. Erlinda Rillo
0930 – 0950	Coconut Research & Development Foundation	Mr. Emile Carandang
0950 – 1010	Research Institute for Coconut and Palmae, Indonesia	Mrs. Nurhaini Mashud
1010 – 1030	Cocoa and Coconut Research Institute, PNG	Mr. Alfred Kembu
1030 - 1050	Coffee break	
1050 – 1110	Central plantation Crops Research Institute, India	Dr. V.A. Parthasarathy
1110 – 1130	Coconut Research Institute, Sri Lanka	Dr L. K. Weerakoon *(by Dr Pons Batugal)*
1130 – 1150	Centro de Investigación Científica de Yucátan, Mexico	Dr. Carlos Oropeza
1150 – 1210	Instituto de Investigaciones de Cítricos Soly otros Frutales, Cuba	Ms. Maricela Capote del

1210 – 1230	Centro de Pesquisa Agropeuaria dos Tabuleiros Costeiros (CPATC), Brazil	Ms. Paula Angelo *(by Dr. Pons Batugal)*
1230 – 1330	Lunch	
1330 – 1350	Mikocheni Agricultural Research Institute, Tanzania	Mr. Raphael Sallu
1350 - 1410	Centre National De Recherche Agronomique, Côte d'Ivoire	Dr. Sie Koffi
1410 - 1430	Secretariat of the Pacific Community, Fiji	Dr. Mary Taylor
1430 – 1450	International Research for Development , France	Dr. Bernard Malaurie
1450 – 1510	Institute of Plant Breeding, Philippines	Dr. Olivia Damasco
1510 – 1530	University of Queensland, Australia	Dr. Steve Adkins
1530 – 1600	Coffee break	
1600 – 1700	Discussion Project proposals – Way Forward Coconut Embryo Culture Project Network – Ways to Sustain It	

Closing session

Brief remarks:

Dr. V. A. Parthasarathy
Dr. Mary Taylor
Mr. Raphael Sallu
Mrs. Erlinda Rillo
Dr. Pons Batugal
Dr. Alfonso Larque

Annex 3

List of speakers and contact institutions

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Annex 4

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14 – 17 March 2000
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Annex 5

List of Acronyms Used in the Text

ABA	Abscisic acid
ACIAR	Australian Center for International Agriculture Research
ANOVA	Analysis of Variance
CCRI	Cocoa & Coconut Research Institute
CICY	Centro de Investigación Científica de Yucatán
CIRAD	Centre Institut de Recherche pour le Développement
CNRA	Centre National de Recherché Agronomique
COD	Chowghat Orange
COGENT	International Coconut Genetic Resources Network
CPCRI	Central Plantation Crops Research Institute
CRISL	Coconut Research Institute, Sri Lanka
CV	Coefficient of Variance
DFID	UK Department for International Development
DMT	Mapanget Tall
DMRT	Duncan Multiple Range Test
EAT	East African Tall
GA_3	Gibberellic Acid
GKN	Nias Yellow Dwarf
ICG-SA	International Coconut Genebank-South Asia
ICG-SP	International Coconut Genebank for the South Pacific
IDEFOR/DPO	Institut de Development de Foret/Department du Plante Oleagineous (formerly CNRA)
IICF	Instituto de Investigaciones de Cítricos y otros Frutales
INIFAP	Instituto Nacional de Investigaciones Forestales Agricolas Y Pecuarias
IPB	Institute of Plant Breeding
IRD	Institut de Recherche pour le Développement
LAGT	Laguna Tall
LCT	Laccadive Ordinary
LY	Lethal yellowing
MAKT	Makapuno (variety)
MARI	Mikocheni Agricultural Research Institute

MGD	Malayan Green Dwarf
MRD	Malayan Red Dwarf
MYD	Malayan Yellow Dwarf
OPI	Oil Plant Institute
PCA	Philippine Coconut Authority
PCA-ARC	Philippine Coconut Authority – Albay Research Center
PCRDF	Philippine Coconut Research and Development Foundation
PEG	Polyethylene Glycol
PRD	Pemba Red Dwarf
RICP	Research Institute for Coconut Palmae
RIT	Rennel Island Tall
RLT	Rennel Tall
SLGD	Sri Lanka Green Dwarf
SLT	Sri Lanka Tall
SRS	Stewart Research Station
TAGT	Tagnanan Tall
UNDP	United Nations Development Programme
UPLB	University of the Philippines at Los Baños
VISCA	Visayas State College of Agriculture
WCT	West Coast Tall
ZRC	Zamboanga Research Center

IPGRI

Coconut Embryo *In Vitro* Culture: Part II

COGENT

DFID
Department for International Development

CICY

Florent Engelmann
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FUTURE HARVEST
<www.futureharvest.org>
IPGRI is a Future Harvest Centre supported by the Consultative Group on International Agricultural Research (CGIAR)
ISBN 92-9043-536-4

Published by the Regional Office for Asia, the Pacific and Oceania (APO)