Technical Bulletin No. 45

COCONUT EMBRYO CULTURE

Protocol for Germplasm Collection

Central Plantation Crops Research Institute
(Indian Council of Agricultural Research)
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Cover:

Front : *In vitro* retrieved Maldives Yellow Tall plantlet (collected from Hanimoodho Island)
Back : Guelle Rose accession collected from Mauritius
1. Germinating embryo 2. Field established plantlet

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FOREWORD

The Central Plantation Crops Research Institute, Kasaragod has a unique achievement in the collection and conservation of genetic resources of coconut as it possess the largest nurnber of accessions in the world. The efforts in this direction started way back in 1940s and at present CPCRI maintain 221 indigenous and 92 exotic coconut accessions in its field genebank. Till the standardization of coconut embryo culture techniques, the germplasm was brought in the form of seed nuts. The process was very tedious, involving huge expenditure besides restricting the size of collections. An alternative is the embryo culture technique.

Experiments to standardize embryo culture techniques at CPCRI were initiated during nineties. Based on a series of experiments, a complete protocol for coconut embryo culture was evolved and is internationally referred to as the CPCRI protocol. In a network experiment for the comparison of four embryo culture protocols conducted in 14 international laboratories supported by the COGENT, the CPCRI protocol was rated better for the production of vigorous plantlets and high percentage of *ex vitro* establishment.

The Institute has employed the embryo culture protocol successfully in five expeditions involving scientists from the disciplines of genetics and plant breeding, pathology and entomology. Adequate training was imparted to the personnel involved in the expedition programme for collection and aseptic inoculation of embryos at site itself. CPCRI is the first Institute in the world to attempt the collection of coconut gemplasm exclusively in the form of embryos alone. That the embryo cultured seedlings could be successfully planted in the field (ICG-SA, Kidu) bears testimony to the success of the technique.

This technical bulletin describes the complete steps involved in the use of CPCRI protocol of embryo culture for germplasm collection and will serve as a reference guide to research personnels in this field. I compliment every member of the team that evolved the protocol and putin practice.

V. Rajagopal

Date: 16.10.2002.

(Dr. V. Rajagopal)
Director

CONTENTS

1. INTRODUCTION

The collection and exchange of coconut germplasm in the early days were in the form of whole seednuts. Being a recalcitrant species, lot of problems were encountered while transporting seed nuts from far off places. Large size of the seed, water content and stringent phytosanitary requirements are the major problems in germplasm collection, transportation and storage of coconut seeds. The successful *in vitro* germination of coconut zygotic embryos provides an efficient way of transportation of coconut germplasm in the form of embryo cultures. This method has the advantage from the point of quarantine regulation also as treatment of seed nuts with insecticide, fungicide and fumigant could be avoided. All the more the size of collection need not be restricted for want of space as embryo cultures occupy very less space. Reduction in the cost of transportation, lowest risk of disease transmission etc. are the additional advantages.

Following the standardization of embryo culture protocol, it is now widely accepted that movement of coconut germplasm should be through embryos (IPGRI Newsletter, 1993). International Coconut Embryo Culture and Acclimatization Workshop at PCA, Philippines (27-31 October, 1997) identified four embryo culture protocols *viz.*, PCA, Philippines, UPLB, Philippines, IRHO, France and CPCRI, India. Among these, the CPCRI protocol is relatively simple and distinct from the rest for short-term storage of embryos, rescue of immature embryos and the higher rate of acclimatization of seedlings. The components of the protocol include direct field collection of 8 to 11 months old coconut embryos, short-term storage, *in vitro* retrieval and *ex vitro* establishment. The protocol was further tested during 1994 by bringing 86 embryos of six coconut accessions maintained at World Coconut Germplasm Centre (WCGC), Andamans to CPCRI, Kasaragod and their subsequent *in vitro* retrieval. The protocol was employed successfully in a germplasm expedition to Indian Ocean Islands during 1997. This is the first attempt where all collections were made in the form of embryo cultures. A comprehensive description of CPCRI protocol is given in this publication.

The Chapter 2 describes the collection of embryos from the field by means of simple and portable tools. In Chapter 3, retrieval medium and culture conditions are given. The procedure for *ex vitro* establishment of embryo-derived seedlings and their performance on field establishment are given in Chapter 4. The last Chapter summarizes the germplasm expeditions in which collections were made in the form of embryos.

2. FIELD COLLECTION OF EMBRYOS

2.1. Introduction

The collection sites of coconut germplasm are often isolated islands where only limited facilities are available. Hence simple and portable tools are to be used for collection and direct inoculation of embryos in the field, which are listed in Section 2.2. Extraction of embryos and surface sterilization of embryos of different age groups are dealt in subsequent sections.

2.2. Field collection kit

Materials and tools for collection of embryos in aseptic condition from the collection site are listed in Table 1. A folding type portable inoculation hood made out of plexiglass (Fig. 1) is used for inoculation of embryos in the field.

Table 1. Items to be carried to the collection site for collecting 2000 embryos

Sl.No.	Name of the items	Quantity
	BOX-1 (75x60x10cm)	
1.	Portable folding hood and folding chair	1 each
	BOX-2 (100x70x60 cm)	
2.	Pressure cooker (7 l capacity)	1 no.
3.	Absolute alcohol	3 l
4.	Sodium hypochlorite Solution	2 l
5.	Chlorine kit: Conical flask (1 l) separating funnel (500 ml), reagent bottle (500 ml), potssium permanganate (500 g) and HCl (500 ml)	1 set
6.	Test tube (20 cm length and 3 cm diameter)	20 nos.
7.	Steel forceps (24 cm)	4 nos.
8.	Stainless steel cork borer (18 cm length and 2 cm diameter)	2 nos.

(continued)

9.	Small knife (17.5 cm length)	1 no.
10.	Big knife (40 cm length)	1 no.
11.	Beakers of capacity 50 ml, 100 ml and 500 ml (preferably polypropylene)	1 each
12.	Self sticking labels	3000 nos.
13.	Scissors	2 nos.
14.	Parafilm	1 roll
15.	Bandage cloth (30 x 45 cm)	500 pieces
16.	Plastic tray (45 x 30 cm)	2 nos.
17.	Rubber bands	500 nos.
18.	Non absorbent cotton	3 bundles
19.	Sterile disposable gloves	200 nos.
20.	Reagent bottles (500 ml and 250 ml)	1 each
21.	Measuring cylinders (100 ml)	2 nos.
22.	Aluminum foil	3 rolls
23.	Polythene bags (45 x 30 cm)	100 nos.
24.	Coconut dehusker	1 no.
25.	Scalpel	2 nos.
26.	Wash bottle	1 no.
27.	conical flasks (50 ml, 100 ml, 500 ml)	5 each
28.	Cello tape (5 cm wide)	1 roll
29.	Market pens (4 assorted colors)	1 set
	BOX -3	
30.	Carry bag (60 x30x30 cm)	1 no.
31.	Sterile vials containing either sterile water or minimal growth medium	2100 nos.

Other items required for field collection of embryos are shown in Figure 2.

For convenience the items may be packed as indicated in table 1. The sealed sterile vials containing 1.5 to 2 ml sterile water may be packed in a carry bag. A set of 100 vials may be bundled while packing.

2.3. Extraction of embryos

Conventionally mature nuts (11 to 12 months after fertilization) are being used for seed nut collection. This restricts the size of collection in a germplasm expedition. On the other hand even 8 month old embryos could be extracted and retrieved *in vitro*. However, the per cent germination of 8 month old embryos was less compared to 10 month and above. The dimension of 8 month old embryos was observed to be less (1-4 mm) compared to the size of 9 to 11 months old (4-5 mm and 7-8 mm respectively). The size of the embryo may be a factor influencing the *in vitro* germination.

The procedure of extraction of embryos does not change with age but the surface sterilization differs. To extract embryos, first remove the husk of the harvested fruit and split open the nut. Embryo is located under the 'large eye' of the coconut embedded in the solid endosperm. This active 'eye' of the coconut is usually depressed due to the non-lignification of the layer of the cells allowing it to soft for the germination. After locating this 'eye', scoop out the embryo along with a portion of the endosperm by means of a cork borer (Fig. 3a). The extracted endosperm cylinders are collected in a beaker containing distilled water. Once all the plugs are collected from the split opened nuts, the embryo is extracted from the endosperm plug (Fig. 3b) using scalpel or small knife. These extracted embryos (Fig. 3c) alone are collected in a beaker containing distilled water .

About 18% embryos may get damaged while extracting from the nut as endosperm plug and 2% while extracting from endosperm plug.

2.4. Surface sterilization

2.4.1. Surface sterilization of inoculation hood: Immediately after the extraction of embryos the folding hood should be kept ready for surface sterilization of embryos (Fig. 4a). Both inside and outside of the hood must be wiped thoroughly with alcohol for

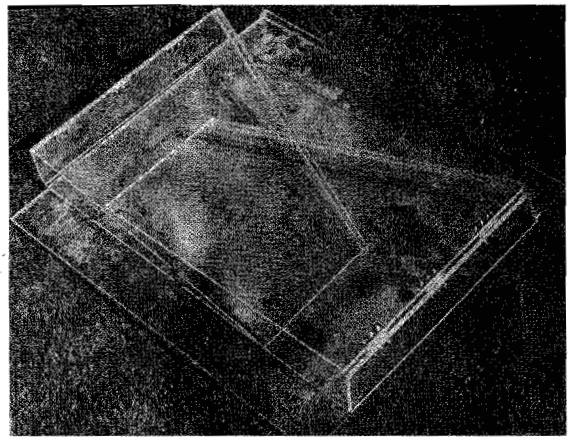

Fig. 1. Foldable inoculation hood

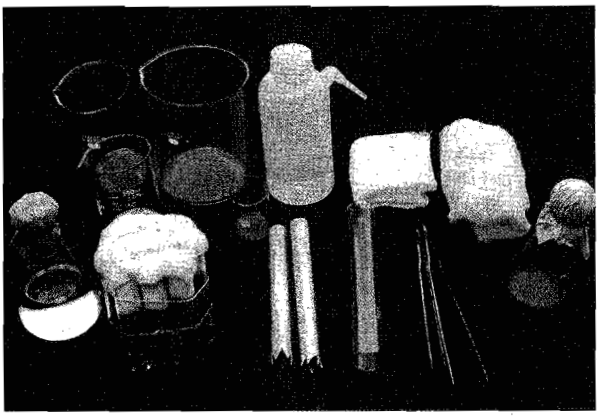

Fig. 2. Laboratory items required for extraction and inoculation of embryos

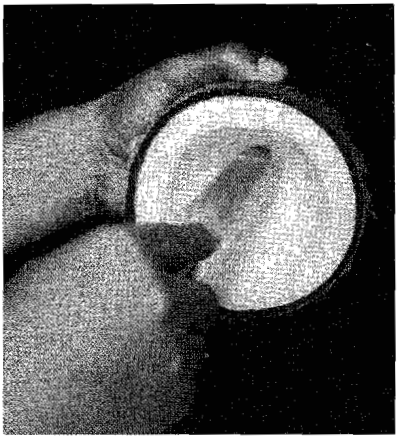

Fig. 3a. Embryo is scooped out using a cork borer

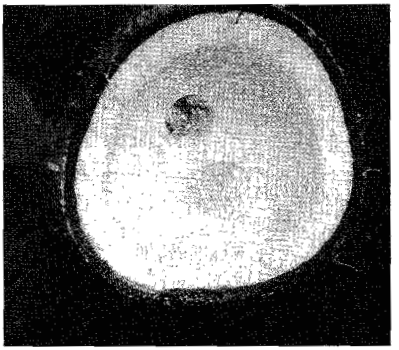

Fig. 3b. The endosperm plug with embryo can be seen inside the split half nut.

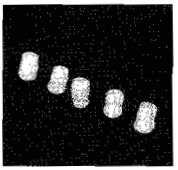

Fig. 3c. The extracted embryos

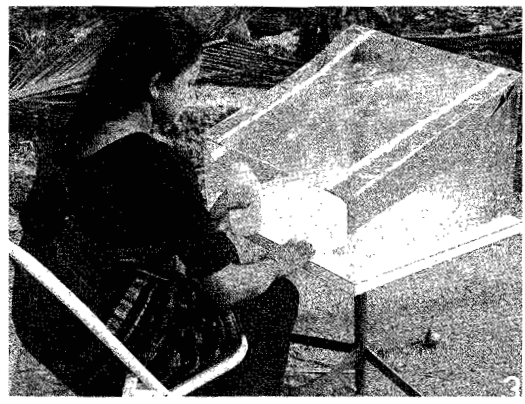

Fig. 4a. Surface sterilization of the inoculation hood

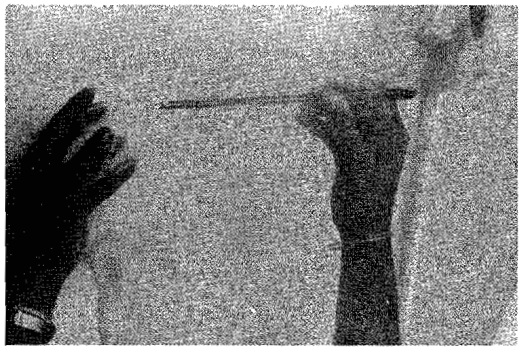

Fig. 4b. Embryo is inoculated inside the hood

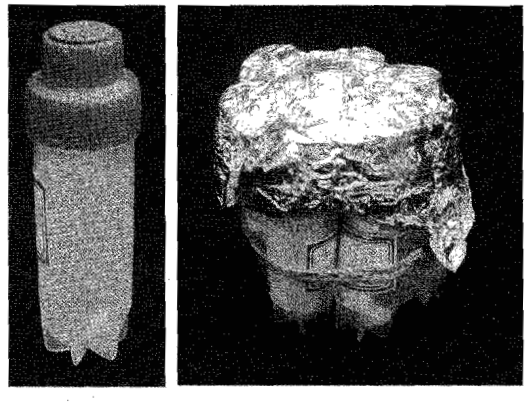

Fig. 5. Screw cap vial containing embryo after labeling (left)and vials bundled accession wise

Fig. 6. Emergence of plumule and radicle

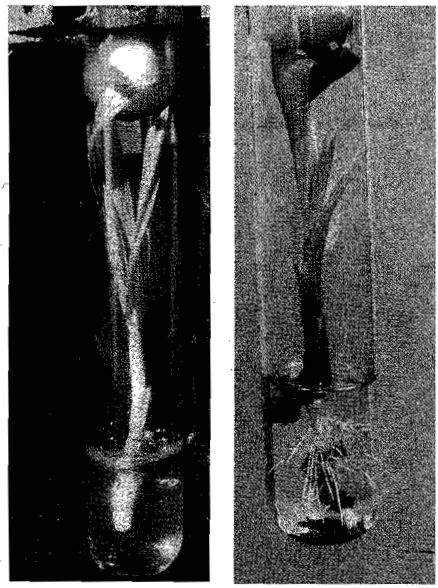

Fig. 7. Plantlet in retrieval medium (solid) ready for transferring to rooting medium (left); plantlet with well developed roots in rooting medium (liquid)

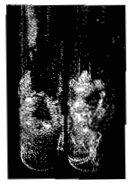

Fig. 8. Abnormalities noticed during *in vitro* retrieval

Fig. 8a. Cessation of growth after germination

Fig. 8b. Plantlet showing necrosis after 6 months of culture

Fig. 8c. Yellowing of leaves

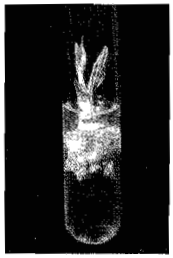

Fig. 8d. Hyperhyricity and necrosis in culture

Fig. 9. *In vitro* retrieved plantlets ready for pot planting

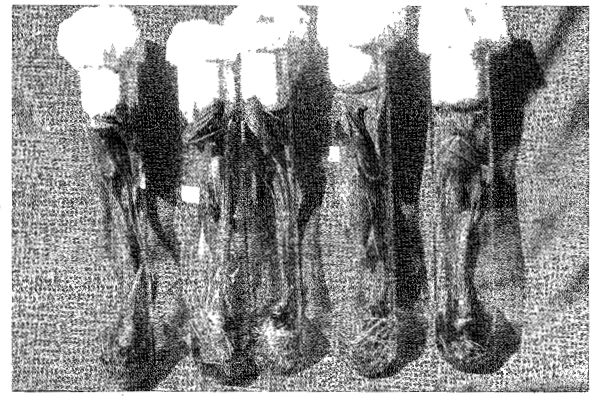

Fig. 10. Humidity control by covering the individual plantlet with polythene bag

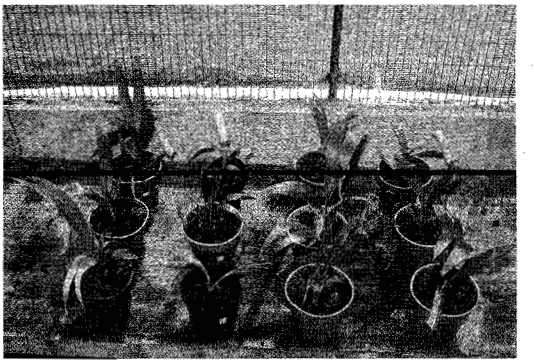

Fig. 11. Potted plants inside the net house

Fig. 12. Plants in poly bags

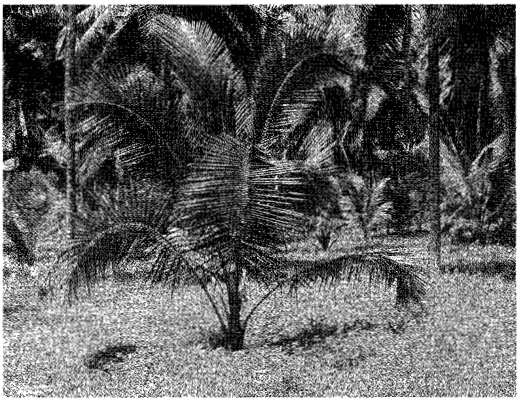

Fig. 13b. Embryo-derived plant after 2 years of field planting

Fig. 13a. Field establishment of embryo-derived plant

disinfection. Disposable gloves must be used while working with bleach to reduce the contamination. Before surface sterilization wash the embryos with absolute alcohol quickly to remove the fat globules on the surface of the embryos.

2.4.2. Surface sterilization of 9 to 11 month old embryos: The surface-sterilization of embryos in the field is done by 50% chlorine water for 20 minutes and washing 4 to 5 times in sterile water till traces of chlorine is removed. Chlorine water can be prepared in the site or use commercial bleach (Sodium hypochlorite solution 2 % concentration).

2.4.3. Surface sterilization of 8 month old embryos: The duration of surface sterilization of immature embryos with 50 % chlorine water should be reduced to 5-10 minutes. These embryos are very sensitive and long duration of surface sterilization leads to death of the embryos.

2.5. Inoculation of embryos

After proper washing, each embryo is inoculated directly into a small screw cap vial (5 ml capacity) containing 1.5 to 2 ml of sterile water. The sterilization of embryos and inoculation are carried out inside inoculation hood (Fig. 4b).

An alternative to sterile water for *in vitro* active storage of embryos is half-strength Y3 medium (without sucrose) supplemented with charcoal. In either of the case the embryos could be stored for two months. Compared to nutrient medium, chance of contamination of cultures is less with sterile water.

Inoculation of embryos in separate vials will reduce the loss of cultures due to contamination. After proper sealing of the cap (air tight) with parafilm, group the vials according to the accession and label them properly (Fig. 5).

3. *IN VITRO* RETRIEVAL OF EMBRYOS

3.1. Re-sterilization

After the embryos are brought into the laboratory, critical examination of individual embryos is essential for discarding the damaged/contaminated cultures. Clear water inside the culture indicates absence of contamination. On observing slight contamination due to bacteria (showing slight turbidity of water), embryos are to be treated with tetracycline (2 ppm) before inoculation into the retrieval medium. Severely contaminated embryos are to be discarded at the beginning.

3.2. Composition of retrieval medium

The basal medium for retrieval of the embryos is Eeuwens' Y3 medium. Constituents of the medium are shown in Table 2. Solid medium is used till the plantlet produced 3-4 leaves and thereafter shifted to liquid medium for rooting. Solidification of medium is done by adding agar 5.5 g/l. The supplementation of the basal medium for different purposes are summarized in Table 3.

Table 2. Composition Eeuwens' Y3 medium

Sl. No.	Chemical	Quantity (mg/l)
1.	NH_4 C1	535
2.	KNO_3	2020
3.	$MgSO_4 .7H_2O$	247
4.	$CaC1_2 .2H_2O$	294
5.	KC1	1492
6.	NaH_2PO_4	312
7.	KI	8.3
8.	H_3BO_3	3.1
9.	$MnSO_4 .4H_2O$	11.2
10.	$ZnSO_4 .7H_2O$	7.2
11.	$Na_2MoO_4 .H_2O$	0.24
12.	$CuSO_4 .5H_2O$	0.16
13.	$CoCl_2 .6H_2O$	0.24
14.	$NiC1_2 .6H_2O$	0.024
15.	Inositol	100
16.	Pyridoxine HCl	0.05
17.	Nicotinic acid	0.5
18.	Thiamine HCl	0.5
19.	Biotin	0.05
20.	Glycine	2.0
21.	Na_2EDTA	37.3
22.	$FeSO_4 .7H_2O$	13.9

Table 3. Supplementation of basal medium for various purposes

Purpose	Sucrose (g/l)	Glucose (g/l)	NAA (mg/l)	BAP (mg/l)	IBA (mg/l)	Charcoal (g/l)	State
9-11 month old embryo For germination							
Tall type	30.0	-	0.05	0.05	-	1.0	Solid
Dwarf type	60.0	-	0.05	0.05	-	1.0	Solid
Immature embryo Culture initiation *	-	60	0.05	0.05	-	1.0	Solid
For rhizogenesis	30.0	-	1.0	-	5.0	1.0	Liquid

* On germination cultures are transferred to the medium as that of mature embryos

3.3. Culture conditions

Initially, the cultures are to be incubated in dark at 27± 2°C and 85% RH till the plumules emerge. After germination, transfer the cultures to an illuminated room (2500 lux; temp. 27 ± 2°C, RH -85 %) with a photoperiod of 16 hours. Subculturing is required once in every 21-25 days.

The *in vitro* germination of coconut embryos is similar to that of seed nuts except for the enlargement of haustorium. In the cultured embryos, the surface of the haustorium becomes dark and brittle. Remove this portion at the time of subculturing. The number of days taken for germination is not the same in all situations. In various experiments, we observed that 9 to 11 months old embryos of WCT germinated within 45 days of inoculation (Fig. 6), while it took 60 to 80 days for the 8-months old embryos to germinate. In case of embryos of dwarf type, the emergence of plumule was noticed on the 15th day after inoculation.

After the emergence of 3 to 4 leaves (Fig. 7), the culture is to be shifted to the rooting medium (Table 3).

3.4. Abnormalities noticed during *in vitro* retrieval

The abnormalities noticed during *in vitro* retrieval (Fig. 8a-d) are (a) cessation of growth after germination (22%); (b) hyperhydricity, necrosis and senesecence (10-46.66%) and (c) no root formation even in the rooting medium (10%). Hyperhydricity is more for red and yellow dwarf accessions.

4. *EX VITRO* ESTABLISHMENT OF PLANTLETS

4.1. Importance

Acclimatization of *in vitro* derived plantlets is a critical step and calls for due attention. At this stage, plantlets are taken out from the culture tubes and pot-planted. It is experienced that vigorous plants acclimatized easily.

4.2. Transfer of plantlets to pot

After 12 months of *in vitro* culture, plants will be ready for transferring to pots (Fig. 9). The plants are to be treated with Carbendazim (1 g/l) and thereafter IBA 1000 ppm solution for one hour each before transferring to flower pots. The potting mixture should consist of sterile soil, sand and coir dust in equal parts.

4.3. Humidity control

Higher humidity in the vicinity of the plant is required. To achieve this cover the potted plantlets with polythene bag for 2-3 weeks (Fig. 10). Reduce the humidity gradually by providing perforations to the polythene bag. After two weeks, the bag may be removed during night. This practice has to be continued for a period of one month and there after remove the bag completely.

4.4. Application of nutrient solution

Apply Eeuwens' Y3 macro nutrients *viz.*, NH_4Cl, Na H_2PO_4 and KCl (Table 2) once in 15 days for the first 2 months of *ex vitro* establishment. At this stage, the plants in pots can be shifted to the net house (50% shade) for further hardening (Fig. 11).

4.5. Establishment of plantlets in pots

A well developed root system is advantageous for the establishment of plants. The correlation between the plant characters at the time of transfer to pots with the height of plants after 8 months in pots was shown in Table 4. The volume of root has a positive effect on the growth of plants in pots.

Seedlings showing satisfactory growth in pots may be transferred to big polybags (Fig. 12). The poly bags are half-filled with sand, soil and vermicompost at equal proportions. While transferring to polybags, proper care should be taken to avoid possible damage of roots. Seedlings in

polybags are found to have better rate of survival. Cowdung and vermi-compost may be applied to plants once in six months. During summer months once in two days water has to be given to plants. The aforesaid acclimatization process has to be carried out under 50% shaded net house to prevent the direct sunlight. After a period of 6 months under the net house, the plants will be ready for field planting.

4.6. Field establishment

Planting procedure followed for the *in vitro* retrieved plantlets is similar to that of normal seedlings (Fig. 13a, b). Field establishment will be very good with these plantlets, mainly due to the well formed roots and the growth of these plantlets in the field will be comparable to that of normal seedlings.

Table 4. Correlation between characters of *in vitro* retrieved plantlets

Characters	West Coast Tall			Different accessions		
	(2)	(3)	(4)	(2)	(3)	(4)
At the time of transfer:						
(1) Plant height	0.16	0.51**	0.51**	0.70**	0.61	0.52
(2) No. of leaves		0.43**	0.16		0.40	0.47
(3) Volume of root			0.34*			0.68**
After 8 months in pots						
(4) Height of plant						

*Significant at 5 %; ** Significant at 1 %

5. GERMPLASM COLLECTION

5.1. Introduction

The CPCRI protocol of coconut embryo culture was successfully used in germplasm expeditions since 1997. The protocol was first tried to bring embryos from six Pacific Ocean accessions maintained in the World Coconut Germplasm Centre of CPCRI at Andaman Islands. The details of the same is provided in Section 5.2. Section 5.3 summarizes the exotic collections made so far by CPCRI using this protocol. The suggestions for improving the rate of retrieval of plantlets are described in the last Section.

5.2. Indigenous collection

Two attempts were made to bring coconut germplasms in the form of embryo cultures from World Coconut Germplasm Centre (WCGC), Andamans to CPCRI, Kasaragod. In the first attempt 15 embryos were brought in liquid Y3 medium during 1992. Eighty percent of the cultures got contaminated. During 1994, 86 embryos of six Pacific Ocean Island accessions were brought from WCGC in sterile water. The embryos were retrieved *in vitro* at Kasaragod and successfully transplanted to the field. Number of embryos collected, percent germination and survival are shown in Table 5. Twenty two embryo-derived plants were field planted during 1995 at ICG-SA (International Coconut Gene Bank for South Asia) at CPCRI Research Centre, Kidu.

The feasibility of using the

Table 5: Percentage of germination, contamination and survivability of field collected embryos from Andamans Islands.

Name of the Accession	No. of embryos inoculated	Germination (%) after 45 days	Contamination (%) during Transit	Survival (%) after 4 months	Plantlets survived in the field
Niu Ui	15	100.00	0.00	60.00	4
Niu Hako	13	100.00	2	92.2	3
Bora Bora (Local tall)	17	58.2	0.00	52.94	4
Rangiroa Tall (Avatoru)	19	100.00	0.00	84.21	7
Rangiroa Tall (Tipura)	9	77.77	0.00	66.66	3
Nikkore	10	100.00	1	80.00	1

protocol for collection, transport and *in vitro* retrieval was established by the aforesaid experiment. It was then recommended for the use in exotic germplasm expeditions.

5.3. Exotic collection

In the year 1997, 15 exotic coconut germplasm from Mauritius, Madagascar and Seychelles were collected using the CPCRI protocol. This was the first attempt to collect coconut germplasm in the form of sterile cultures directly from the field. This expedition made some of the rare collections like Coco Gra (Makapuno type) and Guelle Rose. The developmental stages of *in vitro* retrieval of these accessions are given in the back cover.

In subsequent years, germplasm from Maldives, Comoros and Reunion islands of Indian Ocean, Sri Lanka and Bangladesh were made using the

Table 6. Details of exotic germplasm collected using CPCRI protocol of embryo culture

Place of collection	Year	No. of accessions	No. inoculated	Per cent germination	Per cent survived	No.of plantlets in the field *
Mauritius	Jun 1997	6	374	73.9	48.9	19
Madagascar	Jun 1997	4	422	77.7	72.9	34
Seychelles	Jun 1997	5	465	66.8	68.4	23
Maldives	Jun 2000	8	660	67.0	69.9	85
Comoros	Aug 2000	5	374	56.2	44.9	13
Reunion	Aug 2000	3	156	78.7	49.2	4
SriLanka	Feb 2001	4	350	54.0	44.2	92
Bangladesh	Dec 2001	10	157	82.2	81.9	24
Total		45	2958	69.5	60.0	294

* Field planting is not completed

Protocol. Details of embryos collected and their germination, survival and field establishment are shown in Table 6.

5.4. Conclusion

The experience suggests that 400 to 500 embryos per accession needed to be collected for the establishment of 100 plants in the field gene bank. The size of collection could be reduced by improving the efficiency of collection techniques and personnel skill that has a direct influence on losses due to contamination and damage of embryos . Losses noticed during the *in vitro* conditions are mainly contamination of cultures, poor growth and abnormalities like fasciations, senile and necrosis.

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ACC. 6
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